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ANTIFUNGAL ACTIVITY OF CRUDE EXTRACTS OF SELECTED MEDICINAL PLANTS USED IN COMBINATIONS IN LAKE VICTORIA BASIN, KENYA.

Judith Agot Odhiambo[1]*, George Muyala Siboe[1], Catherine Wanjiru Lukhoba[1], Saiffudin Fidahussein Dossaji[1].

[1]**School of Biological Sciences, University of Nairobi P.O Box 30197-00100 Nairobi, Kenya**

***Corresponding Author:** Email: judyodhis2003@yahoo.co.uk.Tel. +254 723 752 846,
Fax +254 020 4442115.

ABSTRACT

Use of herbs as combinations is a common practice with many herbal practitioners. The main idea behind this usually is the synergistic action expected to take place by the traditional healer hence being able to give better results as compared to one herb and also treat more than one ailment, even those not mentioned by the patient. However, other interactions such as additive and antagonism too take place when herbs are used in combinations. In this study, anti-aspergillus and anti-candida efficacy of crude extracts of five plants used in combination to treat malaria were investigated. *Toddalia asiatica* (root), *Rhamnus staddo* (root) , *Momordica foetida* (shoot), *Podocarpus falcatus* (bark), *Aloe sp* (secculent leaves) used by traditional health practitioners in the Kalenjin community were extracted using water and dichloromethane/methanol (1:1) and the crude extracts tested for *in vitro* antifungal activity singly and in combinations against *Aspergillus niger* and *Candida albicans.* Dichloromethane/methanol extracts of *P. falcatus* showed the highest activity (77.77% inhibition) against *A.niger* while *M. foetida* showed the highest activity (77.78% inhibition) against *C. albicans. Aloe sp.* Showed no activity against *A. niger* when tested singly. *A.niger* was more sensitive to the plants extracts than *C.albicans.* Aqueous extracts did not show any activity. Antagonism, additive and synergism were observed when combinations of the herbal plants were assayed. Findings in this study are a preliminary verification of the usefulness of using herbal plants in combinations as a prevalent practice among the traditional healers.

Keywords: Traditional medicine, herbal combinations, *C. albicans, A.niger.*

INTRODUCTION

Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases (Calixto, 2000). In developing countries, especially among rural populations and because of poverty and lack of access to modern medicine, traditional medicine remains a vital health resource in providing primary healthcare (Palombo, 2006). Medicinal plants have played a key role in world health and in spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care (Calixto, 2000). Through past experience, herbalists have used herbal preparations either from single plants or as combined proportions (Liu *et al.,* 2004).The use of herbal drugs as combinations has existed for centuries in several cultural systems (Gathirwa *et al.,* 2008). A study carried in the Kenyan Lake Victoria basin revealed high usage of herbal drugs in combined proportions to treat ailments such as malaria, sexually transmitted infections, and typhoid. *Toddalia asiatica* (Rutaceae), *Rhamnus staddo* (Rhamnaceae), *Podocarpous falcatus* (Podocarpaceae), *Mormodica foetida* (Cucurbitaceae) and *Aloe sp*.(Aloacaeae) are some of the plants used by local communities in the Kenyan Lake Victoria Basin as a combined proportion to treat malaria. Although the main aim of the study was to conduct an ethnomedicinal survey on plants used to treat fungal infections in the area and thereafter establish their claimed therapeutic activity, it was predicted that diseases such as malaria had similar signs and symptoms to those of systemic fungal infections. This article therefore reports the antifungal activity of crude extracts used in combined proportions against *Candida albicans* and *Aspergillus niger*.

MATERIALS AND METHODS

Plant samples

Prepared decoction of the combined proportion of the plant samples was collected from Moiben in Eldoret Kenya in 2007. The herbal plants making up the combined herbal drug were collected as herbarium voucher specimens. Identification was done using taxonomic keys (Agnew and Agnew, 1994; Beentje, 1994) and by comparison with authentic herbarium materials. Voucher specimens (SLO 252) were deposited at Nairobi University Herbarium (NAI).

