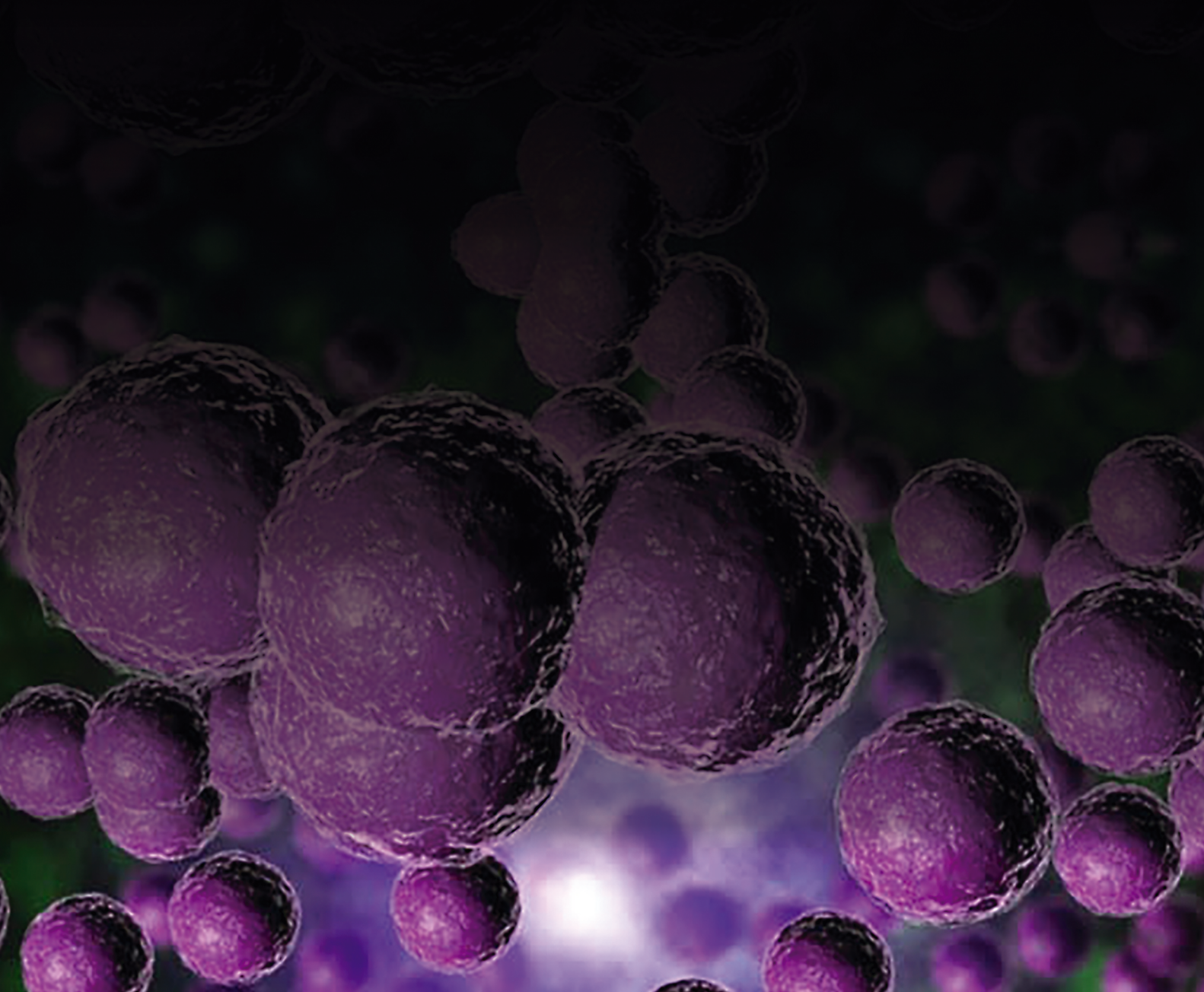# "Compuestos antimicrobianos de origen natural: Una oportunidad para el tratamiento de enfermedades infecciosas resistentes a antibióticos"

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Universidad Miguel Hernández de Elche

Tesis Doctoral 2021

Compuestos antimicrobianos de origen natural: una oportunidad para el tratamiento de enfermedades infecciosas resistentes a antibióticos

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Antimicrobial compounds of natural origin: an opportunity for the treatment of infectious diseases resistant to antibiotics

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Programa de Doctorado en Biología Molecular y Celular

Esta Tesis Doctoral se presenta como un compendio de trabajos previamente publicados que se citan a continuación:

- **Francisco Javier Álvarez-Martínez**, Enrique Barrajón-Catalán, José Antonio Encinar, Juan Carlos Rodríguez-Díaz y Vicente Micol. *Antimicrobial Capacity of Plant Polyphenols against Gram-positive Bacteria: A Comprehensive Review.* **Current Medicinal Chemistry** (2020); volumen 27, número 15, páginas 2576-2606; DOI: 10.2174/0929867325666181008115650.

- **Francisco Javier Álvarez-Martínez**, Enrique Barrajón-Catalán y Vicente Micol. *Tackling Antibiotic Resistance with Compounds of Natural Origin: A Comprehensive Review.* **Biomedicines** (2020); volumen 8, número 10, edición especial Natural Medicine in Therapy; DOI: 10.3390/biomedicines8100405.

- **Francisco Javier Álvarez-Martínez**, Juan Carlos Rodríguez, Fernando Borrás-Rocher, Enrique Barrajón-Catalán y Vicente Micol. *The antimicrobial capacity of Cistus salviifolius and Punica granatum plant extracts against clinical pathogens is related to their polyphenolic composition.* **Scientific Reports** (2021); volumen 11, número 588; DOI: 10.1038/s41598-020-80003-y8d03c117-eb10-4e75-bda9-3a8a60ecb6b2.

Durante el periodo de la presente Tesis Doctoral se colaboró en otros trabajos publicados o enviados no incluidos en este compendio:

- Enrique Barrajón-Catalán, **Francisco Javier Álvarez-Martínez**, Fernando Borrás, David Pérez, Noemí Herrero, Juan J. Ruiz y Vicente Micol. *Metabolomic analysis of the effects of a commercial complex biostimulant on pepper crops.* **Food Chemistry** (2020); volumen 310, número 125818, DOI: 10.1016/j.foodchem.2019.125818.

- Haifa Jebabli, Houda Nsir, Amani Taamalli, Ibrahim Abu-Reidah, **Francisco Javier Álvarez-Martínez**, Maria Losada-Echeberria, Enrique Barrajón-Catalán y Ridha Mhamdi. *Industrial-Scale Study of the Chemical Composition of Olive Oil Process-Derived Matrices.* **Processes** (2020); volumen 8, número 6, edición especial Phenolic Compounds: Extraction, Optimization, Identification and Applications in Food Industry; DOI: 10.3390/pr8060701.

- **Francisco Javier Álvarez-Martínez**, Enrique Barrajón-Catalán y Vicente Micol. *Antibacterial plant compounds: an updated review on their effects and putative mechanisms of action* Enviado a **Phytomedicine**.

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CERTIFICAN que el trabajo de investigación que conduce a la obtención del grado de Doctor, titulado: "Compuestos antimicrobianos de origen natural: una oportunidad para el tratamiento de enfermedades infecciosas resistentes a antibióticos", del que es autor D. Francisco Javier Álvarez Martínez, ha sido realizado bajo su dirección en el Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE) de la Universidad Miguel Hernández de Elche,

Y DAN SU CONFORMIDAD para la presentación de dicha Tesis Doctoral bajo la modalidad de **Compendio de Publicaciones**.

Para que conste a los efectos oportunos, firman el presente certificado en Elche a 12 de marzo de 2021.

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Para que conste a los efectos oportunos, firma el presente certificado en Elche a 12 de marzo de 2021.

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en Biología Molecular y Celular

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"El misterio de la vida no es un problema a resolver,

sino una realidad a experimentar."

Frank Herbert

RESUMEN

La presente Tesis Doctoral nace de la urgente necesidad de hallar nuevos agentes antimicrobianos eficaces como consecuencia del auge de los microorganismos resistentes a antibióticos que causan infecciones cada vez más graves y difíciles de tratar en todo el planeta. Este trabajo describe y profundiza en la capacidad antimicrobiana de los compuestos de origen natural como herramienta para el desarrollo de terapias antibióticas alternativas o complementarias a las existentes en la actualidad. Esta Tesis Doctoral se estructura como un compendio de tres artículos científicos publicados en revistas de alto índice de impacto pertenecientes al primer cuartil (Q1), cada una de ellas correspondiente a un capítulo.

El Capítulo 1 revisa y estudia la capacidad antibacteriana de los polifenoles de plantas frente a especies bacterianas de interés clínico, centrándose especialmente en *Staphylococcus aureus* y sus cepas resistentes a antibióticos. Además, incluye un cribado virtual de moléculas polifenólicas frente a dianas moleculares bacterianas relacionadas con la resistencia a antibióticos. Este capítulo remarca el enorme potencial terapéutico de los fitoquímicos como antimicrobianos y sienta las bases bibliográficas para los estudios *in vitro* desarrollados en el siguiente capítulo.

En el Capítulo 2 se describe el proceso de cribado de diversos fitoquímicos potencialmente antimicrobianos frente a once especies bacterianas extraídas de muestras de pacientes del Hospital General Universitario de Alicante. Los resultados obtenidos apuntan a que los dos extractos de plantas seleccionados poseen actividad antimicrobiana y mecanismos de acción diferentes. Asimismo, se observa que el nivel de susceptibilidad bacteriana a la acción de los extractos puede correlacionarse con su perfil de resistencia a antibióticos de uso clínico. Se muestra una actividad antibacteriana diferencial frente a los aislados de *S. aureus* según su perfil de resistencia a antibióticos y la composición polifenólica de los extractos, observación que podría conducir al desarrollo de terapias combinatorias que incluyan antibióticos y extractos vegetales.

El Capítulo 3 consiste en una ampliación del espectro de estudio compuestos naturales antimicrobianos, pasando a estudiar 68 compuestos diferentes de fuentes animales, bacterianas y fúngicas, además de las vegetales hasta ahora descritas. En este capítulo se incluyen las principales dianas moleculares y mecanismo de acción antimicrobiano propuesto para cada compuesto revisado. El campo de los compuestos naturales antimicrobianos es enorme y su tendencia es creciente. El avance tecnológico y científico permite la identificación

y el redescubrimiento de compuestos naturales prometedores para combatir las infecciones humanas, incluyendo aquellas resistentes a antibióticos.

ABSTRACT

This Doctoral Thesis arises from the urgent need to find new effective antimicrobial agents because of the rise of antibiotic-resistant microorganisms that cause increasingly serious and difficult-to-treat infections throughout the planet. This work describes and deepens the antimicrobial capacity of compounds of natural origin as a tool for the development of alternative or complementary antibiotic therapies to those that currently exist. This Doctoral Thesis is structured as a compendium of three scientific articles published in high impact index journals belonging to the first quartile (Q1), each corresponding to a chapter.

Chapter 1 studies the antibacterial capacity of plant polyphenols against bacteria of clinical interest, focusing especially on *Staphylococcus aureus* and its antibiotic resistant strains. Furthermore, it includes a virtual screening of polyphenolic molecules against bacterial molecular targets related to antibiotic resistance. This chapter highlights the enormous therapeutic potential of phytochemicals as antimicrobials and lays the bibliographic basis for the following *in vitro* studies developed in the next chapter.

Chapter 2 describes the screening process of various potentially antimicrobial phytochemicals against eleven bacterial species extracted from patient samples of the Alicante University General Hospital. The results obtained point to the fact that the two selected plant extracts have antimicrobial activity and have different mechanisms of action. Likewise, it is observed that the level of bacterial susceptibility to the action of the extracts can be correlated with its resistance profile to antibiotics of clinical use. A differential antibacterial activity is shown against *S. aureus* isolates according to their antibiotic resistance profile and the polyphenolic composition of the extracts, an observation that could lead to the development of combinatorial therapies that include antibiotics and plant extracts.

Chapter 3 consists of an extension of the spectrum of study of natural antimicrobial compounds, going on to study 68 different compounds from animal, bacterial and fungal sources in addition to the plants described up to now. This chapter includes the main molecular targets and proposed antimicrobial mechanism of action for each compound reviewed. The field of natural antimicrobial compounds is vast, and its trend is increasing. Technological and scientific advancement allows the identification and rediscovery of promising natural compounds to combat human infections, including those resistant to antibiotics.

LISTADO DE ABREVIATURAS

ABTS, ácido 2,2'-azinobis(3-etilbenzotiazolín)-6-sulfónico

AEE, Área Económica Europea

ATCC, Colección Americana de Cultivos Tipo

CDC, Centros de Control y Prevención de Enfermedades

CMI, Concentración mínima inhibitoria

COX, Ciclooxigenasa

CS, extracto de *Cistus salviifolius*

ECDC, Centro Europeo para la Prevención y Control de Enfermedades

ESI, Ionización por electrospray

GLASS, Sistema Mundial de Vigilancia de la Resistencia a los Antimicrobianos

GLMR, Regresión de modelo lineal generalizado

HPLC, Cromatografía líquida de alta eficacia

IL-4, Interleuquina 4

MCA, Análisis de correspondencia múltiple

MMP-2, Metaloproteinasa-2

MS, Espectrometría de masas

ODS, Objetivos de Desarrollo Sostenible

OMS, Organización Mundial de la Salud

PBP, Proteína de unión a penicilina

PCA, Análisis de Componentes Principales

PDB, Protein Data Bank

PG, extracto de *Punica granatum*

PGE2, Prostaglandina E2

SARM, *Staphylococcus aureus* resistente a meticilina

SASM, *Staphylococcus aureus* sensible a meticilina

TNF-α, Factor de necrosis tumoral alfa

UE, Unión Europea

UV, Ultravioleta

TEAC, Capacidad antioxidante en equivalentes de Trolox

TGH, Transferencia genética horizontal

ÍNDICE

1. INTRODUCCIÓN GENERAL

1.1. LAS INFECCIONES RESISTENTES A ANTIBIÓTICOS

1.1.1. Breve historia de los antibióticos

El descubrimiento y producción masiva de antibióticos fue uno de los mayores hitos de la historia de la medicina [1]. Antes de la aparición de éstos, la humanidad fue asediada por epidemias que segaron millones de vidas que fueron provocadas no sólo por bacterias, sino también por virus: peste, viruela, lepra, tifus, fiebre amarilla, tuberculosis, gripe española, cólera, malaria y sífilis, entre otras. El uso de antibióticos junto con los nuevos conocimientos sobre patógenos y nuevos conceptos sobre higiene produjeron un gran aumento de la calidad y la esperanza de vida de las personas durante el siglo XX [2].

Se estima que la era de los antibióticos comenzó con el descubrimiento del efecto de la penicilina en 1928 y su publicación en 1929 por parte del científico escocés Alexander Fleming [3]. Sin embargo, la producción a gran escala y la elucidación de la estructura química de este antibiótico no se dio hasta el año 1940 gracias a Howard Walter Florey y Ernst Boris Chain. Fleming, Florey y Chain que recibieron el Premio Nobel de Fisiología o Medicina en 1945 por sus trabajos sobre la penicilina. Alexander Fleming, además, aportó una gran herramienta que sería clave para el campo del descubrimiento de nuevos antibióticos: el cribado de sustancias con actividad antibiótica por formación de halos en placas de agar (Figura 1) [4].

Figura 1. Réplica de la placa de Petri en la que Alexander Fleming descubrió la penicilina. Se observa el crecimiento de un parche del hongo *Penicillium* en la parte superior de la placa y diversas colonias de *Staphylococcus* en la parte inferior, evitando el área de crecimiento del hongo. Imagen extraída del libro Antibiotics and Antibiotic Resistance [5].

En el año 1935, el fisiólogo alemán Paul Gerhard Domagk descubrió la actividad antibiótica de las sulfamidas sintéticas [6]. Domagk recibió el Premio Nobel en 1939 por el descubrimiento del antibiótico que bautizó como Prontosil, un antibiótico eficaz contra cocos gram-positivos que ayudó a disminuir drásticamente la mortalidad por neumonía y meningitis [7].

A comienzos de la década de 1940, el bioquímico Selman Abraham Waksman y su equipo descubrieron el potencial productor de antibióticos de las bacterias del género *Streptomyces* empleando el método de inhibición en agar desarrollado por Fleming. El primer compuesto prometedor que identificaron fue el polipéptido actinomicina, producido por *Streptomyces antibioticus* subsp. *antibioticus*. En 1943 descubrieron la estreptomicina, un aminoglicósido producido por *Streptomyces anulatus* subsp. *griseus* que sirvió a Waksman para ganar el Premio Nobel de Medicina en 1952 [8]. Estos acontecimientos impulsarían la búsqueda y descubrimiento de más compuestos antibióticos por científicos de todo el mundo, dando lugar al periodo conocido como la "Era Dorada" del descubrimiento de los antibióticos. En la Figura 2 podemos ver a Paul Gerhard Domagk, Alexander Fleming y Selman Abraham Waksman.
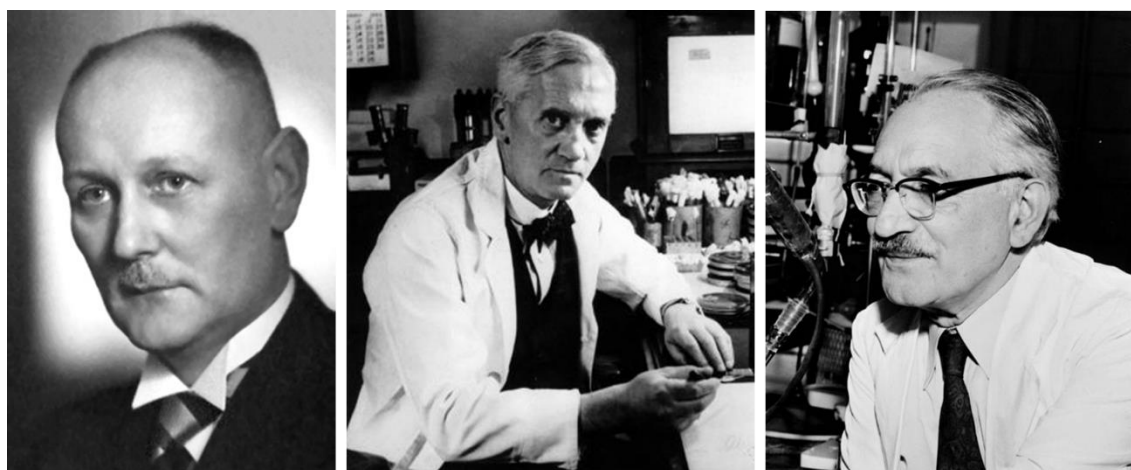
Figura 2. De izquierda a derecha: Paul Gerhard Domagk, Alexander Fleming y Selman Abraham Waksman. Ganadores del Premio Nobel de Medicina en 1939, 1945 y 1952, respectivamente, por sus trabajos en el campo del descubrimiento de antibióticos. Fotografías obtenidas del repositorio online Wikipedia Commons.

Se estima que la gran "Era Dorada" de los antibióticos sucedió entre las décadas de 1940 y 1970, siendo un periodo prolífico en el cual se descubrieron la gran mayoría de los antibióticos que usamos hoy en día [9]. Familias de antibióticos como los aminoglicósidos [10], cloranfenicol [11], tetraciclinas [12], macrólidos [13], glicopéptidos [14], estreptograminas [15], lincosamidas [16], ansamicinas [17] y quinolonas [18] fueron descubiertas durante este periodo. Desde la "Era Dorada" hasta hoy, el descubrimiento de nuevas clases de antibióticos ha descendido drásticamente. Los antibióticos, que una vez fueron tratamientos milagrosos, han perdido gran parte de su eficacia debido a la aparición de bacterias resistentes a éstos. El vertiginoso auge y rápida expansión de estas bacterias resistentes supone, unido al escaso descubrimiento de nuevos antibióticos, una amenaza sanitaria mundial [19].

1.1.2. Bacterias resistentes a antibióticos: una crisis sanitaria global

Las infecciones resistentes a los tratamientos antibióticos clásicos se cobran la vida de miles de personas en todo el mundo cada año [20-23]. Según datos publicados en 2019 por *Centers for Disease Control and Prevention* (CDC), más de 2,8 millones de infecciones resistentes a antibióticos suceden cada año en Estados Unidos, muriendo más de 35.000 personas como consecuencia de ello [24]. Según el informe publicado en 2017 por *European Centre for Disease Prevention and Control* (ECDC), la resistencia a antibióticos supone una grave amenaza sanitaria para Europa [25]. Esta crisis se atribuye, entre otros factores, al uso inadecuado de los antibióticos, la falta de investigación y desarrollo de nuevos antibióticos por la industria farmacéutica y políticas reguladoras deficientes por parte de las entidades gubernamentales [26].

El auge de las bacterias resistentes a antibióticos ha puesto en jaque a las terapias antibióticas que salvaron tantas vidas durante el pasado siglo y que supusieron un hito en la historia de la medicina. Si no se actúa de forma rápida y eficaz, el mundo podría volver al estado de la era pre-antibiótica, en la cual las infecciones comunes pueden desembocar en un destino fatal [27]. El uso adecuado en tiempo y forma de antimicrobianos eficaces resulta crucial para evitar desenlaces fatales para los pacientes [26].

Dada la gravedad de la situación, la Organización Mundial de la Salud (OMS) adoptó en el año 2015 el Plan de acción mundial sobre resistencia a antimicrobianos. Este plan incluyó la creación del Sistema Mundial de Vigilancia de la Resistencia a los Antimicrobianos (Global

Antimicrobial Resistance Surveillance System, GLASS), el cual se encarga de recopilar de forma normalizada los datos sobre la resistencia a los antimicrobianos a nivel mundial para impulsar la adopción de las medidas necesarias en cada región [28]. Debido a la reciente creación del GLASS, todavía no hay datos disponibles de muchos países y regiones, entre ellos España, Estados Unidos, los países latinoamericanos y gran parte de Asia y Oceanía. En la Figura 3 pueden observarse múltiples ejemplos del nivel de resistencia a antibióticos de diversas especies bacterianas en diferentes países cuyos datos sí están disponibles a través del GLASS.
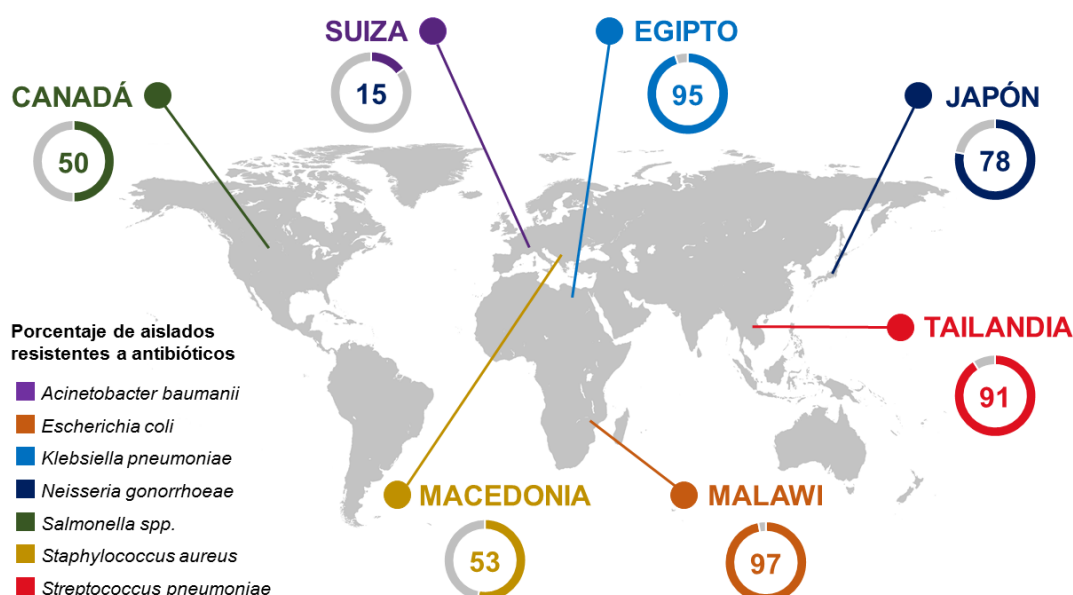
Figura 3. Porcentaje de aislados clínicos resistentes a antibióticos de diversas especies bacterianas en diferentes países del mundo. Datos extraídos del Sistema Mundial de Vigilancia de la Resistencia a los Antimicrobianos (GLASS, OMS). Elaboración propia.

Según la ECDC, las especies bacterianas que más problemas sanitarios causan en Europa en la actualidad debido a su resistencia a antibióticos son: *Klebsiella pneumoniae*, *Escherichia coli*, diversas especies del género *Acinetobacter*, *Enterococcus faecium* y *S. aureus*. Este último patógeno ha cobrado especial relevancia en los últimos años, ya que su fenotipo resistente a meticilina es muy común en gran parte de Europa, especialmente en el sur [25].

1.1.3. Causas del aumento del número de bacterias resistentes a antibióticos

La resistencia a antibióticos es un ejemplo de la enorme capacidad de evolución y adaptación natural de las bacterias frente a diferentes ambientes [29, 30]. Aunque este proceso parece inevitable, los humanos han acelerado esta evolución y adquisición de resistencias mediante el abuso y la inadecuada utilización de los antibióticos durante las últimas décadas [31, 32]. Entre las causas del mal uso de los antibióticos en humanos figuran la falta de conocimiento y conciencia pública, la posibilidad de acceder a antibióticos sin receta, el uso de sobrantes de antibióticos, capacitación médica inadecuada, la promoción farmacéutica y la falta de pruebas de diagnóstico rápidas [33].

Las bacterias usan su plasticidad genética para resistir el ataque de los antibióticos mediante mutaciones, adquisición de material genético y alteración de la expresión de su genoma [34]. De este modo, las bacterias que sobreviven al ataque de un antibiótico se convierten en las precursoras de las próximas generaciones bacterianas, agravando aún más el problema de la resistencia. Una vez se adquieren los genes de resistencia a antibióticos, estos pueden pasar de una bacteria a otra mediante procesos de división o por transferencia genética horizontal (TGH) [35]. Los procesos de TGH puede darse por transformación, transducción o conjugación con otras bacterias (Figura 4).
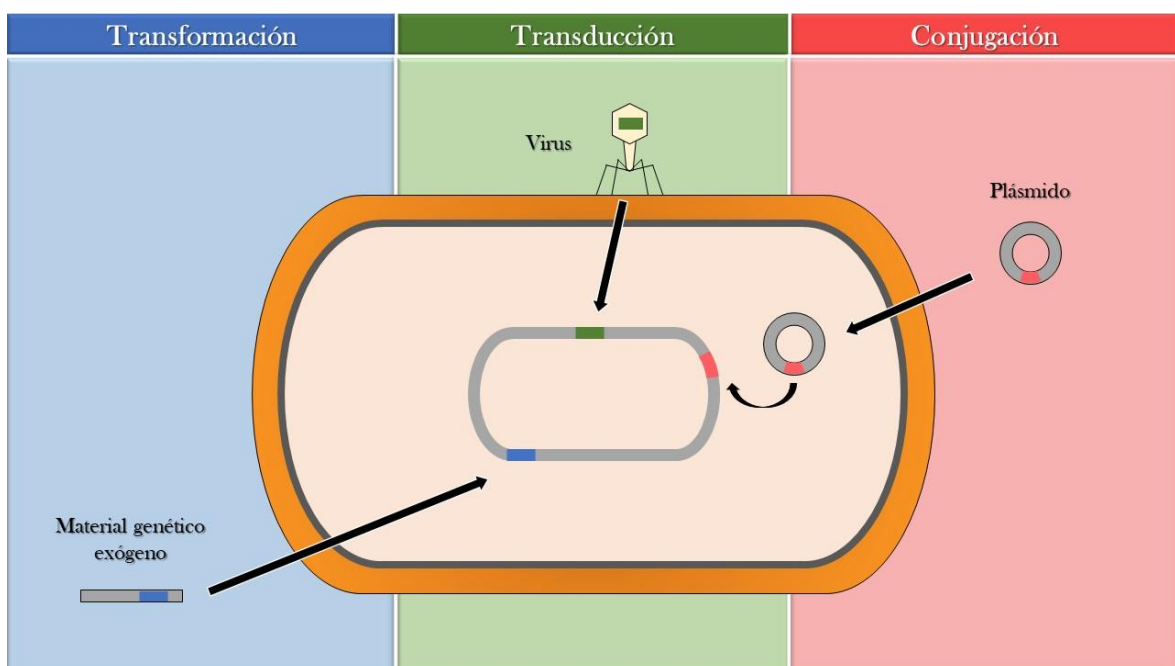
Figura 4. Procesos de transferencia genética horizontal en bacterias. Figura de elaboración propia.

Estos mecanismos pueden transferir resistencia a antibióticos a las bacterias que no han estado sometidas a la presión de selección antibiótica, creando reservorios de bacterias resistentes en el entorno [36]. Además, el fondo genético y la epistasia de las bacterias receptoras posee un rol fundamental en el proceso de adquisición de genes de resistencia, determinando si dichas bacterias son capaces de mantener, acumular y propagar el material genético [37].

Los procesos TGH pueden generar las conocidas como "superbacterias", que son bacterias que incorporan múltiples genes de resistencia frente a antibióticos, convirtiéndolas en patógenos intratables con los fármacos disponibles. Este hecho es especialmente relevante en el ámbito clínico hospitalario, en el cual se usan grandes cantidades de diferentes antibióticos y la aparición de "superbacterias" puede ser fatal para los pacientes que allí se encuentran [38].

Además del uso incorrecto de los antibióticos, existen otras conductas que han producido la aparición acelerada y descontrolada de bacterias resistentes. Una vez surgen bacterias resistentes a antibióticos, el uso continuado de los mismos produce una presión selectiva que ayuda a diseminar los genes de resistencia, sobre todo en zonas que carecen de medidas para controlar infecciones y de condiciones higiénicas óptimas [39]. De esta manera, las medidas de higiene tienen un papel determinante en la velocidad de transferencia y propagación de resistencias. El lavado incorrecto de manos, las malas condiciones de los centros sanitarios y las prácticas deficientes en la manipulación de alimentos aceleran la velocidad de aparición de bacterias resistentes a antibióticos [40]. Las causas mencionadas se resumen en la Figura 5.
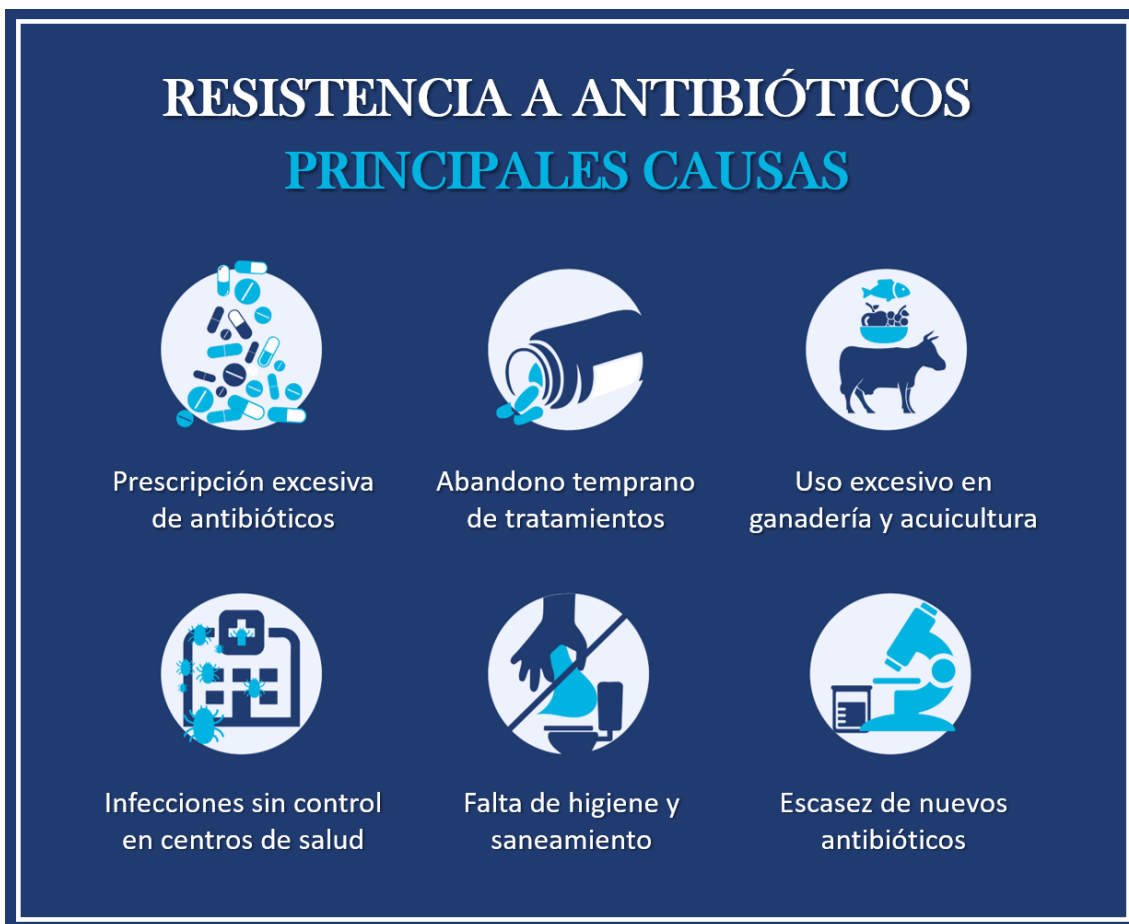
Figura 5. Principales causas del auge en la resistencia a antibióticos. Figura extraída y adaptada de la página web oficial de la OMS.

En las últimas décadas, se ha experimentado una importante escasez en el descubrimiento de nuevos antibióticos (Figura 6) [41]. Durante los últimos 30 años, solamente se han descubierto dos nuevas clases de antibióticos: las oxazolidinonas [42] y los lipopéptidos [43]. Ambas clases están indicadas para el tratamiento de infecciones por bacterias gram-positivas. No se han hallado nuevos antibióticos frente a bacterias gram-negativas. Existen distintos factores que han propiciado este escenario de escasez en el descubrimiento de nuevos antibióticos: un ambiente regulatorio estricto, una alta tasa de fracaso (mayor que para otros fármacos), un bajo índice de retorno económico debido a que las terapias antibióticas se suelen aplicar durante cortos periodos de tiempo y una gran preocupación por la aparición de resistencias que disminuiría el valor del nuevo antibiótico rápidamente [44]. Estos factores han convertido al desarrollo de nuevos antibióticos en un campo poco atractivo para la industria farmacéutica [45].
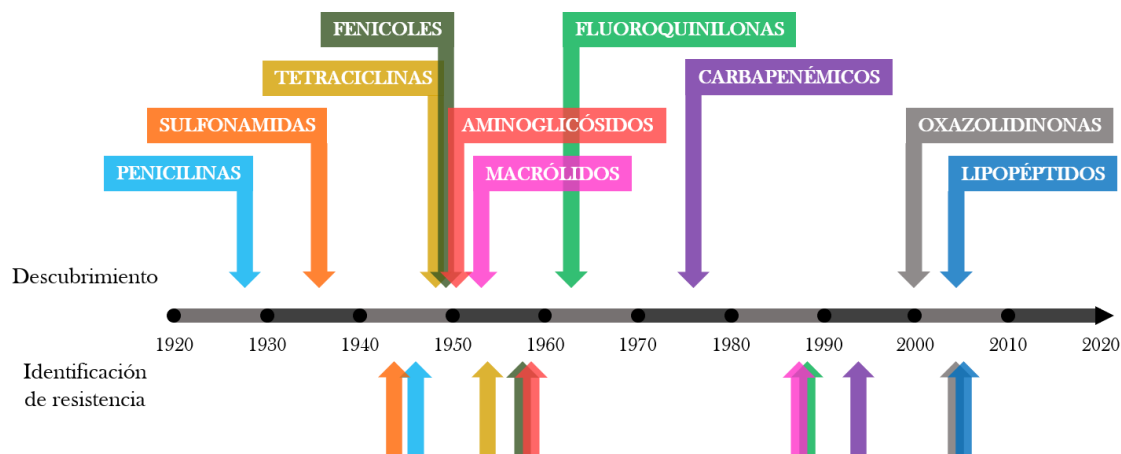
Figura 6. Línea temporal del descubrimiento de nuevas familias de antibióticos y de la identificación de bacterias resistentes frente a estos. Adaptación de la Figura 2 presente en el artículo correspondiente al Capítulo 3 de la presente Tesis Doctoral. Elaboración propia.

1.1.4. *Staphylococcus aureus* resistente a meticilina

Una de las infecciones resistentes a antibióticos más comunes y problemáticas es la causada por cepas de *S. aureus* resistentes a meticilina (SARM) [46]. Las infecciones causadas por SARM se asocian con mayores niveles de morbilidad, mortalidad, tiempo de ingreso y coste sanitario que las producidas por estafilococos no resistentes [47]. *S. aureus* posee múltiples factores de virulencia y la capacidad de adquirir resistencia frente a la mayoría de los antibióticos clínicos, lo que hace que se la catalogue como una "superbacteria" [48].

Las cepas de SARM (Figura 7) surgen cuando cepas susceptibles de *S. aureus* adquieren e incorporan un elemento genético móvil a su genoma denominado *staphylococcal cassette chromosome mec* (SCCmec) [49]. Este elemento incorpora el gen *mecA*, que codifica una proteína de unión a penicilina alterada (PBP2a), menos sensible a la acción de la mayoría de penicilinas semisintéticas y otros antibióticos betalactámicos que tienen como objetivo molecular la biosíntesis de la pared celular [50]. Debido a la acción de PBP2a, el SARM es capaz de llevar a cabo la biosíntesis de su pared celular incluso en presencia de concentraciones de antibióticos betalactámicos que serían inhibitorias para el resto de cepas de *S. aureus* sensibles [51].
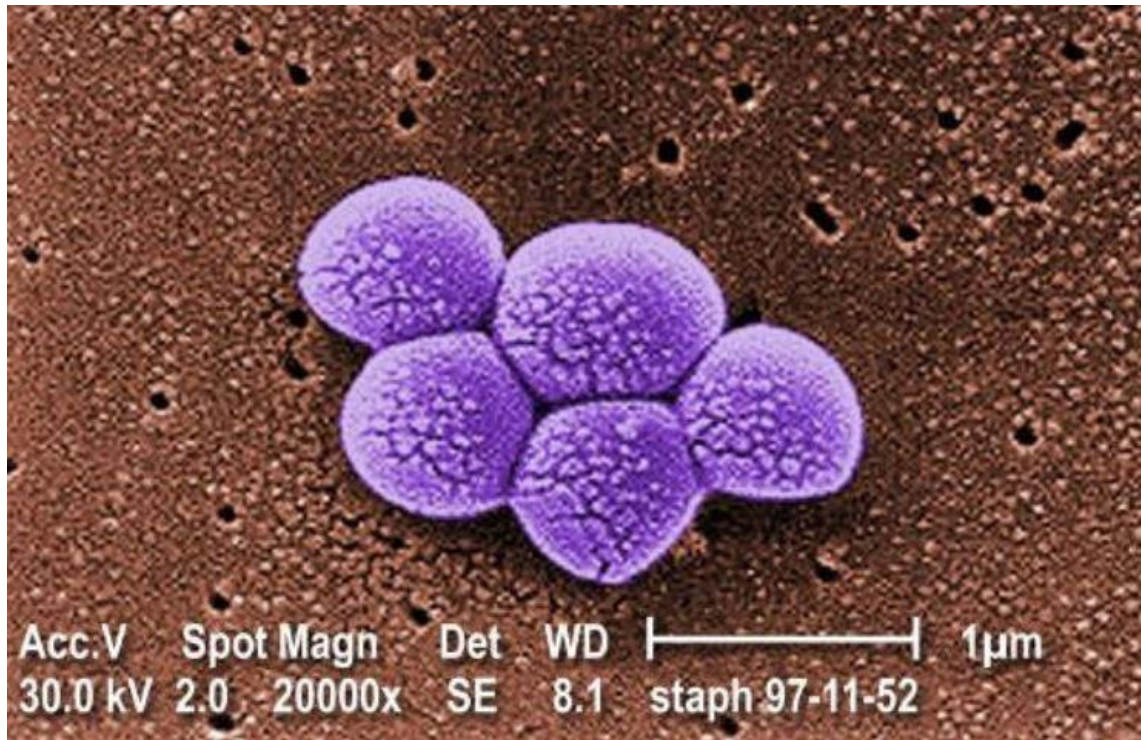
Figura 7. Micrografía electrónica de barrido coloreada de un grupo de SARM. Fuente: Public Health Image Library.

Según la ECDC, el 24,2 % de los aislados de *S. aureus* analizados en España durante el año 2018 presentaron resistencia a meticilina (Figura 8) [52].
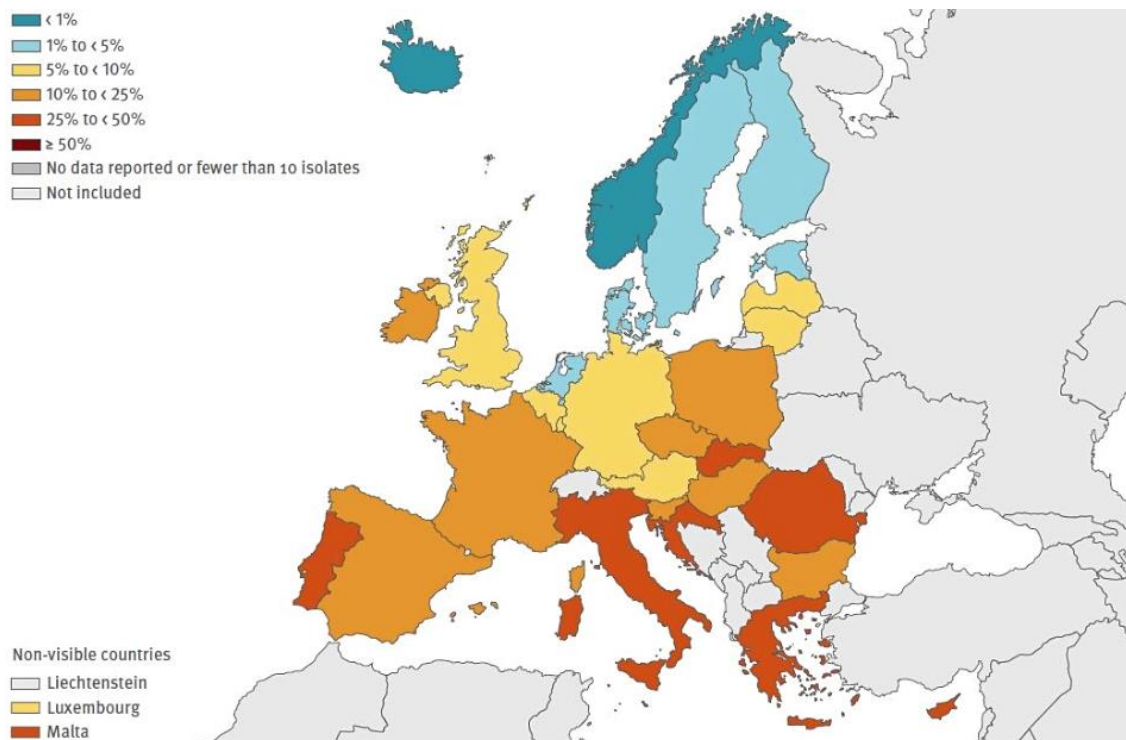
Figura 8. Porcentaje de aislados invasivos de SARM por país en 2018. Se incluye países de la Unión Europea (UE) y del Área Económica Europea (AEE). Figura obtenida del informe "Surveillance of antimicrobial resistance in Europe" publicado en el año 2018 por la ECDC [52].

