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Synergism between clotrimazole and cinnamon oil: An effective (weapon) drug *in vitro* and *in vivo* against some multi drug resistant dermatophytes

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ABSTRACT

Antifungal activity of eight commercially antifungal drugs and cinnamon oil were screened against twenty two dermatophytic isolates of clinical origin. The strains that revealed resistance against maximum number of antifungal drugs (under investigation) were selected for synergistic assay using Checkerboard method. The interaction between clotrimazole and cinnamon oil against twelve of multi-drug resistant dermatophytic isolates *in vitro* revealed that this interaction was synergistic or in-additive while antagonism was not detected; the interaction was synergistic in the most isolates (91.67%) while inadditive was demonstrated in only one isolate. Fifty four patients, twenty five males and twenty nine females suffering from dermatophytic infections were enrolled in this study to evaluate synergism between clotrimazole and cinnamon oil *in vivo*. The dermatophytic infections were confirmed by laboratory mycological method and direct microscopic examination. The patients were randomly divided into three groups, which treated with Closol (commercial clotrimazole topical solution), placebo and Clo-Cin topical solution twice daily for two weeks and then followed up for two weeks. After two weeks of the treatment *in vivo* 50% improvement in clotrimazole (closol) treated patients and 81.82% in Clo-Cin group. The rate of improvement was 25% and 100% in clotrimazole and Clo-Cin groups respectively after four weeks of the dermatophytosis treatment. The recurrence of dermatophytic infection in Closol treated patients representing significant difference between the two groups. Moreover, Clo-Cin also offered significant protection to infected tissue challenged with irritation (clotrimazole side effect) as revealed by fluorescence microscope result.

Keywords: clotrimazole and cinnamon oil**,** multi drug resistant dermatophytes, Synergism, *in vitro* and *in vivo*

INTRODUCTION

Dermatophytic infections are highly prevalent due to the large number of reservoirs (skin, hair, nails), the readiness of transmission from one host to another, and high resistance of the strains to adverse environmental conditions (Robert and Pihet, 2008), So it is not surprising that dermatophytic infections are a major cause of morbidity-associated superficial mycoses (Gupta and Cooper, 2008). Dermatophytes are responsible for serious fungal human pathogenic infections that have been increased during the last decades (Arif *et al.*, 2011).

Treatment of dermatophytosis by both oral and topical formulations mainly include one of the two antifungal drug families: azoles and allylamines (Gupta and Cooper, 2008). Superficial mycosis (e.g. *Tinea pedis, T. mannum*, *T. corporis and T. cruris*) are usually respond to topical antifungals (Andrews and Burns, 2008). The most common agents are azoles (eg. clotrimazole, miconazole, econazole, oxiconazole, tioconazole) and allylamines, (e.g.

terbinafine and naftifine) have been also used (Gupta and Cooper, 2008).

Oral treatment with antifungal represents the treatment of choice for dermatophytoses that fail to respond to topical medication (Monod, 2008). Nevertheless, the use of these medications may result in undesirable side effects in the patient. Terbinafine cause secondary gastrointestinal and cutaneous side effects (Del-Rosso and Gupta, 2000). The use of azoles presents disadvantages such as hepatotoxicity and liver metabolism via cytochrome P450 (CYP), affecting the metabolism of other drugs (Del-Rosso, 2000 & Del-Rosso and Gupta, 2000).

Although conventional antifungal drugs are available, but frequent recurrence, fungi resistance, and side-effects of most antifungal drugs can result in treatment failure, Taking into account treatment costs and duration, therefore an accurate diagnosis is crucial to define which treatments must be applied (Robert and Pihet, 2008). In order to improve cure rates it is absolute necessary to increase the efficiency of treatments, for this purpose a combination of antifungal therapies may help in the fight against these diseases such as the use of several antifungal agents (Evans, 2003 & Khan and Ahmad, 2011), but oral drug interactions must also be evaluated, since some antifungal agents are inhibitors of enzymes involved in the metabolism of other drugs (Del-Rosso, 2000 & Del-Rosso and Gupta 2000).

In the last years, researches in aromatic and medicinal plants particularly their essential oils (EO), has attracted many investigators. Essential oils have traditionally been used during centuries for their antifungal properties (Ríos and Recio, 2005). More recently, several studies have shown evidence of the huge potential of these natural products as antifungal agents (Inouye *et al.,* 2006; Tullio *et al.,* 2007; Jantan *et al.,* 2008; Zuzarte *et al.,* 2009; Vale-Silva *et al.,* 2010 and Lima *et al.,* 2011), Justifying their current use in a number of pharmaceutical, food, and cosmetic products. Therefore, it is not surprising that EO are one of the most promising groups of natural products, for the development of broad-spectrum, safer and cheaper antifungal agents (Lima *et al.,* 2011).

Because of the widespread use of EO as antifungals, so it is not surprising that combining conventional antifungals with EO has shown promising results (Hemaiswarya *et al.,* 2008).

This work aims at evaluating *in vitro* and *in vivo* synergism between clotrimazole (azole commercial antifungal) and cinnamon oil (Essential oil of *Cinnamon* plant) against some multi drug resistant dermatophytes of clinical origin in order to treat dermatophytic infections to improve cure rates and subsequently increase the efficiency of treatments by using more active, safer and cheaper topical solution.

MATERIALS AND METHODS

Fungal isolates:

A total of twenty two patients (who had not clinically responded to (2-3) months of any antifungal treatment) suspected with dermatophytosis were send from several dermatology outpatient clinics according to some dermatology doctors requests to culture and sensitivity unit at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, all suspected patients have been subjected to direct mycological isolation and culture. The purified isolates then identified at the Regional Center for Mycology and Biotechnology (RCMB).In skin dermatophytoses the clinical specimens collected were epidermal scales. The scales were scrapped from near the advancing edges of the lesions after disinfecting the lesions with 70% alcohol. Where the advancing edges were not evident, scrapings were collected from areas representing the whole infected area. In hair dermatophytoses basal root portion of hair was collected by plucking the hair with sterile forceps. In cases with black dot, scalpel was used to scrape the scales and excavate small portions of the hair roots and then cultured on Sabouraud chloramphenicol agar medium (Bio-rad, France) containing

chloramphenicol (0.05%) with and without cycloheximide (0.5%) and incubated at 25°C for 4 to 6 weeks (Robert and Pihet, 2008).

