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Potassium Isomerized Linoleate Satisfies EPA MB-35-00 for Surface Disinfection of *Candida auris* (AR-0381) and *Candida albicans* (ATCC 10231)

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Abstract

Long-standing United States Environmental Protection Agency regulatory perspectives hold that all plant oil salts lack direct antimicrobial capacity. We show that one plant oil salt, potassium isomerized linoleate, a C18 conjugated diene (the potassium salt of isomerized linoleic acid (UNII: 70S2158RCI)) at 0.42M exceeds the required 5 log kill rate within 1 minute for *Candida auris* (AR-0381). Similar testing of this plant oil salt against *Candida albicans* (ATCC 10231) also demonstrated greater than 5 log kill rates at 1 minute of exposure. In the case of *C. albicans*, 5 log kill rates could be obtained at concentrations as low as 98 mM when extending exposures to 10 min. Potassium hexadiene (potassium sorbate), another conjugated diene, had negligible kill rates when tested at the same pH conditions and concentrations as potassium isomerized linoleate. The presence of the conjugated diene alone did not elicit any rapid antimicrobial effects.

Keywords: *C. auris; Potassium Isomerized Linoleate; Surface Sanitization; EPA MB-35-00*

Introduction

Control of the emerging threat *Candida auris* rests with isolation and disinfection [1-9]. Although *C. auris* is primarily associated with severe, life-threatening, invasive infections, including wound and bloodstream infections, it also appears to readily colonize the skin [10]. Person-to-person transmission upon close contact between infected and non-infected individuals is possible, with as little as 4 hours being required for the organism to successfully colonize a new host [11]. Pulmonary colonization of respirator-dependent patients poses serious treatment limitations due to the shared capacity of *Candida* and *Pseudomonas* species to form biofilms [12-16], with antimicrobial resistance further complicating treatment strategies [3,4,11,17].

Efforts to contain *C. auris* include surface [18-21] and human skin disinfection methods, both of which have proven challenging. Long exposures and microbial resistance, with the latter even evolving during the treatment, have made successful treatment difficult [4,7,8,22]. All this contributes to the established 40-60 % lethality in immunocompromised patients. According to the United States Centers for Disease Control and Prevention (CDC), *C. auris* qualifies as an emerging global threat.

Current regulatory guidance from the Environmental Protection Agency (EPA) states: "In 1988, the EPA determined that soap salts have 'no independent pesticidal activity' in antimicrobial products, and must be classified as inert ingredients in those products (please see 40 CFR 153.139.)… Antimicrobials that still contain soap salts as active ingredients are considered misbranded… [23]".

Since this determination in 1992, studies published within the US patent literature have indicated that isomerized linoleate, a plant oil salt, displays broad-spectrum antimicrobial inhibition activity towards gram-positive and gram-negative bacteria, yeast, and fungi [24-26]. The microorganisms it reportedly exerts its antimicrobial efficacy upon include antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus* and vancomycin-resistant *Enterococcus* species, as well as *Candida albicans* [24]. It appears to us that the mechanism of action may be outside of the known mechanisms classically used by microbes to develop resistance [27]. With similar homologies to the Diffusional Signal Factor family of quorum sense agents [43], it is possible to see both the disruptive nature a salt soap with these similar side chains may cause in the rapid action, but also the long-term regulatory effects the degradation products could contribute.

While first identified as a mutagenic inhibitor found in charred hamburger in 1979 [28], isomerized linoleic acid sells throughout the world for its capacity to enhance meat and dairy products [2,29-32]. Sold as a nutritional supplement, people consume the oil alone for weight loss and to increase muscle mass, as well as due to its alleged anti-mutagenic effect [33,34]. We have learned that potassium isomerized linoleate has extraordinary rapid antimicrobial capacity.

We limited our study to the US EPA protocol MB-35-00, which specifies *C. auris* AR-0381 as the sole strain to test for US labeling for *C. auris* disinfection. The current standard requires a 5 log kill rate against this strain of *C. auris* (AR-0381). We adapted the MB-35-00 protocol to *C. albicans* (ATCC 10231), a yeast common in package spoilage of food and cosmetics [35]. A recent publication suggests the use of *C. albicans* as a surrogate for *C. auris*, which led us to include data we obtained on *C. albicans* [18].