Como puede observarse en la Figura 8, existe un gradiente geográfico en el porcentaje de aislados estafilocócicos resistentes a antibióticos, habiendo una menor prevalencia en el norte de Europa que va aumentando conforme se avanza hacia el sur. Además, cabe destacar que en España existe una tendencia creciente en la prevalencia de infecciones adquiridas en la comunidad, es decir, infecciones por SARM que no son contraídas en ambientes clínicos. En España, los clones más comunes de SARM son ST8-IVc, USA300 y ST398-V [53].

1.2. LAS PLANTAS COMO HERRAMIENTA TERAPÉUTICA

1.2.1. Uso terapéutico tradicional de las plantas

Los organismos vegetales constituyen la mayor parte de la biosfera del planeta Tierra. La biomasa total del planeta está establecida en unas 550 gigatoneladas de carbono, de las cuales 450 gigatoneladas pertenecen al Reino Plantae, lo que supone un porcentaje mayor del 80 % de la biomasa total (Figura 9) [54]. La colonización de la tierra por parte de la primera alga

verde hace millones de años constituyó una revolución crucial para la vida en este planeta [55]. Desde su aparición, las plantas han sobrevivido, evolucionado y adaptado a todo tipo de ecosistemas y condiciones adversas, dando lugar a la basta diversidad de especies vegetales que podemos presenciar hoy en día. Este proceso adaptativo las ha llevado a desarrollar sistemas de defensa complejos y eficaces frente a las agresiones externas: depredadores, estrés abiótico y, por supuesto, infecciones. Al ser organismos sésiles que no pueden huir de sus amenazas, las plantas han desarrollado un espléndido arsenal químico en forma de metabolitos secundarios capaz de hacer frente a los más peligrosos patógenos [56]. Esta capacidad defensiva en forma de síntesis de biomoléculas es muy interesante desde el punto de vista del descubrimiento de nuevos agentes antibióticos, ya que estos sistemas de defensa han sido puestos a prueba por la evolución durante millones de años de exposición a todo tipo de infecciones [57].
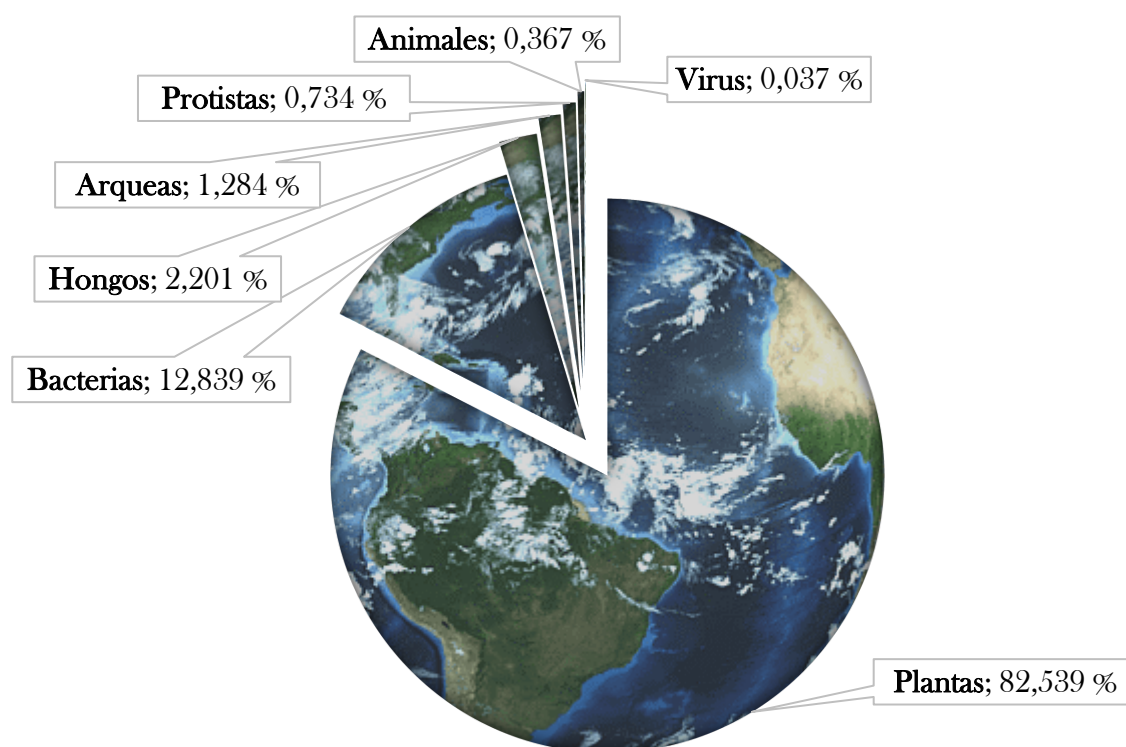
Figura 9. Distribución porcentual de la biomasa en el planeta Tierra medida en gigatoneladas de carbono. Datos extraídos de Bar-On, Y. M. *et al.* 2018 [54]. Figura de elaboración propia.

Las plantas poseen una ingente variedad de moléculas químicas con capacidad antimicrobiana [58, 59]. En el *Dictionary of Natural Products* se recogen aproximadamente 200.000

metabolitos secundarios de plantas, de los cuales 170.000 poseen estructuras químicas únicas [60]. En el caso de las moléculas con capacidad antimicrobiana producidas por las plantas, éstas pertenecen en su gran mayoría a las familias de los péptidos antimicrobianos, los alcaloides, los terpenoides y los polifenoles [61].

La Humanidad ha hecho uso de las propiedades medicinales de las plantas durante miles de años. Existen evidencias de que en el año 5.000 a. C. los sumerios ya usaban el tomillo por sus propiedades beneficiosas para la salud [62]. El Papiro Ebers egipcio (Figura 10) que data de alrededor del año 1.500 a. C. ya atribuía propiedades medicinales a plantas y especias como el aloe vera, el ricino, el ajo, el cáñamo, el anís o la mostaza [63, 64]. Otros textos como el Atharva Veda, el Rig Veda y el Sushruta Samhita pertenecientes a la Ayurveda india también hablaban de las propiedades farmacológicas de sustancias vegetales como la cúrcuma o el cannabis [65, 66].
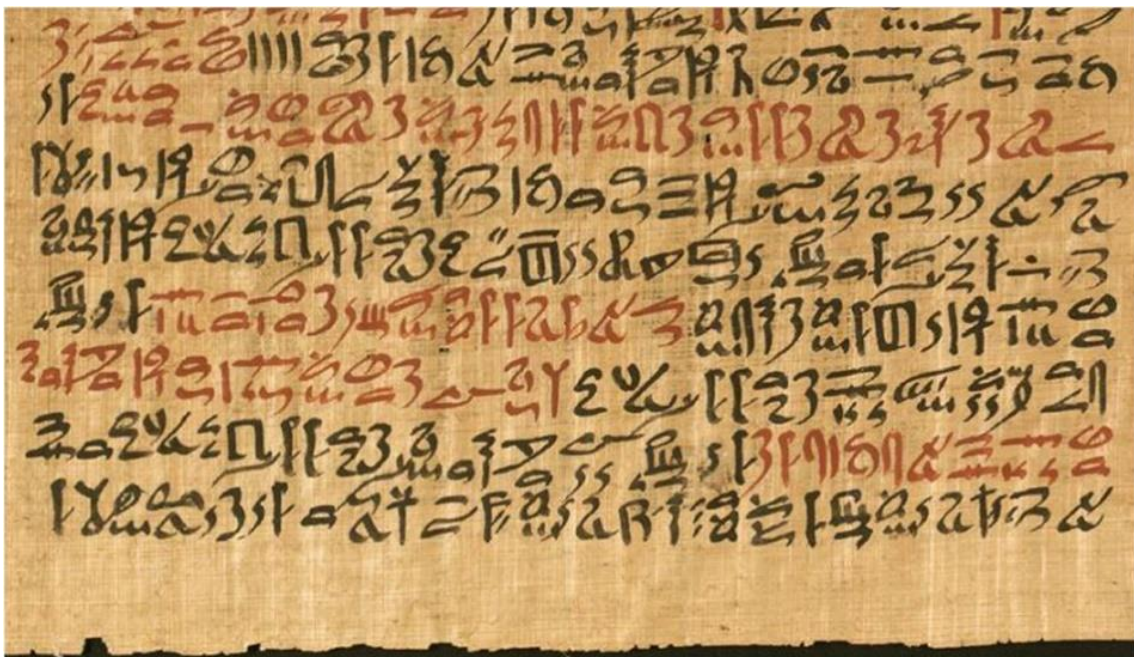
Figura 10. Fragmento del Papiro Ebers (pasaje de texto Eb 251). Resume las propiedades curativas de la planta de ricino (*Ricinus communis*). La tinta roja indica encabezados, información clave y cantidades. Imagen extraída de Franke H. *et al.* 2019, cortesía de la Biblioteca de la Universidad de Leipzig.

En la actualidad, el avance de la ciencia y la medicina nos ha permitido investigar las bases moleculares de estas antiguas prácticas, revelando el mecanismo de acción de muchas de ellas,

arrojando luz sobre prácticas ancestrales que parecían obsoletas [67, 68]. Estos conocimientos arcanos han servido de punto de partida para el desarrollo de terapias basadas en compuestos vegetales eficaces y seguras. De acuerdo con la Organización Mundial de la Salud (OMS), el 80 % de la población mundial todavía emplea fármacos de origen vegetal y muchos de los medicamentos sintéticos actuales tienen como origen las plantas medicinales [69, 70]. Ejemplos de moléculas de origen vegetal presentes en fármacos de la actualidad son la ergotamina (proveniente del centeno contaminado por cornezuelo, *Secale cereale*) [71], la morfina (de la adormidera, *Papaver somniferum*) [72], el paclitaxel (del tejo, *Taxus brevifolia*) [73], la digotoxina y la digoxina (de la dedalera, *Digitalis purpurea*) [74], la quinina (del quino, *Cinchona officinalis*) [75] y los salicilatos (de la corteza de los sauces, *Salix alba* y *Salix fragilis*) [70, 76].

1.2.2. Aplicaciones de los compuestos vegetales en la actualidad

En la actualidad existen multitud de extractos vegetales empleados con distintos fines terapéuticos [77]. La variedad de métodos para obtener extractos a partir de materias primas vegetales y sus características son determinantes para la composición final del extracto, repercutiendo directamente en parámetros clave como su contenido en fitoquímicos o su capacidad antimicrobiana [78]. Extractos obtenidos por distintos métodos a partir de una misma planta pueden poseer cualidades completamente diferentes. El disolvente empleado, el tiempo de extracción, la temperatura, el uso de técnicas como la asistencia por ultrasonidos o microondas, la agitación, el filtrado o el secado son parámetros clave que impactan drásticamente en la composición final del producto [79]. Por ejemplo, existen estudios que demuestran que distintos métodos de secado y de fraccionamiento de extractos de *Cistus salviifolius* (Figura 11) impactan de manera significativa en su composición molecular y capacidad antibacteriana [80]. Además, se ha observado que extractos obtenidos empleando el mismo método de extracción a partir de distintas especies vegetales del género *Cistus* resultan en extractos con una composición molecular diferente, influyendo en su composición factores adicionales como el tipo de suelo, la estación del año o el clima de la zona de crecimiento de la especie [81].

Figura 11. *C. salviifolius* ubicado frente al Edificio Torregaitán de la Universidad Miguel Hernández de Elche, del cual se obtuvo la materia prima vegetal para realizar uno de los extractos empleados en el Capítulo 2.

Los extractos vegetales siguen siendo motivo de estudio gracias a sus propiedades terapéuticas (Figura 12). Su producción y caracterización es fundamental en el proceso de descubrimiento de nuevos agentes activos. Existen evidencias de las propiedades farmacológicas de diversos extractos vegetales o partes de ellos, destacando su aplicación en los siguientes campos: cardiología, inflamación, neurología, oncología y el desarrollo de nuevos antimicrobianos [82].
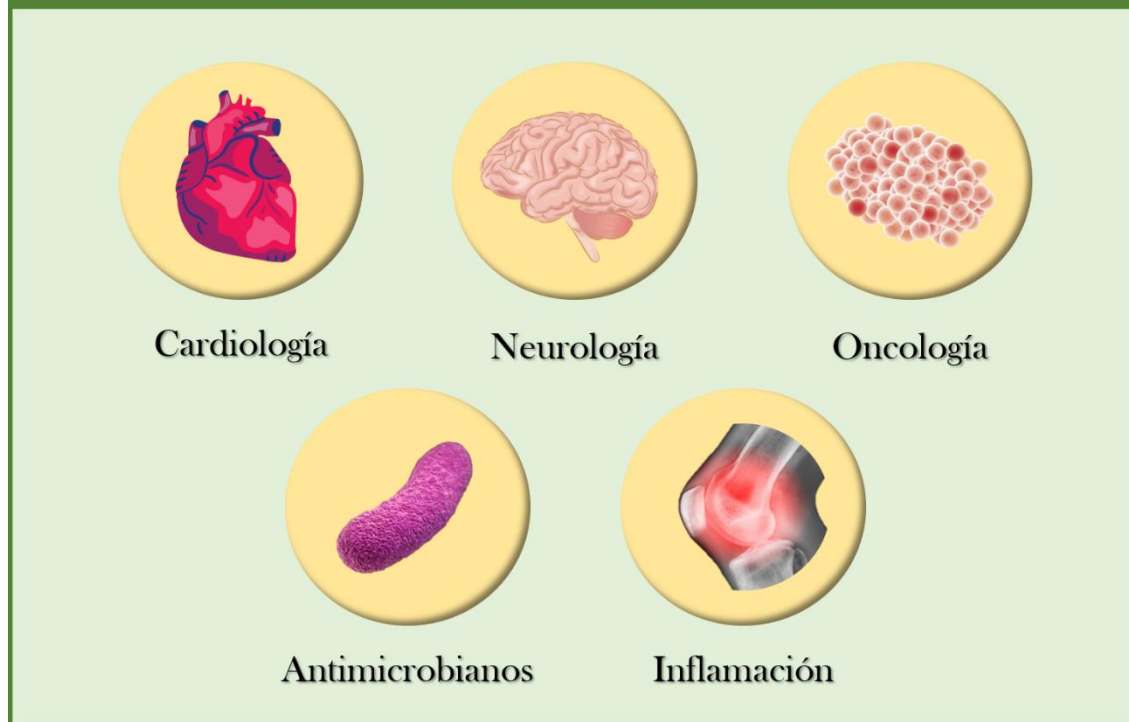
Figura 12. Campos de aplicación terapéutica de los extractos de plantas. Imágenes extraídas de diversos repositorios online. Figura de composición propia.

1.2.2.1. Uso en cardiología

Las patologías cardiovasculares suponen un grave problema sanitario en todo el mundo, independientemente del nivel de desarrollo económico de la región observada. La vida cada vez más sedentaria, una alimentación poco saludable y niveles de estrés elevados son factores de riesgo para el desarrollo de estas patologías. Los extractos de plantas y compuestos puros son usados para tratar estas patologías y su mecanismo de acción suele ser multifactorial. Algunas de las dianas y mecanismos de acción moleculares de estos productos son la producción de NO, la activación canales de potasio, la inhibición de canales de calcio activados por voltaje, la inhibición de fosfodiesterasa, la inhibición de la proteína quinasa C o el secuestro de radicales libres, entre otros [82]. Algunos de los compuestos que muestran estas propiedades cardioprotectoras son los alcaloides [83], los flavonoides [84, 85] y otros polifenoles [86]. Como ejemplo, el extracto de hojas de *Scutellaria baicalensis* contiene baicalina, una flavona glucosilada, con capacidad para bloquear canales de potasio

dependientes de voltaje [87]. El extracto de flores de *Erigeron canadensis* contiene gran cantidad de polifenoles conjugados con polisacáridos que exhiben propiedades anticoagulantes excepcionales, convirtiendo a esta planta en una nueva fuente de compuestos potencialmente útiles como anticoagulantes y antiplaquetarios [88]. Un clásico en cardiología descrito hace más de dos siglos es el uso de la planta dedalera, *Digitalis purpurea*, para el tratamiento de la insuficiencia cardíaca. A día de hoy, los glucósidos cardiotónicos como la digoxina todavía se utilizan ampliamente como inótropos positivos en la insuficiencia cardíaca y por su actividad cronotrópica negativa en la fibrilación auricular [89].

También existen evidencias del uso de extractos vegetales ricos en polifenoles de *Hibiscus sabdariffa* y *Lippia citriodora* para el tratamiento de patologías cardiovasculares relacionadas con la obesidad, como la hipertensión o la diabetes. Estos extractos han demostrado poseer capacidad para potenciar la oxidación de ácidos grasos, disminuir la lipogénesis, activar el sensor energético AMPK, disminuir la presión arterial e inhibir la producción de especies reactivas de oxígeno [90-93].

1.2.2.2. Uso antiinflamatorio

Los compuestos bioactivos aislados de plantas con capacidad antiinflamatoria son muy numerosos. Uno de los ejemplos más famosos lo constituyen los salicilatos, precursores de la aspirina, provenientes de árboles del género *Salix*, que han mostrado una gran capacidad antiinflamatoria en el tratamiento de diferentes patologías desde hace siglos [94]. Otra de las familias de moléculas de plantas más conocidas por sus capacidades analgésicas y antiinflamatorias son los alcaloides. Los alcaloides presentes en plantas como *Sophora subprostrata* y *Alstonia scholaris* son capaces de inhibir las ciclooxigenasas (COX), enzimas relacionadas con la inflamación y el dolor [95, 96]. Los flavonoides también han demostrado excelentes cualidades antiinflamatorias. La luteolina presente entre otras fuentes en la planta *Terminalia chebula* es capaz de interaccionar con dianas moleculares como el factor de necrosis tumoral alfa (TNF-α), la metaloproteinasa-2 (MMP-2), la producción de NO o la interleuquina 4 (IL-4), disminuyendo la inflamación y los signos de la alergia [97]. La naringenina y la hesperitina de la piel de cítricos o el eriodictiol de *Eriodictyon californicum* han mostrado su eficacia antiinflamatoria mediante la interacción con TNF-α, prostaglandina E2 (PGE2) [98, 99]. La quercetina y la quercetina-3-O-glucurónido procedentes de *Hibiscus*

sabdariffa han demostrado capacidad de reducir la inflamación y el estrés oxidativo en adipocitos hipertróficos, aliviando el estrés metabólico inducido por glucolipotoxicidad [100]. Otros polifenoles con actividad antiinflamatoria son la apocinina, el resveratrol o la curcumina, que actúan inhibiendo la enzima NADPH oxidasa [101].

1.2.2.3. Uso en neurología

Es bien sabido que diversas plantas y sus sustancias derivadas son capaces de alterar las funciones cerebrales humanas. Algunas de las plantas más conocidas en todo el mundo con capacidad de modular el sistema nervioso humano son *Cannabis sativa* (marihuana) [102], *Papaver somniferum* (morfina y heroína) [103], *Erythroxylum coca* (cocaína) [104] y *Coffea arabica* (cafeína) [105]. Este potencial puede ser empleado con fines terapéuticos gracias a la basta diversidad de fitoquímicos existentes y sus diferentes efectos usados en las cantidades adecuadas. Un ejemplo es el de la atropina extraída de *Atropa belladona*, un alcaloide antagonista competitivo del receptor muscarínico de la acetilcolina ampliamente empleado farmacológicamente gracias a su efecto supresor del sistema nervioso parasimpático [106]. La efedrina, extraída de *Ephedra sínica*, es una amina simpaticomimética que actúa como agonista adrenérgico y es ampliamente usado como descongestivo nasal gracias a su acción sobre los receptores alfa adrenérgicos de los vasos sanguíneos de la mucosa nasal [107].

Ciertos fitoquímicos son valorados por su efecto protector frente a procesos neurodegenerativos que pueden derivar en patologías tales como la enfermedad de Alzheimer, Parkinson o Huntington. Ejemplo de fitoquímicos neuroprotectores son los terpenos, como los carotenoides presentes en las frutas de color naranja, o los polifenoles, como la quercetina, el kaempferol, las catequinas, la apigenina, la berberina, el resveratrol, la miricetina o la curcumina. Los mecanismos de acción responsables de la actividad neuroprotectora de los fitoquímicos incluyen, entre otros, la prevención de la muerte de las neuronas al disminuir la actividad de las secretasas β y γ, la promoción de la supervivencia de neuronas mediante su interacción con neurotrofinas y la eliminación de radicales libres activando las vías de señalización PI3K/Akt/Nrf2 [108, 109].

1.2.2.4. Uso en oncología

El cáncer supone un problema sanitario que afecta a millones de personas en todo el mundo. Según *Cancer Research Fund International*, se prevé que la cifra de afectados por distintos tipos de cáncer sea de 24 millones para el año 2035. La investigación de productos naturales anticancerígenos se remonta a mediados del siglo pasado, con el descubrimiento de las propiedades de los alcaloides de la vinca (vinblastina, vincristina, vinorelbina, vindesina y vinflunina) [110]. Desde entonces, multitud de fitoquímicos naturales han mostrado eficacia clínica: los taxanos [111], la podofilotoxina [112], la camptotecina [113] y las antraciclinas [114], entre otros. Además, se ha probado que diversas moléculas polifenólicas presentes en la dieta humana como la curcumina [115], la genisteína [116] o el resveratrol [117] poseen propiedades anticancerígenas. La investigación en productos vegetales ha dado lugar a decenas de patentes de extractos vegetales con propiedades anticancerígenas, como por ejemplo el extracto de corteza de árbol *Banyan* (código de patente US6660309B2), el extracto de *Euphorbia antiquorum* (US20030165579A1), el extracto de hojas de *Melissa officinalis* (US20040009244B2) o el extracto de raíces de *Solanaceae dulcamara* (US7250180B2) [82].

1.2.2.5. Uso antimicrobiano

Desde la antigüedad se han preparado cataplasmas e infusiones a partir de plantas locales con fines medicinales, entre ellos, la curación de infecciones [118, 119]. Desde el descubrimiento e implementación de los antibióticos a mediados del siglo XX, el uso de las plantas como antimicrobianos prácticamente ha desaparecido [120]. Sin embargo, el auge de las bacterias resistentes a antibióticos ha obligado a los investigadores a buscar compuestos antimicrobianos en diversas fuentes, revisitando el mundo vegetal. En este contexto, las plantas suponen un gran reservorio de moléculas bioactivas con potencial terapéutico aún por explorar en profundidad. Los fitoquímicos han mostrado una actividad a menudo multifactorial, afectando simultáneamente a diversos procesos del cuerpo y produciendo menores efectos secundarios adversos que los antibióticos químicos tradicionales [121]. Por ejemplo, el extracto de *Hydrastis canadensis* (Figura 13), además de su actividad antimicrobiana, es capaz de aumentar el riego sanguíneo en el bazo, promoviendo su funcionamiento óptimo y promoviendo una recuperación más rápida [122].
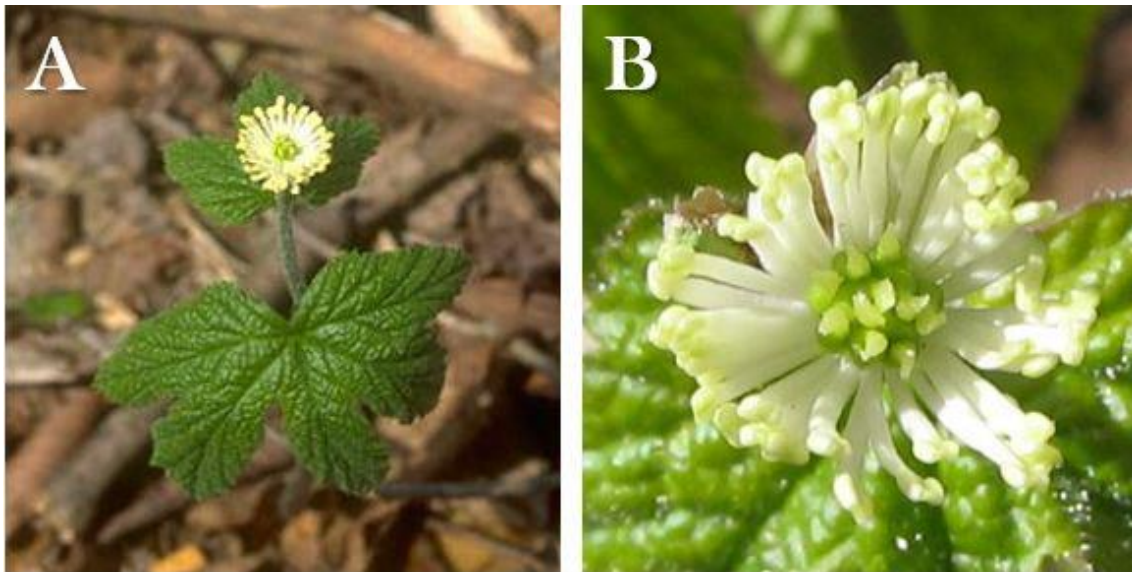
Figura 13. A: Visión general de *Hydrastis canadensis*, también conocida como sello de oro. Fotografía realizada por Luis Coronel. B: Detalle de la flor. Fotografía realizada por Phyzome.

El carácter multifactorial de los extractos vegetales también se hace patente en su mecanismo de acción antimicrobiano, ya que los fitoquímicos presentes en un mismo extracto pueden actuar sobre diferentes dianas moleculares bacterianas, tales como la pared celular, la membrana celular, diversas proteínas o moléculas asociadas a los ácidos nucleicos [77]. Además, se ha observado que ciertos fitoquímicos, como ciertos polifenoles, son capaces de sensibilizar bacterias resistentes a antibióticos consiguiendo revertir sus mecanismos de resistencia y haciéndolas susceptibles a los fármacos tradicionales [123]. La capacidad antimicrobiana de los extractos vegetales y sus componentes es ampliamente desarrollada en los Capítulos 1, 2 y 3 de la presente Tesis Doctoral.

1.2.3. Los polifenoles

Los polifenoles son moléculas que incorporan en su estructura química uno o más grupos fenólicos y se encuentran naturalmente en frutas, verduras, frutos secos, bayas, cacao, té, y vino, entre otros (Figura 14) [124]. Son sintetizados como parte del metabolismo secundario de las plantas a través de la ruta del ácido shikímico, la cual es promovida como respuesta a estreses bióticos y abióticos. Los polifenoles poseen importantes funciones dentro de las

plantas, sirviendo como antioxidantes, pigmentos, señalizadores, elementos estructurales y mecanismo de defensa [125].

Figura 14. Ejemplos de alimentos ricos en polifenoles. Imagen extraída de *Adobe Images*.

Según su estructura química, los polifenoles pueden clasificarse en diferentes familias: ácidos fenólicos, estilbenos, flavonoides, lignanos y taninos hidrolizables. A su vez, los flavonoides pueden subdividirse en diversas subfamilias, siendo las principales: antocianidinas, flavanonas, flavonas, flavonoles e isoflavonas (Figura 15) [77]. Además, los polifenoles pueden hallarse unidos a moléculas hidrocarbonadas como los azúcares, formando moléculas conjugadas con propiedades físicas y químicas diferentes a los compuestos en su forma aglicona, aumentando aún más la posible diversidad química y funcional de los mismos.
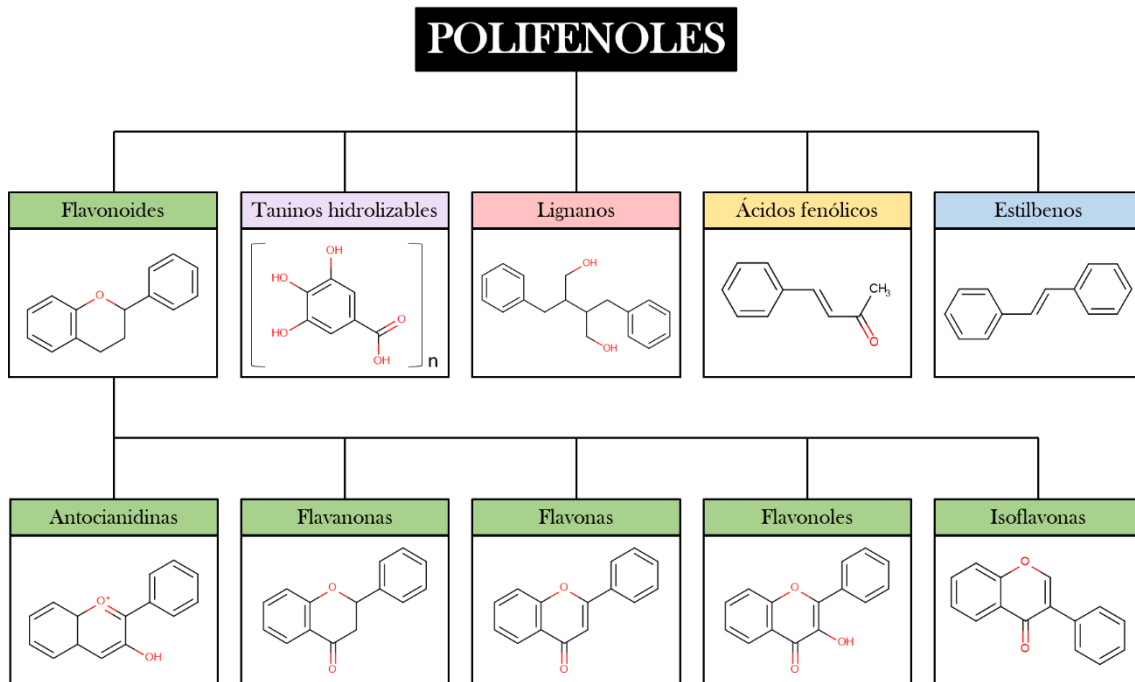
Figura 15. Esquema representativo de las principales familias y subfamilias de polifenoles con sus estructuras químicas fundamentales. Fuente: Álvarez-Martínez *et al.* 2020 [77]. Figura de elaboración propia.

Los polifenoles son moléculas seguras para los humanos. Multitud de estudios relacionan el consumo prolongado de polifenoles en la dieta con efectos beneficiosos para la salud: protección frente a cáncer, enfermedades cardiovasculares, diabetes o procesos neurodegenerativos, entre otros [124, 126, 127]. Estas propiedades medicinales adicionales convierten a los polifenoles en candidatos deseables para ser administrados como agentes antimicrobianos en terapias antibióticas.

1.2.3.1. Flavonoides

Los flavonoides son compuestos polifenólicos abundantes en alimentos como las frutas, el cacao, el té, el vino, los cítricos o las bayas. El interés y la investigación sobre flavonoides ha aumentado de forma exponencial en los últimos años [128]. Este creciente interés surgió de su potente capacidad antioxidante, la cual permitió su aplicación a enfermedades cardiovasculares y cerebrovasculares cuyo origen y patología están relacionadas con el estrés oxidativo [129]. Además, se observó que contribuyen a combatir infecciones gracias a su

capacidad antimicrobiana. Los mecanismos de acción antimicrobiana propuestos para los flavonoides son muy diversos. Estos incluyen la inhibición de la síntesis de ácidos nucleicos, la disrupción de la membrana citoplasmática, la inhibición del metabolismo energético, la inhibición de la formación de biopelículas y la atenuación de la patogenicidad [130]. Esta variedad funcional y multiplicidad de unión a dianas es conocida como promiscuidad molecular y es una característica clave de los polifenoles en general y de los flavonoides en particular para ejercer su actividad multifactorial [131].

1.2.3.1.1. Antocianidinas

Las antocianidinas son pigmentos naturales presentes en las plantas, responsables de los colores rojo, azul y púrpura. Habitualmente se encuentran en su forma glicosilada, dando lugar a moléculas conocidas como antocianinas. Estas moléculas son especialmente abundantes en frutas tropicales, grosellas, uvas y bayas (Figura 16). Una de las antocianidinas más extendidas en plantas es la cianidina, especialmente su forma antocianina, la cianidina-3-glucósido. A este grupo de polifenoles se le atribuyen numerosas propiedades beneficiosas para la salud humana, incluyendo actividad antioxidante, antiinflamatoria, neuro-protectora, preservadora de la visión, antidiabética, anticancerígena y antimicrobiana [132]. Las antocianinas extraídas de la soja negra han demostrado capacidad antiinflamatoria y antimicrobiana en modelos de rata con prostatitis bacteriana crónica, además de una actividad sinérgica con el antibiótico ciprofloxacino [133].

Figura 16. Las fresas, moras y arándanos son grandes fuentes de antocianidinas. Imagen extraída de PickPik.com.

1.2.3.1.2. Flavanonas

Las flavanonas se hallan abundantemente en tomates, cítricos y zumos de éstos (Figura 17). Algunas de las principales moléculas pertenecientes a este grupo son la naringenina, la hesperitina y el eriodictiol. A estos compuestos se les atribuyen diversos efectos beneficiosos para la salud, como la defensa frente al estrés oxidativo o las infecciones víricas y bacterianas, así como la prevención de accidentes cardiovasculares, la ateroesclerosis o el cáncer [134, 135]. Además, en el caso de la naringenina, se ha demostrado que posee capacidad antimicrobiana e inhibidora de la formación de biopelículas frente a *Streptococcus mutans*, lo cual convierte a dicha flavanona en un agente anti-caries natural prometedor [136].

Figura 17. Limones, naranjas y limas, fuentes abundantes de flavononas. Imagen extraída de Flickr.com.

1.2.3.1.3. Flavonas

Las flavonas son polifenoles abundantes en alimentos como el perejil (Figura 18), la menta, las espinacas o el apio, así como en bebidas como el zumo de bergamota, el zumo de mandarina o el vino. Algunas de las flavonas más comúnmente halladas en estos alimentos son la apigenina, la luteolina y la diosmetina. Existen estudios en humanos que apuntan a que el consumo de flavonas se relaciona con una mayor concentración de enzimas antioxidantes en sangre, disminución de biomarcadores relacionados con riesgo cardiovascular y disminución del colesterol total [137]. En cuanto a su actividad antimicrobiana, la apigenina ha mostrado capacidad antifúngica y antibacteriana frente a bacterias de interés clínico como *Acinetobacter baumanii, Enterococcus faecalis, E. coli, K. pneumoniae* o *S. aureus*, entre otros [138, 139].

Figura 18. El perejil es una de las principales fuentes de flavonas. Imagen extraída de Pixabay.com

1.2.3.1.4. Flavonoles

Los flavonoles son una clase de polifenoles que se distinguen por poseer un grupo hidroxilo en el carbono C3 del esqueleto flavonoide. Los flavonoles pueden encontrarse virtualmente en todas las frutas y verduras, destacando las manzanas, la cebolla, el tomate o la lechuga (Figura 19) [140]. Algunos de los flavonoles más estudiados son la quercetina, el kaempferol o la miricetina. Sus propiedades biológicas incluyen la capacidad antioxidante, antimicrobiana, antiinflamatoria y vasodilatadora. Estas propiedades han propiciado la aparición de estudios de actividad de flavonoles en enfermedades cardiovasculares y diabetes, obteniendo resultados positivos que abren la puerta al desarrollo de nuevos fármacos basados en ellos [141, 142].

En cuanto a su actividad antimicrobiana, los flavonoles quercetina, kaempferol y miricetina han mostrado capacidad antimicrobiana frente a distintos aislados clínicos y cepas control de *S. aureus* con concentraciones mínimas inhibitorias (CMI) tan bajas como 1,95 μg/mL, 7,8 μg/mL y 15,76 μg/mL, respectivamente [131, 143].

Figura 19. La lechuga y el tomate representan una fuente importante de flavonoles. Imagen extraída de Pixabay.com.

1.2.3.1.5. Isoflavonas

Las isoflavonas son moléculas polifenólicas calificadas como fitoestrógenos, ya que debido a su estructura molecular y tamaño se asemejan a los estrógenos esteroideos presentes en los vertebrados. Las principales fuentes de isoflavonas en la dieta humana son las legumbres, la soja y sus derivados (Figura 20), que contienen principalmente daidzeína y genisteína. Debido a su condición de fitoestrógenos, las isoflavonas se consideran sustancias quimioprotectoras que pueden ser usadas como terapia alternativa en desórdenes relacionados con las hormonas, incluyendo el cáncer de mama o próstata, la osteoporosis y los síntomas de la menopausia [144].

Las isoflavonas también poseen actividad antimicrobiana. Ciertas isoflavonas aisladas de plantas de soja han mostrado capacidad antimicrobiana frente a bacterias tanto gram-positivas (*S. aureus* ATCC 26112 y *Enterococcus faecium* ATCC 35667) como gram-negativas (*Pseudomonas aeruginosa* ATCC 27853, *Streptococcus pneumoniae* ATCC 49619 y *E. coli* ATCC 87394), con valores de CMI entre 10,6 μg/mL y 22,6 μg/mL [145].

Figura 20. La soja y sus derivados representan una de las fuentes principales de isoflavonas. Imagen extraída de Pixabay.com.

1.2.3.2. Taninos hidrolizables

Los taninos hidrolizables son un grupo heterogéneo de polifenoles de alto peso molecular que se hallan principalmente en cereales, legumbres, verduras y frutas como la granada (Figura 21). Los taninos hidrolizables pueden dividirse en dos subfamilias: galotaninos y elagitaninos, dependiendo de los residuos que tengan esterificados al grupo hidroxilo de la glucosa. Los taninos hidrolizables presentan diversas actividades biológicas beneficiosas para el ser humano. Entre ellas destaca su actividad como antioxidantes, antimicrobianos, captadores de radicales libres, agentes anticancerígenos o cardioprotectores [146, 147]. Su actividad antimicrobiana es elevada y por ejemplo, los elagitaninos punicalina y punicalagina han mostrado valores de CMI frente a *S. aureus* (ATCC 25923) de 12.5 µg/mL [148].

Figura 21. Granada (*Punica granatum*), una gran fuente de taninos hidrolizables, especialmente de punicalagina. Imagen extraída de Pixabay.com.

1.2.3.3. Lignanos

Los liganos son moléculas polifenólicas derivadas de la combinación de dos unidades fenilpropanoides C6 – C3 en el carbono β y β' que a su vez se pueden formar enlaces adicionales de éter, lactona o carbono. Los lignanos se pueden hallarse abundantemente en alimentos como las semillas de lino (Figura 22) y sésamo. En menor cantidad también pueden encontrarse en granos completos, frutas y verduras [149]. Algunos de los más estudiados son el pinoresinol, el matairesinol, el honokiol, el magnolol, el ecoisolariciresinol, el medioresinol y el lariciresinol. Diversos estudios han demostrado que las dietas ricas en lignanos son eficaces para disminuir la aparición de cáncer dependiente de hormonas, accidentes cardiovasculares y diabetes [150, 151]. Los lignanos magnolol y honokiol han demostrado capacidad antibacteriana frente a cepas de SARM, presentando además una acción sinérgica con el antibiótico oxacilina por medio de la inhibición de genes de resistencia bacterianos [152].

Figura 22. Semillas de lino, fuente dietética de lignanos. Imagen extraída de Wallpaperflare.com.

1.2.3.4. Ácidos fenólicos

Los ácidos fenólicos son moléculas polifenólicas que se distinguen por poseer una función carboxilo en su estructura química. Son los componentes mayoritarios de la lignina que forma la lignocelulosa y se hallan de forma abundante en trigo (Figura 23), maíz, café, té y frutas [153]. Algunos de los ácidos fenólicos más estudiados son el ácido ferúlico, el ácido gálico, el ácido cafeico, el ácido cinámico, el ácido *p*-hidroxibenzoico y el ácido *p*-coumárico [154]. Estas moléculas presentan actividad antioxidante y antimicrobiana, lo que ha potenciado la investigación de estos como conservantes alimentarios. La conjugación de los ácidos gálico y cafeico con quitosano ha demostrado la potenciación de su efecto bacteriostático y antioxidante, convirtiendo a estos ácidos fenólicos en candidatos prometedores para fabricar envases biológicos activos [155]. Por otro lado, el ácido *p*-coumárico ha demostrado capacidad antifúngica frente a *Fusarium oxysporum* y *Fusarium verticillioides*, así como actividad antibacteriana frente a bacterias gram-positivas y gram-negativas [156]. Además, se han observado efectos terapéuticos derivados de su consumo en la dieta, incluyendo actividad anticancerígena y antiinflamatoria [157].

Figura 23. El trigo es una de las principales fuentes de ácidos fenólicos en la dieta. Fotografía tomada por Sagarjitkar.

1.2.3.5. Estilbenos

Los estilbenos son moléculas que se caracterizan por poseer dos anillos aromáticos unidos por un puente de etileno. Algunos de los compuestos más conocidos y estudiados son el resveratrol, la combretastatina A-4 y el pteroestilbeno. Los estilbenos pueden hallarse en alimentos como las uvas (Figura 24), las bayas y los cacahuetes, entre otros [158]. Estudios recientes apuntan a que los estilbenos tienen potencial terapéutico gracias a su actividad antioxidante, antiinflamatoria, anticancerígena, antienvejecimiento y antimicrobiana [159-161]. El resveratrol posee capacidad antimicrobiana frente a distintos hongos, virus y bacterias. Concentraciones subinhibitorias de resveratrol son capaces de alterar la producción de factores de virulencia bacterianos, además de presentar una actividad sinérgica con antibióticos de uso tradicional frente a *S. aureus* [162].

Figura 24. Uvas, una fuente abundante de estilbenos. Fotografía tomada por Martin Kozák.

1.3. HERRAMIENTAS PARA EL HALLAZGO DE NUEVAS MOLÉCULAS NATURALES ANTIMICROBIANAS

Existen distintos métodos para el cribado, identificación y análisis de nuevas moléculas bioactivas. Las moléculas bioactivas pueden ser identificadas a partir de mezclas complejas, como los extractos vegetales, en los cuales se observa un efecto activo y se intentan aislar los compuestos responsables de dicha actividad. En otras ocasiones, el cribado comienza con un gran conjunto de moléculas, como una quimioteca, que se van poniendo a prueba para descartar aquellas sin la actividad deseada. Las metodologías empleadas para estos procesos son muy variadas, incluyendo estrategias tanto *in vitro* como *in silico* [60, 163].

En referencia al análisis de los datos obtenidos en los ensayos de capacidad antimicrobiana, resulta crucial destacar la importancia del uso de métodos estadísticos que permitan comprender en profundidad el sentido de los datos. Los métodos estadísticos de análisis de correspondencia múltiple (MCA) o de regresión de modelo lineal generalizado (GLMR) empleados en el Capítulo 2 de la presente Tesis Doctoral son claves para procesar de la

información obtenida, detectar y representar estructuras o comportamientos subyacentes en los conjuntos de datos y unificar otros métodos estadísticos más sencillos.

