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Antifungal activities of *Camellia sinensis* crude extract, mixture with milk, on selected pathogenic and mycotoxic fungi

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Camellia sinensis **extracts have been documented to have antibacterial activity but little knowledge on their antifungal activity. The aqueous extracts of *C. sinensis* (tea) both green and black, mixed with milk in equal ratio parts, referred as mixture were investigated for their antifungal activity and minimum inhibitory concentrations (MICs) against seven fungal species; Green and black tea crude extracts (100 mgmL^{-1}) were evaluated for antifungal activities. Quantitative bioassay was done using disc diffusion method and MIC done using broth dilution methods. The fungal isolates used for bioactivity testing were yeasts. Green tea crude extract mixture showed stronger inhibitory effect against the fungal strains tested than black tea crude extract mixture. There was a significant difference in zone of inhibitions (T=4.09, P<0.05). Zone of inhibition exhibited by green tea crude extracts (8.33±0.87 mm) were higher than black tea crude extracts (6.75±0.66 mm). The pattern of activity by tea crude extracts mixture against ATCC standard fungal strains and clinical isolates strains were similar. *Candida tropicalis, Candida lusitaniae, Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* ATCC 66031 and *Candida famata* were inhibited by green tea crude extracts mixture (IZD≥15±0.50 mm). Clinical isolates of *Candida albicans* (strain 5) showed susceptibility to *C. sinensis* green crude extracts mixture. The MIC of *C. sinensis* crude extracts mixture against fungal isolates tested ranged from 50 to 1.6 mg mL^{-1}, with green tea crude extract mixture showing highest MIC on clinical fungal isolates. The studies on *C. sinensis* have shown remarkable antifungal activity and highlighted its significance as potential health products.**

Key words: *Camellia sinensis,* crude tea extracts, fungal species.

INTRODUCTION

Fungal infections in human pose serious medical issues. There is a general consensus among researchers, clinicians and pharmaceutical companies that new, potent, effective and safe antifungal drugs are needed. *C.*

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sinensis (Tea) is one of the most consumed drinks worldwide where green tea accounts for about 20% of the total tea consumption. In recent years, several studies have shown that green tea consumption can protect against diseases that are associated with free radical damage including atherosclerosis, coronary heart disease and cancer (Leenenet al., 2000). Kenyan black tea has between 7 and 27% more polyphenols when compared with tea from China, Japan and Taiwan (Wachira and Kamunya, 2005).

The Kenyan tea germ plasma 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 to fungi is limited and the majority of work has been conducted on green tea with very little on black and white tea against bacteria. Beneficial effects of tea have been attributed to the strong antioxidative activity of the tea phenolic compounds known as catechins (Fernando et al., 2005).

Tea catechins possess strong antioxidants properties, which protects the body from damage caused by free radical induced oxidative stress. In addition, many reports have presented data on the antimicrobial activity of different types of tea extracts on various pathogenic microorganisms (Luczaj and Skrzydlewska, 2005). Green tea elicits strong antibacterial activity including potential to inhibit gram positive cocci; gram negative bacilli. 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 (Rechner et al., 2002).

Various studies have shown significant suppressive effects of green tea polyphenols against many microorganisms. Black tea, a major source of phenolic, including theaflavins and thearubigins, 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 *C. sinensis* plant product as novel modalities in the line of new drug discoveries.

MATERIALS AND METHODS

Samples of *Camellia sinenensis*

Processed Commercial *C. 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, Kenya.

Test fungal organisms

The standard test fungi of American Type Culture Collection (ATCC) was sourced from Kenya Medical Research Institute (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 niger* and *Penicillium chrysogeneum*. The selection of test strains was based on their significance as opportunistic pathogens and their resistance to conventional drugs.

Experimental design

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 and 30°C for yeast and moulds respectively, for a period of 3 h. They were then sub-cultured onto sterile Sabouraud Dextrose Agar (SDA) and incubated for 72 h at 35 and 30°C 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).