Analysis of essential oil:

The analysis of the essential oil was performed using GC–MS (type Hewlett Packard 5890 USA). The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, NBS75K library data of the GC–MS system and literature data (Adams, 2001).

Evaluation of Minimum Inhibitory Concentration (MIC):

The MIC of cinnamon oil and eight commercially antifungal agents were determined by broth microdilution method according to Hammer *et al.* (2002). Two fold serial dilutions of each sample were placed in eppendorf tubes labeled A to M. Tube A was filled with 100 µl of each sample (stock solution 1024 µg/ml, DMSO). Only 50 µl of the stock solution in tube A was transferred to tube B and diluted with 50 µl of DMSO. The procedure was repeated for solutions in tube B to M. Each tube was diluted with broth to obtain concentrations ranging from 512-0.12 µl /ml. 100 µl from each tube was then transferred into 96-well microtitre plates. Each well was then filled with 100 µl fungal suspension to obtain serial dilution of the test materials ranging from (256-0.01 µl /ml). The mixtures were mixed thoroughly and incubated at 25^{o}C. The final inoculum size for fungi was 2.5 x 10^4 CFU/ml. 1% DMSO served as a negative control, broth as sterility control and broth with fungal suspension as growth control. Turbidity was taken as an indication of growth and the lowest concentration at which it remained clear was recorded as the minimum inhibitory concentration (MIC) of the sample. After reading the MIC, the minimum fungicidal concentration (MFC) was determined. A 100 µl aliquot from the wells in which no growth was observed was transferred to test tubes containing 2 ml of Sabouraud-dextrose broth (Difco, Detroit, MI, USA). A positive control (growth control) and a negative control (sterility control) were included in the test. The tubes were incubated for 7 days at 28 ºC and growth was observed visually. MFC was defined as the minimum concentration at which no fungal growth occurred (Favre *et al.* 2003).

Checkerboard microtitre test:

In vitro antifungal combination assay was performed to investigate the combined effect of cinnamon oil and clotrimazole against multi drug resistant dermatophytes using the checkerboard technique, as described by Davidson and Parish (1989). The assay involved multiple dilutions for both cinnamon oil and clotrimazole in concentrations equal to, above, and below their MIC values for the fungi being tested. Seven serial two-fold dilutions of both cinnamon oil and clotrimazole were prepared in DMSO as described in the broth microdilution procedure. Fifty microlitre aliquots of cinnamon oil solution was dispensed into the wells vertically down the 96-well microtitre plate and 50 µl aliquots of clotrimazole solution was dispensed horizontally. A 100 µl suspension (1-5 x 10^4 CFU/ml) of multi drug resistant dermatophytes was added into each well. The result was that each square in the checkerboard (well) contained a series of combination of the cinnamon oil and clotrimazole being tested. To assess whether synergistic antifungal activity occurred between the two sample solutions as described by Warnock (1989). The fractional inhibitory concentrations (FIC) was calculated which is the concentration of each sample necessary to inhibit growth in a given row or column divided by the MIC value of the sample alone against the test organism. The FIC index was obtained by adding the FIC value of each sample and interpreted as follows: synergistic effect if it was <1, additive if it was = 1 and as antagonistic if it was > 1.

Cinnamon oil - clotrimazole topical solution (Clo-Cin topical solution):

Since all the commercial topical solutions of clotrimazole contain 1 mg/ 100ml clotrimazole, so five different

concentrations of clotrimazole (1, 0.5, 0.25, 0.12, 0.06 mg/ 100 ml) were selected to evaluate for their antifungal activity *in vitro* with substitution of steroids (0.1 %) with cinnamon oil at the same concentration beside gallic acid, propylene glycol and ethyl alcohol according to Barry (1983). The experiment was performed in triplicate. The positive control was Closol (commercial topical solution). The percent inhibition in the radial growth of the colony was calculated by the following formula:

Percent growth inhibition = (C-T) / C×100, where C = Growth in control andT= Growth in treatment accorging to Yadav *et al*. (2011).

Cytotoxicity assay:

The cytotoxicity assay was carried out using 0.1ml of cell suspension, containing 10,000 Vero cells (African's monkey kidney) cells seeded in each well of a 96-well microtitre plate. Serial two-fold dilutions of the Clo-Cin topical solution (1-0.06 mg/100 ml) and Closol topical solution (1 mg/100 ml) were added after 24 h of seeding. Control cells were incubated without the tested samples. The microtitre plates were incubated at 37ºC in a humidified incubator with 5 % CO_2 for a period of 72 h. The morphology of the cells was inspected daily (Qiaoxia *et al*., 2004).

Patients:

The present study was a randomized clinical trial. A total of fifty four patients, twenty three males and twenty nine females, suffering from dermatophytic infection, which have not been received antifungal drugs, were enrolled in this study. All patients examined by dermatologist (dr Hesham Singer). The diagnosis of disease was confirmed according to standard laboratory method (direct microscopic examination). Occupationally, (28.6 %) cases were housewives, (23.8%) students, (14.3%) employees, (23.1%) workers and the rest had free jobs. The range of patient's age was from 10 to 54-years-old. Twenty patients received clotrimazole topical solution (Closol) while twenty two patients received Clo-Cin topical solution twice daily for two weeks, respectively. Also, 12 patients with dermatophytic infection, as positive controls, were run in experiments and received placebo including cinnamon oil, gallic acid, propylene glycol and ethyl alcohol. Clinical and laboratory findings were detected two weeks after starting of treatment and followed up the next two weeks.