1.3.1. Aproximaciones *in vitro*

La metodología clásica de cribado molecular *in vitro* de productos naturales consiste en el aislamiento inicial de la molécula de interés y posterior caracterización de su farmacodinámica y farmacocinética. Aunque esta metodología ha demostrado ser eficaz, requiere una gran cantidad de tiempo y trabajo. Por este motivo, se han desarrollado las técnicas y equipamiento de cribado farmacológico de alto rendimiento (*high-throughput screening,* HTS) que son capaces de identificar compuestos bioactivos con gran eficiencia gracias a procesos automatizados con un uso mínimo de reactivos y volúmenes (Figura 25). Es posible cribar hasta 10.000 moléculas al día empleando los métodos HTS estándar y hasta 100.000 si se emplean métodos de ultra cribado (*ultra high-throughput screening,* UHTS) [164].
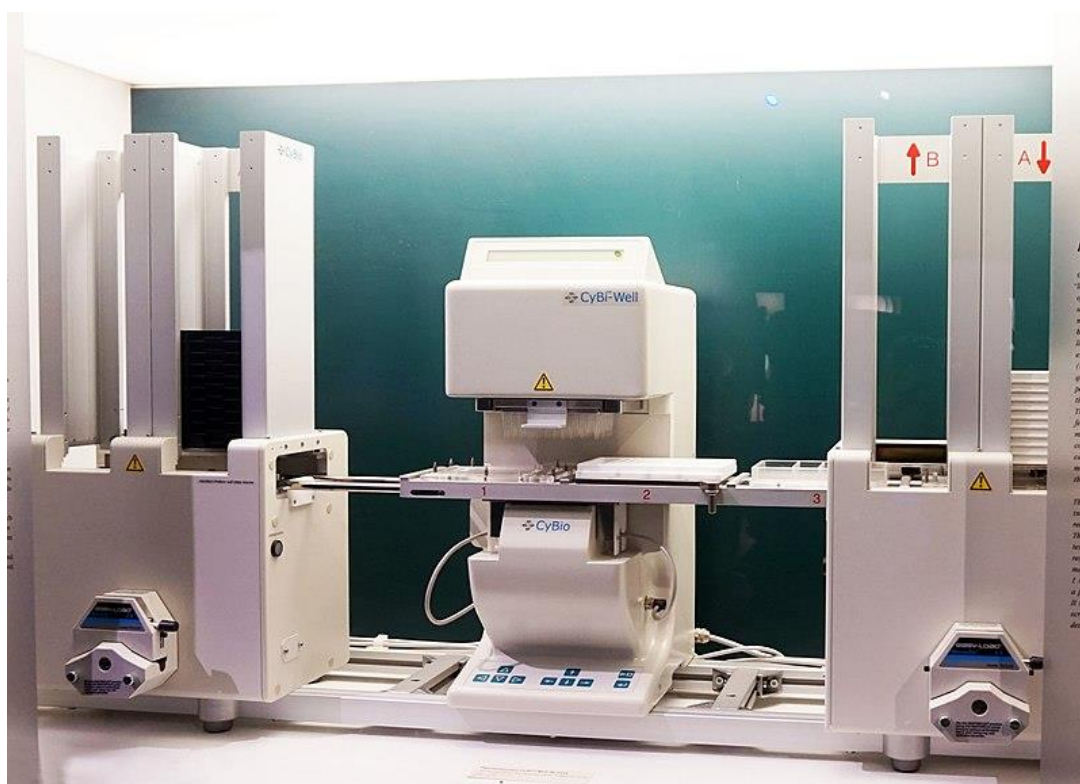
Figura 25. Ejemplo de aparato (CyBio Well) diseñado para realizar cribados farmacológicos de alto rendimiento, capaz de manejar hasta 1.536 muestras líquidas a la vez, localizado en Deutsches Museum, Munich, Alemania. Fotografía tomada por Tila Monto.

Algunas de las técnicas más comúnmente empleadas en los cribados moleculares *in vitro* son el cultivo celular o bacteriano [165], la diálisis de equilibrio [166], la microdiálisis [167], la ultrafiltración [168], la cromatografía [169] y las técnicas de afinidad de ligandos en objetivos inmovilizados [170].

Este tipo de técnicas son muy eficaces para la detección de compuestos bioactivos frente a una diana concreta. Sin embargo, se debe tener en cuenta que los productos naturales bioactivos pueden actuar de forma compleja, interaccionando con múltiples dianas y rutas metabólicas, suponiendo una limitación para las técnicas de cribado molecular que solo emplean una diana única [171].

1.3.2. Aproximaciones *in silico*

El desarrollo de nuevos fármacos es un proceso extremadamente costoso, tanto en tiempo invertido como económicamente. Por este motivo, es crucial reunir toda la información posible sobre una molécula y su comportamiento antes de iniciar el arduo proceso de producción y ensayos clínicos. Los ensayos *in silico* (hechos por computadora o vía simulación computacional) son, por lo general, ensayos económicamente accesibles que pueden aportar gran cantidad de datos que permiten predecir el comportamiento *in vitro* o *in vivo* de una o varias moléculas. Estos datos son esenciales para identificar las moléculas con mayor potencial y aumentar la tasa de éxito del desarrollo de fármacos [172, 173].

Dentro de las técnicas *in silico,* los ensayos de acoplamiento molecular (*molecular docking*) se han convertido en una herramienta clave en el diseño de fármacos en los últimos años. Esta técnica consiste en la predicción del modo de unión y afinidad entre un ligando dado y una proteína diana para la cual exista un modelo de su estructura tridimensional (Figura 26) [174]. De este modo, es posible realizar cribados de grandes quimiotecas de compuestos y seleccionar cuáles son capaces de unirse o interaccionar con la proteína diana con mayor afinidad. El acoplamiento molecular proporciona una gran ayuda para seleccionar compuestos líderes en el proceso de desarrollo de un fármaco [175, 176]. La técnica de acoplamiento molecular es empleada en el Capítulo 1 de esta Tesis Doctoral con el objetivo de realizar un cribado de cientos de moléculas polifenólicas frente a distintas proteínas clave involucradas en la biosíntesis de la pared celular de diversas especies bacterianas con el fin de identificar posibles inhibidores [77].

Proteína diana   Ligando   Acoplamiento molecular
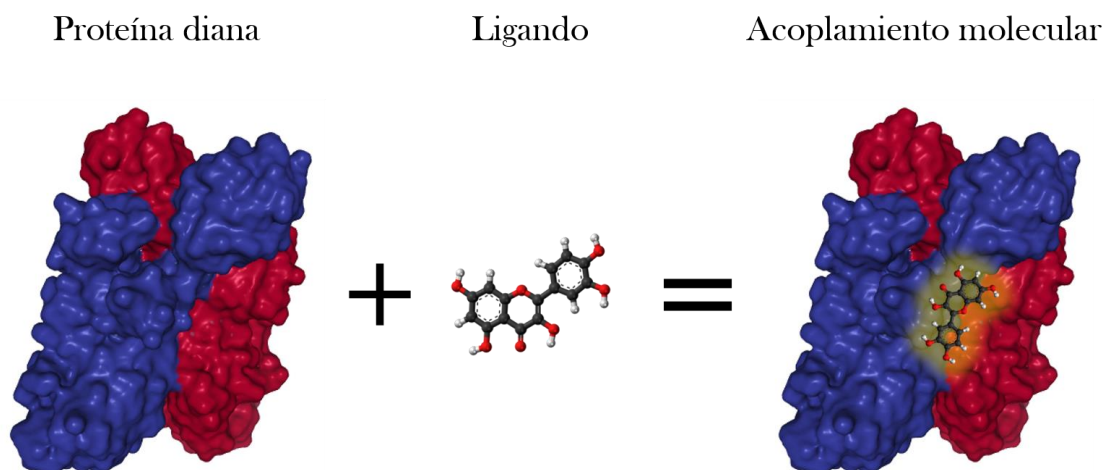
Figura 26. Ejemplo de acoplamiento molecular. La proteína diana escogida es PBP2a de *S. aureus*, cuyo modelo tridimensional ha sido extraído de RCSB Protein Data Bank. El ligando es el flavonoide quercetina. El aura amarilla corresponde al sitio de unión entre diana y ligando. Esquema de elaboración propia.

Los ensayos de acoplamiento molecular nos permiten predecir la estructura de los complejos ligando-receptor en base a cálculos que estiman la variación de la energía libre de Gibbs (Kcal/mol) del proceso de unión de un ligando dado (compuestos naturales de origen vegetal en nuestro caso) a un sitio de unión conocido de una proteína de nuestro interés. Para las enzimas seleccionadas, la información sobre su estructura está disponible a resolución atómica, y se conoce tanto su dominio catalítico como la presencia de los dominios de regulación que posea. Por lo tanto, la detección de aquellos compuestos fenólicos con una alta afinidad de unión al sitio catalítico de la enzima diana se comportarían como inhibidores competitivos de estas enzimas y podrían ser considerados como candidatos para posteriores estudios *in vitro* e *in vivo*. Así mismo, la unión a sitios reguladores permitiría la identificación de posibles moléculas que regularan la actividad de dicha diana sin llegar a interaccionar con el sitio catalítico.

Otro tipo de ensayo *in silico* que puede aportar ingentes cantidades de información predictiva sobre el comportamiento de un candidato a fármaco son las simulaciones de dinámica molecular. Esta aproximación emplea grandes cantidades de recursos computacionales para simular el comportamiento de un sistema a nivel atomístico integrando las leyes físicas del movimiento de Newton y la variable temporal. Los sistemas de dinámica molecular incluyen factores como las uniones flexibles entre moléculas, el papel de las moléculas de agua

presentes en el sistema, la solvatación y el estado de protonación molecular con el objetivo de representar de la forma más fiel y detallada el sistema simulado [177, 178]. Las simulaciones de dinámica molecular pueden arrojar una cantidad ingente de datos sobre el comportamiento de una molécula de interés. El continuo avance tecnológico en capacidad computacional permite realizar simulaciones cada vez más ambiciosas y exhaustivas, dotando a esta técnica de un porvenir prometedor [179].

1.3.3. Métodos estadísticos para el análisis de datos

El análisis estadístico de los datos recabados en los ensayos antimicrobianos es una etapa fundamental para dar sentido a la investigación realizada. Dos de los métodos estadísticos empleados en la presente Tesis Doctoral para el análisis de datos son el análisis MCA y el GLMR.

El análisis MCA toma múltiples variables categóricas y busca asociaciones entre diferentes niveles de esas variables. Puede considerarse un análogo al análisis de componentes principales (PCA) que se emplea para variables cuantitativas. Al igual que otros métodos multivariados, es un método de reducción de dimensiones que presenta los datos como puntos en un espacio bidimensional.

En análisis GLMR generalmente se refiere a modelos de regresión lineal convencionales para una variable de respuesta continua dados predictores continuos y/o categóricos. En el caso de los datos obtenidos en el Capítulo 2, se supone que la variable de respuesta sigue una distribución familiar normal.

Cabe destacar la relevancia de sistematizar la adquisición y el tratamiento de datos, así como de la obtención de resultados a través del uso de la inteligencia artificial con el fin de aumentar la reproducibilidad y facilitar la accesibilidad a los datos para las editoriales y para la comunidad científica en general. Los datos obtenidos en el Capítulo 2 de la presente Tesis Doctoral fueron procesados sistemáticamente empleando cuadernos Jupyter en Google Colab empleando códigos creados en lenguaje de programación Python.

2. OBJETIVOS

OBJETIVOS

El objetivo general de la presente Tesis Doctoral es la caracterización de la capacidad antimicrobiana de distintos compuestos de origen natural y el abordaje de su potencial para lidiar con bacterias resistentes a antibióticos con el fin de evaluar su idoneidad como alternativa o apoyo a las terapias antibióticas existentes en la actualidad.

2.1. OBJETIVOS DEL CAPÍTULO 1

El Capítulo 1 tiene como objetivo específico establecer el papel actual de los compuestos bioactivos de origen vegetal en la terapia antibiótica. Este capítulo revisa la bibliografía publicada hasta la fecha y profundiza en la capacidad antimicrobiana de compuestos polifenólicos frente a bacterias gram-positivas de importancia clínica, especialmente frente al SARM. También se plantea extraer conclusiones sobre las principales dianas moleculares en la actividad antimicrobiana de los polifenoles, así como de su potencial mecanismo de acción. Incluye además un cribado *in silico* de cientos de polifenoles frente a diversas enzimas bacterianas realizado para determinar posibles candidatos para el desarrollo de terapias antibióticas dirigidas empleando estos compuestos naturales.

2.2. OBJETIVOS DEL CAPÍTULO 2

El Capítulo 2 tiene por objetivos específicos la caracterización de la actividad antimicrobiana de varios extractos vegetales y un grupo de polifenoles de plantas con antecedentes de capacidad antimicrobiana y la posterior selección frente a distintas especies bacterianas de interés clínico. Una vez seleccionados aquellos con una mayor actividad, se estudiará su capacidad frente a 100 aislados clínicos de *S. aureus* (50 SARM y 50 sensibles a meticilina) y se explorará la relación entre los niveles de resistencia a antibióticos de uso clínico tradicional y la susceptibilidad a los compuestos naturales.

2.3. OBJETIVOS DEL CAPÍTULO 3

El Capítulo 3 tiene por objetivo específico revisar la bibliografía existente hasta el momento actual y estudiar en profundidad la relación entre los compuestos antimicrobianos de origen

natural de diversas fuentes y el desarrollo de resistencia bacteriana frente a estos. Asimismo, se ahonda en la capacidad y posible mecanismo de algunos compuestos naturales para sensibilizar bacterias resistentes a antibióticos y revertir sus mecanismos de resistencia.

3. MATERIALES Y MÉTODOS

3.1. MATERIALES

3.1.1. Extractos y compuestos puros

El extracto de *C. salviifolius* fue producido en el laboratorio a partir de planta cultivada en la Universidad Miguel Hernández de Elche. Los extractos de *P. granatum* y *Citrus × paradisi* fueron amablemente suministrados por las empresas Monteloeder, S.L. (Elche, Alicante, España) y Nutracitrus S.L. (Elche, Alicante, España), respectivamente. Los compuestos polifenólicos puros: punicalagina, quercetina, quercetina-3-glucurónido, miricetina, naringinina y ácido gálico y ácido elágico fueron comprados a Sigma-Aldrich (Steinheim, Westfalia, Alemania).

3.1.2. Microorganismos

Los aislados clínicos de las siguientes especies bacterianas fueron amablemente recolectados y suministrados por el Servicio de Microbiología del Hospital General Universitario de Alicante (Figura 27): *S. aureus, Enterococcus faecalis, Enterococcus faecium, Streptococcus pneumoniae, E. coli, K. pneumoniae, Enterobacter cloacae, Serratia marcescens, P. aeruginosa, Acinetobacter baumanii* y *Stenotrophomonas maltophilia*.

Figura 27. Hospital General Universitario de Alicante. Fotografía tomada por Diego Delso.

Las cepas control de *S. aureus* fueron adquiridas de la *American Type Culture Collection* (ATCC® 33591, ATCC® 43300 y ATCC® 29213, Manassas, Virginia, Estados Unidos).

3.2. MÉTODOS

3.2.1. Extracción y fraccionamiento

Los extractos de *C. salviifolius* empleados durante la presente Tesis Doctoral han sido elaborados y caracterizados integralmente en el IDiBE a partir de planta cultivada en el terreno de la Universidad Miguel Hernández de Elche. Los extractos se obtuvieron mediante el triturado e infusión de planta fresca en agua a 50 ºC tal y como se describe en trabajos previamente publicados por el grupo de investigación [80, 81, 131, 180]. El extracto se filtró empleando un filtro de placas (Rover Pompe Colombo, Polverara, Padua, Italia) y se fraccionó empleando la resina Amberlite FPX66 comprada a Rohm and Haas (Esslingen am Neckar, Stuttgart, Alemania) y empaquetada en el interior de una columna de vidrio de 75 cm de alto y 5 cm de diámetro. Una vez saturada la resina, se eluyó con etanol absoluto y esta fracción se secó empleando un aparato *spray dryer* (Büchi Mini Spray Dryer B-290, Flawil, Suiza), obteniendo finalmente un polvo de color pardo [80].

El proceso de obtención de los extractos consta de cuatro pasos fundamentales: obtención de la materia prima vegetal, extracción, fraccionamiento en columna y secado. El proceso de caracterización integral de los extractos consta de cinco pasos: determinación de la cantidad de fenoles totales, determinación de la actividad antioxidante, análisis de la composición molecular por HPLC-MS, determinación de la capacidad antimicrobiana y análisis de datos. En la Figura 28 puede observarse un esquema del proceso completo.
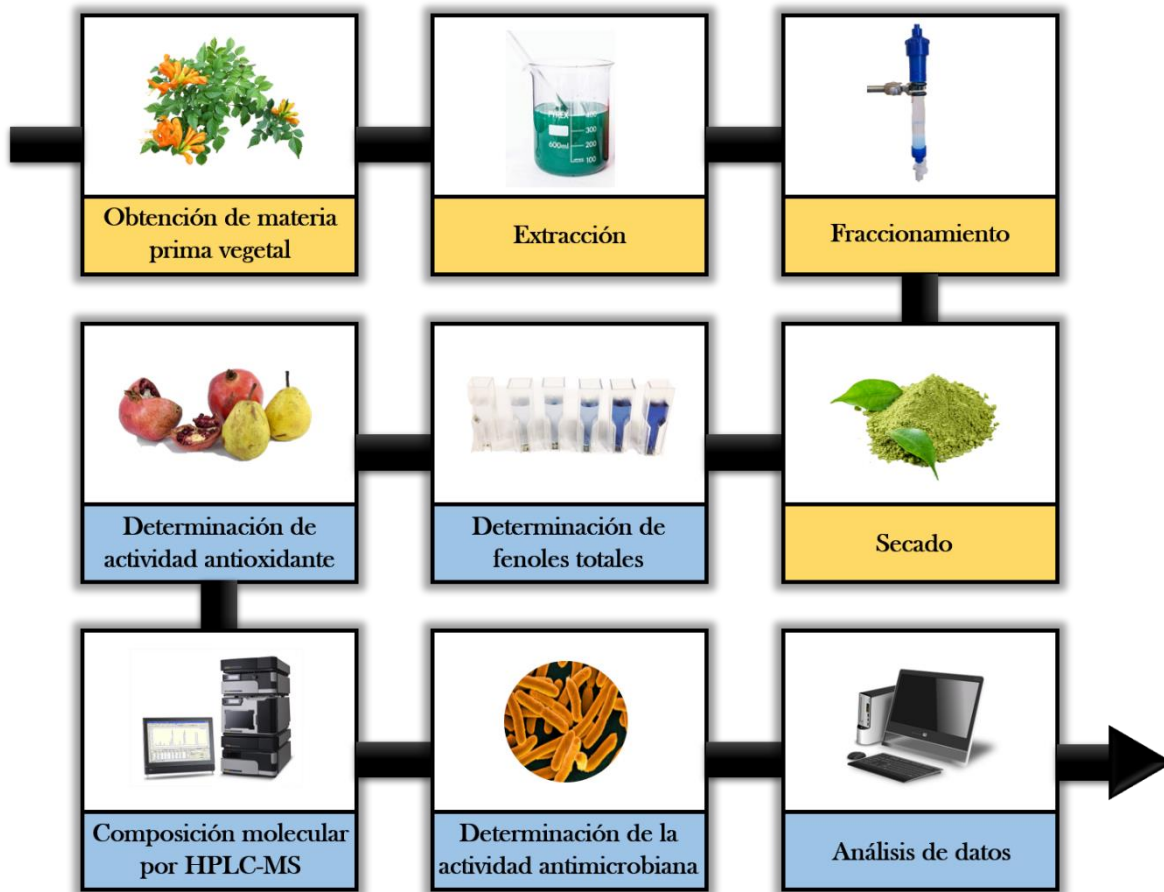
Figura 28. Esquema del proceso de obtención (pasos en color naranja) y caracterización integral (pasos en color azul) de los extractos vegetales. Imágenes extraídas de distintos repositorios online. Figura de composición propia.

3.2.2. Caracterización y cuantificación por HPLC-MS

La caracterización molecular de los extractos de *C. salviifolius* y *P. granatum* fue realizada mediante cromatografía líquida de alto rendimiento acoplada a espectrometría de masas (HPLC- MS) empleando un instrumento Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA). El equipo contaba con una bomba, un inyector automático, un horno de columna, un detector de matriz de diodos UV-vis y fue controlado mediante el software Agilent ChemStation. El instrumento HPLC se acopló a un espectrómetro de masas Esquire 3000+ (Bruker Daltonics, GmbH, Alemania) equipado con un analizador de masas, ionizador por electrospray ESI y trampa de iones, operado por el software de control y análisis de datos Esquire (Figura 29). La columna utilizada fue una Agilent Poroshell 120 RP - C18 4.6 x 150 mm 2.7 μm (Agilent, Santa Clara, California, Estados Unidos).

Figura 29. Cromatógrafo Agilent LC 1100 acoplado a un espectrómetro de masas Esquire 3000+ empleado en la caracterización de los extractos empleados en este trabajo.

La separación de la muestra se llevó a cabo usando un gradiente lineal usando ácido fórmico al 1 % (A) y acetonitrilo (B). El gradiente comenzó con 5 % de B, 25 % a los 30 minutos, 45 % de **B** a los 45 minutos, luego 5 % a los 51 minutos y 5 minutos más para reequilibrar. El caudal fue de 0,5 mL/min. Se obtuvieron las señales específicas a las longitudes de onda de 280 nm, 320 nm y 340 nm a partir del espectro de absorción obtenido mediante el detector de matriz de diodos. El sistema de ionización ESI se usó en modo negativo para generar iones [MH]⁻, en las siguientes condiciones: temperatura de desolvatación a 360 °C, temperatura del vaporizador a 400 °C, gas seco (nitrógeno) con nebulizador a 12 L por minuto y 70 psi, respectivamente. Los datos se recopilaron como espectros de masas de exploración completa entre 50 y 1400 m/z utilizando 200 ms para la recolección de los iones en la trampa. La interpretación de los espectros y la identificación de los principales compuestos se realizó con el software DataAnalysis 3.4 (Bruker, Billerica, Massachusetts, Estados Unidos).

3.2.3. Determinación del contenido fenólico total

El contenido fenólico total de los extractos se midió utilizando el método de equivalencia de ácido gálico (GAE) o método Folin-Ciocalteu [181]. Este método utiliza el reactivo Folin-Ciocalteu (Sigma-Aldrich, Misuri, Estados Unidos), compuesto por una mezcla de fosfomolibdato y fosfotungstato. Este reactivo reacciona con los polifenoles en los extractos en presencia de carbonato de sodio (Na_2CO_3, Sigma-Aldrich, Steinheim, Westfalia, Alemania), dando lugar a una coloración azul proporcional a los fenoles presentes en la muestra (Figura 30) [182]. Esta coloración puede ser medida a una longitud de onda de 600 nm utilizando un lector de placas multimodal (BMG Labtech, Offenburg, Alemania) [183].
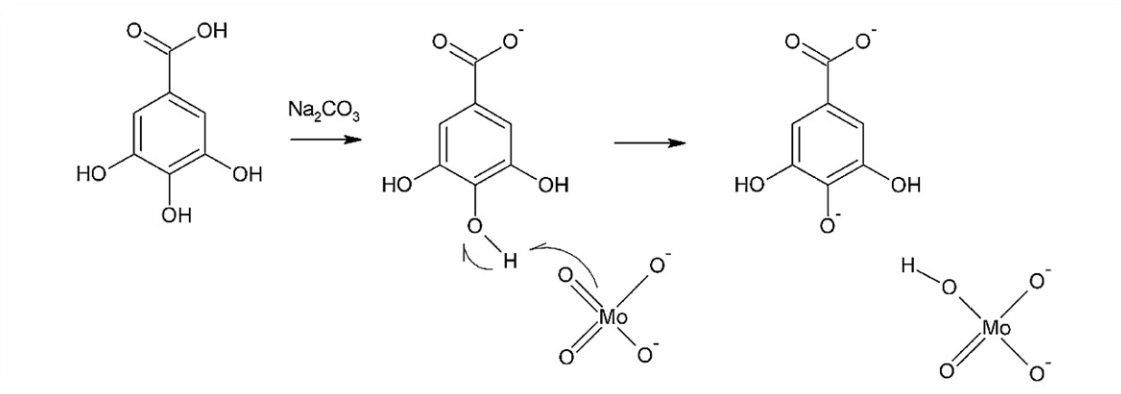
Figura 30. Mecanismo de reacción entre los fenoles de una muestra y el reactivo de Folin-Ciocalteu. Imagen extraída de Muñoz-Bernal O. A. *et al.* 2017 [182].

3.2.4. Determinación de la actividad antioxidante

La capacidad antioxidante de los extractos vegetales fue determinada empleando el método TEAC (*Trolox equivalent antioxidant capacity*) [181]. Este método usa el ácido 2,2'-azinobis(3-etilbenzotiazolín)-6-sulfónico (ABTS) como precursor radicalario tras su exposición al persulfato potásico ($K_2S_2O_8$). El radical catiónico generado en esta reacción se trata de un compuesto de color azul con un espectro de absorción en el UV-visible. Las moléculas antioxidantes de las muestras son capaces de reducir el catión $ABTS^{·+}$, produciendo una pérdida de color que puede ser cuantificada (Figura 31) [184, 185]. La medida de absorbancia se llevó cabo en un en un lector de placas Spectrostar Omega (BMG Labtech, Offenburg, Alemania) a una longitud de onda de 734 nm. Como estándar de referencia, se

utilizó Trolox (Sigma-Aldrich, Steinheim, Westfalia, Alemania), sustancia análoga de la vitamina E de naturaliza hidrófila.
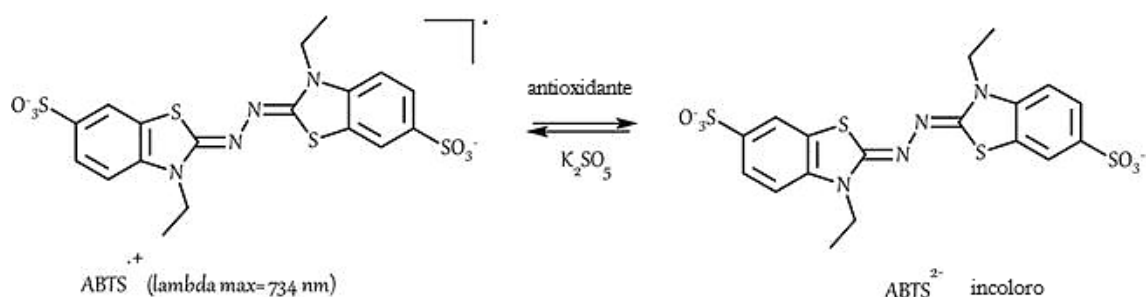
Figura 31. Mecanismo de reacción del método TEAC. El radical ABTS de coloración azulada reacciona con los antioxidantes de la muestra y pasa a un estado incoloro. Imagen extraída y adaptada de Santos-Sánchez N. F. *et al.* 2019 [185].

3.2.5. Ensayo antimicrobiano por el método Kirby-Bauer

El ensayo antimicrobiano Kirby-Bauer o método de difusión en agar o de disco-placa consiste en la aplicación de cantidades conocidas de los compuestos a probar (disoluciones de polifenoles o extractos en nuestro caso) impregnados en pequeños discos de papel colocados sobre un césped bacteriano previamente inoculado en una placa de Petri con agar bacteriológico semisólido. Alrededor de los discos impregnados en compuestos con capacidad antimicrobiana aparece un halo de inhibición del crecimiento bacteriano de tamaño proporcional al nivel de actividad del compuesto (Figura 32) [186]. El medio de cultivo utilizado para el crecimiento bacteriano fue el agar Mueller-Hinton (Merck Millipore, Massachusetts, Estados Unidos). Cada muestra y control fue realizado por duplicado. Los diferentes aislados bacterianos fueron incubados a su temperatura óptima de crecimiento durante 24 horas. Las áreas de los halos de inhibición fueron medidas digitalmente a partir de fotografías de las placas de Petri empleando el software AxioVision 4.8.2. Las áreas se midieron en píxeles cuadrados y se normalizaron con respecto a la superficie total de cada placa.

Figura 32. Ejemplo de ensayo antimicrobiano por el método Kirby-Bauer. El tamaño de los halos de inhibición presentes alrededor de los discos es proporcional a la capacidad antimicrobiana del compuesto que los impregna. Imagen extraída y adaptada de Kaniyarakkal V. *et al.* 2016 [186].

3.2.6. Ensayo antimicrobiano por el método de microdilución en placa

Los ensayos antimicrobianos de microdilución en placa se realizaron en placas de 96 pocillos (Figura 33). Se realizaron microcultivos de diferentes aislados de *S. aureus* con 10 concentraciones diferentes de extracto antimicrobiano en cada placa. Cada placa y control se realizaron por duplicado. El medio de cultivo utilizado fue el caldo Mueller-Hinton (Merck Millipore, Massachusetts, Estados Unidos). Después de 24 horas de incubación a 37 °C, los pocillos con medio y bacterias se tiñeron con cloruro de iodonitrotetrazolio (Sigma-Aldrich, Misuri, Estados Unidos) para teñir de rojo las bacterias viables. Después de 30 minutos de incubación de la placa a 37 °C, se midió la absorbancia a una longitud de onda de 570 nm utilizando un lector multimodal (BMG Labtech, Offenburg, Alemania) para determinar la proliferación microbiana en cada pocillo.
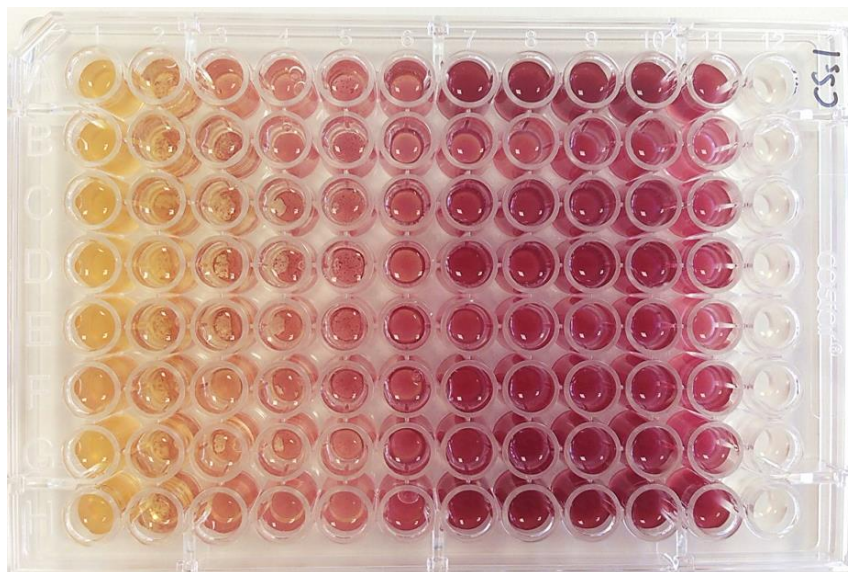
Figura 33. Ensayo antimicrobiano por método de microdilución en placa de 96 pocillos. La intensidad de la coloración roja se relaciona con una mayor concentración bacteriana en el pocillo. Se observa un gradiente creciente de izquierda a derecha, coincidente con la disminución de la concentración del extracto antimicrobiano empleado en el ensayo.

3.2.7. Acoplamiento molecular *in silico*

Los cálculos de variación de la energía libre de Gibbs (ΔG) en la interacción ligando-receptor de los ensayos de acoplamiento molecular o *docking* se llevaron a cabo empleando el software Autodock/Vina ejecutado en un clúster informático de alto rendimiento (**LUSITANIA II**) bajo sistema operativo Linux perteneciente al Centro Extremeño de Investigación, Innovación Tecnológica y Supercomputación [CenitS]. Las estructuras de alta resolución de las enzimas diana se obtuvieron de Protein Data Bank (**PDB**). Para las enzimas no presentes en PDB se realizó un modelado de homología utilizando las secuencias de aminoácidos que se encuentran en la base de datos UniProt. Las estructuras de los compuestos polifenólicos empleados para el cribado se obtuvieron de la base de datos Phenol-Explorer 3.6 [187, 188].

3.2.8. Análisis estadístico

La concentración mínima inhibitoria del 50 % de la población bacteriana (**CMI$_{50}$**) para cada aislado y las diferencias significativas entre tratamientos y conjuntos de datos se calcularon utilizando el software GraphPad Prism 6 procesando los datos obtenidos en los ensayos

antimicrobianos de microdilución. Los datos recopilados en los ensayos se analizaron utilizando un ajuste no lineal con mínimos cuadrados (logaritmo de la concentración de inhibidor frente a respuesta normalizada con pendiente variable, ecuación: $Y = 100/(1 + 10^{\wedge}((LogIC_{50}-X)*HillSlope)))$ para calcular los valores de CMI_{50}. Los gráficos finales se realizaron con Microsoft Excel 2016.

Los análisis MCA se realizaron para analizar datos categorizados y transformarlos en tablas cruzadas para mostrar los resultados de manera gráfica. Los análisis GLMR fueron utilizados para generar modelos de regresión lineal para una variable de respuesta continua dados predictores continuos y/o categóricos. Los análisis MCA y GLMR se realizaron empleando los softwares Microsoft Excel 2016 y Google Colab. Se utilizaron para ellos cuadernos Jupyter y las bibliotecas mca-1.0.3, Pandas v0.25.3 y Matplotlib Python v3.2.0.

4. RESULTADOS

CAPÍTULO 1

Título: Antimicrobial Capacity of Plant Polyphenols against Gram-positive Bacteria: A Comprehensive Review.

Autores: Francisco Javier Álvarez-Martínez, Enrique Barrajón-Catalán, José Antonio Encinar, Juan Carlos Rodríguez-Díaz y Vicente Micol.

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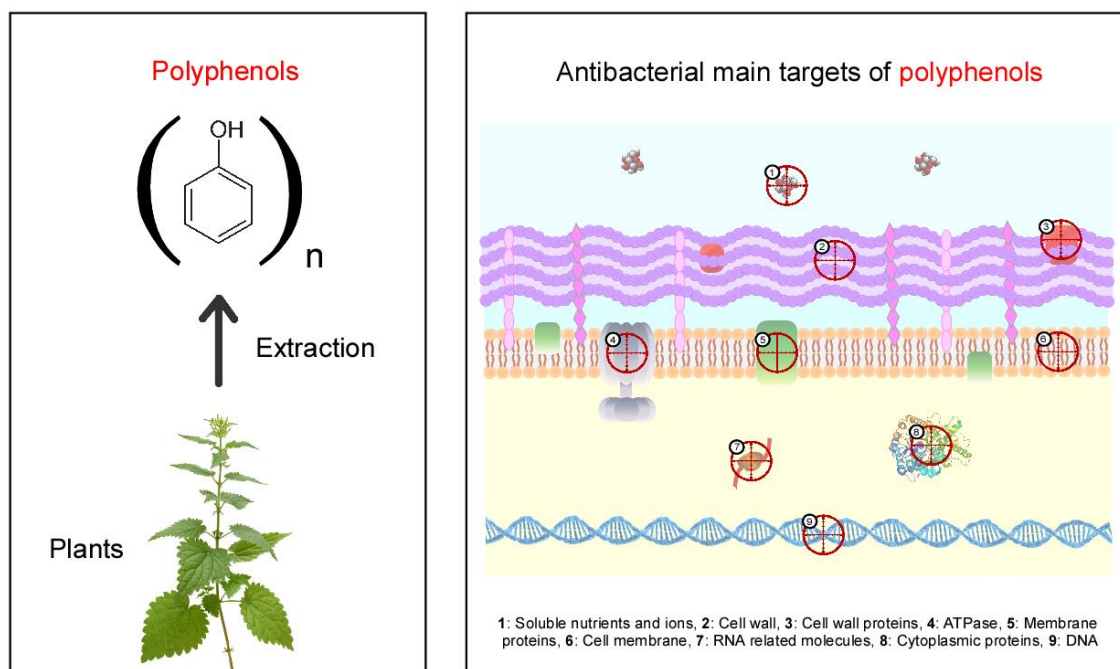
4.1. RESUMEN DE LOS RESULTADOS

La presente revisión, publicada en 2020, recoge los resultados publicados hasta ese momento sobre la capacidad antimicrobiana, mecanismos de acción y dianas moleculares de los polifenoles de plantas frente a bacterias de interés clínico a nivel mundial, centrándose especialmente en bacterias gram-positivas como *S. aureus* y sus cepas resistentes a antibióticos. Los artículos revisados fueron obtenidos de la base de datos MEDLINE vía PubMed. Además, se llevó a cabo un cribado molecular *in silico* de 931 polifenoles presentes en la base de datos Phenol-Explorer 3.6 frente a 9 enzimas involucradas en la síntesis del peptidoglucano de la pared celular de 6 especies bacterianas distintas para determinar afinidades de unión y seleccionar aquellas moléculas susceptibles de ser candidatos para el desarrollo de terapias dirigidas frente a dianas específicas.

Los resultados de la revisión indican que ciertos polifenoles, especialmente los flavonoles, y sus combinaciones en forma de extractos vegetales completos poseen una actividad antimicrobiana significativa frente a bacterias gram-positivas en rangos de CMI de pocos µg/mL. Su mecanismo de acción es complejo e implica diferentes dianas moleculares, tales como la pared celular, la membrana citoplasmática, los receptores de membrana, ciertos metabolitos y la formación de biofilms. Además, se ha comprobado que existe un efecto sinérgico en la capacidad antimicrobiana en el uso de antibióticos como la gentamicina cuando se aplica con polifenoles como las catequinas del té verde. Algunos polifenoles y extractos vegetales poseen la capacidad de sensibilizar bacterias resistentes a antibióticos frente a éstos, revirtiendo la resistencia y volviéndolas vulnerables de nuevo.

Los resultados del cribado *in silico* de polifenoles frente a distintas enzimas involucradas en la síntesis del peptidoglucano de la pared bacteriana apuntan a que existen polifenoles capaces de unirse al sitio catalítico de las enzimas diana con incluso más afinidad que sus inhibidores de referencia. Este es el caso de cinco catequinas (con códigos de Phenol-Explorer 3.6 PE000780, PE000786, PE000787, PE000788 y PE000789) y tres teaflavinas (PE000143, PE000144, PE000149) frente a la enzima glutamato racemasa de *Enterococcus faecium*, *Enterococcus faecalis* y *Acinetobacter baumanii*. Dos daidzeínas (PE000857, PE000859), el ácido litospérmico (PE001041) y el ácido salvianólico (PE001044) mostraron también mayor afinidad de unión por la enzima glutamato racemasa de *Helicobacter pylori* que inhibidores probados experimentalmente. Estos resultados abren la puerta a la realización de ensayos

antimicrobianos *in vitro* empleando los polifenoles con mayor afinidad obtenida, postulándose como candidatos para el desarrollo de terapias antibióticas dirigidas.

En conclusión, los polifenoles y sus mezclas se erigen como agentes prometedores en la búsqueda de nuevos compuestos antimicrobianos que ayuden a combatir el auge de las bacterias resistentes a antibióticos. Gracias a sus propiedades, éstos podrían usarse como agentes únicos o en combinación con terapias ya existentes, reduciendo la cantidad de antibiótico necesaria para tratar las infecciones y con ello, sus efectos secundarios indeseados.

Antimicrobial Capacity of Plant Polyphenols against Gram-positive Bacteria: A Comprehensive Review

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Abstract: ***Background***: Multi-drug-resistant bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) disseminate rapidly amongst patients in healthcare facilities and suppose an increasingly important cause of community-associated infections and associated mortality. The development of effective therapeutic options against resistant bacteria is a public health priority. Plant polyphenols are structurally diverse compounds that have been used for centuries for medicinal purposes, including infections treatment and possess, not only antimicrobial activity, but also antioxidant, anti-inflammatory and anticancer activities among others. Based on the existing evidence on the polyphenols' antibacterial capacity, polyphenols may be postulated as an alternative or complementary therapy for infectious diseases.

Objective: To review the antimicrobial activity of plant polyphenols against Gram-positive bacteria, especially against *S. aureus* and its resistant strains. Determine the main bacterial molecular targets of polyphenols and their potential mechanism of action.

Methodology: The most relevant reports on plant polyphenols' antibacterial activity and their putative molecular targets were studied. We also performed virtual screening of thousand different polyphenols against proteins involved in the peptidoglycan biosynthesis to find potential valuable bioactive compounds. The bibliographic information used in this review was obtained from MEDLINE *via* PubMed.

Results: Several polyphenols: phenolic acids, flavonoids (especially flavonols), tannins, lignans, stilbenes and combinations of these in botanical mixtures, have exhibited significant antibacterial activity against resistant and non-resistant Gram-positive bacteria at low µg/mL range MIC values. Their mechanism of action is quite diverse, targeting cell wall, lipid membrane, membrane receptors and ion channels, bacteria metabolites and biofilm formation. Synergic effects were also demonstrated for some combinations of polyphenols and antibiotics.

Conclusion: Plant polyphenols mean a promising source of antibacterial agents, either alone or in combination with existing antibiotics, for the development of new antibiotic therapies.

1. INTRODUCTION

1.1. Increase of Antibiotic Resistance: The Gram-positive *Staphylococcus aureus* Case

Antibiotic resistance is a significant public health problem nowadays with a tendency to increase world-wide. Worse still, very few therapeutic alternatives are available in the management of some serious infectious diseases. Therefore, many international institutions are urging to find new treatments for these infections, in particular agents that suppress or abrogate the emergence of drug resistance [1, 2]. The causes for antibiotic resistance increase are very varied and distributed in different areas: the massive use in human health, veterinary medicine and in agriculture, tourism and emigration. In fact, the World Health Organization

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(WHO) has pointed out the need to develop a global plan of action called "One health" as a way to indicate the need for the involvement of many actors in different fields [3, 4]. These actions include the prevention of hospital infections, the recommendation of the discriminated use of antibiotics or the promotion of research and development of new drugs [5].

Strategies to combat this phenomenon should start with the prudent use of antibiotics and must implement multidisciplinary stewardship groups that address the infectious processes in an integrated manner. This includes improving microbiological diagnosis, studying the influence of antibiotics in the microbiome and in the environment, controlling the use of antibiotics in animals destined for human consumption, implementing measures of education to the users of antibiotics and to health professionals and promoting measures for infection control in the sanitary field, including the promotion of hand washing of staff. Also, the need to promote basic and clinical research that helps the development of new antibiotics has repeatedly been reported, since the number of drugs available to treat these multiresistant bacteria is extremely low [6-8].

At present, there are numerous bacterial species (both Gram-positive such as *Enterococcus faecium*, *Enterococcus faecalis* or *Staphylococcus aureus* and Gram-negative such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Helicobacter pylori*; among others), that cause infections whose treatment with the usual antibiotic drugs is not effective, that is, they have developed phenomena of resistance to one or several drugs.

Within Gram-positive bacteria, *S. aureus* is a microorganism with high rates of resistance to multiple antibiotics and is frequently associated with very serious infections. Resistant *S. aureus* is viewed as a public health priority. Besides being a risk for patients, it supposes a global health burden due to the additional cost for the health systems. Then, specific plans have been designed and implemented to control these infections [9-11]. In addition to the resistance problems presented by this pathogen, some strains have virulence factors that cause serious infectious processes, such as Panton-Valentine Leucocidin (PVL) that has frequently been associated with necrotizing pneumonia in the out-of-hospital setting [12].

Resistance to methicillin, which affects the activity of most beta-lactam drugs, is the most prominent problem in this strain and results in Methicillin-Resistant *S. aureus* (MRSA). MRSA is frequently associated with infections in hospital environments, disseminates rapidly amongst patients in healthcare facilities and is an increasingly important cause of community-associated infections, which leads to high mortality and morbidity rates. In hospitals, there is also the risk of horizontal transmission of this pathogen, both among patients and with the participation of health personnel since this microorganism can colonize the nasal mucosa asymptomatically [13]. MRSA affects more than 150,000 patients hospitalized annually in the EU, resulting in extra in-hospital costs of EUR 380 million for EU healthcare systems.

Methicillin resistance is associated with the presence of the penicillin-binding protein 2a (PBP2a) protein, with lower affinity to the beta-lactam antibiotics encoded in the mecA gene and in some cases by mecC [14]. Much less frequently, Borderline Oxacillin-Resistant *S. aureus* (BORSA) is isolated with borderline resistance to methicillin (oxacillin MICs equal to 1-8 μg/mL) and without alterations in the penicillin-binding protein; these strains show hyperproduction of beta-lactamases or some mutations in PBP genes. Treatment of severe infections caused by BORSA may be ineffective, even with larger doses of oxacillin [15]. In addition, the existence of strains with mutations in PBP4 that cause high resistance to beta-lactams has recently been reported [16].

