Ethnobotany and the discovery of anti-infectives for the post-antibiotic era

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Quave Research Group

Medical Ethnobotany & Anti-Infective Drug Discovery

Casandra L. Quave, Ph.D.
Personal/Professional Financial Relationships with Industry

External Industry Relationships	**Company Name**	**Role**
Equity, stock, or options in biomedical industry companies or publishers	PhytoTEK LLC Lifestory Health	CEO/CSO SAB
Board of Directors or officer	PhytoTEK LLC	CEO/CSO
Royalties from Emory or from external entity	None	
Industry funds to Emory for my research	The Coca Cola Company; NatureX; Bionorica SE	PI
Other		

Overview

- Challenges presented by antibiotic resistance
- Ethnobotany as a key piece to the toolkit for drug discovery
- Examples of discoveries in this arena:
  - Biofilm inhibitors
  - Quorum sensing inhibitors
  - Resistance modifying agents

A Dance with Death:
Birth defects, amputation & infection

Diagnosis: Congenital absence of the right fibula, shortened tibia and femur, and pseudarthrosis of right ankle

You only live twice: Once when you are born and once when you look death in the face.

-Ian Fleming, *You Only Live Twice*, 1964

Early days of adventure in Florida…from the swamps to the high seas

Nature, science & medicine

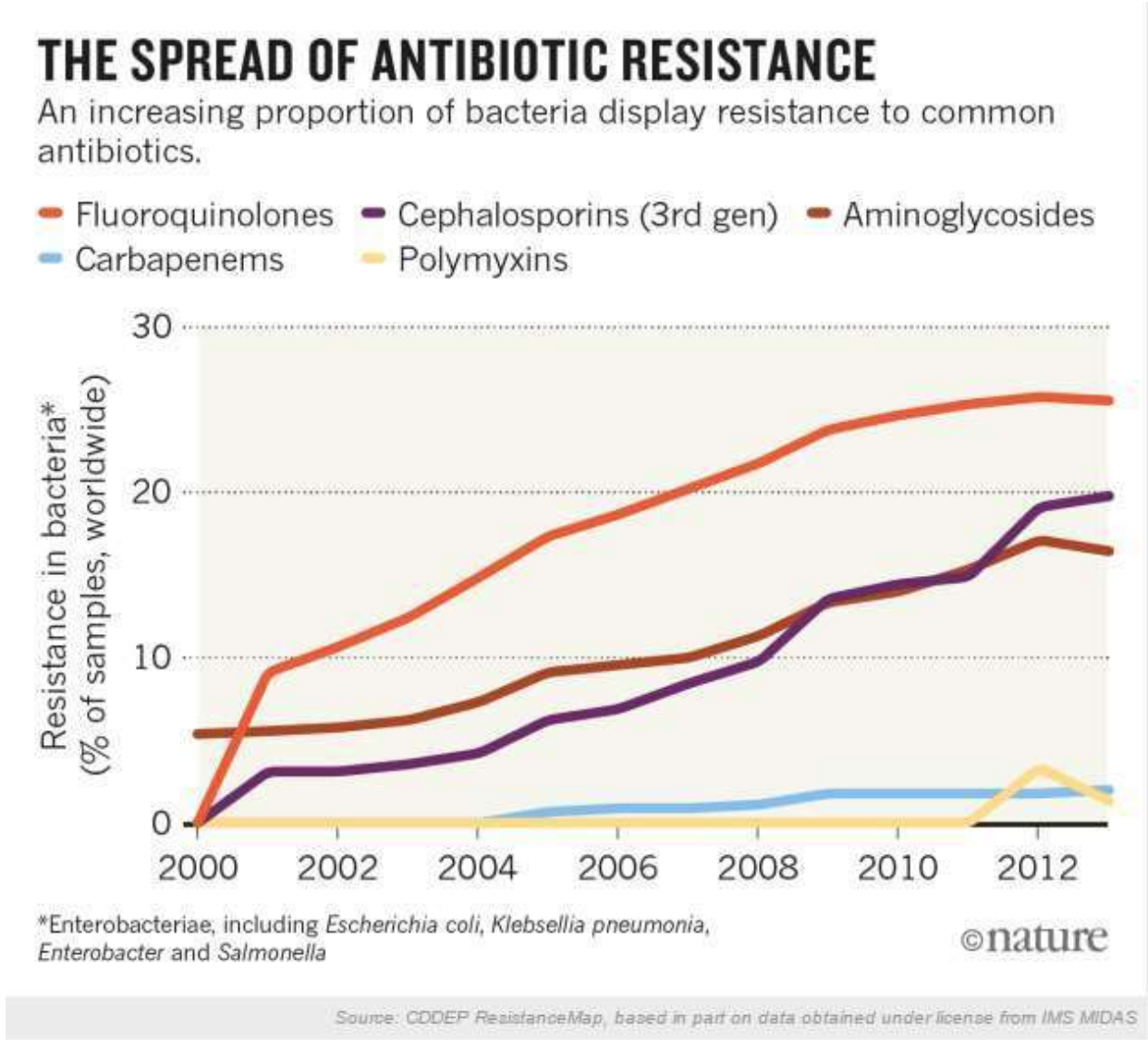
Emergency Room

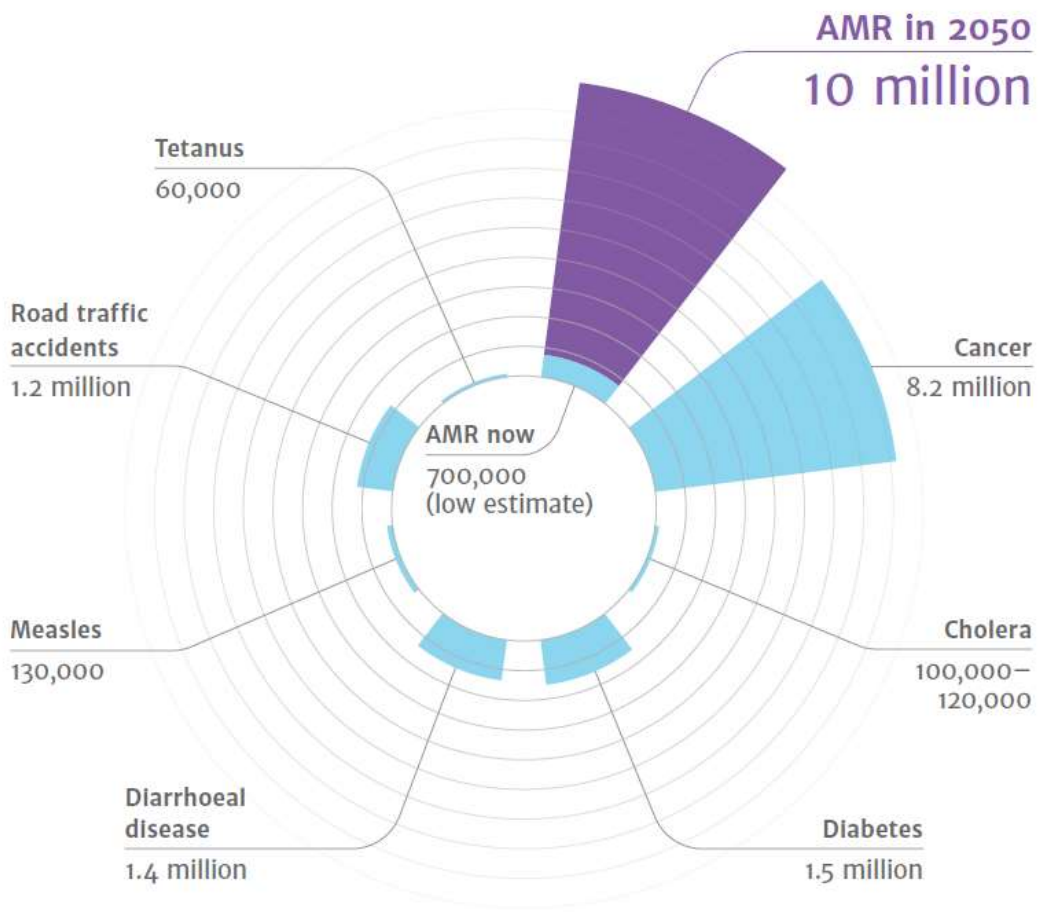
Science fair: microbiology and studies on *E. coli* infections and antibiotic resistance

Land Clearing

Rise of the Post-Antibiotic Era

Review on Antimicrobial Resistance





WHO priority pathogens list for R&D of new antibiotics

Priority 1: CRITICAL
- *Acinetobacter baumannii*, carbapenem-resistant
- *Pseudomonas aeruginosa*, carbapenem-resistant
- *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing

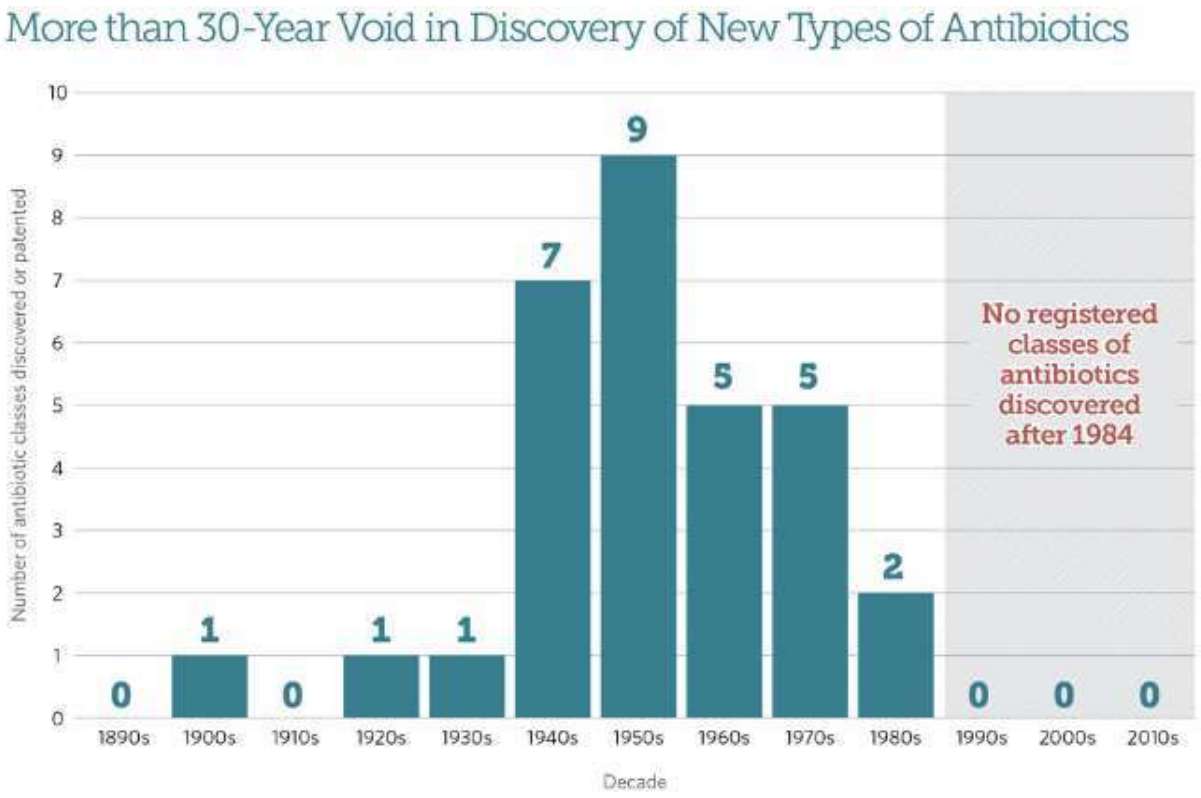
Priority 2: HIGH
- *Enterococcus faecium*, vancomycin-resistant
- ***Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant**
- *Helicobacter pylori*, clarithromycin-resistant
- *Campylobacter* spp., fluoroquinolone-resistant
- *Salmonellae*, fluoroquinolone-resistant
- *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant

Priority 3: MEDIUM
- *Streptococcus pneumoniae*, penicillin-non-susceptible
- *Haemophilus influenzae*, ampicillin-resistant
- *Shigella* spp., fluoroquinolone-resistant

WHO News Release, February 27, 2017

Antibiotic Discovery Void

Shift from natural products to HTS screens of combi-chem libraries & focus on protein targets



New classes introduced into the market but discovered in the past

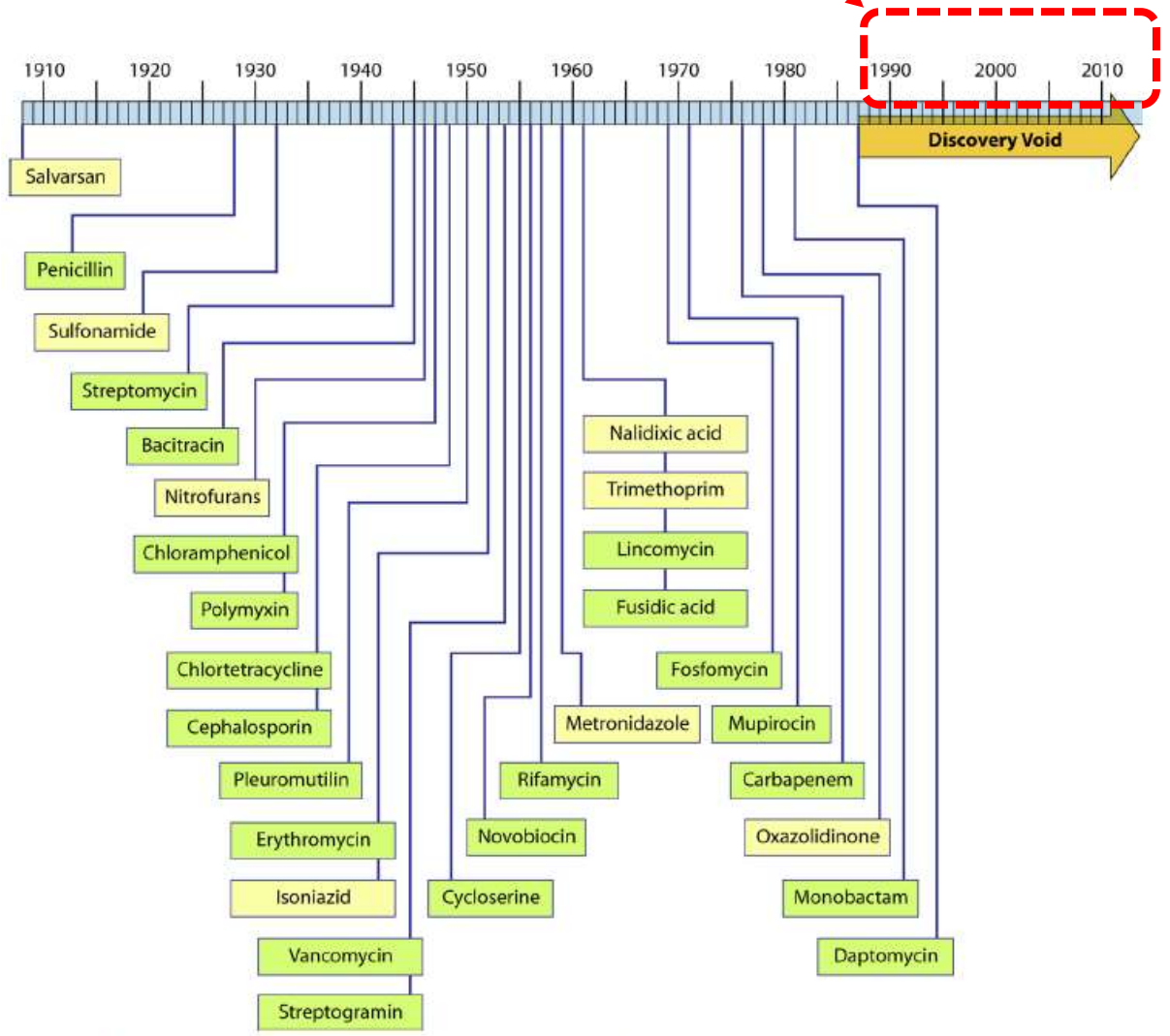
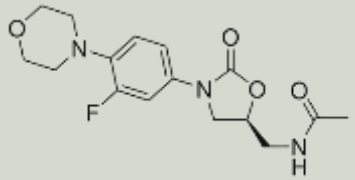
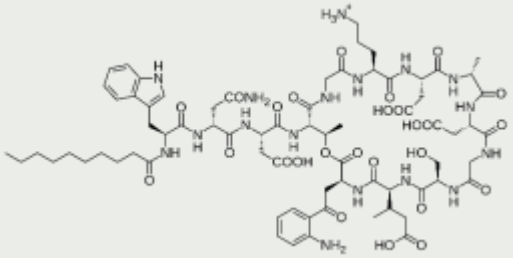
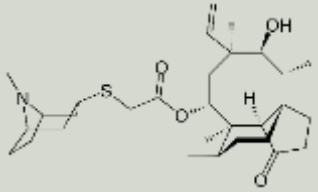


FIG. 1. Illustration of the "discovery void." Dates indicated are those of reported initial discovery or patent.

Adapted from Lynn Silver, "Challenges of Antibacterial Discovery", Clin. Microbiol. Rev. (2011)

Latest representatives of novel antibacterial classes

Structure	Name	Class	Year to Market	Year Class Discovered
	Linezolid	Oxazolidinones	2000	1978
	Daptomycin	Acid lipopeptides	2003	1987
	Retapamulin	Pleuromutilins	2007	1952

Adapted from Lynn Silver, “Challenges of Antibacterial Discovery”, Clin. Microbiol. Rev. (2011)

Where to find new drugs for MDR infections?

- Environmental samples
  - Soil microbes
  - Marine organisms
  - Endophytes
- "Unculturable" microbes
- Genome mining
- Animal proteins
- **Plants and fungi**

Plants as a source of medicine

Willow

Aspirin

Foxglove

Digoxin/Digitoxin

Mayapple

Podophyllin/ Etoposide

Poppy

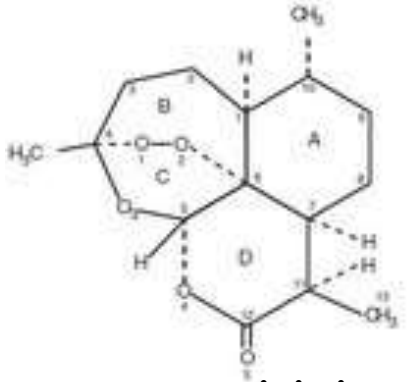
Codeine/ Morphine

Kew Report:
State of the World's Plants 2017

Plant natural products in the Nobel Prize spotlight

Dr. Youyou Tu

Ming dynasty version (1574 CE) of the handbook. “A handful of qinghao immersed with 2 liters of water, wring out the juice and drink it all” is printed in the fifth line from the right.

