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SHRAGA™ Griseofulvin Topical for Treatment of Otomycosis: New Indication

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ABSTRACT

This paper(documented by clinical assay and Susceptibility testing)will discuss, an innovative remedy (SHRAGA™), containing Griseofulvin (GF), for treating Otomycosis , a fungal infection of the ear external auditory canal, by topical use, This composition includes: Tea tree oil (TTO) used for dissolving the (GF)in water , with its additional role of synergism effect to GF. Betamethasone (BM) which is a steroid, a synthetic glucocorticoid, known for its potent anti-inflammatory action, which also suppresses the immune response to GF. GF compound is non soluble in water and therefore was not available so far for use as topical treatment. Our methods make the facility for solubility of Griseofulvin in water for using it as a *topical drug*. *Aspergillus*. and *Candida* species are the most common isolated organisms in fungal external otitis(Otomycosis) .[i] and were eradicated by(SHRAGA™). GF alone has no any effect to *Aspergillus*. and *Candida* species. TTO originally by100% concentration has a negligible effect on *Aspergillus*, and *Candida* species which is toxic. In the contrary (SHRAGA™), using a low concentration of TTO and GF, has safe and almost 3 fold effectiveness. (SHRAGA™) is most effective for treating the majority of fungi such as *Dermatophytes, Molds and Yeasts* by topical use, whereas other remedies available in the market specialize in treating only one or at the most two fungi .

Keywords: Otomycosis, *Aspergillus fumigates, Candida albicans*: Non solubility of GF in water ,TTO,BM , synergism effect.

INTRODUCTION

Otomycosis is defined as fungal infections of the ear external auditory canal. The causative organism includes molds, yeasts *Aspergillus* and *Candida* parapsilosis and rarely dermatophytes.[1] Griseofulvin[2-5] is an antifungal agent that was isolated from the mold *Penicillium griseofulvin* in 1939. . It was the first available oral agent used for the treatment of dermatophytoses for forty years. TTO oil (*Melaleuca alternifolia*) serves also as a vehicle for Griseofulvin, for facilitating its penetration to the **hyphal** filaments and for its synergism effect to GF. The aim was to explore the therapeutic efficacy of Griseofulvin as a solution or ointment and Cream which was topically applied in patients with otomycosis, some of them resistant to treatment with the common available drugs in the market. The solubility of GF was higher in *alcohols* or in the mixtures with *higher molecular weight* carriers, such as polysaccharide.[6] .*Polysaccharide increases the fungal growth and is not compatible for this project.* Also Alcohol is not compatible. "*The severity of interaction between ethanol (alcohol derivatives)/Griseofulvin is major and may result, nausea, vomiting, diarrhea, flushing, tachycardia, and hypotension*. [9] In (SHRAGA™),

TTO with its high *molecular* weight was used for increasing the solubility of Griseofulvin in water. Moreover, tea tree oil is rarely used as a *mono therapy*, but is more effective with other antifungal agents.[7] . Furthermore, Betamethasone suppresses the *allergenicity* of Griseofulvin[8], and has no any fungal effect. .[10]

MATERIALS AND METHODS

Materials:

Shraga Cream: 0.1% Betamethasone 10% Griseofulvin, 35% Lanolin, 20% Petroleum jelly (Vaseline^{R}) , 15% Tea tree oil , and 20%Distill water.

Shraga Ear drops: 0.1% Betamethasone, 10% Griseofulvin, 15% Tea Tree oil , ,25% Petroleum jelly (Vaseline^{R}) and 50%Distill water

Methods:

Clinical assay*:* A total of 30 patients were treated by (SHRAGA™), in a cream form and as ears drops and the clinical cure was achieved after 7-10 days. The clinical evaluation of (SHRAGA™), was conducted with patients of the *department of Otolaryngology/Head & Neck Surgery, Hadassah University Hospital, (H.U.H)Jerusalem, Israel*, which was followed by an in-vitro mycology test*(*Susceptibility testing*)* held by *Department of Clinical Microbiology and Infectious Diseases of the (H.U.H)Medical Center in Jerusalem ,ISRAEL* This project clearly shows that a combination of GF and TTO was 3-fold more effective against *C. albicans ,and A. fumigatus* than pure(100%) TTO. As known GF has no any effect to *C. albicans* and *A. fumigatus* [16,20] . This increase in efficacy is the result of a synergistic effect between the agents comprised in the in vitro Mycological Test Solution, (MTS) and in clinical assay. In the "Clinical Study" section below, this amount (0.5g) of SHRAGA™, once a day is tolerable, and resulted in cure of the condition as soon as one week. Most importantly, *Candidiasis and Aspergillosis* are common fungal infections which especially affect subjects prone to the proliferation of opportunistic fungal infections. For example, subjects who are immunosuppressed. SHRAGA™is especially indicated for these patients, without using a, non-specific, systemic anti-fungal drugs .It is also intended to prevent Aspergillosis related to pneumonia, which is most dangerous and fatal in these patients.