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 ($BaCl_2.2H_20$) 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°C). Exactly 0.5 McFarland gives an equivalent approximate density of fungi 1.5×10^8 Colony Forming Units per ml (CFU) mL^{-1} (Stein et al*.,* 2005).

Crude extraction of C. sinensis (Tea)

The prepared soluble granules of both black and green tea samples sealed in silver lined sachets stored at room temperature were obtained. The mixture of 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 g of tea sample was then weighed and added to the contained conical flask and boiled for 20 min. 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 (4ºC) until use. Only fresh extracts was used in the experiment, as marked chemical changes occurred when tea was allowed to stand (Yam et al*.,*1997).

Preparation of tea extracts stock and working solutions

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

Preparations of antifungal compounds stock and working solutions

The antifungal compounds were removed from storage (-20ºC) 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 stock solutions of azole group of compounds (Fluconazole) used was usually kept at -20ºC until used. Doubling dilutions of stock solutions were made to obtain working solution.

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 clinical and laboratory standards institute (NCLSI, 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 1.5×10^8 Colony Forming Units per ml (CFU/mL). Circular chromatographic paper discs (6mm 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 mixture. The discs were air dried and aseptically transferred into respective inoculated plates (Esimone et al., 2006). Briefly, approximately 1.5×10^8 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 mixture concentrations were aseptically placed on a Muller-Hinton agar plates to which the test fungi had been inoculated. The inoculated plates were incubated at 4ºC for at least 24 h to allow the tea leaves liquors to diffuse into the media.The cultures were then incubated for 72 h at 35 and 30ºC for yeast and mould respectively, before the activity was determined. The activities of the tea crude extracts mixture were established by the presence zones of inhibition which were measured in mm. Fluconazole discs containing (25µg) 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 mixture against test fungal organisms. The procedures were done as recommended by the National Clinical Laboratory Standards institute (NCLSI) 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 100µL. The wells were inoculated with 5µL of microbial suspension. The yeasts were incubated at 35ºC for 24h while molds were incubated 30ºC for 3 to 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.

The experimental work flow was as shown in Figure 1. The samples were analyzed using paired sample T-test to establish the differences in zones of inhibition caused by black tea crude extracts mixture from green tea crude extracts mixture.

RESULTS

The antifungal activities of green and black tea (*C. 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.

From Table 1, yeasts *C. albicans* ATCC 90028, *C. glabrata* ATCC 24433, *C. krusei* ATCC 6258 and moulds. *Penicillium chrysogenium as* well as *Aspergillus niger* showed no inhibition (6 mm) in either of *C. sinensis* green and black crude extraction. Green tea crude extracts mixture, showed maximum antifungal activity for yeasts *C. tropicalis* ATCC 750, followed by *C. lusitaniae*, *C. parapsilosis* ATCC 22019, *C. famata* and *Cryptococcus neoformans* ATCC 66031 respectively. None of the mould tested showed inhibitory above the cut-off point of IZD ≥ 15±0.5 mm. However, black tea crude extract mixture on the other hand showed slightly moderate inhibition of 10±0.50 mm for yeasts *C. lusitaniae, C. famata* and mould *Fusarium moniliforme* with IZD of 12±0.62 mm as compared to break point of IZD ≥ 15±0.5 mm.

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 mixture (8.33 ± 0.87 mm) were higher than inhibition by black tea crude extracts mixture (6.75 ± 0.66 mm).

Among the clinical isolates tested, *C. neoformans* strain 5, *C. albicans* strain 4 and strain 5, showed susceptibility to antifungal activity of green tea extracts mixture with inhibition zone diameter ≥ 10.0±0.5 mm each. This is moderately active as considered highest at 15.0mm and least at6.0mm. 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).

Minimum inhibitory concentration (MIC) to standard fungal test strains

Minimum inhibitory concentrations (MIC) of tea crude extracts mixture to the fungal strains were established. Tested at 15±0.5 mm diameter of inhibitory zone diameter, the MICs of mixture tea crude extracts were recorded in mg/mL. Black tea crude extract was only tested against *C. famata* strain because it was the only one which showed inhibition activity (Table 2).