Microorganism

Candida albicans, a yeast fungus and *Aspergillus niger,* a filamentous fungus, were used in the susceptibility assays. Stocks were maintained on Sabourad's Dextrose Agar (SDA) slants at 4°C prior to use for antifungal tests.

Preliminary screening of the herbal preparation for antifungal activity

The combined herbal preparation was subjected to preliminary antifungal tests using disc diffusion method (Serrano *et al.,* 2004)**.** Discs were cut from Whatman filter paper No.1 using a cock borer with a diameter of 1.2 cm and soaked in the herbal preparation. These impregnated discs were then aseptically transferred into Sabourads Dextrose Agar (SDA) plates freshly inoculated with the test organisms *Candida albicans,* and *Aspergillus niger*. The plates which were prepared in duplicates were then sealed with parafilm to avoid contamination and any possible drying up, and incubated in humid conditions at 37°C and 25°C respectively. The antifungal activities were determined by checking the inhibition zones after 24, 48 and 72 hours for *C. albicans* and 3-6 days for *A.niger* .

Preparation of crude extracts

Crude extracts of the individual plants making up sample 252 were made in order to test their antifungal activity singly and compare to the combined portion. The plant samples were air dried at room temperature, powdered, divided into three portions and extracted using hot water, cold water and organic solvents Dichloromethane/Methanol(1:1) according to standard extraction methods (Harborne 1998). 20g of powdered plant sample was mixed thoroughly with the appropriate amount of solvent, left to stand for 24 hours, and decanted. The liquid portions were filtered using a Buchner funnel. Filtrates from organic solvents were concentrated *in vacuo* using a rotary evaporator at temperature 40° C. All the residues from water extracts were freeze dried. The dry extracts were stored in vials and refrigerated at 4° C prior to antifungal test.

Biological assays of the sample and the individual extracts

A volume of 5ml Stock solution at concentration 1mg/100µl were prepared for each plant extract and the two antifungal drugs (ketoconazole and griseofulvin). These were then subjected to antifungal tests by disc diffusion technique (Serrano *et al.,* 2004). The set plates were incubated as indicated previously. The results were checked after time period indicated previously. Activities of the extracts were determined by measuring the zones of inhibition. Discs with solvents only were used as negative controls while those with the antifungal drugs were used as positive standards.

Direct Bioautography using *A. niger*

In order to identify the active compound/s in each extract, Thin Layer Chromatography (TLC) with commercially prepared silica gel plates were developed in various organic solvent systems and ratios to separate compounds (Table 1.)

Table 1. Solvent systems and ratios used to obtain best separation of compounds on a TLC

CH_2Cl_2 : MeOH (1:1) crude Extract	**Part of the plant**	**Solvent system**	**Ratio**
Toddalia asiatica,	Root	Hexane/Ethylacetate	7:3
Rhamnus staddo	Root	Ethylacetate/Acetone	8:2
Podocarpous falcatus,	Bark	Acetone	100%
Momordica foetida	Shoot	Hexane/Acetone	7:3
Combination of the above five plants		Hexane/Ethylacetate	7:3

The separated spots were seen visible in the UV lamp. Freshly developed TLC plates were then sprayed with previously prepared suspension of *A.niger* in Peptone media. The sprayed TLC plates were incubated under humid conditions at 25° C (Hostettmann and Marston 1994; Horvath *et al.,* 2002). The observation was recorded after 3 days and the inhibition zones noted.

Bioautography agar overlay using *C.albicans*

Preparation of *C. albicans* inoculum

A weight of 9g of NaCl was dissolved in 700ml of distilled water and made up to 1.0 litre using water to make 0.9% NaCl. Previously cultured *C. albicans* on SDA slants was suspended into this sterile 0.9% NaCl. 14ml of the above suspension was added to 1.0 litre of freshly prepared sterile SDA media before solidification to allow fungus to grow. About 22ml of freshly prepared inoculum (culture) was poured into Petri

dishes and left to solidify. TLC plates developed on the same day were carefully laid on to the inoculated medium on the petri-dishes and compounds allowed to diffuse from stationary phase in the TLC plate into in the inoculum in the petri-dish (Runyoro *et al.,* 2006; Hostettmann, and Marston, 1994).The petri dishes were then incubated at 31° C for 20 hours; after which they were sprayed with aqueous solution of thiazolyl blue (3-(4,5 dimethyldiphenyl tetrazolium bromide) (MTT) at concentration of 2.5mg/ml and then incubated further for 4 hours and the inhibition zones which appeared colourless against a purple background (Runyoro *et al.,* 2006) were noted.

