ANTIFUNGAL ACTIVITIES OF *Camellia sinensis* CRUDE EXTRACT ON SELECTED PATHOGENIC AND MYCOTOXIC FUNGI

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DECLARATION

This thesis is my original work and has not been presented for award of degree in any University or any other award.

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DEDICATION

This thesis is dedicated to all my family members; my father the Late Zacharia Kipsigei Bosseck; my mother Emily Tembur and my brothers, all for they have been inspirational to me at all times.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CLSI	Clinical Laboratory Standard Institute
EC	Epicatechin
ECG	Epicatechin Gallate
EGC	Epigallo catechin
EGCG	Epigallocatechin gallate
FLC	Fluconazole
ITC	International Tea Committee
IZD	Inhibition Zone Diameters
KEMRI	Kenya Medical Research Institute
MET	Multidrug Efflux Transporter
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
NAC	Non Albicans Candida
NCCLS	National Committee of Clinical Laboratory Standards
SD	Standard Deviation
SPP	Species
TF	Theaflavins
WHO	World Health Organization
μg	Micro grams

ABSTRACT

Human fungal infections pose serious medical issues. Up to now, more than a hundred thousand fungal species are considered as natural contaminants. During the last decade, the incidence of superficial and deep mycotic infections has continued to increase explosively. There is a general consensus among researchers, clinicians and pharmaceutical companies that new, potent, effective and safe antifungal drugs are needed. Majority of work has been conducted on *Camellia sinensis* extracts against bacterial agent's activity but little work for antifungal activity. In this study, *in vitro* antifungal activities of *Camellia sinensis* crude extracts compared with azole group of compounds on selected pathogenic and mycotoxic fungi were determined. That was done by evaluating the difference in antifungal activities of green and black tea crude extracts having a concentration of 100mg mL^{-1}. Quantitative bioassay was done using disc diffusion method and Minimum Inhibition Concentration was done using broth dilution methods. The fungal isolates used for bioactivity testing were yeasts; *Candida famata*, C. *lusitaniae, C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 24433, *C. krusei* ATCC 6258 and *Cryptococcus neoformans* ATCC 66031; and moulds, *Trichophyton mentagrophytes, Microsporum gypseum, Fusarium monilliforme, Aspergillus* spp and *Penicillium chrysogeneum*. ATCC standard fungal strains and clinical isolates were included. Green tea crude extract showed stronger inhibitory effect against the fungal strains tested than black tea crude extract. There was a significant difference in zone of inhibitions ($T=4.09$, $P<0.05$). Zone of inhibition exhibited by green tea crude extracts (11.92 ± 0.00mm) were higher than black tea crude extracts (8.14 ± 0.56mm). The pattern of activity by tea crude extracts against ATCC standard fungal strains and clinical isolates strains were similar. *C. famata, C. lusitaniae, C. tropicalis* ATCC 750 and dermatophyte, *T. mentangrophyte* were inhibited by green tea crude extract (IZD≥15mm). Clinical isolates of *Candida albicans* (strain 4 and strain 5); *Cryptococcus neoformans* (strain 3, 5 and 12), showed susceptibility to *Camellia sinensis* green crude extracts. The MIC of *Camellia sinensis* crude extracts against fungal isolates tested ranged from 50 mg mL^{-1} to 1.6 mg mL^{-1}. Hot green tea crude extract (mean MIC 12.25mg mL^{-1}) had a higher MIC on clinical fungal isolates than cold green tea crude extract (Mean MIC 12.167 mg mL^{-1}). The concentrates of aqueous *Camellia sinensis* crude extracts showed synergistic activity with conventional antifungal drug. However, level of synergism differed as observed in difference in inhibitory effect. The difference in inhibitory effect was significant ($P<0.05$). The crude tea extract restored the activity of lower concentration of antifungal, Fluconazole below MIC to susceptible breakpoints. Generally, the MFC (Minimum Fungicidal Concentration) of Mixture crude extracts were slightly higher as compared to that of green tea crude extract. These results are suggestive that addition of milk to blend the crude extracts altered the bioactive ingredients resulting to higher concentration for its MFC as compared to crude extracts alone. The studies on *Camellia sinensis* crude extracts (green and black) have shown remarkable antifungal activity against different strains of fungi and highlighted its significance to humans as potential health products.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Human fungal infections pose serious medical issues. Up to now, more than a hundred thousand fungal species are considered as natural contaminants (Kacaniova, 2003). There is a general consensus among researchers, clinicians and pharmaceutical companies that new, potent, effective and safe antifungal drugs are needed (Selitrennikoff, 1992). Historically, most of the substances have been part of natural products. Therefore, it is quite logical that any recent search for new prototype antifungal products should also include a variety of plant extracts. In designing a search for novel prototype antifungal agent, it seems reasonable to assume that if new agents are to be found that have different structures and different activities from those in current use, higher plants are a logical choice. It is chiefly because of their seemingly infinite variety of novel molecules, which are often referred to as secondary metabolite (Clark and Hufford, 1992).

Antifungal agents are widely distributed among higher plants (Caceres *et al.,* 1991), but only a few have been evaluated for their activity against human pathogenic fungi. In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungi to conventional antifungal drugs used in medical practice. Therefore, new prototype antimicrobial agents are needed to address this situation (Sati and Joshi, 2010).

Fungi are one of the most neglected pathogens as demonstrated by the fact that the amphotericin B, a polyene antibiotic discovered as long as 1956, is still used as a gold standard for antifungal therapy (Abad *et al.,* 2007). Exploitation of naturally occurring compounds from plants and microbes has been suggested by Sati and Arya (2010). The majority of clinically used antifungal agents have various draw backs in terms of toxicity, efficacy as well as cost and their frequent use has led to emergence of resistant strains. Hence there is a great demand for novel antifungal drugs belonging to a wide range of structural classes, selectively acting on new targets with least side effects.

Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of their having normally matchless chemical diversity. Natural products are also associated with low levels of toxicity, and in many cases have a fairly broad spectrum of activity (Silver and Bostian, 1990). In addition, edible plants have been proven to be harmless and are economical (Lee *et al*, 2003). It is possible that edible plants and beverages may be a source of new antimicrobial agents. With this in mind, tea is one of the most popular beverages in the world and second to water in its popularity (Kokhar and Magnusdottir., 2002; Yanagimoto *et al*., 2003; Lin *et al*., 2005; Thangapazham *et al*., 2007).

The beverage is an infusion of the leaves of *Camellia sinensis* plant (Higdon and Frei, 2006). Tea farming is the largest agribusiness in Kenya and contributes to over 26% of the total foreign exchange earnings and over 4% of gross domestic product (Economic survey, 2005).

In addition, the country is the third largest producer of black tea in the world after China and India (International Tea Committee, 2006). There are different types of tea from the leaves of *Camellia sinensis* (Higdon and Frei, 2006). Different processing methods produce these types of tea (Higdon and Frei, 2006). A study has revealed that Kenyan black tea has between 7-27 % more polyphenols when compared with tea from China, Japan and Taiwan (Wachira and Kamunya, 2005). The Kenyan tea germplasm has also been observed to be diverse in its polyphenol composition and contents and therefore provides raw material for production of different types of tea products including health drinks (Magoma *et al*., 2000).

However, the state of research on tea regarding its pharmacological properties is limited and the majority of work has been conducted on green tea with very little on black and white tea. Many health benefits have been ascribed to consumption of the tea beverage, including the effects of reduction of cholesterol, protection against cardio-vascular disease and cancer (Zuo *et al*., 2002). Consumption of tea has been associated with reduced risk of major diseases, including coronary heart disease, stroke and cancer (Ramarathnam *et al*., 1995); Robinson *et al*., 1997; Benzie *et al*., 1999; Langley-Evans, 2000; Leenen *et al*., 2000).

Beneficial effects of tea have been attributed to the strong antioxidative activity of the tea phenolic compounds known as catechins (Zuo *et al*., 2002). Tea catechins possess strong antioxidants properties which protects the body from damage caused by free radical induced oxidative stress (Manzocco *et al*., 1998).

In addition, many reports have presented data on the antimicrobial activity of different types of tea extracts on various pathogenic microorganisms (Yam *et al*., 1997; Chou *et al*., 1999). Green tea elicits strong antibacterial activity including potential to inhibit gram positive ccoci; gram negative bacilli (Hamilton–Miller, 1995). Studies have also shown that tea can inhibit and kill a wide range of pathogenic bacteria at or slightly below typical concentrations found in brewed tea (Hamilton-Miller, 1997).

Various studies have shown significant suppressive effects of green tea polyphenols against many microorganisms (Shetty *et al*., 1994). Black tea, a major source of phenolic, (Rechner *et al*., 2002) including theaflavins and thearubigins, (Luczaj and Skrzydlewska, 2005) has also been shown to have antimicrobial properties both *in vivo* and *in vitro* (Bandyopadhyah *et al*., 2005). Screening for antifungal properties of tea products is an important strategy for development of novel drugs or rational ways of managing fungal resistance to azoles group of compounds. This study attempts to facilitate to unravel the potentiality of *Camellia Sinensis* plant product as novel modalities in the line of new drug discoveries.

1.2 Statement of the problem

During the last decade, the incidence of superficial and deep mycotic infections has continued to increase significantly. This is as a result of the AIDS epidemic that has caused a significant increase in the occurrence of fungal infections such as *Mucocutaneous candidiasis, Pneumocystis Carinii (jirovencii) Pneumonia* (PCP), *Cryptoccocal Meningitis, Histoplasmosis, Coccidioidomycosis, Blastomycosis* and

infections due to *Penicillium Marneiffei* among others. The increased use of immunosuppressive agents in association with organ transplants, chemotherapy and improved life-saving medical techniques necessitating indwelling catheters has also led to substantial increase in the occurrence of serious fungal infections.

The situation has been made worse by misuse of broad spectrum antibiotics as well as antifungal drugs resulting in intense selective pressure on fungal populations, thus increasing fungal resistance. This has rendered current antimicrobial agents insufficient to control fungal infections and has created the need for new antifungal agents. It is therefore necessary to evaluate the role of plant products in combating fungal infections. *Camellia sinensis* extracts has been used against bacterial agents but its antifungal activity has not been studied. This study evaluates the role of *Camellia sinensis* in antifungal activity.

1.3 Research Justification

Studies to determine the efficacy of *Camellia sinensis* on bacteria has shown potential for antibacterial activity. However, no studies have been done on fungi despite the emergence of newly identified fungal pathogens and the re-emergence of mycotic infections that were previously uncommon now posing a serious growing public health conern. Moreover, natural products and traditional medicines are now preferred sources of new mycotic drugs because these agents have minimal side effects (Maluventhan and Sangu, 2010). Based on current literature no significant side effects of toxicity have been associated with regular tea consumption.

In addition, numerous environmentally friendly industrial cleaning agents, deodorizes and antimicrobial agents have been formulated using tea (Koenden *et al*., 2011). The outcome from the study will offer valuable information on the need to use tea as either complementary or alternative medicine for management of fungal infections.

1.4 Null Hypothesis

The crude extracts of *Camellia sinensis* have no antifungal activities and synergism with azole antifungal against selected pathogenic and mycotoxigenic fungi.

1.5 Objectives

1.5.1 General objectives

To determine antifungal activities of *Camellia sinensis* crude extract on selected pathogenic and mycotoxigenic fungi

1.5.2 Specific objectives

i) To determine the *in vitro* antifungal activities of crude extracts of green and black Kenyan tea

ii) To determine synergism between crude extracts of Kenyan tea and conventional antifungal drugs on azole resistant fungi.

iii) To determine the effect of temperature (boiling) and addition of milk as a common mode of tea preparation on the fungicidal activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology

Fungi are increasingly recognized as major pathogens in critically ill patients. *Candida* spp. and *Cryptococcus* spp. are the yeasts most frequently isolated in clinical practice. The most frequent filamentous fungi (moulds) isolated are *Aspergillus* spp., but *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp. and Zygomycetes are increasingly encountered (Marr *et al.,* 2002; Husain *et al*., 2003). Several reasons have been proposed for the increase in invasive fungal infections, including the use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, and prosthetic devices and grafts, and more aggressive surgery. Patients with burns, neutropenia, HIV infection and pancreatitis are also predisposed to fungal infection (Eggimann *et al*., 2003). Prophylaxis with azoles antifungal agents may account for this change in epidemiology.

2.2 Fungal infection and treatment options

2.2.1 *Candida albicans*

Disseminated candidiasis is associated with mortality in excess of 25 % (Kibbler *et al.,* 2003). *Candida* is a normal commensal of the skin, and gastrointestinal and genitourinary tracts. *Candida albicans* is the most frequent species isolated from clinical specimens, but other species (Non-*Albicans candida*, NAC) are increasingly seen. The NAC are of special concern, since some are highly virulent and are associated with treatment failure due to reduced susceptibility to antifungal agents.

Although identification to species level and susceptibility testing are technically demanding, it is recommended that they should be performed on all fungi obtained from sterile sites and urine of intensive-care and transplant patients (Denning *et al.,* 2003).

C. albicans is responsible for 79.4% of Candidaemias in intensive-care patients, and only 37.5% in hematology patients (Kibbler *et al.,* 2003). Similar results were seen in a prospective pan-European study (Tortorano *et al*., 2004). *Candida* is capable of causing a wide spectrum of disease. Clinical diagnosis of invasive *candida* infection is challenging. Late or no treatment are independent predictors of death in invasive candidiasis (Denning *et al.,* 2003).

2.2.2 *Candida glabrata*

Historically, *Candida glabrata* has been considered a relatively non–pathogenic saprophyte of the normal flora of healthy individual, rarely causing serious infections in humans (Haley, 1961; Stender *et al*., 1962). However, following the widespread and increased use of immunosuppressive therapy together with broad spectrum antimycotic therapy, there is significant increase of mucosal and systematic fungal infections caused by *Candida glabrata* which is often the second and common in abnormal hosts such as immune compromised persons or diabetic (Mellitus type), (Geiger *et al.,* 1995; Sinnott *et al.,* 1987; Wingard *et al*., 1993). *Candida glabrata* infections are difficult to treat and are often resistant to many azole antifungal agents, especially Fluconazole (Hitchock *et al*., 1993; Komshian *et al* 1989; Willocks *et al*, 1991).

The rise in *C. glabrata* infections has also been showed in two prospective studies from Italy (Tortorano *et al*., 2002; 2004). Consequently, *C. glabrata* infections have high mortality rate in compromised and high risk hospitalized patients.

2.2.3 *Candida krusei*

Early reports of *C. krusei* in human describe the organism as a transient, infrequent isolate of minor clinical significance inhabiting the mucosal surfaces (Samaranayake and MacFarlane, 1990). More recently it has emerged with biological properties that differ from *C. albicans* and has spectrum of clinical manifestations such of which usually occurs in compromised patients in nosocomial setting (Samaranayake *et al*., 1994). It is true that the wide use of newer triazole drug. Studies in both humans and animals have demonstrated prophylactic and therapeutic failure of Fluconazole against *C. krusei* due to increasing resistance of organism to azoles (Wingard *et al*., 1991; Roder *et al*., 1991; Persons *et al*., 1991).

2.2.4 *Cryptococcus neoformans*

Cryptococcus neoformans is the commonest cause of cryptococcosis, and is usually acquired by inhalation. Pulmonary Cryptococcosis may be asymptomatic or may present non-specifically with cough, fever or pleural symptoms. Meningitis is a common feature of infection, especially in HIV-seropositive patients and solid-organ transplant recipients. Human immunodeficiency virus (HIV) epidemic has led to a growing population of immunocompromised patients at risk of contracting opportunistic fungal infections particularly cryptococcosis (Mitchell and Perfect, 1995).

Globally, the risk for cryptococcal meningitis HIV/ AIDS is estimated at 6–8% in adults and 1% in children (Steenbergen and Arturo, 2000; Goldman *et al*., 2001). In sub-Saharan Africa which has the highest burden of HIV/AIDS worldwide, the incidence of meningoencephalitis has increased significantly with mortality higher than meningococcal meningitis caused by *Neisseria meningitides* (Bogaerts *et al.,* 1999; Heyderman *et al*., 1998). There are three varieties of *Cryptococcus neoformans; Cryptococcus neoformans* var. *neoformans, Cryptococcus* var. *grubii* and *Cryptococcus neoformans* var. *gatti* (Franzot *et al*., 1999; Kwon- Chung, 1976). *C. neoformans gattii* is limited to tropics and sub-tropical regions coinciding with the distribution of the host trees *eucalyptus camaldulensis* and *eucalyptus tereticornis*, (Kwon-Chung and Bennett, 1984).

The need for lifelong Fluconazole maintenance therapy due to high relapse rates of cryptococcosis in HIV and AIDS raises conce`rns over antifungal resistance in developing countries including Kenya (Paugam *et al*., 1998, Bii *et al*., 2006). As Fluconazole becomes widely used due to expanding population of HIV/AIDS confounded by irrational use of antibiotics/generic antibiotics in developing countries, emergence of azoles resistance is a mycological challenge (Berg *et al*., 1998).

2.2.5 *Trichophyton mentagrophytes*

Trichophyton mentagrophytes is a very common dermatophyte isolated in laboratories worldwide (Summerbell *et al.*, 2007). There are two main varieties of *T. mentagrophytes* (Kwong-Chung and Bennett, 1992).

Trichophyton mentagrophytes var. *interdigitale* is a cottony strain specifically associated with humans (anthropophilic) that infects all parts of the body surface and is a common causative agent of athlete's foot (Krajden 1997; Nenoff *et al*., 2007). Infections caused by *T. rubrum* are often associated with frequent relapses following cessation antifungal therapy (Mukherjee *et al*., 2003).