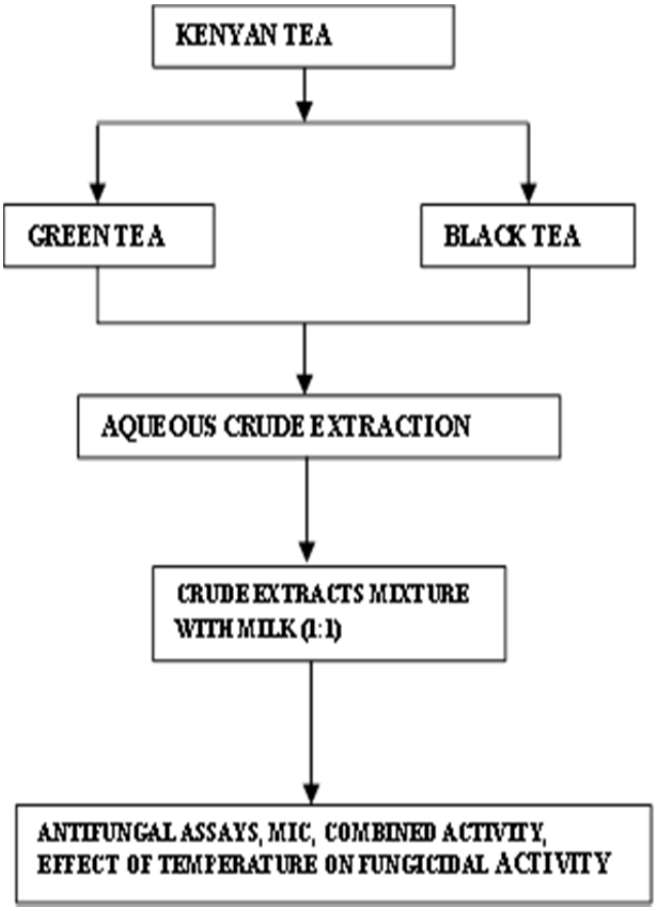

Figure 1. Experimental work flow chart.

Table 1. Zones of inhibitions (mm) by crude tea extracts mixture on the selected pathogenic and mycotoxic fungi.

Fungi	Mixture extract	
	Black tea	Green tea
Candida albicans ATCC 90028	6	6
Candida lusitaniae	10	16
*Candida parapsilosis*ATCC 22019	6	16
Candida glabrata ATCC 24433	6	6
Candida famata	10	15
Candida tropicalis ATCC 750	7	18
Candida krusei ATCC 6258	6	6
Cryptococcus neoformans ATCC 66031	6	15
Microsporugypseum (clinical isolate)	7	12
Aspergillusniger(clinical isolate)	6	6
Fusariummoniliforme(clinical isolate)	12	6
Penicilliumchrysogenium(Clinical isolate)	6	6
Trychophytonmentagrophytes(Clinical isolate)	6	9

The MIC of the *C. sinensis* crude extracts mixture which had inhibition diameters of 15±0.5mm and above (significance activity) was determined. Green tea crude extracts mixture had the least minimum inhibition concentration at 1.6 mg/mL against *C. neoformans* ATCC 66031and *C. famata;* and highest MIC against yeast *C.*

Table 2. The minimum inhibition concentration of tea crude extracts to the standard fungal test strains.

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

The black crude extracts mixture tea showed no detectable inhibitory activity. -, No Inhibition activity.

parapsilosis ATCC 22019 and mould *M. gypseum*. Green tea mixture 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 tea crude extract mixture.

Green tea crude extract mixture minimum inhibition concentration of 50 mg/mL was adequate to inhibit growth of *M. 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.

Synergism/antagonism between crude extracts of Kenyan tea and conventional antifungal drugs on azoles resistant fungi

To establish synergism effect, fluconazole was mixedwith tea crude extracts mixture (blended with milk) and zone of inhibition recorded. The findings show that there were 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 greater than tea crude extracts mixture inhibition zones.