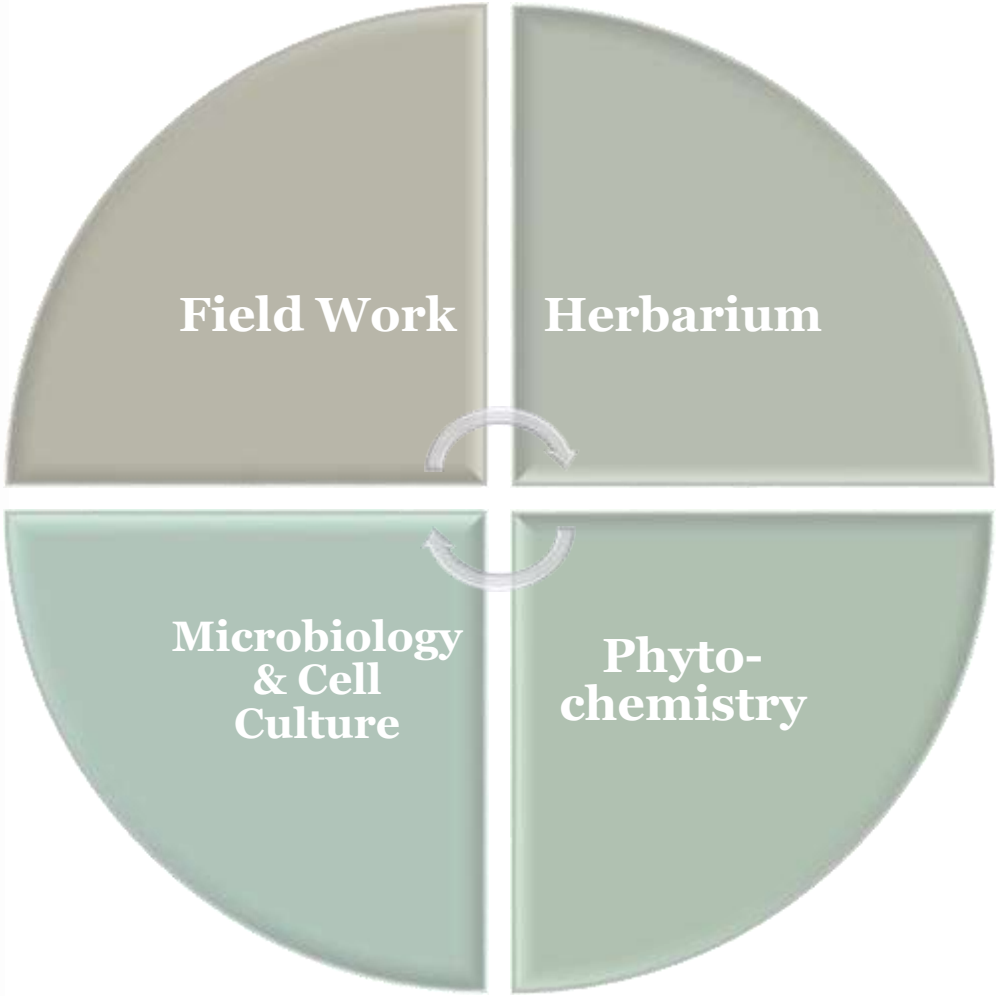


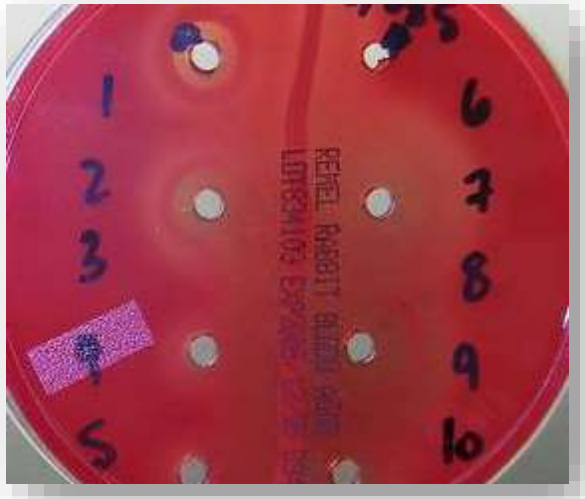
artemisinin

Artemisia annua L., Asteraceae

Tu, Y. 2011. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine *Nature Medicine* 17: 1217–1220

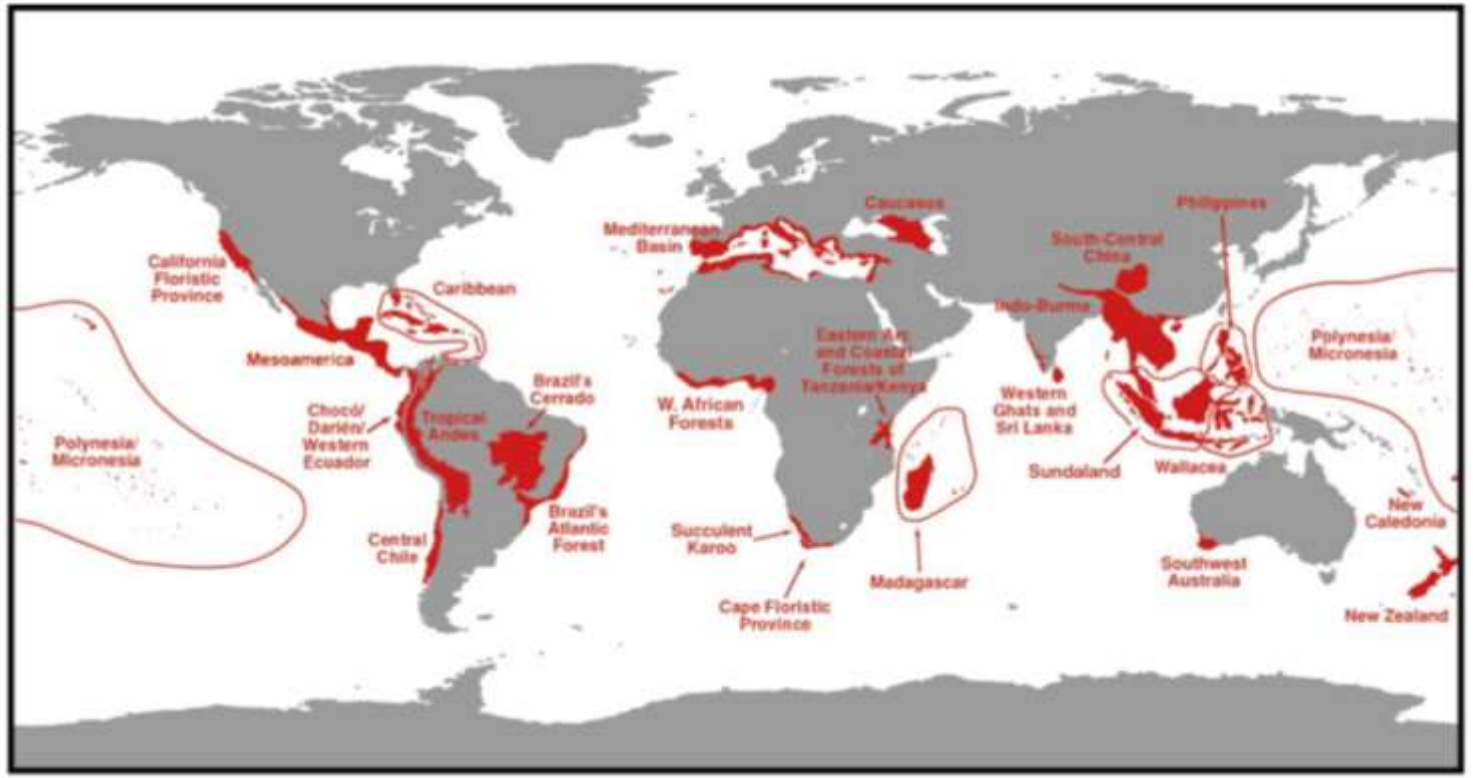
Core Research Approach





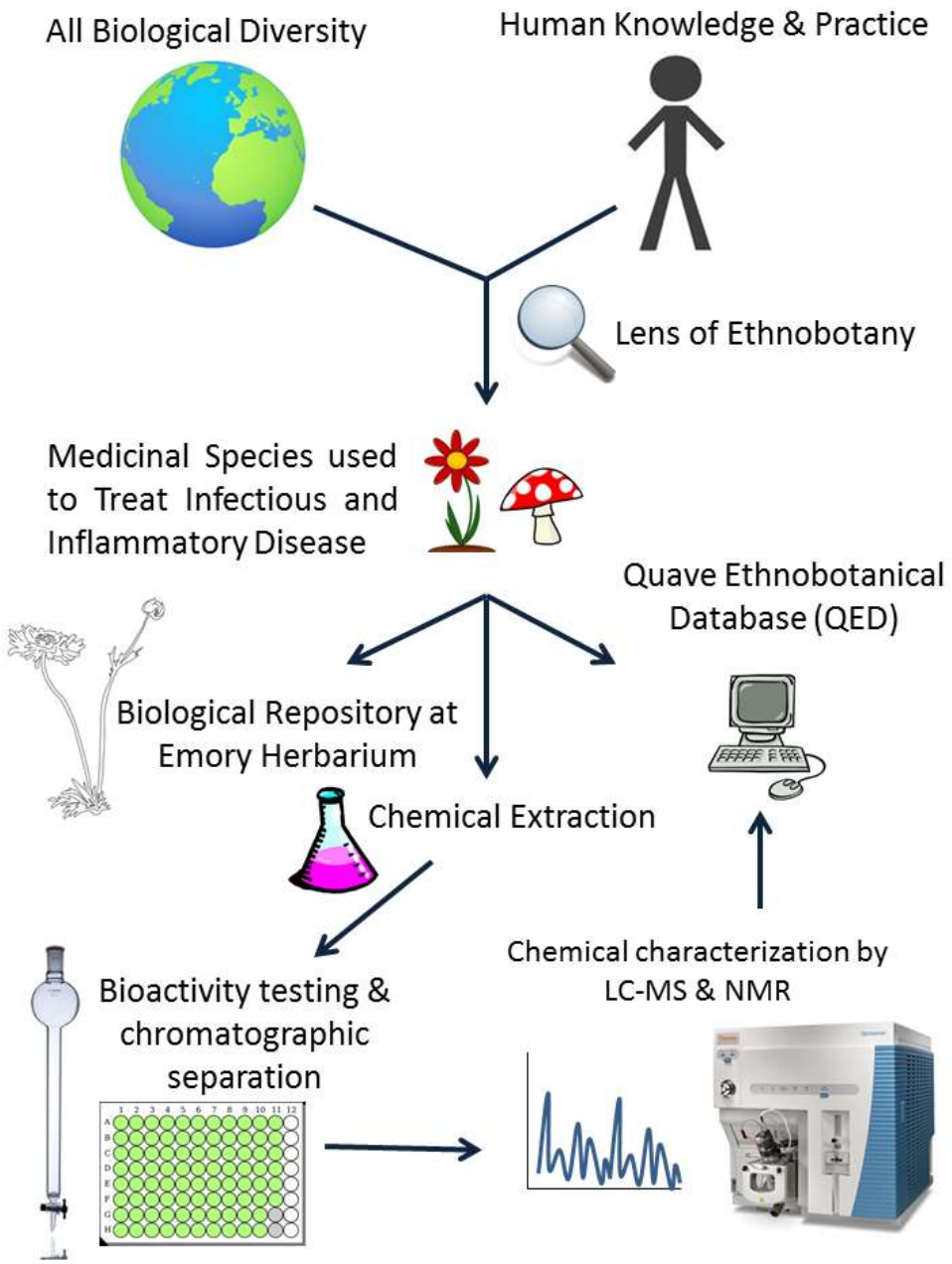
How to pick a field research location?

- Global Hotspots of Biodiversity
  - As many as 44% of all species of vascular plants confined to 25 hotspots comprising 1.4% of Earth's land surface
    - 25,000 plants native to the Mediterranean basin
    - 13,000 of these are **endemic**!



The 25 hotspots of biodiversity.
Myers, N., et al. 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853-858,

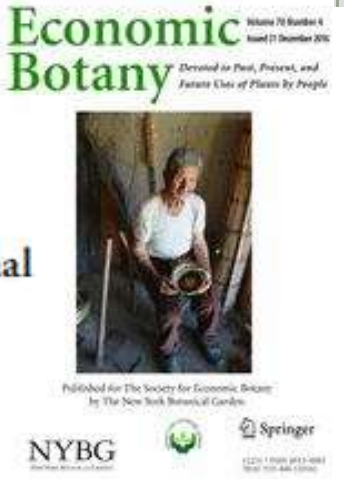
Ethnobotanical Approach to Drug Discovery



Collection sites: USA (Oregon, Florida, Georgia); Italy (Basilicata, Sicily, Aegadian Islands, Pantelleria); Albania (NE), Kosovo (Central and SW)

Why do fieldwork in the Mediterranean?

- High levels of endemism
- High density of biological and cultural diversity
- Different groups may use same ecological resources in very different ways!
- Flora underexplored for drug discovery



Forty-five years later: The shifting dynamic of traditional ecological knowledge on Pantelleria Island, Italy

CASSANDRA L. QUAVE*,[1,2,3] AND ALESSANDRO SAITTA[4]

Journal of Ethnobiology and Ethnomedicine
BioMed Central

Research

Open Access

Dermatological remedies in the traditional pharmacopoeia of Vulture-Alto Bradano, inland southern Italy

Cassandra L Quave*[1], Andrea Pieroni[2] and Bradley C Bennett[1]

nature plants
ARTICLES
PUBLISHED: 2 FEBRUARY 2015 | ARTICLE NUMBER: 14021 | DOI: 10.1038/NPLANTS.2014.21

A reservoir of ethnobotanical knowledge informs resilient food security and health strategies in the Balkans

Cassandra L. Quave[1,2]* and Andrea Pieroni[3]

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Journal of Ethnopharmacology 101 (2005) 258–270
Journal of ETHNO-PHARMACOLOGY
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Traditional pharmacopoeias and medicines among Albanians and Italians in southern Italy: A comparison

Andrea Pieroni[a,b,*], Cassandra L. Quave[c]

Albania, 2012








Pantelleria, 2014

Daphne gnidium L., Thymelaceae

Kosovo, 2015








Aegadian Islands, 2017








Interviews & Plant Collecting

- Prior informed consent
- Follow SEB/ISE Code of Ethics
- Access & Benefit Sharing

DNA barcoding

Herbarium Vouchers

Access and Benefit Sharing

- Returning traditional knowledge to communities:
  - Book in local language
  - Community garden (ethnobotanical)
  - Community workshops
- Fostering training of local students and scientists
  - Research training workshops
  - University capacity building projects
  - Student exchange programs
- Collaboration agreements with local university partners and communities

Plant Extraction

Dried 48-72 hrs

Vacuum-sealed with silica packets and shipped to lab

Pulverized with a grinder

1:10 extraction in 95% EtOH or MeOH for 2 x 72 hrs. or boiled in water for 30 minutes

Plant Extraction

Plant materials separated from extract with vacuum filtration

Solvent removed under reduced pressure with a rotary evaporator

After freezing at -80°C, extracts are lyophilized

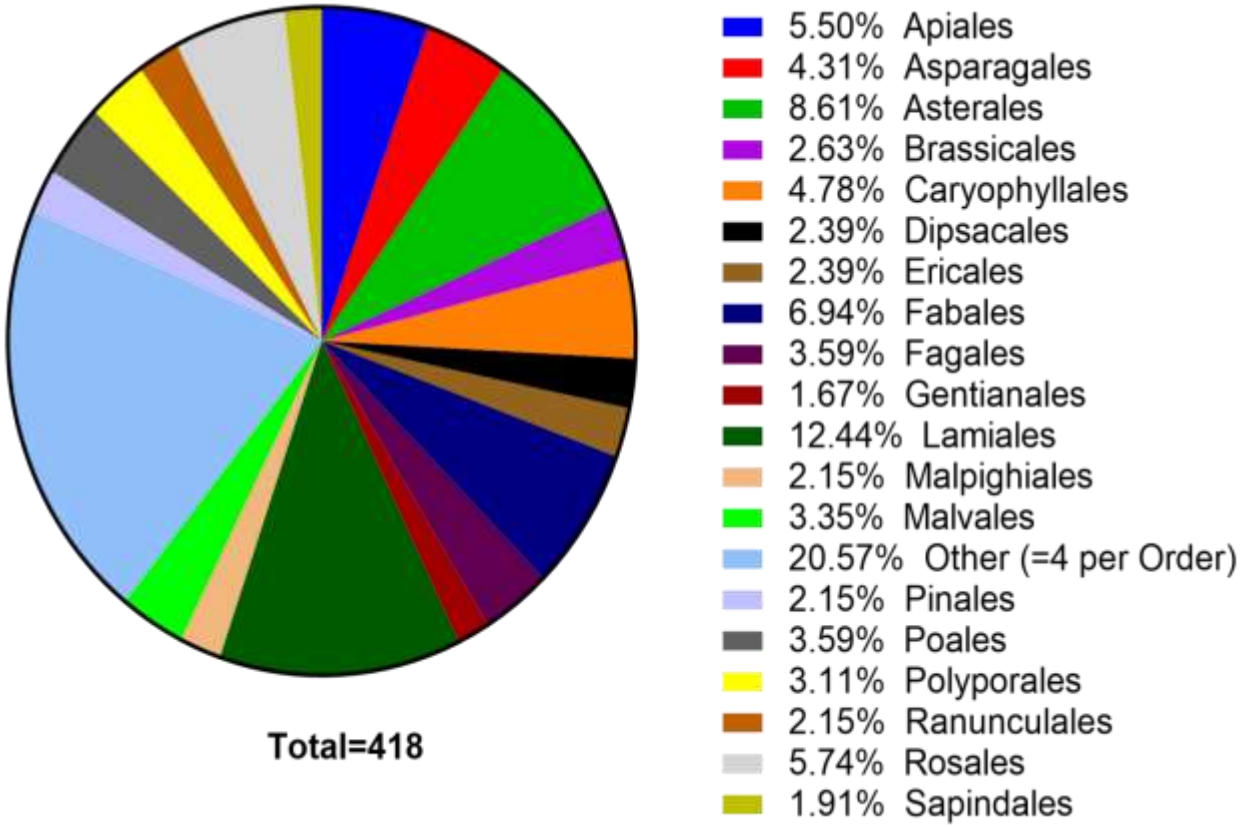
Dried extracts scraped out and weighed

Quave Natural Products Library (QNPL)

Inspired by traditional medicine. Driven by bioactivity.

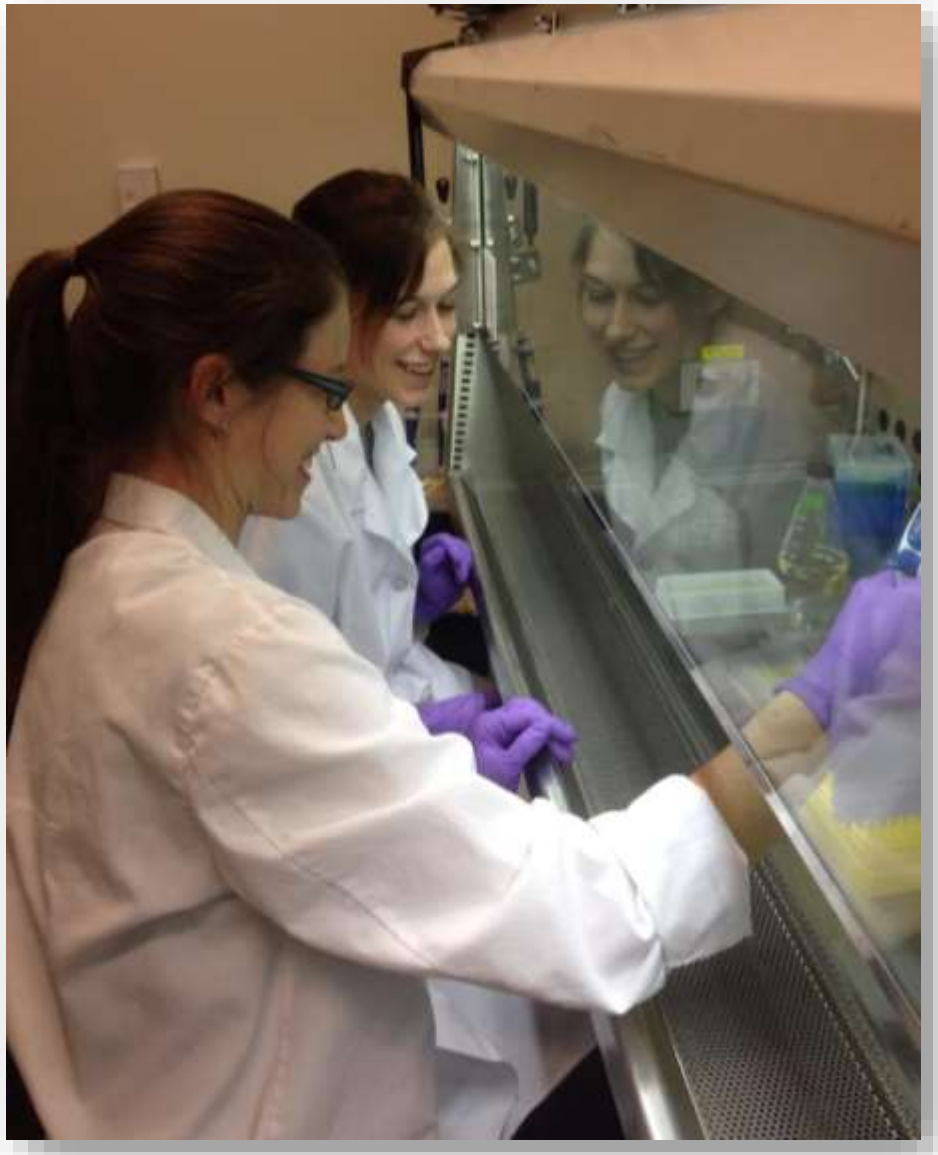
- >1,400 botanical and fungal extracts
  - plus fractions from bioactive leads
- Library uniquely targets plants used in human medicine and food
- Existing extract library is:
  - Biodiverse:
    - 51 orders
    - >400 species
    - Linked to ethnobotanical use data