Chemical analysis in each crude extract using Thin Layer Chromatography plates

In this study, Dichloromethane/Methanol (1:1) crude extracts of the individual plants screened were tested for the presence or absence of five classes of secondary natural compounds at the active spot using standard methods (Chowdhury *et al.,* 2008 ; Harborne, 1998). The five classes of compounds tested were alkaloids, flavonoids, sapogenins, quinones, and terpenoids (Table 2).

Table 2. Thin Layer Chromatography analyses of the antifungal compounds

Phytochemicals	Spraying reagent	Appearance of the positive spot
Flavonoids	25% aqueous solution Basic lead acetate (25g of Pb in 75 ml of water)	Spots fluoresce in long-wave UV light
Alkaloids	Dragendorff ; solution a consist of 0.85g basic bismuth nitrate dissolved in a mixture of 10ml acetic acid and 40ml water; solution b consists of 8g potassium iodide in 20ml of water. Equal volumes of solutions a and b are mixed to make Dragendorff reagent. 1ml is mixed with 2ml acetic acid and 10ml water before use.	Orange
Sapogenins	Antimony chloride in concentrated hydrochloric acid	Violet
Terpenoids	Antimony chloride in chloroform in the ratio 1:4. heated in oven at 120° C. also evaluated in Longwave UV light	Green
Quinones	Exposure to ammonia fumes	Red ,orange ,yellow, brown

(Adapted from Chowdhury *et al*., 2008; Harborne 1998)

Data analysis

In order to analyse data, MS Excel 2003 was used to quantify, sort data, determine % mean inhibitions, and draw bar graphs and tables. One way Analysis Of Variance (ANOVA) was used to determine the means, the standard deviations and the pvalue. Student t- test was used to determine significance of the difference of the antifungal activities of the extracts.

RESULTS

Antifungal activity of the herbal preparation

Preliminary antifungal bioassays on Samples 252 showed antifungal bioactivity and was taken for further bioassay analysis. Sample 252 consisted of *Toddalia asiatica(root), Rhamnus staddo*(root), *Podocarpous falcatus*(bark*), Aloe sp*.(secculent leaves) and *Momordica foetida* (shoot).

Further antifungal bioassays of the crude extracts of Sample 252

Crude extracts obtained from the individual plants in sample 252 were subjected to antifungal tests against *C. albicans* and *A.niger.* Only extracts from organic solvents dichloromethane/methanol showed inhibition activity on one or both test pathogens. Water extracts did not show any antifungal activity. Organic solvents' extracts of all individual plants in sample 252 showed

fungitoxic activity against both *C. albicans* and *A.niger* except *Aloe sp.* which showed no activity against *A.niger.*

Dichloromethane/Methanol (1:1) extracts of *Toddalia asiatica, Rhamnus staddo, Momordica foetida, Podocarpus falcatus, Aloe sp.,* and their combined proportions showed activity against one or both of the test organisms. *Momordica foetida* showed the highest activity (77.78% mean inhibition) against *C. albicans* .

Amongst the five plants together with their combined proportions, *Podocarpus falcatus* showed the highest activity (77.77% mean inhibition) against *A. niger* while *Aloe sp*. Showed no activity when tested singly.

When tested against *C. albicans, Rhamnus staddo*, *Aloe sp*., *Momordica foetida*, and the combined sample were more active than Ketoconazole and Griseofulvin. When tested against *A.niger, Rhamnus staddo*, *Podocarpous falcatus*, *Momordica foetida*, and combined proportions were more active than Griseofulvin but similar in activity to Ketoconazole. Although the combined proportions had no significant difference in activity to *Rhamnus staddo, Aloe sp., Momordica foetida* when tested against *C. albicans* and ; *Rhamnus staddo*, *Podocarpous falcatus*, *Momordica foetida* when tested against *A.niger,* its percentage inhibitions was much lower than for the mentioned individual plants .