Using a combination of green tea crude extracts mixture (blended with milk) to Fluconazole mainly inhibited growth of *C. neoformans* 5, *C. tropicalis* ATCC 750 *and C. albicans*15. The *C. sinensis* crude extracts exhibited diminished activity when combined with Fluconazole (lesser inhibition zone diameters as compared to fluconazole IZD) as compared to activity by Fluconazole alone. This shows antagonism between the crude extracts mixture and conventional antifungal drug, Fluconazole.

Effect of temperature and addition of milk to crude extracts

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

Green tea crude extract mixture at 3.12 mg/mL was effective enough to kill *C. lusitaniae* while at 6.25 mg/mL, *C. tropicalis* ATCC 750 and *C. neoformans* ATCC 66031 was made static and could not grow. At a concentration of 8.25 mg/mL it was fungicidal to *C. parapsilosis* ATCC 22019. When the concentration reached 50 mg/mL all the tested fungi including *C. famata* were killed by the tea crude extract mixture.

DISCUSSION

According to World Health Organizations, WHO (2000) report of infectious diseases, overcoming antimicrobial resistance is the major issue of W.H.O 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 *C. sinensis* (green and black) blended with milk, giving a mixture 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 et al. (2009) also found that water extracts was blended with milk as normal home-made tea giving strength of “normal cup of tea”. The results obtained in this study indicate a considerable difference in antifungal activity of antimycotic activity of *C. sinensis* green and black crude extracts mixture. For all the yeasts tested, *Candida tropicalis* ATCC750 was the most sensitive

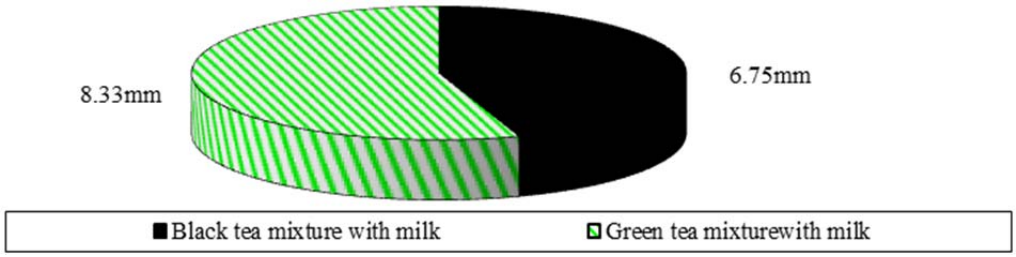

Figure 2. Mean zones of inhibition by green and black crude tea extracts mixture.