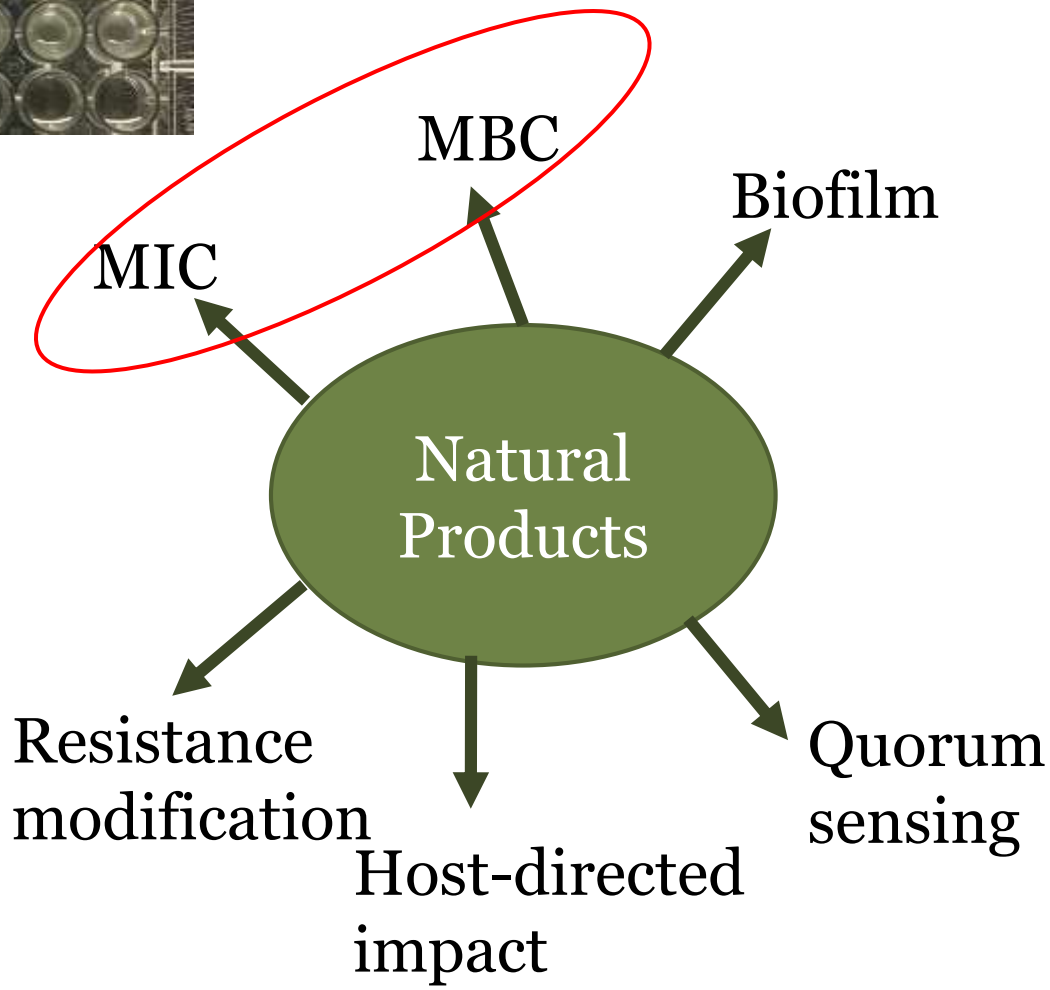
Diversity of the Quave Natural Products Library (QNPL)



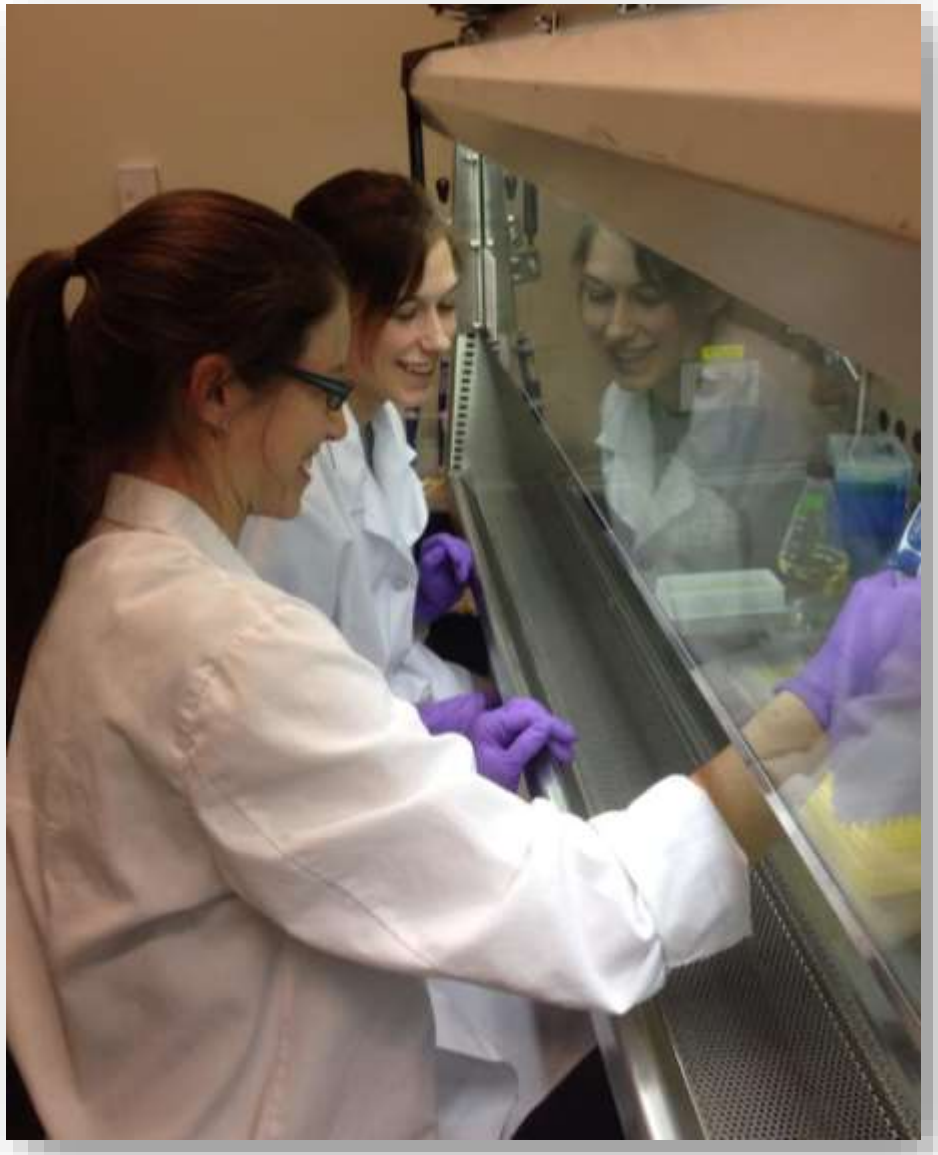
	Species	Genera	Families	Orders
Plants	396	271	106	45
Fungi	22	22	11	6
TOTAL	**418**	**293**	**117**	**51**

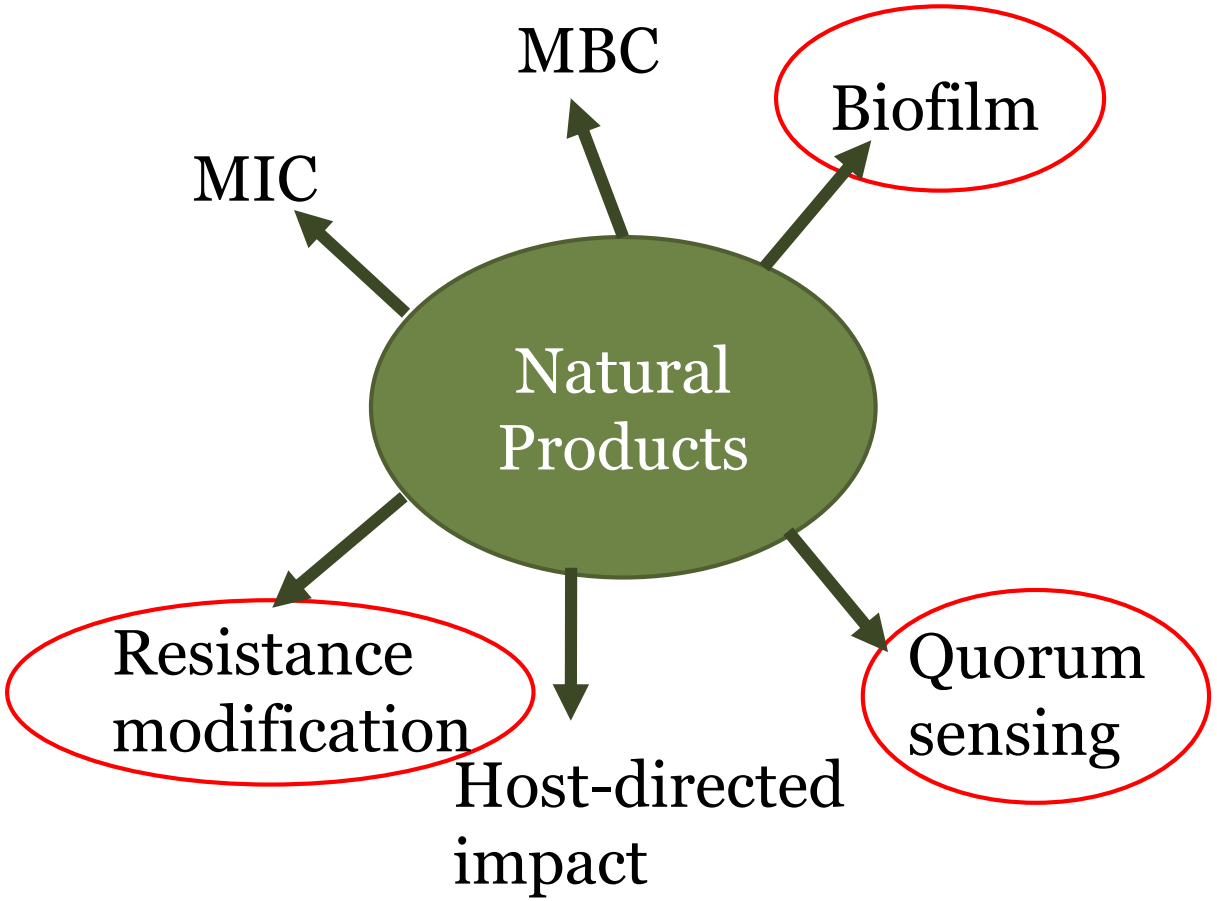
New solutions require innovative & timely screens

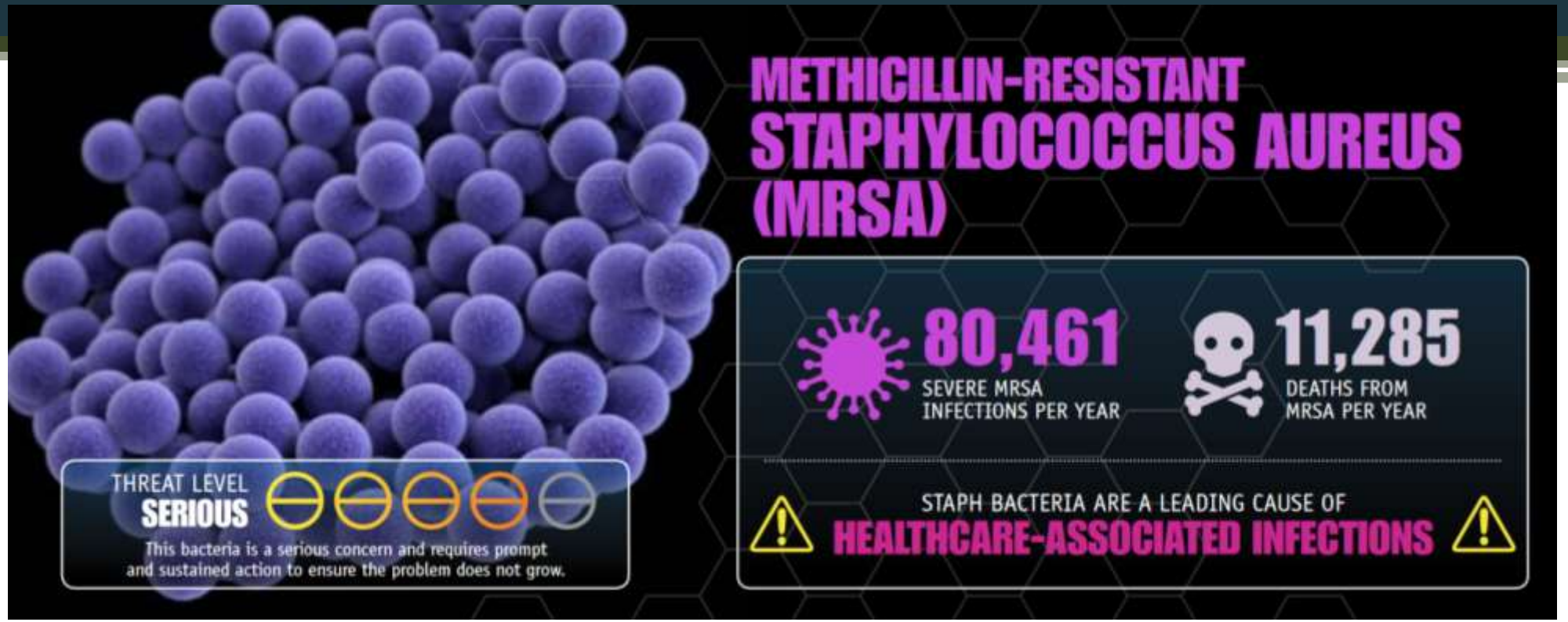




New solutions require innovative & timely screens



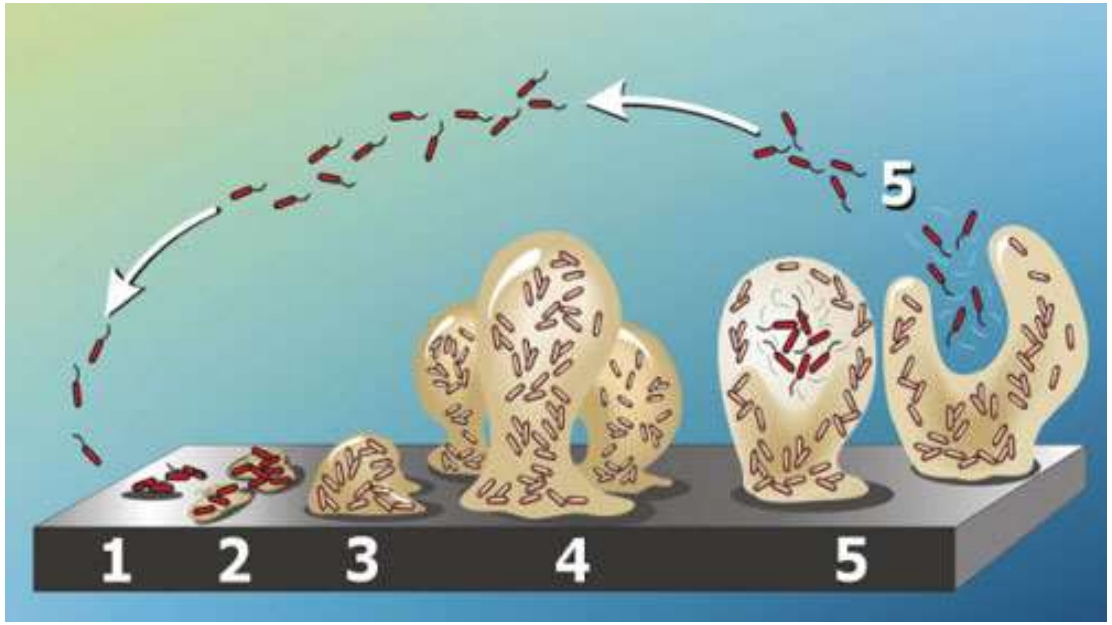


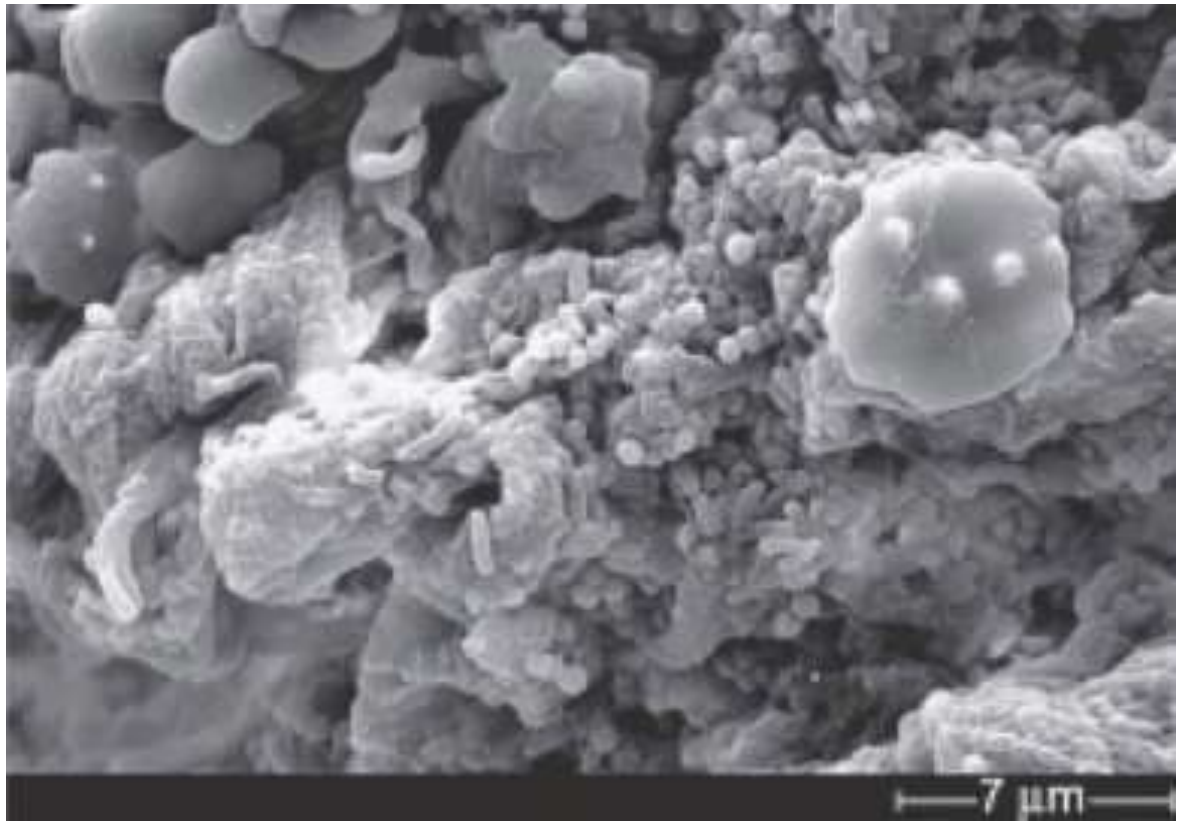


- Opportunistic pathogen
- Leading cause of:
  - Bacteremia
  - Sepsis
  - Brain abscesses
  - Medical device infections
  - Skin and soft tissue infections (SSTI)
- Colonizes nasal passages of 30% healthy adults in US
- Commonly implicated in:
  - Bone and joint infections
  - Surgical site infections
  - Pneumonia
  - Endocarditis
- HA-MRSA vs. CA-MRSA

Intrinisic Resistance: Biofilm

- Uni- or Poly-microbial
- Heightened gene exchange
- Slow growth/metabolism
- Matrix presents a physical barrier to host immune response and antibiotic therapy





Elmleaf Blackberry

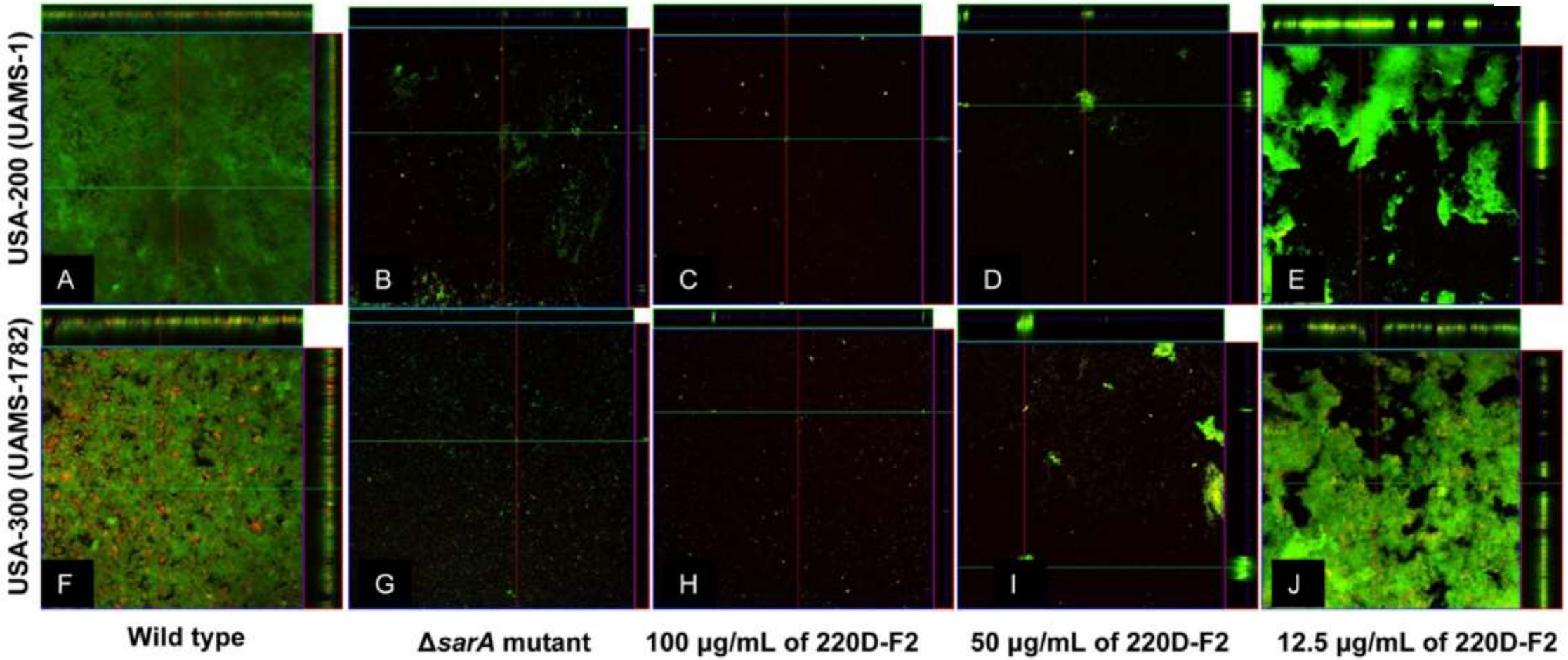
- Traditional uses in S. Italy:
  - Leaves: furuncles, abscesses, and other skin inflammations
  - Roots: hair loss
  - Fruits: eaten fresh and in marmalades
- One of 116 remedies related to SSTIs and other topical dermatological treatments identified
- 168 extracts screened
- Anti-biofilm activity first identified & published in 2008 and # 220 marked as possible lead

Rubus ulmifolius Schott. (Rosaceae): The source of the bioactive composition “220D-F2”.

