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Topical Antifungal Herbal Formulations With Broad Spectrum Activity

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Abstract: ***Creams and Ointments containing extracts of either Mitracarpus scaber or Occimum gratissimum were formulated for possible topical application.***

In-vitro sensitivity study of the ethanol extract of Mitracarpus scaber, Occimum gratissimum oil and standard antifungal drugs (Nystatin, Ketoconazole and Fluconazole) were assessed against the clinical isolates of Candida albicans, Aspergillus niger, Trichophyton spp, Fusarium spp, Cladosporium spp and Penicillium spp using a modified cup-agar diffusion plate method. The antifungal properties of the creams and ointments were evaluated in vitro against isolated organisms using the well-agar diffusion technique. Standard creams and ointments containing clotrimazole and miconazole respectively were evaluated in parallel as controls.

The activity of the antifungal extracts competes well with that of the standard antifungal drugs. Evaluation of the topical formulations showed that the ethanol extract of M. scaber Cream has a lesser activity against the clinical isolates than the standard (clotrimazole); the Ointment could however not diffuse into the agar. On the other hand, the O. gratissimum Oil Creams and Ointments have a great activity against the isolates compared to the standards (clotrimazole and miconazole).

This study confirms the broad-spectrum antifungal effects of herbal creams and ointments containing O. gratissimum Oil.

Keyword: Candida albicans, Mold, Mitracarpus scaber, O. gratissimum Oil, Creams and Ointments.

I. INTRODUCTION

Treatment of fungal infections using conventional antifungals is increasingly facing challenges as a result of resistance of fungi to these drugs and the high cost of effective antifungal drugs[1].Recently, because of the greater concern about the side effects of antifungal, an understanding of ecology and people's desire to take greater responsibility for their own health, herbal medicine is experiencing a notable revival [2,3].

Two herbal antifungal extracts from *Mitracarpus scaber* and *Occimum gratissimum* were studied. *Mitracarpus scaber* is a plant that belongs to the family of Rubiaceae and genus of Mitracarpus. It has a dense inflorescence that is usually thick

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and clustered together with small white flowers at the leaf axils. It reproduces by seed and varies in its habitat. It is found in subtropical regions like Nigeria, Gambia, Senegal and Ghana[4]. It is commonly employed in West African herbal medicine to treat toothache, amenorrhea, hepatic diseases, headaches, dyspepsia, leprosy and venereal diseases. Other works revealed that their different extracts have broad anti-fungal and anti-bacterial activity against isolates of *C. albicans* and *Staphylococcus aureus* that cause skin infections[5,6,7,8].

Occimum gratissimum is a minty scented herbal plant that belongs to the family of Lamiaceae and found throughout the tropics and subtropics. Its different species occurs in tropical Africa and India[9,10]. They appear as a perennial terrestrial shrub of 2 m height. The leaves are oval with an attenuated, decurrent base and a coarsely serrated blade. Their flowers are white with a small calyx while their fruits are 2 millimeters long and made up of 4 spherical capsules[11,12]. It is generally used for cooking as a spice, food condiment or a source of flavor in food preparations. The essential oil of this therapeutic plant also has antibacterial, antifungal and insecticidal effects[13,14,15,16].

Based on these previous investigations, this work was carried out to formulate and evaluate Creams and Ointments containing extracts of either *M. scaber* or *O. gratissimum* oil for possible topical application.

II. MATERIALS AND METHOD

COLLECTION OF ISOLATES

A total of 25 clinical isolates of *Candida albicans* previously isolated from different clinical samples (ECS, Sputum, HVS, Urine, Palm, Groin, and Oral thrush) at Federal Medical Centre (FMC), Owerri, south east Nigeria were used for the work. Whereas, 25 isolates of molds collected from 2 clinical samples (skin and scalp), identified to specie levels at University of Nigeria Teaching Hospital (UNTH), Enugu, south eastern Nigeria were used for the research.

Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (Middle Sex-U.K) were used and prepared following the instructions of the manufacturers.

Nystatin, Ketoconazole, (MERCK, GERMANY), Chloramphenicol, (Clotrimazole cream (FIDSON, Nigeria), Fluconazole (Janssen-Cilag, India) and Miconazole ointment (FIDSON, Nigeria) were the drugs used while Dimethyl Sulfoxide (DMSO) (MERCK, GERMANY), Potassium hydroxide, Ethanol (MERCK GERMANY), Lanette wax, petrolatum, methyl paraben, sesame oil were the reagents used.