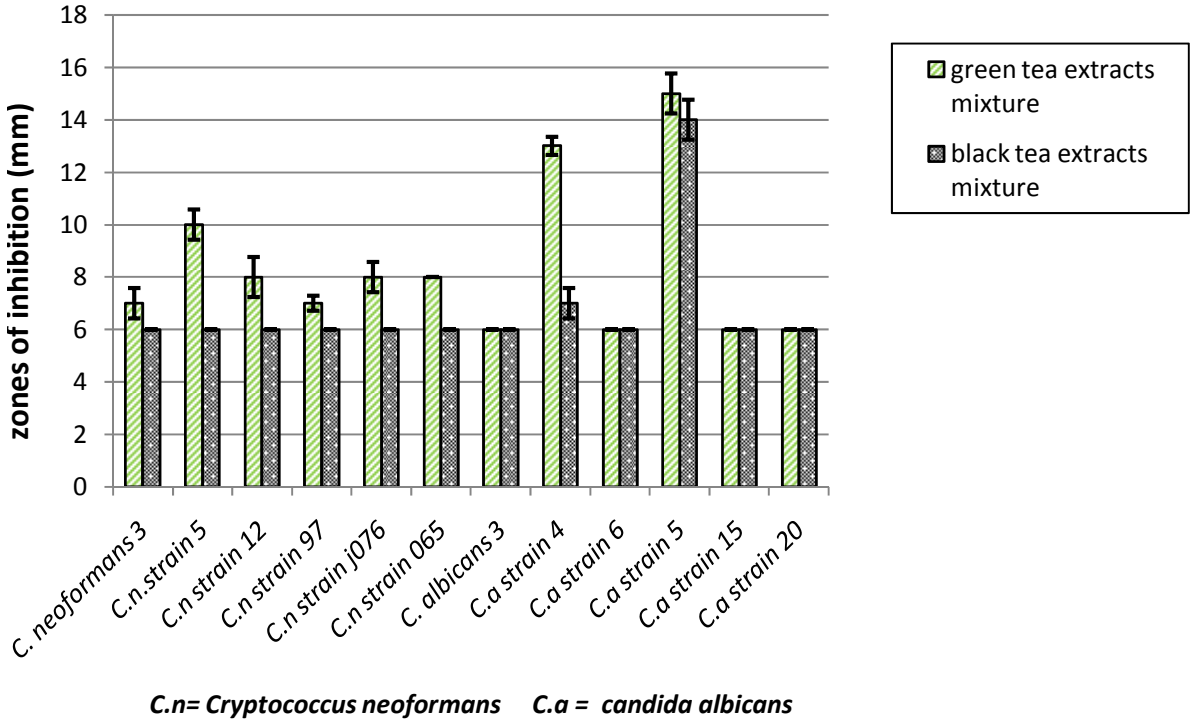

Figure 3. Zones of inhibition of fungi clinical isolate by green and black tea crude extracts mixture. Growth inhibition of fungal clinical isolates by black tea crude extracts mixture were not significantly different ($P > 0.05$), showing lowest sizes zones of inhibition.

fungus to all the crude extracts (Table 1). 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 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 (8.33 ±0.87 mm) were higher than inhibition by black tea crude extracts (6.75 ± 0.66 mm, Figure 2). *C. albicans* strain 4 and strain 5 among all the clinical isolates had greatest susceptibility to antifungal activity of green tea extract with IZD of 15±0.5 mm (Figure 3). *C. neoformans* strain 3, strain 5 and strain 12; showed susceptibility to antifungal activity of green tea extracts mixture with IZD ≥ 10.0±0.5mm each. This is moderately active as considered highest at 15.0mm 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±0.5 mm and the highest was at 18.0±0.5mm in diameter.

The green tea crude extract mixture has shown higher antifungal activity than black tea (Figure 6). This difference in results is probably due to presence of different contents of active substances in the teas. 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 mixture 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*. Catechins are known to have an affinity for proteins; this is clearly shown by a decrease in antibacterial activity of tea. 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. Thus, a number of membrane dependent cellular processes, such as cell signaling and cell cycle, arachidonic acid metabolism and cell proliferation, and apoptosis and mitochondrial functionality may be influenced by interaction of catechins with cellular phospholipid palisade (Caturla et al., 2003).

The resistance of fungal strains (least susceptible) of clinical isolate *C. neoformans* strain 3, strain 5, strain 12, strain 97, strain j076 and strain 065 (Figure 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 *C. albicans* ATCC 90028, *C. glabrata* ATCC 24433*, C. parapsilosis* ATCC 22019that showed less susceptibility to antimycotics of *C. sinensis* crude extract mixture could be due to their outer membrane consisting of chitin binding proteins and 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 broad range of compounds including antifungal agents (Marchetti et al., 2000). But in this study, we found contradicting results among the *Candida* strains of clinical isolates. *C. albican*s strain 3, 6 and 20 showed least or no activity whereas strain 4 and 5 had activity (Figure 3). The disparity in findings could be due to differences in strains of fungi used and their 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 *C. sinensis* which had inhibition zone diameter of 15±0.5 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 *C. sinensis* crude extract against the isolate in question. The green tea crude extract mixture had the least minimum inhibition of 1.6 $mgmL^{-1}$ against yeast *Candida famata,* and *C. neoformans* ATCC 66031 and the highest MIC against yeast *C. parapsilosis* ATCC 22019 of 50 $mgmL^{-1}$ and mould *M. gypseum* (Table 2). When the green tea crude extract was mixed with milk in the ratio of 1:1, the MIC was established to be 1.6 $mgmL^{-1}$ against *C. famata* which also formed the MIC_{50} (Figure 4); whereas, at a concentration of 50 $mgmL^{-1}$, 90 % of the fungal isolates tested were inhibited. At this concentration of mixture green tea crude extract, all the fungi tested were inhibited as shown in Figure 5.