Quave, C.L., A. Pieroni, and B.C. Bennett (2008) **Dermatological remedies in the traditional pharmacopoeia of Vulture-Alto Bradano, inland southern Italy.** *Journal of Ethnobiology and Ethnomedicine* 4:5.

Quave, C.L., L.R.W. Plano, *T. Pantuso, and B.C. Bennett (2008). **Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence in MRSA.** *Journal of Ethnopharmacology* 118: 418-428

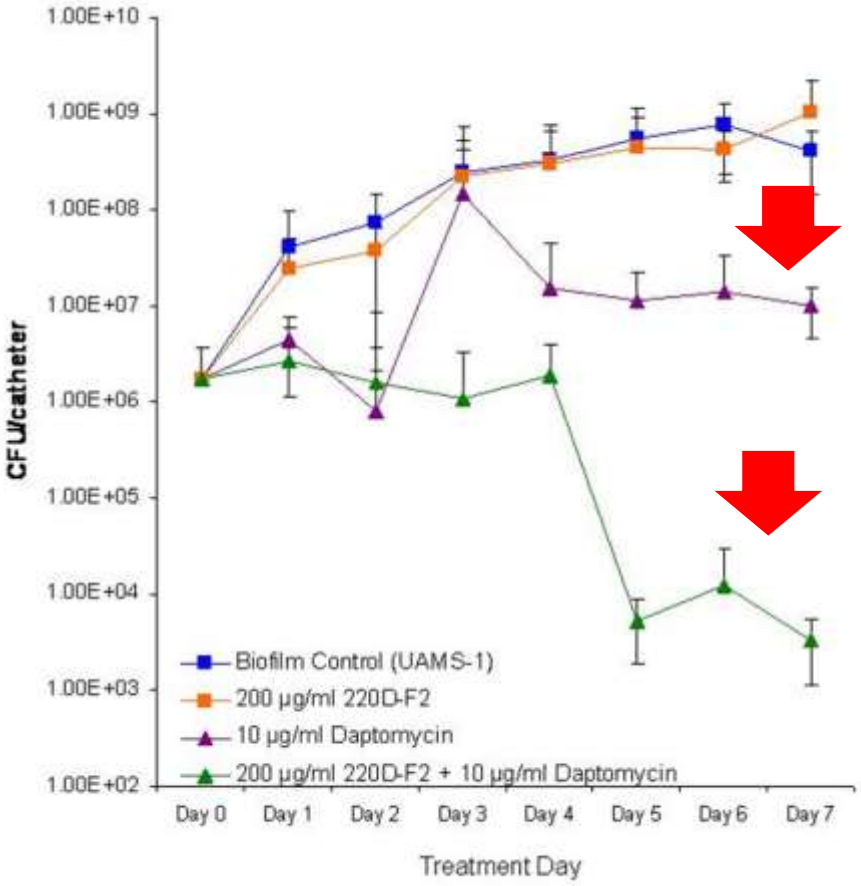
Intrinisic Resistance: Biofilm

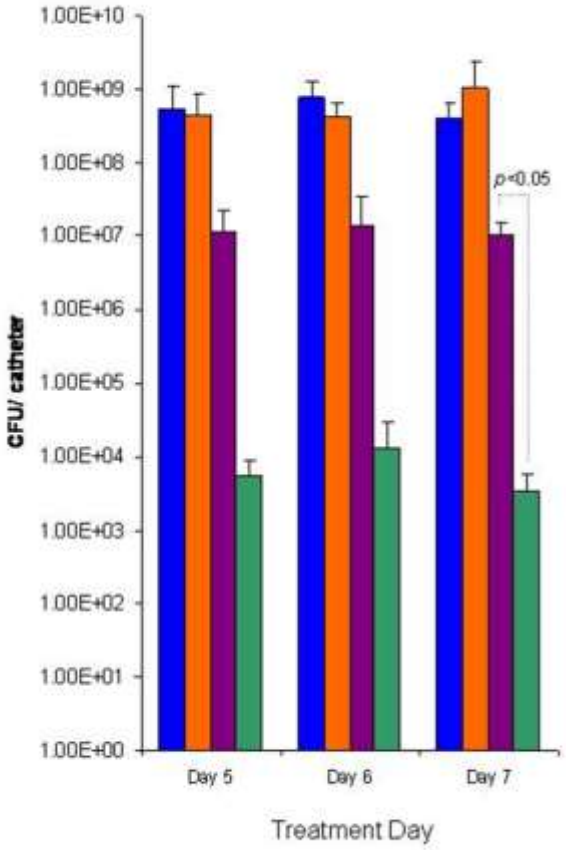


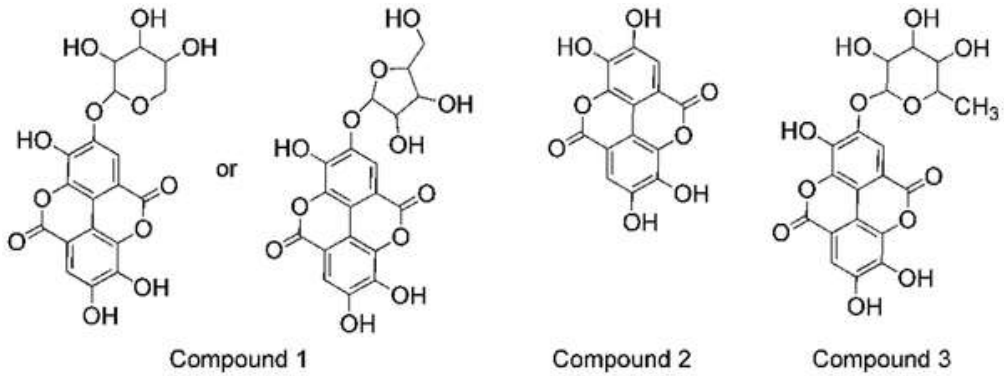
Ellagic acid glycosides from *Rubus ulmifolius* block biofilm formation and potentiate antibiotic clearance of biofilm on catheters.

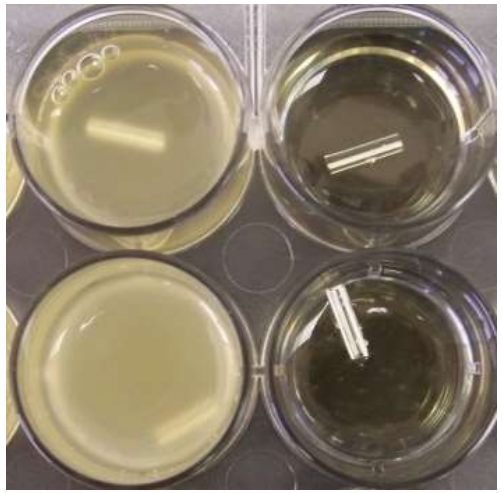
Quave et al. (2012). Ellagic acid derivatives from *Rubus ulmifolius* inhibit *Staphylococcus aureus* biofilm formation and improve response to antibiotics. *PLoS ONE* 7(1): e28737.
Talekar et al.(2014). 220D-F2 from *Rubus ulmifolius* kills *Streptococcus pneumoniae* planktonic cells and pneumococcal biofilms. *PLoS ONE* 9(5): e97314.

220D-F2 improves response to functionally distinct classes of antibiotics, including daptomycin, clindamycin, vancomycin, and oxacillin.



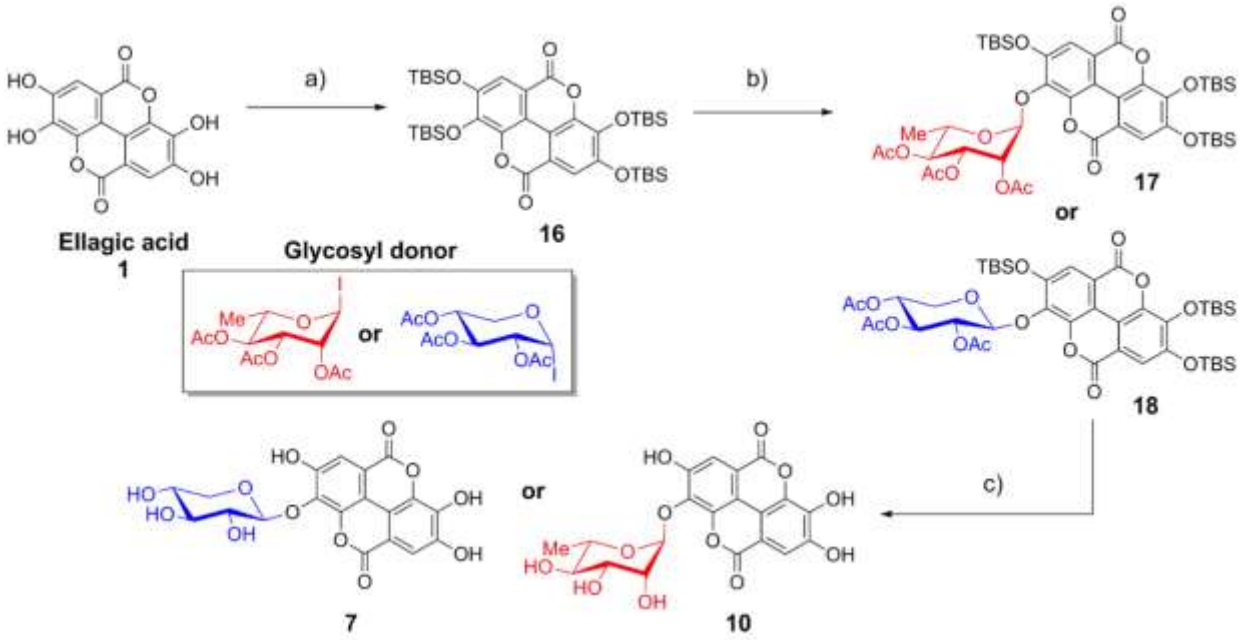




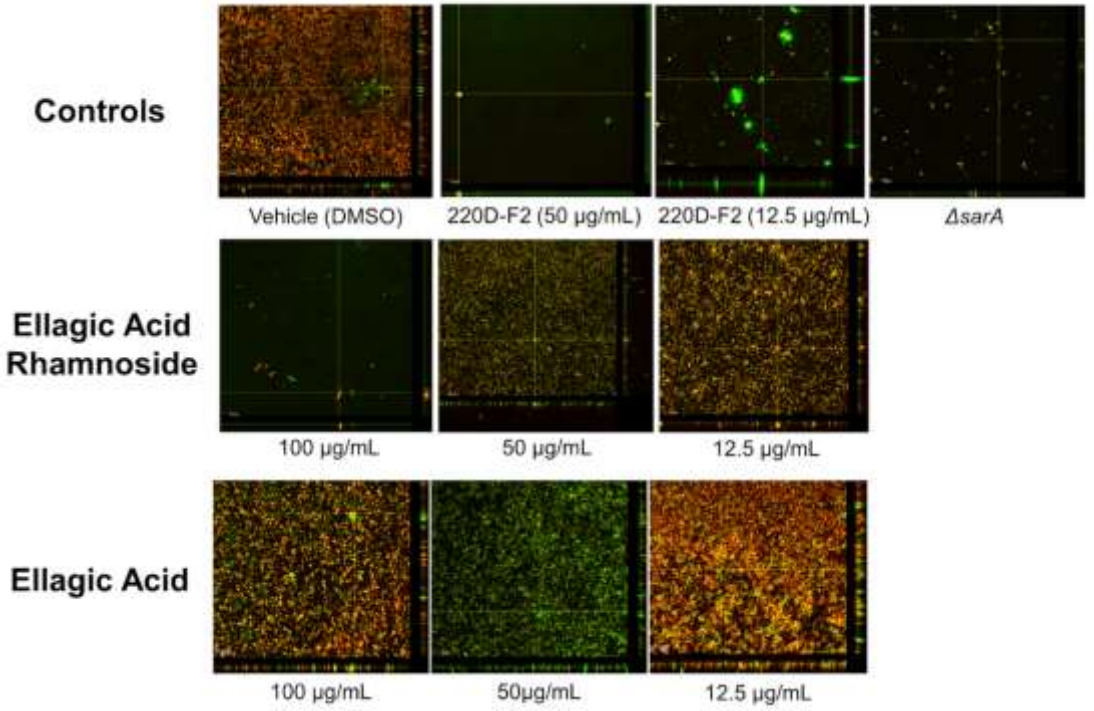


Quave *et al., PLoS One*. 2012: 7(1)

Synthetic library of glycosides (ellagic acid, catechol, phenol)

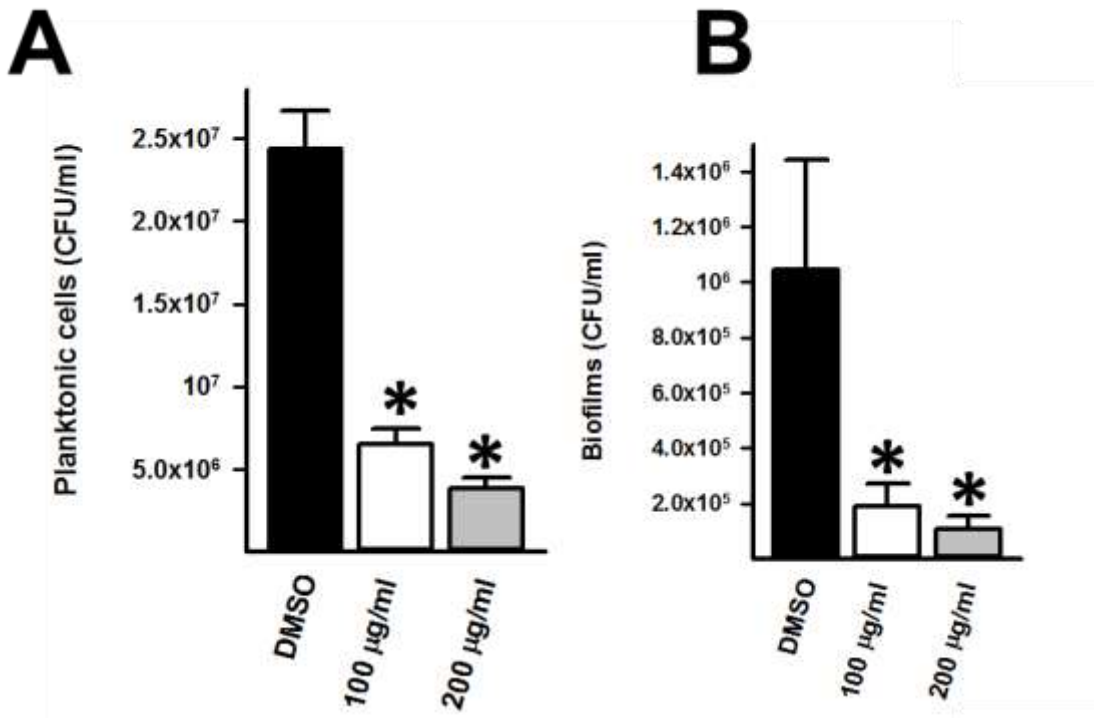


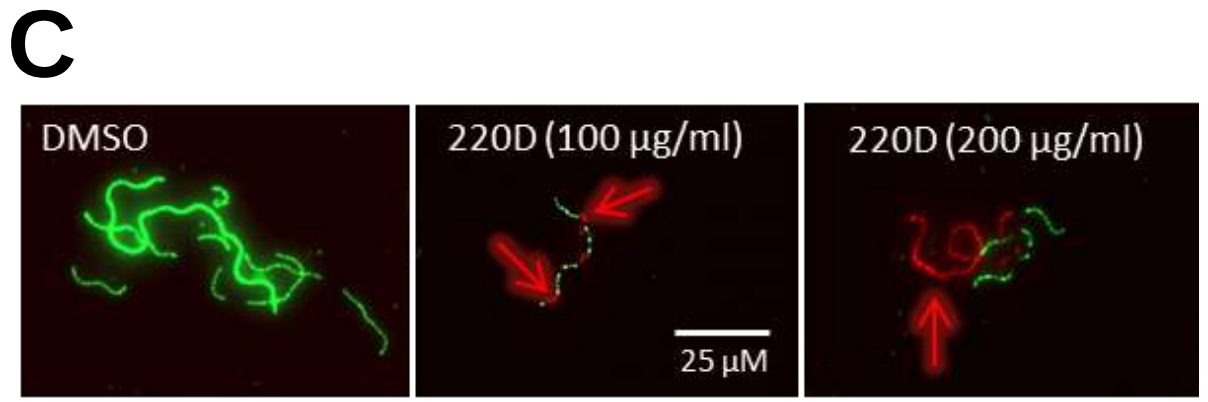
Synthesis of ellagic acid glycosides. a) TBSCl, Im, DMAP, CH_2Cl_2/DMF, 50°C, 36 h, 71%; b) TASF, CH_2Cl_2, room temperature, 1 min; then glycosyl donor, Bu_4NI (xyloside only), reflux, 48 h, 13-15%. c) 1) K_2CO_3, DMF/H_2O; 2) K_2CO_3, MeOH/H_2O, 86-92%.



The sugar is important!