The treatment of these MRSA strains implies vancomycin as the drug of choice, but this drug is associated with risk of renal toxicity for the patients. In addition, the existence of heteroresistant strains with a high minimum inhibitory concentration has been reported in recent years, although within the range considered sensitive (MIC of 1.5 mg/L), so there is much controversy about the most appropriate treatment [17].

Another therapeutic alternative for MRSA are oxazolidinones, such as linezolid and tedizolid. These antibiotics inhibit protein synthesis by binding to the peptidyl transferase center of the 50S bacterial ribosomal subunit and have very low rates of antibiotic resistance, although some clinical isolates have recently been described resistant by mutations in the V domain of 23S rRNA or by cfr plasmids [18-20].

Daptomycin, a cyclic lipopeptide, is also a therapeutic alternative in some of these infectious processes. It is 4-8-fold as active as vancomycin against Methicillin-Susceptible *S. aureus* (MSSA) and MRSA and retains most of this activity against *S. aureus* with reduced susceptibility to vancomycin. Daptomycin binds to the bacterial cytoplasmic membrane, leading to membrane depolarization due to the loss of potassium ions from the cytoplasm. Resistant strains are rarely described,

and resistance mechanisms are often associated with changes in composition, charge, and fluidity of the cell wall [21].

Recently, some new drugs have appeared that improve the perspective in the treatment of this pathogen, such as ceftaroline and ceftobiprole, the only active cephalosporins against MRSA strains and therefore, with great clinical utility in the treatment of some of these infectious processes [22].

Two new lipoglycopeptides have also been commercialized, called oritavancin and dalbavancin. They work as classic glycopeptide drugs (vancomycin and teicoplanin) binding the terminal carboxyl of the d-alanyl-d-alanine residue of the growing peptide chains but differ from their parent glycopeptides by the addition of lipophilic tails. This addition allows these agents to have prolonged half-lives, giving them unique dosing profiles [23, 24].

Despite the above mentioned recent advances, the treatment of *S. aureus* infections and especially if it is resistant to methicillin (MRSA) is a public health problem of the first magnitude that requires a coordinated effort to control it and an urgent need for new more active therapeutic tools with low toxicity for patients.

2. EVOLUTIONARY BASIS OF THE PHARMACOLOGY OF PLANT POLYPHENOLS: MOLECULAR PROMISCUITY

Humans have been using plants as medicinal resources for thousands of years. There are many records from Traditional Chinese Medicine, Ayurvedic medicine, Kampo medicine, European medicine and African medicine among others using herbal products as a crucial medicinal system [25].

Plants are sessile organisms, which cannot escape from environmental stress situations (radiation, climate, predators, *etc.*). They do not possess an immune system to fight microbial infections neither. For these reasons, plants have developed some static defense systems to respond to the environmental stress and survive. These mechanisms include mechanical ones, as spines, thorns, and barks. On the other hand, plants have developed an extensive chemical arsenal of secondary metabolites which serves, among other uses, as an antimicrobial defense system [26, 27].

Plants produce a remarkably diverse array of more than 100,000 different low-molecular-mass secondary metabolites. These metabolites are distinct from the components of primary metabolism because they are generally nonessential for the underlying metabolic processes (photosynthesis, protein synthesis, tissue differentiation, *etc.*) of the plant. This significant molecular diversity results in part from an evolutionary process driven by selection for the acquisition of improved defense against microbial attack or insect/animal predation [28]. In tissues subjected to these stressful situations, high concentrations of various polyphenolic compounds can be observed [29].

Among plant secondary metabolites, polyphenols represent a broad class of natural products, which present a wide biological activity such as anticancer, antioxidant, anti-inflammatory, antiaging, cardioprotective and antimicrobial. This review focuses only on their antimicrobial capacity.

Polyphenolic compounds present a significant structural variability but share common phenolic moieties in their structure (Fig. **1**). In addition, polyphenols usually show conjugated forms with carbohydrates or form esters with organic acids. This phenomenon contributes to the enormous increase in the variety of the chemical forms of polyphenols. These compounds have modulated their diversity throughout evolution to act as ligands of many different molecular targets generating high molecular promiscuity [30, 31]. This multi-target trait is vital in the antimicrobial capacity of plant polyphenols and their synergistic effects with traditional antibiotics too [32].

Polyphenols are well tolerated by the human body. Dietary polyphenols are mostly present in plants as glycosides. After ingestion, these compounds are transformed in the gastrointestinal tract by the microbiota and digestive enzymes. They are usually deglycosylated at the digestive tract becoming aglycones, which exhibit higher bioactivity than their respective glycosides. If not absorbed, they may reach the large intestine where microbial transformation may occur yielding a diversity of bioavailable phenolic acids and lactones. When absorbed in the small intestine, they pass through portal vein towards the liver, where suffer further transformations [33, 34]. *In vivo* intestinal metabolism studies in rats suggested that main metabolic transformation involved were glucuronidation and sulfation, whereas *in vitro* studies highlighted hydrolysis of polyphenols [35]. After these processes, transformed polyphenols circulate towards different tissues and organs, exerting their antimicrobial and other bioactive activities [36]. The digestive and microbiota driven transformations are crucial for polyphenols to become bioactive molecules. For this reason, the administration route of polyphenols has to be optimized to obtain the desired biological activity.

3. MAJOR POLYPHENOLS WITH ANTIMI-CROBIAL CAPACITY

In this section, the antimicrobial capacity of the most representative polyphenols classified by families against different bacteria is reviewed (Table **1**).

3.1. Phenolic Acids

Phenolic or phenol carboxylic acids are substances that contain a phenolic ring and an organic carboxylic acid function in their chemical structure (Fig. **1**). Among the most common phenolic acids present in plants are p-hydroxybenzoic, 3,4-dihydroxybenzoic, caffeic, vanillic, ferulic, *p*-coumaric, syringic and sinapic acids.

Phenolic acids have demonstrated antimicrobial activity against several Gram-positive bacterial species. The antibacterial activity of phenolic acids-enriched peanut extracts against *S. aureus* (ATCC 13565) has been reported with MIC values between 24-301 µg/mL, depending on the type of extract [37, 38]. *Bacillus subtilis* (8649) was also sensitive to phenolic acids with MICs of 2 mM when using *p*-coumaric acid, ferulic acid or sinapic acid and a MIC of 4 mM when using caffeic acid [39]. Pure *p*-coumaric acid also inhibited *Bacillus cereus* (MTCC 1272) growth with a MIC value of 41 µg/mL [40].

3.2. Ellagitannins and Gallotannins

Both ellagitannins and gallotannins belong to the group of hydrolyzable tannins (Fig. **1**). Ellagitannins derive from ellagic acid, while gallotannins derive from gallic acid. Ellagitannins have a common monomeric moiety called Hexahydroxydiphenoyl (HHDP) which is esterified to a polyol and a galloyl residue. Ellagitannins are present in wood-aged wine, walnuts, pecans, berries and fruits, especially abundant in pomegranates, guavas and tropical highland blackberries [41, 42]. Oligomeric ellagitannins have vast structural diversity and varied biological activities depending on their structures. One of the largest groups of oligomeric ellagitannins contains a valoneoyl group that is produced through oxidative C-O coupling between a galloyl group of one monomer and an HHDP group of the other [43]. Some of the most representative ellagitannins commonly found in seeds, leaves, and fruits of plants are punicalagin, punicalin, corilagin, tellimagrandin I, geraniin and furosan. Examples of studied gallotannins are pentagalloylglucose, trigalloylglucose and tannic acid. Gallotannins are often found as complex mixtures [44]. Gallotannins are abundant in bean coats and nuts, especially in red sword bean (*Canavalia gladiata*) coat and witch hazel (*Hamamelis virginiana*) [45, 46].
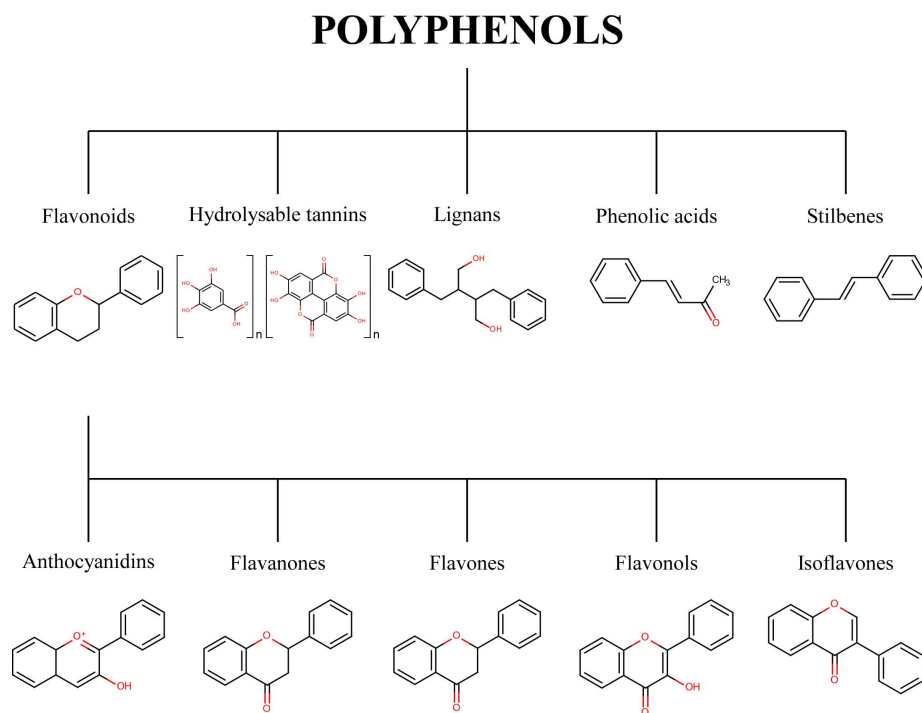
POLYPHENOLS

Fig. (1). Families and subfamilies of polyphenols with their core chemical structures.

It is known that ellagitannins extracted from botanical sources such as *Cistaceae* possess antibacterial activity within micromolar values [47, 48]. As examples, the ellagitannins davidiin and 3-O-galloylgranatin A have shown antibacterial activity against MRSA (OM481 and OM584) with a MIC of 64 μg/mL. They also had activity against VRE (*E. faecium* FN-1 and *E. faecalis* NCTC 12201) with MICs between 16 and 64 μg/mL [49]. Isorugosins are a subclass of ellagitannins that also present antibacterial activity against both MSSA (209P) and MRSA (OM481 and OM505) at MIC concentrations between 23 and 91 μM [43]. Ellagic acid and punicalagin have also demonstrated antimicrobial activity against *S. aureus* (CECT 59) with MIC values of 12.35 and 42.11 μg/mL, respectively [50]. Other study estimated at 12.5 μg/mL the MIC of both punicalagin and punicalin against *S. aureus* (BCRC 10781) [51].

Regarding gallotannins, penta-, hexa-, hepta-, octa-, nona- and deca-O-galloylglucose exhibited antibacterial activity against the Gram-positive *S. aureus*, *B. cereus*, *L. monocytogenes* and *B. subtilis* with MICs between 100 and 600 μg/mL. The degree of galloylation did not seem to affect the antibacterial activity. In general Gram-positive bacteria were generally more susceptible to tannins than Gram-negative [52]. Tannic acid also demonstrated antibacterial activity against *B. subtilis* with a MIC of 30 μg/mL [53].

3.3. Flavonoids

Flavonoids are a group of polyphenolic compounds found ubiquitously in plants. Until now, more than 9000 different flavonoid compounds have been described in plants, where they play important biological roles by affecting several developmental processes. They are structurally composed of a 15C backbone with at least two aromatic rings, which are tailored with diverse hydroxyl, methoxy or glycosyl groups (Fig. **1**) [36]. They form a wide molecular class with interesting antimicrobial properties. Next, the antimicrobial activity of the different sub-classes of flavonoids will be described.

3.3.1. Flavanols (Catechins, Procyanidins and Proanthocyanidins)

Flavanols (sometimes referred to as flavan-3-ols) are derivatives of flavans that bear the 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton. Flavanols exist in monomeric and polymeric forms, may be substituted with gallic acid and present different isomers, so these compounds constitute one of the most numerous sub-

classes of polyphenols. On the contrary, they do not exist in the glycosylated form in nature. The most representative compounds of this class include the monomeric forms catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, theaflavins and the polymeric forms proanthocyanidins and thearubigins.

Flavanols are most abundant in brewed black and green tea and dark chocolate, with more than 100 mg per 100g of foodstuff. Other significant flavanol sources are blackberries, cooked broad beans and pecan nuts [54]. Monomeric catechins are the most abundant polyphenols in green tea extracts. Their antimicrobial activity has been intensely studied, especially against Gram-positive bacteria, either isolated or in synergy with traditional antibiotics [36]. For instance, MICs between 62.5 and 125 μg/mL have been reported for Epigallocatechin (EGC) and epigallocatechin gallate (EGCG) against five different clinical isolate strains of *S. aureus* [55]. Besides the positive interaction with classical antibiotics, researchers have found that EGCG antibacterial effects against *S. aureus* are enhanced by other organic molecules such as ascorbic acid and decreased by others such as casein [56]. Green tea catechins have also shown activity against *B. subtilis* (MTCC1427) with a MIC of 156 μg/mL and a large decrease of its adhesion potential to host cells [57].

Polymeric procyanidins and proanthocyanidins have shown moderate antimicrobial activity against clinical isolates of MRSA (OM481, OM505, OM584 and OM623), but exhibited strong synergic effects and resistance reversion when used with oxacillin or penicillin G [58].

3.3.2. Anthocyanins

Anthocyanins are colored water-soluble molecules belonging to the flavonoid subfamily of polyphenols. Anthocyanins are responsible for the red, purple, and blue colors in fruits and vegetables, being very common in berries, currants and grapes. Among the anthocyanin pigments, cyanidin-3-glucoside is the major anthocyanin found in most of the plants [59].

Anthocyanins are very abundant in bilberries (285.21 mg/100 g foodstuff), aronia (349.79 mg/100 g foodstuff) and elderberry juice concentrate (411.40 mg/100 g foodstuff) [54].

Anthocyanin enriched extracts from bilberry and blueberry have shown antimicrobial activity against some Gram-positive bacteria: *Listeria monocytogenes*, *S. aureus*, *B. subtilis* and *E. faecalis* [36]. These molecules can cause localized disintegration of bacterial

outer membrane causing leaking of cytoplasm [60]. Anthocyanins may also difficult the bacterial uptake of certain oligoelements, inhibiting diverse physiological functions [61, 62].

3.3.3. Flavonols, Flavanones, Flavones and Isoflavones

These subgroup of flavonoids include well-known flavonols such as kaempferol, quercetin, and myricetin; the flavanones naringenin, eriodyctiol and hesperidin [63] the flavones luteolin, apigenin and baicalein and the isoflavones genistein and daidzein as isoflavones [36, 64].

Flavonols are mainly present in fresh capers, dried parsley, and elderberry juice, with concentration over 100 mg per 100 g of foodstuff, but are also widely distributed in many other plants. Flavanones are mainly present in oregano, grapefruit, lemon and oranges. Flavones are extremely abundant in dried parsley and oregano, with concentrations of 4.5 g and 1.0 g per 100 g of foodstuff, respectively [54].

The flavonols quercetin and kaempferol have demonstrated to have high antimicrobial activities with reported MIC values as low as 1.95 μg/mL and 7.8 μg/mL respectively against *S. aureus* (ATCC 6538) and MRSA clinical isolates [65, 66]. Glycosylated forms of these flavonols also showed antimicrobial activity against *S. aureus* with MICs of 250 μg/mL for quercetin-3-O-arabinofuranoside and 130 μg/mL for kaempferol-3-O-rhamnoside. Myricetin has shown a MIC value of 15.76 μg/mL against *S. aureus* (CECT 59) [50]. Glycosylated myricetin derivatives also showed antibacterial activity with MICs of 250 μg/mL against *S. aureus* (ATCC 12600) [67]. Quercetin-3-glucoside obtained a MIC value of 14.37 μg/mL against *S. aureus* (CECT 59) [50].

The flavanone naringenin exhibited antibacterial activity against several *S. aureus* strains (ATCC 29213, ATCC 10832, BAA-1717, 8325-4 and DU 1090) with MIC values between 256 and 512 μg/mL. Moreover, staphylococcal expression of α-toxin was significantly reduced when the organism was treated with 16 μg/mL of naringenin, reducing *S. aureus* virulence [68]. Naringenin derivatives have also proven to be especially effective against Gram-positive bacteria. Concentrations of 0.25 mM of alkyl prunin esters with 10-12C chain lengths diminished viable *S. aureus* (ATCC 29213) with about 6 log orders and *L. monocytogenes* (01 / 155, 99 / 287 and 99 / 287RB6, strains obtained from Dr. Carlos Malbran Microbiology Institute, Bue-

nos Aires, Argentina) with about 3 log orders after two hours [69].

Flavones has shown a remarkable antibacterial capacity against Gram-positive bacteria. Luteolin inhibited clinically isolated *S. aureus* growth with MICs between 31.2 and 125 μg/mL. Apigenin demonstrated MICs ranging 3.9-15.6 μg/mL against 15 MRSA and 5 MSSA strains [66, 70]. The flavone baicalein exhibited weak antimicrobial activity against clinically isolated Vancomycin-Resistant *Enterococcus* (VRE). Nevertheless, baicalein demonstrated great synergy effectiveness with the antibiotic gentamicin against Gram-positive VREs [71] and also synergy with tetracycline against MRSA (OM481 and OM584) [72].

A group of nine isoflavones (2′-hydroxyerythrin A, daidzein-7-O-butenoyl glycoside, 7,4'-dihydroxy-6-methoxyisoflavone, daidzein, daidzin, genistein, formononetin, ononin and isoerythrinin A) was tested against *S. aureus* (ATCC 26112) and *E. faecium* (ATCC 35667). Among them, only 2′-hydroxyerythrin A inhibited bacterial growth significantly with MICs of 13.1 μg/mL for *S. aureus* and 16.5 μg/mL for *E. faecium*, followed by isoerythrinin A with MICs of 18.3 μg/mL for *S. aureus* and 22.6 μg/mL for *E. faecium* [73].

3.4. Lignans

Plant lignans are a polyphenol subclass, which a chemical structure consisting of two phenylpropanoid moieties connected *via* C8-C8′ at their side chain or by additional ether, lactone, or carbon bonds (Fig. **1**) [74]. Some examples of lignans are enterodiol, magnolol, and honokiol. The most abundant natural sources of lignans are flax and sesame seeds. Other secondary sources are grains, fruits and vegetables [75].

The norlignans Hyposoxide (HYP) and rooperol (RO), derived from *Hypoxis rooperi* T. Moore, demonstrated antimicrobial activity against *S. aureus*, showing MIC values of 20 μg/mL and 800 μg/mL for RO and HYP, respectively. These values were lower than their respective MIC values for *E. coli*. A stronger antibacterial effect was also observed against the Gram-positive bacteria when the whole *Hypoxis rooperi* extract was utilized. RO showed a 5 times lower MIC value than the positive control neomycin [76].

Magnolol and honokiol have shown remarkable antimicrobial activity against both methicillin-sensible *S. aureus* (MSSA ATCC 25923) and ten clinical isolates of MRSA. Magnolol showed a MIC of 32 μg/mL against MSSA and a MIC range between 8 and 64

µg/mL against the MRSA clinical isolates. On the other hand, honokiol demonstrated a MIC of 16 µg/mL against MSSA and a range between 16 and 32 µg/mL against MRSA [77].

The antimicrobial activity of some lignans has been proposed to be related to their Multidrug-Resistant Reversal Activity (MDR). This is the case of dibenzylbutane, furofuran, and tetrahydrofuran lignans, which have exhibited comparable or stronger MDR activity than verapamil. Arylnaphtalene lignans exhibited additional moderate antimicrobial activity against *S. aureus* [78]. Melaleucin A and melaleucin C are recently discovered lignans showing a potent antimicrobial activity against MSSA (ATCC 6538) and MRSA (JCSC4788) with MICs of 8 µg/mL and 16 µg/mL, respectively [79].

Virgatusin, a tetra-substituted tetrahydrofuran lignan, has shown stereoselective anti-Gram-positive bactericidal activity in disc-plate assays, affecting *B. subtilis*, *S. aureus* and *Listeria denitrificans*. This compound seemed to damage the cytoplasmic membrane, leading to membrane-related cell death [80].

3.5. Stilbenes

Stilbenes chemical structure consists of two aromatic rings and phenolic hydroxyl groups with double bonds that makes two *cis-* and *trans-* forms of isomers (Fig. **1**) [81]. Stilbenes are typically present in wine, grapes, tree nuts and berries. Resveratrol is the most famous integrant of this polyphenol subclass [82].

Resveratrol has demonstrated a significant antibacterial activity against some Gram-positive bacteria. Resveratrol exhibited MICs of 16.5 µg/mL against *B. subtilis* and 32 µg/mL against *S. aureus* [83]. The resveratrol efficacy has been also proven against MRSA clinical isolates with MICs between 250 µg/mL and 1000 µg/mL [66]. The chemical stilbene backbone has been proven to reduce *S. aureus* virulence factors, *e.g.* hemolysis [84]. It also inhibits *Mycobacterium tuberculosis* (H37Rv) and *E. faecalis* (ATCC 29212) growth with a MIC of 100 µg/mL each [85].

Other stilbenes such as pterostilbene have shown strong antibacterial activity against *S. aureus* (ATCC 25923) with a MIC of 25 µg/mL [86]. It is also reported that prenylation of stilbenes enhance their antibacterial activity against MRSA (18HN) and *L. monocytogenes* (EGD-e) [87].

3.6. Herbal Mixtures and Botanical Extracts

There is a great deal of research related to the antibacterial properties of herbal mixtures and plant ex-

tracts. Botanical extracts can be obtained in many ways, such as infusion, fractioning, ultrasound-assisted extraction, using supercritical fluids, *etc.* There is extensive literature reporting the antimicrobial activity of plant extracts, however, in this review, only data derived from well characterized extracts, with clear evidences on their putative correlation between their composition and their antimicrobial activity have been included (Table **2**). One of the most widely studied botanical extracts worldwide is the green tea extract derived from *Camellia sinensis* [88].

Green tea extract is well known for its traditional therapeutic properties: antioxidative, anti-inflammatory or antimicrobial among others. The chemical composition of green tea is complex and varies depending on variety and extraction procedure. The most abundant phytochemicals present in green tea are polyphenols, and flavonoids in particular: catechins, catechin gallates and proanthocyanidins [89]. Green tea extract has shown antimicrobial activity against *S. aureus* (ATCC 6538p and four clinical isolates) with MICs ranging from 250 to 1000 µg/mL [55]. Green tea extract also demonstrated antibacterial activity against other Gram-positive bacteria with MIC values of 156 µg/mL against both *B. subtilis* (MTCC1427) and *Staphylococcus epidermis* (MTCC435) and a MIC of 313 µg/mL against *Brevibacterium linens* (MTCC268). Moreover, skin pathogens such as *S. epidermidis, Micrococcus luteus, Brevibacterium linens, Pseudomonas fluorescens* and *B. subtilis* were found to be sensitive to green tea extract *via* disc diffusion assay [57]. MRSA growth was also especially sensitive to green tea extract, being inhibited by the equivalent of a 1:10 dilution of a cup of tea [89].

Cistaceae extracts have demonstrated antimicrobial activity against *S. aureus* (CECT 59). Aqueous extracts from *Cistus ladanifer* and *Cistus populifolius* have shown MIC values of 154 and 344 µg/mL, respectively [47]. Hydroalcoholic extract of *C. ladanifer* showed a MIC value of 144 µg/mL. Aqueous spray-dried extracts of *Cistus albidus* and *Cistus clusii* showed MIC values of 60 µg/mL and 91 µg/mL, respectively. The most active extract was the hydroalcoholic spray-dried *Cistus salviifolius* one after column fractionation, with a MIC value of 11 µg/mL [48]. Several botanical extracts from *Hibiscus sabdariffa, Hibiscus arnottatianus, Lippia citriodora, C. albidus, C. ladanifer, C. clusii* and *Hypooxis rooperi* were tested against three pathogenic model microorganisms: *E. coli, S. aureus* and *C. albicans* [90]. At the lowest concentrations tested, *i.e.* 1 mg/mL, only *C. salviifolius* extract re-

tained significant growth inhibitory activity against *S. aureus* (533R4 Serovar 3) among the extracts tested. At higher concentrations, 1 and 2 mg/mL, most extracts showed significant antimicrobial activity against *S. aureus*. The results suggest that extracts enriched in ellagitannins and flavonols revealed promising antibacterial activity agents both against Gram-positive and Gram-negative bacteria. Phenolic acids, anthocyanidins, and flavonols are plausibly more related to the antifungal activity [91-98].

3.7. Synergic Mixtures

3.7.1. Combination of Polyphenols

There is wide evidence of the synergistic effects of the combination of different polyphenols with traditional antibiotics. Pharmacological synergic interactions are often expressed as FICI (fraction inhibitory concentration index) value for a certain pair of substances in combination. For a paired combinations of antimicrobial agents, a FICI value ≤ 0.5 represents synergy, 0.5 ≤ FICI ≤ 1 represents additivity, 1 ≤ FICI ≤ 2 represents indifference and FICI ≥ 2 represents antagonism [99]. The FICI value is calculated by adding up the FIC (fraction inhibitory concentration) values of both antimicrobial agents. The FIC value of a compound in a combination is calculated by dividing the MIC value of the whole combination between the MIC value of the compound alone [100].

Several studies have proposed that different polyphenolic compounds may have synergic antibacterial capacity in complex botanical mixtures. In many cases, complex polyphenolic mixture loses its efficacy after purification. This loss of functionality has been attributed to the decrease in the synergistic interactions between the phytochemicals previously present in the sample [93, 101]. The possible explanation to this behavior may be due to the capacity of polyphenols to interact with multiple molecular targets such as lipid membranes, membrane receptors, enzymes, ion channels, transport proteins and others [102].

Several examples of the synergy between a pair of polyphenols have been reported. Combinations of rutin with quercetin, morin, kaempferol, myricetin or eriodictiol strongly decreased MIC values against *B. cereus*, even when rutin had no antibacterial activity by itself [103, 104]. Two-drug combinations between luteolin, quercetin and resveratrol have shown synergistic effects against two MRSA strains (SA0929, SA1056) [66]. The flavonols quercetin and kaempferol have shown synergy when applied together or in combination with caffeic acid against *S. aureus* (ATCC

6538). The strongest synergistic effect was observed for the combination containing 7.8 µg/mL of quercetin and 31 µg/mL of caffeic acid with a FICI value of 0.37 [65].

The proportions of each polyphenol in the combination seem of key importance in synergy. Strong synergy against *S. aureus* (CECT 59) has been also found between the combinations punicalagin + ellagic acid (FICI value of 0.038), quercetin-3-glucoside (FICI of 0.31) or myricetin (FICI of 0.17). Quercetin-3-glucoside also showed synergy when paired with ellagic acid (FICI of 0.21) or myricetin (FICI of 0.05). The synergy of all these mixtures depended directly on the ratio of each component [50].

Due to the vast variety of polyphenolic structures present in nature, it is difficult to propose specific synergistic mechanisms. Nevertheless, some mechanisms have been proposed for some polyphenolic mixtures, in which partially hydrophobic phenolics interact with bacterial membranes, disrupting them to the point that small phenolic acids can enter the cells and trigger hyperacidification and electronic quenching, leading to cell death. That is the proposed mechanism for the synergy found between rosmarinic acid, a partially hydrophobic biphenyl and small phenolic acids such as gallic or caffeic acid [105, 106].

3.7.2. Interaction between Polyphenols and Antibiotics

Isolated plant polyphenols and whole extracts are currently being used to sensitize multidrug-resistant bacterial strains (MRSA, VRE, *etc.*) to traditional antibiotics with promising results [58, 107]. Green tea catechins have shown synergistic activities with gentamycin against *S. aureus* standard strains (ATCC 6538p) and a clinical isolate with FICI values of 0.56 and 0.75, respectively [55]. Baicalein has also shown strong synergy with gentamycin against VRE (VRE-70, VRE-940, VRE-096, and VRE-721) [71]. EGCG has also demonstrated powerful synergy with tetracycline to revert resistance in both resistant and sensitive *S. aureus* and *S. epidermis via* inhibiting specific Tet(K) efflux pump [108].

Some green tea catechins have demonstrated high MRSA sensitizing capacity towards methicillin, oxacillin and other β-lactam antibiotics in addition to its intrinsic antimicrobial capacity. For instance, galloyl catechins at concentrations between 6.25 and 25 µg/mL have reduced MIC of β-lactams against *S. aureus* (BB568, EMRSA-16 and EMRSA-15) from high resistance levels to below the common resistance break-

point [109]. Moreover, EGCG and especially EGC at concentrations of 25 µg/mL can heavily reduce the resistance of *S. aureus* clinical isolates towards other β-lactam antibiotics besides oxacillin: flucloxacillin, cefotaxime, cefepime, imipenem and meropenem [110].

The lignans magnolol and honokiol have demonstrated antimicrobial synergy with a broad spectrum of antibiotics: amikacin, gentamycin, fosfomycin, levofloxacin, etimicin, piperacillin, ciprofloxacin, and norfloxacin, against several clinically isolated MRSA strains with FICI values ranging between 0.25 and 0.5. These polyphenols were especially effective in reversing resistance against amikacin and gentamycin. Moreover, magnolol and honokiol have shown great synergy with oxacillin, chloramphenicol, cefoxitin and other traditional antibiotics [77, 111].

The ellagitannins corilagin and tellimagradin I have proven to inhibit the activity of PBP2a in MRSA, allowing β-lactam antibiotics to increase their activity [112]. Anti-VRE activity of tannins have also been recently reported [49]. Isoflavones isolated from *Lupinus argenteus* have potentiated the activity of norfloxacin and berberine *via NorA* multidrug resistance pump inhibition [113]. Pterostilbene has shown great synergy with the antibiotic gentamicin with a FICI value of 0.125 [86].

Extracts from guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), "carqueja" (*Baccharis trimera*) and mint (*Mentha piperita*) were tested in combination with thirteen antibiotics for antimicrobial synergism against clinically isolated *S. aureus*. All of them showed synergy with tetracycline and each extract showed synergy between two and eleven antibiotics [107]. Other study showed that a fraction of an extract from *Duabanga grandiflora* had synergy with ampicillin against MRSA (ATCC 43300) *via* PBP2a inhibition [114].

4. FOOD PRESERVATION

Besides being an important human pathogen, *S. aureus* is also one of the largest producers of foodborne illnesses. Because of its resistance, this bacterium can grow in many different types of foods, producing problematic heat-resistant toxins that can severely affect human health [115]. To avoid this, several studies have assessed the possibility of using polyphenols enriched formulations as natural biopreservatives of food due to its antimicrobial properties and low toxicity in humans. Besides their antimicrobial action, polyphenols can be

used in new trends of active packaging, edible films, fortification of products to extend shelf life or even turn some traditional foods into functional foods [116].

Nowadays, there exist many compounds to preserve food, but not all are equally effective. A recent study compared the *S. aureus* enterotoxin I production, bacterial growth and toxin gene expression in the presence of four different food preservatives: sodium nitrite, polylysine, chitosan, and tea catechin. Results showed that tea catechins were the most efficient among the four preservatives studied exhibiting a higher antimicrobial activity and toxin suppression [117].

Researchers studied the effect of several polyphenol-derived food additives against the production of toxins and biofilms by foodborne pathogens. They found that many polyphenols such as gallic, rosmarinic and ellagic acids, catechins and epigallocatechin gallate, had strong biofilm inhibition capacity against *S. aureus* at growth sub-inhibitory concentrations. It is important to notice that polyphenols could also affect organoleptic properties in food, especially the flavor. Nevertheless, the effective concentrations proposed in the study did not affect any food properties while protecting it against staphylococcal toxins and biofilms [118].

Polyphenols like catechin or tannic acid have also demonstrated quality and organoleptic improvements when added to fish and seafood. In addition to protecting food from bacterial attack, some polyphenols have the ability to retard food browning and texture degradation *via* antioxidant capacity and protein cross-linking, respectively [119].

5. MOLECULAR TARGETS AND POTENTIAL MECHANISM OF POLYPHENOLS

In this section, the main molecular targets of polyphenols and the proposed mechanisms of action are reviewed and discussed. these molecular targets are also shown in Fig. (**5**).

5.1. Cell Wall Components and Synthesis

The development of antimicrobial drugs is a transcendental fact in modern medicine that saves the lives of many people infected by pathogenic bacteria. Most of these drugs have molecular targets enzymes involved in the biosynthesis of the bacterial cell wall and cellular proteins and DNA. Since the bacterial cell wall is for these microorganisms unique, using drugs that prevent their biosynthesis will have an enormous therapeutic value. In fact, more than 60% of antimicrobial drugs for clinical use prevent the formation of the bacterial cell wall [120].

The primary component of the bacterial cell wall is a highly cross-linked polysaccharide (alternating ß-1,4-linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine) with a pentapeptide that includes D-amino acids: the peptidoglycan. Peptidoglycan maintains the shape of the bacterial cell, acts as an anchoring point to extracellular structures such as flagella and allows the bacterial cell not to explode as a consequence of the greater osmolarity of its interior with respect to the hypoosmotic external environment. In Gram-negative bacteria, peptidoglycan resides in the periplasm between the cytoplasmic and external membranes, whereas in Gram-positive bacteria is a thicker layer interconnected with other polymers such as teichoic acids [121, 122]. The main difference between Gram-positive and Gram-negative bacterial cell wall is the presence of an additional polysaccharide outer membrane in the Gram-negative ones [123].

The different localization of the peptidoglycan biosynthesis machinery has allowed distinguishing three phases in this process. Most drugs for clinical use target enzymes involved in Phase III, in which the cross-linking and final maturation of this biopolymer occurs [124]. In the so-called Phase I, nucleotide-activated precursors (UDP-N-acetylglucosamine and UDP-N-acetylmuramyl pentapeptide) are synthesized in the bacterial cytoplasm. During Phase II, which occurs in the inner half of the inner membrane, the precursors are assembled with undecaprenyl phosphate to form the lipid-anchored disaccharide-pentapeptide (Lipid II), which must be flipped across the inner membrane to polymerize with other disaccharide-peptide units [121, 124]. Most of the enzymes involved in Phases I and II have not been validated as therapeutic targets, perhaps except for glutamate racemase (coded by the MurI gene) for which several inhibitors have been developed.

Polyphenols can target multiple bacterial locations and structures. However, the bacterial cell wall seems to be the main molecular target for the antimicrobial action of most polyphenols. Gram-positive bacteria seem to be more susceptible to the antimicrobial action of phenolic compounds as the outer membrane of Gram-negative bacteria acts as a permeability barrier, reducing the uptake of the phenolic compounds [61].

Polyphenols can cause morphological damage to bacterial cells or destroy the structural integrity of the cell wall and intracellular matrix. Phenolic compounds may cause cell deformation, breakage of the cell wall, and membrane condensation of cellular material with the presence of cytoplasmic material and membrane

debris outside affected cells [125, 126]. Leakage is explained by the increase of the bacterial membrane and cell wall permeability caused by polyphenols [127, 128]. Some specific polyphenols such as ellagitannins, catechins or nor-lignans have demonstrated high affinity for bacterial membranes and great disruption capacity [49, 76, 129]. It is stated that catechins have a high affinity for bacterial membranes, specifically for the membranes of Gram-positive bacteria [88]. Other polyphenols such as the norlignans rooperol and hyposoxide have been proposed to perturb bacterial membranes enriched in negatively charged phospholipids (phosphatidylglycerol or cardiolipin), such as those of Gram-positive bacteria [76].

One potential mechanism involved in the degradation of the bacterial wall could be the inactivation by aggregation of certain essential surface proteins. Galloylated catechins seem to bind and cause the aggregation of at least 73 different proteins, including PBPs (penicillin-binding proteins), which are key in the wall formation, transporter proteins including ABC transporter (Oppa), PTS system transporter and phosphate ABC transporter and others [130]. It is also proposed that catechins can intimately interact with lipids in biological membranes, modifying their physical properties and causing membrane phase separations [129]. Moreover, this ability to interact and the eventual penetration of membranes may be potentially linked to apoptosis mechanisms and other cellular responses [131]. Studies demonstrated that ECG binds the MRSA cell membrane reducing its fluidity by penetrating deep into the hydrophobic region. To overcome these changes, the MRSA cell membrane undergoes molecular lipid transformations that affect peptidoglycan biosynthetic machinery, affecting also to the cooperation between PBP2 and PBP2a to overcome β-lactam antibiotics, decreasing the bacterial viability and antibiotic resistance [132].

Due to the discovery and usage of β-lactam antibiotics such as penicillin, which targets cell wall formation, bacteria developed β-lactamases to avoid the action of this antibiotic. Polyphenolic compounds have demonstrated interesting β-lactamase inhibitory properties. For instance, epicatechin, tannic acid, epigallocatechin gallate and quercetin showed significant β-lactamase inhibitory activity. Interestingly, the high antibacterial performance of tannic acid was predicted computationally based on the favorable docking assays between the polyphenol and TEM-1, a β-lactamase [133].

MRSA is a very important β-lactamase producer, conferring it with an annoying antibiotic resistance.

MRSA produces PBP2a (penicillin-binding protein 2A) which has a low affinity for β-lactam antibiotics enabling transpeptidase activity in the presence of β-lactams, preventing them from inhibiting cell wall synthesis. Nevertheless, some polyphenols such as kaempferol and quercetin have demonstrated high β-lactamase inhibition capacity and a great synergy with antibiotics like ciprofloxacin and rifampicin [134]. Natural polymeric proanthocyanidins strongly suppressed MRSA resistance to β-lactam antibiotics against MRSA and reduced cell membrane stability and β-lactamase activity at sub-MIC concentrations [135]. Other polyphenolic compounds such as epicatechin gallate, licoricidin, corilagin and tellimagrandin I have also demonstrated β-lactam antibiotic activity potentiation through PBP2a inhibition in MRSA. These findings could lead to alternative pharmaceutical treatments for resistant infections. Nonetheless, it is worth to notice that polyphenols bind to serum proteins and others, which limits their intravenous use. For this reason, polyphenolic therapy would be preferably indicated for skin, digestive tract and lung infections [136].

All of the mechanisms described above can modify the properties of bacterial membranes, facilitating other small polyphenols or antibiotics to enter the cytoplasm and cause metabolic damage [106].

5.1.1. Virtual Screening of Polyphenols on Bacterial Putative Protein Targets

If we consider that the development of a new antibiotic drug can take ten or more years and therefore is very costly economically, we can understand that the search for antimicrobial substances from products of natural origin, such as polyphenols, is gaining both scientist and medical interest. In this regard, it is necessary to clarify that the interest for phytochemical compounds does not completely lie in its use as an alternative therapy to conventional antibiotics since phytochemicals show inhibitory effects at concentrations of several orders of magnitude higher than antibiotics of current clinical use. However, there is abundant scientific evidence that adequate combinations of phytochemicals and antibiotics modify the mechanisms of resistance in pathogenic bacteria, playing a synergistic role (as we have shown earlier in this review) that allows reducing the dose of antibiotics.

In this context, we have considered opportune trying to look for possible polyphenolic phytochemicals that can modulate the activity of enzymes involved in Phases I and II of peptidoglycan biosynthesis. For this purpose, we have selected several enzymes: MraY,

MurA, MurB, MurC, MurD, MurE, MurF, MurG, and MurI (see Fig. **1** of the Supplementary Information available on the website http://dockingfiles.umh.es/bcwall/) and with them we have performed molecular docking experiments with the phenolic compounds stored in the base of data Phenol Explorer 3.6 [137].

Molecular docking calculations allow us to predict the structure of ligand-receptor complexes based on calculations that estimate the variation of Gibbs free energy (Kcal/mol) [138, 139] of the binding process of a given ligand (phenolic compounds in this case) to a known binding site of a protein of our interest. For the enzymes selected above, information on their structure is available at atomic resolution, and its catalytic site is known. Thus, we have carried out the screening of those phenolic compounds that could bind with high affinity to the catalytic site and, therefore, behave as competitive inhibitors of these enzymes.

Molecular docking calculations have been carried out with the Autodock/Vina software [140] executed in a high performance computing cluster under a Linux operating system belonging to the Research, Technological Innovation, and Supercomputing Center of Extremadura [CenitS] (http://www.cenits.en/cenits/ lusitania-II/lusitania-ii-specifications). For those enzymes without high-resolution structures available at Protein Data Bank (see Table **1** of the Supplementary Information) we have carried out a homology modeling [141] using the amino acid sequences found in the UniProt database (http://www.uniprot.org/). All this information about the amino acid sequences and the generated 3D models is also available at http:// dockingfiles.umh.es/bcwall/.

After carrying out the molecular docking experiments, we made the first selection of compounds that had a free energy variation (ΔG, Kcal/mol) threshold less than or equal to the corresponding value for the co-crystallized inhibitor in each type of enzyme. Fig. (**2**) shows an example of the molecular architecture of several enzymes involved in the biosynthesis of peptidoglycan with some of the polyphenols coupled in the catalytic center that have the highest affinity. The Gibbs free energy variation values of the 931 compounds stored in the Phenol-Explorer 3.6 database docked to the nine enzymes analyzed for the six selected bacterial species are available at http://dockingfiles.umh.es/bcwall/phenol/.

Panels **A)** and **B)** of Fig. (**3**) show the phenolic compounds with ΔG values less than or equal to the inhibitor compound co-crystallized with the enzyme

Table 1. Reported MIC values for selected polyphenols against different *S. aureus* strains.