Generally, the MIC of the *C. sinensis* crude extracts mixture was as high as 50 $mgmL^{-1}$ as compared to the standard drugs which is 0.5 $mgmL^{-1}$ for yeasts and 1.0 $mgmL^{-1}$ 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 mixture could be attributed to the differential processing methods of green and black tea and the blending with milk as well as boiling which affect/alter their composition (cold and hot water extraction).

Absence of bioactivity does not warrant disapproval of ethno botanical utilization of the *C. 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 antagonistic antifungal activity of the combination of tea crude extracts mixture and antimycotic, Fluconazole against tested fungal isolates (Table 3). This is in contrary to earlier studies, since the arrival of azole antifungal agents as first-line drugs; Fluconazole-resistant *C. albicans* has begun to appear. Similar studies have been reported by Hirasawa et al. (2003), on the combined use of EGCG and Fluconazole effective against Fluconazole resistant *C. albicans.*

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 (Zhao et al., 2001). EGCG synergizes the activity of β-lactam antibiotics against *Staphylococcus aureus* by

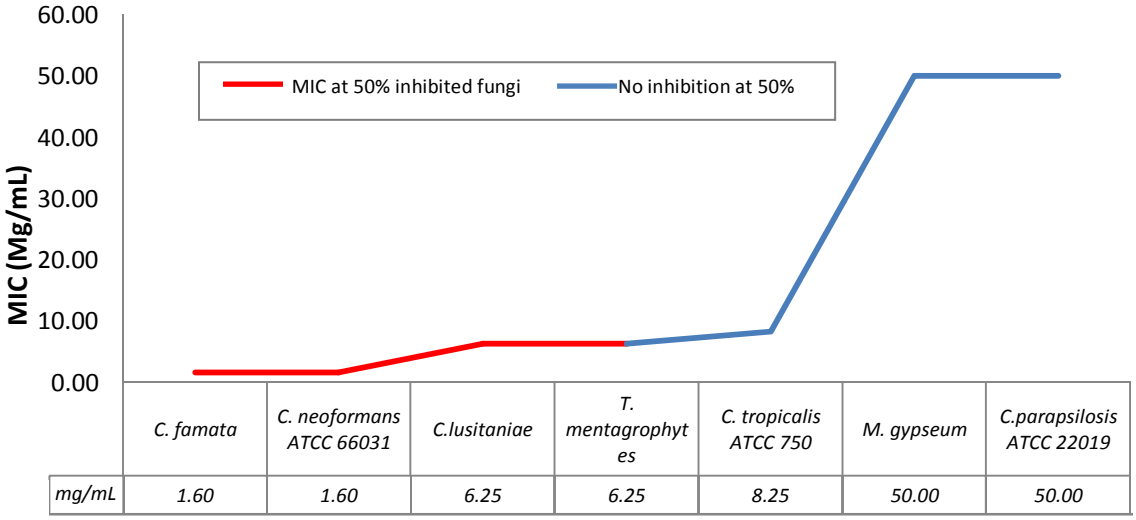

Figure 4. MIC_{50} inhibition by green tea crude extract mixture.