Examination of epidermal scales treated with Closol and Clo-Cin topical solution by fluorescence microscope:

Specimen collection:

A housewife suffering from ringworms in her both arms, one of those arms (right) treated with closol topical solution while the other treated with Clo -Cin topical solution at the same dose. After two weeks very thin scales of glabrous skin which present an active inflammatory border will be scraped from the both arms separately.

Microscopic examination:

Scraped scales stained by adding combining of stains fluorescein diacetate (FDA) and propidium iodide and then have been incubated for 10 min at 37 °C in dark and then examined using fluorescence microscope (LEICA CTR 5000 with DFC 280) at 488 nm (Oparka and Read, 1994).

RESULTS AND DISCUSSION

Dermatophytic infections are the most common cutaneous fungal infections in humans (Chinelli, *et al* 2003). Over the past few decades, the number of antifungal agents used in clinical practice for the treatment of dermatophytoses has increased (Barchiesi *et al.,* 2001). Nevertheless, not all species have the same susceptibility pattern and there is evidence that dermatophytes have become resistant to some antimycotic agents (Fernàndez-Torres *et al.,* 2002).

In vitro sensitivity profile of eight commercially available antifungal agents against different species of dermatophytes isolated from patients was evaluated using the broth microdilution method. Of the azole antifungal agents, the best results in terms of MIC values were found with itraconazole

followed by miconazole (Table 1). On the other hand, the activity of fluconazole and clotrimazole were weak against all the species (Table 1). Of the non-azole antifungal agents tested, terbinafine was found to be the most effective antimycotic agent (Table 1), This finding is in agreement with the results reported by Favre *et al* (2003) who demonstrated that terbinafine was the most active antifungal agent.

The MFC values of azole antifungal agents confirm that high concentrations of these medications are required to obtain their fungistatic effect and that may be due to the mechanism of their antifungal activity according to Carrillo-Munõz *et al.* (2010).

Table 1: *In vitro* susceptibility testing in the term of MIC&MFC of isolated dermatophytes against eight commercially available antifungal agents and cinnamon oil.

Islates	TEF		KTZ		GSF		ITZ		MCZ		Amp		Fcz		CLZ		Cinn.oil	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
M. canis																		
8F	0.03	0.03	64	>128	16	32	0.1	8	2	16	2	8	64	>128	16	64	16	4
21M	0.01	0.01	0.5	2	2	4	0.2	0.5	0.2	8	0.1	0.2	8	32	4	16	0.5	0.01
22F	0.01	0.01	2	8	4	8	0.2	8	0.1	8	0.5	1	16	64	2	8	1	0.03
7F	0.03	0.03	32	>128	64	>128	8	32	0.2	8	0.1	0.2	64	>128	64	>128	0.5	0.03
13M	0.06	0.06	0.5	2	2	4	0.1	8	0.2	8	0.1	0.5	8	16	16	64	0.5	0.06
14M	0.01	0.01	32	128	128	>128	8	32	8	32	4	8	64	>128	32	128	32	16
T. rubrum																		
1 **F**	0.06	0.06	4	8	2	4	0.1	8	0.1	8	0.2	1	32	>128	1	4	0.5	0.06
15 **F**	0.03	0.03	32	>128	16	32	0.2	8	0.5	32	8	32	8	32	8	16	16	4
6 **F**	0.01	0.01	4	32	2	4	1	16	0.2	8	2	16	16	64	4	16	8	2
16 **F**	0.01	0.01	16	64	32	64	8	64	8	32	8	32	64	>128	32	128	2	4
T. verrucosum																		
2 **M**	4	4	4	8	4	8	4	16	0.5	32	0.5	4	32	>128	4	16	0.5	0.5
19 **F**	1	1	8	32	16	16	8	32	0.5	64	0.5	8	64	>128	16	64	0.5	0.2
18 **M**	2	2	16	32	16	32	8	32	0.5	32	4	16	64	>128	16	128	16	16
E. floccosum																		
5**F**	4	4	8	32	16	32	2	8	0.2	16	4	8	64	>128	64	128	32	32
T.mentagrophytes																		
3 M	1	1	4	8	8	16	4	32	0.1	8	0.1	1	16	64	4	16	0.2	0.2
4 M	0.1	0.1	4	16	16	32	8	32	0.5	16	4	8	8	32	32	128	8	4
11F	1	1	4	16	2	4	4	16	0.5	16	4	8	64	128	64	128	8	8
9F	2	2	2	4	1	8	2	16	0.2	8	0.2	4	64	>128	8	64	0.5	0.5
12F	2	2	8	16	32	64	8	32	0.2	16	4	8	64	>128	16	32	16	4
17F	0.2	0.2	4	16	8	16	4	16	0.2	16	4	16	16	64	16	32	8	2
10M	0.1	0.1	8	64	16	64	8	32	0.2	16	2	4	64	>128	16	64	16	8
20M	0.5	0.5	0.5	2	8	16	16	32	8	32	0.5	1	64	>128	8	32	8	2

ITZ =Itraconazole, KTZ =Ketoconazole, CLZ =Clotrimazole, Fcz, =Fluconazole, CLZ =Clotrimazole, MCZ= Miconazole, Amp =Amphotericin, TEF =Terbinafine* and GSF=Gresiofulvin, MIC= minimum inhibitory concentration, MFC= minimum fungicidal concentration

To determine the resistance of these fungi to the different antifungal agents, in this study the parameters established in the Clinical and Laboratory Standards Institute (2002) for filamentous fungi were taken into consideration, which establish MIC resistance values for each antifungal agent and subsequently the resistance profiles of isolated dermatophytes were demonstrated in Table2, Furthermore, these results highlight the problems involved in treating patients with azole antifungal agents, since resistance to ketoconazole (50%), fluconazole (63.64%), itraconazole (36.36%) and clotrimazole was 72.73% (Table 2).