2.2.6 *Microsporum gypseum*

The geophilic dermatophyte *Microsporum gypseum* is considered an infrequent cause of cutaneous mycosis, usually causing self-limited inflammatory lesions on the glabrous skin and scalp. Cases of dermatophytosis caused by *M. gypseum* in HIV infected patients have been described in association with severe immunodeficiency, presenting as disseminated lesions of varied clinical appearance, refractory to long term use of imidazoles (Fernandes *et al*., 1998).

2.2.7 *Aspergillus species*

Aspergillus spp. is the most commonly isolated invasive moulds (Denning, 1998). Only a few of the 200 or so species are pathogenic to man, primarily *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger*. *A. fumigatus* remains the mould most frequently isolated, but the epidemiology appears to be changing. *A. fumigatus* accounted for 82% of cases of invasive aspergillosis in 1985, compared to 66% in 1999 in stem cell transplant patients (Marr *et al.,* 2002). *Aspergillus terreus* is increasingly recognized as a pathogen, accounting for 15% of isolates in 2001, compared to < 2% in 1996 in one study (Baddley *et al.,* 2003).

Aspergillus spp. can lead to invasive aspergillosis, trachea bronchitis, aspergilloma and chronic necrotizing aspergillosis, but colonization without infection can occur. Infections caused by *Aspergillus* species have significantly increased in recent years. This probably is as a result of increasing numbers of patients at risk, including the HIV immunocompromised individuals. Despite a better understanding of the epidemiology of *Aspergillus* infections, important diagnostic limitations persist. *Aspergillus* spp. can cause disease in humans by colonization and subsequent allergic reactions, colonization of pre-existing cavities or tissue invasion.

Aspergillus flavus is the second leading cause of invasive and non-invasive aspergillosis (Hedayati *et al*., 2007) and is more virulent than *A. fumigatus*. *Aspergillus flavus* is a common cause of fungal sinusitis and cutaneous infections Chronic conditions such as chronic cavitary pulmonary aspergillosis and sinuses fungal balls have rarely been associated with *A. flavus*. However, infection by *Aspergillus fumigatus* is the most prevalent species in the genus (Guinea *et al*., 2005). Amongst the several secondary metabolites produced by *A. flavus* are aflatoxins, the most toxic and potent carcinogenic natural compounds ever characterized (Hedayati *et al*., 2007).

Aflatoxin B1, B2, G1 and G2 are produced *by A. flavus* and *A. parasiticus* while gliotoxin is one of the most abundant metabolites produced by *A. fumigatus* during invasive hyphal growth. The toxin exerts a broad spectrum of immunosuppressive effects *in vitro*, including inhibition of cytokine production, antigen presentation and production

of reactive oxygen species by macrophages, and reduced cytotoxicity in T-cells (Kupfahl *et al*., 2007).

2.2.8 *Fusarium species*

Fusarium is a ubiquitous fungus widely distributed in the soil, plants and different organic substrates. During recent years, they have been increasingly associated with humans and now represent the second most frequent mould causing invasive fungal infections in immunosuppressed patients associated with high morbidity and mortality rates (Consigny *et al*., 2003; Nucci and Anaissie, 2002). It is capable of causing a wide variety of infections.

Most cases of disseminated infection occur in immunocompromised patients, particularly in stem-cell and solid-organ recipients (Marr *et al.,* 2002; Husain *et al.,* 2003). Other risk factors include diabetes and renal failure (Ribes *et al.,* 2000). It can cause localized disease including keratomycosis, endopthalmitis, and peritonitis due to implanted catheters for chronic ambulatory peritoneal dialysis, paronychia, invasive nasal infection and post-traumatic lesions of the bone, joint or skin.

Fumonisins are carcinogenic mycotoxins produced by *Fusarium verticillioides* (telemorph Gibberella Moniliforme) and other *fusaria* (Anonymous, 2006) and are among the most important toxins regarding food and feed safety. Fumonisins B1, B2 and B3 are all carcinogenic and regarded as most important (Birzele *et al*., 2002). *Fusarium spp.* are resistant to many of the antifungal compounds licensed to treat fungal infections

and among them, *F. solani* is considered the most resistant. However, some data pointed out that the resistance could be species and even isolate dependent (Cuenca–Estrella *et al.*, 2006). *Fusarium moniliforme* has been reported as an agent of cutaneous disease in man, as a new agent of mycetoma in Europe, as an agent of keratitis, septic arthritis.

2.2.9 *Penicillium speecies*

Many species of *penicillium* are common contaminant on various substrates and commonly found in house dust. Some species grow in-door on dry wall, decaying fabrics, moist chip boards and paint. It is also found in dried foodstuff, cheese fresh herbs, dry cereals, nuts, decaying vegetation among others. It may cause pneumonitis, asthma and allergic alveolitis in susceptible individuals. Type I allergies (hay, fever, asthma) and Type III hypersensitivity has been associated with *Penicillium* spp.

The genus *Penicillium* has several species; the most common ones include *P. chrysogenum, P. citrinum, P. janthinellum, P. marneffei* and *P. purpurogenum*. Patients who do not receive the appropriate antifungal treatment have a poor prognosis; however, primary treatment with amphotericin B and secondary prophylaxis with itraconazole are effective (Supparatpinyo *et al*., 1998).

2.3 Azole (Fluconazole) Resistant Fungi

Most of the currently available drugs are directed against the ergo sterol moiety in the fungal membrane (polyene antimycotics) or against enzymes involved in the biosynthesis of ergosterol (azole antimycotics); there is a threat of cross resistance and a clear demand

for a new class of antimycotics with a different cellular target. Fluconazole and Amphotericin B resistance has been reported (Verweiji *et al.*, 1998).

2.4 Antifungal Drugs

2.4.1 Azoles group of compounds (Fluconazole)

Fluconazole is the first of a new subclass of synthetic triazole antifungal agents (Pfizer, 2010). It is available as tablets for oral administration as a powder for oral suspension and as sterile solution for intravenous use in glass and in via-flex plus plastic containers. It is designated chemically as 2,4-difluoro-α,α-bis (1H–1,2,4-triazol-1-methyl1) benzyl alcohol with empirical formula of $C_{13}H_{12}F_2N_6O$ and molecular weight of 306.3. Fluconzole is active against most strains of *Candida albicans*, *Cryptococcus neoformans, Candida tropicalis, Candida parapsilosis* and others.

Its mode of action is that it is a highly selective inhibitor of fungal cytochrome P_{450} dependent enzyme lanosterol 14–α-demethylase. This enzyme functions to convert lanosterol to ergosterol, and its inhibition disrupts membrane synthesis in the fungal cell. Resistance can arise from a modification in the quality or quantity of the target enzyme, reduced access of the drug to the target, or some combination of these mechanisms. In the first instance, point mutations in the gene (*ERG11*) encoding the target enzyme, 14-α-demethylase, lead to an altered target with decreased affinity for azoles. Over expression of *ERG11* results in the production of high concentrations of the target enzyme, creating the need for higher intracellular Fluconazole concentrations to inhibit all of the enzyme molecules in the cell.

Loss of allelic variation in the *ERG11* promoter may result in a resistant strain that is homozygous for the mutated gene.

The second major mechanism involves active efflux of Fluconazole out of the cell through the activation of two types of multidrug efflux transporters (MET): the major facilitators (encoded by *MDR* genes) and those of the ATP-binding cassette super family (encoded by *CDR* genes). Up regulation of *CDR* genes leads to resistance to multiple azoles. These mechanisms may act individually, sequentially, and in combination. It is also now well established that the mechanism of resistance to Fluconazole, and other azoles, in *C. glabrata* involves up regulation of the *CDR1* and *CDR2* genes, resulting in resistance to multiple azoles. Thus, exposure of *C. glabrata* to sub-therapeutic doses of Fluconazole may result in resistance not only to Fluconazole but to other azoles (that is itraconazole and voriconazole) as well.

Fluconazole resistance in *C. krusei* appears to be mediated by reduced sensitivity of the target enzyme to inhibition by the agent. The subsequent loss of normal sterols correlates with the accumulation of 14-α-methyl sterols in fungi and may be responsible for the fungistatic activity. Fluconazole is thought to be fungistatic when given in lower doses, and fungicidal when given in higher doses.

2.5 New approaches to combat resistance

Development of new antibiotics and antifungal agents can provide solution to the problem of resistance but is expensive and may have side effects (Elopoulos, 1982).

Alternatively, current researchers have focused on strengthening the antimicrobial action of the existing antibiotics through combined antibiotic therapy and combined antibiotic-herb therapy (Elopolous, 1982). This can as well be demonstrated in antifungal therapy and combined antifungal herb therapy.

2.5.1 Combined antifungal therapy

Combined antibiotic therapy has been shown to delay the emergence of bacterial resistance and may also produce desirable synergistic effects in the treatment of bacterial infection (Elopolous, 1982). The combined use of tea and antibiotics could be useful in fighting emerging drug-resistance problem especially among enteropathogens, (Abascal and Yarnell, 2002). The side effects normally encountered in combined antimicrobial therapy have led to shift in focus to combined herb drug therapy (Nwafor *et al.*, 2003; Esimone *et al*., 2003). The potential herb drug interaction could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome).

One of the most herbs that are widely consumed concomitantly with most drugs is tea. Recent works on tea have shown that it has medicinal properties including antimicrobial effect against a wide range of bacteria and viruses (Sakanka *et al*., 1989, Toda *et al.*, 1991). Many plants and their extracts used against microbial infections due to the presence of secondary metabolites such as phenols (Kazmi *et al*., 1994); essential oils (Cosentino *et al*., 1999; Daferera *et al*., 2003); terpenoids (Habtemariam *et al*., 1993; Taylor *et al*., 1995); alkaloids (Omulokoli *et al*., 1997) and flavanoids (Batista *et al*., 1994).

Natural products either in extract from or pure compounds provide unlimited opportunities for the development of new drugs due to the availability of chemical diversity (Cos *et al*., 2006). The screening of plant extracts and their products for antimicrobial activity has shown that higher plants represent a potential source of novel therapeutic agents (Afolayan, 2003).

Higher plants as a source for new potential drugs is still largely unexplored and only a small percentage of them has been subjected to phytochemical investigation and the fractions submitted to pharmacological screening is very low. Such screening of various natural organic compounds and identifying active agents is urgently needed. Successful prediction of lead molecule and drug like properties at the onset of drug discovery will pay off later in drug development. Therefore combined antifungal therapy needs to be ascertained against emergence of antifungal drug resistance. There are several reports that show antifungal activity by natural products (Mondello *et al*., 2003). *Camellia* spp. is a natural substance that is commonly drunk worldwide.

2.6 *Camellia sinensis*

Tea is an evergreen shrub plant that belongs to the family *Camellia sinensis* L; and genus *Camellia,* that includes some 82 species (Banerjee, 1992). The *Camellia* spp. is the most important of all both commercially and taxonomically and is cultivated to produce a stimulant brew. The two main varieties are *Camellia sinensis* var. *assamica* with relatively large leaves and *Camellia sinensis* var. *sinensis* with small semi erect leaves (Higdon and Frei, 2006).

The *Assamica* tea originated from the forests of Assam in North Eastern India and the *sinensis* tea from Sichuan Province, South Western China (Van der Vossen and Wessel, 2000). Tea can be cultivated in regions that have high humidity, fair temperatures and acidic soils, from sea level to high mountains (Hara *et al*., 1995); Gutman and Ryu, 1996).

Tea is an infusion of the leaves of *Camellia sinensis* plant, and is one of the most widely consumed beverage in the world (Higdon and Frei, 2006). Herbal teas are infusions of herbs of plants other than *Camellia sinensis*. There are different types of tea from the leaves of *Camellia sinensis* (Higdon and Frei, 2006). Different processing methods produce the types of tea. White tea is made from apical buds and immature leaves, technically called "flush", which are steamed to inactivate polyphenol oxidase and then dried. Green tea is processed from withered and steamed fresh tea leaves. Semi fermented teas (Oolongo tea) is processed by macerating tea leaves and it is fermented before heating dry to stop further biochemical changes. Fully fermented tea (black tea) is made by macerating tea leaves and then allowed to ferment completely before drying (Wilson *et al*., 1999; McKay and Blumberg, 2002).

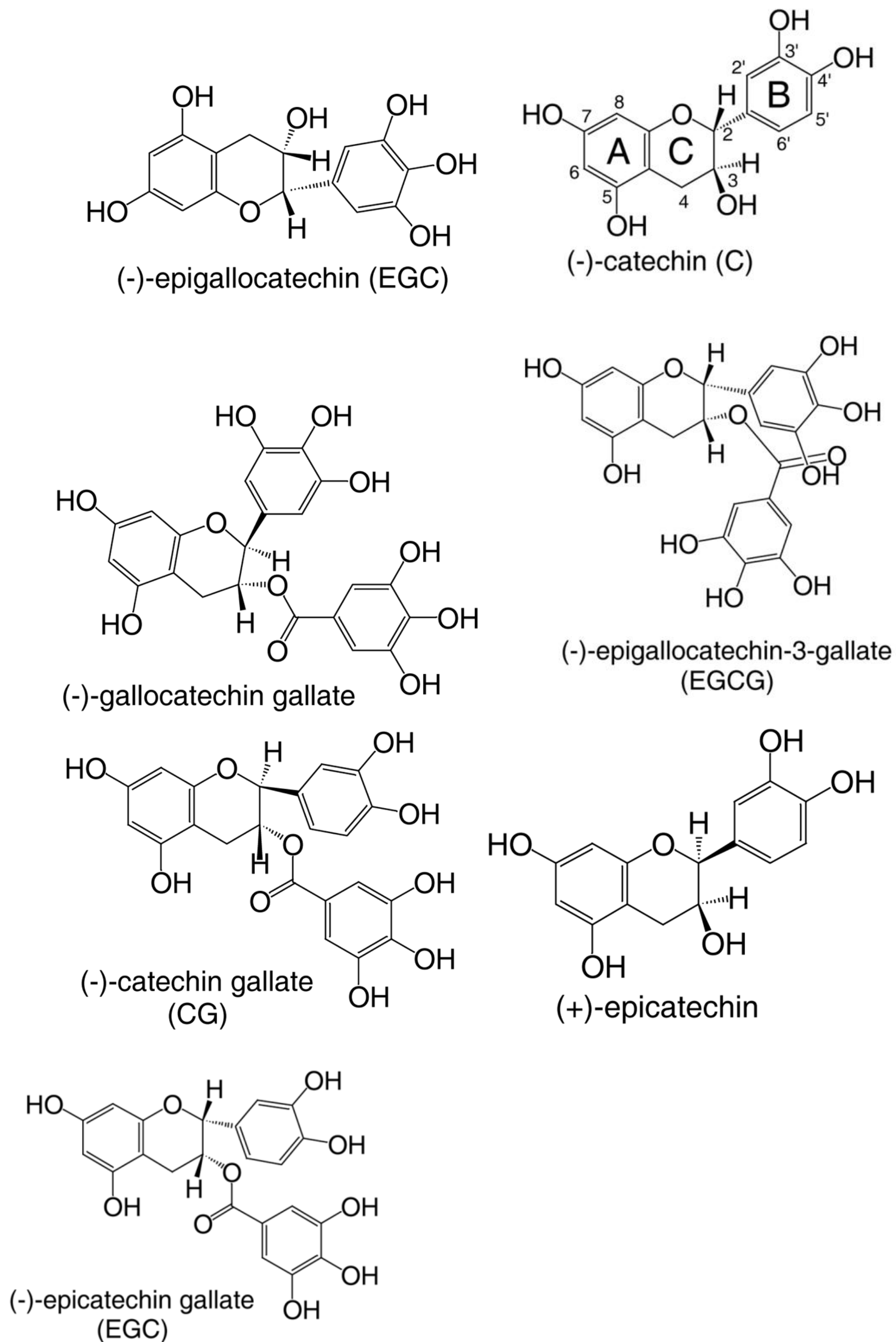
2.6.1 *Camellia sinensis* and health

The tea plant has unique secondary products including polyphenols which are pharmacologically active and with potential to promote human health. There is a growing body of knowledge on health benefits of tea (McKay and Blumberg, 2002). This has been largely generated from studies using green tea (non-fermented).

Little work has been carried out on black (fermented) tea, yet this is the predominant type of tea product produced and consumed in Kenya. Tea contains a number of bioactive chemicals. The phytochemical screening of tea revealed the presence of alkaloids, saponins, tannins, catechin and polyphenols (Sofowara 1984; Opara, 1992). Recent researchers mainly focus on the potential health benefits of a class of compounds in tea known as flavanoids (Lai *et al.*, 2001). Different types of teas have different biochemical profiles. Studies have demonstrated that, processed Kenyan black tea has residual catechin levels that are as high as those found in green teas produced in Asia and therefore may be more efficacious as green tea in enhancing human health (Obanda *et al*., 1997).