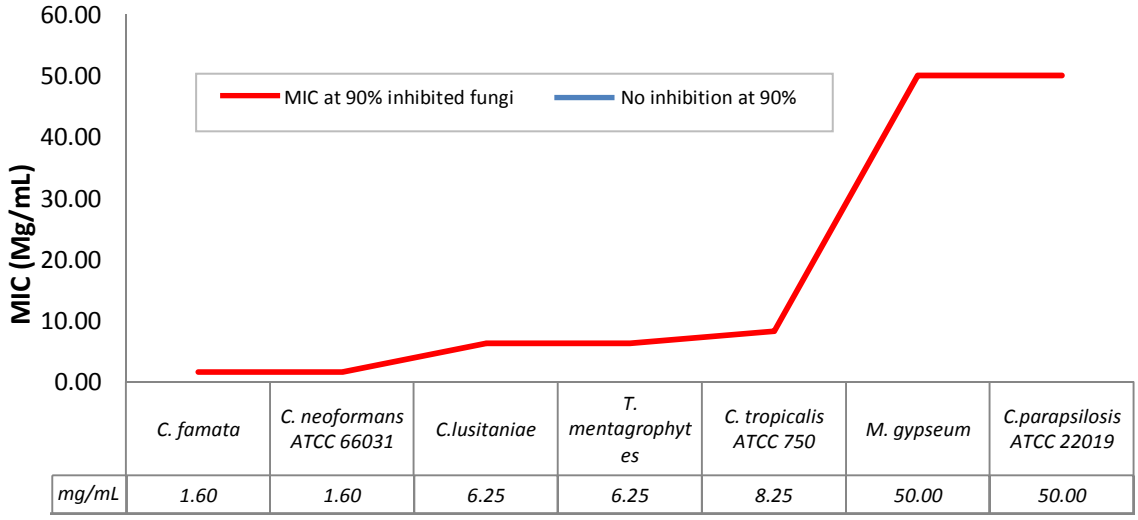

Figure 5. MIC_{90}inhibition by green tea crude extract mixture.

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).

However, findings of the present study established that using a mixture of tea crude extracts to Fluconazole mainly diminished inhibitory effect to fungal species. In terms of effects of inhibition as a result of difference in extraction temperatures, the present study revealed that higher temperatures reduces the polarity of water, thus increasing its extraction efficiency and capability to dissolve polar compounds (Hassas-Roudsariet 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 green tea crude extract mixture (mean 0.017±0.008 mm) was higher than black tea (0.0143±0.007 mm). When green tea crude extract was mixed with milk, the mixture crude extracts at a concentration of 3.12 $mgmL^{-1}$was fungicidal to *C. lusitaniae* but fungi static to other fungal isolates tested (Table 4). But at concentration of 6.25 $mgmL^{-1}$ 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 fungi static to *C. famata*. The MFC of *C. famata* was 50 $mgmL^{-1}$. These results are

Table 3. Synergism/antagonism between *C. sinensis* crude extracts mixture and Fluconazole.

Fungal test strain	Mixture extract + Fluconazole (mm)	Fluconazole alone (mm) (positive control)
Candida albicans 4	20	22
Candida albicans 15	20	22
*C. tropicalis*ATCC 750	21	22
Cryptococcus neoformans 3	10	22
C. neoformans 5	26	22
F. moniliforme	12	18
M. gypseum	16	18
Mean ± SE	17.86 ± 2.10	20.00 ±1.29

Table 4. Minimum fungicidal concentration of green tea crude extract mixture.

Fungi	Green tea crude extract mixture (mg/mL)
C. tropicalis ATCC 750	6.25
C. neoformans ATCC 66031	6.25
C. lusitaniae	3.12
C. famata	50.00
C. parapsilosis ATCC 22019	8.25