Nevertheless, the sensitivity profile of some isolates was found to vary within the same species (Table 2). This result reinforces the importance of analyzing sensitivity at least in all the fungal cultures obtained from patients with superficial mycoses in whom therapy has failed and, in view of their severity and this result in agreement with Manzano-Gayosso *et al.* (2008). Therefore, knowing that fungal infections are naturally progressive and may advance to potentially

severe stages in patients, identification of the species that is causing the infection in patients with dermatophytosis in order to select the optimal treatment is not enough, since sensitivity to a single antimycotic agent may vary between species and this result is in the same line with that of Dyachenko *et al*. (2007).

Table 2: Resistance profile of multi-drug resistant dermatophtes isolates.

Microorganism [a)]	Site of Infection	Resistance Pattern of Antifungals	Isolates [b)]
***Trichophyton mentagrophytes* (8)**	Face	GSF	3 M
	Toe nails	KTZ, GSF, ITZ, CLZ	4 M
	Toe nails	Fcz, CLZ	11 F
	Finger nails	CLZ, Fcz	9 F
	Toe nails	KTZ, GSF, ITZ, Fcz, CLZ	12 F
	Face	GSF, CLZ	17 F
	Scalp	ITZ, MCZ, Fcz, CLZ	20 M
	Scalp	KTZ, GSF, ITZ, Fcz, CLZ	10 M
***Microsporum canis* (6)**	Scalp	KTZ, Fcz, CLZ	8 F
	Exposed skin	----------	21 M
	Eyebrows	CLZ	13M
	Hand	KTZ, GSF, ITZ, Fcz, CLZ	7 F
	Hand	----------	22F
	eyebrows	KTZ, GSF, ITZ, MCZ, Fcz, CLZ	14 M
***Trichophyton rubrum* (4)**	Hand	Fcz	1 F
	Toe nails	KTZ, GSF, Amp, CLZ	15 F
	Toe nails	----------	6 F
	Face	KTZ, GSF, Fcz, ITZ, MCZ, Amp, CLZ	16 F
***Trichophyton verrucosum* (3)**	Beard	Fcz	2 M
	Toe nails	KTZ, GSF, ITZ, Fcz, MCZ, CLZ	19F
	Finger nails	KTZ, GSF, ITZ, Fcz, CLZ	18 M
***Epidermophyton floccosum* (1)**	Feet	KTZ, GSF, Fcz, CLZ	5 F

a)= No. of isolates tested in parentheses, **b)** = Code of the strains studied, **Antifungal Agents:** ITZ =Itraconazole, KTZ =Ketoconazole, CLZ =Clotrimazole, Fcz, =Fluconazole, , Amp =Amphotericin, MCZ= Miconazole, TEF =Terbinafine and GSF=Gresiofulv.

All isolates were susceptible to cinnamon oil which consists of twenty one compounds identified by means of GC and CG/ MS analyses (Table3), the strong antifungal activity of cinnamon oil is not surprising because some of its constituents namely: cinnamaldehyde (68.90% w/w), Cinnamic acid (9.74% w/w) and some terpenic compounds, such as α− pinene and caryophyllene, have strong antifungal activity according to Sen-Sung *et al*. (2008).

Table 3: Percentage composition of the essential oils of *Cinnamomum verum*

Phytochemical constituents	%	Phytochemical constituents	%	Phytochemical constituents	%
Styrene	0.53	γ-Terpinene	0.13	Tetradecanal	2.6
α-Thujene	0.32	Terpinolene	0.50	δ-Cadinene	0.52
α-Pinene	0.62	Linalool	3.84	Cinnamic acid	9.74
Benzaldehyde	0.33	Terpinen-4-ol	0.99		
Sabinene	.,012	α-Terpineol	1.52		
β-Pinene	0.23	Cinnamaldehyde	68.90		
Myrcene	0.14	Eugenol	2.32		
α-Phellandrene	1.94	Methyl (E)-cinnamate	0.32		
***p*-Cymene**	2.62	α -Copaene	3.6		
β-Phellandrene	1.74	Methyl eugenol	0.24		

With the increased use of antifungal agents there is an increase in the number and variety of fungal strains resistant to these drugs. Also the present antifungal therapeutics is often toxic. Alternative therapy needs to improve efficacy besides reducing both toxicity and development of resistance of antifungal agents. This can be achieved by the use of combinations between existing agents and plants essential oils which can exhibit synergy with drugs to perform safer and more effective agents (Hemaiswarya *et al*., 2008).

Clotrimazole and cinnmon oil synergy was evaluated against multi drug resistant isolated strains (12 isolates), namely; *Trichophyton mentagrophytes* (4M &20M), *Microsporum canis* (8F, 10M, 7F &14M) and *Trichophyton rubrum* (15F). Synergy (∑FIC ≥ 0.5) was found in 11/12 (91.6%) of isolates under investigation but there was in-additive interaction between clotrimazole and cinnamon oil against *Epidermophyton floccosum* (∑FIC =1) by Checker Board Microtitre test (Table 4).

Table 4: MICs tested alone and in combination by Checkerboard method: SYN: synergistic interaction, IND: inadditive interact.