Fontaine BM, Nelson K, Lyles JT, Jariwala PB, Garcia-Rodriguez JM, Quave CL and Weinert EE (2017). Identification of Ellagic Acid Rhamnoside as a Bioactive Component of a Complex Botanical Extract with Anti-Biofilm Activity. *Front. Microbiol.* **8**:496

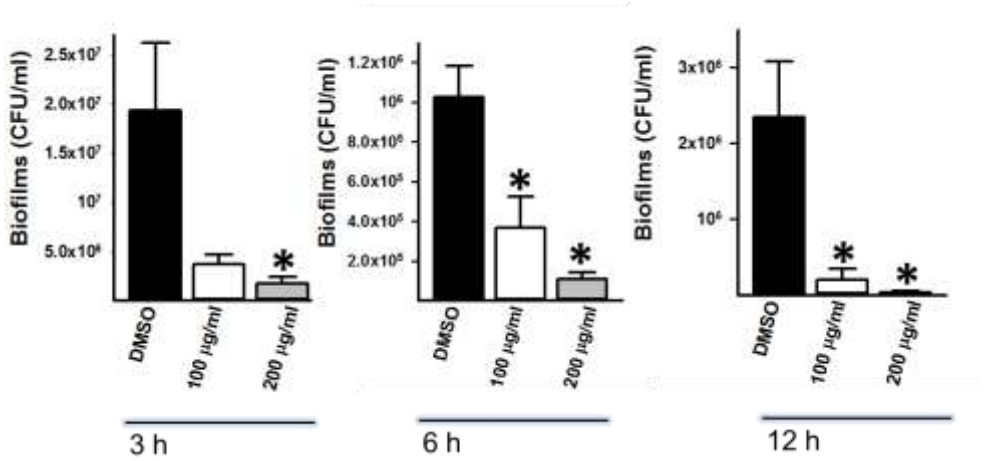




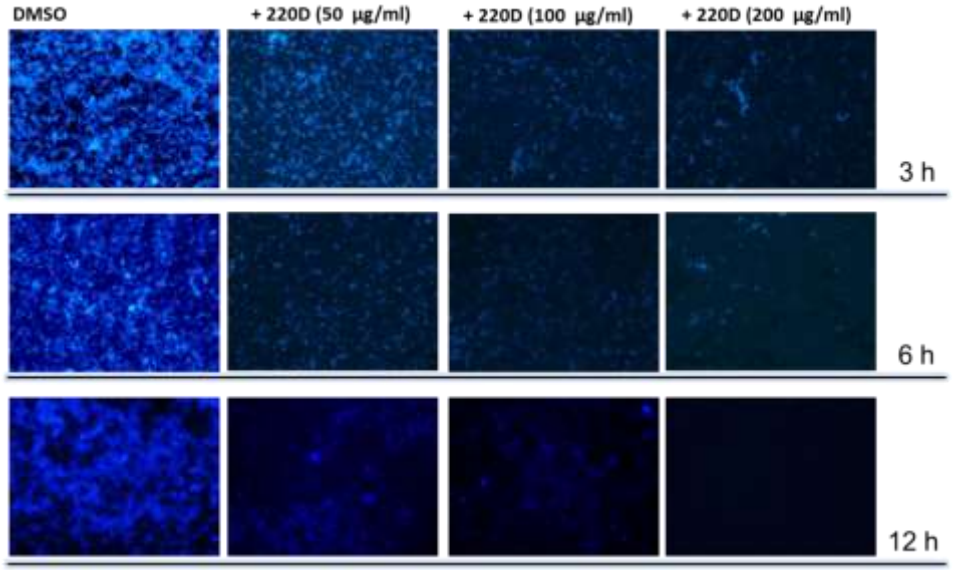
Killing of planktonic pneumococci by 220D-F2. *Streptococcus pneumoniae* strain D39 was inoculated in 24 well-plates containing THY and treated with DMSO or the indicated concentration of 220D-F2; treated cultures were incubated for 3 h at 37°C. Planktonic cells were removed (**A**) and then biofilms were washed and removed (**B**). Both populations were diluted and plated onto BAP to obtain CFU/ml. (**C**) Planktonic pneumococci treated for 3 h were also stained by the LIVE/DEAD assay and imaged using a fluorescent microscope. (Data: Vidal Lab)

Talekar, S.J., S. Chochua, K. Nelson, K.P. Klugman, C.L. Quave and J.E. Vidal (2014). 220D-F2 from *Rubus ulmifolius* kills *Streptococcus pneumoniae* planktonic cells and pneumococcal biofilms. PLoS ONE 9(5): e97314.

Killing of mature pneumococcal biofilms by 220D-F2. *S. pneumoniae* D39 was inoculated and incubated for 8 h at 37°C after which mature biofilms were washed and added with fresh THY containing the indicated concentration of 220D-F2 or DMSO. Treated biofilms were incubated for (A) 3, (B) 6, or (C) 12 h at 37°C and then washed, diluted and plated onto BAP to obtain CFU/ml. (Data: Vidal Lab)



Micrographs of 220D-F2 incubated with mature pneumococcal biofilms. *S. pneumoniae* D39 was inoculated and incubated for 8 h at 37°C after which biofilms were washed and added with fresh THY containing the indicated concentration of 220D-F2 or DMSO. These treated mature biofilms were incubated for 3, 6 or 12 h and after washes, the biofilm structure was stained with DAPI (100 nM). Stained biofilms were imaged by fluorescence. (Data: Vidal Lab)



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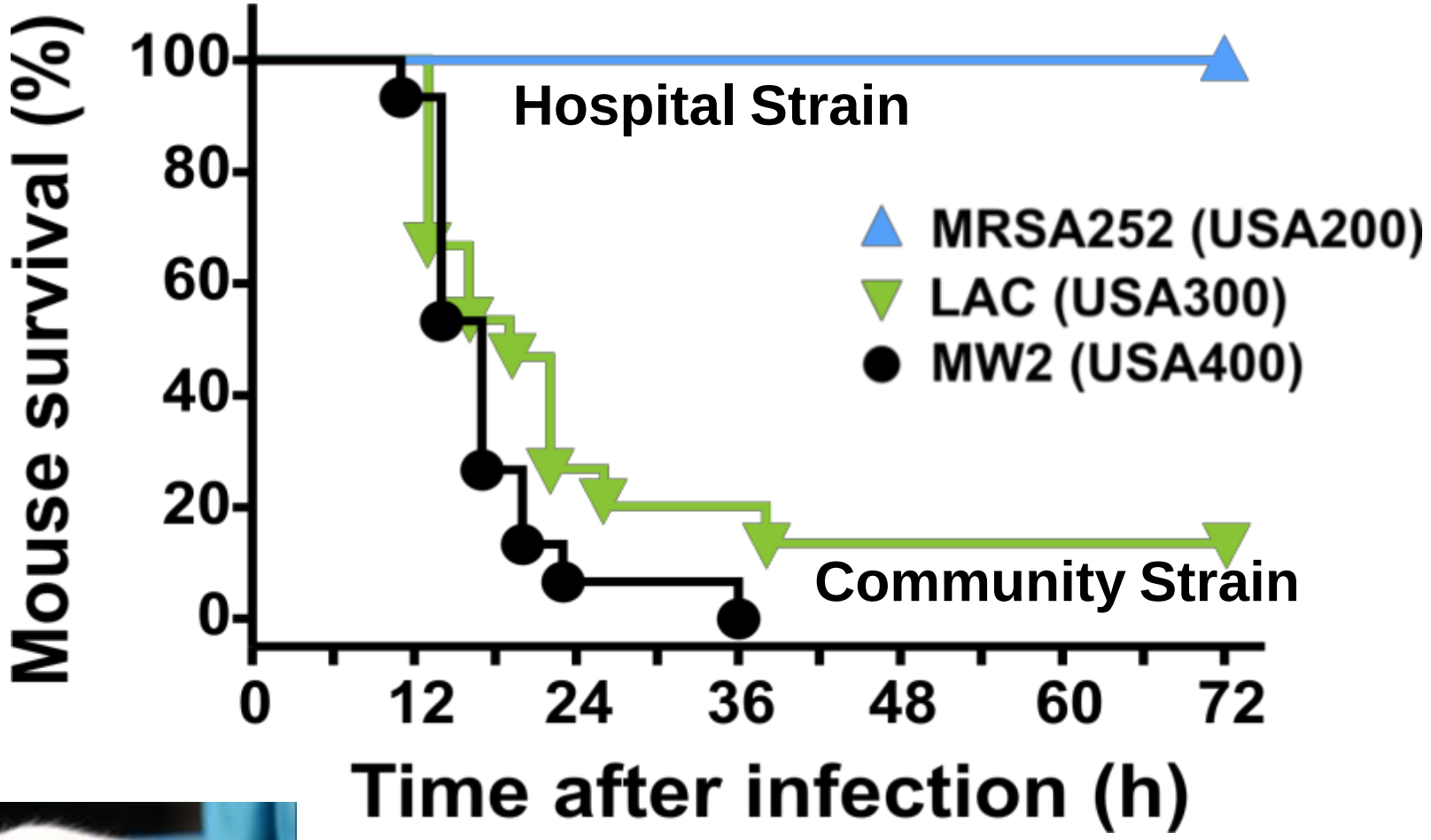
CA-MRSA Epidemic

- Causes disease in otherwise healthy individuals
- Predominantly skin and soft tissue infections (~75%), but invasive disease is severe
- The most prevalent CA-MRSA isolates in the United States are USA400 (MW2) and USA300 (Los Angeles County clone, LAC)





Prominent CA-MRSA are Highly Virulent*



Voyich *et al*., *J Immunol,* 2005; *J Infect Dis*, 2006

*First observed in humans

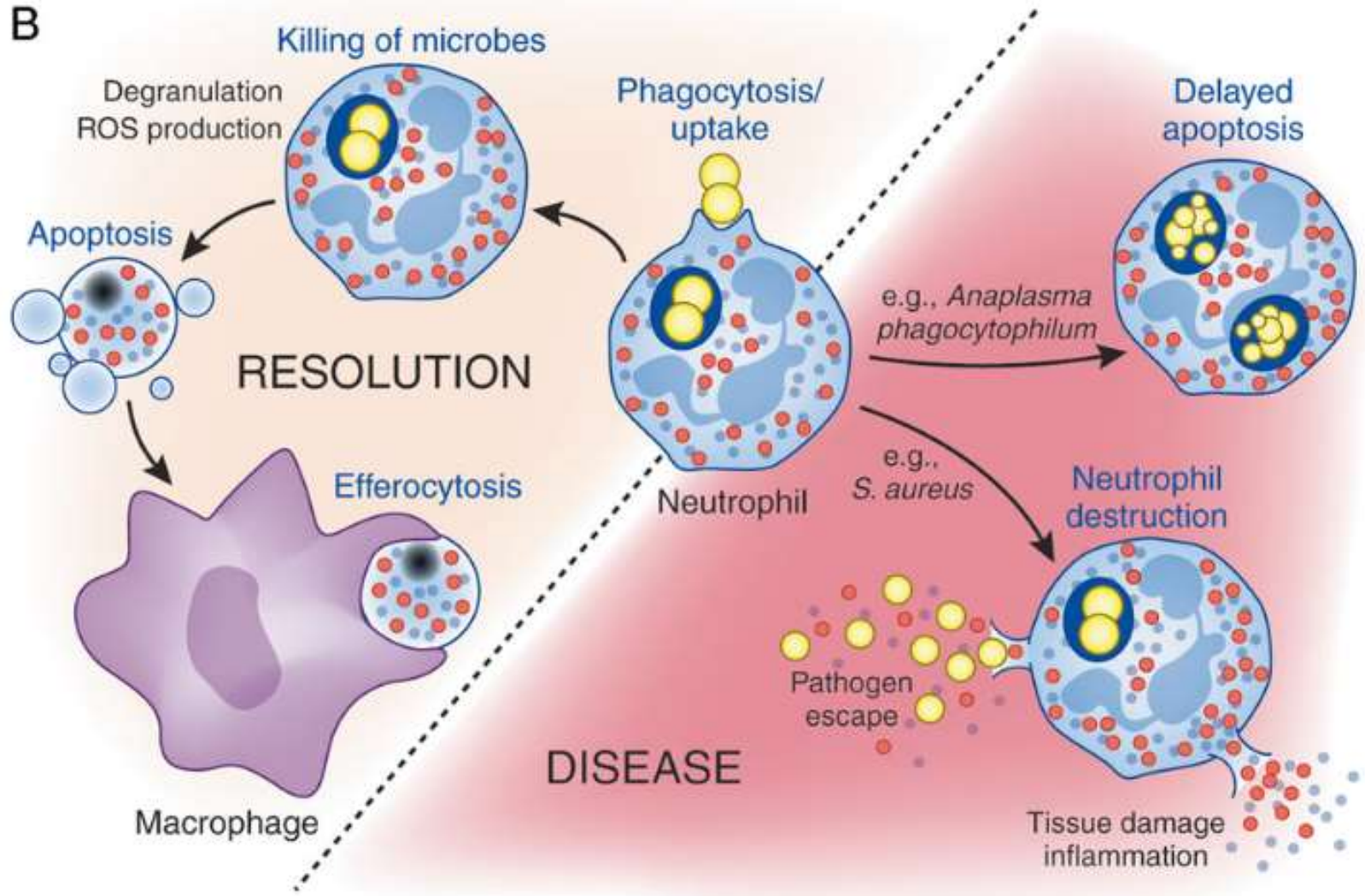
S. aureus exotoxins cause serious disease

Toxic Shock Syndrome Toxin (TSST-1)
Pyrogenic Toxin Superantigens

Scalded Skin Syndrome
Exfoliative Toxins

Abscesses, Necrosis, Sepsis
Hemolytic Toxins, Proteases, Lipases

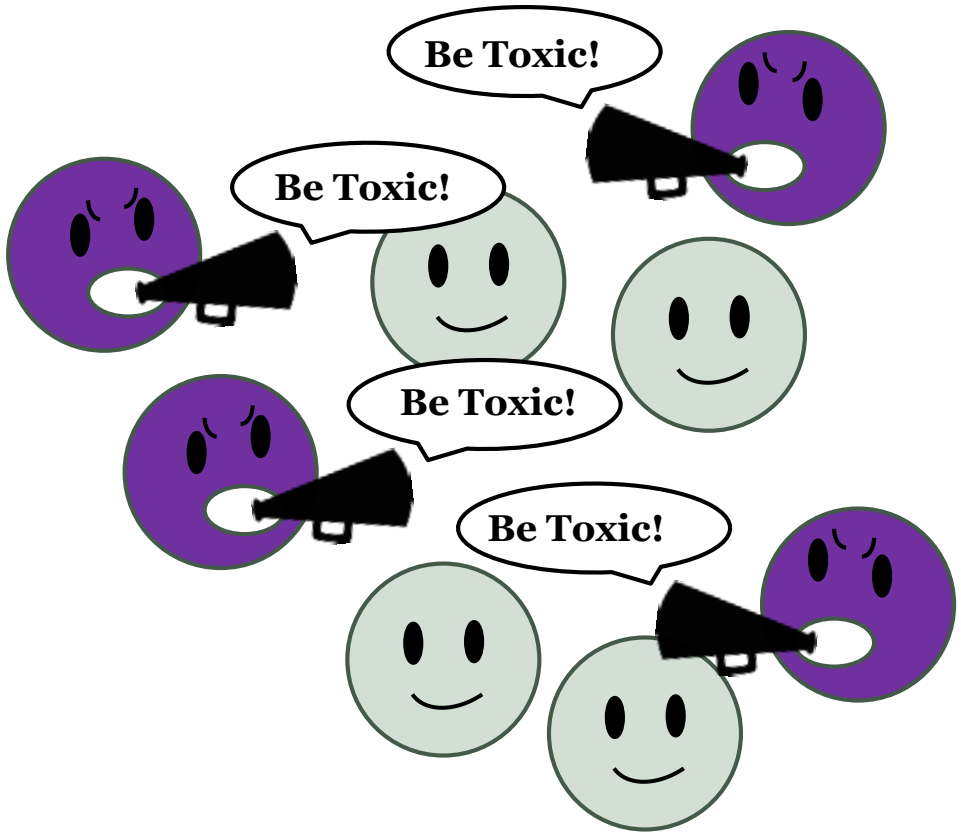
S. aureus immune evasion mechanisms



Rigby and De Leo. 2012 Semin. Immunopathology 34:237-29

Quorum Quenching Approach

- **Quorum quenching**
  - "Disarming" bacteria
  - Protect the host
  - Adjuvant to existing lines of antibiotics
- Accessory gene regulator (***agr)*** system
  - controls virulence



Quave & Horswill. (2014) Flipping the switch. *Frontiers in Microbiology*. 5(706):1-10

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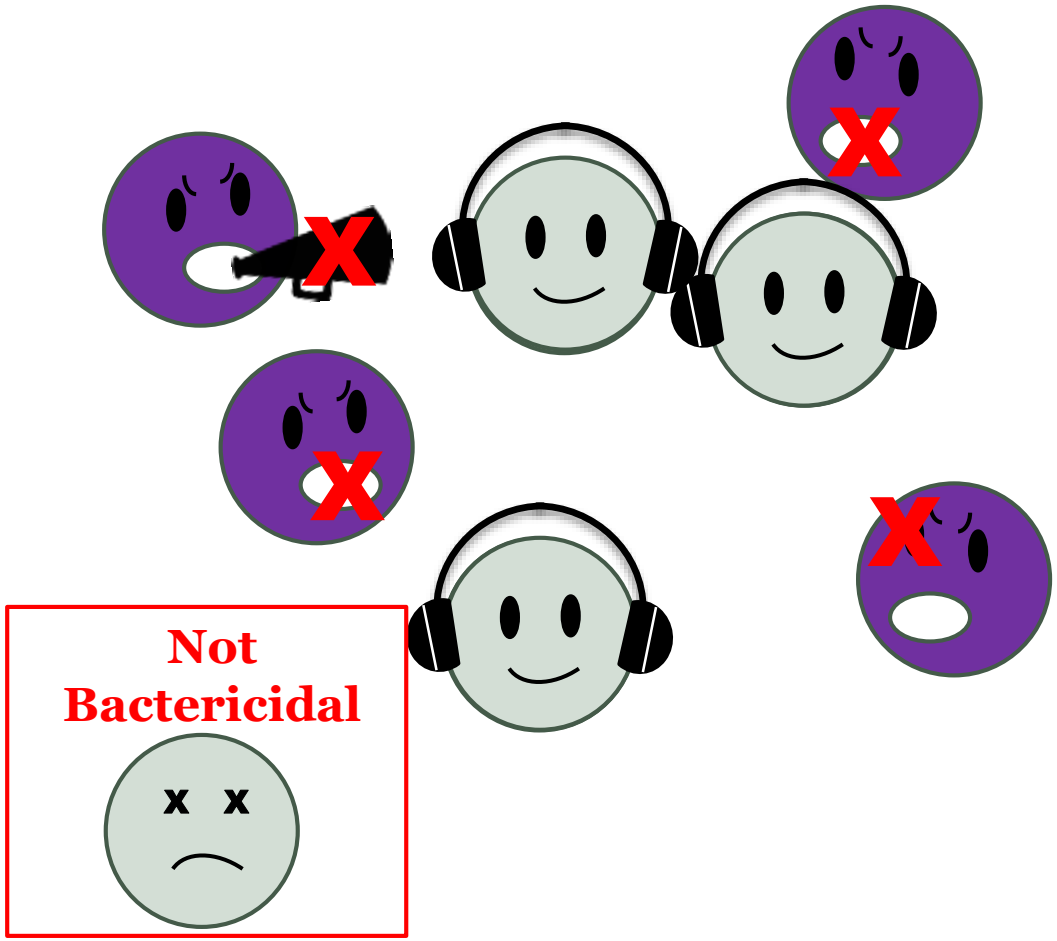
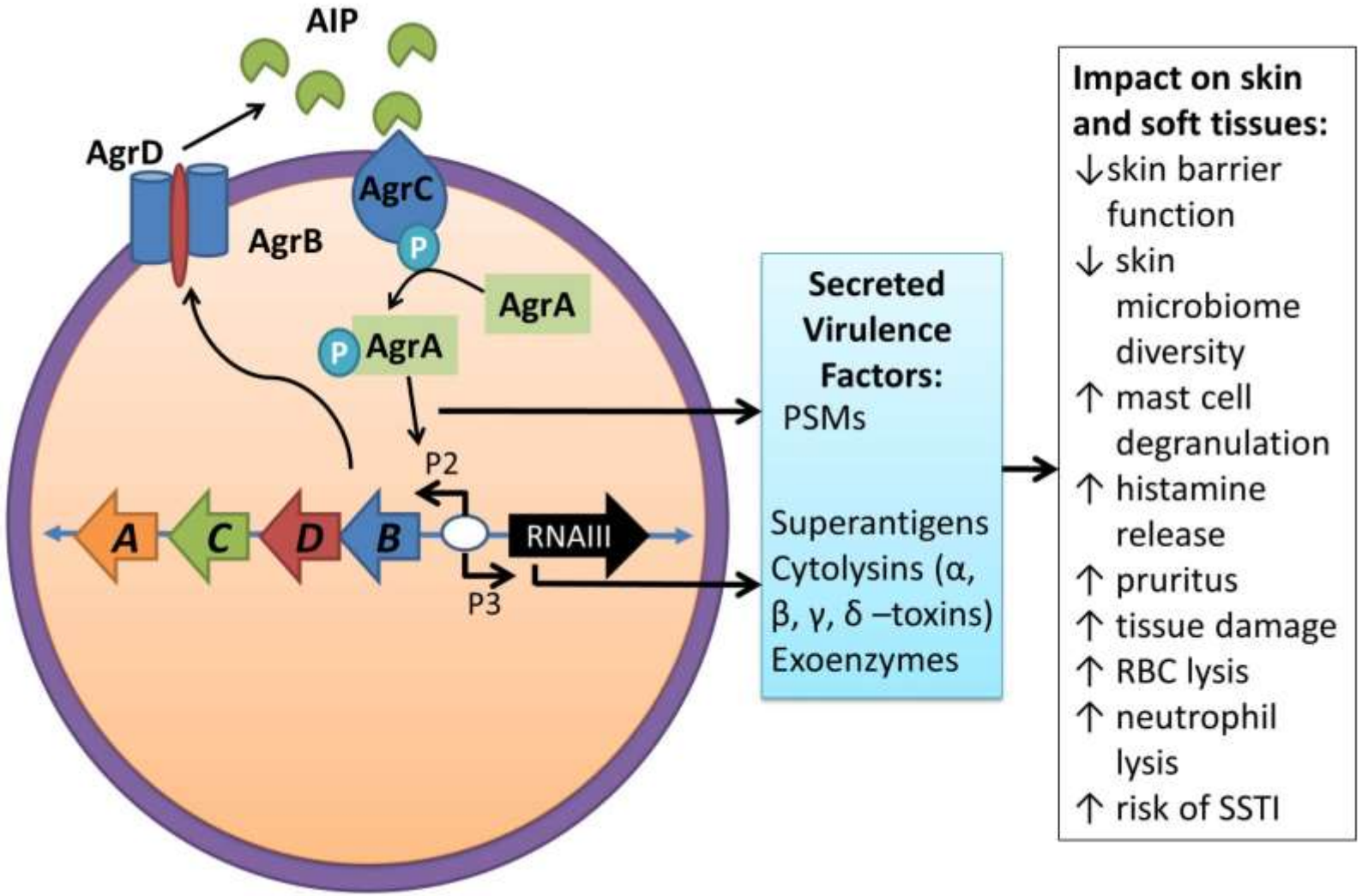