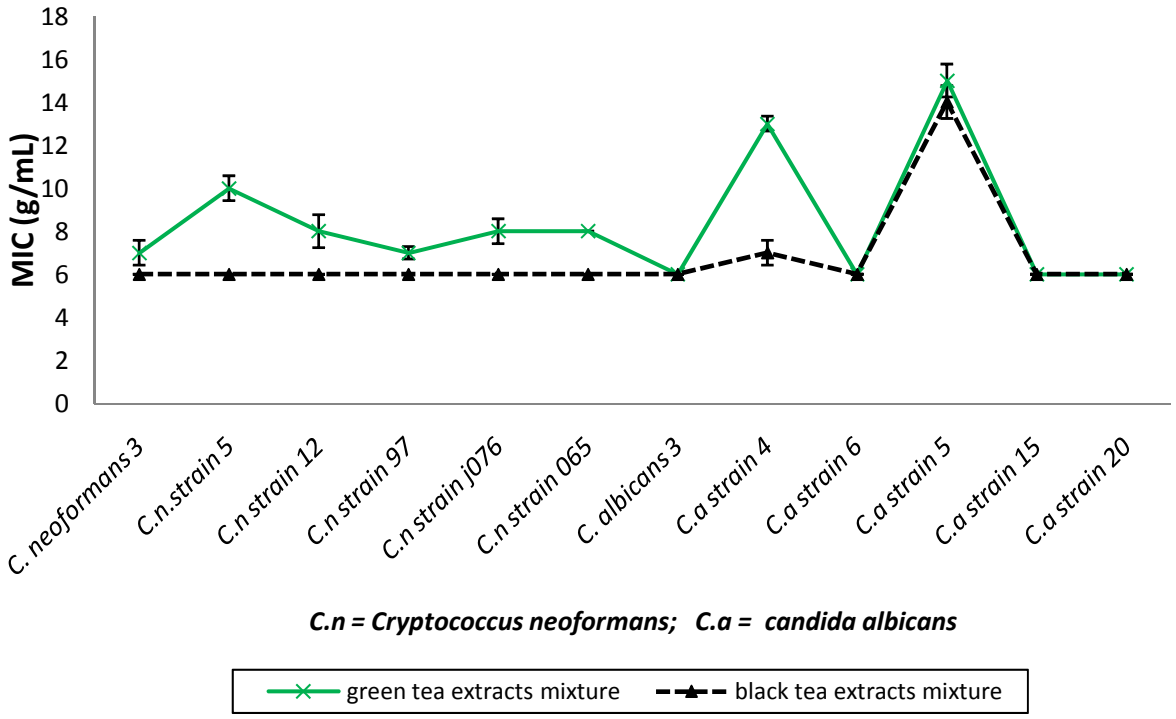

Figure 6. MICs of green and black tea crude extracts mixture on clinical fungal isolates.

suggestive that addition of milk to blend the crude extracts altered the bioactive ingredients resulting in higher concentration for its MFC as compared to crude extracts alone. These results conform to previous studies by Wachira et al. (2011) that milk decreases antioxidant activity of *C. 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}(Muto et al., 2001; Yang and Raner, 2005). 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.

CONCLUSION AND RECOMMENDATIONS

The *C. sinensis* crude extracts possess antifungal activity. In the present study, the crude extracts of *C. sinensis* (green and black) produces inhibitory actions against the fungal test strains. The Minimum Fungicidal Concentration (MFC) of the *C. sinensis* crude extracts mixture with milk was slightly higher as compared to that of fluconazole drug. Therefore, addition of milk to blend the crude extracts alters the bioactive ingredients resulting in higher concentration for its MFC as compared to azole drug alone (it diminishes fungicidal activity).The plant based crude extracts represents unlimited sources of modern therapies therefore; a continued and regular exploration of *C. sinensis* for antifungal agent is required. Tea is an infusion of the leaves of *C. sinensis* plant, and is one of the most widely consumed beverages in the world. For potential antifungal beneficial effects, the green tea should be consumed in preference to black tea. The green tea as beverage should also be consumed purely without blending with milk so as to achieve maximum health benefits. 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/antagonism. Assayed antifungal were tested *in vitro*, but practically in human aspect both antifungal and polyphenolic compounds of *C. sinensis* undergo metabolic processes in the body; there is no information on the interaction of the related metabolites. This needs further studies to examine directly in human populations under carefully controlled conditions to get positive results.

Conflict of Interests

Authors declare that the present work was done by the authors and there is no any financial support from any agency and there are no conflicts of interest.

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