PLANT MATERIAL

Mitracarpus scaber and *Occimum gratissimum* were collected from Nsukka, Enugu State, Nigeria. The plants were identified and authenticated in the Department of Botany, University of Nigeria, Nsukka, Nigeria. Voucher specimen was deposited accordingly.

EXTRACTION OF PLANTS

Sun-dried powdered material of *Mitracarpus scaber* (500g) was extracted using cold maceration method[5] with 2000ml of ethanol. The filtrate was then exposed to air till the solvent vaporized to dryness. Thereafter, the extract (residue seen after drying) was collected, weighed and kept in a container until needed.

VOLATILE OIL EXTRACTION

Fresh leaf samples of *Occimum gratissimum* were subjected to steam distillation in a modified Clevenger-type apparatus for 3 hrs. The oil obtained was stored in a sealed glass vial. The yield was 0.3% per 100g.

MAINTENANCE AND STANDARDIZATION OF STOCK CULTURE

Isolates of *C. albicans* and molds were inoculated onto Sabouraud Dextrose Agar plates containing 500 mg/L chloramphenicol and incubated at 28˚C for 24 hours and 7 days for *C albicans* and molds respectively. The colonial growths were stored in Sabouraud Dextrose Agar (SDA) slants at 4˚C. Before use, the cultures were reactivated by culturing again into SDA containing 500 mg/L chloramphenicol and incubated at 28˚C for 24 hours and 7 days for *C albicans* and molds respectively. For standardization of *C. albicans*, overnight (18 hours) sub-cultures in Sabouraud Dextrose broth were adjusted to 90% transmittance at 530nm using distilled water. For molds, a 40mm diameter of the mycelia growth was inoculated into 2.0mL SDA plate and processed[6,17].

SCREENING STUDIES

In-vitro sensitivity study of the ethanol extract of *Mitracarpus scaber* and *Occimum gratissimum* oil and standard antifungal drugs (Nystatin, Ketoconazole and Fluconazole) were assessed against the isolated organisms using a modified cup-agar diffusion plate method[18]. A small portion of extract was dissolved in 2ml DMSO and the resulting solution diluted to a concentration of 50µg/ml using sterile distilled water. Molten SDA (20ml each) were seeded with 0.1ml of standardized cultures of fungi (Molds and *C. albicans*) respectively. A total of 5 wells, 8 mm in diameter were made in the agar using a sterile cork borer. Two drops of each of the extract and standard drugs were carefully placed into each of the wells. Two drops of 2-fold diluted DMSO was put in the center as control. The plates were left for 1 hr. at room temperature for diffusion, after which they were incubated at 28˚C for 24 hours and 7 days as the case may be. Diameters of the zones of inhibition (IZD) were measured at the end of the incubation period. The mean of duplicate determination was taken[18].

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DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) USING AGAR-DIFFUSION METHOD

These involved making two-fold serial dilutions of the herbal extracts and standard drugs with distilled water or dilute DMSO. Thereafter, 0.04ml of the different dilutions were introduced into wells bored in SDA plates, which are seeded with a standardized inoculum of the test micro-organism. After the incubation at 28˚C for 24 hrs, and 7 days for C. albicans and mold respectively, the zones of inhibition (1ZD) were measured and the MIC obtained from the intercepts on the log concentration axis of a graph of squares of inhibition zone diameter ($1ZD^2$) against log concentration[18].

FORMULATIONS

Creams and Ointments containing extracts of either *Mitracarpus scaber* or *Occimum gratissimum* were formulated following the formula in Tables 1 and 2 for possible topical application. In preparing the creams, required amount of lanette wax (emulsified wax) was melted in a glass jar (wide mouthed) and placed on a water bath. Thereafter, petrolatum and 2mls of sesame oil were added into the glass jar and melted too. The extracts were dissolved in distilled water and methyl paraben added to the mixture. These mixtures were heated to a temperature of 66˚C and then added to the melted oils at the same temperature. The mixtures (oil and water) were stirred with glass rod continuously until homogenized and cooled.

In preparing the ointments, required amount of lanette wax and petrolatum were melted in a glass jar on a water bath. The extracts were dissolved in 3.0ml of sesame oil and added. These mixtures were stirred until homogenized.

