REPORT

ASSESSMENT OF THE IN VITRO EFFICACY OF CHD-FA (CarboHydrate Derived - Fulvic Acid) AGAINST MALASSEZIA AND DERMATOPHYTE ISOLATES

FOR

Fulhold Ltd.

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CONFIDENTIAL

1.1 PHYSICAL PROPERTIES OF CHD-FA ALSO KNOWN AS FULVIC ACID

Euprotec has received 2 bottles of CHD-FA (henceforth called CHD-FA) reconstituted as a 4% solution. The solutions have been stored at room temperature in the dark since delivery. All experiments in this report have been completed using the second bottle which was received August 2008 directly from South Africa

The 4% CHD-FA solution is a yellow/brown slightly viscous solution with a strong odour and a pH of 2.1 at 25^{o}C.

2.1 METHODS

2.1.1 *Malassezia* and *Dermatophyte* Isolates

Susceptibility tests were performed on the following isolates: *Malassezia dermatis, Malassezia furfur, Malassezia globosa, Trichophyton rubrum, Trichophyton mentagrophytes, Trichophyton mentagrophytes interdigitale, Epidermophyton and Microsporum canis.*

The Malassezia strains were grown on modified Leeming Notman agar (MLNA) containing 1% peptone, 1% glucose, 0.2% yeast extract, 0.8% ox bile, 1% glycerol (v/v), 0.5% tween 60 (v/v), 1.5% agar and overlaid with olive oil to support their growth at 30°C for 96 hours. Whereas, the Dermatophytes were grown on Sabouraud dextrose agar (Oxoid; 6.5% w/v) for 7 days at 30°C.

2.1.2 Media

The Malassezia strains were prepared in Leeming Notman broth (1% peptone, 1% glucose, 0.2% yeast extract, 0.8% ox bile, 1% glycerol (v/v), 0.5% tween 60 (v/v) which was also supplemented with olive oil (2% v/v) to support the growth of these lipophilic yeasts. The Dermatophyte isolates were prepared in Iso-sensitest medium (Oxoid) as per the manufactures instructions.

2.1.3 Preparation of the Inoculum

a) All Malassezia strains were cultured in ambient air at 30°C on modified Leeming Notman agar for 96-120 hours before testing, to ensure their purity. All Dermatophyte isolates were cultured on Sabouraud dextrose agar in ambient air at 30°C for 7 days before testing.

b) The inoculum for each strain was prepared by picking distinct colonies from the culture plates and suspending them in 4 ml of either phosphate buffered saline (PBS) for the Malassezia strains or Iso-sensitest media for the Dermatophyte strains.

c) The inoculum was completely resuspended by vigorous shaking on a vortex mixer for 15s. Any remaining particulates were then removed by filtering the inoculum through a sterile fine weaved gauze.

d) The yeast count was then determined using a counting chamber and adjusted to give a final count of $1x10^4$ conidia/spores per ml of suspension.

2.1.4 Assay Conditions

Sterile plastic, disposable, microtitration plates with 96 flat-bottom wells were used.

STEP 1 Addition of CHD-FA

The stock solution of CHD-FA contains 4% of the native compound.

For each strain tested, 100µL of media was added to each well 1-12. 100µL of 4% CHD-FA was then added to wells in column 1 to give a final dilution of 2%. 100µL amounts were then taken from wells in column 1 and diluted two fold by transferring them to column 2 with a multichannel pipette (± 2% coefficient of variation). 100µL samples were then removed from wells in column 2 and transferred to column 3, and so on through to column 10. The last 100µL of drug is discarded. Column 11 is a positive control containing no CHD-FA and column 12 is a negative control containing diluent only. CHD-FA has a native pH of 2.1. In some instances, this was adjusted using hydrochloric acid to give a stock solution of 4% CHD-FA with a buffered pH of either 5 or 7.

STEP 2 Addition of *Malassezia* or *Dermatophyte* strains

100µL of the diluted inoculum suspension in either modified Leeming Notman broth or Iso-sensitest broth is added to the appropriate wells. This produces a well containing 200 µL final volume (made up of 100µL diluted CHD-FA or diluents and 100µL of inoculum at $1x10^4$/ml in the appropriate broth for the strain or broth alone).

STEP 3 Incubation of Plates

All plates were incubated at 30^oC in an air and darkened incubator for 96-120 hours.

STEP 4 Reading of Plates

Plates were read visually with the endpoint taken as the lowest concentration of drug that inhibited growth by 50% of that of the drug free control.

3.1 RESULTS

3.1.1 MICs against CHD-FA

MICs demonstrated that CHD-FA at 4%, inhibited the growth of *Malassezia* and *dermatophytes* for at least 96 hours. The MIC values for CHD-FA are detailed in table 1.

Table 1 Efficacy of CHD-FA against *Malassezia* and *Dermatophytes*

Isolate Species	Isolate number	pH	MIC (%) CHD-FA
Malassezia dermatis	1	2.1	0.06
Malassezia dermatis	1	5.0	0.125
Malassezia dermatis	1	7.0	0.25
Malassezia furfur	1	2.1	0.125
Malassezia furfur	1	5.0	0.5
Malassezia furfur	1	7.0	1.0
Malassezia globosa	1	2.1	0.031
Malassezia globosa	1	5.0	0.06
Malassezia globosa	1	7.0	0.125
Trichophyton rubrum	1	2.1	0.2
Trichophyton rubrum	2	2.1	0.4
Trichophyton mentagrophytes	1	2.1	0.2
Trichophyton mentag/interdigitale	1	2.1	0.2
Epidermophyton floccosum	1	2.1	0.2
Microsporum canis	1	2.1	Resistant

SUMMARY

- CHD-FA inhibits the growth of *Malassezia dermatis*, *Malassezia furfur* and *Malassezia globosa* when used as a 0.125% solution at its native pH of 2.1 *in vitro*

- CHD-FA is effective as an antifungal agent against *Malassezia* strains *in vitro* whether examined at its native pH of 2.1 or buffered to pH 5.0 or 7.0. However, CHD-FA activity is best observed at its native pH of 2.1.

- CHD-FA inhibits the growth of all dermatophyte strains tested, except *Microsporum canis*, when used as a 0.2-0.4% solution.

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