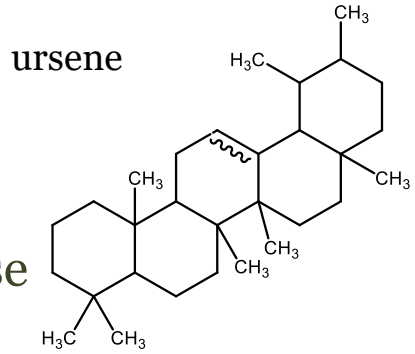
Quave & Horswill. (2014) Flipping the switch. *Frontiers in Microbiology*. 5(706):1-10

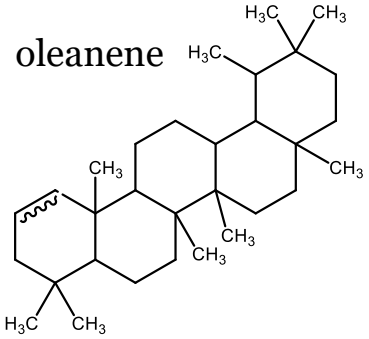
Accessory Gene Regulator (*Agr*) System



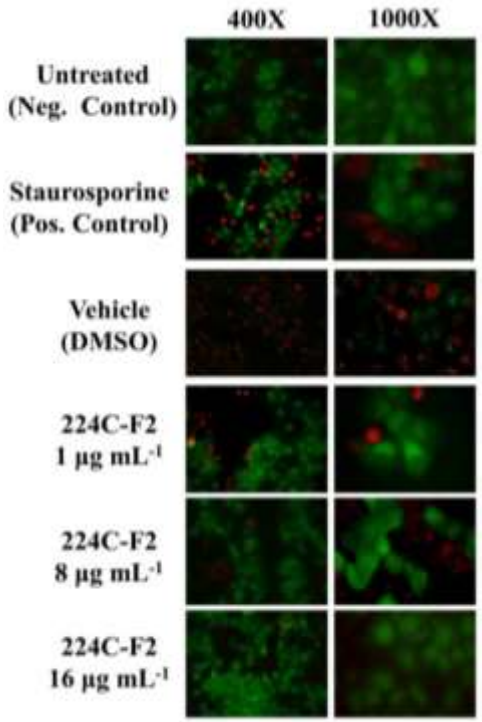
Chestnut

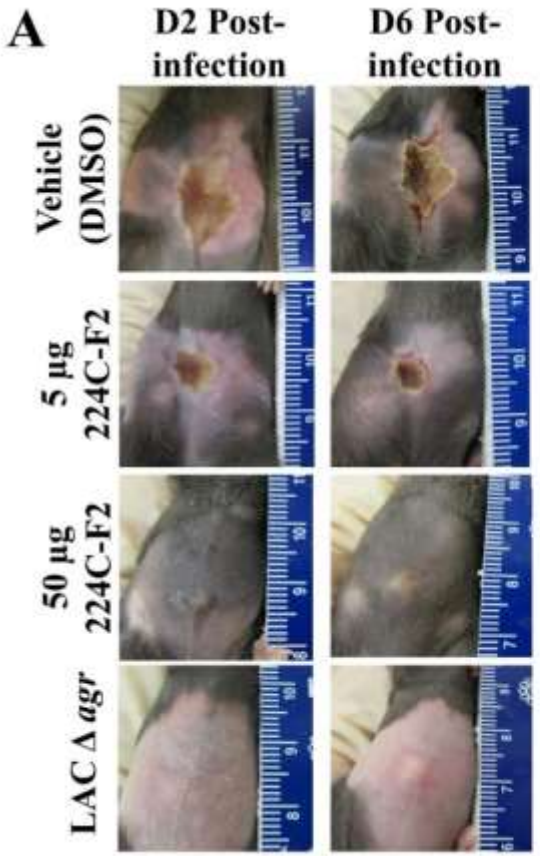
- Virulence Factors
  - Attack host immune response
  - Cause tissue damage
  - Contribute to clinical failure in antibiotic therapy
  - Controlled by cell-cell communication

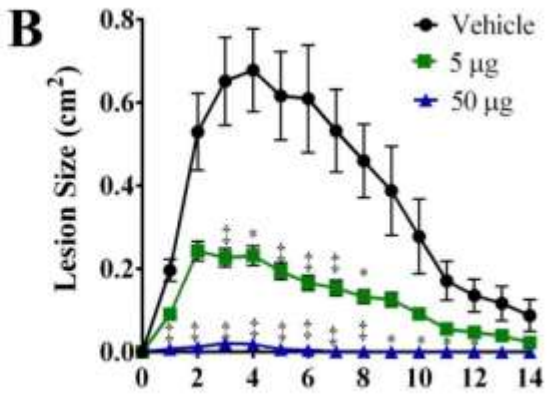


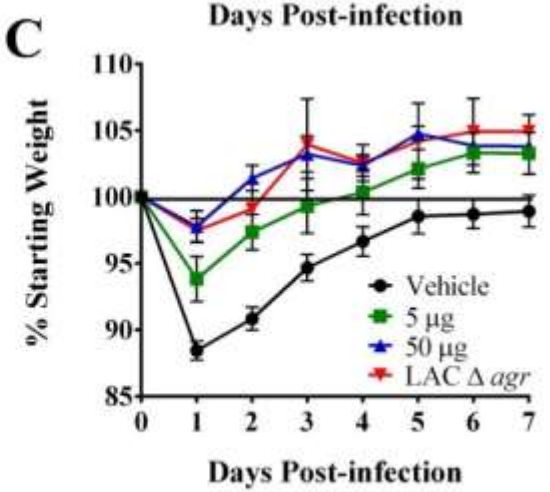


Quave et al. (2015) *Castanea sativa* (European Chestnut) leaf extracts rich in ursene and oleanene derivatives block *Staphylococcus aureus* virulence and pathogenesis without detectable resistance. PLoS ONE 10(8): e0136486.

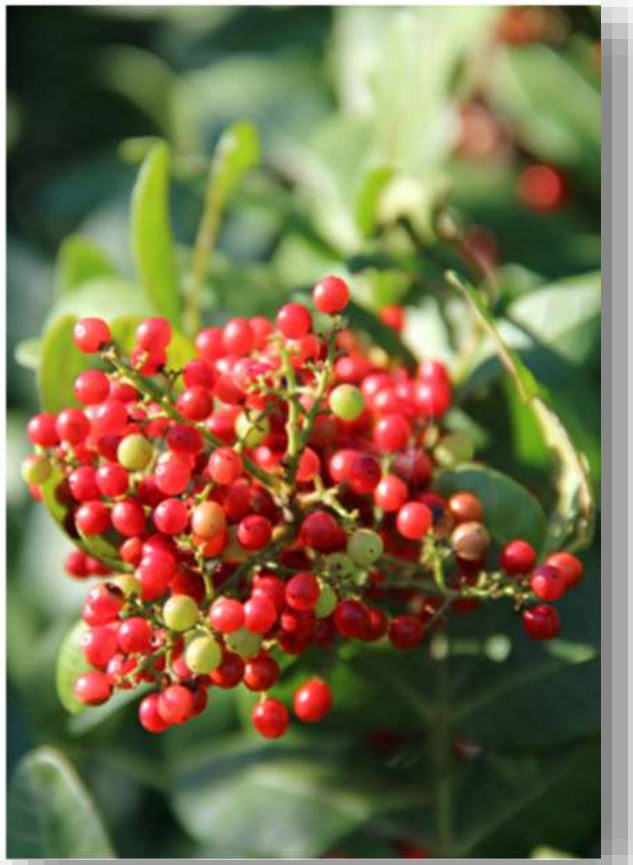


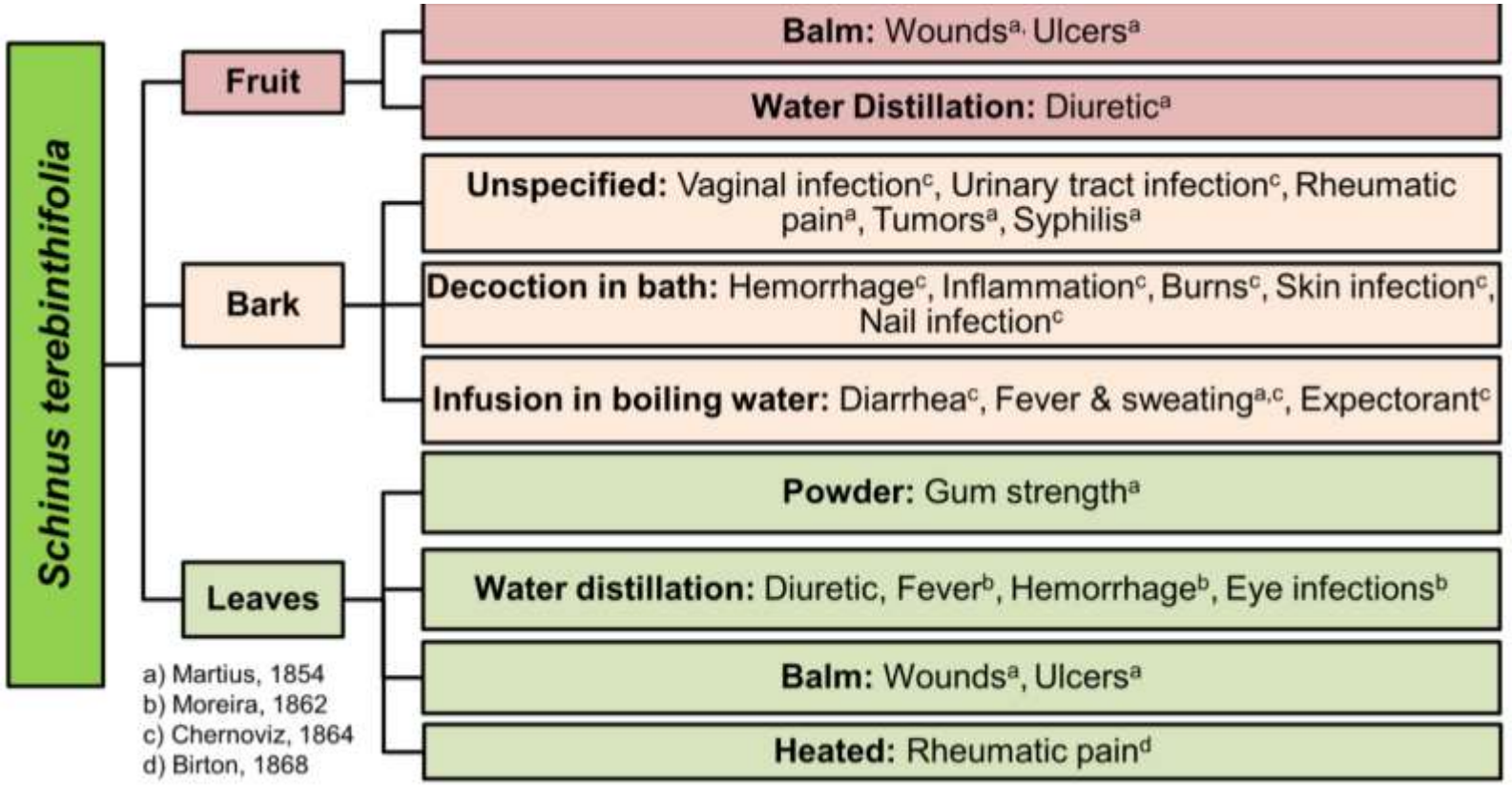






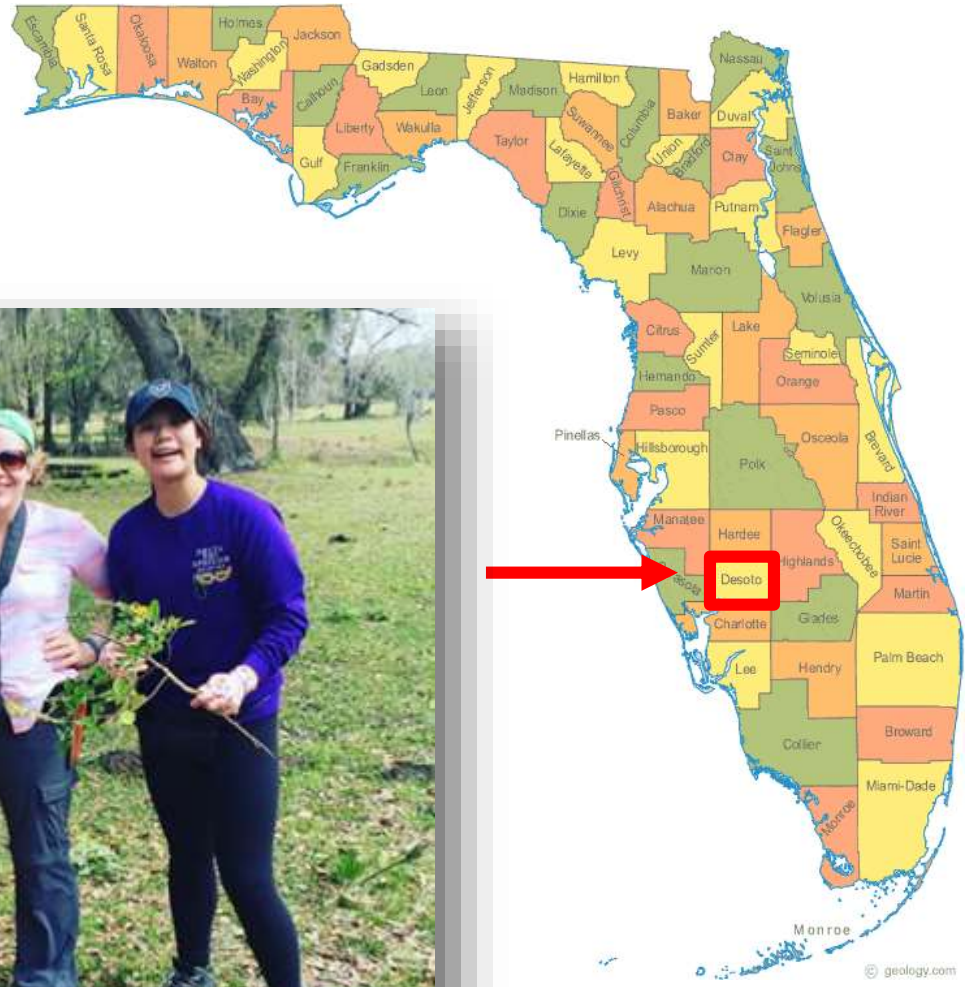
Brazilian Peppertree



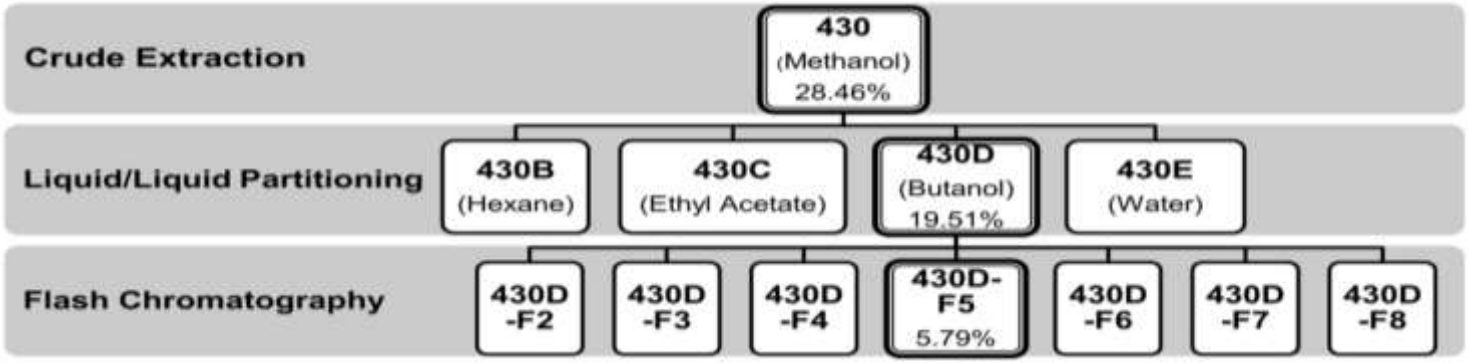


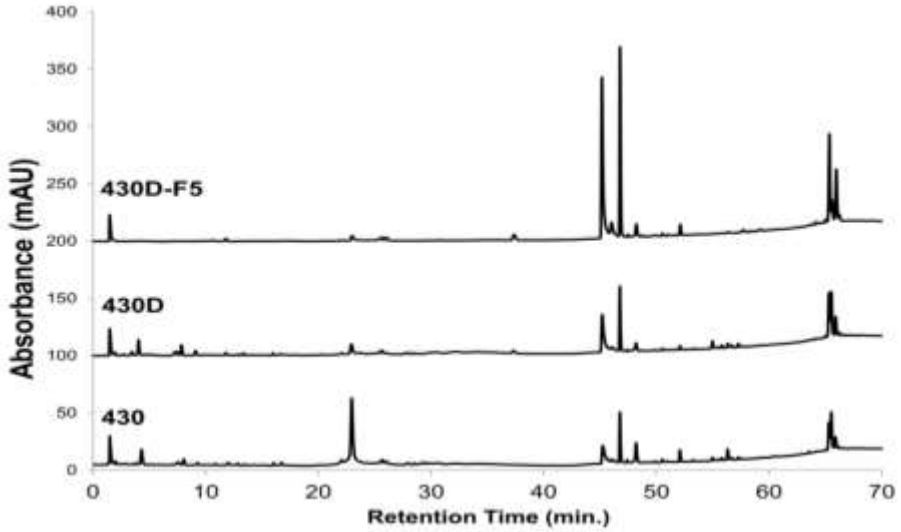
An exotic pest plant to some, a valued source of medicine to others. *Schinus terebinthifolia* Raddi is classified as a **Category I pest plant** by the Florida Exotic Pest Plant Council. Efforts to remove it from the United States have included the use of the herbicides triclopyr and glyphosate. On the other hand, its value as a medicinal plant has been broadly reported in South America

Where to collect?