RESULTS AND DISCUSSION

Clinical studies: A Clinical evaluation[13] of the (SHRAGA™) was done in 30 patients who had been suffering from *otomycosis* of the *department of Otolaryngology/Head & Neck Surgery (H.U.H), Jerusalem, Israel*. This study was approved by the local committee on Humans Experimentations (*Helsinki*) and each patient has signed on informed consent.

Patents: Shraga.S..USA Patent, 8,192,750, June5, 2012

PCT: WO2007/144888-June.14, 2007

Clinical protocol:

Otomicroscopic examination with typical finding of otomycosis fluffy white to off-white discharge, white hyphea, black or yellow (spores) and various degrees of inflammation sample of the discharge was taken for culture. Cleaning of the external canal was done under direct visualization using suction tips no. 5 & 7.Application of 0.5 g. of Shraga) cream by thin cotton swab. Continuation of the treatment with 0.5 cc of drops once a day, for a week. (Self administered by the patient). Reevaluation of the patient after a week by otomicroscopical examination and a sample from the EAC (external auditory canal) was taken for culture. If the clinical examination at this point showed complete recovery – the treatment was stopped. If there was still sign of infection, the treatment continued for a few days more.

Results of table C: Three subjects of group 1, were resistant to the common topical anti fungal agents of the market. But they were treated by SHRAGA ™ successfully and the clinical cure was achieved after 7 days,. One of these 3 patients (infected by both *aspergillus* and *candida*) was waiting as a candidate for *STAPEDECTOMY* for 6 months, and thanks to SHRAGA ™ had a successful surgery. Subject number 2, who had been treated unsuccessfully in a general public clinic, was recovered after seven days of treatment with SHRAGA. Clinical cure supported by mycological examination was achieved in all patients. Within 7-12 days of treatment by SHRAGA ™. There was no evidence of any side effect.

In vitro assay (Susceptibility testing):

1. Susceptibility testing for SHRAGA, Tea tree oil (TTO) against *Aspergillus fumigatus* ATCC 64086 according to the CLSI guidelines (M38-A2 molds).

Results for A: The MIC (Minimum Inhibitory Concentration) and MEC (Minimal Effective Concentration) value of Shraga drops was lower than

this of the TTO by more than 3 fold, indicating that SHRAGA is more active than the TTO alone against Aspergillus fumigatus.

2. Susceptibility testing of SHRAGA, Tea Tree Oil (TTO) against Candida *albicans* ATCC 90028 by CLSI guidelines. (M27-A3)

Results for B: TheMIC value of SHRAGA ear drops was lower than this of the TTO, by 3 fold, indicating that SHRAGA is more active than the TTO alone.

3. Susceptibility testing of Griseofulvin against *Aspergillus fumigates* and *Candida albicans*:
The drug solvent: (Griseofulvin 10%w/w , benzyl alcohol 20%w/w, dimethyl phthalate 30%w/w, ethanol 40, %w/w) compared to positive control with amphotericin B. The solvent control in this special test (water 10 % w/w, benzyl alcohol 20% w/w, Dimethyl phthalate 30% w/w, ethanol 40 % w/w) ** . **Benzyl alcohol and Ethanol, was used just for in vitro testing of GF, according to Bird et al USA Patent 3,899,578.

Results forexamination no' 3:Non antifungal activity, [16,20] measured as inhibition zone diameter, was observed in both, *C. albicans* and *A. fumigates*. (Inhibition zone diameter of 15 mm for *A. fumigates*) and fluconazole (inhibition zone diameter of 30 mm for *C. albicans)*. Please see the protocol of Susceptibility testing and table A, B, C in Annex A in the end of article ***

Susceptibility testing Methods: [18]CLSI **(Clinical and Laboratory Standards Institute**) (Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline-Second Edition. CLSI, Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline-Second Edition. CLSI document M44-A2 .Wayne,PA. Clinical and Laboratory Standards Institute. 2009. CLSI, Method for Antifungal Disc Diffusion Susceptibility Testing of nondermatophyte Filamentous Fungi; Approved guidline. CLSI document M51-A. Wayne, PA: Clinical and Laboratory Standards Institute[18]

CONCLUSION

THE NOVELTY OF THIS SHRAGA™ INVENTION:

- Anti-Dermatophytes infection for treatment of fingernails and toenails onychomycosis.
- Anti-molds infection for otomycosis
- Anti-yeasts infection for otomycosis
- Anti-inflammatory effect
- A short period of treatment, by a little amount of drug the cure was completed..
- No side effects
- Non Specific, Systemic anti-fungal drugs is needed

**** *ANNEX .A**

In vitro testing protocol

Mycology:

1.Susceptibility testing for SHRAGA ear drops, Griseofulvin, Tea tree oil (TTO) against *Aspergillus fumigatus* according to the CLSI guidelines (M38-A2 molds)

Method of testing

Griseofulvin-
Stock- 21.30 mg/ml in DMSO (21.3 mg in 1 ml DMSO).
The stock were diluted to 1:50 in RPMI (Sigma, 20µl+980µl RPMI) and 200µl of the diluted
The drugs were added to wells A1, B1.
The drug will be diluted 1:1 to final concentrations of: 213,106.5,53.25,26.6,13.3,6.6,3.3,1.6,0.8,0.4 µg/ml.
TTO (Tea Tree Oil):
200 µl (of the drug itself – not diluted) were added to the wellsC1, D1
SHRAGA ear drops container#2:

200 µl (of the drug itself – not diluted) were added to the wells E1, F1
* The substance appears to be in 2 phase forming emulsion - white opaque layer and clear transparent layer, even after vortex of 2 minutes.
Amphotericin B (AMB) – AMB (Alpharma) were checked also as positive control for the MIC procedure. 5 mg/ml (prepared on 03/08/2010) was diluted 1:312.5 in RPMI (20µl+ 6230µl RPMI) this gave a concentration of 16µg/ml then 200µl from this dilution were added to the wells G1, H1
Each well of 2-12 were filled with 100µl RPMI, then from each sample A-D column1 and serially diluted 100µl from column 1 to the 100µl RPMI in column 2, in this way I diluted serially all the samples 1:1 .
The final concentrations of AMB were:
8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156 µg/ml
The final dilutions of SHRAGA were:
1:2,1:4,1:8, 1:16,1:32,1:64,1:128,1:256,1:512, 1:1024, after the drugs were serially diluted. Then 100µl of the Candida working solution were added to each well (except the 2 negative control columns 10,12 that were added instead with 100µl RPMI).
Aspergillus fumigatus ATCC 64086: was grown for 7 days on SDA at 35^0C and then recultured on SDA plate for 7 days more prior the test in order to produce spores.
To prepare the inoculums 5 colonies (1 mm diameter) were added to 8 ml saline or sterile PBS contains 2 loops of Tween 20 and pour on top of the cultured SDA plate. The fluid was spread all over the plate and then collected into 10 ml sterile tubes, vortexed for 20 seconds and then let settle down for 5 minutes. Finally the supernatant was transfer to another tube containing RPMI and adjusted to density of 0.09-0.13 OD at 530 nm.
We got OD=0.521, this concentration was diluted 1:5 in PBS (500µl + 2000 µlPBS) and then 1:50 in RPMI (300µl + 14700µlRPMI)
Plate I plan – drug dilutions/concentrations in µg/ml .
Controls – Drug alone (without mold), No Drug (*Aspergillus* without drugs), RPMI
AMB – control for drug with MIC

	1	2	3	4	5	6	7	8	9	10	11	12
A Gris	213	106.5	53.25	26.625	13.3	6.6	3.3	1.6	0.8	0.4 D.alone	No Drug	RPMI
B Gris	213	106.5	53.25	26.625	13.3	6.6	3.3	1.6	0.8	0.4 D.alone	No Drug	RPMI
C TTO	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 D.alone	No Drug	RPMI
D TTO	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 D.alone	No Drug	RPMI
E SHRAGA	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 D.alone	No Drug	RPMI
F SHRAGA	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024 D.alone	No Drug	RPMI
G AMB	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.0156 D.alone	No Drug	RPMI
H AMB	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.0156 D.alone	No Drug	RPMI

MIC against *Aspergillus fumigatus* ATCC 64086 – Susceptibility testing for SHRAGA, , Tea tree oil (TTO) against *Aspergillus fumigatus* ATCC 64086 according to the CLSI guidelines (M38-A2 molds).Tab.le A

PRODUCT	MIC – 5DAYS	MIC (mg/ml)	MEC – 5DAYS	MEC (mg/ml)
TTO	1:16	62.5	1:32	31.25
SHRAGA	1:8	18.75	1:16	9.375
AMB	0.5 µg/ml		0.5 µg/ml	

Results:The MIC and MEC value of Shrga drops was lower than this of the TTO by more than 3 fold ,indicating that it is more active than the TTO alone against Aspergillus fumigatus.

Susceptibility testing of SHRAGA ear drops and Tea Tree Oil (TTO) against *Candida albicans* ATCC 90028 by CLSI guidelines (M27-A3)
Method of testing

Griseofulvin: Stock- 21.30 mg/ml in DMSO (21.3 mg in 1 ml DMSO). The stock were diluted to 1:50 in RPMI (Sigma, 20µl+980µl RPMI) and 200µl of the diluted

The drugs were added to wells A1, B1.