Microorganism	Minimum inhibitory concentration (µg/ml)						∑ FICs	
	Clotrimazole			cinnamon oil				
	alone	After combination	Fold Decrease	alone	After combination	Fold Decrease		
Microsporum canis 4 M	32	4	8	8	1	8	0.25	(SYN)
Microsporum canis 20 M	8	2	4	8	2	4	0.5	(SYN)
Trichophyton mentagrophytes 8F	16	4	4	16	4	4	0.5	(SYN)
Trichophyton mentagrophytes 10M	16	4	4	16	2	8	0.38	(SYN)
Trichophyton mentagrophytes 7F	64	16	4	0.5	0.125	4	0.5	(SYN)
Trichophyton mentagrophytes 14M	32	8	4	16	2	8	0.38	(SYN)
Trichophyton rubrum 15F	8	1	8	8	2	4	0.38	(SYN)
Trichophyton rubrum 16 F	32	2	16	2	0.25	8	0.19	(SYN)
Epidermophyton floccosum 5 F	64	32	2	32	16	2	1	(IND)
Trichophyton verrucosum 18 M	16	2	8	16	2	8	0.25	(SYN)
Trichophyton mentagrophytes 12F	16	2	8	16	2	8	0.25	(SYN)
Trichophyton mentagrophytes 19 F	16	2	8	8	2	4	0.38	(SYN)

By plotting an isobologram with axis representing fixed ratio values of both clotimazole and cinnamon oil, the lowest concentration of combined sample showing fungal growth inhibition was plotted. In the case of *Trichophyton rubrum* 16 F (Fig 1) with the lowest ∑ FICs value the line is shifted to the left while in the case of *Epidermophyton floccosum* 5 F (Fig. 2) with the highest ∑ FICs value the line is straight. The result in the same line with Meadowas *et al.* (2002) who demonstrated that the combination effect of the mixture is considered synergistic if the line was shifted to the left, antagonistic if it was shifted to the right and in-additive if the line was straight.

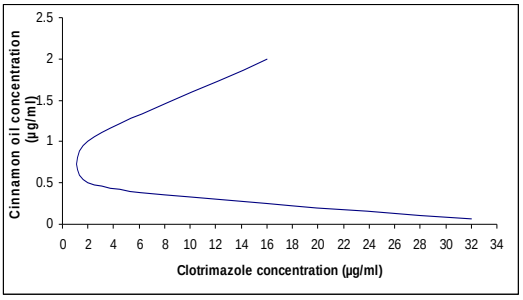

Fig 1: Isobologram shifted to the left revealing the synergistic effect between clotimazole and cinnamon oil against *T. rubrum* 16

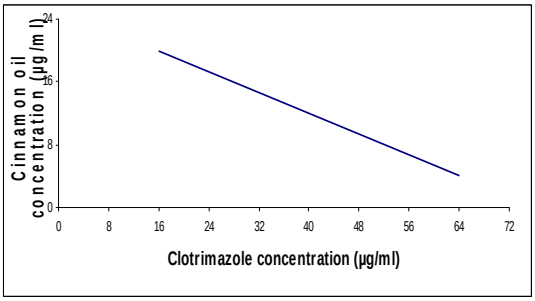

Fig 2: Straight line isobologram revealing the in-additive effect of clotimazole and cinnamon oil against *E. floccosum* 5 F

Clo-Cin topical solution have been used in vitro with five different concentration of clotrimazole (1, 0.5, 0.25, 0.12, 0.06 mg/ 100 ml) to detect the least concentration able to be used *in vivo* against the most resistant isolated dermatophyte (*Trichophyton mentagrophytes* 8F) according to the ∑ FICs values and the result revealed that Clo-Cin with 0.12 mg clotrimazole/ 100 ml is more active than closol alone with 1 mg clotrimazole/ 100 ml and The rest component of Clo-Cin topical solution without clotrimazole or cinnamon oil as negative control (Table 5).

Table 5: Percent of growth inhibition against *T. mentagrophytes* 8F and cytotoxicity against Viro cells as affected by different Clo-Cin topical solution concentrations and Closol topical solution:

Sample / Against	Clo- Cin topical solution with clotrimazole (mg/ 100 ml)					Closol	Negative control
	1	0.5	0.25	0.12	0.06	1	0
T. mentagrophytes 8 F	Growth inhibition Percent (%)						
	100	100	81	75	50	59	0
Viro cells	Cell viability (%)						
	72	84	93	100	100	62	100

As shown in Table 6, the most frequent clinical sign in the three groups was ringworm. The clinical findings showed improvement two weeks after starting treatment, but many of cases had recurrence of symptoms after four weeks of Closol treatment (Figs 3, 4, 5 and6). A prominent improvement of clinical findings had been occurred in patients during anther two weeks after treatment with Clo-Cin topical solution and this improvement was continued after two weeks follow up.

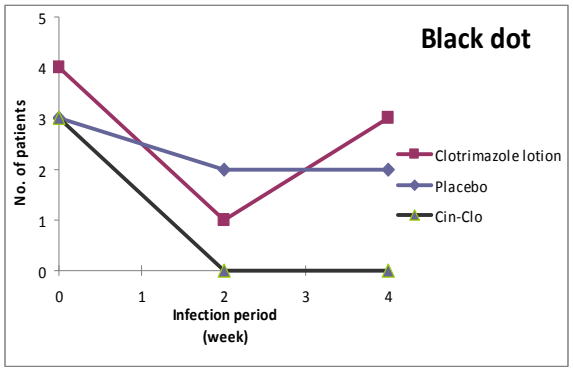

Fig. 3: Histogram showing no. of patient suffering from Black dot after subjected to treatment by Clotrimazole topical solution, Placebo and Clo cin during different time intervals

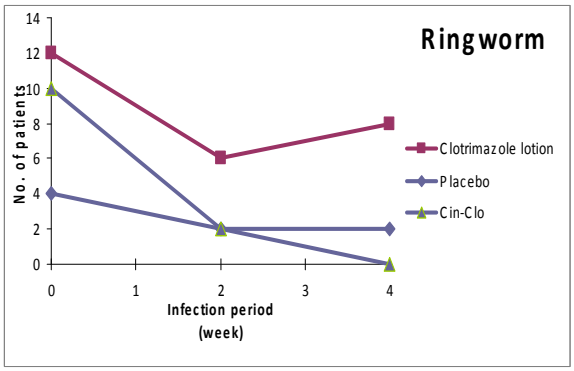

Fig. 4: Histogram showing no. of patient suffering from Ringworm after subjected to treatment by Clotrimazole topical solution, Placebo and Clo cin during different time intervals