Batches	**1**	**2**	**3**	**4** (control)	**Batches**
Lanette wax (g)	2.35	2.25	2.25	2.25	Lanette wax (g)
Petrolatum (g)	3.38	3.38	3.38	3.38	Petrolatum (g)
Herbal extracts (g)	0.50	1.00	1.50	_	Herbal extracts (g)
Methyl paraben (g)	0.04	0.04	0.04 0.04		Methyl paraben (g)
Water to 15g (ml)	6.73	6.33	5.83	7.33	Water to 15g (ml)

Key (-) - No extract. Clotrimazole cream was used as the standard.

Table 1: Composition of formulated creams containing herbal extracts

Batch	**1**	**2**	**3**	**4 (control)**
Lanett wax (g)	4.5	4.5	4.5	4.50
Herbal extract (g)	0.50	1.00	1.50	-
Petrolatum (g)	7.00	6.50	6.00	7.50

Key: (-) - No Extract. Miconazole was the standard ointment used.

Table 2: Composition of formulated ointment containing herbal extract

ANTIFUNGAL EVALUATION OF THE FORMULATED CREAMS AND OINTMENTS

The antifungal properties of the various batches of Creams and Ointments were evaluated in vitro against clinical isolates of *Candida albicans*, *Aspergillus niger*, *Trichophyton spp*, *Fusarium spp*, *Cladosporium spp* and *Penicillium spp* using the well-agar diffusion technique (described as follows).

Holes were bored into already prepared SDA plates seeded with the test organisms. The different batches of formulated Creams and Ointments were introduced into separate holes. The plates were left for 1 hour at room temperature to allow for pre-diffusion, after which they were incubated at 28˚C for 24 hours (for *C. albicans)* and 7 days (for molds) respectively. Standard creams and ointments containing clotrimazole and miconazole were evaluated in parallel as controls. The inhibition zone diameters (1ZDs) of all the Cream and Ointment batches, including the controls were measured[19].

III. RESULTS AND DISCUSSION

The preliminary results (Table 3) showed that the clinical isolates were sensitive to the herbal extracts.

No	**Strain**	**Nyst**	**Keto**	**Fluco**	**A**	**B**
1	*C. albicans*	13	20	22	65	14
2	*C. albicans*	15	20	20	44	15
3	*C. albicans*	17	14	16	64	15
4	*C. albicans*	17	-	-	70	15
5	*C. albicans*	17	30	25	54	-
6	*C. albicans*	15	25	25	60	14
7	*C. albicans*	22	20	27	60	13
8	*C. albicans*	15	-	-	55	10
9	*C. albicans*	19	33	37	53	-
10	*C. albicans*	16	18	15	64	10
11	*T. soudanense*	13	35	30	54	15
12	*T. mentagrophytes*	30	35	27	55	15
13	*P. linacinum*	22	-	-	66	13
14	*Fusarium*	15	17	15	40	13
15	*Cladosporium*	11	18	17	44	15
16	*Cladosporium*	15	20	17	50	14
17	*Curvularia*	14	18	22	40	15
18	*A. niger*	15	18	15	61	15

Key: Antifungal drugs-50µg/ml each, A (O. gratissimum oil) - 50µg/ml, B (Mitracarpus scaber) -50µg/ml.

Table 3: Preliminary test on different strains of Candida albicans and molds

One is isolated from sputum, 2- oral thrush, 3- ECS, 4- HVS, 5- sputum, 6- groin, 7-palms, 8- HVS, 9-10- urine.

The MIC of ketoconazole and *M. scaber* extract showed a decrease in activity indicating antagonisms against the isolates of both molds and *C. albicans* correspondingly (Table 4). The exception is on one particular isolate of *C. albicans* where ketoconazole has activity and one mold where it has no activity at all.

The MIC of *O. gratissimum* oil against both organisms showed that it has great activity against both test organisms (Table 4). *O. gratissimum* oil exhibited a lower MIC against

C. albicans than against molds showing a high degree of potentiation. On one isolate of molds, *O. gratissimum* oil inhibited the organism completely.