Identification of antifungal compounds

Phytochemical components responsible for the antifungal activity of the plants were identified as alkaloids, flavonoids, terpenoids, quinones and sapogenins. Appearing as colourless regions against a purple background are the inhibition zones showed by the active components of individual plants of sample 252 against *C.albicans* by bioautography agar overlay.

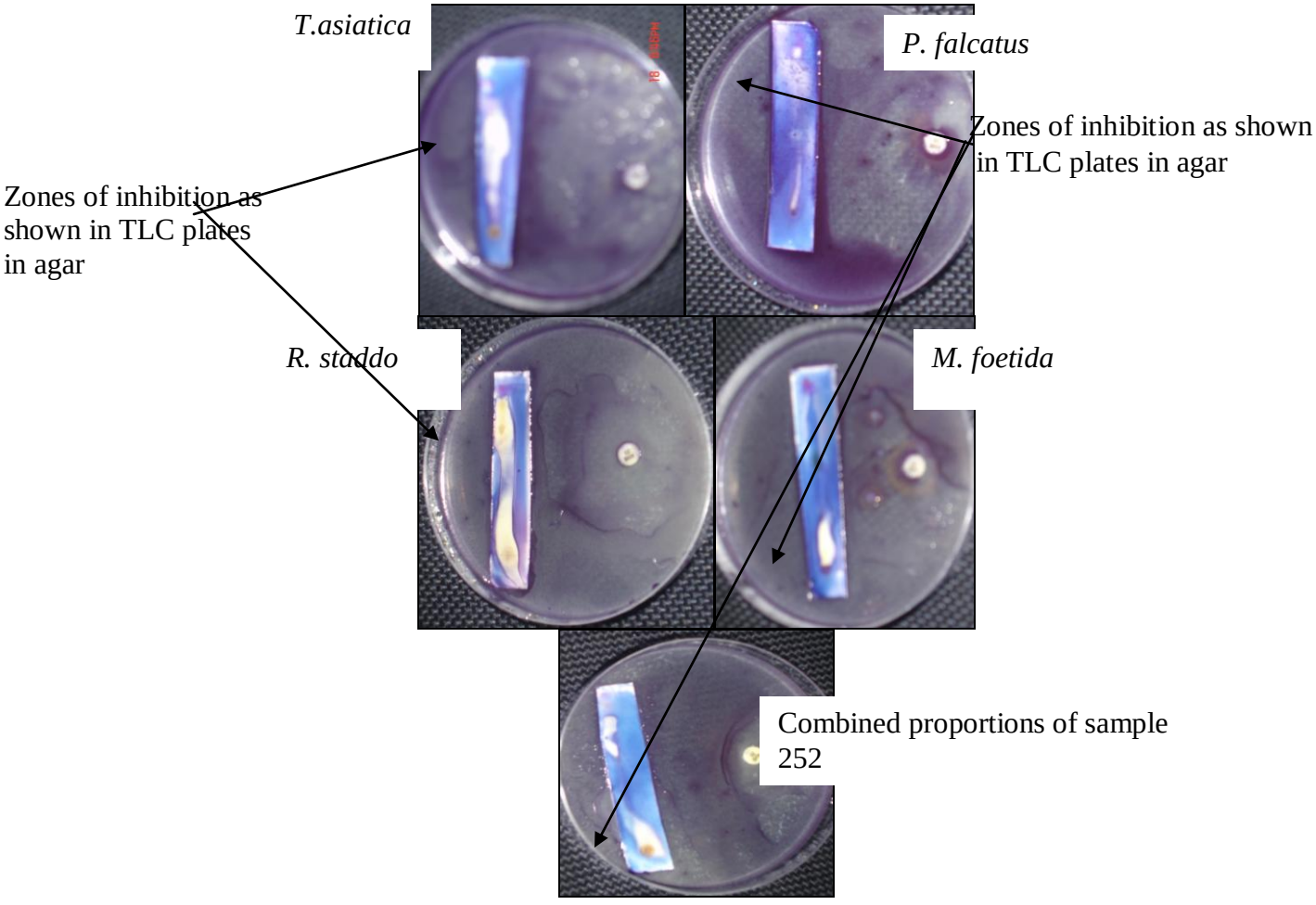

Figure 1. Inhibition zones caused by Dichloromethane/Methanol (1:1) crude extracts against *C.albicans*