Polyphenolic Agent	Class	Source	*S. aureus* Strain	MIC µg/mL	References
Davidiin	Ellagitannin	*Davidia involucrata*	OM481	64	[49]
			OM584	64	
			OM481	64	
			OM584	64	
3-O-galloylgranatin A	Gallotannin	Mango kernels	ATCC 6538	< 100	[52]
Hexa-O-galloylglucose					
Hepta-O-galloylglucose			ATCC 6538	< 100	
Octa-O-galloylglucose			ATCC 6538	< 100	
Nona-O-galloylglucose			ATCC 6538	< 100	
EGC	Catechin	Purchased from Sigma	ATCC 6538p	62.5	[55]
			Clinical isolate 1	62.5	
			Clinical isolate 2	125	
			Clinical isolate 3	125	
			Clinical isolate 4	62.5	
EGCG	Catechin	Purchased from Sigma	ATCC 6538p	125	
			Clinical isolate 1	62.5	
			Clinical isolate 2	62.5	
			Clinical isolate 3	62.5	
			Clinical isolate 4	62.5	
Punicalagin	Ellagitannin	Purchased from Phytolab	CECT59	42.1	[50]
		Pomegranate dried peels	BCRC 10781	12.5	[51]
Punicalin			BCRC 10781	12.5	
Quercetin	Flavonol	Purchased from Sigma	ATCC 6538	31	[65]
			Clinical isolate 8	3.9	
			Clinical isolate 14	3.9	
			Clinical isolate 26	125	
			Clinical isolate 32	1.95	
			Clinical isolate 319	3.9	
			Clinical isolate 550	7.8	
			MRSA ATCC 43300	125	[66]
			MSSA ATCC 29213	125	
			Clinical isolate SA1053	31.2	
Kaempferol			ATCC 6538	15.6	[65]
			Clinical isolate 8	15.6	
			Clinical isolate 14	15.6	

Polyphenolic Agent	Class	Source	*S. aureus* Strain	MIC μg/mL	References
			Clinical isolate 26	15.6	
			Clinical isolate 32	15.6	
			Clinical isolate 319	7.8	
			Clinical isolate 550	15.6	
Myricetin			CECT59	15.76	[50]
Quercetin-3-glucoside	Glycosylated flavonol	Purchased from Sigma	CECT59	14.37	
Quercetin-3-O-arabinofuranoside		Isolated from *Searsia chirindensis*	ATCC 12600	250	[67]
Kaempferol-3-O-rhamnoside			ATCC 12600	130	
Myricetin-3-O-rhamnoside			ATCC 12600	250	
Myricetin-3-O-arabinopyranoside			ATCC 12600	250	
Naringenin	Flavanone	Purchased from NICPBP	ATCC 29213	256	[68]
			ATCC 10832	256	
			BAA-1717	512	
			8325-4	256	
			DU 1090	256	
Luteolin	Flavone	Purchased from Sigma	MRSA ATCC 43300	125	[66]
			MSSA ATCC 29213	125	
			Clinical isolate SA0922	31.2	
Apigenin	Flavone	*Scutellaria barbata*	15 MRSA and 5 MSSA laboratory strains	62.5 - 125	[70]
2′-hydroxyerythrin A	Isoflavone	Soya beans	ATCC 26112	13.1	[73]
Isoerythrinin A			ATCC 26112	18.3	
Magnolol	Lignan	Purchased from XXST	ATCC 25923	32	[77]
			10 MRSA clinical isolates	8 - 64	
Honokiol			ATCC 25923	16	
			10 MRSA clinical isolates	16 - 32	
Hypoxoside		Provided by Monteloeder SL	CECT59	20	[76]
Rooperol			CECT59	800	
Melaleucin A	Neolignan	Isolated from *Mucuna bracteata*	ATCC 6538	8	[79]
			MRSA (JCSC4788)	8	
Melaleucin C			ATCC 6538	16	
			MRSA (JCSC4788)	16	
Resveratrol	Stilbene	Isolated from *Bacillus cereus*	MTCC 902	32	[83]

(Table 1) contd….

Polyphenolic Agent	Class	Source	*S. aureus* Strain	MIC µg/mL	References
		Purchased from Sigma	MRSA ATCC 43300	1000	[66]
			MSSA ATCC 29213	1000	
			Clinical isolate SA1056	250	
Pterostilbene		Purchased	ATCC 25923	25	[86]
Eupomatenoid-5	Neolignan	Extracted from *Piper regnellii*	MRSA (32 clinical strains)	1 - 8	[182]
			MSSA (32 clinical strains)	1 - 8	

Table 2. **Reported MIC values for selected botanical extracts against different *S. aureus* strains.**

Polyphenolic Agent	Extraction Type / SolvenT	Vegetal Source	*S. aureus* Strain	MIC µg/mL	References
Green tea extract	Water	*Camelia sinensis* leaves	ATCC 6538p	500	[55]
			Clinical isolate 1	250	
			Clinical isolate 2	1000	
			Clinical isolate 3	500	
			Clinical isolate 4	500	
Cistus populifolius extract	Water	Flowers and leaves	CECT 59	344	[47]
Cistus ladanifer extract			CECT 59	154	
	Hydroalcoholic		CECT 59	144	[48]
Cistus albidus extract	Water		CECT 59	60	
	Hydroalcoholic		CECT 59	292	
Cistus clusii extract	Water		CECT 59	91	
	Hydroalcoholic		CECT 59	304	
Cistus salviifolius extract	Water		CECT 59	50	
	Hydroalcoholic		CECT 59	45	

MraY. It should be noted that most of these compounds have been selected for *S. aureus* (121 compounds), while for the other species only 30 to 40 have been selected, except for *E. faecalis*, for which there are only 7 compounds. We can find several examples of compounds that show a high affinity for the catalytic site of the MraY enzyme for all six bacterial species, and that could, therefore, be considered as potential broad-spectrum inhibitors. This is the case of flavanols PE000133, PE000134, PE000136, PE000143, PE000149, PE000157, PE000161, PE000169, PE000170, PE000174, PE000189, PE000198, PE000322 and PE000450.

Several studies have shown the antibacterial activity of several theaflavins against *S. aureus* [142], *P.*

aeruginosa [143], *H. pylori* [144] or *E. faecalis* [145]. As we can see in (Fig. **3C**), only some polyphenols show high affinity for the catalytic site of the enzyme MurA for the six bacterial species studied, including several theaflavins PE000143, PE000149, and PE000151. Numerous compounds show high affinity to the binding site studied, especially for the enzymes of the species *A. baumannii* and *P. aeruginosa*, and with ΔG values lower than -12 Kcal/mol. Likewise, several proanthocyanidins show high affinity for MurA, as is the case of PE000150, PE000151, PE000152, PE000153, PE000154 and PE000155. Except for the case of *H. pylori*, numerous compounds show high affinity against the catalytic site of the MurB enzyme (Fig. **3D**), with values lower than -11 Kcal/mol.
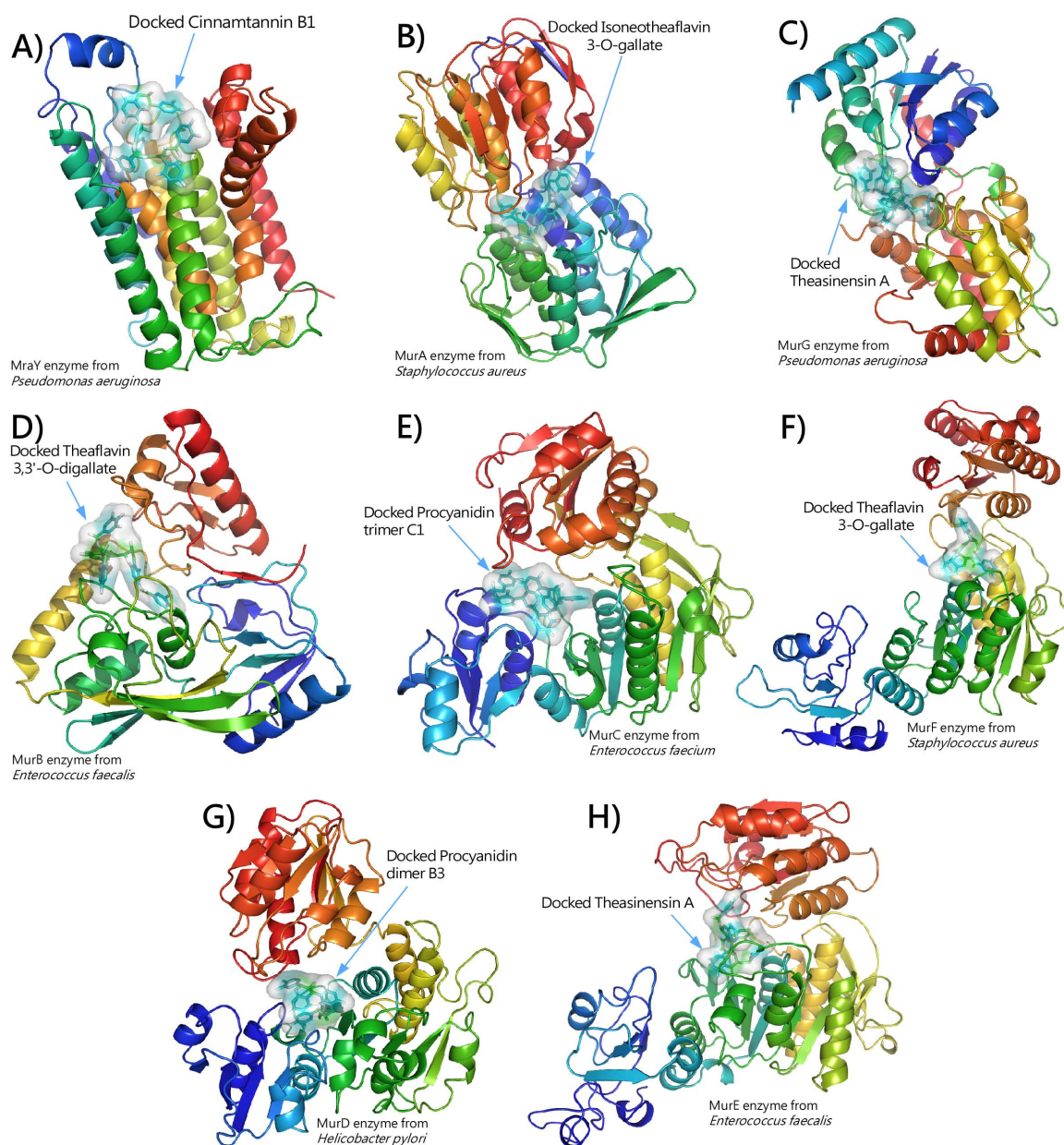
Fig. (2). Secondary structure model of eight enzymes involved in Phases I and II of peptidoglycan biosynthesis with some of the phenolic compounds resulting from molecular docking experiments at the catalytic site of the enzyme. Each panel includes the name of the enzyme, the bacterial species, and the docked compound.

Among these compounds are several theaflavins again. Also, theaflavins (PE000134, PE000136, PE000143) and proanthocyanidins (PE000157, PE000161, PE0174) show high affinity for the catalytic site of the MurC enzyme (Fig. **3E**). In the case of the MurD enzyme (Fig. **3F**), in addition to theaflavins and proanthocyanidins, the high affinity shown by several epicatechins is noteworthy (PE000193, PE000194 and PE000175). Against the catalytic site of MurE enzyme (Fig. **3G** and **3H**) abundant phenolic compounds show lower ΔG than the crystallographic ligands (NADP$^+$

and FAD), especially for the species *E. faecium*, *E. faecalis* and *Acinetobacter baumannii*; although these compounds are usually different in the three species. In the same way as against MurD, also proanthocyanidins (PE000174, PE000176, PE000188 and PE000189) and epicatechins (PE000193, PE000194) show a high affinity towards the catalytic site of Mur E of all six analyzed species. Theaflavins (PE000133, PE000134, PE000136, PE000143, PE000149) and proanthocyanidins (PE000157, PE000174, PE000189, PE000198) also show high affinity against MurF enzyme for several of

the bacterial species analyzed (Fig. **3I**). Up to 90 phenolic compounds show high affinity compared to the crystallographic ligand against the catalytic site of the MurG enzyme (Fig. **3J** and **3K**) of *P. aeruginosa*. As compared to other enzymes, proanthocyanidins are remarkable for their affinity to this enzyme in most of the selected species.

As we can see in (Fig. **2**), the enzymes MurC, MurD, MurE and MurF show an identical pattern of secondary structure; probably this explains why several phenolic compounds show similar affinity to the four enzymes. Similarly, the MurA and MurG enzymes also have high similarity in their secondary structure (Fig. **2B** and **2C**, respectively).

Finally, we analyzed the data of the molecular docking of polyphenols against the catalytic and regulatory site of the glutamate racemase (MurI); an amino acid racemase that has been widely studied as a pharmacological target (Fig. **4**), in whose active center there are two thiol groups. This enzyme shows a different quaternary structure in *H. pylori* (head-head dimer, Fig. **4A**) of the remaining species analyzed in this study, which forms a tail-tail dimer (Fig. **4B**). This enzyme, in addition to catalyzing the conversion of L-Glu to D-Glu, has been shown to be a potent inhibitor of DNA gyrase [146]. Both glutamate analogs [147, 148] and allosteric inhibitors with affinity in the nanomolar range against *H. pylori* have been designed against this enzyme [149-151]. The ΔG values calculated from molecular docking data of different polyphenols against the catalytic and allosteric sites of the glutamate racemase are shown in (Fig. **4C** and **4D**), and we can observe that many polyphenols show better affinity than the reference inhibitor compounds, especially against the *E. faecium* enzyme (Fig. **4C**). It must be highlighted the high affinity of various catechins (PE000780, PE000786, PE000787, PE000788 and PE000789) against the glutamate racemase of *E. faecium*, *E. faecalis* and *A. baumannii*. Again, certain theaflavins (PE000143, PE000144, PE000149) have a high affinity for the catalytic site of this enzyme. In the case of the *H. pylori* racemase there are also numerous phenolic compounds that show lower ΔG than the experimentally tested inhibitors. Such is the case of the daidzeins (PE000857, PE000859) and the lithospermic (PE001041) and salvianolic acid (PE001044).

5.2. Other Bacterial Protein Targets of Polyphenols

Besides their capability of forming non-covalent multiple hydrogen bonds, hydrophobic interactions and van der Waals attractions with proteins and other molecules, polyphenols can also bind covalently to proteins. The sulfhydryl groups of cysteine and the ε-amino groups of lysine, as well as α-terminal amino groups, appear to combine most readily with quinones derived from polyphenols [152]. Possible targets for polyphenols are cell surface adhesion proteins, membrane-bound enzymes, and cell wall polypeptides [61].

PBPs are critical molecular targets for antibiotics. These proteins are part of the peptidoglycan synthesis machinery, key in the cell wall formation [153]. Changes in these proteins allow bacteria to avoid antibiotic effects, *e.g.*, PBP2a in *S. aureus* that confers resistance to methicillin, penicillin and other penicillin-like antibiotics [154]. Some polyphenols such as flavonoids and tannins have been proposed to form non-specific interaction with PBPs (including PBP2a) eventually leading to MRSA growth inhibition [114].

The lignan 3′-demethoxy-6-O-demethylisoguaiacin from *Larrea tridentate* has been shown to interact with the cellular membrane where this polyphenol represses the activity of some proteins of the ABC transport system of MRSA. As a consequence, the bacteria could not release the phytochemical causing bacteria death [155]. This is proposed as a novel target mechanism for the development of novel antibacterial agents. Other related target protein is the oligopeptide ABC transporter binding lipoprotein (Oppa), a component of the oligopeptide permease that capture peptides ranging in size from 2 to 18 amino acids from the environment and pass them on to the other components of the oligopeptide transport system for internalization [156]. It has been reported that the galloylated catechin EGCG binds Oppa at the bacterial inner wall and inhibits its function in *B. subtilis*. EGCG strongly binds Oppa in its open conformation and prevents it from changing to closed conformation [130].

5.3. Gene Expression Regulation

Certain polyphenols are able to modify gene expression leading to major metabolic changes in resistant bacteria. Although the mechanism of this activity is unknown, it may be presumed that the multiple targets reached by polyphenols may indirectly modulate the activity of transcription factors that result in gene expression regulation. Nevertheless, either direct interaction with DNA or epigenetic regulation by polyphenols through the modulation of the activity of DNA methyltransferases cannot be discarded [157].

The lignan magnolol has shown the ability to significantly reduce the expression of the antibiotic resistance genes *mecA*, *mecI*, *femA* and *femB* in mRNA

Best docked polyphenols to MraY enzyme

Best docked polyphenols to MraY enzyme

Best docked polyphenols to MurA enzyme

Best docked polyphenols to MurB enzyme

Best docked polyphenols to MurC enzyme

Best docked polyphenols to MurD enzyme
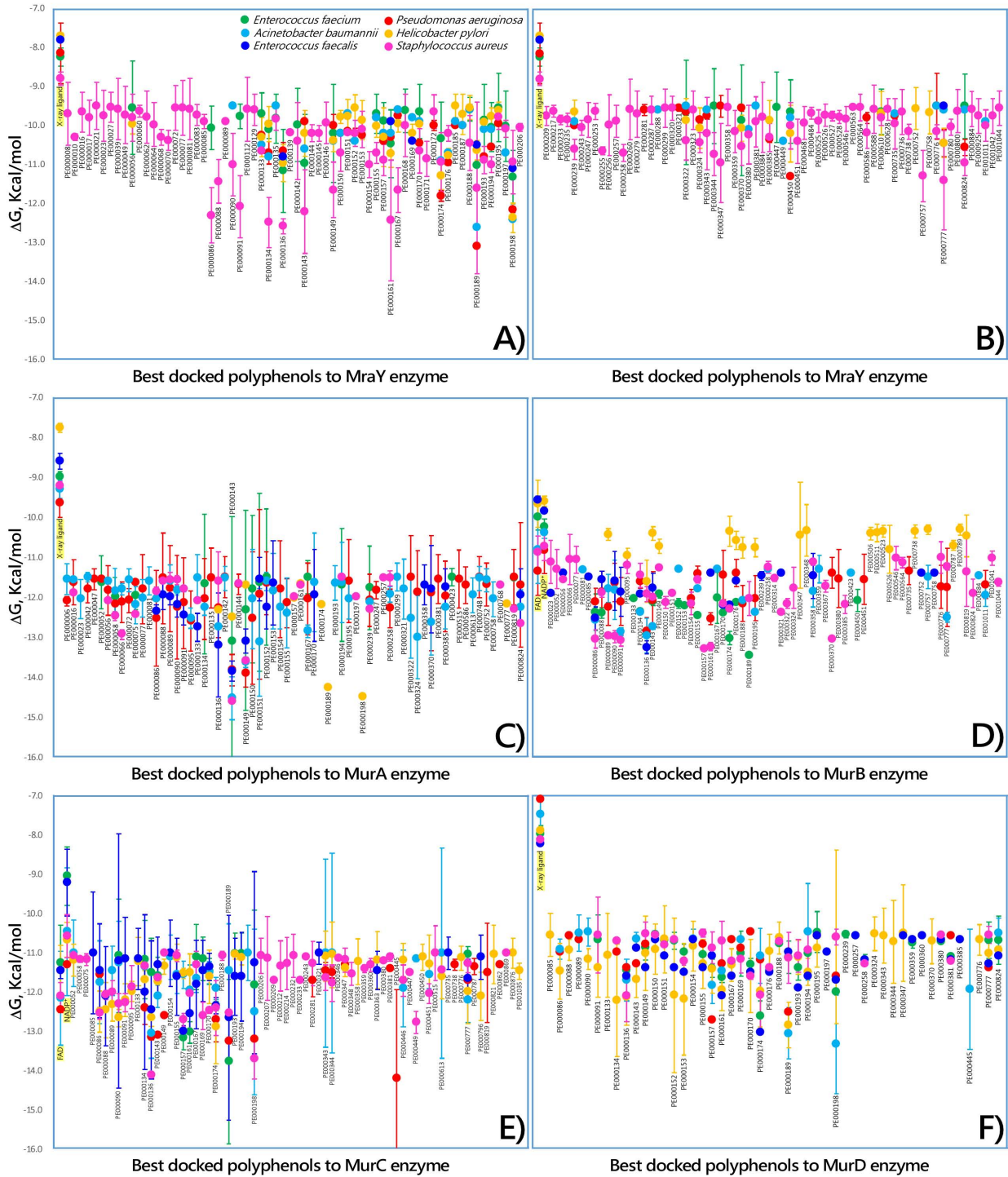
(Fig. 3) contd….
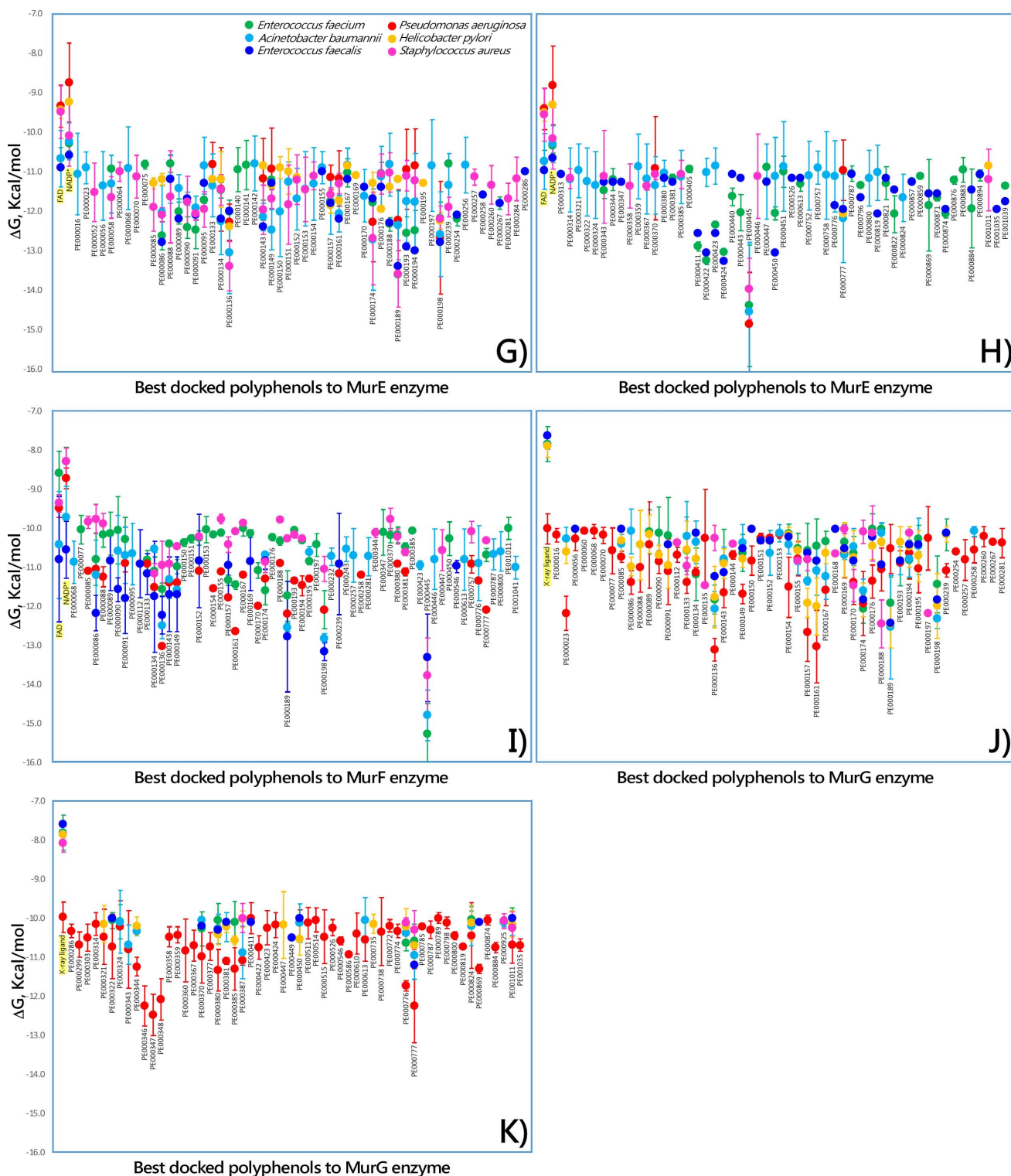
Fig. (3). Comparison of the free energy variation of the selected Phenol-Explorer database 3.6 polyphenols with ΔG less than or equal to the corresponding value for the crystallographic ligands for each bacterial species. In each panel, the enzyme is indicated, below each calculated value the name of each phenolic compound is included with the terminology used by the Phenol-Explorer 3.6 database. The color code is the same in all panels: green, *Enterococcus faecium*; red, *Pseudomonas aeruginosa*; light blue, *Acinetobacter baumannii*; orange, *Helicobacter pylori*; dark blue, *Enterococcus faecalis*, and pink, *Staphylococcus aureus*.
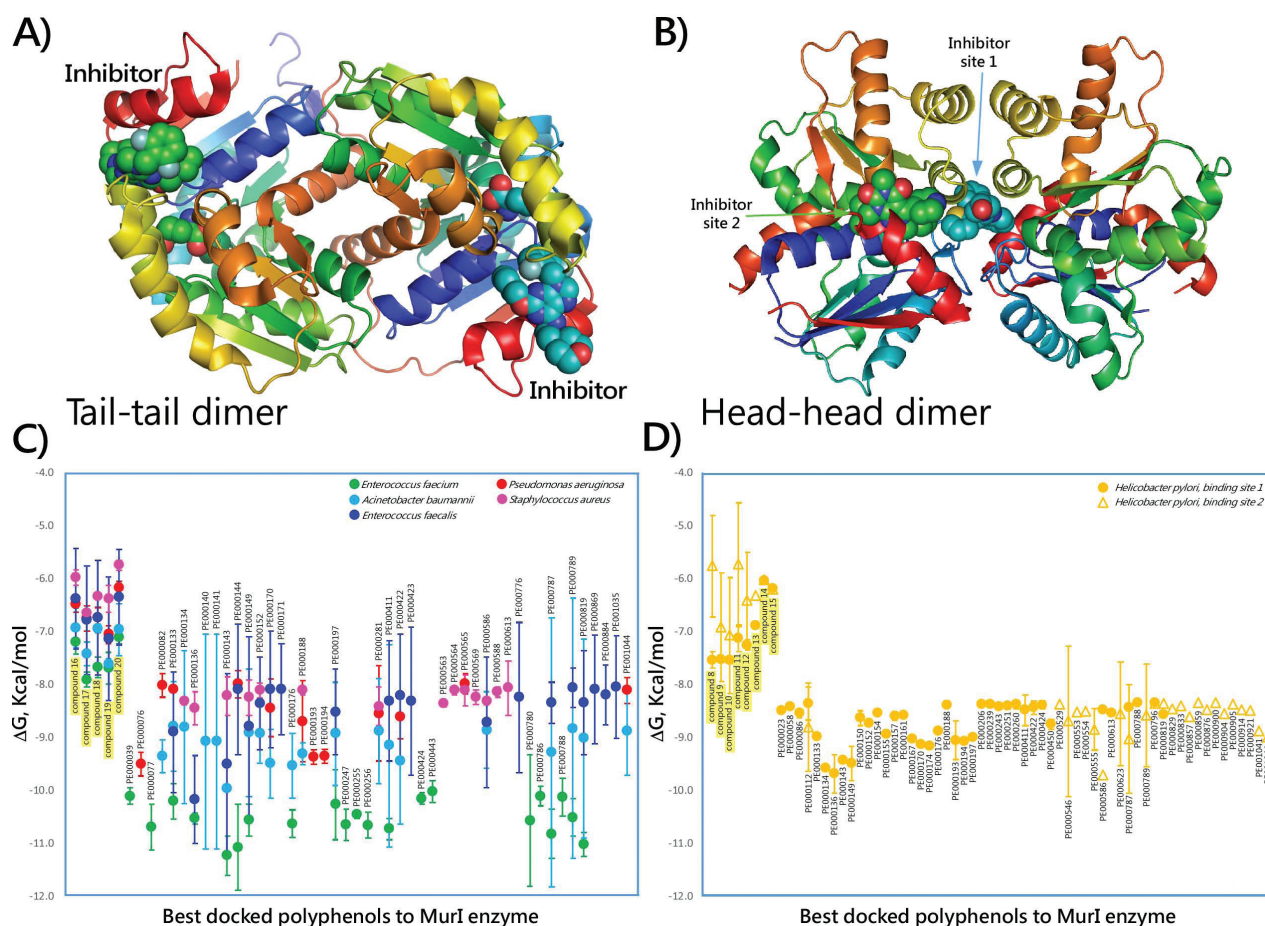
Fig. (4). Quaternary structure of the glutamate racemase with tail-tail dimers for *E. faecalis* (Panel **A**) and head-head dimers for *H. pylori* (Panel **B**). Comparison of calculated free energy variation (Panels **C** and **D**) of phenolic compounds with some known compounds inhibitors (yellow name).

[111]. Other lignan, 3′-demethoxy-6-O-demethylis-oguaiacin have been proven to modulate MRSA (ATCC BAA-44) genetic expression, affecting more than 200 genes. From this pull, it downregulated 6 key genes involved in antibiotic resistance making MRSA unable to pump out antibiotic molecules [155].

Galloyl catechins, especially (-)-epicatechin gallate (ECg), are able to abrogate beta-lactam resistance in MRSA and prevent biofilm formation with profound changes in cell morphology. ECg binds to the bacterial membrane eliciting major alteration in the structure and the thermotropic behavior of the bilayer. All these changes induce the up-regulation of genes responsible for protection against cell wall stress and maintenance of membrane integrity and function and reverse the MRSA resistant phenotype [158]. The antibacterial activity of Caffeic Acid Phenethyl Ester (CAPE) against *E. faecalis*, *L. monocytogenes*, and *S. aureus* has been also related to its capacity to target RNA and DNA related molecules [159].

The flavonoid naringenin can bind A-T base pairs regions of the DNA of *S. aureus* (ATCC 6538) *via* groove mode, provoking changes on its molecular conformation and altering its secondary structure [128]. Certain flavonoids such as quercetin, dihydrorobinetin and Epigallocatechin (EGC) inhibited RNA synthesis in *S. aureus* (FDA 209 PJC-1). These activities are explained because of the structure of the flavonoid 3',4',5'-trihydroxy B-ring coupled with the 3-OH may interrupt the intercalation or hydrogen bonding with the stacking of nucleic acid bases [160]. This fact was corroborated later in structure-activity relationship studies by the finding that the presence of at least one hydroxyl group in rings A or B in flavonoids at C-3,5,7 was crucial for their antibacterial activity. Compounds without hydroxyl groups in ring B (pinocembrin, chrysin, galangin) or compounds in which the hydroxyl group was replaced with a methoxy group (kaempferide, tamarixetin) turned out to be inactive against MRSA and VRE [161].

Last, it is worth to mention that is possible to enhance the antimicrobial properties of plant extracts using molecular genetics technology. It has been reported that plants overexpressing the γ-tocopherol methyltransferase gene (γ-tmt) showed larger concentrations of polyphenolic compounds (phenolic acids and flavonoids) leading to an increased antimicrobial activity against *B. subtilis* (KCTC 3728) [162].

5.4. Biofilm Formation

Most of the bacteria live as biofilms in their natural habitats, so this feature is crucial for their survival. Furthermore, most of the staphylococcal diseases are related to biofilm formation. In this regard, many polyphenols have demonstrated to have anti-biofilm properties against *S. aureus* [163], even when biofilms can be much more resistant to antimicrobial than planktonic cells [164].

Biofilm producing Gram-positive bacteria usually cause Urinary-Tract Infections (UTIs) in people with high-risk factors, such as elderly or pregnant. Even if some UTIs are polymicrobial, the main bacteria isolated in these cases are *Staphylococcus saprophyticus*, *E. faecalis*, and *Streptococcus agalactiae* [165]. To treat UTIs is essential that the antimicrobial agents penetrate biofilms and cellular membranes to act into the infecting cells. For these reasons, polyphenols and plant extracts with antimicrobial and antibiofilm capacity can be effective tools for improving medical treatments against UTIs.

Recent studies point out that many polyphenols can inhibit the formation of streptococcal biofilms. This capacity is important for preventing human pharyngitis, which is caused by well-organized attached bacterial biofilms. After the polyphenol application, the target *Streptococcus pyogenes* bacteria appeared less aggregated and showed morphological changes when observed by scanning electron microscope. The generation of Reactive Oxygen Species (ROS) by polyphenols has been proposed as the underlying mechanism that affects cell wall integrity, adhesion molecules and quorum sensitivity [166].

The choice of delivery system is essential for the polyphenols in order to exert their antibacterial and anti-biofilm activity. For example, curcumin is known to have high antibacterial and anti-quorum sensing activity, but its use is very limited because of its poor aqueous solubility and quick degradation. To overcome these problems, researchers have created curcumin quantum dots using acetone as a solvent, which are hundreds of times smaller than regular curcumin particles. Consequently, they significantly improved its durability and efficacy. These particles show better penetration and interaction with cells and biofilm matrix, resulting in increased uptake by the bacteria [167].

5.5. Bacterial Metabolites, Proton and Ion Equilibrium

Polyphenols can also exert their antibacterial activity by modulating the level of some essential metabolites or by impairing ionic strength equilibrium or proton gradient leading to cell death. Nevertheless, most of these effects may derive from the capability of polyphenols to interact with phospholipid membranes or directly with the receptors and ion pumps on the cell membrane. For example, the inhibitory effect of the gallotannins on bacterial growth is in part associated with its role as iron chelators with high affinity, besides their capacity to inactivate membrane proteins of bacterial cells [52].

A synergistic effect in a combination of oregano and cranberry extracts has been proposed against *Helicobacter pylori*, exerting a stronger antibacterial capacity compared to the isolated extracts. The authors postulate that, in a first stage, some polyphenols of the combination damage cell membrane or affect ion pumps, making cells more sensitive to other compounds [168]. As a consequence, hyperacidification takes place at the bacterial plasma membrane interface and cytosol, due to proton donation of acidic polyphenols and cell membrane disruption, inhibiting ATP synthesis. Moreover, it is stated that soluble polyphenols can also quench free electrons from the electron transport chain at the bacterial membrane. All these effects could reduce cytochrome activity and thus the oxidative phosphorylation, inhibiting bacterial growth. Some evidences point out that these effects may be mediated through the modulation of proline dehydrogenase by polyphenols at the plasma membrane. Polyphenolic moieties may mimic proline structure and can cause enzyme inhibition, which can be reverted by proline. This mechanism is proposed for the antibacterial activity against *S. aureus* of small soluble polyphenols such as gallic or caffeic acid [106, 169].

Researchers have found that oolong tea extract (semifermented tea leaves of *Camellia sinensis*) and isolated polymeric catechins have an inhibitory action on the insoluble glucan synthesis from sucrose by the Glucosyltransferases (GTFs) of *Streptococcus mutans* (MT8148R) and *Streptococcus sobrinus* (6715). This activity leads to inhibition of sucrose-dependent cell adherence of these bacteria, avoiding caries formation
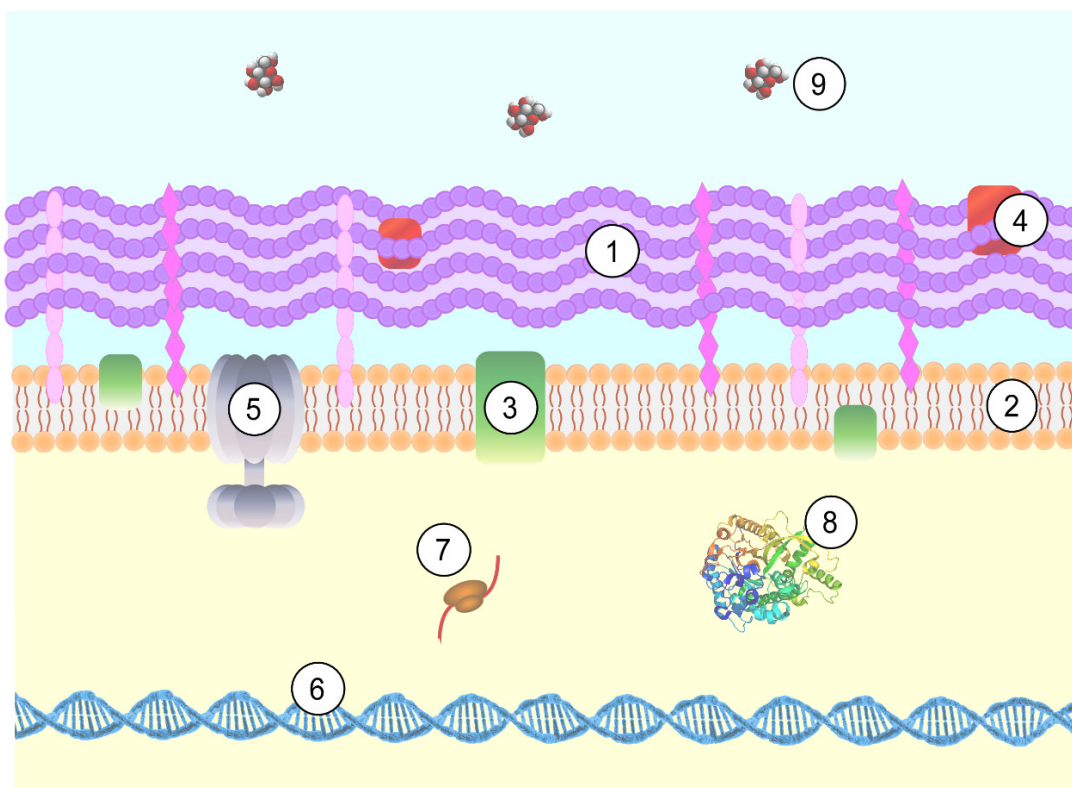
Fig. (5). Scheme of a bacteria and the putative molecular target of polyphenols. 1: Cell wall, 2: Cell membrane, 3: Membrane proteins, 4: Cell wall proteins, 5: ATPase, 6: DNA, 7: RNA related molecules, 8: Cytoplasmic proteins, 9: Soluble nutrients and ions.

in animal models [170]. Some polyphenols from cranberry have also shown GTFs inhibitory capacity against *S. mutans* (UA159), such as quercetin-3-arabinofuranoside, myricetin and procyanidin A2. Furthermore, these polyphenols reduced biofilm formation and surface adsorption by inhibiting F-ATPases and acid production [171, 172]. Catechins have also demonstrated to neutralize staphylococcal enterotoxin B (SEB), a superantigen that aggravates atopic dermatitis [173].

Other flavonoids such as rutin, naringenin, quercetin and sophoraflavanone G can affect the fluidity of both internal and external bacterial membranes. This activity results in a membrane potential nullification decreased ATP production and cell motility [162, 174, 175]. Polymeric tannins are also capable of making complexes with certain nutrients and minerals rendering them unavailable for bacteria to intake, and affecting their metabolism [176].

5.6. Resistance to Natural Antimicrobials

To date, literature about bacteria acquiring resistance to botanicals is limited. One example is a study that relates genetic changes (deletion of *sigB* gene) in

L. monocytogenes with enhanced resistance to carvacrol [177]. It may be possible for bacteria to develop resistance against specific polyphenols with a specific molecular target involved. However, it seems less likely to develop resistance when complex mixtures of polyphenols that affect several molecular targets at the bacterial cell are utilized [178, 179]. Since plant extracts are a complex mixture of numerous phytoactive components, development of bacterial resistance to such synergistic combinations may be much slower than those for single chemical compounds.

A "tannin-resistant" Gram-positive bacteria (*Streptococcus sp.*) has been identified in places with high exposure to this kind of polyphenols, such as goat, sheep and deer rumens [180]. This kind of bacteria is supposed to protect ruminants from anti-nutritional effects. The proposed mechanisms by which bacteria can overcome growth inhibition by tannins include modification of the substrate, dissociation of tannin-substrate complexes, extracellular polysaccharide formation, cell membrane modifications and metal ion chelation [181]. It is worth to highlight that bacteria, which are predominant in tannin-rich mediums of the gastrointestinal tract of ruminants, may not be resistant *per se*. Likely,

this resistance may be more related to higher nutrient accessibility of the bacteria in the particular microenvironment of the ruminant stomach [176].

CONCLUSION AND PERSPECTIVES

Resistance to antibiotics has now become a public health problem worldwide. Drug-resistant infections kill around 700,000 people worldwide each year, and this figure could increase to several million by 2050, according to experts. If at the individual level it causes loss of human lives, at a collective level it can lead to the collapse of public health systems, since it involves high-cost therapies (if they exist) due to the more extended hospitalization of the patients compared to treatments of non-resistant strains. Poor sanitary conditions together with deficient diet in developing countries and the indiscriminate and inappropriate use of beta-lactam antibiotics (both for human and veterinary use) in the developed countries make it possible to understand the emergence of resistance phenomena to antimicrobial drugs.

Although research is continuously increasing our knowledge and adding new therapeutic alternatives, no new effective antibiotics against resistant strains have been developed in nearly 30 years. Only five of top fifty pharmaceutical companies are developing new antibiotics, and only a few projects for drug discovery are based on antibiotics development among more than five hundred. In this context of peremptory need of new treatments and therapeutic solutions, natural compounds have been underestimated since pharma companies are mostly focused on more profitable synthetic compounds. Evolution has selected these natural compounds along millennials providing them with molecular promiscuity and polypharmacological properties.

Among plant compounds, polyphenols are probably the most important family of natural compounds, both in number and relevance. Throughout this review, we have highlighted a number of polyphenolic compounds (phenolic acids, flavonoids, tannins, lignans, stilbenes and combinations of these in botanical mixtures) that have exhibited significant antibacterial activity against resistant and non-resistant Gram-positive bacteria at low microg/mL range MIC values. Interestingly, the synergic interaction of some of these polyphenols with selected antibiotics that allows diminishing resistance to the antibiotic deserves further research.

The mechanism of action of the antibacterial capacity of polyphenols is quite diverse in agreement to their multitargeted character. Bacterial membrane and cell wall seem to be one of the main targets of polyphenols.

Some polyphenols have exhibited the capacity to interact or even integrate into the phospholipid bilayer causing membrane disruption or lipid phase separation affecting the activity of several protein receptors and channels and cell wall assembly machinery. Alternatively, some of these small molecules can interact directly with proton or ion pumps causing the impairment of membrane-related processes such as proton gradient, ATP synthesis or oxidative phosphorylation leading to bacteria cell death. Alternatively, polyphenols may interact with bacteria nucleic acids either directly or through epigenetic regulation leading to compromised bacteria cell viability. Some polyphenols have also exhibited the capacity to affect cell wall integrity and/or adhesion molecules that are essential for microbial surface colonization and biofilm formation.

Other putative molecular targets for polyphenols may be those proteins involved in the peptidoglycan biosynthesis such as PBP, Mra or Mur protein families. To this respect, the results of virtual screening techniques for the docking of the 931 compounds of the Phenol-Explorer 3.6 database against nine enzymes for six selected bacterial species are fully available. Among all the compounds tested, some theaflavins, proanthocyanidins, and catechins showed promising results that may deserve further attention. This is just an example of the power of *in silico* drug screening, as a complementary technique, to accelerate the discovery of novel antibiotics.

To date, just a few *in vivo* experiments using polyphenols as antibiotics have been clinically relevant, thus much work needs to be done. One of the major tasks will be to find the right polyphenolic combinations or combinations polyphenol-antibiotic that enable to reduce resistance in resistant strains. For that purpose, a pharmacological approach will be required in order to look for synergistic therapeutic effects.

Bioavailability, administration route, delivery and galenic formulation are still significant issues. Due to the metabolism of polyphenols both in the gastrointestinal tract and in the liver, polyphenolic antibacterial therapy would be preferably indicated for skin, digestive tract and lung infections. Anyway, based on the existing evidence, plant polyphenols suppose a promising source of antibacterial agents, either alone or in combination with existing antibiotics, for the development of new antibiotic therapies.

LIST OF ABBREVIATIONS

µg/mL	= Micrograms Per Milliliter
ABC	= ATP-Binding Cassette

ATCC	=	American Type Culture Collection
ATP	=	Adenosine Triphosphate
B. cereus	=	*Bacillus cereus*
B. subtilis	=	*Bacillus subtilis*
C. albidus	=	*Cistus albidus*
C. clusii	=	*Cistus clusii*
C. ladanifer	=	*Cistus ladanifer*
C. salviifolius	=	*Cistus salviifolius*
CECT	=	Colección Española de Cultivos Tipo
CFU	=	Colony-Forming Unit
DNA	=	Deoxyribonucleic Acid
E. faecalis	=	*Enterococcus faecalis*
E. faecium	=	*Enterococcus faecium*
EGC	=	Epigallocatechin
EGCG	=	Epigallocatechin Gallate
FICI	=	Fractional Inhibitory Concentration Index
GTFs	=	Glucosyltransferases
KCCM	=	Korean Culture Center of Microorganisms
KCTC	=	Korean Collection for Type Cultures
L. monocytogenes	=	*Listeria monocytogenes*
LPS	=	Lipopolysaccharide
MDR	=	Multi-drug Resistance
MIC	=	Minimum Inhibitory Concentration
MRSA	=	Methicillin-Resistant *Staphylococcus aureus*
MSSA	=	Methicillin-Sensitive *Staphylococcus aureus*
MTTC	=	Microbial Type Culture Collection
NCTC	=	National Collection of Type Cultures
PBP	=	Penicillin-Binding Protein
PTS	=	Phosphotransferase System
RNA	=	Ribonucleic Acid
ROS	=	Reactive Oxygen Species
S. aureus	=	*Staphylococcus aureus*
VRE	=	Vancomycin-Resistant *Enterococcus*

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Functional and structural information of selected enzymes involved in the peptidoglycan biosynthesis (Phase I and II) of the cell wall of some medically relevant bacteria available at http://dockingfiles.umh.es/bcwall/.