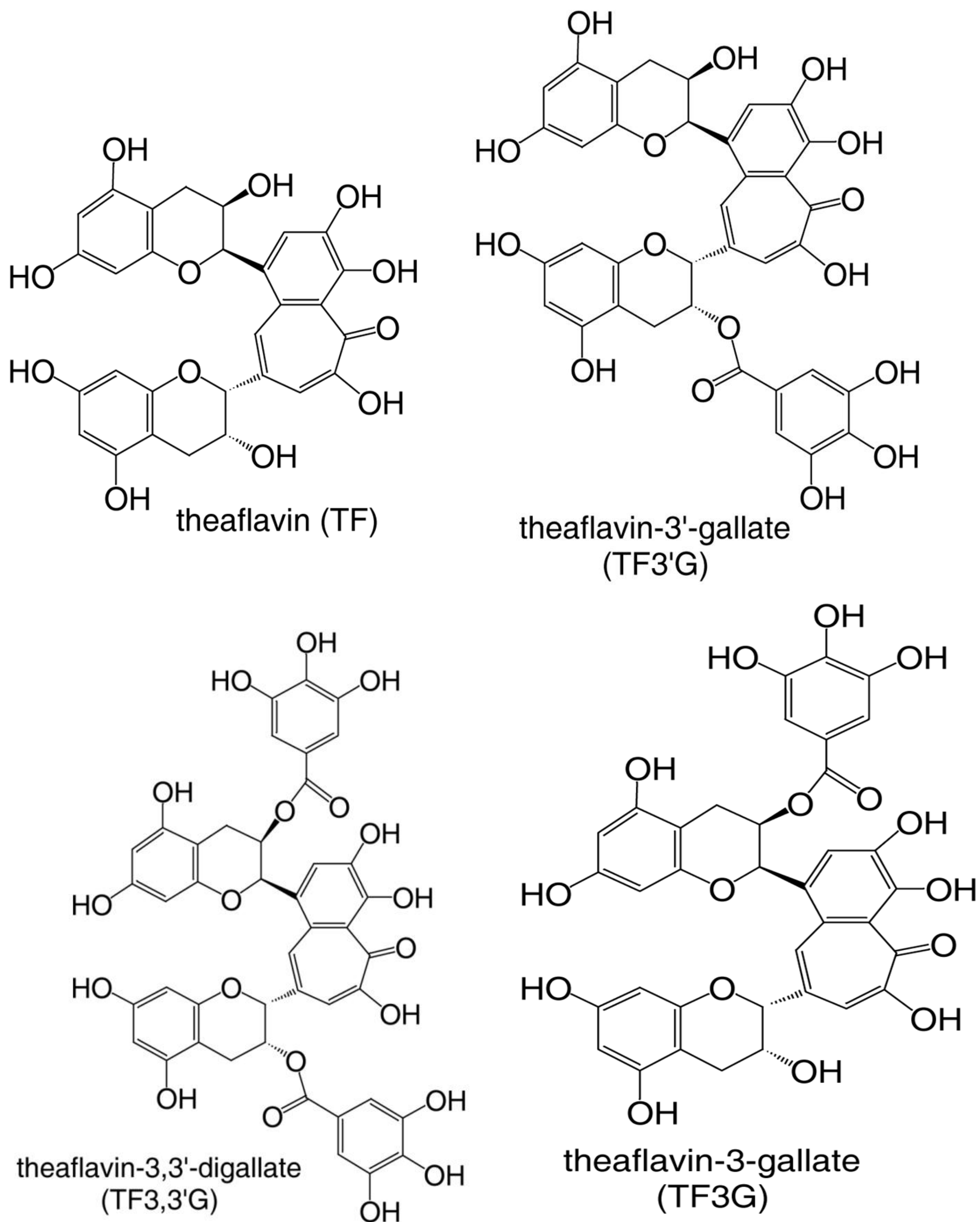
The catechins in black tea have been oxidized to coloured polyphenols called theaflavins and the thearubigins (Owour and Mc Dowell, 1994). Catechins have four main derivatives, including (-)-epilagallo catechin (EGC, (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). Catechins account for 6 -16% of the green tea leaves.

Figure 2.1 Polyphenols in *Camellia sinensis* (Green Tea)



Chemical structures of polyphenol compounds found in tea (Yang *et al.,* 2000)

Figure 2.2 Polyphenols in Black tea



Chemical structures of polyphenol compounds found in tea (Yang *et al.,* 2000)

Theaflavins are formed from polymerization by catechins due to oxidation by polyphenol oxidase at fermentation stage during the manufacture of black tea. Theaflavins contribute to the characteristics bright orange-red color of black tea, accounting approximate 2g/100ml of the dried water extract of black tea. The bioactivity of these oxidized polyphenols is different from that of catechins (Karori *et al*., 2007). The polyphenols from green as well as black tea have been associated with amelioration of inflammation (Maeda, 1989; Yamada, 1995; Karori *et al*., 2008); inhibition of diabetes including hyperglycemia (Vinson *et al*., 2001; Sabu *et al*., 2002) prevention of intestinal damage and anti-diarrhea properties (Asfar *et al*., 2003). It is also implicated with enhancement of oral health (Wu and Wei, 2002) antiparalytic effects at the skelemotor junction (Das *et al*., 1994) and potential to improve spatial cognitive learning ability (Hague *et al*., 2006). In addition, it reduces cholesterol against cardiovascular disease and cancer (Zuo *et al*., 2002).

Human and animal studies have also demonstrated that EGCG has ability to block inflammatory responses to ultraviolet A and B radiation and inhibit neutrophil migration that occurs during inflammatory process. In addition, it is a powerful antagonist of human immunodeficiency virus reverse transcriptase (Nakane and Ono, 1990, Matsui *et al*., 1999; Hofbauer *et al*., 1999). The chemical composition includes polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins chlorophyll, volatile compounds, minerals, trace elements and other unidentified compounds.

Food stuff can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way which is relevant to either the state of well being and health or the reduction of the risk of a disease (Wu *et al*., 2002; Diplock *et al*., 1999; Roberfroid, 2002).

2.7 Antifungal activities of tea extracts

Differences in antimicrobial activities of tea have been found to be related with the kind and degrees of fermentation of tea (Chou *et a*/., 1999). Aqueous (crude) extracts of tea (*Camellia sinensis*) of different types and from various sources inhibit a wide range of microbial pathogens (Saroj and Hamilton-Miller, 1997). Testing of pure tea compounds and closely related chemicals suggested that antimicrobial activity of extracts of green tea was due to catechins (Saroj and Hamilton-Miller, 1997). Catechins from the tea extracts have been observed to be cytotoxic to microbial pathogens and therefore may be useful as antibacterial and antifungal agents (Katsuhiko *et al*., 1991); Hamilton- Miller, 1995; Yam *et al*, 1997).

In black tea extracts, theaflavin-3,3-digallate (TF3) has been reported to have antifungal activity against *Trichophyton mentangrophytes, Trichophyton rubrum, Candida albicans* and *Cryptococcus neoformans*. This was in dose and contact time dependent manner (Okubo *et al*., 1991). Despite the valuable data generated so far from green tea, no data has been generated of drug resistant fungi as well as from black tea. Similarly efficacy of tea extracts on significant clinical fungal strains species is yet to be determined.

2.8 Synergistic activity

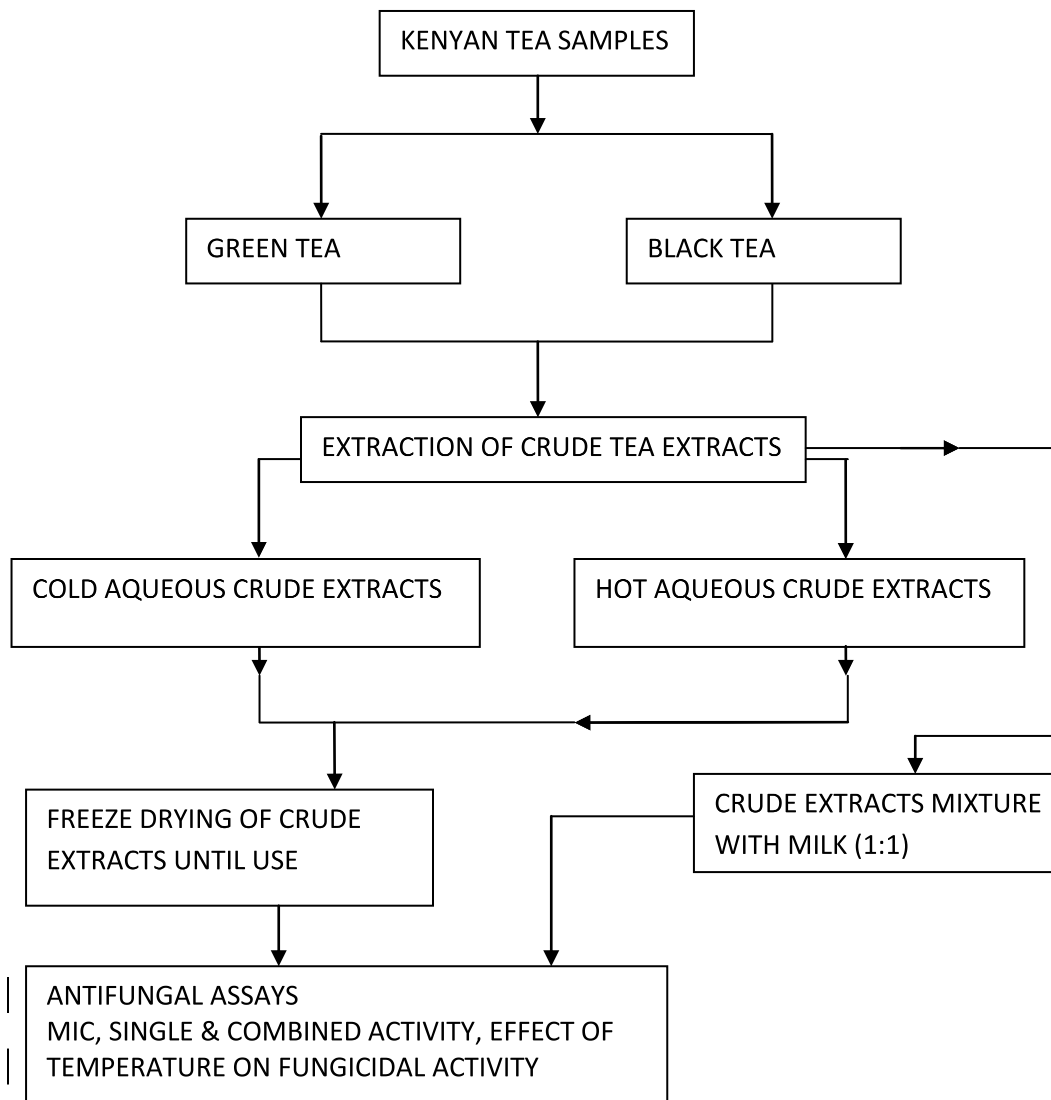
Bacteria and fungi resistance to antimicrobial drugs has continued to grow in the last decades (Cohen, 1992; Nascimento *et al*., 2000). Tea extract has showed synergistic activity with chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against test strains (Tiwari *et al*., 2005). Green tea containing high concentrations of catechins such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG) and (-)- epigallocatechin gallate (EGCG) has reported synergy with levofloxacin against enteroahaemorrhagic *Escherichia coli* (Isogai *et al*., 2001). Antifungal activity of green tea catechins against *C. albicans* has been shown (Hiraswa and Takada, 2004). The convenience of combined treatment with catechins and lower doses of antimycotics may help to avoid the side effects of antimycotics.

Tea polyphenols have particularly proven to synergistically enhance the antimicrobial activity of antimicrobial agents (Zhao *et al.,* 2001; Hu *et al*., 2002). This development has led to increased search to unfold new, broad spectrum, potent antifungal agents. Antimicrobial resistance to anti microbial agents has lead to treatment failure and the shift of medical care from orthodox to herbal medicine. Most of the herbal medicines in use await validation of their claimed effects and possibly the development of novel antimicrobial drugs from them. Virtually all synthetic antifungal are associated with serious adverse effects and for some fungal infections there is still no effective cure. Medicinal plants are valuable natural resources and regarded as potentially safe drugs and have been tested for biological, antimicrobial activity with significant role in the modern medicine (Hassawi and Kharma, 2006; Bhat *et al*., 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental design



3.1.1 Samples of *Camellia sinenensis*

Processed Commercial *Camellia sinensis* (black and Green tea) produced and packed by James Finlay (K) Ltd were purchased off shelf in retail outlet at the factory in Kericho County.

3.1.2 Test Fungal Organisms

The standard test fungi of American Type Culture Collection (ATCC) was sourced from KEMRI and included: *Cryptococcus neoformans* ATCC66031, *Candida albicans* ATCC 90028, *Candida krusei* ATCC6258, *Candida glabrata* ATCC24433, *Candida tropicalis* ATCC750, *and Candida parapsilosis* ATCC22019 as standard organisms. Clinical isolates included: *Cryptococcus neoformans, Candida albicans, Candida famata*, *Candida lusitaniae*, *Trichophyton mentangrophytes, Microsporum gypseum.* Mycotoxigenic fungi included: environmental pathogenic isolates *Fusarium moniliforme, Aspergillus flavus, Aspergillus niger* and *Penicillium chrysogeneum*. The selection of test strains was based on their significance as opportunistic pathogens and their resistance to conventional drugs.

3.1.3 Preparation of fungal strains

Viability tests were carried out by picking the organism from the stock using sterile loop and inoculating into RPMI 1640 media and then incubated at 35°C and 30°C for yeast and moulds respectively, for a period of 3 hours. They were then sub-cultured onto sterile Sabouraud Dextrose Agar (SDA) and incubated for 72 hours at 35ºC and 30ºC for yeast

and moulds respectively. Distinct pure colonies were picked and used for bioactivity testing. The test fungi were confirmed using macro and micro morphological characteristics with up to date identification keys (Lorene *et al.,* 2002).

3.1.4 Preparation of McFarland standard

McFarland standard is used as a reference to adjust the turbidity of fungal suspension so that fungal organisms will be within a given range. Exactly 0.5 McFarland equivalent turbidity standards was prepared by adding 0.6 ml of 1% barium chloride solution ($BaCl_{2}.2H_{2}0$) to 99.4 ml of 1% sulphuric acid (H_2SO_4) and mixed thoroughly. A small volume of the turbid solution was transferred to cap tube of the same type that was used to prepare the test and control inocula. It was then stored in the dark at room temperature (25^0C). Exactly 0.5 McFarland gives an equivalent approximate density of fungi 1.5 x 10^8 Colony Forming Units per ml (CFU) mL^{-1} (Stein *et al.,* 2005).

3.1.5 Crude Extraction of Camellia sinensis (Teas)

The prepared soluble granules of both black and green tea samples sealed in silver lined sachets stored at room temperature were obtained. Cold aqueous crude extracts were done by soaking weighed amount of dry soluble granules of tea (10 grammes) in 100 mL of sterile distilled water and shaken for half an hour in an electric shaker. The extracts were filtered using Whatman No.1 filter paper to exclude any suspending granules. Crude extract, supernatant, was then transferred to sterile screw cap bottles, labeled and stored under refrigerated condition (4^0C) until use.

For hot aqueous crude extraction, 10 grammes of dry soluble granules of tea samples were extracted with 100 mL of boiling water and filtered using sterile Whatman filter paper No.1 to give a solution that contains 100 mg/ml (Yam and Hamilton-Miller, 1997). Crude extract, filtrate, was then transferred to sterile screw cap bottles, labeled and stored under refrigerated condition (4^0C) until use.

The mixture aqueous crude extract for each tea was prepared by mixing (50 ml) fresh milk with (50 ml) water in the ratio of 1:1, in a 250 ml conical flask. 10 grammes of tea sample was then weighed and added to the contained conical flask and boiled for 20 minutes. The aqueous mixture tea extract obtained was approximately more in the strength of normal "cup of tea". The extracts were then filtered using sterile Whatman filter paper No.1 to exclude any suspending granules and filtrate of 100 mg/ml allowed to cool, then transferred to sterile screw cap bottles, labeled and stored under refrigerated condition (4^0C) until use. Only fresh extracts was used in the experiment, as marked chemical changes occurred when tea was allowed to stand (Spiro, 1995; Yam. *et al.,* 2002).

3.1.6 Preparation of tea extracts stock and working solutions

A two fold dilutions were obtained (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.5625 mg/ml) concentrations. Antifungal activities of the above concentrations were determined.

3.1.7 Preparations of antifungal compounds stock and working solutions

The antifungal compounds were removed from storage ($-20^{0}C$) and allowed to come to room temperature. Each 250 µg of antifungal compound (Fluconazole) was weighed and dissolved in sterile distilled water to make a final 10 mL solution.

The following formula was used;

$$W = \frac{1000 \text{ x } V \text{ x } C \ (\mu gmL^{-1}}{P \ (\mu gmL^{-1})}$$

Where P = potency given by manufactured in relation to base

C= Final concentrations of the solution

W = Weight of antifungal in mg dissolved in V

V- Volume required in ml

The stock solutions of azole group of compounds (Fluconazole) used was usually kept at $-20^{0}C$ until used. Doubling dilutions of stock solutions were made to obtain working solution.

3.2 Antimicrobial assay

The antimicrobial activities of the extracts were evaluated by the disc diffusion method (Muanza *et al*., 1994). The use of agar disc diffusion method to screen for antimicrobial activities of the crude tea extracts was done according to the National committee of clinical and laboratory standards (NCCLS, 2007) now CLSI.

The fungal inoculums for susceptibility test were standardized using barium sulphate standard equivalent to McFarland No 0.5, giving a cell density of 1.5 x 10^8 Colony Forming Units per ml (CFU/ml). Circular chromatographic paper discs (6 mm diameter) were prepared with the aid of an office paper perforator. The discs were placed in a Petri dish and sterilized in an autoclave. Dilutions of several concentrations of the crude tea extracts and azole group of compounds, Fluconazole, were then made in a test tube using sterile distilled water. Positive and negative standard controls were used.