Test organism	MIC (µ g/ml)		
	M. scaber	O. gratissimum oil	Keto
T. soudanense	40	0.63	1.25
T.mentagrophytes	20	0.63	5.00
A. niger	20	0.16	2.50
T. soudanense	40	-	+
C. albicans	40	0.16	5.00
C. albicans	40	0.31	2.50
C. albicans	40	0.16	2.50
C. albicans	20	0.18	0.31

Key: (+) - growth,(-) - no growth

Table 4: MIC of Extracts and Ketoconazole against selected fungal isolates

Evaluations of the topical formulations are presented in Tables 5-8. The results showed that the ethanol extract of *M. scaber* Cream has a lesser activity against the clinical isolates than the standard (Table 5); the Ointment could however not diffuse into the agar making the release rate very negligible (Table 6). In the results of the release study of Cream and Ointment containing *O. gratissimum* oil against the selected isolates, both Creams and Ointments released well and exhibited greater zones of inhibition that were even greater than the standard (Tables 7 and 8).

Isolates	Inhibition zone diameter (mm) of various cream batches				
	1	2	3	4	5(standard)
1	15	+	15	+	25
2	10	10	11	10	35
3	11	12	21	10	30
4	10	12	16	12	25
5	12	10	17	8	28
6	11	8	17	+	28
7	10	10	21	12	30
8	11	15	18	10	35
9	13	10	30	15	28
10	11	10	25	+	28
11	8	9	15	9	35
12	+	10	19	+	35
13	16	12	21	12	40
14	10	11	17	11	50
15	13	14	21	10	40
16	13	15	20	12	35
17	+	18	10	+	30
18	6	10	20	+	25
19	20	20	30	15	50
20	8	6	18	6	50
Average limit	9.65	11.6	19.1	7.6	34.10

Key: (+) – growth. Standard - Clotrimazole cream

Table 5: Inhibitory effect of Creams containing ethanolic extract of M. scaber against Candida albicans and molds

Isolates 1-10 - Candida albicans (1 is isolated from sputum, 2- oral thrush, 3- ECS, 4-HVS, 5- sputum, 6- groin, 7-palms, 8- HVS, 9-10- urine).

Isolates 11-12 - *Penicillium lilacinum*, 13-14 - *Aspergillus niger*, 15 - Fusarium specie, 16-17- Trichophyton species, 18-19 - Cladosporium specie, 20 - Curvularia specie.

Isolates	Inhibition zone diameter (mm) of various batches of Ointment				
	1	2	3	4	5 (standard)
1	+	+	20	25	30
2	+	+	+	+	25
3	+	+	+	+	30
4	+	+	+	+	35
5	+	+	+	+	35
6	+	+	+	+	25
7	+	+	+	+	30
8	+	+	+	+	25
9	+	+	+	+	30
10	+	+	+	+	40
11	+	+	+	+	45
12	+	+	+	+	35
13	+	+	+	10	30
14	+	+	+	+	25
15	+	+	+	+	28
16	+	+	+	+	40
17	+	+	+	+	27
18	+	+	+	+	30
19	+	+	+	+	50
20	+	+	+	15	45
Average activity	+	+	+	15	45

Key: (+) – growth. Standard - Miconazole ointment

Table 6: Inhibitory effect of Ointment containing ethanolic extract of M. scaber on Candida albicans and molds

Isolates 1-10 - Candida albicans (1 is isolated from sputum, 2- oral thrush, 3- ECS, 4-HVS, 5- sputum, 6- groin, 7-palms, 8- HVS, 9-10- urine).

Isolates 11-12 - *Penicillium lilacinum*, 13-14 - *Aspergillus niger*, 15 - Fusarium specie, 16-17- Trichophyton species, 18-19 - Cladosporium specie, 20 - Curvularia specie.

Isolates	Inhibition zone diameter (mm) of various cream batches				
	1	2	3	4	5 (standard)
1	22	30	39	19	20
2	30	40	42	23	24
3	21	22	30	+	20
4	29	40	45	+	21
5	32	38	41	+	22
6	30	30	40	+	23
7	29	30	41	25	25
8	28	32	38	+	23
9	28	30	30	+	24
10	30	40	35	20	23
11	29	40	35	20	22
12	22	40	39	+	20
13	31	28	40	+	25
14	25	35	36	16	25
15	25	38	40	17	23
16	27	35	40	17	20
17	30	38	41	10	24
18	27	30	40	13	25
19	28	40	43	18	20

20	20	35	38	10	26
Average activity	27.65	34.55	38.65	10..4	22.75

Key: (+) – growth. Standard - Clotrimazole cream

Table 7: Inhibitory effect of Cream containing O. gratissimum oil against Candida albicans and molds Isolates

Isolates 1-10 - Candida albicans (1 is isolated from sputum, 2- oral thrush, 3- ECS, 4-HVS, 5- sputum, 6- groin, 7- palms, 8- HVS, 9-10- urine).