The Gibbs free energy variation values of the 931 compounds stored in the Phenol-Explorer 3.6 database docked to the nine enzymes analyzed for the six selected bacterial species are available at http://dockingfiles.umh.es/bcwall/phenol/.

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CAPÍTULO 2

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Título: The antimicrobial capacity of *Cistus salviifolius* and *Punica granatum* plant extracts against clinical pathogens is related to their polyphenolic composition.

Autores: Francisco Javier Álvarez-Martínez, Juan Carlos Rodríguez, Fernando Borrás-Rocher, Enrique Barrajón-Catalán, y Vicente Micol.

4.2. RESUMEN DE LOS RESULTADOS

Los microorganismos multirresistentes a fármacos representan una amenaza para la salud humana a escala mundial. Los antibióticos están perdiendo eficacia debido a muchos factores, como su uso excesivo, escaso control de las terapias y la escasez en la producción de nuevos antibióticos por la industria farmacéutica. Por estas razones y dada la actividad demostrada por algunos compuestos naturales, los extractos vegetales y los compuestos polifenólicos que los componen y que poseen capacidad antimicrobiana, se presentan como posibles alternativas o complementos a las terapias antibióticas tradicionales.

En el presente trabajo, diferentes extractos vegetales y compuestos puros se cribaron en una primera etapa usando el método antimicrobiano Kirby-Bauer de difusión en disco-placa frente a diversos aislados clínicos de especies bacterias patógenas de relevancia clínica. Los dos mejores agentes antimicrobianos, codificados como CS (extracto de *C. salviifolius*) y PG (extracto de *P. granatum*) fueron seleccionados para una posterior fase de ensayos antimicrobianos por el método de microdilución en placa multipocillo. Su composición molecular se caracterizó completamente usando cromatografía líquida de alta resolución acoplada de espectrometría de masas (HPLC-MS). Finalmente, se determinó la capacidad antimicrobiana de cada extracto frente a 100 aislados bacterianos diferentes de *S. aureus* (50 SARM y 50 SASM) y se correlacionó con el perfil de resistencia a antibióticos de cada aislado bacteriano.

El extracto CS demostró una mayor actividad frente a los aislados de SARM (CMI_{50} de 51,21 μg/mL) que frente a SASM (CMI_{50} de 80,70 μg/mL), mientras que el extracto de PG fue más efectivo frente a los aislados de SASM (CMI_{50} de 51,67 μg/mL) que frente a SARM (CMI_{50} de 72,89 μg/mL). La actividad de los extractos frente a *S. aureus* varió según el perfil clínico de resistencia a antibióticos de cada aislado. Se observó una mayor actividad antimicrobiana del extracto CS contra bacterias resistentes a los antibióticos betalactámicos. Por otro lado, la actividad del extracto PG fue mayor contra bacterias sensibles a oxacilina y a antibióticos del grupo quinolonas. Los componentes polifenólicos mayoritarios de ambos extractos fueron los taninos hidrolizables. Sin embargo, el extracto CS también contó con la presencia de diversos flavonoides no presentes en el extracto PG.

Ambos extractos demostraron actividad antimicrobiana contra las bacterias probadas. Los resultados apuntan a que es necesario realizar más estudios sobre la posible relación entre

resistencia a antibióticos clínicos y sensibilidad a extractos vegetales, ya que estos agentes podrían modular los mecanismos de resistencia o debilitar las bacterias expuestas para volverlas vulnerables nuevamente.

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The antimicrobial capacity of *Cistus salviifolius* and *Punica granatum* plant extracts against clinical pathogens is related to their polyphenolic composition

Francisco Javier Álvarez-Martínez[1], Juan Carlos Rodríguez[2], Fernando Borrás-Rocher[3], Enrique Barrajón-Catalán[1,5✉] & Vicente Micol[1,4,5]

Antimicrobial resistance poses a serious threat to human health worldwide. Plant compounds may help to overcome antibiotic resistance due to their potential resistance modifying capacity. Several botanical extracts and pure polyphenolic compounds were screened against a panel of eleven bacterial isolates with clinical relevance. The two best performing agents, *Cistus salviifolius* (CS) and *Punica granatum* (GP) extracts, were tested against 100 *Staphylococcus aureus* clinical isolates, which resulted in average MIC$_{50}$ values ranging between 50–80 µg/mL. CS extract, containing hydrolyzable tannins and flavonoids such as myricetin and quercetin derivatives, demonstrated higher activity against methicillin-resistant *S. aureus* isolates. GP extract, which contained mostly hydrolyzable tannins, such as punicalin and punicalagin, was more effective against methicillin-sensitive *S. aureus* isolates. Generalized linear model regression and multiple correspondence statistical analysis revealed a correlation between a higher susceptibility to CS extract with bacterial resistance to beta-lactam antibiotics and quinolones. On the contrary, susceptibility to GP extract was related with bacteria sensitive to quinolones and oxacillin. Bacterial susceptibility to GP and CS extracts was linked to a resistance profile based on cell wall disruption mechanism. In conclusion, a differential antibacterial activity against *S. aureus* isolates was observed depending on antibiotic resistance profile of isolates and extract polyphenolic composition, which may lead to development of combinatorial therapies including antibiotics and botanical extracts.

The increasing number of multidrug-resistant microorganisms represents a serious threat to human health worldwide, and unless drastic measures are taken, this number will continue to increase. There is an imminent risk of having very few therapeutic alternatives in the management of some serious infectious processes. The prognosis for the year 2050 is 10 million deaths each year and a global economic cost of 86 trillion dollars derived from antibiotic resistance[1]. Moreover, few new antibiotics are being discovered by the scientific community at present and in recent years. Pharmaceutical companies are uncertain when investing in the development of new antibiotics due to the possibility of rapid bacterial resistance development, resulting in an inability to recover their investment[2]. This concerning trend can be observed in Fig. 1.

As traditional drug therapies are losing efficacy, novel therapies based on natural antimicrobial compounds are emerging as alternative or complementary treatments against nosocomial infections. Natural combinations such as plant extracts containing a wide range of different molecules, including polyphenols, have demonstrated antimicrobial activity. These combinations can act against many different bacterial molecular targets, sometimes

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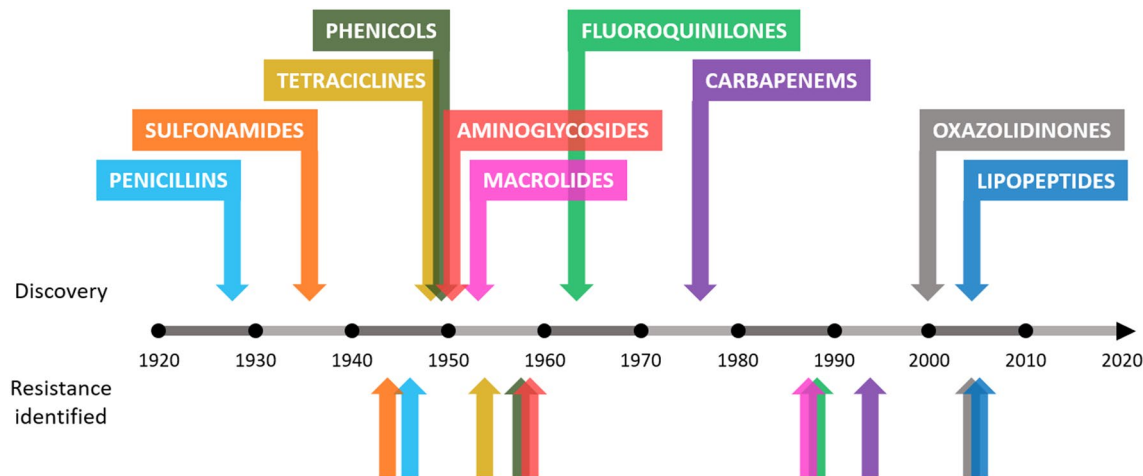
Figure 1. Approximate dates of discovery of new classes of antibiotics and identification of bacterial resistance.

potentially avoiding common antibiotic resistance mechanisms[3]. There is evidence of polyphenols and plant extracts capable of disrupting the bacterial plasma membrane, inhibiting efflux pumps, inhibiting the formation of biofilms and inhibiting the action of proteins related to antimicrobial resistance such as PBP2a[4].

Polyphenols are compounds with large structural variability but common phenolic moieties in their structure (Supplementary Fig. S1). In addition, polyphenols usually appear as conjugated forms with carbohydrates or form esters with organic acids, contributing to a considerable increase in their chemical diversity. Polyphenolic compounds have modulated their diversity throughout evolution to act as ligands of many different molecular targets, generating high molecular promiscuity[5]. This multitarget trait is key in the antimicrobial capacity of plant polyphenols and in their synergistic effects with traditional antibiotics[6]. Examples of polyphenols with demonstrated antimicrobial capacity are the flavonols quercetin and kaempferol, with reported MIC values as low as 1.95 µg/mL and 7.8 µg/mL respectively, against *S. aureus*. Botanical extracts rich in polyphenols have also been shown to possess significant antibacterial capacity. For instance, extracts obtained from *Cistus ladanifer*, *Cistus albidus*, *Cistus clusii* and *Cistus salviifolius* have shown MIC values under 100 µg/mL against *S. aureus*[3]. The chemical composition of botanical extracts derived from different *Cistus* species and that of *P. granatum* have been fully reviewed in the past[7,8]. Moreover, the relationship between the antimicrobial activity against *S. aureus* and *Escherichia coli* and the presence in these extracts of hydrolyzable tannins and flavonoids has been previously reported[9–11].

The objective of the present work was to test the antimicrobial capacity of complex botanical extracts, such as *P. granatum* and *C. salviifolius* with previously reported antimicrobial capacity, and some selected pure polyphenolic compounds to make a selection of the best performing ones for further resistant bacteria profiling. In addition, it was intended to explore whether a relationship existed between the antimicrobial activity of the selected extracts and the antibiotic resistance profile of a panel of clinical isolates of *S. aureus*. Moreover, by using different statistical approaches, we also investigated if the polyphenolic composition or the type of extract might play a role in the bacterial susceptibility, according to the antibiotic resistance profile and mechanism involved in the resistance.

Results

First screening: disk diffusion assay. A panel consisting of 3 different plant extracts (*CS, C. salviifolius*; *PN, Citrus paradisi*; *GP, P. granatum*) and 7 pure polyphenolic compounds (GA, gallic acid; P, punicalagin; Q3G, quercetin-3-glucuronide; M, myricetin; N, naringenin; EA, ellagic acid) was initially selected for the first antimicrobial screening based on existing literature and previous experiments of the research group in which these agents showed activity against bacterial models[9,12]. The screening was performed using various clinical isolates of the following microbial species: *Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., *Serratia marcescens, Salmonella* spp., *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Stenotrophomonas maltophilia*.

A disk diffusion antimicrobial assay was performed as the first screening to choose the most active compounds or extracts for further exhaustive antibiograms. The percentage of sensitive isolates of each bacterial species for each extract or pure compound is displayed in Table 1. The isolate that had an inhibition halo for a given compound was considered a susceptible isolate to that compound, no matter its diameter. Differences in the number of clinical isolates used for each bacterial species were due to the variability in hospital bacterial collection derived from patients during the selection period of 30 days.

Based on the obtained results, further investigation was conducted to determine the activity of the most effective agents in this first screening against the most sensitive species of clinical relevance. According to this reasoning, CS and GP were chosen for use against *S. aureus* due to their crucial clinical importance, ease of laboratory culture and good results obtained in the first screening. Although compound P (pure punicalagin) was also effective against several bacteria, it was rejected for further tests due to its high economic cost and because it was the main component of the GP and CS extracts, so its activity would be somewhat covered by these extracts.

	CS	GP	PN	GA	Q3G	Q	M	P	N	EA	Tested isolates
E. faecalis	0	0	0	0	0	0	0	0	0	0	**7**
E. faecius	0	0	0	0	0	0	0	0	0	0	**4**
*S. aureus**	**65.5**	**62.1**	0	**24.1**	**3.4**	0	0	**72.4**	0	0	**29**
K. pneumoniae	**12.5**	**12.5**	0	0	0	0	0	**25**	0	0	**14**
Enterobacter spp	**25**	**25**	0	0	0	0	0	**25**	0	0	**4**
E. coli	0	0	0	0	0	0	0	0	0	0	**20**
S. marcescens	0	0	0	0	0	0	0	0	0	0	**4**
Salmonella spp	0	0	0	0	0	0	0	0	0	0	**1**
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	**12**
S. maltophilia	**62.5**	**50**	0	**37.5**	0	0	0	**37.5**	0	0	**8**
A. baumanii	0	0	0	0	0	0	0	0	0	0	**2**

Table 1. Percentage of sensitive isolates of gram-positive cocci (rows 1–3), Enterobacteria (rows 4–8) and nonfermentative Gram-negative bacilli (rows 9–11) species to each extract or pure compound. The dotted lines separate the different groups of bacteria. *S. aureus* includes both MRSA and MSSA isolates indistinctly.
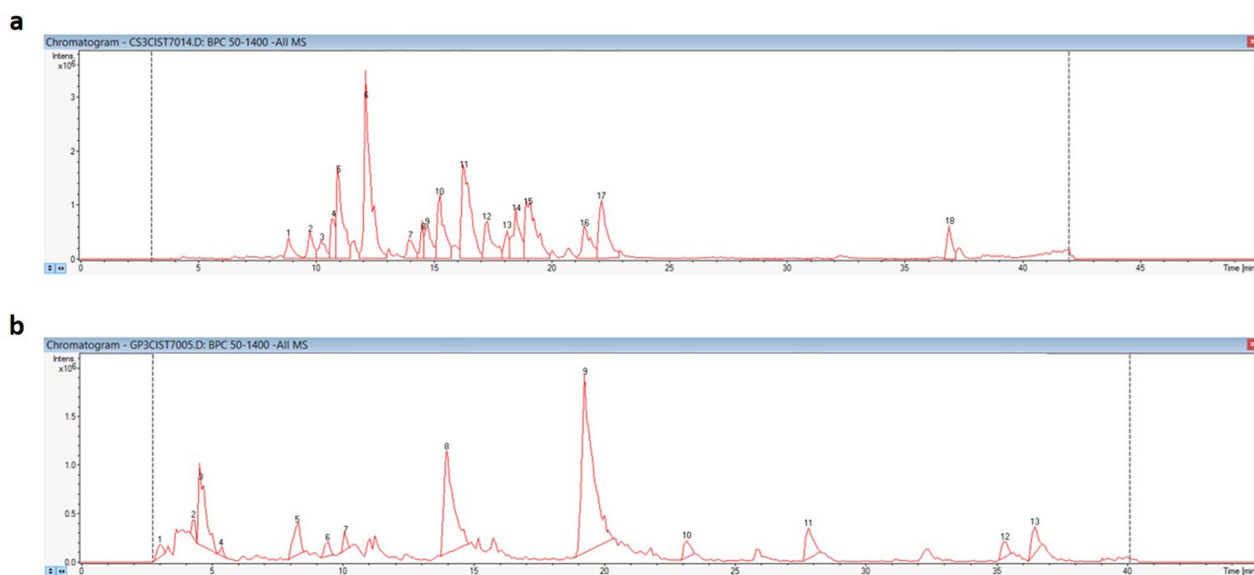
Figure 2. Base peak chromatograms of the CS (**a**) and GP extract (**b**) obtained by HPLC analysis.

Phenolic content and HPLC–MS molecular characterization of the extracts. After the first screening, the total phenolic content of the selected extracts was then measured by using the gallic acid equivalence (GAE) method and their fully composition were characterized by HPLC–MS as described in the following section. The CS extract showed a total phenolic content of 60.1 ± 5.5 mg GAE/g extract. In contrast, the GP extract showed a lower total phenolic content with 35.9 ± 2.5 mg GAE/g extract.

The CS and GP extracts were characterized using HPLC–MS using methods specifically designed for these botanical extracts as described in the Materials and Methods section. Base peak chromatograms are shown in Fig. 2, and the identified compounds and chromatographic and mass spectral data are included in Table 2 for CS and Table 3 for GP extracts. The quantification of the main compounds in the extracts was done by construction of standard curves of punicalagin and quercetin as representative compounds for hydrolyzable tannins and flavonoids respectively. Standard curves for both compounds are included in Supplementary Fig. S2.

The hydrolyzable tannins identified in the CS extract and quantified using the punicalagin standard were punicalagin (peaks 5 and 6 as isomers I and II respectively) and pedunculagin (peak 2). These compounds

Peak	RT (min)	Relative area (%)	[M−H]⁻	MS/MS	Proposed compound	References
1	8.8	2.101	609	423, 441	(−)-(Epi)gallocatechin-(epi)gallocatechin dimer	[21]
2	9.8	2.243	783	275, 301, 451, 481	Pedunculagin I	[22]
3	10.3	2.036	337	161	Coumaroylquinic acid	[23]
4	10.7	3.022	761	423, 609	Prodelphinidin B2-3′-O-gallate	[12]
5	10.9	8.626	1083	301, 601, 781	Punicalagin isomer I	[12]
6	12.1	17.557	1083	301, 601, 781	Punicalagin isomer II	[12]
7	14.0	1.941	343	181	*Not identified*	
8	14.5	1.807	341	179	Caffeoyl-hexose	[21]
9	14.7	3.548	453	151, 169, 313	Ligstroside derivate	[24]
10	15.2	6.656	631	317, 479	*Not identified*	
11	16.3	13.326	479	179, 271, 316	Myricetin hexoside	[21]
12	17.2	5.055	615	301	Dehydrated tergallic-C-glucoside	[23]
13	18.1	1.824	449	179, 271, 316	Myricetin 3-arabinoside isomer I	[12]
14	18.5	5.761	449	179, 271, 316	Myricetin 3-arabinoside isomer II	[12]
15	19.0	10.097	463	151, 301	Quercetin glucoside	[12]
16	21.4	4.174	433	301	Ellagic acid-7-xiloside isomer I	[25]
17	22.1	7.676	433	301	Ellagic acid-7-xiloside isomer II	[25]
18	36.9	2.549	593	285, 447	Kaempferol diglycoside	[12]

Table 2. Identified compounds in the CS extract using HPLC–MS.

Peak	RT (min)	Relative Area (%)	[M−H]⁻	MS/MS	Proposed compound	References
1	3.0	1.531	193	149	*Not identified*	
2	4.3	1.659	481	275, 301	HHDP glucoside isomer	[26]
3	4.6	12.315	481	275, 301	HHDP glucoside isomer II	[26]
4	5.4	0.712	331	169, 271	Galloyl glucose	[23]
5	8.3	4.932	781	271, 601, 721	Punicalin	[27]
6	9.4	1.674	783	275, 301, 481	Pedunculagin I	[22]
7	10.1	1.377	1083	301, 601, 781	Punicalagin isomer I	[12]
8	14.0	21.511	1083	301, 601, 781	Punicalagin isomer II	[12]
9	19.2	40.990	1083	301, 601, 781	Punicalagin isomer III	[12]
10	23.1	2.411	801	347, 649	Punigluconin	[22]
11	27.8	4.977	463	301	Quercetin glucoside	[12]
12	35.3	2.247	447	300	Ellagic acid rhamnoside	[28]
13	36.4	3.662	301	229, 257	Ellagic acid	[25]

Table 3. Identified compounds in the GP extract using HPLC–MS.

exhibited concentrations of 0.299 and 0.608 and 0.077 mg/mL punicalagin equivalents respectively. The total hydrolyzable tannin concentration in the CS extract was 0.984 mg/mL punicalagin equivalents, corresponding to 32.800% w/w of the extract. The hydrolyzable tannins identified in the GP extract were punicalagin (peaks 7, 8 and 9), punicalin (peak 5), pedunculagin I (peak 6) and punigluconin (peak 10). The determined concentrations of these compounds were 0.017, 0.270, 0.514, 0.062, 0.021 and 0.030 mg/mL punicalagin equivalents, respectively. The total hydrolyzable tannin concentration in the GP extract was 0.914 mg/mL punicalagin equivalents, corresponding to 30.460% w/w of the extract.

Quercetin was used as the representative flavonoid to quantify this polyphenolic compound group. The flavonoids identified in the CS extract were myricetin hexoside (peak 11), myricetin 3-arabinoside isomer I (peak 13), myricetin 3-arabinoside isomer II (peak 14), quercetin glucoside (peak 15) and kaempferol diglycoside (peak 18). The determined concentrations for these compounds were 0.0154, 0.0021, 0.0066, 0.0116 and 0.0029 mg/mL quercetin equivalents, respectively. The total flavonoid concentration in the CS extract was 0.0386 mg/mL quercetin equivalents, corresponding to 1.286% w/w of the extract. There was only one flavonoid identified in the GP extract, being quercetin glucoside (peak 11) with a determined concentration of 0.0057 mg/mL which corresponds to 0.190% w/w of the extract, approximately 10 times less than CS flavonoid content. The structures of the main compounds on each extract are shown in Supplementary Fig. S3 online.

Second screening: antimicrobial assays using the microdilution method. Microdilution in the p96 plate method was used for the second screening in which the antimicrobial activity of CS and GP extracts against 100 *S. aureus* clinical isolates (50 MRSA and 50 MSSA) was studied. Values for the minimum concen-
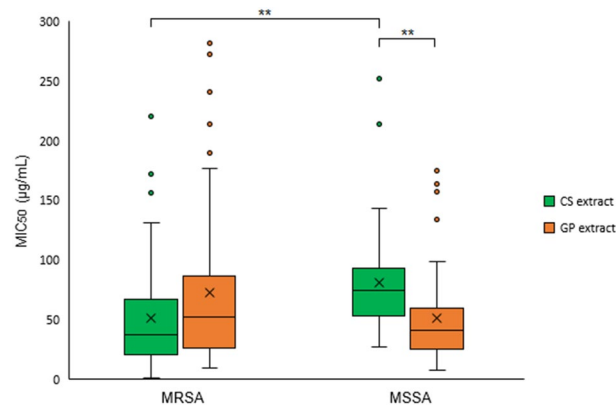
Figure 3. Box charts of the MIC_{50} distribution of the CS extract (green) and GP extract (orange) against MRSA and MSSA. The X within the box indicates the value of the mean, and the horizontal line indicates the median.

tration that inhibit the bacterial growth by 50% (MIC_{50}) were obtained for every single isolate as explained in methods section. The distribution of the MIC_{50} values of the two extracts against each of the 100 tested isolates of *S. aureus* is shown in Fig. 3. The specific MIC_{50} values for each extract can be seen in Supplementary Tables S1 and S2 online.

The mean MIC_{50} value of the CS extract against MRSA was 51.21 μg/mL, while that of the GP extract was 72.89 μg/mL. No significant differences between the MIC_{50} values of the two extracts were observed in this case (two-tailed p value = 0.0657 > 0.05). The average MIC_{50} value against MSSA of the CS extract was 80.70 μg/mL, while that of the GP extract was 51.67 μg/mL. There was a significant difference in the antimicrobial activity against MSSA between the two extracts (two-tailed p value = 0.0012 < 0.01, **).

On the contrary, when the activity of each extract against MRSA and MSSA isolates was compared, we encountered significant differences in relation to CS extract, which was more effective against MRSA (two-tailed p value = 0.0019 < 0.01, **) than against MSSA. No significant differences were found when comparing the activity of the GP extract against both MRSA and MSSA (two-tailed p value = 0.0709 > 0.05). The MIC_{50} values were independent for each isolate, and there was no direct correlation between CS and GP MIC_{50} values for the studied isolates (data not shown).

Antibiotic resistance and extract activity relationship study. All isolates of *S. aureus* were characterized by their profiles of resistance to various antibiotics commonly used clinically to determine whether there was a relationship between strain resistance to antibiotics and the antimicrobial action of the extracts through their MIC_{50} values. These profiles are included for each single isolate in Supplementary Table S3 online.

In Fig. 4, the mean MIC_{50} values of the CS (a) and GP (b) extracts are represented for every sensitive or resistant isolate of *S. aureus*. Unlike the previous Fig. 3, now mean MIC_{50} values were grouped based on the resistance or sensitivity of the *S. aureus* isolates against the panel of clinical antibiotics previously used to characterize those isolates (see Supplementary Table S3). Individual comparisons for each antibiotic are also shown in Supplementary Fig. S4 online.

When grouped MIC values of CS and GP extracts for *S. aureus* isolates were analyzed by resistance to particular antibiotics, it could be observed that significant differences between certain groups of treatments existed depending on the antibiotic. The CS extract was generally more active against resistant bacteria than against sensitive ones (Fig. 4a). On the contrary, GP extract was more active against sensitive bacteria than against resistant ones (Fig. 4b) (red squares are located below blue circles but grey squares are above golden circles regardless of the resistance). CS extract showed a significantly higher antimicrobial capacity against *S. aureus* isolates that were resistant to beta-lactam antibiotics (methicillin and oxacillin), than that against sensitive isolates ($p < 0.01$, **). Similar results were obtained for other antibiotics, such as fosfomycin, daptomycin and mupirocin, but without showing statistical significance ($p > 0.05$). In contrast, the GP extract showed greater activity against sensitive isolates compared to resistant ones regardless the type of antibiotic (circles are below squares), with the single exception of fosfomycin. These differences were statistically significant ($p < 0.05$, *) for oxacillin and quinolones (ciprofloxacin and levofloxacin).

Generalized linear model regression (GLMR). GLMR was used to give a consistent explanation of the behavior of the MIC_{50} values of the CS and GP extracts against *S. aureus* isolates based on their profile of resistance to clinical antibiotics overall. Supplementary Tables S4 and S5 online show the results of the GLMR analysis, obtained as described in the methods section.

The results indicate that it was possible to predict the MIC_{50} value of the CS extract based on the resistance or sensitivity of each isolate to the antibiotics methicillin ($p = 0.014$, *), oxacillin ($p = 0.014$, *), ciprofloxacin ($p = 0.003$, **) and levofloxacin ($p = 0.007$, **). In contrast, this did not seem to be possible for the GP extract, since no parameter could significantly explain its contribution to the MIC_{50} value. In both cases, sensitivity to teicoplanin appeared to be significant, but this was because no isolates were found to be resistant to teicoplanin,
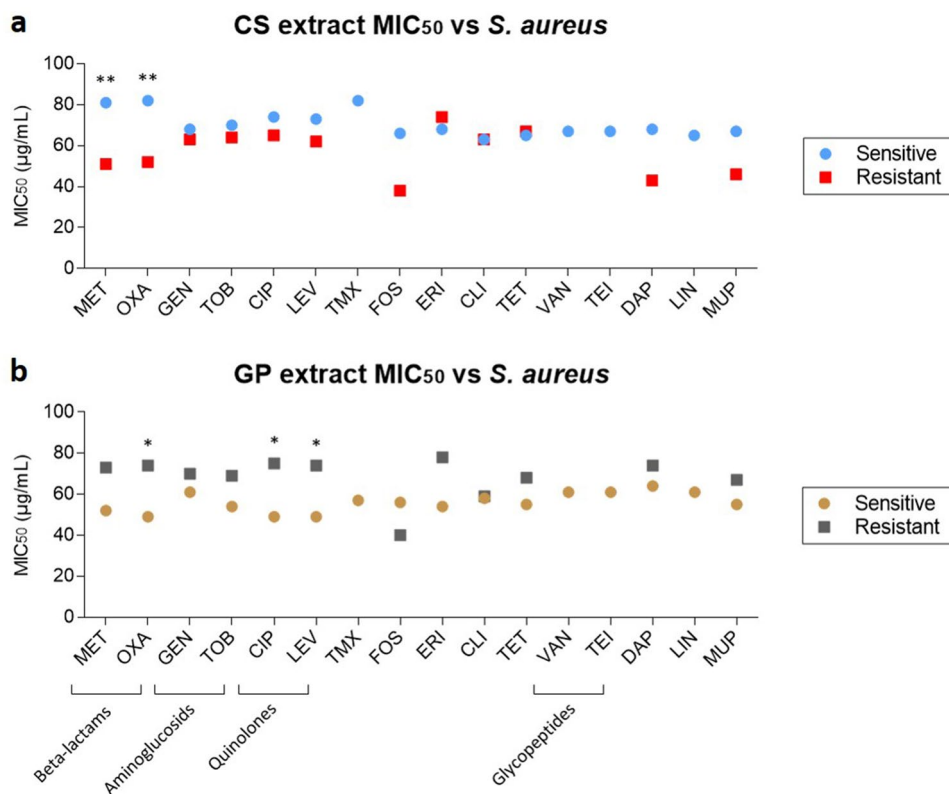
Figure 4. Comparative MIC$_{50}$ values of the CS extract (**a**) and GP (**b**) extracts against S. aureus isolates grouped by antibiotic sensitivity (circles) or resistance (squares). *MET* methicillin, *OXA* oxacillin, *GEN* gentamicin, *TOB* tobramycin, *CIP* ciprofloxacin, *LEV* levofloxacin, *TMX* trimethoprim/sulfamethoxazole, *FOS* fosfomycin, *ERY* erythromycin, *CLI* clindamycin, *TET* tetracycline, *VAN* vancomycin, *TEI* teicoplanin, *DAP* daptomycin, *LIN* linezolid, *MUP* mupirocin. Error bars are not included in this figure to minimize the symbols included on it but are included in Supplementary Fig. S4 online.

making it impossible to compare them in the analysis and yielding values exactly equal to the value of the initial interception.

Multiple correspondence analysis (MCA). MCA was conducted to further determine the relationship among the antimicrobial activity of extracts and the susceptibility to a given antibiotic for each bacterial isolate. In MCA, relations between row and column variables and relations between different levels of each variable can both be obtained. This analysis allowed for categorical/categorized data to be transformed into cross tables and two-dimensional images of data, simplifying their interpretation[13]. Based on these criteria, two different solutions were explored with two MCA dimensions.

The first solution was based on a direct representation of individual isolates as points. According to this approach, the first dimension accounted for 38.73% of the variance, and the second accounted for 7.22%, yielding a total of 45.94% of the variance being accounted for. The results are shown in Fig. 5.

As seen in this figure, there is a clear grouping of the MRSA isolates, with positive factor 1 values (red circles), while the MSSA group is mainly negative for this factor (blue squares). This grouping indicates that the different isolates behaved differently based on their methicillin resistance or sensitivity, confirming the results obtained in the previous sections in which the resistance against methicillin was determinant of the extracts' activity (see Figs. 3 and 4). Furthermore, additional groupings can be observed in Fig. 5 (black capital letters). The A group contains a series of heterogeneous strains with resistances of up to four antibiotics, but without common characteristics, because it is close to the origin point. The B group contains 32 isolates mainly sensitive to most of the tested antibiotics, so it can be considered the most sensitive population of the study. The C group contained 9 isolates also presenting sensitivity to most of the antibiotics in group B, but 8 of them showed resistance to ERI and CLI antibiotics, whereas only intermediate resistance was obtained for some of the isolates contained in the B group. The D group contained only 6 isolates; up to 5 of them share resistance to beta-lactams (MET and OXA) and total or intermediate sensitivity to quinolones (CIP and LEV). Group E includes 15 isolates that have common resistance against beta-lactams and quinolones, accompanied by ERY resistance in 14 of them. Finally, the F group contained 25 isolates with resistance against beta-lactams and quinolones, but 24 isolates did not have resistance against ERY. Additionally, the F group contained most of the isolates with relatively low MIC$_{50}$ values for the CS extract, with 17 of the isolates among the 50 most sensitive isolates to the CS extract.
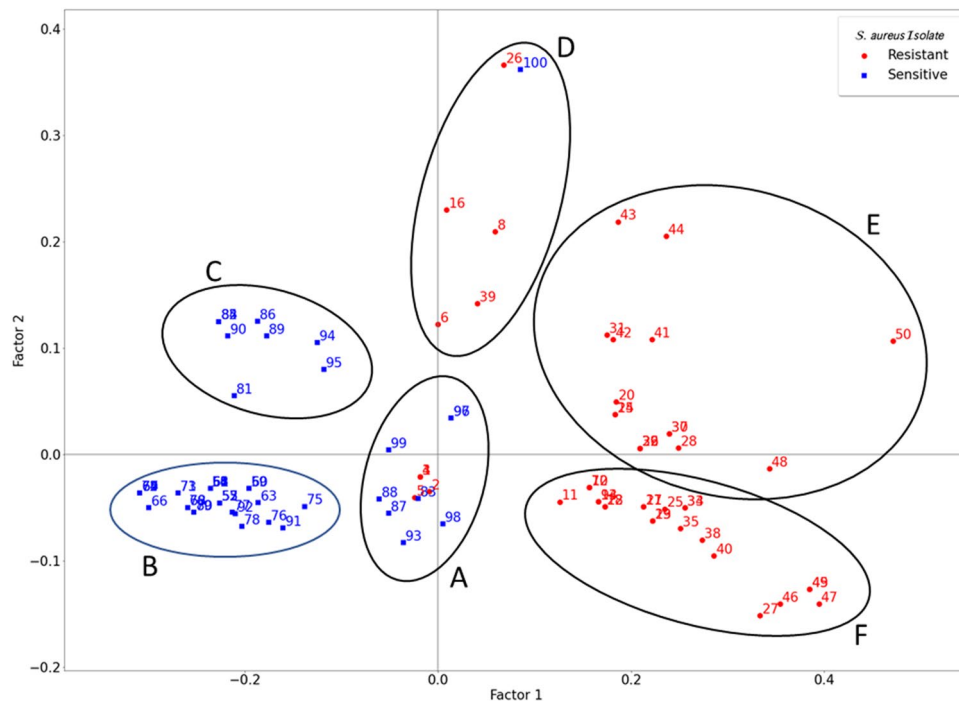
Figure 5. MCA results for individual isolates as points. Distribution of the different clinical isolates of *S. aureus* based on their profiles of resistance to antibiotics for clinical use and extracts.

The second approach was based on a direct representation of categorical values (resistant or not to a single antibiotic/extract) as points. Intermediate sensitivity was also included for vancomycin (VAN), clindamycin (CLI), levofloxacin (LEV) and ciprofloxacin (CIP). No data for VAN-resistant or teicoplanin (TEI)-resistant isolates were included, as none of the isolates presented these characteristics. For this analysis, the susceptibility to CS and GP extracts was determined by comparing the individual MIC_{50} value for each isolate with the mean MIC_{50} value for the whole 100 isolates population (65.34 and 62.96 μg/mL for CS and GP respectively). If the individual MIC_{50} was higher than the mean value for all the isolates, that single isolate was considered as resistant. Otherwise if the value was lower, the isolate was classified as sensitive. In this analysis, the first dimension accounted for 44.17% of the variance, and the second accounted for 9.85%, accounting for a total of 54.02% of the variance. A joint plot of category points was obtained, and is shown in Fig. 6. Category quantification plots constitute an alternative method of displaying discrimination of variables that can identify categorical relationships.

The results shown in Fig. 6 clearly group most of the points close to the horizontal axis (Factor 2 = 0), suggesting that this second dimension has little influence on the point distribution, and shows a greater influence of the F1 factor on this distribution. Additionally, most of the points are close to the axis intercept (0:0 point), indicating that they are not influenced by any of the values of either dimension. After this preliminary conclusion, some interesting results could be extracted from this analysis. Most of the points showing resistance to an antibiotic are on the right side of the plot (positive F1 values), whereas the majority of points associated with antibiotic sensitivity are on the left side. There are only two exceptions to this general conclusion, clindamycin (CLI), whose resistant point (CLI-R) is on the left side of the graph, and CS extract, whose points are also inverted. This last result indicates that the sensitivity of the isolates to the CS extract is related to their resistance to almost all of the antibiotics, confirming the results obtained in Fig. 4. In other words, the CS extract is especially active against antibiotic-resistant isolates.

Another relevant result was related to the mechanism of action of the different antibiotics. The antibiotics and extracts were divided into five categories according to their putative mechanism: protein synthesis inhibitors (GEN, TOB, ERI, CLI, TET, LIN and MUP), cell division inhibitors (CIP and LEV), plasmatic membrane disruptors (DAP) and cell wall disruptors (MET, OXA, FOS, VAN and TEI) and other mechanisms (TMX and the CS and GP extracts). Supplementary Fig. S5 online shows a complementary version of the MCA plot showing these mechanisms. Surprisingly, antibiotics presenting plasmatic membrane and cell wall disruption mechanisms are aligned with the F1 axis, with F2 values close to 0. This profile is similar for both the CS and GP extracts, suggesting that their putative mechanisms may be related to membrane or cell wall alterations. On the other hand, antibiotics that inhibit both protein synthesis and cell division showed a wider distribution, hampering our ability to draw any conclusions in addition to their relationship with both the F1 and F2 dimensions.
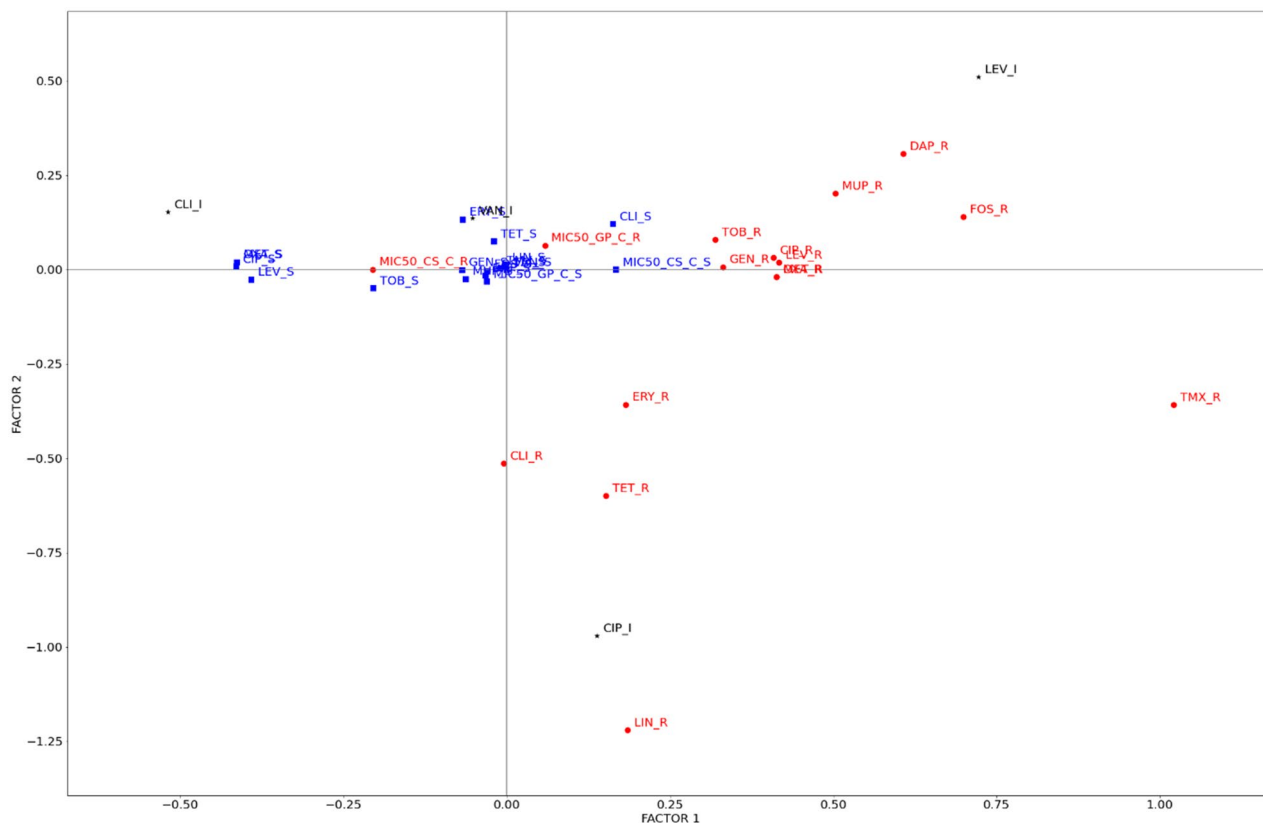
Figure 6. MCA results for categorical values (resistance, sensitiveness or intermediate) as points. Distribution of the different groups of isolates divided based on their resistance or sensitivity to the different clinical antibiotics and extracts tested. R (red text and red circles), I (black text and black * symbols) and S (blue text and blue squares) indicate resistant, intermediate and sensitive, respectively. Two magnified inserts and connections between antibiotics have been included for clarification in Supplementary Fig. S5.

Discussion

In the present work, natural pure compounds and extracts, previously selected for their potential antimicrobial capacity, have been challenged against different microorganisms with clinical relevance to study their putative use as new antimicrobial agents. A two-step screening design was used to select the best candidates for further studies. This first screening utilized 105 clinical isolates of 11 different strains (3 Gram-negative and 8 Gram-positive bacteria), and it revealed that three pure compounds and two extracts were active against *S. aureus* and *S. maltophilia.* As the number of available isolates of *S. maltophilia* was very low in our geographical area, the study was therefore focused on *S. aureus.* When the results for this microorganism were analyzed, only three pure compounds (P, Q3G and GA) and two extracts (CS and GP) presented positive results, especially the compound P and the CS and GP extracts. In addition, P was also the main compound in both extracts. These compounds and extracts have been studied previously because of their antimicrobial activity on *S. aureus*, confirming their relevance for future developments[3].

In general, it was observed that the CS extract was more effective against antibiotic-resistant bacteria than against antibiotic-sensitive bacteria, while the GP extract was more effective against bacteria sensitive to common clinical antibiotics. This tendency was evidenced when the CS extract was used against bacteria resistant to beta-lactam antibiotics, with statistical significance ($p < 0.05$, *). Significant differences were also observed in GP extract against bacteria sensitive to quinolones and oxacillin ($p < 0.05$, *). These results were reinforced by using GLMR and MCA analysis. These results were also consistent with those obtained by Atef M. et al., in which differential antimicrobial activity of plant extracts against several strains of *P. aeruginosa* was observed and correlated with different antibiotic resistance profiles[14].

In the present study, the average MIC_{50} value of the CS extract against clinical isolates of MSSA was 80.67 μg/mL, whereas it was 51.21 μg/mL against MRSA isolates. The antimicrobial capacity of different *C. salviifolius* extracts has been reported previously by our group and others, resulting in similar MIC_{50} values: 11 μg/mL[12] and between 45 and 50 μg/mL[9], both against *S. aureus* CECT 59 (MSSA). Regarding MRSA, a previous study quantified the MIC_{50} of a CS extract against a clinically isolated MRSA strain from Libya at 25 mg/mL[15]. This MIC_{50} value was much higher than that obtained from our CS extract. This difference may be due to the different methods utilized to obtain the CS extract. The extract used by Abdurrezagh E. et al. was obtained by conventional extraction using a mixture of methanol and water (8:2, v/v) filtered and dried without any further purification step and without providing any other analytical data. Our extract was obtained without using alcohol and was subjected to a final column fractionation step using FPX66 resin to obtain the polyphenol-enriched (60% w/w)

extract fraction. The different antimicrobial capacity might also be due to the different *C. salviifolius* raw material utilized to produce the extract, since the level of polyphenols in the plants can vary based on the environmental conditions, stresses or even the time of year in which collection occurred[16].