Blank sterile paper discs measuring 6mm were impregnated with 20 µL of test concentration of crude tea extract. The discs were air dried and aseptically transferred into respective inoculated plates (Esiome *et al.*, 2006). Briefly, approximately 1.5 x 10^8 cells of freshly grown fungal suspension were uniformly spread in the sterile Muller-Hinton agar dishes using sterile cotton swabs. The discs with respective crude tea extract concentrations were aseptically placed on a Muller-Hinton agar plates to which the test fungi have been inoculated. The inoculated plates were incubated at 4ºC for at least 24 hours to allow the tea leaves liquors to diffuse into the media.

The cultures were then incubated for 72 hours at 35ºC and 30ºC for yeast and moulds respectively, before the activity was determined. The activities of the tea crude extracts were established by the presence zones of inhibition which were measured in mm. Fluconazole discs containing (25 µg) were used as antifungal reference standards. Similarly the sterile distilled water was set as negative controls.

Extracts with activity was serially diluted and re-tested to determine the minimum inhibitory concentrations (MIC). All the assays were carried out in triplicates, average result calculated and recorded against corresponding concentrations as described by Elgyayyar *et al*., (2001). Assays were subjected to quality control procedures recommended by clinical laboratory standard institute (CLSI). Fluconazole disc was prepared as described by (Klevay *et al*., 2005).

Minimum inhibitory concentrations were determined by Broth micro dilution method for the active crude extracts against test fungal organisms. The procedures were done as recommended by the National Committee for Clinical Laboratory Standards (NCCL) now Clinical Laboratory Standard Institute (CLSI) (Ferraro, 2003). The tests were performed in 96 well-micro-titer plates. Crude tea extracts were transferred into micro-titer plates to make serial dilutions ranging from 10^1, 10^2, 10^3 up to 10^{10}. The final volume in each well was 100 µl.

The wells were inoculated with 5 µl of microbial suspension. The yeasts were incubated at 35°C for 24 h while molds were incubated 30°C for 3-7 days in ambient air. The MIC was recorded as the lowest extract concentration demonstrating no visible growth as compared to the control broth turbidity (Michael *et al.*, 2003). Wells that were not inoculated were set to act as control. All the experiments were done in triplicates and average results were recorded.

3.3 Antifungal test concentration

A series of two fold dilutions was made using MIC as the starting concentration. These varying concentrations were combined with concentrates of tea crude extracts at 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.500 mg/ml, 6.250 mg/ml, 3.125 mg/ml, and 1.5625 mg/ml.

3.3.1 Preparation of Combined Test Concentrates

Concentrations of azoles' antifungal were made from manufacturer's instructions (Pfizer Company) to obtain doubling dilutions (25 ug/ml, 12.5 ug/ml, 6.250 ug/ml, 3.125 ug/ml, 1.5625 ug/ml, 0.780 ug/ml and 0.390 ug/ml) of minimum inhibitory concentrations of antifungal. These concentrations were then combined with concentrations each crude extract of teas (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.500 mg/ml, 6.250 mg/ml, 3.125 mg/ml, and 1.5625 mg/ml)). Sterile discs were impregnated with 20 uL of test combination of the respective concentration soaked and then air dried at room temperature.

3.3.2 Determination of combined activity of crude tea extracts and azole group of compounds (Fluconazole)

The corresponding concentrations of both tea extracts and antifungal compounds obtained from standard dose response curves were used to prepare stock solution of both tea and antifungal agents.

The stock solutions were then mixed in such a way that each mixture contained from zero parts of the antifungal agent and ten parts of tea to ten parts of antifungal agents to zero parts of tea. The discs were then prepared as described above and then placed on the seeded agar plates. Triplicates of these process were reproduced for each fungal strain/ species tested. After 24 hours at 4ºC pre- diffusion time interval, the plates were then incubated at 35ºC and 30^0C for yeast and moulds respectively for 24 to 72 hours , after which the inhibition zone diameters (IZDs) surrounding the disc were measured in mm.

3.4 Data analysis

The results from antifungal activity and synergistic activity of the test fungal organisms were expressed as the mean ± SD, student's t-test and ANOVA were used to determine the differences in antifungal and synergistic activity of crude tea extracts and Fluconazole antifungal agent among the test fungal organism. The corresponding drug mass of the target IZDs were obtained by linear regression analysis. Data obtained was analyzed by determining the confidence interval to ascertain if the differences in the mean diameters of the zones of inhibition are statistically significant. Probability limits were set as $P \leq 0.05$. Correlation was used to determine the relationship between antifungal activity and synergistic activity. All statistical analyses were done using the windows/SPSS version 11.5 package. Data was presented as pie charts, tables and / or graphs.

CHAPTER FOUR

4.0 RESULTS

4.1 *In-vitro* antifungal activities of Crude Extracts of green, black and mixture *Camellia sinensis*

The antifungal activities of green and black tea (*Camellia sinensis*) crude extracts having a concentration of 100 mg/ml of extracting solvent (sterile distilled water) and from the same tea mixture with milk (with ratio of milk to extracting solvent is 1:1) are presented in the tables below. Their inhibitory effects against selected pathogenic and mycotoxic fungi were then compared.

4.1.1 Antifungal activities of Crude Extracts on different Extraction treatments

The antifungal activities of black tea, green tea and mixture tea on selected yeasts and moulds are shown in table 4.1.

Table 4.1: Zones of inhibitions (mm) by crude tea extracts on the selected pathogenic and mycotoxigenic fungi

	Cold extract		Hot extract		Mixture extract	
FUNGI	**Black tea**	**Green tea**	**Black tea**	**Green tea**	**Black tea**	**Green tea**
Candida albicans ATCC 90028	6	6	6	6	6	6
Candida lusitaniae	8	18	10	20	10	16
Candida parapsilosis ATCC 22019	6	15	6	16	6	16
Candida glabrata ATCC 24433	6	6	6	6	6	6
Candida famata	12	22	18	22	10	15
Candida tropicalis ATCC 750	6	22	8	20	7	18
Candida krusei ATCC 6258	6	14	6	12	6	6
Cryptococcus neoformans ATCC 66031	6	17	6	17	6	15
Microsporu gypseum (clinical isolate)	9	17	10	15	7	12
Aspergillus niger (clinical isolate)	6	6	6	6	6	6
Fusarium monilliforme (clinical isolate)	12	6	13	6	12	6
Penicillium chrysogenium (Clinical isolate)	6	6	6	6	6	6
Trychophyton mentagrophytes (Clinical isolate.)	8	16	19	18	6	9

From the table 1 above, yeasts *C. albicans* ATCC 90028, *C. glabrata* ATCC 24433 and moulds *Penicillium chrysogenium* as well as *Aspergillus niger* showed no inhibition (6mm) in either of *Camellia sinensis* green and black crude extraction or in the different extraction mode, that is cold, hot and mixture. Green tea crude extract irrespective of extraction mode, showed greater antifungal activity for yeasts *Candida lusitaniae*, *C. parapsilosis* ATCC 22019, *C. famata, C. tropicalis* ATCC 750 and moulds *Microsporum*

gypseum and *Trichophyton mentagrophytes*. For *Candida krusei* ATCC 6258, the green tea both cold and hot crude extract showed greater activity compared to black tea crude extract except under mixture extraction mode. *Cryptococcus neoformans* ATCC 66031 was inhibited by green tea crude extracts under different treatments but resistant to black tea crude tea extracts. However, black tea crude extract on the other hand showed greater antifungal activity than green tea in all the different treatments of extraction. For *Trichophyton mentagrophytes*, under cold extraction, green tea elicited more activity than black tea crude extract but when subjected to hot extraction, black tea had greater zone of inhibition.

The samples were analyzed using paired sample T-test to establish the differences in zones of inhibition caused by black tea crude extracts from green tea crude extracts. The results revealed that there was a significant difference in zones of inhibitions ($T = 4.09$, $P < 0.05$). Zones of inhibition caused by green tea crude extracts (11.92 ± 0.99 mm) were higher than inhibition by black tea crude extracts (8.14 ± 0.56 mm).

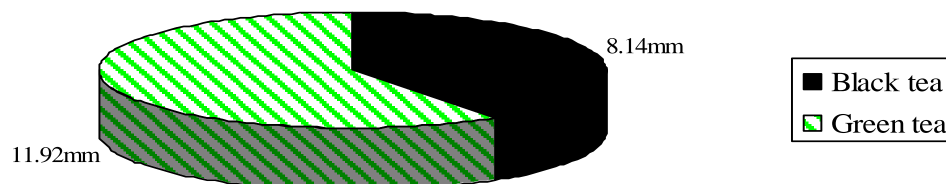


Figure 4.1: Mean zones of inhibition by green and black crude Tea Extracts

From the figure 4.1, the mean zone of inhibition by green tea crude extracts (11.92 mm) is greater as compared to inhibition by black crude tea extracts (8.14 mm).

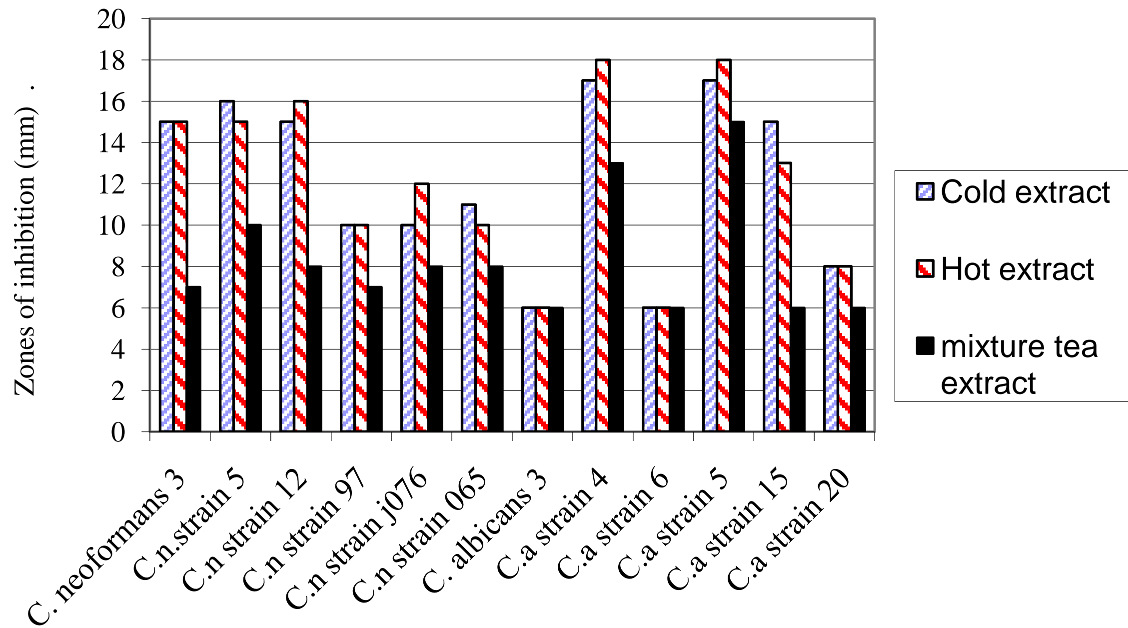
4.1.2 Zones of inhibition for clinical isolates

Fungal clinical isolates used were: *C. neoformans* strain 3, strain 5, strain 12, strain 97, strain J076 and strain 065. *Candida albicans* strain 3, strain 4, strain 6, strain 5, strain 15 and strain 20. Growth inhibitions of these clinical isolates by green tea crude extract of different extraction treatments (Cold, hot and mixture) were tested.

The result showed that at different treatments of green tea crude extract, there was a significant difference in inhibition of these clinical isolates ($F = 4.1$, $df = 2$, $P = 0.026$). The Mixture Green tea crude extract (mean zone of inhibition 8.33 ± 0.87 mm) was lower than Cold green tea crude extract (12.17 ± 1.20 mm) and hot green tea crude extract (12.25 ± 1.24 mm). The green tea crude extracts inhibition on the clinical isolates were as shown in figure 4.2 below.

The hot extract of green tea showed greater inhibition zone diameter as compared to cold or mixture tea extract. *Candida albicans* strain 4 and strain 5 among all the clinical isolates showed greatest susceptibility to antifungal activity of green tea extract with inhibition zone diameter of 18 mm. Among the *Cryptococcus neoformans* clinical isolates tested, *C. neoformans* strain 3, strain 5 and strain 12 showed susceptibility to antifungal activity of green tea extracts (cold, hot and mixture) with inhibition zone diameter ≥ 10.0 mm each.

This is moderately active as considered highest at 18.0 mm and least 6.0 mm. mixture tea extract showed the least activity in all the fungal organisms tested.



Key: *C.n = Cryptococcus neoformans; C.a = candida albicans*

Figure 4.2: Zones of inhibition of fungi clinical isolates by green tea

Growth inhibition of fungal clinical isolates by black tea crude extracts at different extraction treatments were not significantly different ($P > 0.05$). However mixture black tea crude extracts had the lowest sizes zones of inhibition (Mean 6.75 ± 0.66 mm) than cold black tea crude extracts (mean 8.25 ± 0.99 mm) and hot black tea crude extracts (mean 8.17 ± 0.94 mm).

The black tea crude extracts inhibition on the clinical isolates were as shown in figure 4.3.

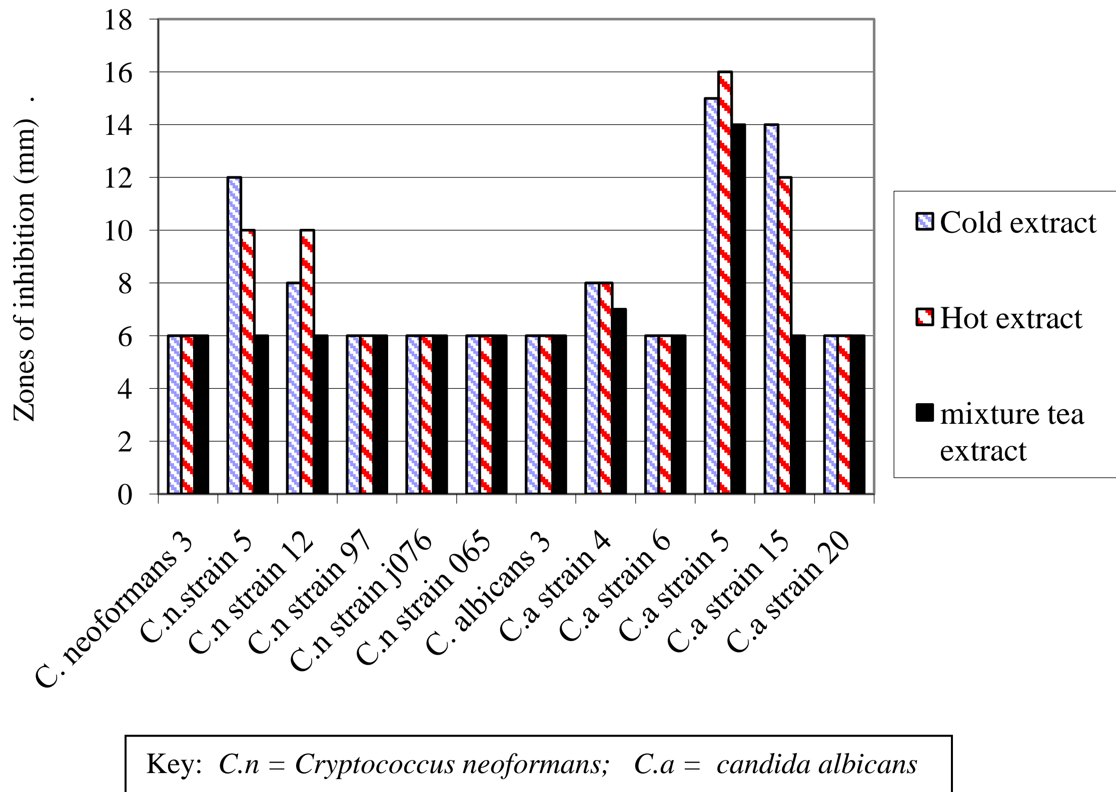


Figure 4:3 Zones of inhibition of fungi clinical isolates by black tea

From the figure 4.3 above, the hot black crude tea extract showed greatest activity only to *Candida albican* strain 5 with inhibition zone diameter of 16.0 mm as compared to cold extract.

All the clinical isolates showed resistance to activity by black tea crude extract (IZD 6.0 mm) irrespective of extraction mode; except *Cryptococcus neoformans* strain 5 and 12 and *Candida albicans* strain 4, 5 and 15 respectively which had activity. Mixture tea extract showed the least activity in all the fungal organisms tested.

Table 4.2: Zones of inhibitions of clinical isolates by green and black tea crude extracts of different extraction treatments

Tea crude Extracts	**Inhibition of clinical fungal isolates by the extracts**		
	Cold Extract	**Hot extract**	**Mixture tea**
Green tea	12.17 ± 1.20 mm	12.25 ± 1.24 mm	8.33 ± 0.87 mm
Black tea	8.25 ± 0.99 mm	8.17 ± 0.94 mm	6.75 ± 0.66 mm

Hot green tea crude extract showed greater inhibition (mean inhibition value 12.25 ± 1.24 mm) than cold or mixture crude extract. But for the black tea crude extract, the cold crude extract had greater inhibitory effect (mean inhibition value 8.25 ± 0.99 mm) as compared to other extraction treatments.

4.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the crude tea extracts both green and black under the different extraction treatments (cold and hot) was established. The MIC to the standard fungal test strains and clinical isolates was evaluated.