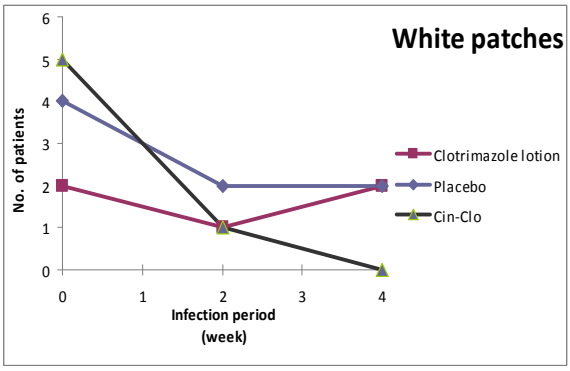

Fig. 5: Histogram showing no. of patient suffering from White patches after subjected to treatment by Clotrimazole topical solution, Placebo and Clo cin during different time intervals

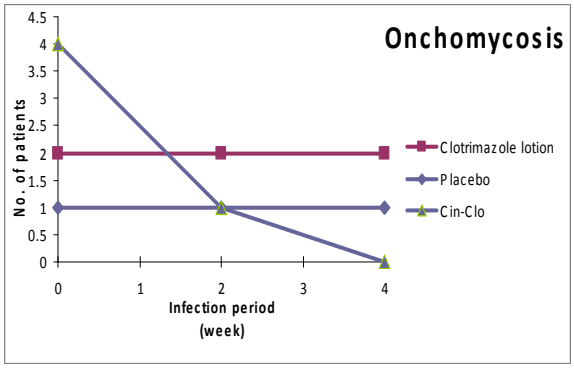

Fig. 6: Histogram showing no. of patient suffering from Onchomycosis after subjected to treatment by Clotrimazole topical solution, Placebo and Clo cin during different time intervals

It was obvious that two weeks after treatment, the improvement rates in clotrimazole and Clo-Cin groups was 50% and 81.82%, respectively, whereas four weeks after treatment, the improvement rate was 25% in clotrimazole group and 100% in Clo-Cin group.

The improvement rate in clotrimazole topical solution group had decreased by 25%, this result exhibited the recurrence of disease. While in the case of Clo-Cin group, the improvement rate was 81.82% two weeks after treatment and reached to 100% after two weeks

follow up. This result showed 18.18% improvement in the course of disease. Of 12 patients with dermatophytosis receiving placebo (positive controls), the improvement rates was 41.7% and still the same after four weeks of treatment. Some clinical pictures with different infected sites, namely Ringworm (Fig.7), *tinea corporis* (Fig. 8), Blackdot ringworm(Figs 9, 10), *Tinea capitis* (Fig. 11) represented as A; before treatment and B; after treatment with Clo-Cin topical solution form 2-4 weeks of treatment.

Table 6: Frequency distribution of clinical signs and laboratory results of dermatophytosis by clotrimazole, Placebo and Clo-Cin Topical solutions .

Sign	Clotrimazole topical solution (Closol)			[a]Placebo			Clo-Cin Topical solution		
	No.								
	[b] Start	[c] 2 W	[d] 4 W	[b] Start	[c] 2 W	[d] 4 W	[b] Start	[c] 2 W	[d] 4 W
Black dot	4	1	3	3	2	2	3	0	0
Ringworm	12	6	8	4	2	2	10	2	0
White patches	2	1	2	4	2	2	5	1	0
Onchomycosis	2	2	2	1	1	1	4	1	0
(%)of uncured cases	100	50	75	100	58.3	58.3	100	18.18	0
(%) of improvement	0	50	25	0	41.7	41.7	0	81.82	100
Laboratory Test									
Positive(%)	100	50	75	100	58.3	58.3	100	18.18	0
Negative(%)	0	50	25	0	41.7	41.7	0	81.82	100

[a]Placebo: topical solution without clotrimazole as positive control
[a] Start: Start of treatment., [b] 2 W: Two weeks after treatment., [c] 4 W: Four weeks after treatment

It is very useful to test the viability of fungal cells and cell Population, combining fluorescein diacetate (FDA) and propidium iodide works extremely well for this purpose (Oparka and Read, 1994). FDA is readily taken up by living cells (fungal cells or skin cells) and is hydrolysed to fluoroscein which fluoresces green. Propidium iodide, however, is only taken up by cells which had their plasma membrane damaged. Only damaged cells, therefore, take up propidium iodide and these fluoresce red. By simultaneously imaging at the appropriate emission wavelengths (488 nm.) it is thus possible to determine which cells are living (green) and which damaged are (red) (Haugland, 2002).

In order to investigate the inflammatory effect of Clo- Cin topical solution and Closol topical solution on skin scales before and after treatment with Clo-Cin topical solution and Closol topical solution separately. The three skin scales samples have been stained with FDA and PI and then viewed under fluorescent microscope at 20X. The skin scales sample before treatment revealed dense damaged scales (fluoresce red) infected with viable dermatophytic fungus (fluoresce green) Fig.12A. It was found that Clotrimazole alone resulted in the elimination of the organism; however, the cells were still damaged and in irregular shape (fluoresce red) Fig.12B and that allow re-infection with pathogenic fungi. On the other hand, treatment by Clo- Cin topical solution led to elimination of fungus as well as skin scales cells returned to their normal viable status (fluoresce green) Fig.12C.

It's worth mentioning that the results of the current study has shed light on the importance of the combination between clotrimazole and cinnamon oil as antifungal agents due to three reasons:

First, antifungal activity of clotrimazole was significantly enhanced when combined with cinnamon oil and the minimal effective dose of clotrimazole was also reduced (by four folds), hence minimizing possible side-effects according to Pyun and Shin (2006). who demonstrated the side effects of azole antifungals.