Table 3. Active chemical components identified in the different extracts

CH_2Cl_2:MeOH crude Extract	Chemical compounds identified in the active spots in TLCs									
	ALKALOIDS		**FLAVONOIDS**		**SAPOGENINS**		**QUINONES**		**TERPENOIDS**	
	C.a	*A.n*	*C.a*	*A.n*	*C.a*	*A.n*	*C.a*	*A.n*	*C.a*	*A.n*
Toddalia asiatica,	+	+	+	+	-	+	-	-	-	-
Rhamnus staddo	+?	+?	+	-	-	-	-	-	+	-
Podocarpous falcatus,	-	+	+	+	-	-	-	-	-	-
Momordica foetida	+	+	-	-	-	-	-	-	+	+
Combination of the above five plants	+	+	+	+	-	+	-	-	-	-

C.a….. *Candida albicans* , *A.n*…..*Aspergillus niger,* +….. Present and active
__….. May have been absent or present but not active,
+?...(Not clear) may have been an artifact.

Table 4. Active chemical components identified in different crude extracts

	ALKALOIDS	**FLAVONOIDS**	**SAPOGENINS**	**QUINONES**	**TERPENOIDS**
CH_2Cl_2:MeOH crude Extract					
Toddalia asiatica,	+	+	+	-	-
Rhamnus staddo	+?	+	-	-	+
Podocarpous falcatus,	+	+	-	-	-
Momordica foetida	+	-	-	-	+

+…..presence, -…..absence /inactive, +?.....(Not clear)May have been an artefact.

Although only five groups of chemical components were successfully tested in each active extract in this study, the presence of other antifungal compounds not tested in these plants cannot be ruled out. Active groups of compounds identified in these plants are shown in Table 3

In this study, the major antifungal compounds were found to be alkaloids and flavanoids. Plant extracts containing flavonoids and alkaloids were active against both the yeast and the filamentous fungus (Table 4).

DISCUSSION

In most cases to the herbalist, using a combination of more than one herb gives 'strength' to the herbal drug and henced increased efficacy against the ailment. In many other cases the herbalist not being sure of the specific ailment from the patient's condition gives a drug in combination form to clear the potential illnesses the patient could be suffering from, still the patient could be having several health conditions and so herbal drugs known to cure specific conditions differently are provided together by the herbalist.

This finding reported antifungal activity of sample 252. The combination showed activity against both C. *albicans* and *A. niger* however *Aloe sp.* one of the plants in the mixture showed no activity against *A. niger* when tested singly. Similar case was reported by Olembo *et al.,* (1995) in which *Aspilia pluriseta* used in combination with *Microglossa pyrifolia* and *Indigofera arrecta* was reported to be a good remedy for fungal infections of the skin but showed no activity when tested singly against dematophytic fungi as reported by Kariba (2000).

Claims by the traditional medicine practitioner to treat malaria using Sample 252 is supported by previous bioassay reports which have shown antimalarial activity of all the plants in the mixture except *P. falcatus.* (Muregi *et al.*, 2007; Kuria *et al.,* 2001; Gessler *et al.,* 1994 ; Orwa *et al.,* 2008 ; Gakunju *et al.,* 1995; Clarkson *et al.,* 2004).

Only organic solvent extracts showed activity against the tested fungi. This observation is of particular interest, given that traditionally, the preparation of herbal remedy is often with water. Fungitoxic activity of water extracts may have been too low to be detected at concentrations 1mg/100µl. This is supported by Fawole *et al.,* (2008), Parekh and Chanda, (2007), Boer *et al.,* (2005), and Kariba (2000) who found out that water extracts showed no/poor fungitoxicity than those made using organic solvents, among other reasons they reported could be that same active substances were present in water extracts but at concentrations at which bioactivity was not longer detectable, another reason could be that the active substances were soluble in organic solvents and basically not present in water extracts. This finding is also in agreement with that of Clarkson *et al.,* (2004) who reported that the inactivity of water extracts may have been because they (extracts) were not prepared according to the traditional methods, which in some cases involved boiling for several hours. It is therefore worth noting that the traditional practitioners use water because that is all they have at their disposal, and success may be due to administration of the concoctions/decoctions in large quantities for instance in basins, cups, water glasses, and in all or most cases, the treatment involves using the extracts' decoction or concoction for a long period of time, (Yineger *et al.,* 2008 ; Lulekal *et al.,* 2008 ; Erasto *et al.,* 2005).