4.2.1 Minimum inhibitory concentration (MIC) to standard fungal test strains

Minimum inhibitory concentrations (MIC) of tea crude extracts to the fungal strains were established. Tested at 15 mm diameter of inhibitory zone diameter, the MIC of green cold, green hot and mixture tea crude extracts were recorded in g/ml.

Black hot tea crude extract was only tested against *C. famata* strain which was the only which showed inhibition activity (table 4.3 below).

Table 4.3: The Minimum Inhibition Concentration of tea crude extracts to the standard fungal test strains

	Tea crude extract			
Fungal test strain	**Green cold** (mg/ml)	**Green hot** (mg/ml)	**Black hot** (mg/ml)	**Green mixture** (mg/ml)
C. lusitaniae	25.00	25.00	-	6.250
C. famata	6.250	1.600	50.00	1.600
C. parapsilosis ATCC 22019	50.00	50.00	-	50.00
C. tropicalis ATCC 750	3.125	3.125	-	8.250
C. neoformans ATCC 66031	6.250	3.125	-	1.600
M. gypseum	6.250	6.250	-	50.00
T. mentagrophytes	3.125	3.125	-	6.250

NB: The black cold crude extracts and black mixture tea had no inhibitory activity

KEY: - No Inhibition activity

The MIC of the C*amellia sinensis* crude extracts which had inhibition diameters of 15 mm and above (significance activity) was determined. Green mixture tea crude extracts had the least minimum inhibition concentration at 1.6 mg/ml against *Cryptococcus neoformans* ATCC 66031 and *C. famata,* and highest MIC against yeast *C. parapsilosis* ATCC 22019 and mould *Microsporum gypseum*. The results show that cold green tea crude extract, at 3.125 mg/ml inhibited growth of C. *tropicalis* ATCC 750.

A concentration of 6.25 mg/ml inhibited growth of 50% of the fungal isolates tested; this therefore gives the MIC_{50} of the test fungi when using cold green tea crude extract. Hot green tea at 1.6 mg/ml inhibited the growth of *C. famata*. A concentration of 3.125 mg/ml inhibited growth of a 50% of the tested fungi; this gives the MIC_{50} of the test fungi when using hot green tea crude extract. Green mixture tea minimum inhibition concentration of 1.6 mg/ml was adequate to inhibit growth of *C. famata*. However, at a concentration of 6.25 mg/ml of mixture green tea, 50% of the tested fungi were inhibited in growth; this gives the MIC_{50} of the test fungi when using green mixture crude extract.

4.2.1.1 Minimum Inhibition Concentration (MIC) at 50% of cold green, hot green and Green mixture tea crude Extracts

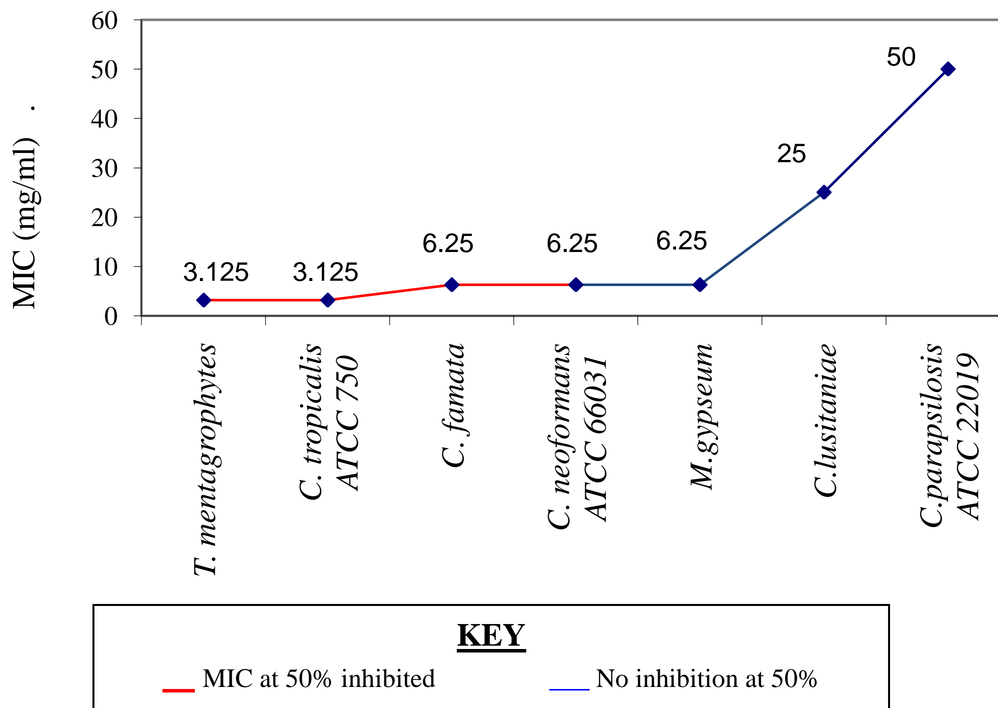


Figure 4.4a: MIC_{50} Inhibition by Cold Green Tea Crude Extract

The MIC_{50} under cold green tea crude extract includes: *T. mentagrophytes, C. tropicalis* ATCC 750*, C. famata* and *C. neoformans* ATCC 66031 respectively. At a concentration of 6.25 mg/mL, 50% of the fungal test strains were inhibited. However, *C. lusitaniae* and *C. parapsilosis* ATCC 22019 were inhibited at higher concentrations. From the above figure, dermatophyte *T. mentagrophytes* and yeast *C. tropicalis* ATCC 750 had the lowest MIC of 3.25 mg/m L.

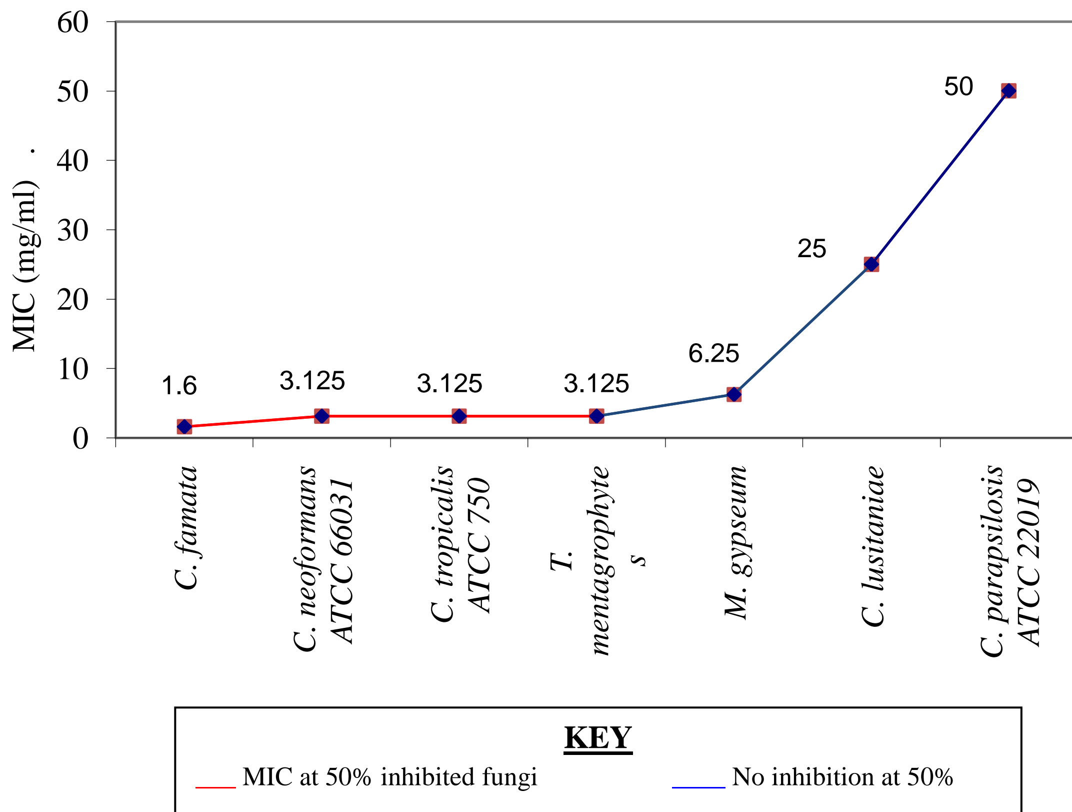


Figure 4.4b: MIC_{50} Inhibition by Hot Green Tea Crude Extract

The MIC_{50} under Hot green tea crude extract includes: *C. famata, C. neoformans* ATCC 66031, *C. tropicalis* ATCC 750 and *T. mentagrophytes* respectively. From the above figure, the lowest MIC was at 1.6 mg/mL against *C. famata.* At a concentration of 3.25 mg/mL, 50% for the test fungal strains were inhibited.

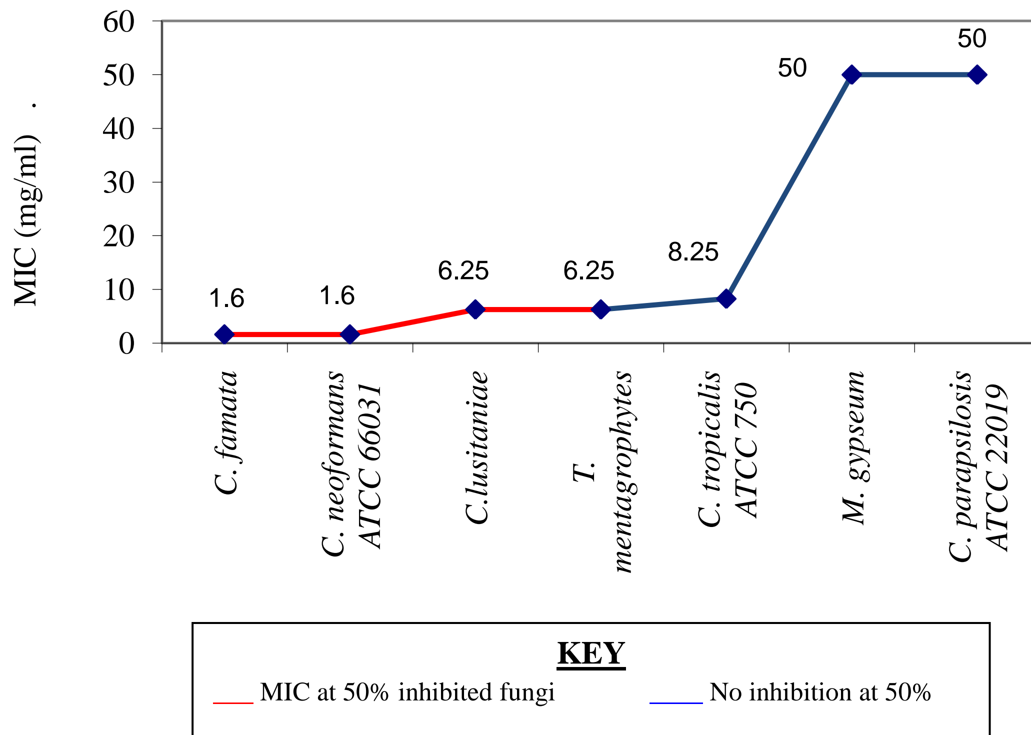


Figure 4.4c: MIC_{50} Inhibition by mixture Green Tea Crude Extract

The MIC_{50} under Mixture green tea crude extract includes: *C. famata, C. neoformans* ATCC 66031, C. *lusitaniae* and *T. mentagrophytes* respectively. From the figure 4.4c, the lowest MIC achieved was at 1.6mg/mL against *C. famata* and *C. neoformans* ATCC 66031 while at a concentration of 6.25 mg/mL, 50% of test fungal strains were inhibited including the yeast *C. lusitaniae* and dermatophyte *T. mentagrophyte*. This concentration gave the $MIC_{50.}$

4.2.1.2 Minimum Inhibition Concentration (MIC_{90}) of cold green, hot green and Green mixture tea crude Extracts

Cold green tea crude extract, at 25 mg/ml was adequate to inhibit growth of *C. lusitaniae*, while at a concentration of 25 mg/ml inhibited growth of 90% of the fungal isolates tested. At this concentration of cold green tea crude extract, only *C. parapsilosis* ATCC

22019 was not inhibited. Similarly, hot green tea crude extract at 25 mg/ml, inhibited growth of *C. lusitaniae*. This concentration of 25 mg/ml also inhibited growth of 90% of the tested fungi. At this concentration of hot green tea crude extract only *C. parapsilosis* ATCC 22019 was not inhibited. Green mixture tea minimum inhibition concentration of 50 mg/ml was adequate to inhibit growth of *Microsporum gypseum*. At this concentration of 50 mg/ml of mixture green tea, 90% of the tested fungi were inhibited, that is all fungi tested were inhibited.

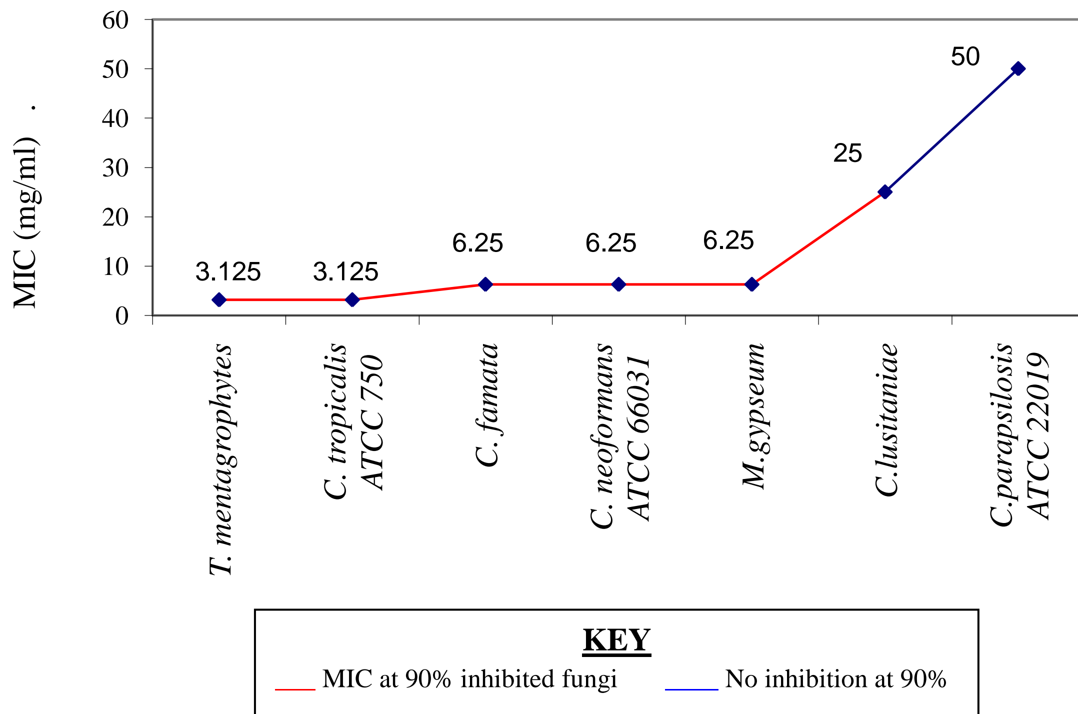


Figure 4.5a: Fungi growth inhibition by Cold green tea crude extract

The MIC_{90} under cold green tea crude extract includes: *T. mentagrophytes, C. tropicalis* ATCC 750*, C. famata, C. neoformans* ATCC 66031, *M. gypseum* and *C. lusitaniae* respectively.

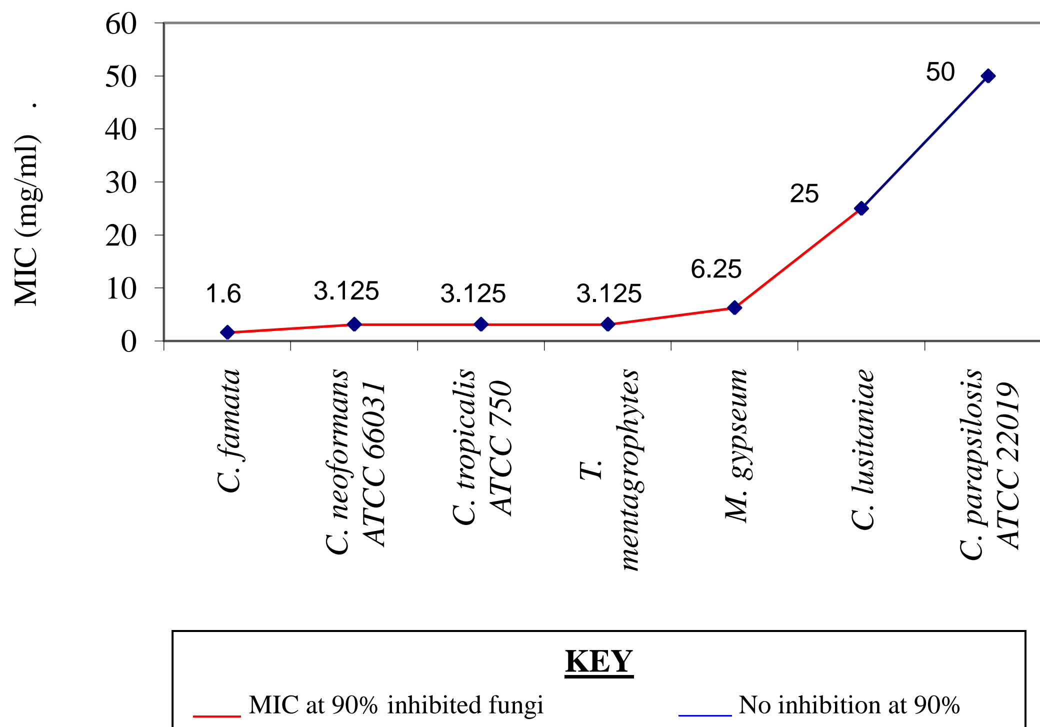


Figure 4.5b: Fungi growth inhibition by hot green tea crude extract

The MIC_{90} of Hot green tea crude extract includes for *C. famata, C. neoformans* ATCC 66031, C. *tropicalis* ATCC 750, *T. mentagrophytes, M. gypseum* and *C. lusitaniae* respectively was 1.6mg/ml, 3.125mg/ml, 3.125mg/ml, 3.125 mg/ml, 6.25mg/ml, 25mg/ml, 50mg/ml. From the above figure 4.5b, a concentration of 25mg/mL, 90% of the test fungal strains were inhibited. The yeast *C. parapsilosis* ATCC 22019 was the only fungal organism inhibited at much higher concentration of 50mg/mL.