The drug will be diluted 1:1 to final concentrations of: 213,106.5,53.25,26.6,13.3,6.6,3.3,1.6,0.8,0.4 µg/ml.
SHRAGA ear drops container#2:1:2 – 2X= 1:1 = 500µl = 500µl SHRAGA
1:3 - 2X= 1:1.5 = 500µl = 333.3µl SHRAGASHRAGA + 166.7µl RPMI
1:3.5 – 2X= 1:1.75 = 500µl = 285.7µl SHRAGASHRAGA + 214.3µl RPMI
200 µl of this concentration was added to the wells A1-A6 as appeared in the plate plan below.

* The substance appears to be in 2 phases forming emulsion - white opaque layer and clear transparent layer, even after vortexing for 2 minutes.

TTO (Tea Tree Oil):
1:12 – 2X= 1:6 = 500µl = 83.3µl TTO + 416.7µl RPMI
1:14 - 2X= 1:7 = 500µl = 71.4µl TTO + 428.6µl RPMI
1:16 – 2X= 1:8 = 500µl = 62.5µl TTO + 437.5µl RPMI

This concentration was added (200µl each) to wells A7-A12 as appears in the plate plan below.
AMB – AMB (Alpharma) were prepared in DMSO and used as positive control for the MIC procedure.
5mg/ml of the AMB stock solution was diluted 1:312.5 in RPMI (20µl+ 6230µl RPMI) to give a concentration of 16 µg/ml. 200 µl (of this concentration) were added to wells G1, H1
Each well of B-D 1-12, and G-H 2-12 was filled with 100 µl RPMI medium (Sigma), then from each sample in row A 100 µl was taken and serially diluted in row B (and repeated for rows C and D) in this way the samples were serially diluted 1:1 .

The final dilution of SHRAGA/TTO appears in the plate plan below.
AMB were serially 1:1 diluted in rows G, H
The final concentrations of AMB was:
8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156 µg/ml
SHRAGA ear drop was also serially diluted as shown in the Table.
After the drugs were all serially diluted, then 100 µl of the *Candida albicans* working inocula were added to each well (except to the controls of RPMI and drug alone).

Candida albicans ATCC 90028: a fresh SDA plate that was prepared a day before the test was used. To prepare this inoculum 5 colonies (1 mm diameter) were added to 5 ml saline or sterile water to yield 0.5 MF (1-5X10^6 cells/ml).

A working suspension is made by in RPMI 1640 to 1:100 followed by dilution of 1:20. This was resulted in $5X10^2$-$2.5X10^3$ cells/ml. The best is to calculate to $2x10^3$ yeasts/ml so the final will be 10^3 yeasts+--+/ml.
In this MIC assay the inoculum concentration (in sterile RPMI) was about 0.5 MF, from the concentration 10µl were taken and added the to 990 µl RPMI 1640 (1:100 dilution) from this dilution the working solution was prepared 500µl added to 9.5ml RPMI (1:20 dilution).
Plate I plan – drug dilutions (for TTO and SHRAGA)/concentrations in µg/ml (For AMB).
Controls – Drug alone (without *Candida*), No Drug (*Candida* without drugs), RPMI
AMB – control for drug with MIC

	SHRAGA						TTO					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:2	1:2	1:3	1:3	1:3.5	1:3.5	1:12	1:12	1:14	1:14	1:16	1:16
B	1:4	1:4	1:6	1:6	1:7	1:7	1:24	1:24	1:28	1:28	1:32	1:32
C	1:8	1:8	1:12	1:12	1:14	1:14	1:48	1:48	1:56	1:56	1:64	1:64
D	1:16	1:16	1:24	1:24	1:28	1:28	1:96	1:96	1:112	1:112	1:128	1:128
E												
F												
G AMB	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.0156 D.alone	No Drug	RPMI
H AMB	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.0156 D.alone	No Drug	RPMI

Susceptibility testing of SHRAGA , Tea Tree Oil (TTO) against *Candida albicans* ATCC 90028 by CLSI guidelines (M27-A3) :(25) .Table .B

PRODUCT	MIC – 24H	MIC -24H	MIC – 48H	MIC – 48H
TTO	1:32	31.25 mg/ml	1:24	41.66 mg/ml
SHRAGA	1:12	12.5 mg/ml)	1:8	18.75 mg/ml
AMB		0.25 mg/L	0.25 mg/L	

Results: *The MIC value of SHRAGA ear drops was lower than this of the TTO by **3, fold** indicating that SHRAGA is more active than the TTO alone*

Table C: Results of clinical treatment

Group	Patient number	Duration of treatment (days)	Ear canal infection (before treatment)	Culture results (after treatment)
1	8	12	*Aspergillus* niger+ *Candida albicans*	Negative
2	4	10	*Candida albicans*	Negative.
3	18	7	*Aspergillus niger*	Negative.

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