We followed the required EPA protocol MB-35-00 for testing disinfectants against *C. auris* (US EPA, 2017, 2020) using the EPA-specific isolate AR-0381. To develop this protocol, we used *C. albicans* (ATCC 10231). Potassium hexadiene, also known as potassium sorbate, a common preservative according to E-200 classification, more specifically, E-202 based on the European system, appears in food products, beverages (wine), and skin care products [39]. This class of sorbate salts contains a central four-carbon conjugated diene structure that lacks the carbon arms of potassium isomerized linoleic acid. To identify whether the conjugated carbons themselves were sufficient, we tested this compound using the same protocol.

Materials and Methods

Potassium isomerized linoleate production

Potassium isomerized linoleic acid was prepared using potassium hydroxide purchased from Spectrum Chemical (New Brunswick, NJ, USA) and isomerized linoleic acid from Quanao Biotech Co., Ltd. (Shaanxi, China) and Stepan Co. (Northfield, IL, USA). We also prepared and tested this salt using in-house isomerized linoleic acid prepared from safflower purchased from Jedwards (Stockton, MA, USA). The final solution contained 0.42 M potassium isomerized linoleate, 15 mM aspartic acid, and 2.2 M ethanol. We tested five batches produced with different sources of isomerized linoleic acid (test substances 1-5).

Antimicrobials against C. auris based on the MB-35-00 protocol

The EPA test protocol for testing disinfection of hard surfaces contaminated with *C. auris* (AR-0381) with antifungal resistance included an overnight culture of *C. auris* (AR-0381) shaken at 200 rpm and 30°C. From this culture, 8 mL were harvested by centrifugation, re-suspended in 2 - 3 mL phosphate-buffered saline (PBS), and mixed with a 3-part soil load including bovine serum albumin (BSA), yeast extract, and mucin. Metal carriers (10 mm) were inoculated (10 µL culture + soil load) and dried in a desiccator under vacuum (~70 min). The disk surface was coated with the test substance at 0.42 M (50 µL) for up to 10 min. Following incubation, the carriers were transferred to 10 mL neutralization solution (Sabouraud Dextrose Broth [SDB], a common yeast-mold growth medium) for subsequent harvesting on membrane filters. These were then transferred to Sabouraud's dextrose Emmon's agar (SDEA) plates and cultured for 120h at 31°C. Controls consisted of PBS (50 µL) applied to inoculated metal disk carriers, subsequently transferred to 10 mL neutralization solution (SDB), serially diluted in PBS, and plated directly onto SDEA plates, as indicated in figure 1.

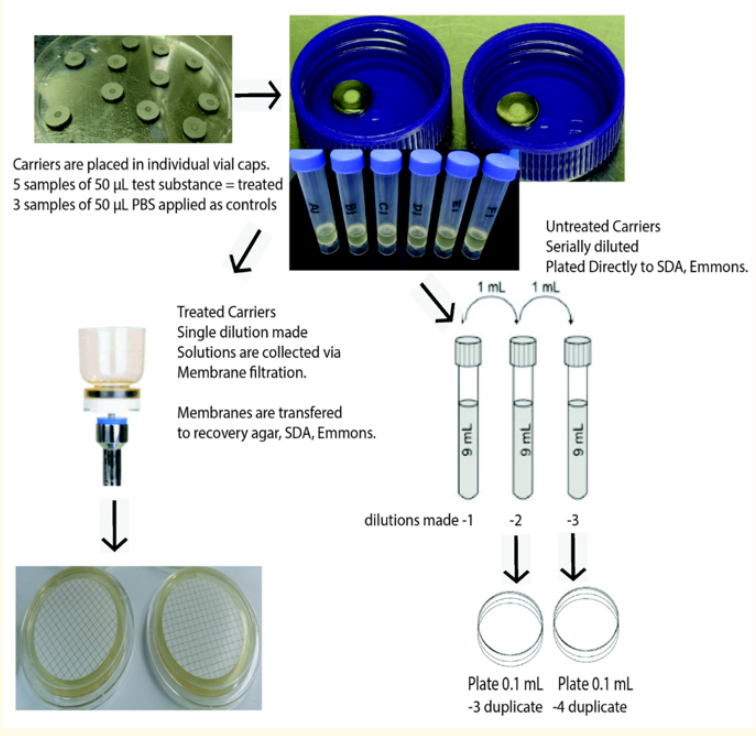

Figure 1: *EPA protocol SOP MB-35-00 begins with 10 mm stainless steel carriers inoculated with 10 µL culture in soil load. This protocol comprises all neutralization media for the carriers tested, but only a fraction of the control.*

Strains and materials

The CDC Isolate Bank (Atlanta, GA, USA) graciously provided *C. auris* (AR-0381). We purchased *C. albicans* (ATCC 10231) from ATCC (Gaithersburg, MD, USA).