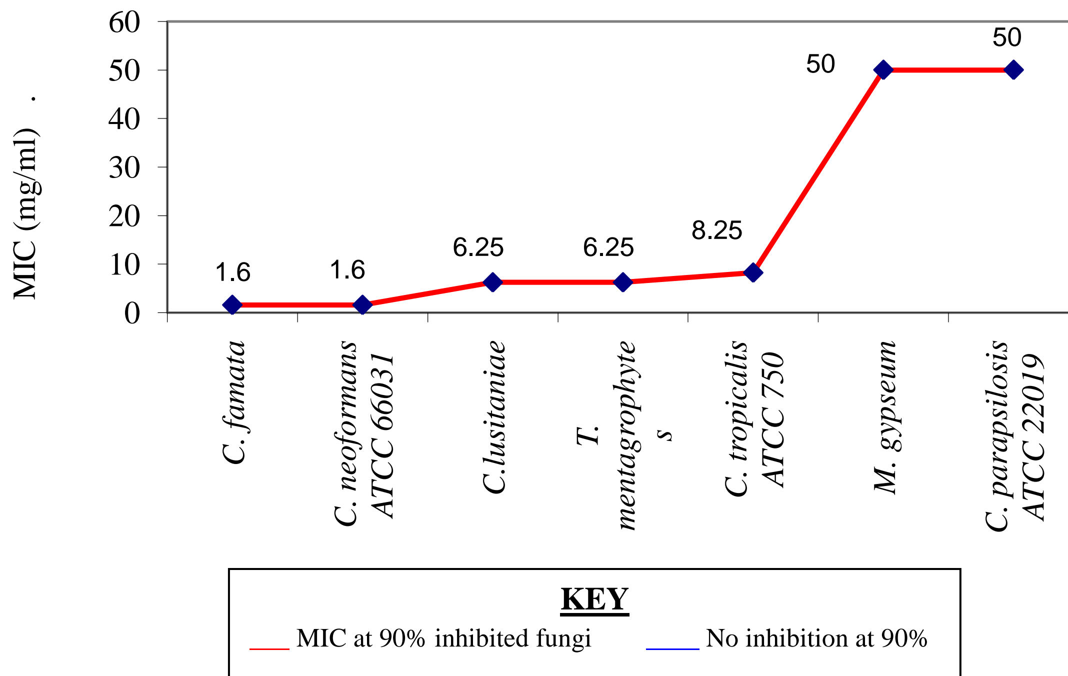


Figure 4.5c: Fungi growth inhibition by Mixture green tea crude extract

The MIC_{90} of Mixture green tea crude extracts for *C. famata, C. neoformans* ATCC 66031, *C. lusitaniae, T. mentagrophytes, C. tropicalis* ATCC 750*, M. gypseum* and *C. parapsilosis* ATCC 22019 respectively.

4.2.2 MIC for tea crude extracts to the fungal clinical isolates

When tea crude extracts were tested against fungal clinical isolates, the green tea crude extract mean 0.0165 ± 0.0139 mm had less zones of inhibition than black tea crude extract mean 0.0518 ± 0.0279 mm.

Table 4.4: The Minimum Inhibition Concentration of tea crude extract against clinical isolates

Clinical isolates	Green tea mg/ml	Black tea mg/ml
Cryptococcus neoformans 065	0. 78	-
Cryptococcus neoformans 3	1.5625	-
Cryptococcus neoformans 5	1.5625	100
Cryptococcus neoformans 12	3.125	-
Candida albicans 4	3.125	6.25
Candida albicans 5	100	0.1
Candida albicans 15	0.78	0.78

KEY: - No Inhibition activity

The MIC of the C*amellia sinensis* crude extracts which had inhibition diameters of 15 mm and above (significance activity) was determined. Green and black tea extracts had the least minimum inhibition concentration of 0.78 mg/ml against *Cryptococcus neoformans 065* and *Candida albicans* strain15 and the highest MIC against *Candida albicans* strain 5 for green tea and *C. neoformans* 5 against black tea. The results are as shown in table 4.4.

Hot green tea crude extract (mean MIC 12.25 mg mL^{-1}) had a higher MIC of fungi clinical isolates than cold green tea crude extract (Mean MIC 12.167 mg mL^{-1}) and mixture green tea crude extract (mean MIC 8.33 mg mL^{-1}).

Green tea crude extract had highest MIC on *C. albicans* strain 4 and strain 5 than other clinical isolates as shown in figure 4.6.

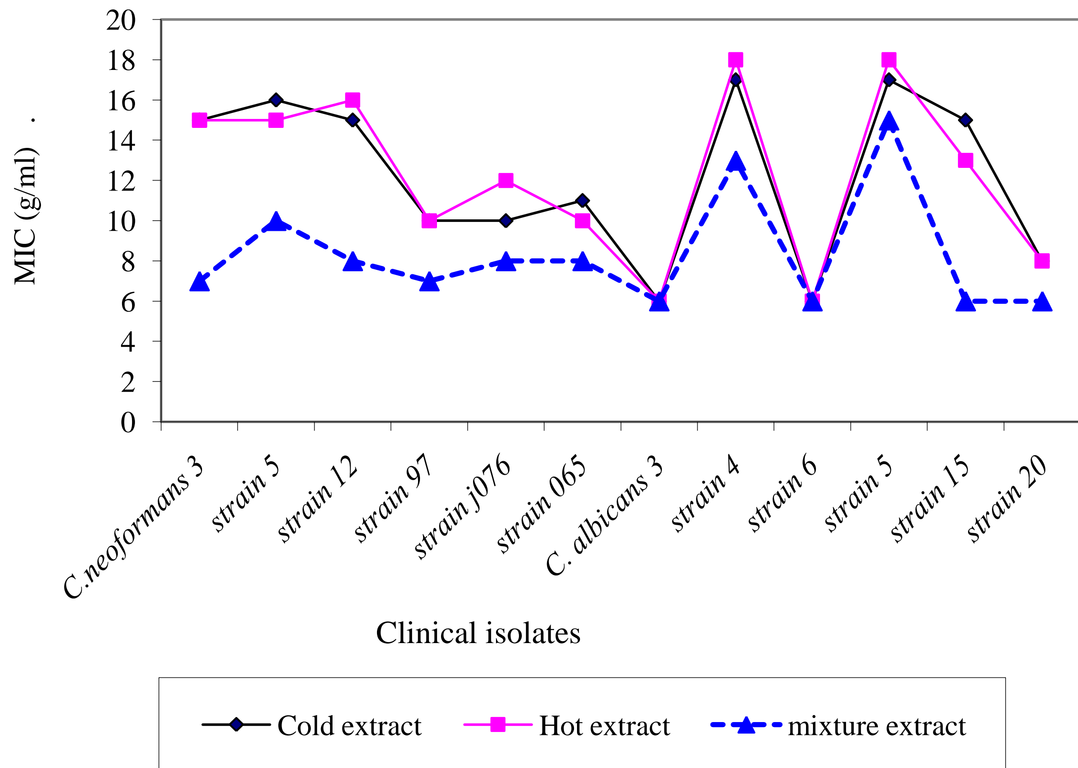


Figure 4.6: MIC of cold green, hot green and mixture tea crude extracts on clinical fungal isolates.

Cold black tea crude extracts (mean MIC 8.25 mg mL^{-1}) was found to have greater MIC of fungi clinical isolates than hot black tea crude extracts (Mean MIC 8.167 mg mL^{-1}) and mixture black tea crude extracts (mean MIC 6.75 mg mL^{-1}).

Black tea crude extract had the highest MIC on *C. albicans* strain 5 and strain 15 more than it had on other fungal clinical isolates.

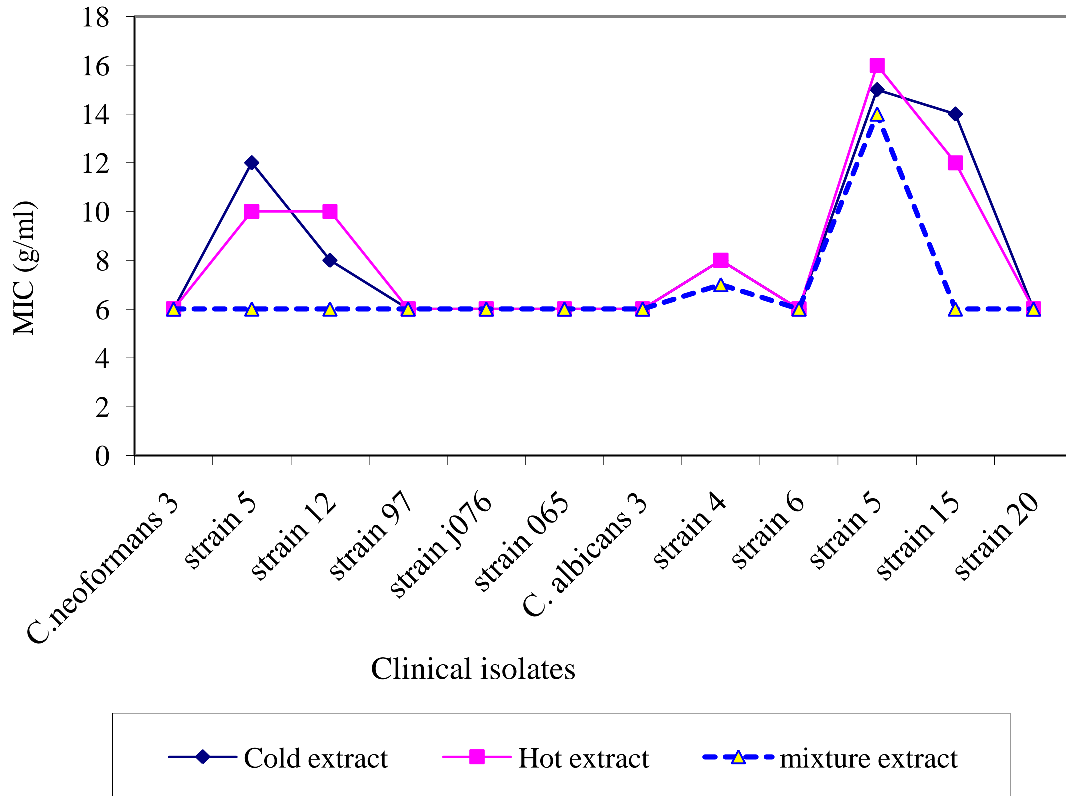


Figure 4.7: MIC of cold black tea, hot black tea and mixture tea crude extracts on fungal clinical isolates.

4.3 Synergism between crude extracts on Kenyan tea and conventional antifungal drugs on azoles resistant fungi

To establish synergism effect, fluconazole was mixed with tea crude extracts and zone of inhibition recorded. The findings shows that there was no significant difference in zones of inhibitions ($F = 0.90$, $df = 3$, $P = 0.455$). However, fluconazole alone (mean inhibition zone 20.00 ±1.29 mm) was less than tea crude extracts inhibition zones.

The highest inhibition zone recorded was noted in cold extract (22.86 ± 2.70 mm) as indicated in table 4.5

Table 4.5: Synergism between Crude Extracts on Camellia sinensis & Fluconazole

Fungi	Cold Extracts + F (mm)	Hot Extract +F (mm)	Mixture Extract +F (mm)	Fluconazole alone (mm) (positive control)
Candida albicans 4	26	20	20	22
Candida albicans 15	25	23	20	22
C.tropicalis ATCC 750	30	24	21	22
Cryptococcus neoformans	15	15	10	22
Cryptococcus neoformans	32	32	26	22
Fusarium moniliforme	15	15	12	18
Microsporum gypseum	17	16	16	18
Mean ± SE	22.86 ± 2.70	20.71± 2.35	17.86 2.10	20.00 ±1.29

Using a combination of tea crude extracts (Cold/hot) to Fluconazole mainly inhibited growth of *C. neoformans* 5, *C. tropicalis* ATCC 750 *and C. albicans 15.* Cold tea extract and hot tea extracts enhanced inhibition by Fluconazole whereas mixture teas tend to reduce inhibition by Fluconazole. In all the treatments, inhibition of C. *neoformans* 5 was high in all the extracts tested. The *camellia sinensis* crude extracts exhibited greater activity when combined with Fluconazole (enhanced inhibition zones) as compared to activity by Fluconazole alone. This shows synergism between the crude extracts and conventional antifungal drug, Fluconazole.

Both cold and hot crude extracts of *Camellia sinensis* exhibited greater synergistic activities as compared to mixture crude extracts. *C. neoformans* had the showed greatest synergism among the clinical isolates tested.

4.4 Effects on inhibition as a result of the differences in extraction temperatures

Three crude extracts were used in this experiment green (Cold, Hot and mixture tea extract) tested on *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 24433, *C. tropicalis* ATCC 750, *C. krusei* ATCC 6258 and clinical isolates *C. lusitaniae, C. famata, M. gypseum, F. monilliforme, P. chrysogenium and T. mentagrophytes* (Clinical isolate.)

Using a one way ANOVA to establish the effects of temperature on inhibition caused by tea crude extracts, the findings showed that; On Black tea; there was no significant difference on zones of inhibition exhibited by cold black tea crude extract (mean 7.58 ± 0.95 mm) from hot black tea crude extract (mean 9.5 ± 0.95 mm) and mixture tea crude extract (7.33 ± 0.95 mm). However, this result showed that black tea crude extract is more effective in fungal inhibition when hot crude extract is used.

On Green tea, there was no significant difference on zones of inhibition caused by cold green tea crude extract (12.75 ± 1.73 mm) from hot green tea crude extract (12.83 ± 1.73 mm) and mixture green tea crude extract (10.17 ± 1.73 mm). Green tea crude extract was therefore more effective in inhibition of fungal growth when used as a hot crude extract.

Using t-test to establish any variation in zones of inhibition in green tea crude extract, the results showed that there was no significant difference in zones of inhibitions. Hot Green tea crude extract (mean inhibition 12.83 ± 1.73 mm) was however higher than cold green tea crude extract zone of inhibition (12.75 ± 1.73 mm).

4.4.1 Effect of Temperature and Addition of Milk to Crude Extracts

To test for effect of temperature on MIC of green tea crude extract, a pair sample T-test was used to compare the MIC values of cold green tea to hot green tea crude extracts. The result showed that there was no significant difference in MIC ($t = 1.51$, $P = 0.182$). Mean MIC of hot green tea crude extract (0.0143 ± 0.007 mm) was slightly higher than Mean MIC of cold green tea crude extract (0.013 ± 0.007 mm). MIC of mixture tea crude extract (mean 0.017 ± 0.008 mm) was higher than both cold green and hot green tea. Minimum fungicidal concentration (MFC) of hot green tea was tested on *C. tropicalis* ATCC 750, *C. neoformans* ATCC 66031, *C. lusitaniae, C. famata and C. parapsilosis* ATCC 22019.

Table 4.6: Minimum fungicidal concentration of Green hot tea extract

Fungi	**Green hot** (mg/ml)
C. tropicalis ATCC 750	25
C. neoformans ATCC 66031	50
C. lusitaniae	100
C. famata	50
C. parapsilosis ATCC 22019	25

Green hot tea at 25 mg/ml was effective enough to kill *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019 alone. At a concentration of 50 mg/ml green hot tea was fungicidal to *Cryptococcus neoformans* ATCC 66031 and *C. famata.* At 100 mg/ml concentration of hot green tea all the tested fungi including C. *lusitaniae* were killed by the tea extract.