Fractionation Scheme

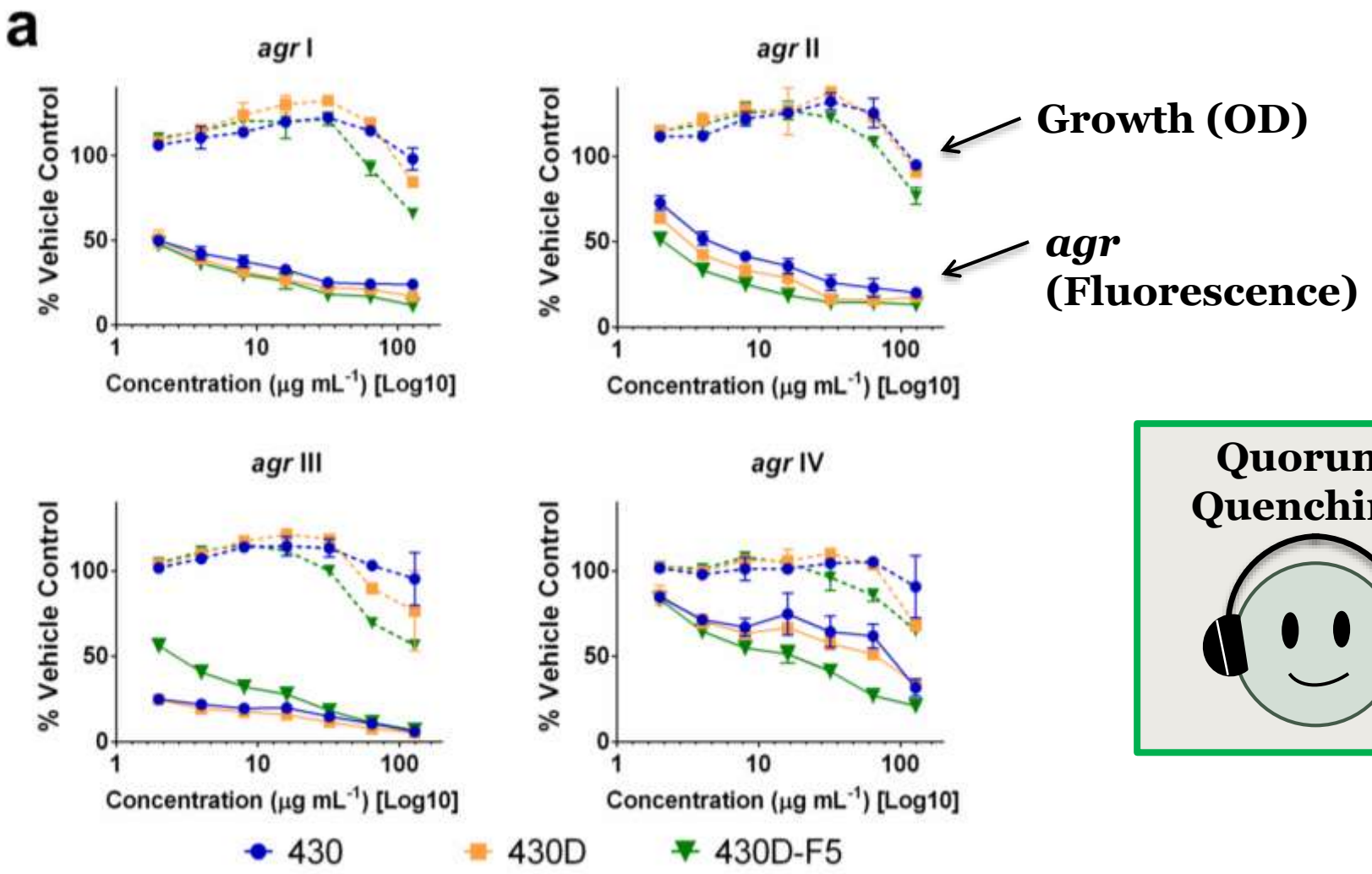




agr P3-GFP reporters used to guide fractionation

430D-F5 inhibits *agr* in a non-biocide manner

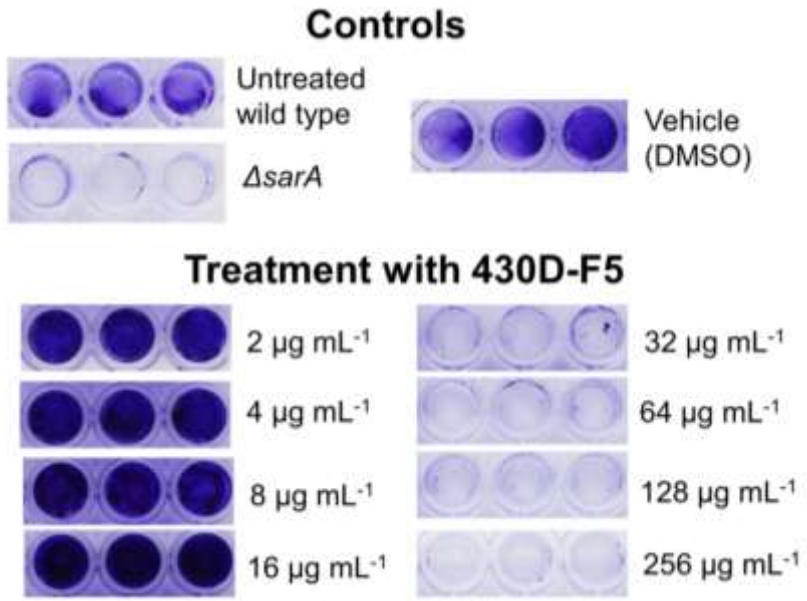
Not Bactericidal



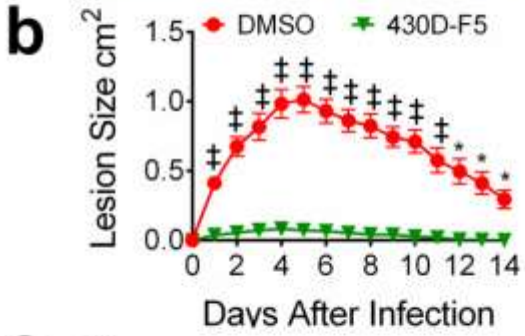
Quorum Quenching

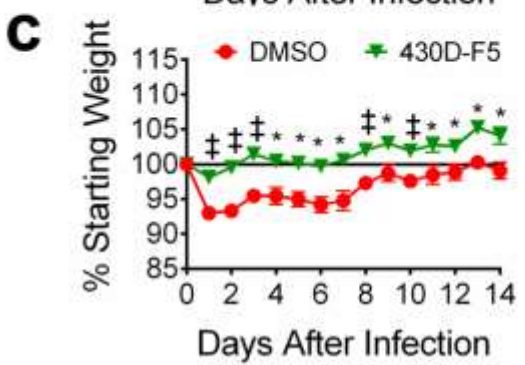
Muhs et al. (2017) Virulence inhibitors from Brazilian Peppertree block quorum sensing and abate dermonecrosis in skin infection models. *Scientific Reports* 7: 42275 doi:10.1038/srep42275

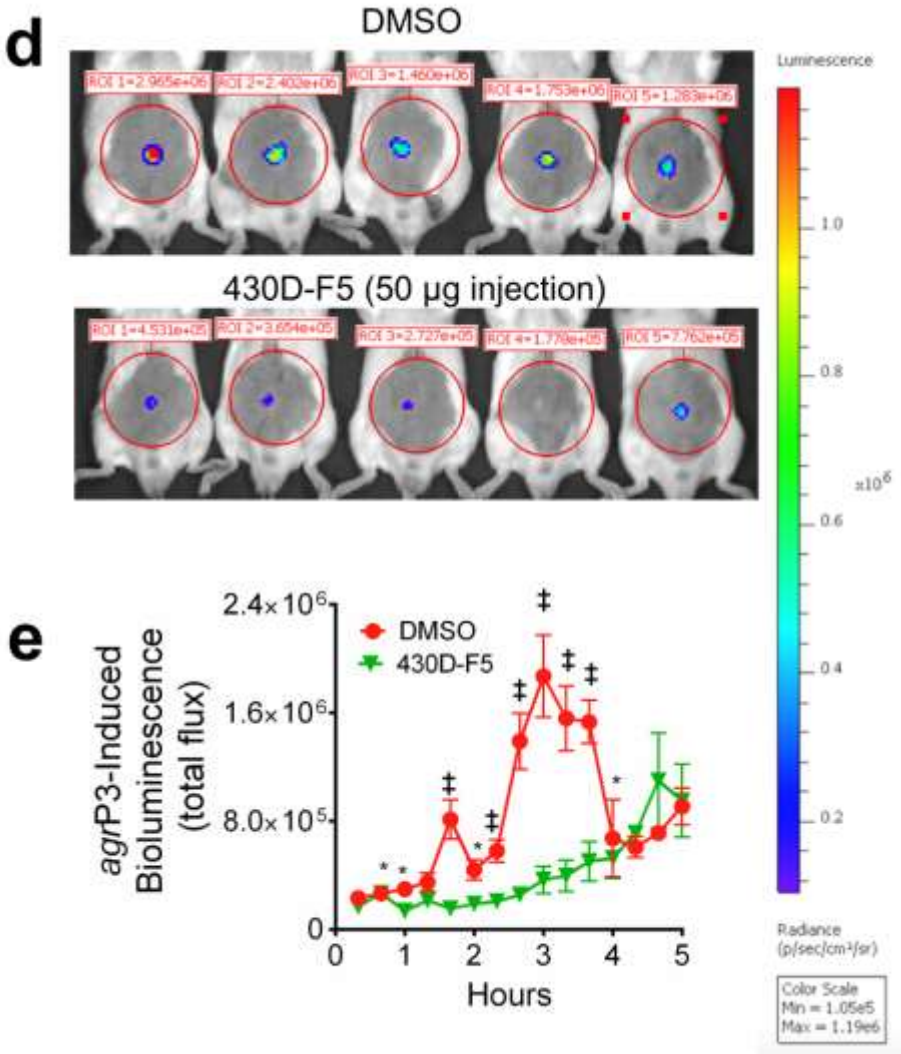
430D-F5 mediates quorum quenching *in vivo* and attenuates MRSA-induced dermatopathology in a murine model of skin and soft tissue infection



430D-F5 impacts biofilm formation



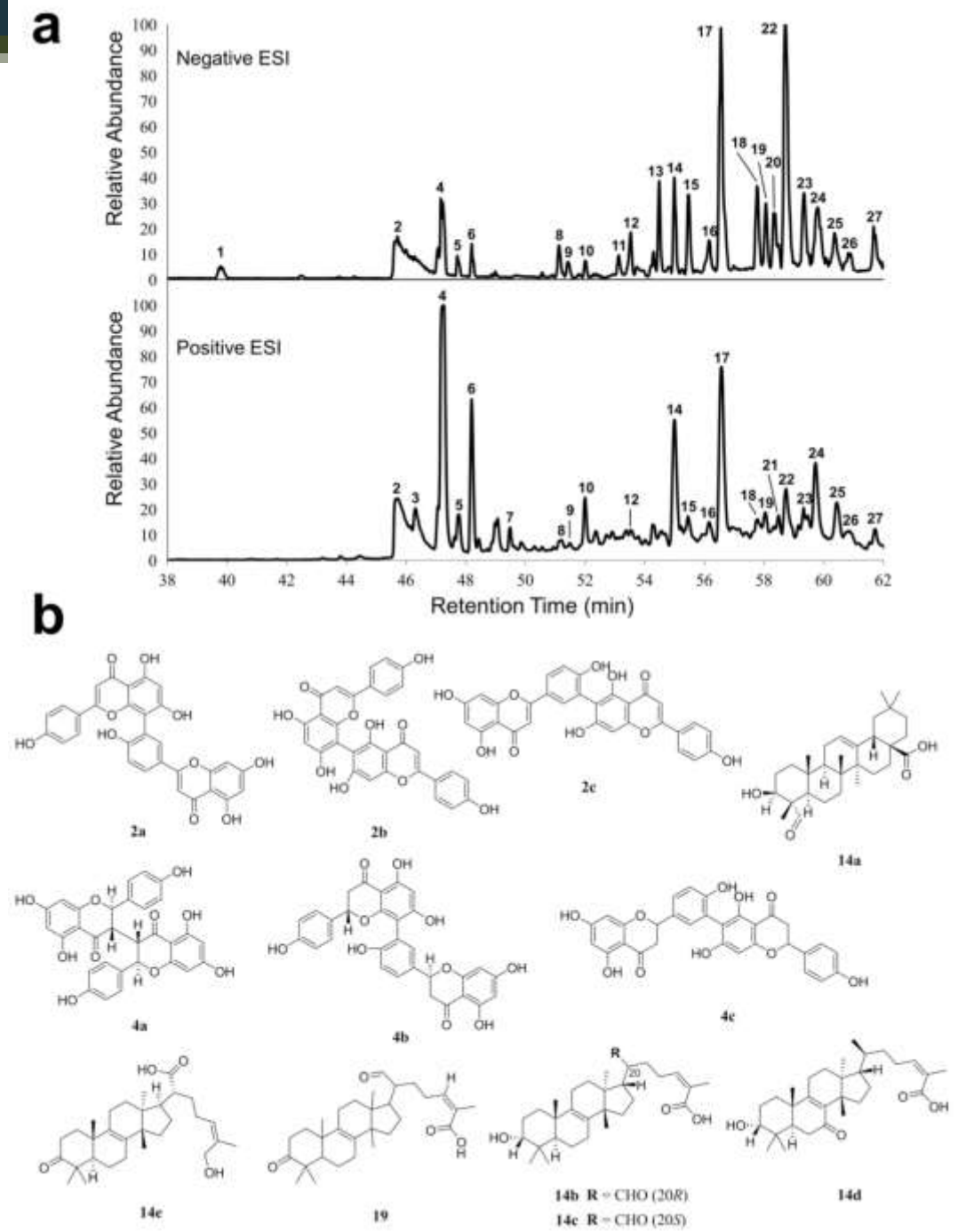




Muhs et al. (2017) *Scientific Reports* 7: 42275

Characterization of 430D-F5 major constituents

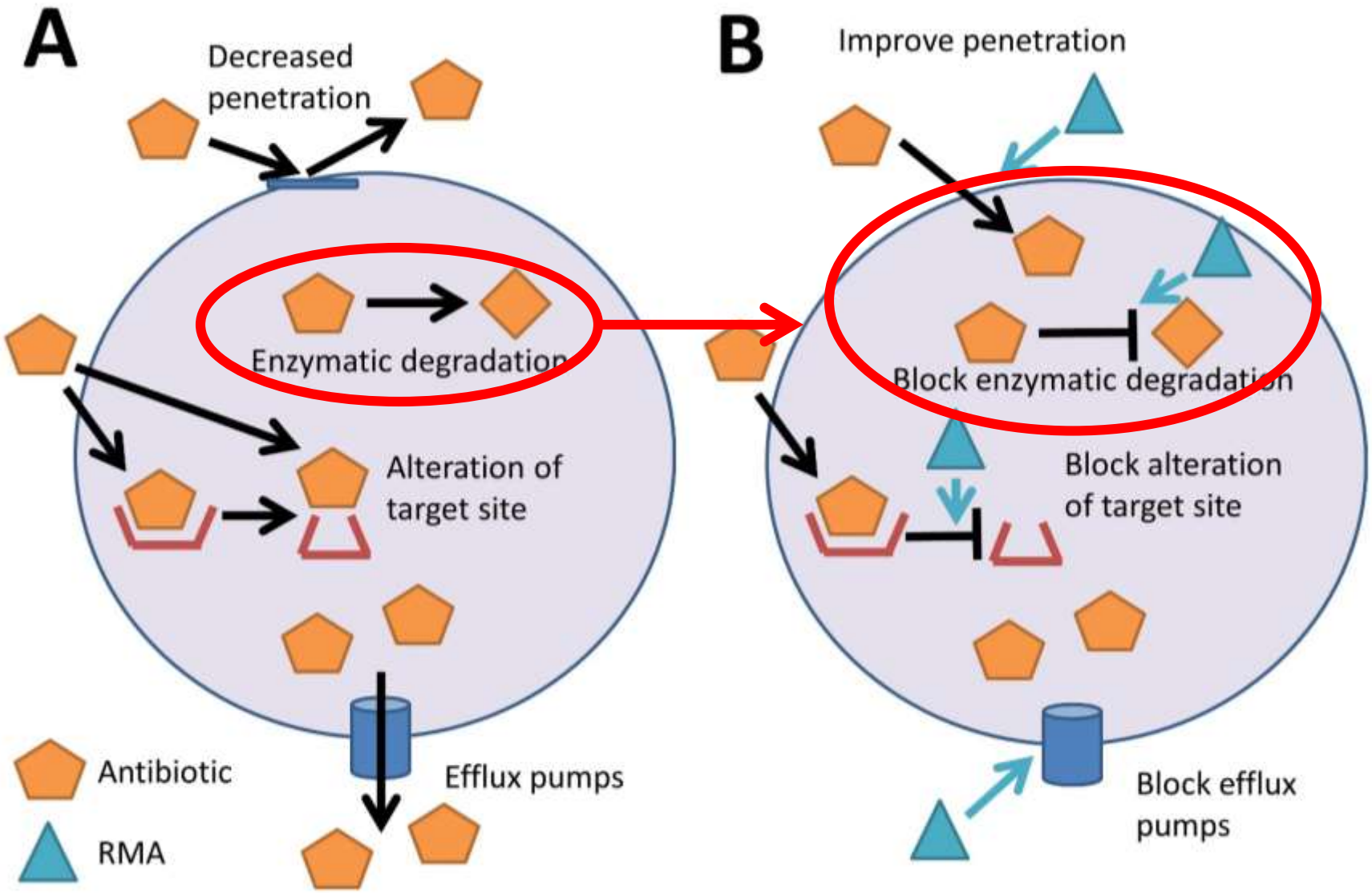
(**a**) LC-FTMS ESI negative and positive base peak chromatograms for 430D-F5. (**b**) Putative structural matches are listed by peak number. Peak **2** was determined to be $C_{30}H_{17}O_{10}$ and putative structural matches include: (**2a**) amentoflavone, (**2b**) agathisflavone, and (**2c**) robustaflavone. Peak **4** was determined to be $C_{30}H_{21}O_{10}$ and putative structural matches include: (**4a**) chamaejasmin, (**4b**) tetrahydroamentoflavone, and (**4c**) tetrahydrorobustaflavone. Peak **14** was determined to be $C_{30}H_{45}O_4$ and putative structural matches include: (**14a**) albsapogenin, (**14b**) (13α,14β,17α,20R,24Z)-3α-hydroxy-21-oxolanosta-8,24-dien-26-oic acid, (**14c**) (13α,14β,17α,20S,24Z)-3α-hydroxy-21-oxolanosta-8,24-dien-26-oic acid, (**14d**) (3α,13α,14β,17α,24Z)-3-hydroxy-7-oxo-lanosta-8,24-dien-26-oic acid, and (**14e**) mollinoic acid. Peak **19** was determined to be $C_{30}H_{45}O_4$ and putative structural matches include (**19**) isomasticadienonalic acid.



430D-F5 has limited impact on growth of commensal skin microflora

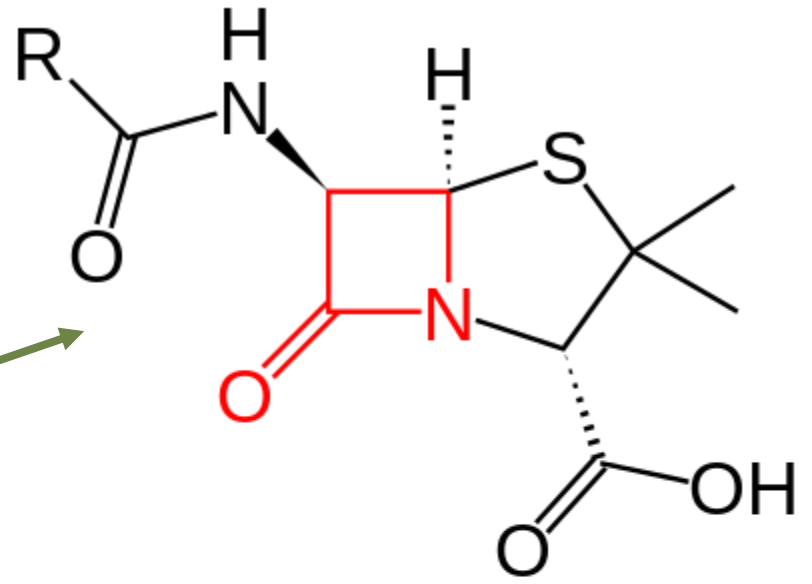
Species	Strain	MIC	430D-F5	Antibiotic Controls*			
				Amp	Clin	Erm	Van
Corynebacterium amycolatum	SK46	MIC_{50}	ND (512)	0.0625	-	0.00781	0.5
		MIC_{90}	ND (512)	2	-	2	2
Corynebacterium striatum	FS-1	MIC_{50}	ND (512)	ND (16)	-	1	0.5
		MIC_{90}	ND (512)	ND (16)	-	2	0.5
Micrococcus luteus	SK58	MIC_{50}	64	0.125	0.125	0.0625	0.25
		MIC_{90}	128	0.125	0.5	0.0625	0.25
Propionibacterium acnes	HL005PA 2; HM-493	MIC_{50}	16	-	0.125	0.125	-
		MIC_{90}	256	-	0.125	0.5	-
Staphylococcus epidermidis	NIHLM00 1; HM896	MIC_{50}	64	0.03125	-	-	1
		MIC_{90}	ND (512)	0.0625	-	NT	1
Staphylococcus haemolyticus	NRS116	MIC_{50}	64	ND (32)	-	ND (32)	1
		MIC_{90}	ND (512)	ND (32	-	ND (32)	2
Staphylococcus warneri	SK66	MIC_{50}	64	0.0625	-	-	0.5
		MIC_{90}	ND (512)	0.0625	-	-	1
Streptococcus mitis	F0392	MIC_{50}	64	0.03125	-	0.00781	0.5
		MIC_{90}	ND (512)	0.0625	-	0.03125	0.5
Streptococcus pyogenes	MGAS1525 2	MIC_{50}	ND (512)	0.0156	0.125	0.0625	-
		MIC_{90}	ND (512)	0.0313	0.125	0.0625	-