Flat metal carriers were purchased from Pegen Industries (part number #430-107L). We prepared and qualified these per EPA protocol (2017).

We prepared soil stocks with yeast extract powder (Ref RM027) purchased from Himedia, BSA (A2153) purchased from Sigma Life Science, and gastric mucin (cat# HY-B2196/CS-7626) purchased from Med Chem Express per SOP MB-35-00 (US Environmental Protection Agency, 2017).

Cells were removed from growth culture media at 10,000 × g for 10 minutes in screw-top-cap vials purchased from Heathrow Scientific (Item HS100600) using a micro-centrifuge obtained from Changzhou Jintan Sanhe Instrument Co., Ltd. (Model TG16-W). The optical density of the inoculate at 600 nm (OD600) was determined to be between 15 - 19 with an Ultrospec10 Cell Density Meter from BioChrom (Holliston, MA, USA) prior to dilution in soil load. Samples were diluted 1:10 in PBS to obtain absorbance readings between 1.5 and 1.9.

Treated yeasts were collected using a combination of a Rocker magnetic filter holder (Cat # 200300-01) and 47 mm polyethersulfone (PES) membranes with a 0.45 micron pore size obtained from Sterlitech (Catalog # PES4547100). Magnets were used to hold the carriers in place during pouring.

We purchased anhydrous dextrose (Item NCM01216A), casein peptide type 1 (Item NCM0120A), and meat peptone no 3 (NCM0246A) from Neogen Culture Media (Lansing, MI, USA). We purchased agar from Himedia (RM201) (L. B. S. Marg, Mumbai, India). To prepare SDEA, 20.0 g of dextrose, 5.0g of casein peptone, 5.0g of meat peptone, and 15.0g of agar were dissolved in water and filled up to 1L, fused and sterilized per the common method.

Common reagents

We generated stock solutions of PBS (10× and 1×) using sodium chloride (IS28090) from Innovating Science (Avon, NY, USA), potassium chloride from Bearclaw Sales (Cooke City, MT, USA), as well as dibasic anhydrous sodium phosphate (89-1442) and monobasic potassium phosphate (88-4250) from Carolina Biological Supply Company (Burlington, NC, USA). We purchased SDB (Himedia Ref GM033) from Weber Scientific (Hamilton, NJ, USA) and CHROMagar Candida (Ref CA222) from local distributors.

Purified water that fulfilled the USP standards for endotoxin-testing <USP85>, bulk water <USP645>, and total organic carbon content <USP643> was produced in-house and was used for all media and reagent preparations.

Results and Discussion

Potassium isomerized linoleate showed greater than 5 log kill rates for both *C. albicans* and *C. auris* for all carriers at 1 min, using two lots manufactured separately. This was in accordance with the US EPA standards for *C. auris* disinfection (EPA, 2020). A 10-min test period, the longest time allowed per SOP, was associated with greater than 5 log kill rates. In fact, kill rates ranged from 5 to 7 logs in all runs during testing (Figure 2-4). Potassium sorbate kill rates were limited to less than 1 log in the case of both organisms.

Similar testing of this plant salt against *C. albicans* (ATCC 10231) also demonstrated greater than 5 log kill rates at 1 minute. In the case of *C. albicans*, 5 log kill rates could be obtained at concentrations as low as 98 mM when extending exposures to 10 min, as shown in figure 2-4. Kill rates of potassium isomerized linoleate prepared in-house were like those of potassium isomerized linoleate made from starting materials purchased from Quanao Biotech Co., Ltd., China or Stepan, USA.