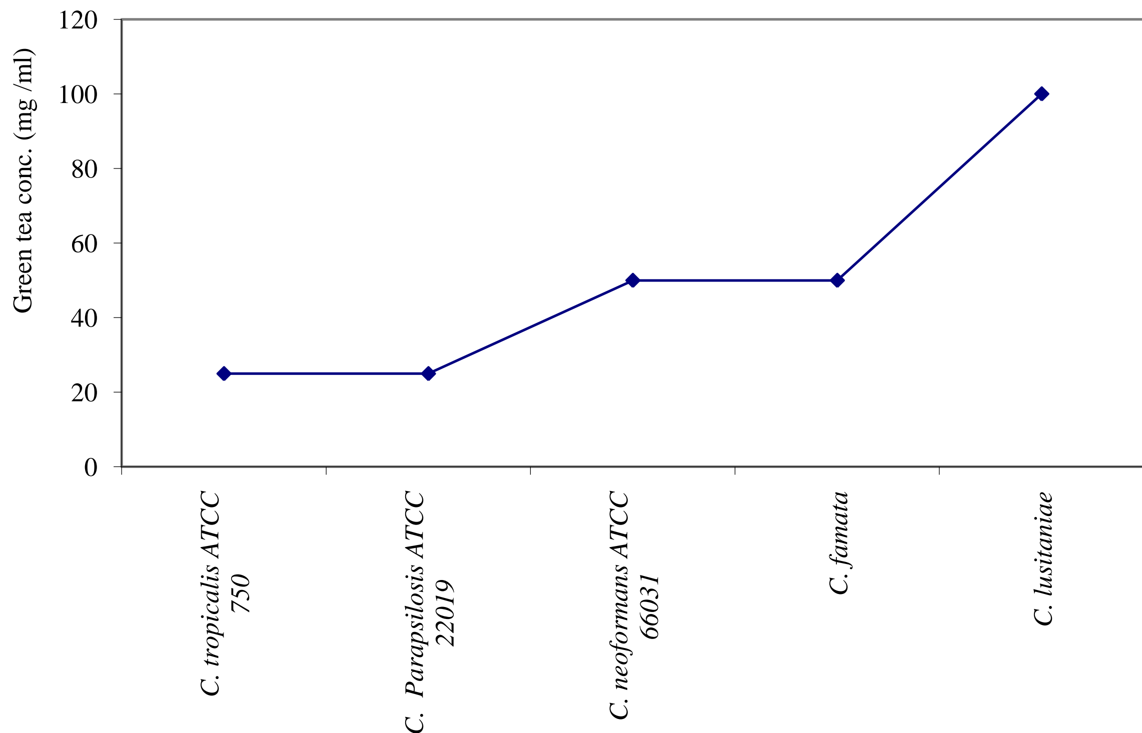


Figure 4.8: Minimum Fungicidal Concentration of Hot Green tea crude extract

Levels at which hot green tea were fungistatic to the organisms and the levels to which they were fungicidal to the organisms tested showed that at minimal concentration of 1.5 mg/ml. *C. neoformans* ATCC 66031 was made static and could not grow.

When the concentration of hot green tea reached 25 mg/ml, all the tested fungi including *C. famata* were static in growth and *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019 were already not able to survive at this concentration.

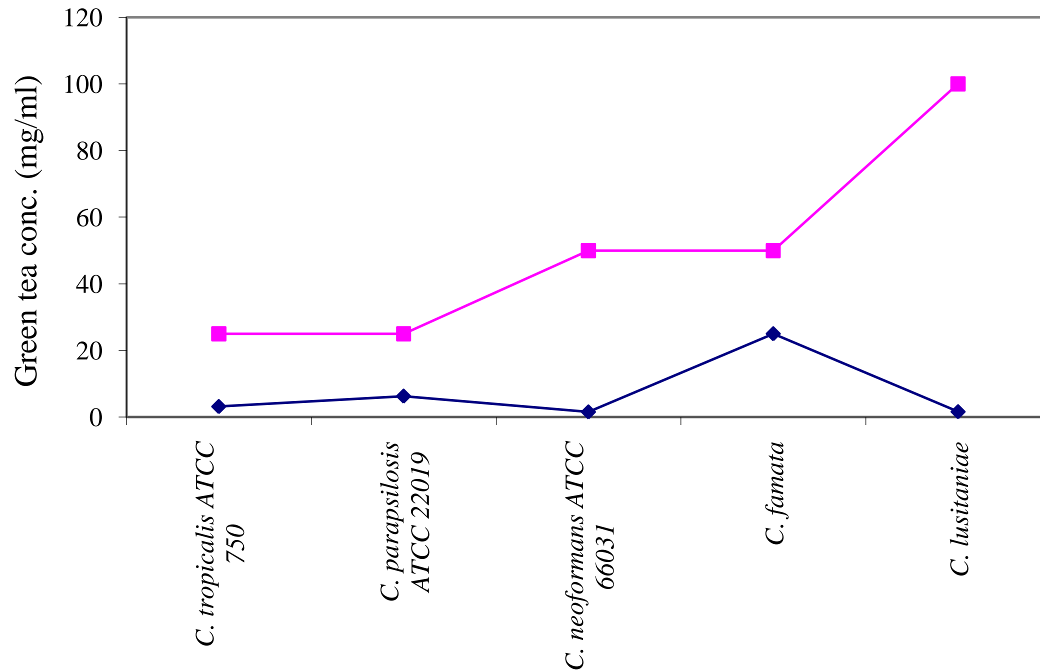


Figure 4.9: Levels at which Green Tea Crude Extracts were Fungistatic and Fungicidal

Similarly, minimum fungistatic concentration (MFC) of hot green tea and mixture tea was tested on *C. tropicalis* ATCC 750, *C. neoformans* ATCC 66031, *C. lusitaniae, C. famata and C. parapsilosis* ATCC 22019 fungi.

Table 4.7: Minimum fungicidal concentration of hot green tea and mixture green tea extract

Fungi	**Green hot** (mg/ml)	**mixture extracts** (mg/ml)
C. tropicalis ATCC 750	3.12	6.25
C. neoformans ATCC 66031	1.50	6.25
C. lusitaniae	1.56	3.12
C. famata	25.00	50.00
C. parapsilosis ATCC22019	6.25	8.25

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

According to world health report of infectious diseases (2000), overcoming antimicrobial resistance is the major issue of WHO for the next millennium. Hence, the last decade witnessed an increase in the investigation of plants as a source of human disease management. In the present study, the crude extracts of *Camellia sinensis* (green and black) produced inhibitory activity against pathogenic and mycotoxigenic fungi. The water crude extraction produced yields enough for the experimental study and is the most commonly used and cost effective method of tea preparation. The choice for water extraction was due to the fact that water is very polar than organic solvents hence it is able to extract more polar compounds from a plant material. Kigondu (2007) also found that water extracts produced the highest yields as compared to organic solvents.

The results obtained in this study indicate a considerable difference in antifungal activity of antimycotic activity of *Camellia sinensis* green and black crude extracts. For all the yeasts tested, *C. famata* was the most sensitive fungus to all the crude extracts (table 4.1). In cold extraction mode, cold green tea crude extracts showed greater antifungal activity as compared to cold black tea crude extracts. Yeasts *C. famata, C. tropicalis* ATCC 750*, C. lusitaniae, C. neoformans* ATCC 66031*, C. parapsilosis* ATCC 22019 and moulds *Microsporum gypseum* and *Trichophyton mentagrophytes* showed greater inhibition zone diameter of ≥ 15 mm. *C. krusei* ATCC 6258 was moderately active with IZD of 14 mm

while yeasts *C. albicans* ATCC 90028, *C. glabrata* ATCC 24433 and moulds *P. chrysogeneum* and *F. moniliforme* were resistant with IZD 6 mm. This conform to earlier studies that extracts of green tea have been reported to be more effective in inhibiting bacterial growth than black tea (Tiwari *et al.*, 2005).

The yeasts *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 24433, *C. tropicalis* ATCC 750, *C. krusei* ATCC 6258, *C. neoformans* ATCC 66031 and moulds *A. niger* as well as *P. chrysogeneum* were resistant to cold black tea extract with IZD 6 mm. However, *C. lusitaniae, C. famata, M. gypseum, F. moniliforme* and *T. mentagrophytes* were moderately inhibited (table 4.1). Okubo *et al*., 1991 reported that 2.5% of black tea extract completely inhibited the growth of *T. mentagrophytes* and *T. rubrum*; however, even at 10% concentration, this extract did not inhibit the growth of *C. albicans* or *Cryptococcus* (*filobasidiella*) *neoformans*.

Similarly, for hot water crude extraction mode, the hot green tea crude extract had greater inhibitory effects. From the study, *C. famata*, *C. lusitaniae*, *C. tropicalis* ATCC 750 and *T. mentagrophytes* were more sensitive to the extract with IZD ≥15 mm. However, yeasts *C. albicans* ATCC 90028, *C. glabrata* ATCC 24433 and moulds *A. niger, F. moniliforme* and *P. chrysogeneum* were resistant with IZD 6 mm. *C. parapsilosis* ATCC 22019, *C. neoformans* ATCC 66031, *C. krusei* ATCC 6258 and *M. gypseum* were moderately active with IZD of ≥ 10 mm.

The black tea hot crude extract showed the least number of fungal test organisms having activity. *C. famata* and *T. mentagrophytes* were the most susceptible while *C. lusitaniae*, *M. gypseum* and *T. mentagrophytes* were moderately active. The yeasts *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 24433, *C. krusei* ATCC 6258, *C. neoformans* ATCC 66031 and moulds *A. niger* as well as *P. chrysogeneum* were resistant with no activity. The results from the present study revealed that there was a significant difference in zones of inhibitions ($T=4.09$, $P<0.05$).

Zone of inhibition caused by green tea crude extracts (11.92±0.99) mm were higher than inhibition by black tea crude extracts (8.14±0.56) mm (figure 4.1). *Candida albicans* strain 4 and strain 5 among all the clinical isolates had greatest susceptibility to antifungal activity of green tea extract with IZD of 18mm (figure 4.2). *C. neoformans* strain 3, strain 5 and strain 12 showed susceptibility to antifungal activity of green tea extracts (cold and hot) with IZD ≥ 10.0 mm each. This is moderately active as considered highest at 18.0mm and least 6.0 mm. These findings are in line with Bii *et al*., (2010) which indicates that the lowest activity was at 7.0 mm and the highest was at 18.0 mm in diameter.

The solvents used for extraction (sterile distilled) were used as negative control while Fluconazole were used as positive control. The standard drug inhibition zone diameter was between 18 and 22 mm which compared well with that of the *Camellia sinensis* crude extract.

When the antifungal activities of antimycotics of these *Camellia sinensis* crude extract (green and black) were compared to that of control, green tea crude extract at a concentration of 100 mg mL-1 was found to have almost comparable activity to standard Fluconazole against yeast *C. famata* and *C. tropicalis* ATCC 750 and mould *T. mentagrophyte.* Similarly, black tea crude extract at concentration of 100 mg mL-1 showed comparable activity against the fungi yeast *C. famata* and mould *T. mentagrophyte* to that of Fluconazole. Although different treatments of crude extraction (both cold and hot water extracts) of *Camellia sinensis* produced inhibitory actions, hot water extracts showed more inhibitory effects than cold water extracts. This tends to show that active ingredients in tea were better extracted with hot water. Significantly higher yields of hot water than cold water extraction of green tea has been reported (Lin *et al*., 2008 and Su *et al.*, 2006).

The green tea has shown higher antimicrobial activity than black tea. This difference in results is probably due to presence of different contents of active substances in the teas (Wang *et al.,* 1992). Several studies have shown that the antimicrobial property is due to presence of polyphenols. Specific antioxidant polyphenols called catechins play an important role in green tea's inhibition of microbial growth. Several significant catechins include: EGCG, EGC, ECG, EC and GCG (Isogai *et al*., 2001). Antimicrobial activities of tea extracts are very selective. This difference in their activity depends upon the concentration and type of the extracts. These effects may also differ depending on (microbe) fungal species so that they may be either growth inhibitory or stimulatory (Tiwari *et al*., 2005).

Green tea and black tea crude extracts tested in current study have also shown varying activities against fungal organisms. Hirasawa *et al*., (2003) showed that the actions of catechins ECGG, EGC were fungicidal. Studies of the antibacterial activity of catechins against phytopathogenic bacteria showed similar results to those against *C. albicans* (Fukai *et al*., 1991). Catechins are known to have an affinity for proteins; this is clearly shown by a decrease in antibacterial activity of tea (Yam *et al.*, 1997). This property is referred to as "astringency" contributes to the sensation known as "mouth feel" experienced when drinking tea. The mode of action involves inducing rapid leakage of small molecules entrapped in the intraliposomal space and aggregation of the liposomes (Ikigai *et al*., 1993). Toyoshima *et al*., (1993) examined the mechanism of the effects of green tea catechins on *T. mentagrophytes* using electron microscopy and suggested that catechins attacked the cell membrane and caused lysis of the conidia and hyphae.

Catechins dimer theaflavins and its gallates play a part in black tea (Yam *et al.,* 1997) for antifungal activity. Evidence has recently emerged, however, to suggest that these molecules have the capacity to modulate the physical structure of cell membranes. Thus, a number of membrane dependent cellular processes, such as cell signaling and cell cycle (Chung *et al.*, 1999), arachidonic acid metabolism and cell proliferation (Agarwal *et al.,* 1992), and apoptosis and mitochondrial functionality (Spencer *et al.*, 2001) may be influenced by interaction of catechins with cellular phospholipid palisade (Caturla *et al.*, 2003). The resistant (least susceptible) fungal strains of clinical isolate *Cryptococcus neoformans* strain 3, strain 97, strain j076 and strain 065 (figure 4.3) was most probably because of the presence of mucopolysaccharide capsule.

The polysaccharide capsular material in some of the pathogenic microorganisms is responsible for virulence and antimicrobial resistance (Hooper, 2001). The *Candida* species such as *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 24433, *Candida parapsilosis* ATCC 22019 that showed less susceptibility to antimycotics of *Camellia sinensis* crude extract could be due to their outer membrane consisting of chitin binding proteins thus able to regulate the access of antifungal properties into the underlying structures. *Candida* species expresses multidrug efflux transporter (MET), which mediates the efflux of a broad range of compounds including antifungal agents (Marchetti *et al.*, 2000). But in this study, we found contradicting results among the *Candida* strains clinical isolates (figure 4.2 and figure 4.3). *C. albican*s strain 3, strain 6 and strain 20 showed least or no activity whereas strain 4, strain 5 and strain 15 had activity. The disparity in findings could be due to differences in strains of fungi used and susceptibility to antifungal drugs.

The preliminary screening assays for antifungal activity can largely be considered as qualitative assays and are used for identifying the presence or absence of bioactive constituents in the extracts. However, these methods of assays offer little information on these compounds. The minimum inhibition concentration (MIC) is a quantitative assay and provides more information on the potency of the compounds present in the extracts. Thus, the MIC values of the crude extracts of *Camellia sinensis* which had inhibition zone diameter of 15 mm and above was determined so as to demonstrate the potency of the extracts against the selected strains of fungi. The least the MIC the better the *Camellia sinensis* crude extract against the isolate in question.

The cold green tea crude extract had the least minimum inhibition of 3.125 mg mL^{-1} against yeast *C. tropicalis* ATCC 750 and mould *T. mentagrophyte* and the highest MIC against *C. parapsilosis* ATCC 22019 of 50 mg mL^{-1} (table 4.4). The MIC^{50} of the test fungi using cold green tea crude extract was 6.25 mg mL^{-1} (figure 4.4a), while at concentration of 25 mg mL^{-1}, it was the least minimum inhibition concentration that inhibited 90% of the fungal isolates tested, MIC^{90} (figure 4.5a). The hot green tea crude extract had the least MIC of 1.6 mg mL^{-1} against *C. famata* (table 4.4) and the highest MIC of 50 mg mL^{-1} against *C. parapsilosis* ATCC 22019. At a concentration of 3.125 mg mL^{-1}, 50% of the fungal isolates tested were inhibited, giving MIC^{50} (figure 4.4b), while a concentration of 25 mg mL^{-1} inhibited 90% of the test fungal isolates giving MIC^{90} (figure 4.5b). When the green tea crude extract was mixture with milk at a ratio of 1:1, the MIC was established to be 1.6mg mL^{-1} against *C. famata* and same concentration formed the MIC^{50} (figure 4.4c); whereas, at a concentration of 50 mg mL^{-1} ,90% of the fungal isolates tested were inhibited. At this concentration of mixture green tea crude extract, all the fungi tested were inhibited (figure 4.5c).

From figure 4.6 above, hot green tea crude extract (mean MIC 12.25 mg mL^{-1}) had a higher MIC on of fungi clinical isolates than cold green tea crude extract (Mean MIC 12.167 mg mL^{-1}) and mixture green tea crude extract (mean MIC 8.33 mg mL^{-1}). Green tea crude extract had greatest MIC on *C. albicans* strain 4 and strain 5 and *C. neoformans* strain 12 than other clinical isolates. Contrary to green tea crude extracts on clinical isolates strains, Cold black tea crude extracts (mean MIC 8.25 mg mL^{-1}) was found to have greater MIC of fungi clinical isolates than hot black tea crude extracts (Mean MIC

8.167 mg mL^{-1}) and mixture black tea crude extracts (mean MIC 6.75 mg mL^{-1}). Black tea crude extract thus had highest MIC on *C. albicans* strain 5 and *C. neoformans* strain 5 more than other fungal clinical isolates (figure 4.7).

Generally, the MIC of the *Camellia sinensis* crude extracts were as high as 50 mg mL^{-1} as compared to the standard drugs which is 0.5 mg mL^{-1} for yeasts and 1.0 mg mL^{-1} for dermatophytes at 95% confidence interval (P=0.05 level of significance). Although this was significantly lower than that of Fluconazole ($P<0.01$), the extracts are promising since they are crude extracts compared to pure compound of Fluconazole. This is a clear indication that the active ingredient is present in low quantities which necessitate the use of large amounts of crude extracts to gain the desired therapeutic effects. The difference in bioactivities of green and black tea crude extracts could be attributed to the facts that plants differ phytochemically and the extraction procedures also affect/alter their composition (cold and hot water extraction).

Absence of bioactivity does not warrant disapproval of ethno botanical utilization of the *Camellia sinensis,* simply because it may suggest that the extracts are acting in an indirect way where active ingredient exists as a precursor requiring activation *in vivo*. The present study also showed synergic antifungal activity of the combination of tea crude extracts and antimycotic, Fluconazole against tested fungal isolates. Since the arrival of azole antifungal agents as first-line drugs (Edwards 1997), Fluconazole-resistant *C. albicans* has begun to appear (Denning *et al.*, 1997).