Second, due to the well-known inflammatory effects of clotrimazole including skin rash, hives, blistering, burning, itching, peeling, redness, stinging, swelling, or other sign of skin irritation, So combination between steroid/antifungal agent (clotrimazole- betamethasone)

indicated for the treatment of dermatophytosis but unfortunately the use of steroid must be justified because of the potential complications associated with steroid use are atrophy, steroid-induced acne, rosacea, and striae (Katz *et al.,* 1984). Since cinnamon oil and its major constituents such as trans-cinnamaldehyde, caryophyllene oxide, L-borneol, L-bornyl acetate, eugenol, b-caryophyllene, E-nerolidol, and cinnamyl acetate have excellent anti-inflammatory activities according to Yu-Tang *et al* (2008), So by replacing steroid with cinnamon oil, will give double efficiency as an antifungal and an anti-inflammatory than clotrimazole topical solution alone.

Third, re infection has occurred with patients treated with clotrimazole and this may be due to the resistance mechanism of azole comounds namely, Target site alteration as demonstrated by Loffler *et al* (1997) who demonstrated that mutations in *ERG11*, the gene encoding for the target enzyme lanosterol C14a-demethylase, prevents binding of azoles to the enzymatic site and subsequently resistant to azole compounds. Antifungal activity of cinnamon oil including lysis of cell wall and plasma membranes, endoplasmic reticulum expansion near cell membrane, excessive vacuolization, abnormal distribution of polysaccharides and leakage of cytoplasmic contents as well as several invasive targets, allowing all together inhibition of fungal infection. (Zuzarte *et al.*, 2011a). In this study re infection hasn't occurred with patients treated with Cin-C1o Topical solution and that may be due to cinnamon oil contains many compounds capable of eliminating fungi; hence it is hard to overcome the antifungal activity of this oil and result is in the same line with Zuzarte *et al.* (2011b) who demonstrated that the antifungal activity of essential oils is not due to a single mechanism of action but it results from the effect of different compounds on several cell targets

RECOMMENDATION

The emerging and rapid dissemination of high-risk multidrug resistant dermatophytes have become a global threat to public health. It is well recognized that misuse, overuse and inadequate use of antifungal drugs in human clinical therapy and in livestock production contributes to the drug resistance by killing the susceptible fungi and selecting the resistant strains. Therefore, combination between conventional antifungal drugs and essential oils (complementary therapeutic) has been known for many years. The collaboration between clotrimazole and cinnamon oil is promising and can expedite the efficacy of dermatophytic treatment using conventional antifungal drug by reducing the dose of antibiotic required in order to decrease the associated side effects to improve cure rates and subsequently increase the efficiency of treatments. So, interest in this category is recommended to be on rise. Overall it seems that, the combination between clotrimazole and cinnamon oil rendered a quick and effective treatment of multidrug resistant dermatophytes, which is required in eliminating this kind of fungi, as well as its economic and medical importance.

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The researcher would like to express her sincere thanks to all the volunteer patients who participated in this research for their full cooperation. Also, I would like to thank all the dermatologists who sent the patients to culture and collection unit at the regional center for mycology and biotechnology- Al Azhar University in order to apply a microbiology examination on them. I would like to especially thank Dr. Hesham Singer, specialist of Dermatology and Venereology, for investigating the patients and determining the extent of their response to treatment, without whose support this work would not have been possible.

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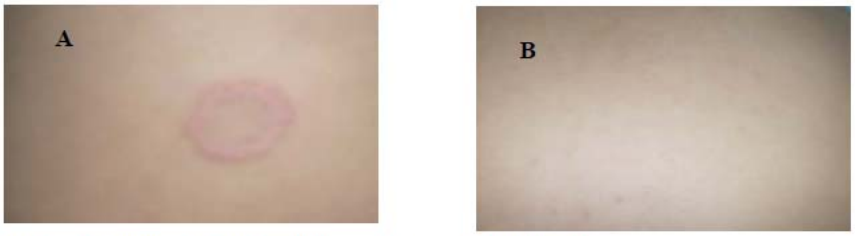

Figure 7. (A) Ringworm due to dermatophytic infection, (B) after treatment.

Figure 8. (A) tinea corporis (B) after treatment.

Figure 9. (A) Blackdot ringworm due to dermatophytic infection, (B) after treatment.

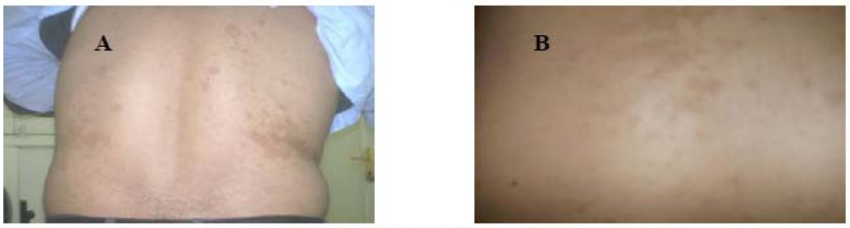

Figure 10. (A) Blackdot ringworm due to dermatophytic infection, (B) after treatment.

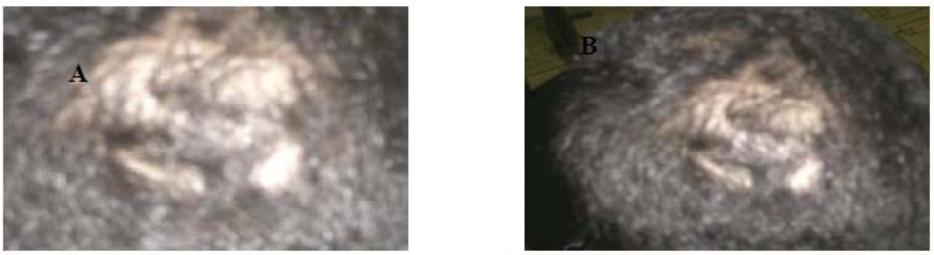

Figure 11. (A)*Tinea capitis*. Highly inflammatory form (kerion) due to dermatophytic infection, (B) after treatment.

