Technical Report:

Spira-Wash Gel™ Wound Care Base – an Antimicrobial Evaluation

Abstract: Spira-Wash™ Gel is a water-washable wound care base containing Meadowsweet Flower Extract, which is rich in phenolic compounds, salicylates and flavonoids. They can aid in the wound healing process due to their anti-inflammatory and antimicrobial properties. This study examined the antimicrobial activity of Spira-Wash Gel by using two different *in vitro* antimicrobial assays: zone of inhibition (ZOI) and time-kill. Wound pathogens (*C. albicans, E. coli and P.aeruginosa*) and antibiotic resistant strains (MRSA and VRE) were tested. The ZOI assay indicated that Spira-Wash Gel was more effective against bacteria over fungi, with similar ZOI values for all four tested bacteria (average of 6-8 mm). It reduced the initial inoculum of *E. faecium* and *S. aureus* by 2-$\log_{10}$ in the first 2h of treatment, promoting death within 24h. *E. coli* and *P. aeruginosa* were completely eliminated within 1h of the time-kill assay. These results support the use of Spira-Wash Gel for wound management due to its high potential for inhibiting the growth of bacteria, with stronger action against Gram-negative bacteria.

Introduction:

Wounds are physical injuries that result from an opening or break in the skin. Wound healing starts from the moment of injury with a complex process aimed at restoring the function and integrity of damaged tissues (Rawat *et al*., 2012). It is categorized into three overlapping phases: inflammation (0-3 days), cellular proliferation (3-12 days) and remodeling (3-6 months) (Schmidt *et al*., 2009).

For centuries, plants have been used to treat several diseases worldwide. Recently there has been a dramatic increase in the usage of natural medicines in the United States. The application of plant extracts or plant-derived compounds has gained valuable momentum in the treatment and management of wounds (Suntar *et al*., 2012).

Many plants have been reported to have antimicrobial activity against a wide range of skin pathogens and are beneficial for wound healing. However, only a few studies have successfully incorporated natural ingredients into wound dressings, among which only a minority have led to commercially-available antimicrobial dressings, such as AmeriGel® (oak extract), MediHoney™, l-Mesitran® and Activon (honey) (Tan *et al*., 2013).

Over the last few years, there has been an increasing interest in Meadowsweet Flower Extract (Filipendula ulmaria (L.) Maxim) due to its high content of phenolic compounds, which show the potential to inhibit activity of various pathogenic microorganisms (Gniewosz *et al.*, 2014). The phenolics together with salicylates and flavonoids also make the extract an excellent anti-inflammatory agent (Harbourne *et a*l., 2009). It has been shown that many plants with anti-inflammatory properties most likely act through an antioxidant protection mechanism to facilitate wound healing (Suntar *et al*., 2012).

Spira-Wash Gel is a blend of Meadowsweet Flower Extract in a polyethylene glycol (PEG) ointment base. SpiraWash-Gel is a soft, adherent gel, which is easily water washable. It has potential use in dermatologic formulations for different applications, such as wound care, an occlusive agent, a topical humectant or infection treatment (PCCA, 2013).

The objective of this study was to examine the antimicrobial efficacy of Spira-Wash Gel, against selected bacteria and fungi through various *in vitro* microbiological assays. These specific pathogens were chosen due to their high incidence of occurrence in a variety of wound types.

Methodology:

Materials: Spira-Wash Gel (lot number 6149240) was obtained from PCCA (Houston, TX, USA) as a 100 mg base. Ciprofloxacin, fluconazole (both from Sigma-Aldrich) and 0.01% HOCl were supplied by EPS.

Microbial Strains: *Candida albicans* ATCC 10231, *E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853, *S. aureus* (MRSA) ATCC 33591 and *E. faecium* (VRE) ATCC 51559 were obtained from American Type Culture Collection (Manassas, VA), while *E. faecium* (VRE) 1674614 clinical isolate was from the EPS collection (used only in the time-kill assay in place of the ATTC strain). All strains were maintained as frozen glycerol stocks at -80°C. Working stocks were streaked onto agar plates with the appropriate media (Tryptic Soy Agar for bacteria and Sabouraud Dextrose Agar for fungi) and grown at 37°C. Liquid cultures were inoculated from working stocks and grown at 35°C on an orbital shaker (ZOI assay) or isolated colonies were suspended in 3 mL of sterile water to approximately 10^8 CFU/mL (time-kill assay).