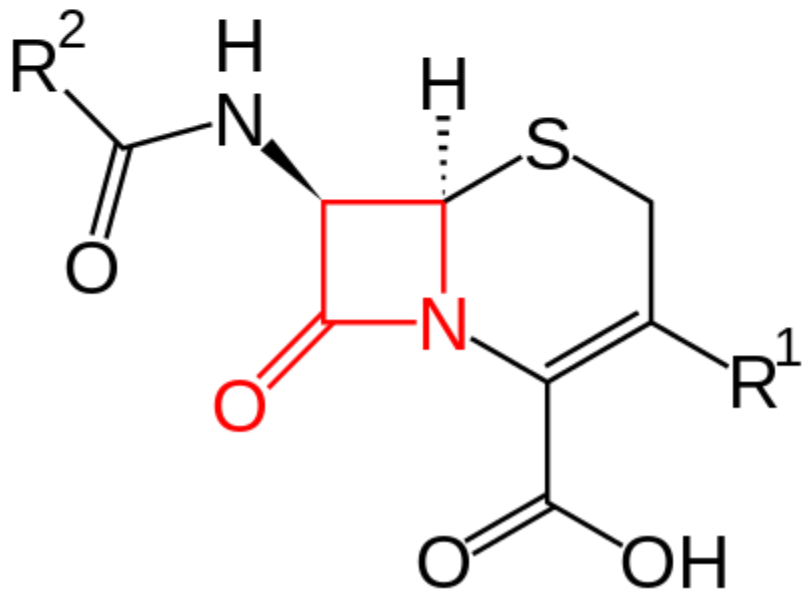
Resistance Modification



Beta-lactam antibiotics

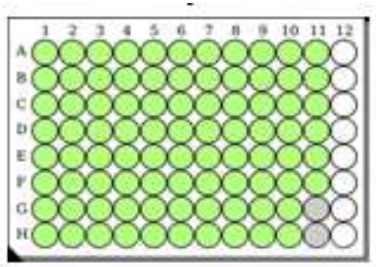
- Class of broad-spectrum antibiotics, all which have a beta-lactam ring in their molecular structures
  - Penams (penicillin derivatives)
  - Cephems (cephalosporins)
  - Monobactams
  - Carbapenems
- Act by inhibiting synthesis of peptidoglycan layer of cell wall
  - Bactericidal
- Resistance occurs when enzymes breakdown the beta-lactam ring





Screening Platform

QNPL



>900 extracts
(25 µg/mL)

Test +/- ¼ MIC for Oxacillin in MRSA isolates

Test actives in 2D-checkerboard assay with Oxacillin & calculate FIC

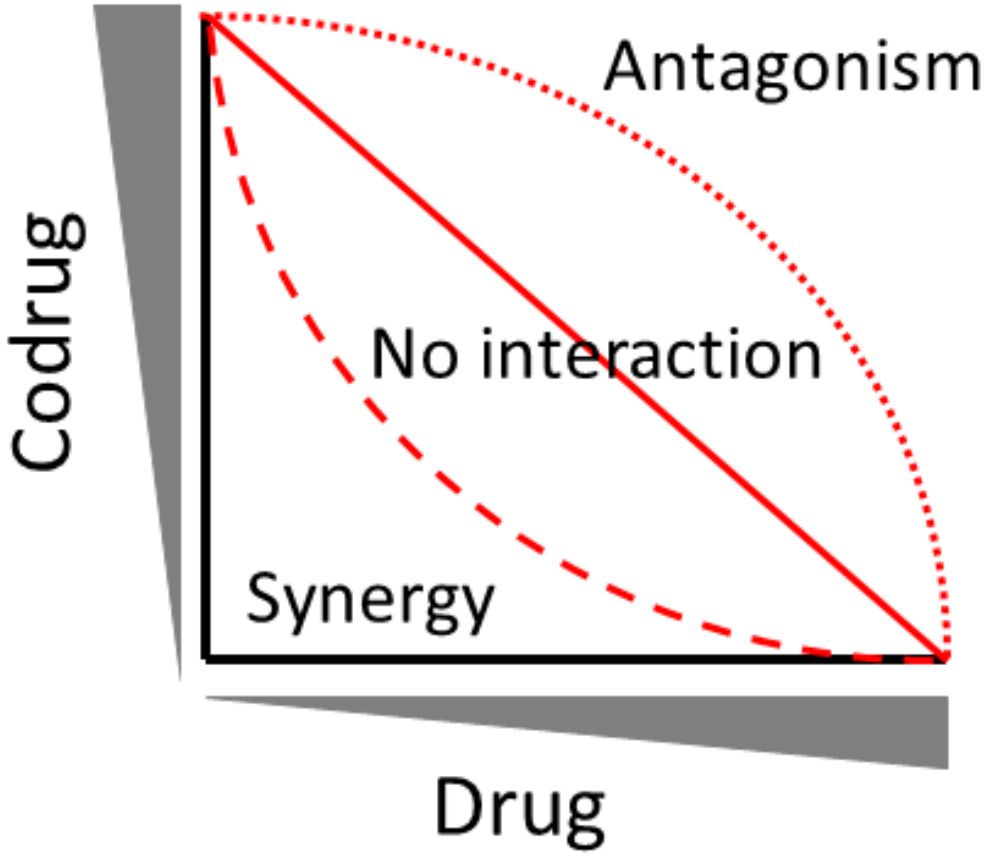
Partitioning of active extract 649

Retest against multiple MRSA strains in checkerboard

Characterize by LC-FTMS

Mechanistic studies

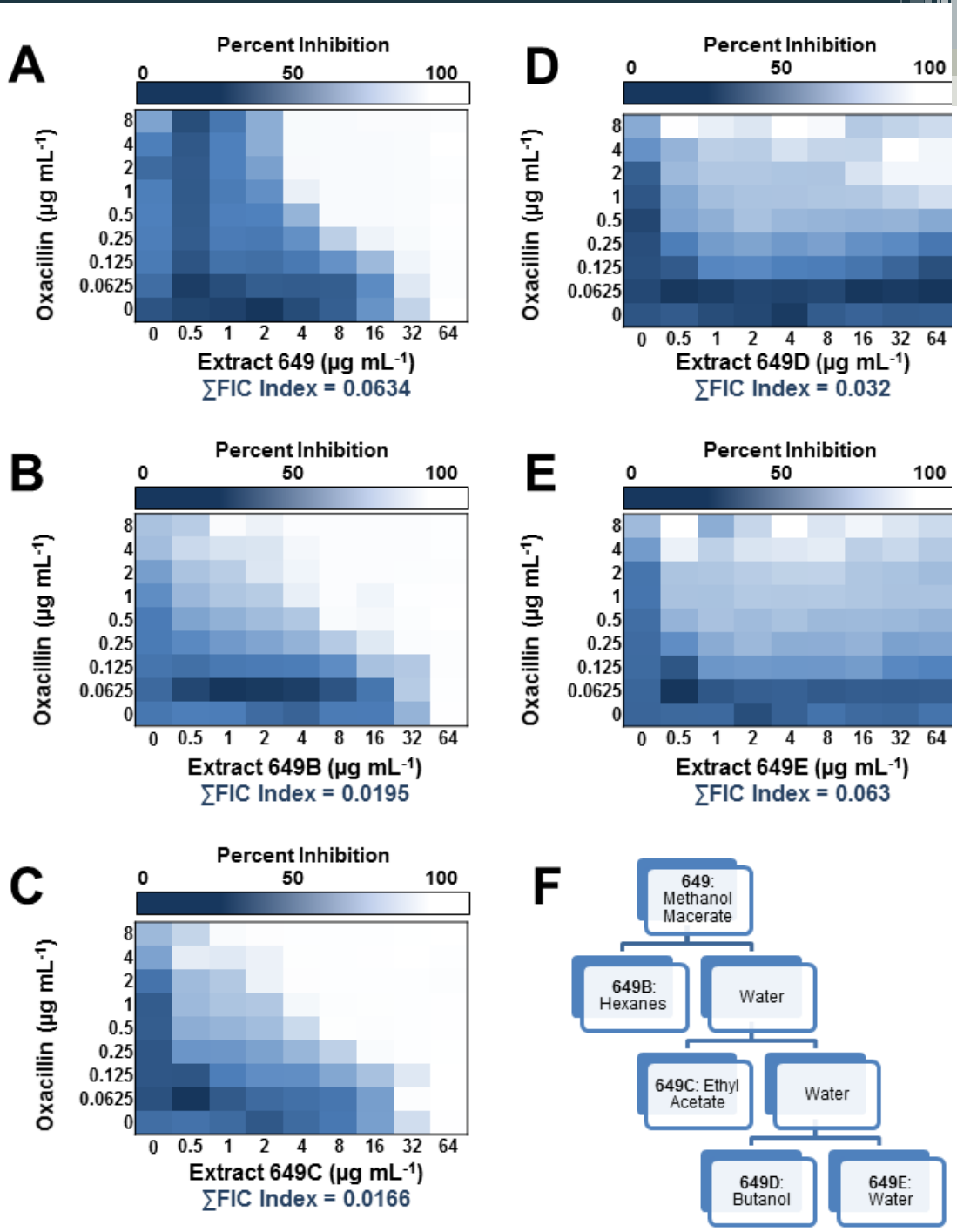
Fractional Inhibitory Concentration (FIC)



- FIC = MIC drug [in presence of codrug] / MIC drug alone
- FIC Index = $\sum FIC_x + FIC_y$
- FICI
  - <0.5 is synergistic
  - 0.5-4 additive or no interaction
  - >4 antagonistic

Resistance Modifying Agents

- 25/900 extracts showed β-lactam sensitization (**3% hit rate from QNPL**)
- Extract 649 pursued due to high potency and lack of toxicity to human skin cells
  - History of use in Native American medicine as poultice for infected wounds and ulcers



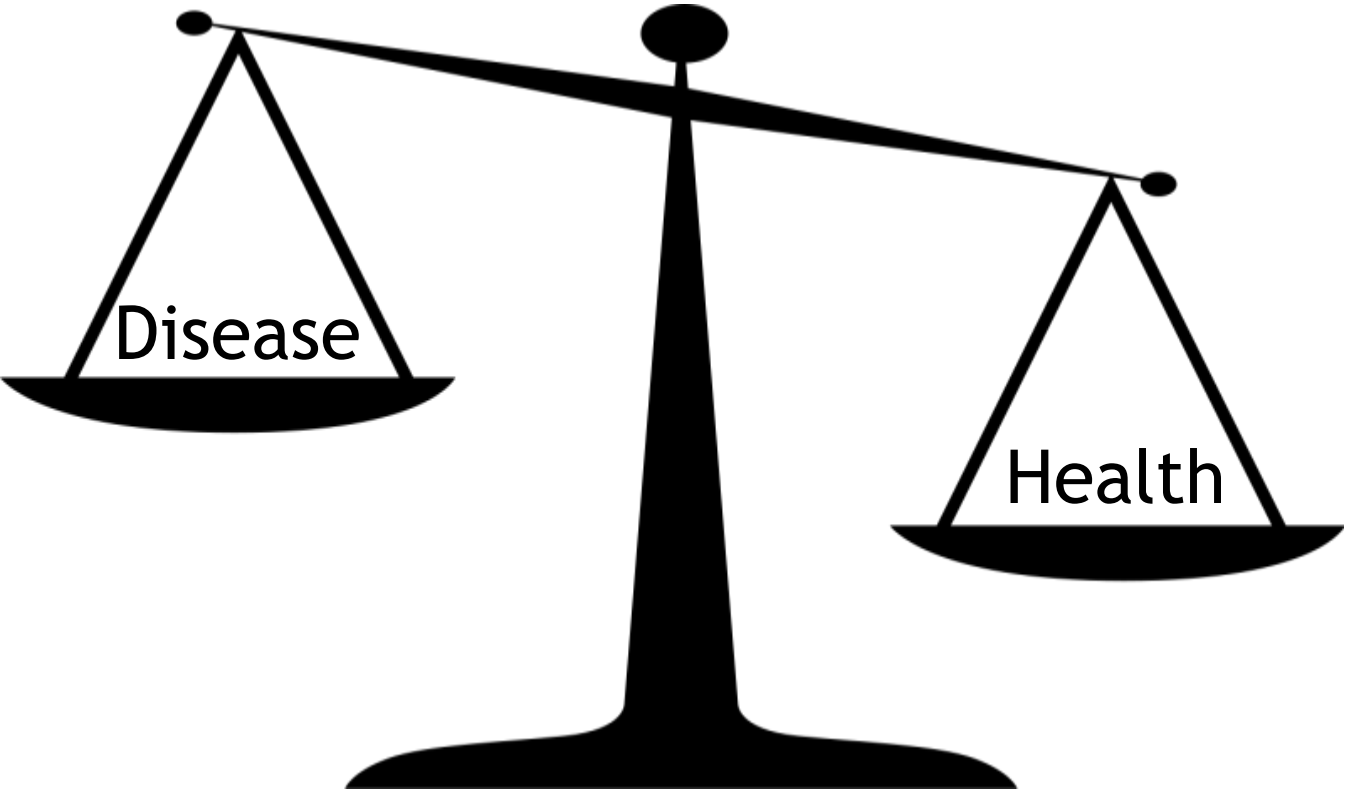
MIC Table

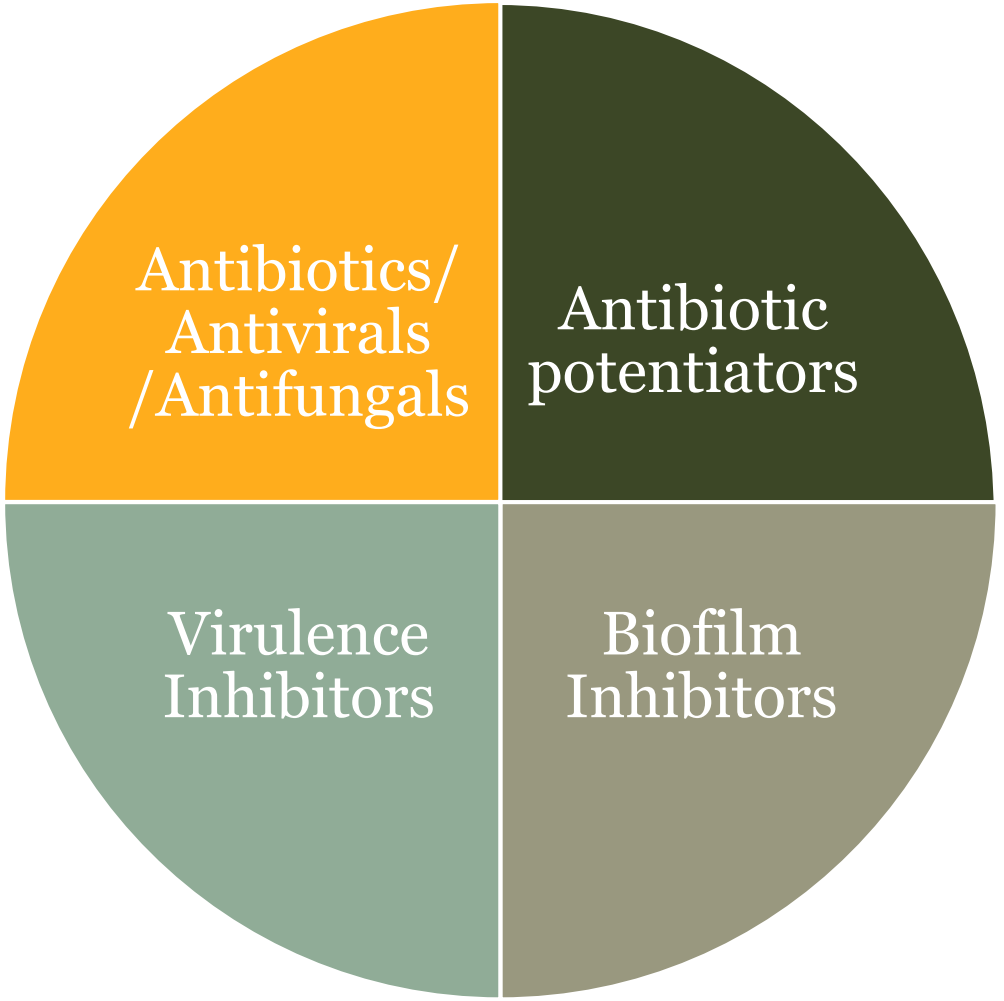
Ox breakpoint MIC = 2 µg/mL

Extract (µg/mL)	649		649B		649C		649D		649E	
	LAC	MW2	LAC	MW2	LAC	MW2	LAC	MW2	LAC	MW2
0	64	32	64	32	64	32	64	32	64	32
1	>8	>8	8	>8	8	>8	8	>8	>8	>8
2	>8	>8	8	>8	2	>8	>8	>8	>8	>8
4	1	>8	1	>8	1	>8	8	>8	8	>8
8	0.5	4	0.5	8	0.5	8	8	>8	>8	>8
16	0.25	2	0.5	2	0.25	2	>8	>8	8	>8
32	0.063	0.25	0.25	1	0.25	1	>8	>8	>8	>8

We achieved a **1,000-fold drop** in Oxacillin MIC (from 64 µg/mL to 0.063 µg/mL) with Extract 649

Health in the balance





Ethnobotany in the post-antibiotic era

- How can we use traditional knowledge of anti-infective remedies to innovate the next generation of therapeutics?
- Looking beyond 1940's paradigm of kill, kill, kill….
  - Anti-virulence
  - Evasion of pathogen defenses (e.g. biofilm)
  - Potentiation of existing therapeutics that have lost activity
  - Host-directed therapies
  - Achieving balance….
- Can we develop the right questions? ….only then can we find the right answers.

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Roberta Melander, PhD

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Andrea Pieroni, PhD (U. Gastr. Sci., Italy)
Alfonso La Rosa (Silene, Italy)

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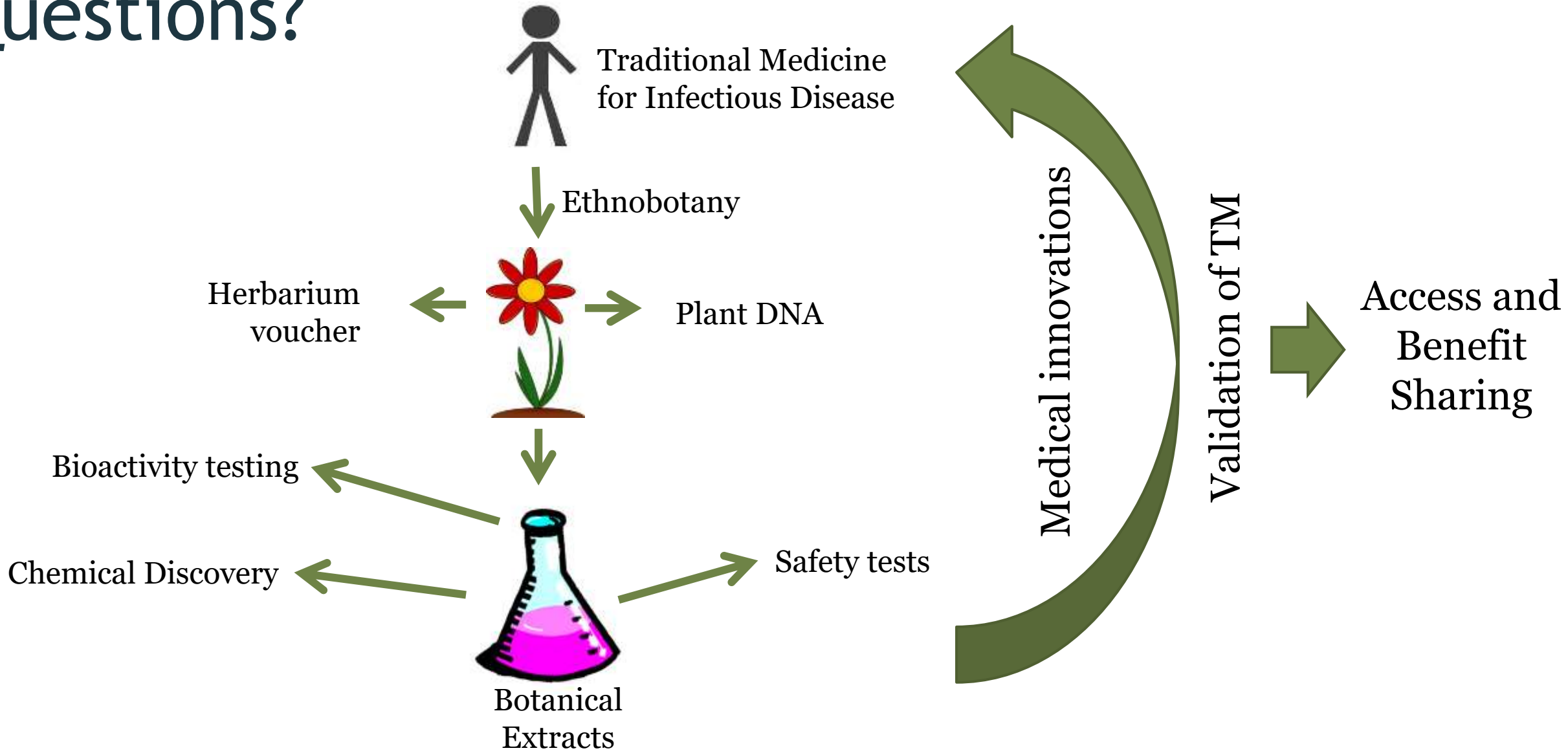
Georgia Research Alliance

Questions?



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