Isolates 11-12 - *Penicillium lilacinum*, 13-14 - *Aspergillus niger*, 15 - Fusarium specie, 16-17- Trichophyton species, 18-19 - Cladosporium specie, 20 - Curvularia specie.

Isolates	**Inhibition zone diameter (mm) of various batches of Ointment**				
	1	2	3	4	5 (standard)
1	+	20	22	22	25
2	20	25	30	+	25
3	26	26	30	+	20
4	20	20	32	+	20
5	20	22	30	20	25
6	20	20	22	+	20
7	25	20	20	25	21
8	22	23	+	+	22
9	22	30	30	+	24
10	20	20	35	20	23
11	25	30	35	20	22
12	20	22	29	+	20
13	22	28	20	+	25
14	25	30	26	16	25
15	25	28	30	+	23
16	27	30	35	+	20
17	22	28	21	10	24
18	25	20	30	13	25
19	20	20	33	+	20
20	20	25	38	10	20
Average Activity	21.30	24.35	27.40	7.80	22.45

Key: (+) – growth. Standard - Miconazole ointment

Table 8: Inhibitory effect of Ointment containing O. gratissimum oil against Candida albicans and molds

Isolates 1-10 - Candida albicans (1 is isolated from sputum, 2- oral thrush, 3- ECS, 4-HVS, 5- sputum, 6- groin, 7- palms, 8- HVS, 9-10- urine).

Isolates 11-12 - *Penicillium lilacinum*, 13-14 - *Aspergillus niger*, 15 - Fusarium specie, 16-17- Trichophyton species, 18-19 - Cladosporium specie, 20 - Curvularia specie.

The activity of the antifungal extracts competes well with that of the standard antifungal drugs. These observations are consistent with the findings of other workers[20]. The findings of Bisignano *et al* reveals that the phenolic compound isolated from *M. scaber* extracts are used for the treatment of skin infections caused by *Staphylococcus aureus* and *C. albicans.* Sanogo *et al* also showed that different extracts of *M. scaber* exhibited broad antibacterial and antifungal activity against standard strains and clinical isolates of *Staphylococcus aureus* and *C. albicans* responsible for common skin infections. Several workers have extensively investigated on the anti-microbial activities including anti-fungal activity of *O. gratissimum* oil. Their findings reveal that the oil has activity against both molds and *Candida albicans*[21]. Our study also shows that *O. gratissimum* oil has the greatest level of activity against all the test fungal isolates.

Essential oils as well as compounds derived from them possess wide range of activities with the antimicrobial as the most studied[22]. In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria[23]. One of the potent anti-microbial compounds isolated from essential oils are lipophilic monoterpens such as thymol, carracrol, linalool, citral, geraniol and 1-8-cineole. Their applications as preservatives are widely studied[24].

It is deduced from MIC result that *O. gratissimum* oil has much greater activity on the test organisms than other test agents. Also, the *O. gratissimum* oil is more effective against *C. albicans.* This finding is in line with the findings of other researchers[25,14] and corresponds with the traditional medicinal use of the plant in some parts of the world.

The effect of Creams and Ointments containing *O. gratissimum* oil corroborated with the MIC result which revealed the great activity of *O. gratissimum* oil against the selected fungi isolates. Our work is in agreement with other similar reports[26,27,28,1]. It can be inferred from the result that creams released the extracts faster than ointments and their activity depends on the quantity of extracts released.

IV. CONCLUSION

Our present study demonstrated the antifungal potentials of ethanolic extract of *M. scaber* and *O. gratissimum* oil. *O. gratissimum* oil has a wonderful activity against fungal isolates. This preliminary data has confirmed the broad-spectrum antifungal effects of herbal creams and ointments containing *O. gratissimum* oil.

We recommend the modification of these formulations and further studies to be carried out in healthy animals using a larger population.

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