From the present study, the combination of black tea crude extracts with Fluconazole enhanced growth inhibition (IZD 26 mm) against clinical isolate *C. albican* strain 4 (figure 4.8) compared to activity by Fluconazole alone (22 mm) or crude extract alone (figure 4.3). Similar studies have been reported by Hirasawa *et al.*, 2003, on the combined use of EGCG and Fluconazole was effective against Fluconazole resistant *C. albicans*. When cold crude tea extracts were combined with Fluconazole, fungal isolates yeast *C. albicans* strain 4, strain 5, NAC *C. tropicalis* ATCC 750 and *C. neoformans* strain 5 showed synergy evidenced by enhanced IZD compared to Fluconazole alone. Among the dermatophytes, *M. gypseum* showed enhanced inhibition (synergy) activity under combination of crude extract and Fluconazole alone (figure 4.8). The present study findings established that using a mixture of tea crude extracts (cold/hot) to Fluconazole, mainly inhibited growth of *C. neoformans* strain 5, *C. tropicalis* ATCC 750, *C. albican* strain 4 and *C. albican* strain 15.

Cold tea extract and hot tea crude extracts enhanced inhibition by Fluconazole where as mixture teas tend to reduce inhibition by Fluconazole. The findings further established there was no significant difference in zone of inhibition (F=0.09, d.f=3, P=0.455). However, Fluconazole alone (mean inhibition zone 20.00±1.29 mm, table 6) was less than tea crude extracts tested, greatest synergism. Similar findings of synergism have been previously reported. Combinations of green tea with butylated hydroxyanisole were more effective against bacteria and fungi than green tea alone (Simonetti *et al*., 2004). Also combination of various teas with antibiotics such as gentamicin and nalidixic acid acted synergistically against *Shigella dysentriae* (Tiwari *et al.*, 2005).

More detailed studies by Hirasawa and Takada 2004 revealed that ECGC enhanced the antifungal activity of the drug Amphotericin B; and the combined use of ECG and antifungal drug Fluconazole inhibited Fluconazole-resistant strains of this fungus. It is suggestive to have converted Fluconazole resistant phenotypes to sensitive ones. Earlier studies showed that ECG converted a Methicillin-resistant phenotype to a Methicillin-sensitive one. EGCG synergizes the activity of β-lactam antibiotics against *S.aureus* by binding to the peptidoglycan component of the bacterial cell wall (Zhao *et al.*, 2001). The wide ranging effects that catechins gallates have on modulation of drug resistance has recently been emphasized by the novel observation that sub-inhibitory concentrations of EGCG are able to reverse resistance by inhibition of efflux pump, in addition to further sensitizing susceptible isolates to antibiotic (Roccaro *et al.,* 2004).

These cited beneficial effects of combinations of flavonoids and medicinal antibiotics suggested the synergism effect observed with Fluconazole could be acting in the same manner for fungi as with the bacteria inhibited. These results suggest that the combined use of catechins in aqueous extract (infusions) and antifungal drugs may be useful in the treatment of Mycotic infections. In terms of effects of inhibition as a result of difference in extraction temperatures, the present study revealed that black tea crude extract was more effective in fungal inhibition when hot crude extract was used (mean 9.5±0.95 mm) compared to cold crude extract (mean 7.58±0.95 mm). Similarly, green tea crude extracts also showed more effective inhibition of fungal growth when used as a hot crude extract (mean 12.83±1.73 mm) compared to cold extract (12.75±1.73 mm).

Several studies have shown that water temperature is an important factor when extracting tea. Significantly higher yields of hot water than cold water extraction of green tea reported (Lin *et al*., 2008 and Su *et al*., 2006). For green and black teas, extraction with water at 100^0C for 3 minutes yielded higher total flavanol content than extraction with water at 60^0C & 80^0C (Horžič *et al*., 2009). It has been reported that higher temperatures reduces the polarity of water, thus increasing its extraction efficiency and capability to dissolve polar compounds (Hassas-Roudsari *et al*., 2009). Raising the temperature of water also reduces its surface tension and viscosity, which increases the diffusion rate and the rate of mass transfer during extraction. The mean MIC of hot green tea crude extract 0.0143±0.007 mm was slightly higher than the mean MIC of cold green tea crude extract (0.03±0.007 mm).

The Minimum Fungicidal Concentration (MFC) of hot green tea crude extract was tested on *C. tropicalis* ATCC 750, *C. neoformans* ATCC 66031, *C. lusitaniae*, *C. famata* and *C. parapsilosis* ATCC 22019 fungi. The findings revealed that green tea hot crude extract at 25 mg mL^{-1} was effective enough to kill *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019 alone. At a concentration of 50 mg/ml green hot tea was fungicidal for *Cryptococcus neoformans* ATCC 66031 and *C. famata.* At 100 mg/ml concentration of hot green tea all the tested fungi including *C. lusitaniae* were killed by the tea extract (figure 9). Levels at which hot green tea were fungistatic to the organisms and the levels to which they were fungicidal to the organisms tested showed that at minimal concentration of 1.5 mg/ml *C. neoformans* ATCC 66031 was made static and could not grow.

When the concentration of hot green tea reached 25 mg/ml, all the tested fungi including *C. famata* were static in growth and *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019 were already not able to survive at this concentration (figure 4.9). Similarly, minimum fungistatic concentration (MFC) of hot green tea and mixture tea was tested on *C. tropicalis* ATCC 750*, C. neoformans* ATCC 66031*, C. lusitaniae, C. famata and C. parapsilosis* ATCC 22019 fungi. When green tea crude extract was mixture with milk, the mixture crude extract at a concentration of 3.12 $mgmL^{-1}$was fungicidal to *C. lusitaniae* but fungistatic to other fungal isolates tested. But at concentration of 6.2 $mgmL^{-1}$ was fungicidal to *C. tropicalis* ATCC 750 and *C. neoformans* ATCC 66031; while at 8.25 $mgmL^{-1}$ was fungcidal to *C. parapsilosis* ATCC 22019 but fungistatic to *C. famata*. The MFC of *C. famata* was 50 mg mL^{-1}.

Generally, the MFC of mixture crude extracts were slightly higher as compared to that of green tea crude extract (table 4.7). These results are suggestive that addition of milk to blend the crude extracts altered the bioactive ingredients resulting to higher concentration for its MFC as compared to crude extracts alone. These results conform to previous studies by Wachira *et al.,* 2011 describing milk known to decrease antioxidant activity of *Camellia sinensis*. The mechanistic aspect of fungicidal brought about by tea crude extracts is suggestive to be due to catechins and gallates. The bioactive ingredients in crude tea extracts binds to ergosterol, one of the cell membrane sterols, and damages the cell membrane directly, leading to fungicidal activity against fungi. Catechins regulate expression of the gene(s) coding for Cytochrome P_{450}.

Detailed physiochemical studies suggest that fungicidal activities of galloylated tea catechins at the cell membrane level may be due to their specific perturbations of ordered structure of chitin binding proteins, a nitrogen containing polysaccharide constituting fungal cell wall. Differential effects of catechins on fungal cell walls compared to membrane of human cells may be due to differences in structures of the respective walls (membranes). The fungicidal action of EGCG may depend on hydrogen peroxide derived from the reaction EGCG with oxygen (Prooxidative activity) (Arakawa *et al*., 2004). These observations suggest that antifungal activity of antimycotic effect seem to arise from the interactions of catechins in crude extract with oxygen, genes, cell membranes and enzymes. This aspect merits further study. This predominantly *in vitro* information has ramifications for Mycotic disease prevention in humans.

5.2 CONCLUSION

1. The *Camellia sinensis* crude extract possess antifungal activity. In the present study, the crude extracts of *Camellia sinensis* (green and black) produced inhibitory actions against the fungal test strains.
2. Hot aqueous green tea crude extracts showed greater antifungal activity than mthe black hot tea crude extracts. Zones of inhibition caused by green tea crude extracts were higher than inhibitory by black tea crude extracts. This showed that bioactive ingredients in *Camellia sinensis* are better extracted in hot aqueous mode.

3. For all the yeasts tested, *C. famata* was the most sensitive fungus to all the crude extracts. *C. tropicalis* ATCC 750*, C. lusitaniae, C .neoformans* ATCC 66031*, C. parapsilosis* ATCC 22019 and moulds *Microsporum gypseum* and *Trichophyton mentagrophytes* also showed greater inhibition. *C. krusei* ATCC 6258 was moderately active while yeasts *C. albicans* ATCC 90028*, C. glabrata* ATCC 24433 and moulds *P. chrysogeneum* and *F. moniliforme* were resistant.

4. The combined formulation of tea crude extracts and antimycotic, Fluconazole against tested fungal isolates showed synergic antifungal activity. Cold crude tea extracts synergized with Fluconazole against fungal isolates yeast *C.albicans* strain 4, strain 5, NAC *C. tropicalis* ATCC 750 and *C. neoformans* strain 5 compared to Fluconazole alone. Among the dermatophytes, *M.gypseum* showed enhanced inhibition (synergy) activity under combination of crude extract and Fluconazole.

5. The Minimum Fungicidal Concentration (MFC) of the *Camellia sinensis* crude extracts mixture with milk were slightly higher as compared to that of green tea crude extracts. Therefore, addition of milk to blend the crude extracts altered the bioactive ingredients resulting to higher concentration for its MFC as compared to crude extracts alone (it diminishes fungicidal activity).

5.3 RECOMMENDATIONS

1. The Plant based crude extracts represents unlimited sources of modern therapies therefore, a continued and regular exploration of *Camellia sinensis* for antifungal agent is required.
2. Tea is an infusion of the leaves of *Camellia sinensis* plant, and is one of the most widely consumed beverage in the world. For potential antifungal beneficial effects, the hot green tea should be consumed in preference to black tea.
3. The green tea as beverage should also be consumed purely without blending with milk so as to achieve maximum health benefits.
4. The fractionation of crude extracts and purification of active compounds is needed to isolate these bioactive compounds to establish their mechanistic aspect of action against the fungal isolates and elucidate mechanism of synergism.
5. An *in vitro* study on combination with Fluconazole showed synergy, thus, further studies on different combined formulations is required including other conventional antifungal to determine synergism and toxicity.
6. Assayed antifungal were tested *in vitro*, but practically in human aspect both antifungal and polyphenolic compounds of *Camellia sinensis* undergo metabolic processes in the body; there is no information on the interaction of the related metabolites. This needs further studies.

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APPENDIX I

Protocols for materials and media preparation

These include the selection of sample materials, equipments, and the preparation of media according to approved formulation by the manufacturers and the use of well characterized test fungal strains.

Main equipments:

- Electronic kettle with capacity of 1.5 litres
- Thermos flask
- Suction or vacuum filter
- Spectrophotometer
- Separating funnel
- Autoclave
- Clean bench
- Bio-safety class II cabinet (hood)
- Incubator
- Mechanical shaker
- Vortex mixer
- Spatula
- Digital electronic weighing balance
- Refrigerator maintained at $4^{0}C$

Supplies:

- 1000ml conical flasks
- Petri dishes
- Measuring cylinder
- Micropipette, 100 µl capacity with sterile disposable tips
- Sterile 13x100 mm, polystyrene, screw cap test tubes,
- 500 ml capacity filtration units with 0.22-µm-pore-size Millipore filters
- Sterile swabs
- Sterile distilled water
- Disposable gloves

Preparation of enrichment broth

RPMI 1640 media

RPMI-1640 cell culture reagent with phenol red pH indicator and 0.2% glucose.

MOPS buffer as a 0.16M solution

Agar (Difco)

The broth supports growth of fungi and was used to rejuvenate fungi from the stock. The broth was also used to test viability of the fungi.

Preparation

1. 34.53 g of MOPS was weighed and added to one litre of deionised water,
2. then to 900 ml of MOPS buffer, the following were added;

a) 10.4 g of RPMI-1640 reagent

b) 18 g of glucose (final concentration 2 g/100 ml)

c) 15 g of agar

d) The pH was then adjusted to 6.9-7.1 by addition of 1N NaoH or 1N HCL

e) MOPS buffer was then added to a final volume of 990 ml

3. Sterilization was done by autoclaving at 121^0C for 15 minutes. After cooling, 0.3 g L-glutamine in 10 ml deionised water (filter sterilized) was added to cooled broth medium. The solution can be prepared and frozen until needed. Glutamine redissolves when thawed in a 35°C water bath or incubator.
4. 25-30 ml was poured into 15 X 100mm sterilized Petri dishes to a thickness of 4mm on a level, horizontal surface to give uniform depth. They were let to solidify on a cold surface with the covers partially removed in order to avoid the formation of water droplets on the surface of the agar, a phenomenon which could deteriorate the diffusion qualities of the medium.

Quality Control Tests Acceptable Performance Range

Candida albicans ATCC 90028:= 32-43 mm for fluconazole

Candida parapsilosis ATCC 22019:= 26-37 mm for fluconazole

Candida krusei ATCC 6258:= 6-17 mm for fluconazole

Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar is a peptone glucose medium enabling the growth (cultivation) of yeasts and molds, in particular dermatophytes. It is a classical medium for the culture, isolation and identification of pathogenic molds and yeasts. It is recommended primarily for the isolation of molds from samples with low bacterial loads, sterility tests of pharmaceutical, cosmetic or food products, and the culture of molds for their identification.

Preparation

65g of powder medium was weighed and suspended in 1 litre of purified water. The mixture was heated with frequent agitation, boiled for one minute and swirled to completely dissolve the medium. Sterilization was done by autoclaving at 121^{0}C for 15 minutes. After cooling to 50°C, 20 ml was poured into 15 X 100mm sterilized Petri dishes to a thickness of 4mm on a level, horizontal surface to give uniform depth.

They were let to solidify on a cold surface with the covers partially removed in order to avoid the formation of water droplets on the surface of the agar, a phenomenon which could deteriorate the diffusion qualities of the medium.

Quality Control:

Prepared Medium Appearance: Trace to slightly hazy and pale yellowish white to light amber.

Expected Cultural Response Testing: Cultural response on Sabouraud Dextrose Agar tested at specified temperatures and incubation times;

Microorganism	Approx. Inoculum (CFU)	Response
Candida albicans ATCC 90028	10 - 100	Growth
Aspergillus niger ATCC 16404	Point Inoculation	Growth
Penicillium roquefortii ATCC 10110	Point Inoculation	Growth
Trichophyton mentagrophytes ATCC 9533	Point Inoculation	Growth

Mueller-Hinton Agar (MHA)

Mueller Hinton Agar is the recommended medium used for standardized antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS).

Preparation

38g of powder medium was weighed and suspended in 1 litre of distilled water. The mixture was heated with frequent agitation, boiled for one minute and swirled to completely dissolve the medium. Sterilization was done by autoclaving at 121^0C for 15 minutes. After cooling to 50°C, 20 ml was poured into 15 X 100mm sterilized Petri dishes to a thickness of 4mm on a level, horizontal surface to give uniform depth.

They were let to solidify on a cold surface with the covers partially removed in order to avoid the formation of water droplets on the surface of the agar, a phenomenon which could deteriorate the diffusion qualities of the medium.

Quality Control:

Candida albican (ATCC 90028) standard strain for antifungal susceptibility testing was used for each new lot prepared. The PH of the medium was 7.2.

Appendix II: Preparation of 0.5M McFarland Turbidity Standard and Inoculums

0.5M McFarland Turbidity Standard

0.5 ml of 0.048M $BaCl_{2,}$ 1.175% W/V $BaCl_{2.}2H_{2}0$ was added to 99.5 ml of 0.36M $(NH_3)_2S0_4$ (1%V/V) and mixed. 5mls were dispensed to screw capped test tubes. The turbidity of actively growing fungi in alkaline peptone water was adjusted to turbidity of 0.5 McFarland standards.

Inoculums preparations

1. Streaking of QC strain *C. albicans* (ATCC 90028) and test isolates on SDA was done. They were incubated overnight in an ambient-air incubator at 35^0C, and purity checked.
2. Three to five isolated colonies of similar colony morphology were picked, and passed again to SDA. The plate was used for initial inoculum preparation.
3. The tip of a sterilized applicator stick was used to pick five isolated colonies of similar colony morphology at least 1 mm in diameter, and 5 ml of sterile 0.85% NaCl added. Vortexing was done for 15 to 20s.
4. The cell density was adjusted to the density of a 0.5 McFarland standard by measuring the absorbance in a spectrophotometer at 85% transmittance wavelength of 530 nm and adding sterile distilled water as necessary.
5. Resulting suspension = 1 x 10^6 to 5 x 10^6 CFU/ml.
6. 1 ml of suspension was added to 9 ml of RPMI-1640. Resulting suspension equals 1 x 10^5 to 5x 10^5 CFU/ml
7. Tubes of the suspension were held at 2 to 8 C for up to 3 h.

Appendix III: Preparation of antifungal stock solution

Antifungal drug solution was prepared after taking the potency of the lot of antifungal drug powder into account. The amount of powder or diluent required to prepare a standard solution was calculated as follows:

$$W = \frac{1000 \text{ x } V \text{ x } C\ (\mu gmL^{-1}}{P\ (\mu gmL^{-1})}$$

Where P = potency given by manufactured in relation to base

C= Final concentrations of the solution (multiple of 1000)

W = Weight of antifungal in mg dissolved in V

V- Volume required in ml (1000ml)

Fluconazole weight was 250mg

Appendix IV: Interpretative standards and equivalent breakpoints for *Candida* spps (CLSI)

Fluconazole (25μg)	Zone Diameter	MIC
Susceptible Intermediate Resistant	> 22 mm 15-21 mm <14 mm	<8.0 mg/ml 16-32 mg/ml >64 mg/ml