Some of the investigated plants within the sample did not show strong antifungal activity. Active compound(s) may have been present in insufficient quantities in the crude extracts to show activity with the dose levels employed. This is in agreement with Parekh and Chanda, (2007), who reported that alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents. The plant extracts may have been active but against other fungal or non fungal pathogens which were not tested.

Promising anti-candida results were obtained with extracts from *Momordica foetida*, *Aloe. Sp, Rhamnus staddo* and the combined proportions. Same extracts except *Aloe sp.,* together with *P. falcatus*, gave promising anti-aspergillus results, which all to a varying extent inhibited growth of these pathogens. Other tested plant extracts also inhibited the growth of the test organisms but to a lesser extent e.g *Toddalia asiatica.*

The finding of anti yeast activity of *M. foetida* seems to be in agreement with that reported by Boily and Van, (1986) who found out that methanolic extracts of leaf of this herb were active against *C. albicans*, however no reported work on the activity of this plant against *A. niger* was found in the literature.

Although reported as significantly active against the two fungi in this study, reports of the antifungal activity of *R. staddo* were not found, however a lot of studies on the antimalarial activity have been reported (Kuria *et al.,* 2001; Muregi *et al.*, 2007).

This study showed that *P. falcatus* was active against *C. albicans*. This agrees with report by Abdillahi *et al.,* (2008) who tested the activity of leaves and stems of this plant using several organic solvents and found that they were active. Amongst the plants tested in this study, *P. falcatus* showed the highest activity against *A.niger*. However no literature reports were found on the activity of this plant against the same fungus and therefore may have been reported in this study for the first time.

A relatively low activity of *T. asiatica* extract against *C. albicans* was reported. Duraipandiyan *et al.,* (2006) and Gurib-Fakim *et al.,* (2005) reported activity of this plant against the same fungus, however their report contrasts with those of Rajakaruna *et al*., (2002) and Kar *et al.,* (2005) who found no activity of this plant against the same yeast fungus. The discrepancy could be due to factors such as time of collection of plant materials, environmental factors, geographical and ecological location of the plants and extracting solvents (Runyoro *et al.,* 2006).

The insignificant activity of *T. asiatica* against *A. niger* is in agreement with Gurib-Fakim *et al.,* (2005) who reported no activity, but contrasts with those of Saxena and Sharma (1999) who reported that essential oils from leaves of this plant was active against this fungus.

Synergy, antagonism and additive interactions may have been exhibited in Sample 252. From the results, it was clear that although the activities of individual extracts of *M. foetida*, *R. staddo* and *Aloe sp.* against *C.albicans* were significantly similar to that of the mixture (combined proportions), the % inhibitions of these individual extracts were higher when tested singly. On the other hand individual extracts of *T. asiatica* and *Aloe* sp. against *A.niger* were comparatively lower than that of the mixture. These findings are in agreement with that of Bella (2005) who reported that synergistic or antagonistic interactions between antimicrobial drugs is a reflective of relationships between their actions on cellular components, and that synergism between two antimicrobial drugs might result from (i) binding to the same target protein such that a conformational change caused by the binding of drug A enhances the binding of B, (ii) binding of drug A to a transporter causing

increased uptake of B into the cell or the subcellular compartment in which it acts, (iii) formation of a complex between A and B of enhanced toxicity, (iv) stimulation by A of the conversion of B to a more active form. Converse examples could be constructed for antagonism and also when the active compounds have a common target. The individual plants may have had compounds which had antagonistic effects which made the mixture to have comparatively lower activity when tested against *C.albicans.* This finding seems to be also in line with the observation by Gathirwa *et al.,* (2008), that marked antagonism, synergy and additive interactions are observed when combinations of the drugs are assayed *in vitro.* The plants that showed lower activity than the mixture when tested singly such as *T. asiatica* and *Aloe* sp. against *A.niger* may be acting as antipyretics or immune stimulants to relieve the symptoms of the disease, rather than having direct activity as reported by Phillipson *et al.,* (1993). Use of herbal remedies as mixtures of different herbs have also been reported in Chinese traditional medicine by Xiao (1983), who observed that determination of their pharmacological effects and the isolation of their active principles are much more difficult than is the case of single medicinal plants owing to the interaction of various constituents.