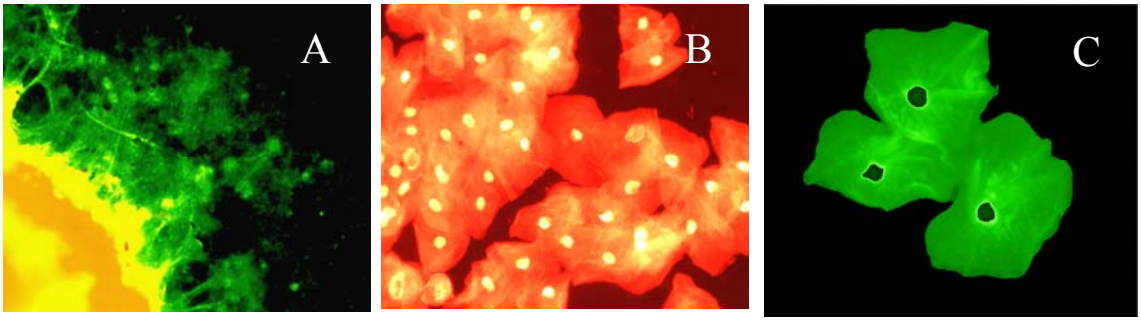

Fig. 12: Fluorescent microscope micrographs of epidermal scales stained with FDA and PI when viewed under at 20 X. {A, Condensed damaged scales infected with dermatophytic fungus which appear in green; stained with FDA as a sign of its viability. B, scales stained with PI. Note irritation, irregular and high damage appearance of cells after treatment for two weeks with clotrimazole alone (Closol). C, scales treated with Clo- Cin stained with FDA as a sign of their healthy}.

ARABIC SUMMARY

التآزر بين الكلوترمازول وزيت القرفة: سلاح (علاج) فعال معملياً وعملياً ضد بعض الفطريات الجلدية المقاومة للأدوية المتعددة

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استهدف هذا البحث تقييم تآزر كلا من الكلوترمازول وزيت القرفة ضد بعض الفطريات الممرضة لجلد الانسان والمقاومة للعديد من المضادات الفطرية المتداولة تجاريا وذلك معملياً وعملياً على بعض المرضى المتطوعين.

من أجل ذلك تم عزل اثنى وعشرون عزلة من الفطريات الممرضة للجلد واختبار حساسيتها ضد ثمانى مضادات فطرية هامة وكذلك ضد زيت القرفة ومن ثم تم اختيارأكثر العزلات مقاومة للعديد من المضادات الفطرية (عددهم اثنى عشر عزلة) وذلك لتقييم كفاءة تآزر كلا من الكلوترمازول وزيت القرفة معمليا ثم تطبيق ذلك عمليا على بعض المرضى المتطوعين وعددهم اثنين وخمسون مريضاً تم تقسيمهم عشوائيا الى ثلاث مجموعات، أولهم: مجموعة الكلوسول (محلول موضعى للكلوترمازول متداول تجاريا)، ثانيهم: مجموعة الكلو- سن (محلول موضعى يحتوى على كلوترمازول - بتركيز أقل اربعة أضعاف من المحلول التجارى- اضافة الى زيت القرفة) وثالثهم :المجموعة الضابطة وهى تتكون من الكلو- سن باستثناء الكلوترمازول، وقد تم أخذ عينتين مختلفتين من سيدة مريضة كلتا ذراعيها مصابان قد تم علاج احداهما بالكلوسول والأخرى بالكلو- سن لبيان تاثير العلاج على الأنسجة المصابة.

وقد أسفرت النتائج عن الاتى :أن التآزر بين الكلوترمازول وزيت القرفة معملياً كان مثيراً للاهتمام من حيث الكفاءة العالية ومن ثم أقل تركيز قاتل للفطريات الممرضة تحت الاختبار وذلك لاغلب العزلات(91.76%) ولم يتحقق ذلك لعزلة واحدة فقط، هذا وعلى الصعيد العملى وجد أن نسبة شفاء المرضى الذين تم علاجهم بالكلوسول 50% بينما هؤلاء الذين تم علاجهم بالكلو- سن86.4% وذلك بعد أربعة عشر يوماً من تلقى العلاج وبعد تلقى العلاج أربعة عشر يوماً أخرى وجد أن نسبة شفاء المرضى الذين تم علاجهم بالكلوسول قد انتكست بنسبة 25% مما يعنى عودة الاصابة مرة أخرى لبعض المرضى الذين تم شفاؤهم بينما الذين تم معالجتهم بالكلو- سن وجد تحسن فى نسبة الشفاء حتى100% كما وفر الكلو- سن حماية للانسجة المعالجة من التهيج وهذا ما هو معروف بأنه أحد الأعراض الجانبية لاستخدام الكلوترمازول مقارنة بتلك المعالجة بالكلوسول كما أظهرت نتائج الميكروسكوب الفلورسنتى.

وعلى هذا قد خلصت الدراسة الى الأتى: أن التآزر بين الكلوترمازول وزيت القرفة فى صورة المحلول الموضعى (الكلو- سن) كان ذا كفاءة ثلاثية، أولهم أن تركيز الكلوترمازول قد قل بأربعة أضعاف عن المحلول الموضعى(الكلوسول) المتداول فى الأسواق وثانيهم حماية الأنسجة المصابة من التهيج وثالثهم عدم عودة الإصابة بالمرض في وقت لاحق.

ولذلك فان البحث يوصى باختبار التآزر بين الكلوترمازول وزيت القرفة ضد العديد من الفطريات الممرضة الأخرى لما فى ذلك من أهمية اقتصادية وطبية.