Assay	Batch	Exposure (min)	Control Disks	Density (log)	Test Disks	Density (log)
1	1	10	n = 2	6.2, 6.6	n = 2	All No Growth
2	2	2	n = 3	6.2, 6.1, 6.3	n = 3	All No Growth
	3	2			n = 7	All No Growth
3	3	2	n = 3	6.6, 6.5, 6.6	n = 7	All No Growth
4	4	1	n = 3	5.2, 5.4, 5.4	n = 5	All No Growth
	5	1			n = 5	All No Growth
Potssium Sorbate		1			n = 2	4.8, 4.9

Figure 2: *Shown are the five separate batches of potassium isomerized linoleate (442 mM) exhibiting 5 log suppression of Candida albicans at exposures of 1 to 10 min. Potassium sorbate shows less than 1 log suppression upon 10 min of exposure.*

Assay	Batch	mM	Exposure (min)	Control Disks	Density (log)	Test Disks	Density (log)
1	1	148	10	n = 2	7.2, 7.1	n = 2	No Growth
	1	126	10	n = 2	7.2, 7.1	n = 2	No Growth
	1	98	10	n = 2	7.2, 7.1	n = 2	2.4, 1.0
	1	88	10	n = 2	7.2, 7.1	n = 2	4.6, 5.2
	1	63	10	n = 2	7.2, 7.1	n = 2	5.7, 5.5

Figure 3: *Shown are varying molarities of potassium isomerized linoleate. Five log average suppression of Candida albicans is seen after 10 min of exposure at concentrations as low as 92 mM.*

Assay	Batch	Exposure (min)	Control Disks	Density (log)	Test Disks	Density (log)
5	1	10	n = 3	5.0, 5.0, 5.5	n = 9	All No Growth
6	1	10	n = 3	5.7, 5.7, 5.5	n = 7	All No Growth
7	1	10	n = 3	6.1, 5.9, 5.9	n = 7	All No Growth
	2	10			n = 7	All No Growth
8	1	2	n = 3	5.7, 6.8, 5.9	n = 5	All No Growth
	2	2			n = 5	All No Growth
9	4	2	n = 3	5.7, 5.7, 5.7	n = 5	All No Growth
	5	2			n = 5	All No Growth
Potssium Sorbate		2			n = 3	5.0, 4.9, 4.9
10	1	1	n = 3	5.7, 5.9, 5.9	n = 5	All No Growth
	2	1			n = 5	All No Growth
	4	1			n = 5	All No Growth
	5	1			n = 5	All No Growth

Figure 4: *Shown are five separate batches of potassium isomerized linoleate (442 mM) with 5 log suppression of Candida auris at exposures of 1 to 10 min.*

Conclusion

Potassium isomerized linoleate possesses the capacity to combat a growing number of antibiotic-resistant species, and it has been demonstrated to be effective against organisms with at least three separate mechanisms of antibiotic resistance [25,26]. This surfactant may offer an effective disinfectant devoid of the need for hazmat precautions.

The capacity of potassium isomerized linoleate as a soap may involve undiscovered mechanisms. Potassium hexadiene (potassium sorbate), a preservative commonly used in food, beverage, and cosmetic applications, also contains a centrally located, conjugated diene; however, it had negligible kill rates at the same pH and concentrations as the potassium isomerized linoleic acid tested. The presence of the conjugated diene and cation-carboxyl groups did not produce a rapid antimicrobial effect.

Micelle formation of the soap in various conformations depending on the lipid portion of the oil, may contributes to the rapid membrane permeability and cell lysis. Detergent lysis along is often slow, however with the added alkalinity, we provide the membrane with more pressure to balance the ions, leading to lysis. The permeability through the various outer cell protection systems, such as chitin, and the known concentration of mannan in yeasts cell walls seems to define its usefulness for both rapid and long-term effects.

While we limited these studies to surface disinfection of non-porous surfaces, we know that similar preparations are cosmetically acceptable and sold on the open market. Cosmetic uses include application to the hair, face, skin, mouth, nasal cavity, and vagina [42]. These qualities would suggest a more pleasant experience for patients, if ever employed to treat infections with *C. auris* in humans.

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Conflict of Interest

The authors declare that this study received funding from David G. Changaris, MD, PSC, through Ceela Naturals, LLC, both of which are wholly owned by David G. Changaris, MD. The funder had the following involvement with the study: David G. Changaris, MD, BS, chose the EPA guidelines, and he was involved in the study design of the remaining components. The funder helped with the analysis and interpretation of the data, the writing of this article, and the decision to submit it for publication. Ms. Carenbauer was responsible for the implementation of the EPA protocols and collection and analysis of the data presented herein. She is employed by Ceela Naturals, LLC, and David G. Changaris, MD, PSC.

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