The present study determined that the average MIC_{50} value against MSSA of the GP extract was 51.67 µg/mL, and its value against MRSA was 72.89 µg/mL. Other studies determined the antimicrobial effect of dried pomegranate peel extracts against MSSA (ATCC 11632) and MRSA (ATCC 33591), presenting MIC_{50} values between 100 and 250 µg/mL for both MSSA and MRSA[17]. According to the manufacturer's information, the GP extract was also obtained after a purification process using an affinity resin, similar to that utilized to obtain CS extract. This process ensures a high polyphenolic content that can be, as occurred with the CS extract, the explanation for the better results of this study when compared with those in the literature.

Another aspect that deserves discussion is the fact that the two polyphenolic extracts utilized in the present study showed very different activities depending on the bacteria tested. This was very likely due to their different polyphenolic composition. The characterization of the extracts performed by HPLC–MS determined that the most abundant quantitative compounds in both extracts were hydrolyzable tannins, which showed 32.800% and 30.460% relative contents in the CS and GP extracts, respectively. Therefore, the differences in activity should depend on the less abundant compounds. The antimicrobial activity of punicalagin alone was tested against 7 different *S. aureus* isolates (ATCC 29213, ATCC 25923 and 5 laboratory isolates from food) and showed a MIC value of 250 µg/mL[18]. While the GP extract only contained hydrolyzable tannins (ellagitannins and gallotannins) and one flavonol at very low concentration, the CS extract contained more flavonoids (flavones, flavonols and flavanols), phenolic acids and a coumarin. Previous studies related the structure of certain polyphenolic compounds to their differential activity against sensitive and resistant antibiotic bacteria. Regarding MRSA, polyphenols presenting certain chemical groups, such as COOH and OH groups in the *ortho* and *para* positions, or an O–CH_3 group in the *meta* position of the benzene ring, seemed to have increased specific anti-MRSA activity[19]; the flavonoids presented in the CS extract but not in the PG extract present these characteristics. The higher activity of the CS extract against antibiotic-resistant bacteria could also be explained by the synergistic activity of flavonoids and hydrolyzable tannins at certain concentrations as previously described by our group[12]. The presence of punicalin and punigluconin, hydrolyzable tannins not present in the GP extract, could also explain part of the increased activity of the CS extract, although it is unlikely since these were present at low concentrations.

Based on the results obtained by using GLMR and MCA statistical approaches, we can conclude that the bacterial susceptibility to the studied extracts is related to the antibiotic resistance profile of the clinical isolates of *S. aureus*. Specifically, it was observed that bacterial isolate resistance to beta-lactam antibiotics and quinolones was directly related to their susceptibility to the action of the CS extract. In contrast, PG extract was more effective on bacteria sensitive to quinolones and oxacillin. On the other hand, MCA based on categorical values suggested a mechanism related to plasmatic membrane or cell wall disruption. This result is consistent with that previously reported which postulated these mechanisms as the main molecular target for polyphenols such as hydrolyzable tannins[3]. Nevertheless, further mechanistic studies must be developed to confirm this hypothesis.

In conclusion, CS and GP extracts demonstrated the highest antimicrobial activity, among all the natural antimicrobial agents used, against a panel of eleven different microbial species with clinical relevance. Moreover, CS extract exhibited higher antimicrobial capacity against resistant *S. aureus* clinical isolates, whereas PG was more effective against sensitive ones. The use of generalized linear model regression and multiple correspondence statistical analysis revealed that extracts antimicrobial capacity depended on the clinical antibiotic resistance profile of each bacterial isolate. The CS extract, which contained hydrolyzable tannins and flavonoids, was more effective against beta-lactam-resistant bacteria, while the GP extract, which contained mostly hydrolyzable tannins, was more effective against quinolone and oxacillin-sensitive bacteria. We postulate that the superior activity of the CS extract against antibiotic-resistant bacteria may be related either to the presence of flavonoids or to a synergistic interaction between hydrolyzable tannins and flavonoids. A link between bacterial susceptibility to GP and CS extracts with the resistance profile based on cell wall disruption mechanism is also proposed. These observations open further possibilities for future studies focused on the relationship between bacterial resistance to certain classes of antibiotics and natural molecules with an enhanced antimicrobial activity. This approach may enable to develop personalized combined antibiotic therapies for treating resistant infections with greater efficacy and less dependence on chemically synthesized molecules.

Materials and methods

Ethical statement.   All the procedures and methods used in this study were performed in accordance with the relevant guidelines and regulations and were previously approved by the Universidad Miguel Hernández Ethics and integrity for Research Committee with the reference UMH.IBM.VMM.05.15.

This committee waived the need for an additional informed consent for this study because all the samples were obtained during the usual clinical practice at the University General Hospital of Alicante and were covered by its corresponding informed consent. These consents remain in the custody of the hospital and were incorporated to the clinical history of each patient. Those informed consents explicitly include future uses for research purposes always maintaining patient's anonymity.

Botanical extract and pure compound selection and procurement.   The following pure compounds were purchased from Sigma-Aldrich (Misuri, USA): gallic acid, punicalagin, quercetin-3-glucuronide, myricetin, naringenin and ellagic acid *Punica granatum* and *Citrus paradisi* fruit extracts were obtained from the companies Monteloeder S.L. and Nutracitrus S.L., respectively. *C. salviifolius* extract was produced in the laboratory by aqueous extraction of leaves followed by a column chromatography purification process using AmberLite FPX66 resin (DuPont, Delaware, USA) and characterized as described in[8,9]. *C. salviifolius* specimens

were grown at Universidad Miguel Hernández facilities in Elche, Alicante (Spain) and harvested after being identified by the authors. Representative samples were deposited and preserved in their sample collection as CS190723-EBC.

Antimicrobial assays. For the disk diffusion assay, a known amount of compound or extract (40 µg) diluted in 5 µL of distilled water was applied on small discs of paper (5 mm of diameter) placed in a petri dish with microorganisms previously sown extensively over a semisolid Mueller–Hinton agar (Merck Millipore, Massachusetts, USA). Positive (discs with antibiotic) and negative (disc with phosphate-buffered saline adjusted to pH 7.4) were included in all the dishes. A halo of inhibition was generated around the discs impregnated with antimicrobial activity. Every test was performed in duplicate. These antimicrobial assays were performed on isolates of the following microbial species: *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Serratia marcescens*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. These species were chosen for their great importance in the health field due to their clinical impact and development of drug resistance. The microorganisms were obtained from clinical samples of patients at the General University Hospital of Alicante.

The antibiotic susceptibility of each single isolate was tested following EUCAST procedures and CLSI standards. All the isolates were classified as resistant (R), sensitive (S) or intermediate (I) according to the EUCAST classification and listed on Supplementary Table S3.

Plate microdilution assays were performed in 96-well plates. Microcultures of different isolates of *S. aureus* with 10 different concentrations ranging between 2 mg/mL and 0.0004 mg/mL of antimicrobial extract were carried out in each plate. Positive (ciprofloxacin) and negative (extracts vehicle and non-inoculated sample) controls were included in all the plates. Each plate was performed in duplicate. The culture medium used was Mueller–Hinton broth (Merck Millipore, Massachusetts, USA). After 24 h of incubation at 37 °C, the wells with medium and bacteria are stained with iodonitrotetrazolium chloride (Sigma-Aldrich, Misuri, USA) to stain viable bacteria red. After 30 min of plate incubation at 37 °C, the absorbance at 570 nm was measured using a spectrophotometer (BioTek Synergy HTX, Vermont, USA), to determine the microbial proliferation in each well. The absorbance values obtained were proportional to the number of viable bacteria stained in each well. The data obtained were analyzed using GraphPad Prism 6 software.

Total phenolic content determination. The total phenolic content of the extracts was measured by using the gallic acid equivalence (GAE) method in 96-well plates[20]. First, 10 µL of each sample was mixed with 50 µL of Folin–Ciocalteu's phenol reagent. After 1 min, 100 µL of Na_2CO_3 solution (20%, w/v) and 840 µL of distilled water were added to the mix. The reaction was kept in dark for 30 min. Plate absorbance was measured at 700 nm using a spectrophotometer (BioTek Synergy HTX, Vermont, USA). A standard curve of gallic acid (Sigma-Aldrich, Misuri, USA) was previously prepared using solutions of a known concentration in water. The absorbance data obtained were analyzed using GraphPad Prism 6 software. Results were expressed in terms of gallic acid equivalents (g GAE/100 g of dry matter of plant).

High-performance liquid chromatography analysis. The molecular composition of the extracts selected in the first screening was analyzed by high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) using an Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer as described previously[12]. Briefly, HPLC instrument was equipped with a pump, autosampler, UV–vis diode array detector, and column oven. The HPLC instrument was controlled by Chemstation software. The mass spectrometer instrument was equipped with an electrospray ionization (ESI) source and ion-trap mass analyzer. Mass spectrometer was operated by Esquire Control and DataAnalysis 3.4 software. The chromatographic column used was an Agilent Poroshell 120 RP—C18 column (4.6×150 mm, 2.7 µm).

The method used for sample separation consisted of a linear gradient of 1% formic acid (A) and acetonitrile (B). Gradient started at 5% of B, increasing to 25% of B at 30 min, to 45% of B at 45 min, then 5% of B at 51 min and for an additional 5 min for column re-equilibration purposes. The flow rate was constant at 0.5 mL/min. The diode-array detector was set at 280, 320 and 340 nm. The ESI ionization source was operated in negative mode to generate [M−H]⁻ ions. ESI conditions were set as follows: desolvation temperature, 360 °C; vaporizer temperature, 400 °C; dry gas (nitrogen), 12 L/min; nebulizer, 70 psi. Full scan mode from 50 to 1400 m/z with an ion trap collection time set at 200 ms was used for data acquisition.

Identification of the main compounds was performed by HPLC–DAD analysis using a home-made library of phenolic compounds and comparing the retention times, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in the literature[7]. The interpretation of the spectra and identification of the main compounds was carried out using DataAnalysis 3.4 software (Bruker Daltonics, GmbH, Germany).

Quercetin was used as the representative flavonoid to quantify this polyphenolic compound group. Punicalagin was used as the representative hydrolyzable tannin to quantify this polyphenolic compound group. Both punicalagin and quercetin used for the standard curves in the quantification of polyphenolic compounds of the extracts were purchased from Merck (Germany). The software ChemStation for LC 3D (Agilent Technologies Life Sciences and Chemical Analysis, Waldbronn, Germany) was used for quantitation purposes. The linearity range of the responses was determined on eight concentration levels (ranging from 0.25 to 0.25 mg/mL) with three injections for each level. Calibration graphs for the quantitative evaluation of the compounds were performed by means of a six-point regression curve ($r^2 > 0.996$)[7].

Statistical analysis. The minimum concentration that inhibits the bacterial growth by 50% (MIC_{50}) for each isolate and significant differences between treatments and data sets were calculated using GraphPad Prism 6 by processing the data obtained in the microdilution antimicrobial assays. The data gathered in the assays were analyzed using a nonlinear fit with least squares (log inhibitor vs normalized response with variable slope, equation: $Y = 100/(1 + 10\wedge((LogIC_{50} - X) \times HillSlope)))$ to calculate MIC_{50} values. Final graphs were generated using Microsoft Excel 2016. Generalized linear model regression (GLMR) and multiple correspondence analysis (MCA) were performed using Microsoft Excel and Google Colab with Jupyter Notebooks, libraries mca-1.0.3, Pandas v0.25.3, and Matplotlib Python v3.2.0. On the one hand, the term GLMR usually refers to conventional linear regression models for a continuous response variable given continuous and/or categorical predictors. In this case, the response variable is assumed to follow a normal family distribution. On the other hand, MCA takes multiple categorical variables and seeks to identify associations between levels of those variables. It can be thought of as analogous to principal component analysis for quantitative variables. Similar to other multivariate methods, it is a dimension reducing method resenting the data as points in a 2-dimensional space.

Data availability

Most data generated or analyzed during this study are included in this published article (and its Supplementary Information files). The datasets generated during and/or analyzed during the current study that are not present in the article or its Supplementary Information files are available upon justified request to the corresponding author.

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Author contributions

Conceptualization, J.C.R., E.B.-C. and V.M.; Data curation, F.J.Á.-M. and F.B.-R.; Formal analysis, F.J.Á.-M. and E.B.-C.; Funding acquisition, E.B.-C. and V.M.; Investigation, F. J.Á.-M.; Methodology, F.J.Á.-M. and F.B.-R.; Project administration, E.B.-C. and V.M.; Supervision, J.C.R., E.B.-C. and V.M.; Validation, F.J.Á.-M. and E.B.-C.; Visualization, F.J.Á.-M., F.B.-R. and E.B.-C.; Writing—original draft, F.J.Á.-M.; Writing—review and editing, J.C.R., F.B.-R., E.B.-C. and V.M.

Competing interests

The authors declare no competing interests.

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CAPÍTULO 3

Título: Tackling Antibiotic Resistance with Compounds of Natural Origin: A Comprehensive Review.

Autores: Francisco Javier Álvarez-Martínez, Enrique Barrajón-Catalán y Vicente Micol.
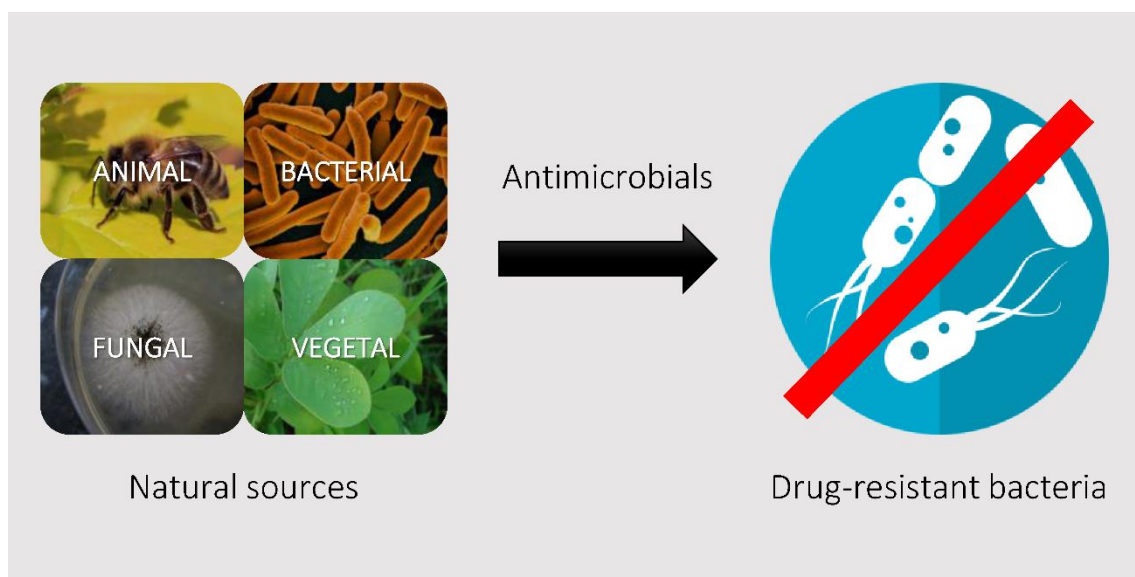
4.3. RESUMEN DE LOS RESULTADOS

Los compuestos antimicrobianos de origen natural poseen un enorme potencial en el descubrimiento de nuevas terapias antimicrobianas debido a su vasta diversidad molecular que es producto de la adaptación de los seres vivos a lo largo de la evolución natural ocurrida durante milenios. El presente trabajo describe los principales compuestos antimicrobianos de origen natural extraídos de cuatro fuentes distintas: animal, bacteriana, fúngica y vegetal. Asimismo, se destacan aquellos más eficaces frente a bacterias resistentes a antibióticos o capaces de sensibilizarlas y revertir sus mecanismos de resistencia.

Se ha revisado un total de 68 compuestos antimicrobianos de origen natural, recopilando datos sobre su origen, especificidad microbiana y potencial mecanismo de acción. Los resultados obtenidos del análisis de los datos presentes en la bibliografía apuntan a que la fuente más prolífica de productos naturales antimicrobianos de los últimos años han sido las propias bacterias, especialmente las del género *Actinomyces*. Como ejemplos notables de productos naturales antimicrobianos de origen bacteriano encontramos la vancomicina, la eritromicina o la fosfomicina.

Entre los productos naturales antimicrobianos destacan aquellos que además son eficaces frente a bacterias resistentes a antibióticos tradicionales. La mayoría de éstos son de origen bacteriano, como por ejemplo la albomicina, la dalbavancina o la teicoplanina. Sin embargo, también destacan algunos de origen animal como la mucroporina y la vejovina, otros de origen fúngico como el ácido fusídico o la mirandamicina y otros de origen vegetal, como la quercetina o la luteolina. Los resultados del análisis de datos revelan que diversos compuestos naturales, especialmente los fitoquímicos, han mostrado capacidad sinérgica con antibióticos tradicionales frente a bacterias resistentes a antibióticos. Los mecanismos de sinergia más comunes hallados en fitoquímicos para promover o restaurar la actividad de los antibióticos son la inhibición de bombas de eflujo, la dispersión de biofilms y la desestabilización de membranas con el consecuente aumento de su permeabilidad.

Los mecanismos de acción antimicrobiana más comúnmente hallados en compuestos naturales están relacionados con la disrupción de la biosíntesis de proteínas y la alteración de las paredes y membranas celulares. También se pone de manifiesto que existe escasa literatura sobre el desarrollo de mecanismos de resistencia específicos frente a compuestos antimicrobianos naturales.

La proyección futura de la investigación y uso de compuestos antimicrobianos de origen natural es muy favorable. Las nuevas tecnologías -ómicas, la farmacología y la biología de sistemas y la biología computacional poseen el potencial para identificar y caracterizar multitud de nuevos y eficaces compuestos antimicrobianos naturales en el futuro. Este conocimiento podría ser clave para el desarrollo de futuras estrategias terapéuticas frente a infecciones resistentes.

Tackling Antibiotic Resistance with Compounds of Natural Origin: A Comprehensive Review

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Abstract: Drug-resistant bacteria pose a serious threat to human health worldwide. Current antibiotics are losing efficacy and new antimicrobial agents are urgently needed. Living organisms are an invaluable source of antimicrobial compounds. The antimicrobial activity of the most representative natural products of animal, bacterial, fungal and plant origin are reviewed in this paper. Their activity against drug-resistant bacteria, their mechanisms of action, the possible development of resistance against them, their role in current medicine and their future perspectives are discussed. Electronic databases such as PubMed, Scopus and ScienceDirect were used to search scientific contributions until September 2020, using relevant keywords. Natural compounds of heterogeneous origins have been shown to possess antimicrobial capabilities, including against antibiotic-resistant bacteria. The most commonly found mechanisms of antimicrobial action are related to protein biosynthesis and alteration of cell walls and membranes. Various natural compounds, especially phytochemicals, have shown synergistic capacity with antibiotics. There is little literature on the development of specific resistance mechanisms against natural antimicrobial compounds. New technologies such as -omics, network pharmacology and informatics have the potential to identify and characterize new natural antimicrobial compounds in the future. This knowledge may be useful for the development of future therapeutic strategies.

Keywords: natural antimicrobial; antimicrobial resistance; polyphenols; future medicine; natural origin; antibacterial compound; phytochemicals

1. Introduction

Antimicrobial resistance (AMR) and the inexorable advance of superbacteria poses a great threat to human health worldwide. If this problem is not tackled, the antibiotics we have used with great success so far could become substances unable to help us against infections caused by bacteria, going back to a worrying pre-antibiotic era. According to data from the United Kingdom government [1], 10 million deaths could happen annually due to antibiotic resistance by 2050, becoming one of the leading causes of death in the world (Figure 1).

This problem is known to scientists and institutions around the world, which are organizing to establish protocols to address the problem of antibiotic-resistant microbes. Proof of this was the 2012 Chennai Declaration of India, in which international experts and representatives of medical entities met to draw up action plans in the face of the inexorable advance of the superbugs [2]. Similar initiatives have been promoted from private and public institutions worldwide.
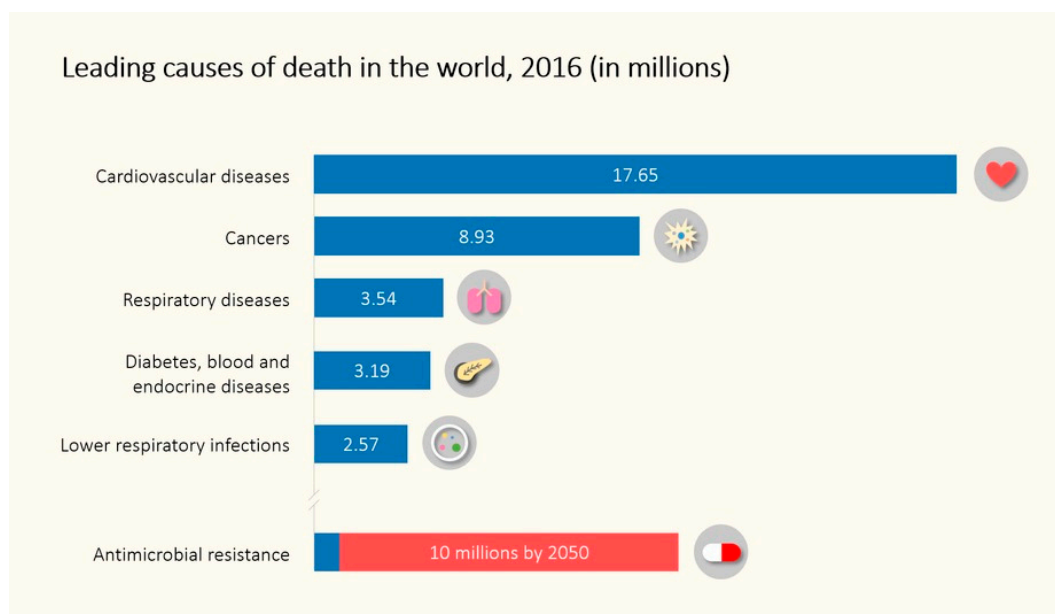
Figure 1. Leading causes of death in the world in 2016 (blue bars) and prognosis for antimicrobial resistance (AMR) related deaths in 2050 (red bar).

Bacteria use their genetic plasticity to resist attack by antibiotics through mutations, acquisition of genetic material, and alteration of the expression of their genome [3]. In this way, bacteria that survive the attack of an antibiotic become the precursors of the next bacterial generations, further aggravating the problem of resistance. Once antibiotic resistance genes are acquired, they can be passed from one bacterium to another through division processes or by horizontal gene transfer [4]. Horizontal gene transfer processes can occur by transformation, transduction or conjugation with other bacteria. These mechanisms can transfer antibiotic resistance to bacteria that have not been subjected to antibiotic selection pressure, creating reservoirs of resistant bacteria in the environment [5]. In addition, the epistasis of the receptor bacteria plays a fundamental role in the process of acquisition of resistance genes, determining whether these bacteria are capable of maintaining, accumulating and propagating the genetic material [6].

Antibiotic resistance is an example of the enormous capacity for natural evolution and adaptation of bacteria to different environments [7,8]. Although this process seems inevitable, humans have accelerated it through various anthropogenic activities [9,10]. The causes behind the increase in the number of antimicrobial-resistant bacteria in recent years include the misuse of antibiotics in humans and animals, inadequate control of infections in hospitals and clinics or poor hygiene and sanitation [9–11]. In addition to the causes mentioned, the problem worsens as there is a drought in the discovery of new antibiotics. The increase in resistance rates in bacteria leads to a decrease in the effectiveness of existing antibiotics, making research in this field unattractive to companies that decide to invest in other types of fields with greater chances of success and benefits [12,13]. This concerning trend can be observed in Figure 2.

In view of this scenario, research on alternative or complementary therapies to traditional antibiotics has emerged strongly. Antimicrobial products of natural origin have been positioned as compounds of great scientific interest due to their enormous chemical variety and intrinsic properties that have promoted their study as a possible therapeutic tool in recent years.
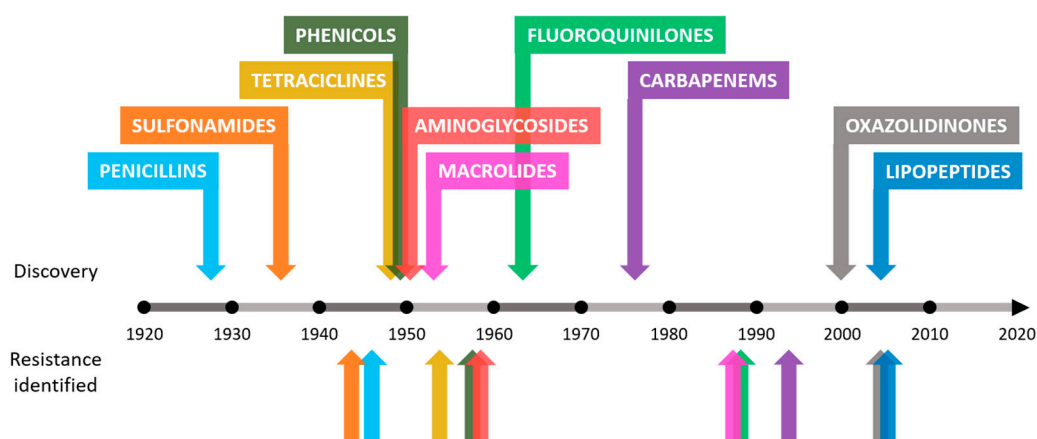
Figure 2. Approximate dates of discovery of new classes of antibiotics and identification of bacterial resistance.

2. Methodology

Electronic databases such as PubMed, Scopus and ScienceDirect were used to search scientific contributions until September 2020, using relevant keywords. Search terms included "natural antimicrobial", "antimicrobial resistance", "polyphenols", "future medicine", "natural origin", "antibacterial compound", "phytochemical" and their combinations. Literature focusing on the antimicrobial activity of natural origin compounds against bacteria focusing on antibiotic-resistant strains were identified and summarized.

The term "antimicrobial activity" is used throughout this work to refer to the process of killing or inhibiting the growth of microbes. Usually, this activity is expressed as MIC (minimum inhibitory concentration) values for a given agent. The methods to test microbial susceptibility compiled in this work are in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and The Clinical and Laboratory Standards Institute (CLSI). Following the EUCAST guidelines for the reproducibility and reliability of antimicrobial assays, broth dilution or microdilution methods should be used to test microbial susceptibility [14].

3. Results

3.1. Use of Natural Products as Antimicrobials

Natural products (NPs) make up a heterogeneous group of chemical entities that possess diverse biological activities with various uses in fields such as human and veterinary medicine, agriculture and industry. Molecules from the secondary metabolism of animals, vegetables, bacteria and fungi are classified as NPs, which are not crucial for the producer's survival under laboratory conditions, but which give him a clear advantage over his competitors in his native habitat [15]. Since the discovery of penicillin, more than 23,000 new NPs have been characterized, many of which have proven to be valuable tools in the field of pharmacology, herbicides, insecticides and more [16].

One of the main sources of antimicrobial NPs is plants. Plant organisms make up most of the biosphere on planet Earth, whose biomass accounts for a percentage greater than 80% of the total biomass [17]. Since their appearance, plants have survived, evolved and adapted to all types of ecosystems and adverse conditions. This adaptive process has led them to develop complex and effective defense systems against external aggressions: predators, abiotic stress and, of course, infections. Being sessile organisms that cannot escape their threats, plants have developed a splendid chemical arsenal in the form of secondary metabolites capable of coping with the most dangerous pathogens [18]. Humanity has made use of the medicinal properties of plants for thousands of years. There is evidence that in the year 5000 BC. the Sumerians already used thyme for its beneficial health properties [19]. The Egyptian Ebers Papyrus dating from around 1500 BC already attributed medicinal properties to

plants and spices such as aloe vera, castor bean, garlic, hemp, anise or mustard [20,21]. Other texts such as the Atharva Veda, the Rig Veda and the Sushruta Samhita belonging to Indian Ayurveda, also spoke of the pharmacological properties of plant substances such as turmeric or cannabis [22,23]. Current technology allows us to study the bases of this ancestral knowledge and find therapeutic applications adapted to our time, making plants a source of invaluable therapeutic potential.

Bacteria are another of the main sources of antimicrobial NPs with radical importance during the 20th century. Most of the antibiotics used today in the clinic were discovered thanks to the Waksman platform in the 1940s. Waksman and his students dedicated themselves to growing soil microorganisms to detect and isolate antimicrobial substances. Through this method, they discovered very important antibiotics such as neomycin or streptomycin, for which Waksman received the Nobel Prize in 1952 for Physiology or Medicine [24]. Despite these successes, it should be noted that most existing bacteria are not cultivable in the laboratory using traditional methods. We could find an immense amount of opportunities for the isolation of new antibiotic compounds using a method like Waksman's combined with new technologies not present decades ago. From this idea, the Small World Initiative was born in 2012, a project in which students from all over the world collect soil samples and look for antibiotic-producing microorganisms in them [25].

Many of the NPs with antibiotic activity have been isolated from bacteria, especially from the genus actinomycetes. In the so-called "Golden Age" of the discovery of new antibiotics, which began in the 40s of the twentieth century, natural products were the star. The isolation of streptomycin from *Streptomyces griseus* in 1944 caused a worldwide surge in which numerous research groups struggled to identify new NPs, especially from samples of soil bacteria. The media were very limited, both in technology and in access to soil samples from remote places. However, another great milestone occurred in 1952, when a sample of soil sent from Borneo allowed *Streptomyces orientalis* to grow, from which vancomycin was extracted. Six years later, vancomycin was used in patients with great success. Unfortunately, this prolific period of discovery of valuable compounds ended the appearance and spread of bacteria resistant to these NPs, such as methicillin-resistant *Staphylococcus aureus* (MRSA) or glycopeptide-resistant enterococci (GREs), since the compounds that worked in the past stopped working with the desired efficiency [26], as observed in Figure 2.

In the 1990s, the pharmaceutical industry concentrated its efforts on other more sophisticated methods of identifying antimicrobial compounds, such as high-throughput screening of synthetic chemical libraries against specific therapeutic targets, many of them discovered from the Human Genome Project. Currently, there is a renewed interest in the discovery of new NPs of different sources since it has a much more advanced technology than that available during the "Golden Age". Advances in genomics, bioinformatics and mass spectrometry, among others, have elucidated that many of the sources of classical NPs were surprisingly under-exploited and have an enormous and unknown potential for the discovery of new NPs to be used for the discovery of present and tomorrow's antibiotics [15].

Given the existing problems in the field of antibiotics, in recent years alternative and complementary therapies have emerged that make use of different strategies to deal with new generations of resistant bacteria. The growing interest in this area is reflected in the ascending number of publications related to natural antimicrobials available in the PubMed search engine over the past recent years (Figure 3).

As abovementioned, the molecules with antimicrobial function present in nature have been molded by thousands of years of evolution to maintain their efficacy and selectivity, since they are a key piece for the development of the life of any organism exposed to bacteria. Thanks to these processes of continuous physicochemical adaptation driven by selective pressure, it has been demonstrated that antimicrobial compounds of natural origin generally have a greater capacity for cell penetration, being able to use active bacterial transporters and, in addition, passively pass through the cell membrane [27]. These and other properties that will be discussed below, make NPs a tool of great potential value for the development of novel and effective antibiotic therapies against AMR bacteria.
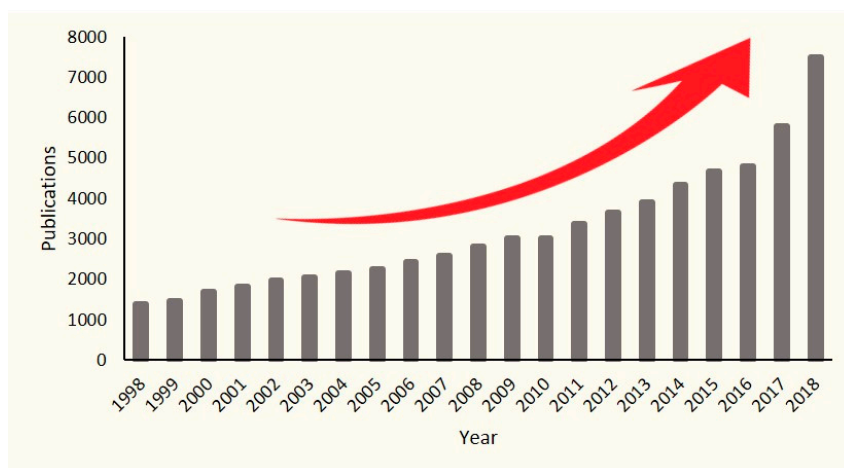
Figure 3. The number of research articles available in PubMed by searching "Natural Antimicrobial" from 1998 to 2018. The red arrow represents a growing trend.

3.2. Main Classes of Natural Antimicrobial Products

NPs are extremely diverse in terms of their chemical structures, properties and mechanisms of action. These agents can be classified according to their original source: animal, bacterial, fungal or vegetal.

3.2.1. Animal Origin

Animals have colonized virtually the entire planet Earth. For thousands of years, they have lived closely with different kinds of bacteria and have faced not a few pathogenic microorganisms. Evolution has shaped animal defense systems to deal with these microscopic threats. In recent years, attention has been focused on identifying which molecules confer resistance and allow certain animals to live in hostile environments with high pollution and pathogenic load, as is the case with certain insects such as cockroaches.

Currently, animals, and especially insects, are one of the main sources of antimicrobial proteins or peptides (AMPs). Since the discovery of AMPs in 1974, more than 150 new AMPs have been isolated or identified, the majority being cationic peptides between 20 and 50 residues in length. These molecules mainly have antimicrobial capacity mediated by disruption of the bacterial plasma membrane, most probably by forming pores or ion channels [28]. Some AMPs also have shown antifungal, antiparasitic or antiviral properties [29]. These AMPs can be divided into four subfamilies with different structures and sequences: the α-helical peptides, such as cecropin, which has a broad spectrum of antimicrobial activity against bacteria of both Gram-positive and Gram-negative bacteria; cysteine-rich peptides, such as insect defensins, which are mainly active against Gram-positive bacteria; proline-rich peptides, such as lebocins, which are active against both Gram-positive and Gram-negative bacteria and some fungi; and finally glycine-rich peptides or proteins, such as attacin, which are effective against Gram-negative bacteria and especially against *Escherichia coli*. These AMPs present a promising basis for the development of medical therapies, however, additional work must be developed to make them more powerful and stable [30]. Moreover, the intrinsic antimicrobial capacity of AMPs can be enhanced by a fusion of peptides to create more potent hybrid ones, such as in the case of attacin from *Spodoptera exigua* and a coleoptericin-like protein from *Protaetia brevitarsis seulensis*, which, when fused, exhibited a greater antimicrobial capacity than its two original peptides [31].

The study of antimicrobial molecules existent in cockroaches (*Periplaneta americana*) has revealed that extracts derived from its brain have a great antimicrobial capacity against MRSA and neuropathogenic *E. coli* K1. Although not all the components of the extract could be accurately identified, a great variety of molecules with known biological activity were found, such as isoquinolines, flavanones, sulfonamides and imidazone among others. A hypothesis about the production of this antimicrobial cocktail in the cockroach brain suggests that there could be a constitutive expression of

these antimicrobials to protect the animal's neural system, since it is the central axis of its survival and a key piece to protect when it is lived in an environment of high pollution and exposure to pathogens and even superbugs [32]. Another example of insect producing antimicrobial molecules against resistant bacteria is *Lucilia cuprina* blowfly maggots. The extract obtained from excretions and secretions from maggots showed mild bacterial growth inhibition. However, using subinhibitory concentrations of this extract in combination with the antibiotic ciprofloxacin enhanced its activity, further delaying the appearance of bacteria resistant to it. The properties of this extract, including the presence of defensins and phenylacetaldehyde, make maggot debridement therapy a promising tool in the treatment of MRSA-infected wounds acquired in hospital [33].

One of the most popular insect-related products worldwide is honey. In addition to its nutritional properties and culinary values, it has antimicrobial capacity against Gram-negative bacteria, such as *E. coli* or *Pseudomonas aeruginosa*, and against Gram-positive bacteria, such as *Bacillus subtilis* or *S. aureus*, including MRSA. The key factors of honey's antimicrobial activity appear to be the presence of H_2O_2, bee defensin-1 and methylglyoxal. The diverse molecular composition of the different honey types that depends on the producing species and the raw material used, exerts also different antimicrobial activities and mechanisms [34]. Another substance produced by bees is propolis, a resinous substance produced by honeybees from plant matter, such as buds or sap. This substance has been used since ancient times, up to 3000 years BC in Egypt thanks to its various biological properties. The main components responsible for its activity are flavonoids, terpene derivatives and phenolic acids, although its composition is variable depending on the geographical area where it occurs. Ethanol extract of propolis produced by *Apis mellifera* in Brazil has demonstrated significant antibacterial capacity against *S. aureus*, *E. coli* and *Enterococcus sp*. [35]. Canadian propolis has also been shown to possess antibacterial capacity against *E. coli* and *S. aureus*, being more effective against the latter [36]. Another product with antimicrobial properties derived from honeybees is royal jelly. It is produced from the mandibular salivary and hypopharyngeal glands of bees aged between 5 and 14 days. Its composition is based on a complex mixture of carbohydrates, proteins, lipids, vitamins and minerals that varies with regional conditions, season, bee's genetics and postharvest storage conditions. Royal jelly shows antimicrobial activity against both Gram-positive and Gram-negative bacteria, including MDR bacteria such as MRSA. The compounds isolated from royal jelly with activity against Gram-positive bacteria are the peptide royalisin [37], the peptide family of jelleines and 10-hydroxy-2-decenoic acid (10-HDA), also known as queen bee acid [38]. Melittin, a major component from the venom of *A. mellifera*, has also shown interesting antimicrobial activity, including in in vivo experiments with mice infected with MRSA [39].

Other animals that can live in contaminated environments and exposed to infections are reptiles, such as snakes that are able to ingest rodents infected with germs and not develop a disease. Results suggest that animals exposed to huge amounts of pathogens can be a valuable source of antimicrobial molecules. However, to further study and identification of the key molecules responsible for the activity, it is necessary to know if they would be candidates for drugs with real applicability in therapies [40]. There are studies in Black cobra (*Naja naja karachiensis*) that show that plasma lysates and certain organs have a potent antimicrobial capacity against *E. coli* K1, MRSA, *P. aeruginosa*, *Streptococcus pneumoniae*, *Acanthamoeba castellanii*, and *Fusarium solani*. Against *E. coli* K1, solutions containing 25% and 50% of plasma from the blood of the Black cobra showed a bactericidal activity of 85% and 93% respectively with respect to the effect of the antibiotic gentamicin. Against MRSA, concentrations of 25% and 50% of plasma showed activity of 90% and 93%, respectively. Lung and gallbladder lysates also showed high antimicrobial capacity against MRSA. Antimicrobial molecules can also be extracted from the venom produced by certain species of snakes, such as cathelicidines or toxins. A cathelicidin-like antimicrobial peptide (cathelicidin-BF) isolated from the venom of *Bungarus fasciatus* has shown high antimicrobial activity, including drug-resistant bacteria [41]. *Crotalus adamanteus* toxin-II (CaTx-II) exerted a strong antimicrobial effect against *S. aureus*, *Burkholderia pseudomallei* and *Enterobacter aerogenes* by causing pores and damaging their membranes. Interestingly, this compound showed no cytotoxicity against lung (MRC-5), skin fibroblast (HEPK) cells or treated mice [42].

Molecules with great antimicrobial capacity have also been found in crustaceans, coming from their immune system. The anti-lipopolysacchride factor of red claw crayfish *Cherax quadricarinatus* has shown low minimum bactericidal concentrations (MBC) against Gram-negative *Shigella flexneri* (MBC < 6 µM) and Gram-positive *S. aureus* (MBC < 12 µM), meaning a high antimicrobial capacity. Studies showed that the mechanism of action of this compound does not appear to be related to the bacterial plasma membrane alteration, requiring more studies to find its specific mechanism [43].

The venom of *Vaejovis mexicanus*, a mexican scorpion, has an AMP called vejovine, which presents a high antimicrobial capacity against MDR Gram-negative bacteria with MIC values between 4.4 µM and 50 µM [44].

3.2.2. Bacterial Origin

Bacteria are the most prolific source of NPs with antimicrobial activity found so far, especially those of the actinomycetes class. Their great diversity, competitiveness and colonization capacity have led them to the development of secondary metabolites capable of giving them great advantages over other bacterial species. As described in previous sections, the detection and isolation of these bacterial antimicrobial NPs propelled medical science vertiginously in the middle of the last century. Some of the most relevant are described below.

Some of the most important antimicrobial molecules produced by bacteria of the actinomyces class are: vancomycin, baulamycin, fasamycin A and orthoformimycin. Vancomycin is a naturally occurring tricyclic glycopeptide extracted from *Streptococcus orientalis* that has reaped great success as an antibiotic against Gram-positive bacteria, especially against threats that are resistant to other treatments such as MRSA and penicillin-resistant pneumococci among others [45]. Vancomycin forms hydrogen bonds with the terminal dipeptide of the nascent peptidoglycan chain during biosynthesis of the bacterial cell wall. This union prevents the action of penicillin-binding proteins (PBPs), interrupting further wall formation and finally activating autolysin-triggered cell rupture and cell death [46]. Another important bacterial NP is produced by actinomyces is baulamycin, which is an isolated molecule of the marine bacterium *Streptomyces tempisquensis* that can inhibit the biosynthesis of iron-chelating siderophores in *S. aureus* (targeting staphylopherrin B) and *Bacillus anthracis* (targeting petrobactin), helping to treat MRSA and anthrax infections, respectively. In addition, it was also able to inhibit the growth of Gram-negative bacteria such as *S. flexneri* and *E. coli*, turning baulamycin and its derivatives into potential broad-spectrum antibiotics [47]. Fasamycin A is a polyketide isolated from *Streptomyces albus* that shows specific antimicrobial activity against Gram-positive bacteria such as vancomycin-resistant Enterococci (VRE) and MRSA with MIC values of 0.8 and 3.1 µg/mL, respectively. This molecule targets FabF in the initial condensation step of the elongation cycle from the lipidic biosynthetic bacterial metabolism [48]. Orthoformimycin is a molecule produced by *S. griseus* which can inhibit bacterial translation by more than 80% in the case of *E. coli*. Although the mechanism of action is not clear now, one hypothesis is the decoupling of mRNA and aminoacyl-tRNA in the bacterial ribosome [49].

The actinobacteria class is also prolific in the production of antimicrobial molecules. One example is kibdelomycin, which is a potent inhibitor of DNA synthesis that was isolated from *Kibdelosporangium* sp., MA7385. Its complex structure and its infrequent function as an inhibitor of bacterial DNA gyrase and IV topoisomerase make kibdelomycin the first bacterial type II topoisomerase inhibitor discovered from natural sources in more than 60 years [50]. This molecule has a broad-spectrum antimicrobial activity against aerobic bacteria, including antibiotic-resistant bacteria such as MRSA, with a MIC value of 0.25 µg/mL. In addition, this molecule has a very low resistance development rate due to its structure and way of binding with its target, at levels of other successful antibiotics such as ciprofloxacin [51]. Another example is pyridomycin, a molecule isolated from *Dactylosporangium fulvum* which has a great antimicrobial capacity against mycobacteria, a bacterium that causes tuberculosis. This disease is becoming relevant due to the appearance of bacteria resistant to the main antibiotics used for its treatment such as the InhA inhibitor isoniazid. Pyridomycin acts on the cell wall of *Mycobacterium tuberculosis* by inhibiting the production of mycolic acid by targeting NADH-dependent enoyl- (Acyl-Carrier-Protein)

reductase InhA even in strains resistant to isoniazid. Pyridomycin showed minimum bactericidal concentration (MBC) values between 0.62 and 1.25 µg/mL against *M. tuberculosis* [52].