Zone of Inhibition (ZOI) Assay: Antimicrobial activity was measured using the method designed by Holder & Boyce (1994) with minor modifications. The tested microorganisms were sub-cultured from agar plates into liquid media (5 mL) and grown at 37°C for 4h. The suspensions were adjusted to an optical density (O.D.) matching a 0.5 MacFarland standard solution (Abs. = 0.13 at 600 nm; ~10^8 CFU/mL) using sterile phosphate buffered saline (PBS). Bacterial lawns were grown on appropriate agar plates and inoculated using the diluted cultures. The inoculated agar plates were punched to form wells (diameter = 6 mm) where 100 µL of reference antibiotics and Spira-Wash Gel were placed, as one sample/well. After 16h of incubation at 37°C, the diameter of the zone of inhibition for each well was measured in millimeters with a metric ruler. The assay was undertaken in triplicate for Spira-Wash Gel and three concentrations of the reference antibiotics were tested as dose-responsive positive controls (1 µg/mL,10 µg/mL, and 100 µg/mL).

Time-Kill Assay: Bacterial working stocks were added to tubes containing Spira-Wash Gel (90% in sterile water) and the antimicrobial control (0.01% HOCl) to achieve a final inoculum of 10^6 CFU/mL. The tubes were incubated at 37ºC and duplicate aliquots were taken from each tube at specific intervals of 1, 2, 4, 8 and 24 hours. Each aliquot (100µL) was serially diluted in PBS and plated onto Brain Heart Infusion (BHI) Agar for *E. faecium* or onto Tryptic Soy Agar (TSA) for

S. aureus, E.coli, or P. aeruginosa. Plates were incubated for 24 hours at 37ºC and colonies were counted. Final colony counts are reported as CFU/mL and graphed on a Log_{10} scale. The quantification limit (LOQ) was 100 CFU/ml (Gregoire *et al*., 2010).

Results and Discussion:

Zone of Inhibition (ZOI) qualitatively evaluates the ability of antimicrobial substances to inhibit microorganism growth. It is a quick and easy way to compare inhibitory activity. Spira-Wash Gel showed excellent antibacterial activity, and provided similar average ZOI values against all four bacterial strains. No ZOI was detected for *C. albicans* (Table 1). Considerably weaker activity of Meadowsweet Flower Extract against fungi was reported by Gniewosz *et al.* (2014). Results for the reference antibiotic are in accordance with literature, validating the assay (Tables 2 and 3) (NCCLS M27-A, M100-S21).

When assessed by the time-kill assay, Spira-Wash Gel presented a gradual decrease in viable cell counts of *E. faecium* and *S. aureus*, producing a reduction of 2-log_{10} in the initial inoculum of both bacteria in the first 2h of treatment, and completely killing them at 24h. *E. coli* and *P. aeruginosa* were completely eliminated within 1h (Figure 1). The antimicrobial control (0.01% HOCl) exterminated all bacteria within 1h of test initiation (data not shown).

Table 1: *Zone of inhibition (ZOI) values for Spira-Wash Gel vs. bacteria and fungi.*

Spira-Wash Gel ZOI (mm)	Average	S.D.	*n*
E. facecium	6	1	3
E. coli	8	1	3
S.aureus	8	2	3
P. aeruginosa	7	1	3
C. albicans	0	0	3

Tables 2 and 3: *Zone of inhibition (ZOI) values for ciprofloxacin vs. bacteria and for fluconazole vs. fungi.*

	Ciprofloxacin ZOI (mm)		
	1 µg/mL	**10 µg/mL**	**100 µg/mL**
E. faecium	0	6	12
E. coli	19	22	29
S. aureus	11	22	29
P. aeruginosa	16	24	32

	Fluconazole ZOI (mm)		
	1 µg/mL	**10 µg/mL**	**100 µg/mL**
C. albicans	0	7	22

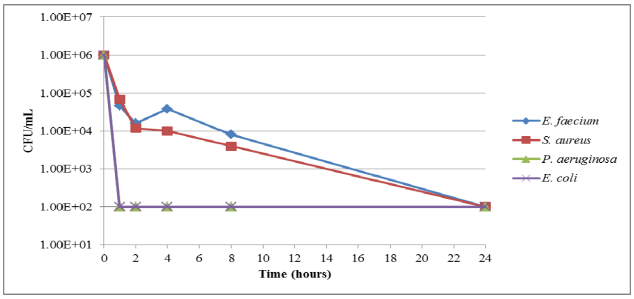

Figure 1: Average CFU/mL values for *Spira-Wash Gel vs. bacteria.*

Conclusions:

The antibacterial activity of Spira-Wash Gel was presumably caused by high levels of phenolic acids and flavonoids in its plant extract ingredient. Moreover, Gram-negative bacteria demonstrated greater susceptibility to the base than Gram-positive bacteria. Spira-Wash Gel, which is incorporated with Meadowsweet Flower Extract, is a promising anti-infective base vehicle. It exhibits positive antimicrobial properties, inhibiting the growth of bacteria to a higher degree than fungi. This study demonstrates the effectiveness of Spira-Wash Gel as an ideal vehicle, for treating a variety of bacterial wound infections.

Financial Disclosure: PCCA contracted Emeryville Pharmaceutical Services (EPS, Emeryville, CA) to conduct this study. EPS has no proprietary or financial interests in the test products, or equity interest in PCCA, the sponsor of the study.

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