It was observed that *R. staddo*, *Aloe sp*., *M. foetida*, and the combined proportions against *C. albican*s had higher antifungal activity than Griseofulvin and Ketoconazole . The same extracts except *Aloe sp*. together with *P. falcatus*, were more active than Griseofulvin when tested against *A.niger*. Griseofulvin and Ketoconazole are both commercial antifungal drugs. This suggests that extracts from *R. staddo P. falcatus*, *Aloe* sp. *M. foetida* as well as their combined extracts could be used successfully for the treatment of Candidiasis and / or Aspergillosis. Results showing higher activity of herbal drugs compared to commercial drugs have been reported by Moundipa *et al.,* (2005), who found higher activity of some medicinal plants extract compared to that of metronidazole against *Entamoeba histolytica* and Samie *et al*., (2005) who reported higher antibacterial activity of some medicinal plants compared to commercially used antibiotics.

The higher effectiveness of these plant extracts and Ketoconazole drug against the filamentous fungus *A niger* compared to the yeast fungus *C albicans* may be due to differences in cell wall composition, as cell-wall synthesis is the target for various groups of drugs (Gooday 1993).Yeast fungi contain glucans and mannan proteins in their cell walls compared to chitin and glycan in the cell walls of filamentous forms (Murrey *et al*., as in Kariba 2000). Depending on composition, the cell wall can act as a barrier preventing drugs from reaching the site of action (Matthison 1977 as in Kariba 2000). This finding is in agreement with that Espinel-Ingroff and Flynn (1996), who reported that yeast fungus, are less susceptible to the few available antifungal agents due to their continuously new emergence.

Although this study reported the major antifungal components against both the yeast and the filamentous fungus as alkaloids and flavonoids, the fact that there could have been other antifungal compounds which were not tested in this study cannot be ruled out.

Although alkaloid test in *R.staddo* showed positive, this plant has never been reported to have alkaloids (Pourveura, 1973) and so this could have been an artefact.

Edeoga *et al.,* (2005) also reported alkaloids and flavonoids as being among the most important bioactive plants constituents. He reported the presence of flavonoids and alkaloids from all the ten medicinal plants used in traditional medicine to treat various ailments among others ringworms, diarrhoea, coughs, chest pains, bronchial asthma, fever, malaria, vomiting, inflammation, sore throat, boils, sores and wound healing. This finding of alkaloids and flavonoids as major important medicinal plant phytochemicals is also in agreement with reports by Adegboye *et al.,* (2008) and Palombo, *et al.,* (2006) that they play wide range of biological activities amongst others antidiarrhoeal, anti-inflammatory, anti-allergic effects , and have analgesic properties.

The antifungal activity of these plants makes them potential source of antifungal agents and may be of economic importance as source of antifungal natural plant products. This study therefore is in agreement with Chowdhury *et al.,* (2008); Mbwambo *et al.,* (2007) ; Duraipandiyan *et al.,* 2006 ; Fabricant and Farnsworth 2001 ; Kariba *et al.,* 2001 ; De Smert, 1997 ; Kubo and Taniguchi, 1993; that the information from ethnomedicinal sources may offer potential leads to new active natural products.

CONCLUSIONS AND RECOMMENDATIONS

It is important to carry out scientific studies on medicinal plants having traditional claims of effectiveness in ethnomedicinal uses which offer immense potential for development of new and valuable pharmaceutical products. Further investigation using bioassay guided fractionations are recommended to isolate and identify the pure compounds responsible for the antifungal activity. Investigations of pharmacological effects and the isolation of the active principles of plant compounds when combined are recommended.

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