In addition to the two classes mentioned above, there are other classes of bacteria such as deltaproteobacteria, cyanophyceae or betaproteobacteria from which antimicrobial molecules have also been isolated. Myxovirecin is a macrocyclic secondary metabolite isolated from myxobacteria (deltaproteobacteria class) that possesses broad-spectrum antibacterial capacity. It seems to inhibit the production of type II signal peptidase by blocking Lpp lipoprotein processing. Myxovirecin showed very potent activity against *E. coli* DW37 with a MIC of 0.063 µg/mL [53]. Spirohexenolide A is a natural spirotetronate originally isolated from *Spirulina platensis* of the cyanophyceae class that shows antimicrobial activity against methicillin-resistant *S. aureus* by disrupting the cytoplasmic membrane, collapsing the proton motive force [54]. Teixobactin is a naturally occurring molecule produced by *Eleftheria terrae* of the betaproteobacteria class that possesses antibacterial capacity against antibiotic-resistant pathogens in infection animal models. It acts by binding to the precursors of the bacterial wall teicoic acid, causing the digestion of the cell wall by autolysins [55].

Lypoglycopeptides isolated from different bacteria show antimicrobial activity by inhibiting signal peptidase type IB (SpsB), which is a membrane-localized serine protease that cleaves the amino-terminal signal peptide from most secreted proteins. One example is actinocarbasin, a molecule isolated from *Actinoplanes ferrugineus* strain MA7383. Moreover, this molecule enhances the activity of β-lactam antibiotics against MRSA, sensitizing it to those drugs. Arylomycin is another lipoglycopeptide with bacterial type I signal peptidase inhibitory capacity which showed antibacterial activity witch MIC values in the range of 4–64 µM against Gram-positive and 8–64 µM against Gram-negative bacteria. Krisynomycin is also a lypoglycopeptide, isolated from *Streptomyces fradiae* strain MA7310, with the capacity of inhibition of SpsB [56].

In addition to the natural bacteria molecules with direct antimicrobial activity, there are also others capable of attacking the virulence factors caused by bacterial infections. Skyllamycins B and C are cyclic depsipeptides isolated from marine bacterial fractions with *P. aeruginosa* biofilm inhibition and dispersal activity. The ability to prevent the formation of biofilms or to disperse those already formed is of great importance since these biofilms are one of the major causes of drug resistance in nosocomial infections. These molecules do not possess a bactericidal capacity per se, but they are effective in combination with antibiotics that are not able to act in the presence of biofilms, causing them to recover their activity as in the case of azithromycin [57].

3.2.3. Fungal Origin

Fungi are eukaryotic-type living things, such as mushrooms, yeasts, and molds. Currently, the existence of some 120,000 species of fungi has been accepted, however, it is estimated that the number of different species of fungi present on earth could be between 2.2 and 3.8 million [58]. This relatively unexplored kingdom is a source of antimicrobial NPs and has great potential to be studied in the future as new species are discovered and identified.

Aspergillomarasmine A is a polyaminoacid naturally produced by *Aspergillus versicolor* capable of inhibiting antibiotic resistance enzymes in Gram-negative pathogenic bacteria, such as *Enterobacteriaceae*, *Acinetobacter spp.*, *Pseudomonas spp.* and *Klebsiella pneumoniae*. This compound has been used successfully to reverse resistance in mice infected with meropenem-resistant *K. pneumoniae* thanks to the NDM-I protein, making the bacterium sensitive to the antibiotic and ending the infection [59].

Mirandamycin is a quinol of fungal origin capable of inhibiting the growth of both Gram-negative and Gram-positive bacteria, being more effective against the latter group, including antibiotic-resistant strains such as MRSA or carbapenemase-producing *K. pneumoniae*. Its mechanism of action consists in the inhibition of the bacterial metabolism of sugars, interfering with their fermentation and transport [60].

There is evidence of the antibacterial capacity of various fungal species against Gram-positive bacteria. Extracts of *Ganoderma lucidum*, *Ganoderma applanatum*, *Meripilus giganteus*, *Laetiporus sulphureus*,

Flammulina velutipes, *Coriolus versicolor*, *Pleurotus ostreatus* and *Panus tigrinus* demonstrated antimicrobial activity in Kirby–Bauer assays against Gram-positive bacteria, such as *S. auerus* and *B. luteus* [61].

In recent times, molecules produced by various species of marine fungi have been studied, especially those that cohabit with sponges or corals. Fungal compounds with activity against antibiotic resistant bacteria have been isolated, such as lindgomycin and ascosetin, with MIC values of 5.1 μM and 3.2 μM against MRSA, respectively. These molecules were isolated from the mycelium and the *Lindgomycetae spp* culture broth from sponges found in the Baltic and Antarctic Sea [62]. Another marine fungus capable of producing antimicrobial molecules is *Pestalotiopsis* sp., isolated from the coral *Sarcophyton* sp. This fungus produces (±) -pestalachloride D, a chlorinated benzophenone derivative, which has shown antibacterial capacity against *E. coli*, *Vibrio anguillarum* and *Vibrio parahaemolyticus* with MIC values of 5, 10 and 20 μM, respectively [63]. *Trichoderma sp.* is a sponge-derived fungus from which different aminolipopeptide classes, called trichoderins, have been isolated. These molecules have a potent antimycobacterial capacity showing MIC values between 0.02 and 2.0 μg/mL against *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG, and *M. tuberculosis* H37Rv in different aerobic and hypoxic conditions [64].

3.2.4. Plant Origin

Plants are a great source of biomolecules with various interesting properties for humans thanks to their enormous diversity and proven safety for human health [65]. Being sessile organisms, evolution has shaped its metabolism to produce certain molecules to cope with external aggressions and infections, since they cannot flee or defend themselves [66]. The Dictionary of Natural Products lists approximately 200,000 secondary plant metabolites, of which 170,000 have unique chemical structures [67]. Some of the families of molecules with antimicrobial capacity produced by plants are alkaloids, terpenoids, and polyphenols [68].

Plants that have been used in traditional medicine in various countries of the world for thousands of years. They are currently being studied at the molecular and functional level, rediscovering their properties and explaining their mechanisms of action.

Alkaloids have been shown to possess antimicrobial capacity against various bacterial species. Although studies of the antimicrobial capacity of pure alkaloids are limited, there are several studies on the antimicrobial activity of plant extracts that contain alkaloids as their main components. Different extracts rich in alkaloids obtained from *Papaver rhoeas* have shown activity against *S. aureus*, *Staphylococcus epidermidis* and *K. pneumoniae*, the main active component being roemerine [69]. Raw alkaloid-rich extracts of *Annona squamosa* seeds and *Annona muricata* root have also shown moderate antimicrobial capacity against *E. coli* and *S. aureus* [70].

Terpenoids, along with other families of compounds, are part of plant essential oils, many of which possess antimicrobial activity. Various in vitro studies affirm that terpenoids do not possess significant antimicrobial activity per se [71]. However, they can contribute to the antimicrobial activity of complete essential oils thanks to their hydrophobic nature and a low molecular weight that allow them to disrupt the cell wall and facilitate the action of the rest of the active components [72].

Polyphenols are molecules present in plants with a function of defense against stress and have one or more phenolic groups in their chemical structure as a common feature. There is abundant literature on the antimicrobial capacity of polyphenols and extracts of plants rich in them that have bactericidal and bacteriostatic capacity against many pathogens, both Gram-positive and Gram-negative. The potential use of polyphenols as antimicrobials is widely studied to be applied in different areas such as agriculture [73], food preservation [74] and medicine [75].

There are several subfamilies within the group of polyphenols according to their differentiated chemical structures: flavonoids, hydrolyzable tannins, lignans, phenolic acids and stilbenes. In turn, the flavonoid group can be subdivided into other subfamilies: anthocyanidins, flavanones, flavones, flavonols and isoflavones [76]. Examples of flavonoids with antimicrobial activity are quercetin [77], kaempferol [78], morin [79], myricetin [80] epigallocatechin gallate [81] or galangin [82] among many others [76,83]. Other known polyphenols with good antimicrobial activity are punicalagin, which

exerts both antibacterial and antibiofilm effect against *S. aureus* [80,84], and resveratrol, which has antimicrobial activity against a wide range of bacteria [75].

The growing relevance of the study of polyphenols in the clinical setting is due to their antimicrobial synergy between polyphenols and antibiotics for clinical use. Polyphenols in subinhibitory concentrations enhance the action of an antibiotic against a bacterium that was originally resistant to its effect. For example, kaempferol and quercetin, two flavonols with antimicrobial activity on their own, have also shown to increase the efficacy of the rifampicin antibiotic against rifampicin-resistant MRSA strains by 57.8% and 75.8%, respectively. The study authors blame this increase in the activity to which these polyphenols are able to inhibit the catalytic activity of topoisomerases, inhibiting DNA synthesis, with a mechanism similar to that of the ciprofloxacin antibiotic, with which they have also shown to have a synergistic activity [85]. Epicatechin gallate (ECg), a flavanol, is capable of sensitizing strains of MRSA against β-lactam antibiotics such as penicillin or oxacillin. This polyphenol can bind to the MRSA cytoplasmic membrane and cause large changes in its structure and reducing its fluidity, decoupling the functioning mechanism of the enzyme PBP2a, which is the protein responsible for resistance to β-lactam antibiotics. In addition, ECg can reduce biofilm formation and protein secretion associated with virulence factors [86]. (-)-Epigallocatechin gallate (EGCg) is another flavanol with a great capacity to enhance the effect of antibiotics that acts mainly on the cell wall directly or indirectly and on some virulence factors, such as the production of penicillinases [87].

Another example of synergy between polyphenols and antibiotics is the case of the combination of catechin and epicatechin gallate extracted from *Fructus crataegi* and ampicillin, ampicillin/sulbactam, cefazolin, cefepime, and imipenem/cilastatin antibiotics, which are usually ineffective against MRSA. These combinations were effective against MRSA in both in vitro and in vivo assays using mice with an established infection model. The authors stressed that the possible mechanism of action of the combination of these two polyphenols to enhance the effect of antibiotics was the accumulation of antibiotics inside the cell thanks to the inhibition of the efflux pump gene [88].

In addition to synergy with antibiotics, there are also studies that point to the synergy between the polyphenols themselves, such as that between EGCg and quercetin against MRSA, attributed to a co-permeabilization process that would facilitate the activity of the compounds inside of the cell [89]. Synergic activity has also been found between the polyphenols quercetin-3-glucoside, punicalagin, ellagic acid and myricetin in different proportions and combinations against *S. aureus* CECT 59 [80].

Apart from the antimicrobial use of concrete molecules of plant origin, the use of complex extracts made from different parts of plants is common and effective. Plant extracts have a great diversity in their composition, since even from the same plant multiple completely different extracts can be obtained varying the extraction conditions. Time, temperature, solvents, pressure and other parameters such as the use of ultrasound or microwave have a huge impact on the final extract composition [90]. There is numerous evidence of the antimicrobial activity of plant extracts [76,91] and the synergistic effect that exists between different phytochemicals [80] when acting against different bacteria. An example of a plant extract with potent activity against AMR bacteria are extracts from *Lantana camara* leaves against clinical isolates of MRSA, *Streptococcus pyogenes*, VRE, *Acinetobacter baumannii*, *Citrobacter freundii*, *Proteus mirabilis*, *Proteus vulgaris* and *P. aeruginosa* [92]. The ethanolic extracts of *Anthocephalus cadamba*, *Pterocarpus santalinus* and *Butea monosperma* Lam. they have also demonstrated antimicrobial activity against MDR clinical isolates of 10 different microbial species: *S. aureus*, *Acinetobacter sp.*, *C. freundii*, *Chromobacterium violeceum*, *E. coli*, *Klebsiella sp.*, *Proteus sp.*, *P. aeruginosa*, *Salmonella typhi* and *Vibrio cholerae* [93,94]. In the case of *B. monosperma* Lam., antimicrobial activity was also found in the extract made with hot water from leaf.

3.2.5. Summary

As a summary, Table 1 contains all the NPs mentioned above together with their producing organism, type, target bacteria, mechanism of action, main use and references. Figure 4 shows the main molecular targets of the most relevant antimicrobial NPs.

Table 1. Alphabetically ordered natural products (NPs) with their properties and capabilities. Grey shaded cells mean effectiveness against AMR bacteria. Asterisk (*) means no antimicrobial activity alone.

Natural Product	Producter Organism	Type of Organism	Activity Against	Mechanism of Action	Main Use	Reference
Actinorhodin	*Streptomyces coelicolor*	Actinomycete	Gram-positive, including multidrug-resistant *S. aureus*	ROS production inside bacterial cells	Research	[95]
Albomycin	*Streptomyces sp. ATCC 700974*	Actinomycete	Gram-negative and Gram-positive, including MRSA	Seryl t-RNA synthetase inhibition	Medicine	[96,97]
Amphomycin	*Streptomyces canus*	Actinomycete	Gram-positive, including MRSA, VRE and MDR *S. pneumoniae*	Inhibition of peptidoglycan and wall teichoic acid biosyntheses	Medicine	[98]
Apramycin	*Streptoalloteicus hindustanus*	Actinomycete	Gram-negative, including MDR *A. baumannii* and *P. areuginosa*	Inhibition of protein synthesis	Veterinary	[99]
Arlomycins	*Streptomyces sp.* Tü 6075	Actinomycete	Gram-positive and Gram-negative	Inhibition of type I bacterial signal peptidase	In research for medical use	[100]
Aspergillomarasmine A *	*A. versicolor*	Fungus	Sensitivizes carbapenem-resistant bacteria	Inhibition of bacterial metallo-β-lactamases	In research for medical use	[59]
Carbomycin	*Streptomyces halstedii*	Actinomycete	Gram-positive and *Mycoplasma*	Inhibition of protein synthesis	Medicine	[101]
Cathelicidin-BF	*Bungarus fasciatus*	Reptile	Mainly Gram-negative, including MDR strains	Damage in microbial cytoplasmic membrane	Research	[41]
CaTx-II	*C. adamanteus*	Reptile	Gram-positive and Gram-negative	Membrane pore formation and cell wall disintegration	Research	[42]
Cecropin A	*Aedes aegypti*	Insect	Gram-negative	Disruption of the cytoplasmic membrane	In research for medical use	[102]
Cephalosporin	*Cephalosporium acremonium*	Fungus	Gram-positive and Gram-negative	Inhibition of cell wall synthesis	Medicine	[103]
Cephamycin C	*Streptomyces clavuligerus*	Actinomycete	Gram-positive and Gram-negative	Inhibition of cell wall synthesis	Medicine and veterinary	[104]
Chloramphenicol	*Streptomyces venezuelae*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Medicine and veterinary	[105]
Chloroeremomycin	*Amycolatopsis orientalis*	Actinomycete	Gram-positive, including VRE	Inhibition of bacterial cell wall formation	Medicine	[106]
Clavulanic acid *	*S. clavuligerus*	Actinomycete	Sensitivizes β-lactam-resistant bacteria	β-lactamase inhibitor	Medicine and veterinary	[107]
Clorobiocin	*Strteptomyces roseochromogenes*	Actinomycete	Gram-positive	Inhibitors of DNA gyrase	Medicine	[108]
Coumermycin	*Streptomyces rishiriensis*	Actinomycete	Mainly Gram-positive	Inhibition of DNA gyrase	Research	[109,110]
Dalbavancin	*Nonomuraea sp.*	Actinomycete	Gram-positive, including MRSA	Inhibition of cell wall synthesis	Medicine	[111]
Daptomycin	*Streptomyces roseosporus*	Actinomycete	Gram-positive	Inhibition of protein, DNA and RNA synthesis	Medicine	[112]
Epigallocatechin gallate	Abundant in *Camellia sinensis*	Plant	Gram-positive and Gram-negative	Damage in microbial cytoplasmic membrane	In research for medical use	[81,113]

Table 1. *Cont.*

| Natural Product	Producer Organism	Type of Organism	Activity Against	Mechanism of Action	Main Use	Reference
Erythromycin	*Saccharopolyspora erythraea*	Actinomycete	Gram-positive	Inhibition of protein synthesis	Medicine	[114]
Fosfomycin	*Streptomyces wedmorensis*	Actinomycete	Gram-positive and Gram-negative	Inhibition of cell wall synthesis	Medicine	[115]
Fusidic acid	*Fusidium coccineus*	Fungus	Gram-positive, including MRSA	Inhibition of protein synthesis	Medicine	[116]
Gentamicin	*Micromonospora purpurea*	Actinomycete	Gram-negative	Inhibition of protein synthesis	Medicine	[117]
Gramicidin S	*B. subtilis*	Bacillales	Gram-positive and Gram-negative	Delocalizes peripheral membrane proteins involved in cell division and cell envelope synthesis	Medicine	[118]
Hc-CATH	*Hydrophis cyanocinctus*	Reptile	Gram-positive and Gram-negative	Damage in microbial cytoplasmic membrane	Research	[119]
Hygromycin	*Streptomyces hygroscopicus*	Actinomycete	Gram-positive	Inhibition of protein synthesis	Veterinary and research	[120]
Josamycin	*Streptomyces narbonensis*	Actinomycete	Gram-positive, certain Gram-negative and *mycoplasma*	Inhibition of protein synthesis	Medicine	[121]
Kanamycin	*Streptomyces kanamyceticus*	Actinomycete	Mainly Gram-negative and certain Gram-positive	Inhibition of protein synthesis	Medicine	[122]
Kirromycin	*Streptomyces collinus*	Actinomycete	Anaerobes, *neisseriae* and *streptococci*	Inhibition of protein synthesis	Research	[123,124]
Lincomycin	*Streptomyces lincolnensis*	Actinomycete	Gram-positive	Inhibition of protein synthesis	Medicine	[125]
Lipiaramycin	*Dactosporangium aurantiacum*	Actinomycete	Gram-positive and *Mycobacterium*, including MDR strains	Inhibition of early transcription	Medicine	[126]
Melittin	*A. mellifera*	Insect	Gram-positive and Gram-negative, including MDR strains	Damage in microbial cytoplasmic membrane	Medicine	[39]
Mirandamycin	Endophytic fungus isolated from the twig of *Neomirandea angularis*	Fungus	Gram-negative and Gram-positive, including MRSA	Inhibition of bacterial quinol oxidase/ROS production	In research for medical use	[60]
Moenomycin	*Streptomyces ghanaensis*	Actinomycete	Gram-positive	Inhibition of cell wall synthesis	Veterinary	[127]
Morin	*Moraceae* family	Plant	Gram-positive and Gram-negative	Inhibition of adhesion to host tissue and DNA helicase	Food technology	[79]
Mucroporin	*Lychas mucronatus*	Arachnid	Gram-positive and Gram-negative, including MDR strains	Damage in microbial cytoplasmic membrane	Research	[128]
Neomycin	*S. fradiae*	Actinomycete	Gram-positive and Gram-negative	Inhibition of ribonuclease P	Medicine	[129]
Orthoformimycin	*S. griseus*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	In research for medical use	[49]
Oxytetracycline	*Streptomyces rimosus*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Aquaculture	[130]

Table 1. *Cont.*

Natural Product	Producer Organism	Type of Organism	Activity Against	Mechanism of Action	Main Use	Reference
Penicillins	*Penicillium crysogenum*	Fungus	Gram-positive and Gram-negative	Inhibition of cell wall synthesis and activation of the endogenous autolytic system	Medicine	[131]
Pleuromutalin	*Clitopilus scyphoides*	Fungus	Gram-positive, Gram-negative and *Mycoplasma*	Inhibition of translation	Veterinary	[132]
Polymyxin	*Paenibacillus polymyxa*	Bacillales	Mainly Gram-negative (including MDR) and certain Gram-positive	Disruption of the cytoplasmic membrane	Medicine	[133]
Pristinamycin	*Streptomyces pristinaespiralis*	Actinomycete	Gram-positive, including MRSA	Inhibition of protein synthesis	Medicine	[134]
Punicalagin	Abundant in *Punica granatum*	Plant	Gram-positive and Gram-negative	Damage in microbial cytoplasmic membrane	Food technology	[80,84]
Quercetin	Ubiquitous in plants	Plant	Gram-positive and Gram-negative	Damage in the structure of the bacterial cell wall and cell membrane	In research for medical use	[135]
Ramoplanin	*Actinoplanes sp.* ATCC 33076	Actinomycete	Gram-positive, including MDR strains	Inhibition of cell wall synthesis	Medicine	[136]
Resveratrol	Abundant in grapes, berries and legumes	Plant	Gram-positive and Gram-negative, including MDR strains	Inhibition of motility, adhesion, quorum sensing, biofilm formation, flagellar gene expression and hemolytic activity	Medicine	[75]
Rifamycin	*Amycolatopsis mediterranei*	Actinomycete	Gram-positive and certain Gram-negative	Inhibition of DNA-dependent RNA synthesis	Medicine	[137]
Ristocetin A	*A. orientalis*	Actinomycete	Gram-positive, including MRSA	Inhibition of cell wall synthesis	Medicine	[138]
Royalisin	*Apis melifera*	Insect	Mainly gram-positive	Damage in the structure of the bacterial cell wall and cell membrane	Research	[37]
Skyllamycins	*Streptomyces sp.* KY 11784	Actinomycete	Gram-positive	Inhibition of biofilm formation	In research for medical use	[139]
SlLebocin1	*Spodoptera litura*	Insect	Gram-positive and Gram-negative	Damage in microbial cytoplasmic membrane or cell division inhibition	Research	[140]
Spectinomycin	*Streptomyces spectabilis*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Medicine	[141]
Spiramycin	*Streptomyces ambofaciens*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Medicine	[142]
Streptothricin	*Streptomyces (multiple species)*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Veterinary and plant production	[143]

Table 1. *Cont.*

Natural Product	Producer Organism	Type of Organism	Activity Against	Mechanism of Action	Main Use	Reference
Teicoplanin	*Actinoplanes teichomyceticus*	Actinomycete	Gram-positive, including MRSA	Inhibition of bacterial cell wall synthesis	Medicine	[144]
Teixobactin	*Eleftheria terrae*	Betaproteobacteria	Gram-positive, including MRSA	Causes digestion of the cell wall by autolysins	Medicine	[55]
Tetracycline	*Streptomyces rimosus*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Medicine	[145]
Thienamycin	*Streptomyces cattleya*	Actinomycete	Gram-positive and Gram-negative	Inhibition of bacterial cell wall synthesis	Derivates used in medicine	[146]
Thiostrepton	*Streptomyces azureus*	Actinomycete	Gram-positive and Gram-negative	Inhibition of protein synthesis	Veterinary and research	[147]
Tobramycin	*Streptoalloteicus hindustanus*	Actinomycete	Gram-negative	Inhibition of protein synthesis and membrane destabilization	Medicine	[148]
Tunicamycin	*Streptomyces chartreusis*	Actinomycete	Gram-positive	Inhibition of peptidoglycan and lipopolysaccharide synthesis	Research	[149]
Tylosin	*S. fradiae*	Actinomycete	Gram-positive and *Mycoplasma*	Inhibition of protein synthesis	Veterinary	[150]
Vancomycin	*S. orientalis*	Actinomycete	Gram-positive, including MRSA	Inhibition of bacterial cell wall synthesis	Medicine	[45]
Vejovine	*V. mexicanus*	Arachnid	Gram-negative, including MDR	Damage in microbial cytoplasmic membrane	Research	[44]
Viomycin	*Streptomyces sp. 11861*	Actinomycete	MDR *Mycobacterium*	Inhibition of protein synthesis	Medicine	[151]
Virginiamycin	*Streptomyces virginiae*	Actinomycete	Gram-positive	Inhibition of protein synthesis	Agriculture and industry	[152]
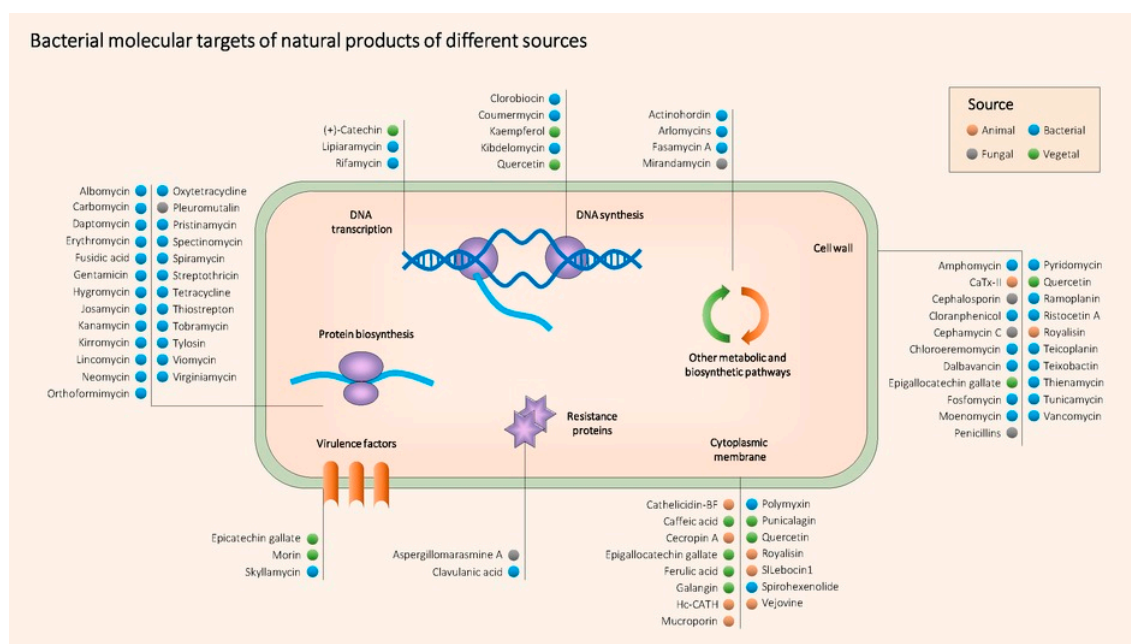
Figure 4. Main known molecular targets of antimicrobial NPs described in this review.

3.3. Antibiotics and Plant Compounds Combinations to Get around AMR

The synergic combination of antibiotics and phytochemicals represents a promising strategy with numerous clinical and developmental benefits. Some plant compounds have direct antimicrobial activity against antibiotic-resistant bacteria, while others can sensitize resistant bacteria against antibiotics, reversing the resistance as mentioned and exemplified in the previous section. Some of these NPs can enhance the effect of antibiotics in different ways, such as facilitating their entry into the cell by destabilizing the cytoplasmic membrane [153,154], inhibiting efflux pumps (EPs) [155] or dispersing biofilms [156] among other mechanisms of action (Figure 4). Some of the synergistic interactions between phytochemicals and antibiotics include increased efficiency, lower antibiotic doses, reduced side effects, increased bioavailability and increased stability [157]. The multidimensional and multifactorial activity of phytochemicals studied by network pharmacology is crucial for synergy with clinical antibiotics, opening the door to many different potential combinations. Moreover, the use of molecules that have already passed the relevant clinical controls, as in the case of antibiotics, in combination with innocuous natural compounds facilitates the process of research and development of new potential therapies [158].

There is clear evidence of NPs capable of inhibiting efflux pumps of AMR bacteria, specifically, phytochemicals. These molecules can inhibit various efflux pumps in different pathogenic bacterial species, both Gram-positive and Gram-negative. As an example, the NorA efflux pump of *S. aureus* SA-1199-B has been effectively inhibited using baicalein plant molecules [159], capsaicin [160], indirubin [161], kaempferol rhamnoside [162] and olympicin A [163]. NorA of *S. aureus* NCTC 8325-4 was inhibited using sarothrin [164]. Cumin demonstrated antimicrobial activity on its own and also resistance modulation properties against MRSA by inhibiting LmrS efflux pump [155]. Plant molecules inhibiting the ethidium bromide efflux pump (EtBr) have also been found: 1′-S-1′-acetoxyeugenol acetate inhibits it in *Mycobacterium smegmatis* [165], catechol and catharanthine inhibits it in *P. aeruginosa* [166,167] and galotannins inhibit it in MDR uropathogenic *E. coli* [168]. The YojI efflux pump of MDR *E. coli* has been shown to be inhibited by molecules such as 4-hydroxy—tetralone, ursolic acid and its derivatives [169] and lysergol [170]. Berberine and palmatine inhibit MexAB-OprM from clinical isolates of MDR *P. aeruginosa* [171]. There are also complete extracts of plants with EPs inhibitory activity with clear synergistic effects with antibiotics in the treatment of MDR bacterial infections. The extract made from *Rhus coriaria* seeds have shown an obvious synergistic effect with

oxytetracycline, penicillin G, cephalexin, sulfadimethoxine and enrofloxacin against MDR clinical isolates of *P. aeruginosa*. This effect is mainly attributed to the inhibitory capacity of EPs of the phytochemicals present in the extract [172]. The activity of these plant molecules as inhibitors of microbial efflux pumps can act as restorers of antimicrobial susceptibility and open the door to combined antibiotic treatments, since these could exert their action more easily by not being expelled from the bacterial interior, allowing relive obsolete or discarded therapies due to this resistance mechanism [173]. A catechin, (-)-epigallocatechin gallate (EGCg), has shown sensitizing activity in *S. aureus* against tetracycline by inhibiting EPs such as Tet (K), increasing intracellular retention of the antibiotic and enhancing its effect [174]. Stilbenes also act as EPs inhibitors against antibiotic-resistant *Arcobacter butzleri*, reducing its resistance. Resveratrol and pinosylvin have also shown activity as resistance modulators being able to even reverse the resistance completely [175].

There are studies that state that certain polyphenols, such as catechins, can enter deeply into the structure of the lipid bilayer of bacterial membranes, causing significant thermotropic changes. Lipophilic hydrocarbons present in plant extracts are known to destabilize the cellular structure of the cytoplasmic membrane, increase its permeability and interact with hydrophobic portions of proteins [176]. This could explain the potentiation in the effect of certain antibiotics against resistant bacteria, as these compounds could increase antibiotic intake and interact with resistance proteins, hindering their activity. Specifically, (-)-epicatechin gallate (ECg) has a great affinity for the staphylococcal wall and its binding to it produces biophysical changes in it that are capable of dispersing the biosynthetic machinery responsible for resistance to β-lactam antibiotics [177]. This activity would explain the restoration of the sensitivity of bacteria resistant to traditional antibiotics through the use of polyphenolic compounds capable of interacting with bacterial membranes, as in the case of catechins capable of sensitizing MRSA against oxacillin and other β-lactam antibiotics thanks to its ability to integrate and interact with the cell membrane [178,179].

Plant extracts are also capable of exert antimicrobial activity against AMR bacteria and synergize with antibiotics. For instance, extracts of *Duabanga grandiflora* can restore MRSA's sensitivity to ampicillin. The mechanism proposed by the researchers is that the components of this extract can decrease the expression of the mecA gene that gives rise to the resistance protein PBP2a [180]. Extracts of *Acacia nilotica*, *Syzygium aromaticum* and *Cinnamum zeylanicum* exhibited antimicrobial capacity against a panel of AMR bacteria including clinical isolates and ATCC strains. Extract of *A. nilotica* showed MIC values as low as 9.75 μg/mL against *K. pneumoniae* ATCC-700803, *Salmonella typhimurium* ATCC-13311 and *E. faecalis* ATCC-29212 [181]. Extracts of *Salvia spp.* and *Matricaria recutita* have shown great synergy with the antibiotic oxacillin [182]. The multifactorial and multi-target character of the compounds that make up plant extracts can hinder the development of resistance by bacteria [80]. The molecular promiscuity of polyphenols, their multarget activity, the possibility of obtaining complex extracts containing multiple different polyphenols, and their synergistic effect in combined use with clinical antibiotics make natural antimicrobial compounds of plant origin ideal tools to be studied from the point of view of network pharmacology in the future. The evidence found in the combination studies between plant extracts and clinical antibiotics shows a synergistic enhancement that may be key to the fight against AMR bacteria. Although the development of new synthetic antibiotics is essential to continue the fight, the sensitization of resistant bacteria by phytochemicals is also crucial to achieving effective and long-lasting therapies [158].

Infections caused by bacteria forming biofilms are extremely difficult to treat and are much less susceptible to antibiotics [183,184]. One way to enhance the effect of an antimicrobial agent is to disrupt the biofilm that certain resistant bacteria form. Studies on *P. aeruginosa* showed that many natural products can inhibit biofilm formation or disrupt the previously formed biofilm: alginate lyase [185], ursolic acid [186], zingerone [187], cranberry proanthocyanidins [188], casbane diterpene [189], manoalide [190], solenopsin A [191], catechin [192], naringenin [193], ajoene [194], rosmarinic acid [195], eugenol [196], bergamottin [197], emodin [198] and baicalein [199] among others. These natural biofilm disrupting compounds could be a very valuable tool to be incorporated into joint

therapies with traditional antibiotics when treating infections caused by AMR bacteria. For example, cranberry proanthocyanidins enhanced the activity of gentamicin in an in vivo model of infection using *Galleria mellonella* [188]. In addition, some of these compounds have intrinsic antimicrobial activity on its own, which could further increase the potency of the treatment.

3.4. Development of Resistance to Natural Products

Historically, bacteria have managed to develop resistance to a greater or lesser extent against most antimicrobial agents used in medicine. Nevertheless, the ability of bacteria to develop a resistance mechanism against natural products is not well documented [200]. Due to the huge chemical and structural diversity among antimicrobial products of natural origin, it is often stated the difficulty for bacteria to avoid the action of NPs [201,202]. However, there are some recent studies that suggest that bacteria can develop certain levels of resistance against plant compounds, especially enteric bacteria [203]. The mechanisms of resistance behind these observations remain unknown and literature on the subject is scarce.

There are multiple mechanisms by which a bacterium can get rid of the action of an antimicrobial molecule: target alterations, expulsion or modification of the antibiotic, inactivation, reduced permeability and biofilm formation among others [204]. These resistant mechanisms can be spontaneously developed (mutations) or acquired (by transduction, transfection or conjugation processes) as shown in Figure 5. Understanding the mechanism by which bacteria can circumvent the action of antibiotics and how they acquire these capabilities is crucial to developing effective and lasting therapies.
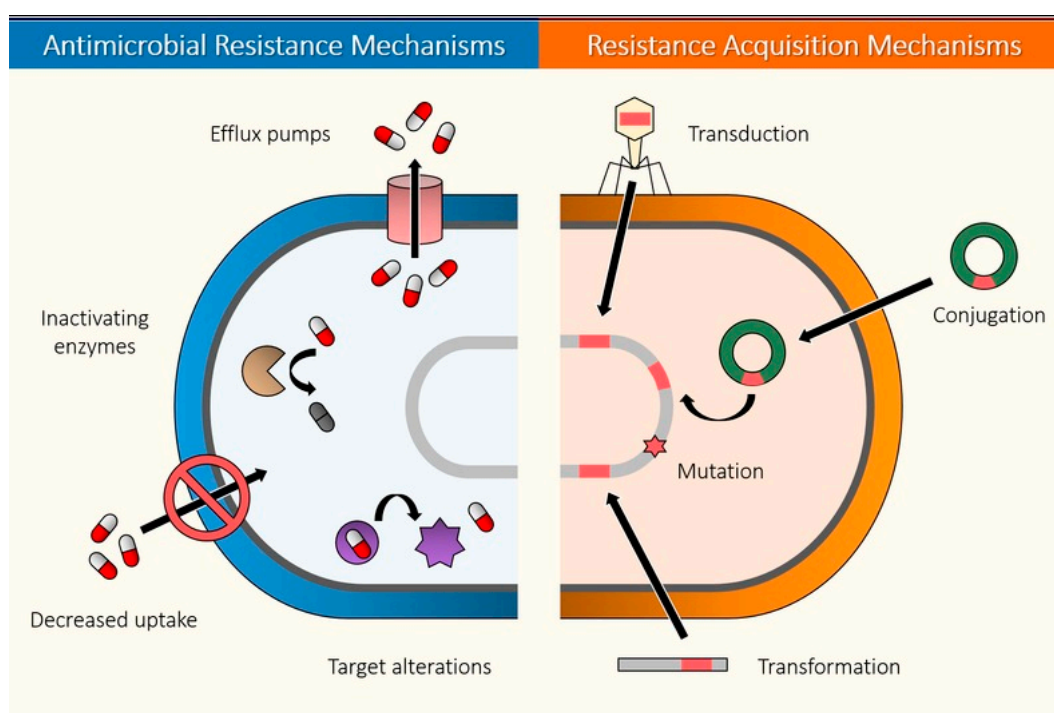
Figure 5. Antimicrobial resistance mechanisms and acquisition mechanisms in bacteria.

Depending on their properties, some products are more susceptible than others to the appearance of bacteria resistant to them. Molecules that attack highly conserved targets are less conducive to the appearance of bacteria with mutations in said targets that confer resistance to the antimicrobial in question, since modifying one or more fundamental routes or targets can imply an unbearable fitness cost for the bacteria [205].

On the other hand, molecules against less conserved molecular targets are more likely to promote the development of resistance mechanisms against them. Modification of less evolutionarily conserved or non-essential targets is easier for bacteria to assimilate since they have greater flexibility to modify the molecular target or adapt their metabolism without paying a high fitness cost. Although the acquisition of antimicrobial resistance mechanisms is often accompanied by reduced fitness in the absence of a selective environment, this loss of adaptive efficacy can be counteracted by compensatory mutations or modifications in epistasis [206].

Thanks to the multifactorial nature of the molecular promiscuity of naturally occurring antimicrobial compounds, bacteria experience difficulties in changing several molecular targets simultaneously [80]. Multiple simultaneous molecular changes in a bacterium to overcome the action of a multifactorial antimicrobial agent would very negatively affect its metabolism, that is, it would have a high fitness cost potentially unacceptable for its development. Likewise, mutations that carry a high fitness cost are less likely to persist in bacterial populations once the selective pressure disappears [207]. This cost would be higher if the molecular targets of the antimicrobial were highly evolutionary conserved molecules or routes, since they would be more difficult to change while maintaining the metabolic efficiency necessary for survival and competition with other living beings. Furthermore, there are studies that affirm that many of the natural antimicrobial compounds attack macromolecular structures such as the membrane or the bacterial wall and that this fact could hinder the appearance of resistance, given that they are very difficult targets to vary as a whole [208,209].

Despite the multiple possible mechanisms for acquiring existing resistances, the use of new technologies in NPs can help prevent their development. Based on new laboratory bacterial culture techniques, it has been possible to identify and isolate interesting natural compounds such as teixobactin. This molecule displays a mechanism of action that is capable of using the bacteria's own machinery to kill itself, in a similar way to how vancomycin, a really successful antibiotic, works. No resistant mutants have been found against teixobactin. Theoretically, the generation of resistant mutants to this compound is difficult, since its target is very conserved among the eubacteria, in addition to being exposed in the outermost part of Gram-positive bacteria. In addition, as teixobactin is produced by a Gram-negative bacterium, the molecule cannot re-enter the cell and exert its action due to the presence of the outer envelope characteristic of Gram-negative bacteria. This fact is crucial in the process of the eventual development of resistance, since the producing microorganism does not use a different metabolic route to avoid the action of the antibiotic it produces. Thus, in the absence of an intrinsic resistance mechanism in the producer, horizontal transfer of resistance genes to other species susceptible to teixobactin cannot occur [55].

Vancomycin, discovered in 1958, enjoyed a period of 30 years in which no bacterium resistant to its antibiotic action was identified, thanks to its potent and unusual mechanism of action. However, during the last 20 years, *S. aureus* strains resistant to this antibiotic have been detected [210]. One of the resistance mechanisms identified is the incorporation of D-Ala-D-lactate instead of the usual D-Ala-D-Ala at the dipeptide termini of nascent peptidoglycan, considerably reducing its binding affinity and formation disruption capacity of the bacterial wall. Other resistant strains identified have a thicker cell wall with free D-Ala-D-Ala ends that can sequester vancomycin and removing it from the place where the biosynthesis of the wall occurs [211]. Despite the emergence of these and other resistance mechanisms, researchers are currently working on vancomycin derivatives that have promising qualities that allow them to circumvent these resistance mechanisms and exert their antibiotic action. An example of this is the discovery of a new vancomycin resistance mechanism mediated by the activity of Atl amidase. This inhibition produces cellular morphological changes that reduce the action of vancomycin on the main target in the biosynthesis of the wall, increasing the tolerance of the pathogen against the antibiotic without any changes at the genetic level. The discovery of this target opens the door to the design of derivatives of vancomycin with a reduced affinity for Atl, resulting in greater efficacy against MRSA [46]. Another resistance mechanism found in *S. aureus*

against vancomycin is based on the thickening of the bacterial wall, which slows the penetration of vancomycin into the bacteria [212].

A possible strategy to prevent or slow the appearance of antimicrobial-resistant bacteria is the combined use of various agents that act against different molecular targets. In this way, the bacteria will have to adopt different resistance mechanisms, which would imply a greater and less likely adaptive cost. This hypothesis could support the use of plant extracts and essential oils in traditional medicine used for millennia, since these may be composed of dozens of different phytochemicals with different mechanisms of action. The combined activity of these molecules would hinder bacterial adaptation and extend the therapeutic shelf life of antimicrobial plant extracts.

Although the idea of the difficulty of acquiring resistance against complex plant extracts is widespread, some studies go in the opposite direction. It has been observed that certain antimicrobial extracts used against enterobacteria isolated from geckos from various environments in India have reduced effectiveness. The authors attribute this resistance to the variability and changing environment that has shaped the isolates collected and used in the assay. They suggest that exposure of geckos to medicinal plants may have caused a process of selecting the bacteria present in them, resulting in strains more resistant to plant compounds [203]. Mechanisms of possible resistance are not mentioned.

3.5. New Methodologies to Find Antimicrobial Compounds against AMR Bacteria

Currently, there are many methodologies capable of having a very positive impact on the discovery of new natural molecules with antimicrobial capacity against AMR bacteria. Some of these methodologies are the use of -omics technologies, network pharmacology, synergy studies and in silico trials.

Thanks to the -omics technologies, today it is known that genomes of bacteria such as actinomycetes are much more complex than previously thought in the mid-twentieth century and that there are multiple secondary metabolite gene clusters (SMGCs) that could produce new NPs. It is estimated that under the conditions of the classic fermentation studies for NP isolation, less than 10% of the SMGCs are active, which could be activated using genetic techniques and varying the culture conditions to reveal potential new NPs hidden inside of the "biosynthetic dark matter" [213]. By combining the progressive lowering of the massive sequencing of bacterial genomes and the advancement of the analysis and prediction software it will be possible to identify new SMGCs and their products [214,215]. The discovery and deepening of knowledge of NP-producing modular macroenzymes such as non-ribosomal peptide synthetases and polyketide synthetases open the door to new NPs production strategies based on combinatorial biosynthesis [15]. Scientists now have greater access to soil samples and other potential sources of NPs, which significantly increases the likelihood of finding new compounds. The use of non-laboratory-dependent metagenomic techniques and the heterologous expression of DNA extracted directly from complex samples will allow the identification and production of new NPs hitherto unknown or impossible to produce [216].

Other new technologies such as molecular docking or virtual simulations open the door to the effective discovery of new natural antimicrobial compounds unknown so far using computers [76,217]. In silico assays allow hundreds of thousands of molecules to be screened to efficiently select leaders, greatly reducing the cost of new drug development processes. Prediction via molecular docking or virtual simulation makes it possible to predict the interactions of a molecule with its target, obtaining huge amounts of valuable information and allowing the screening of drug libraries in a short time if the necessary computing capacity is available [218,219].

Emerging studies based on network pharmacology that expand the classic single-ligand-target viewpoint provide excellent opportunities for the development of new antimicrobial compounds. The study of the network pharmacology of phytochemicals based on their molecular promiscuity and multi-target capacity can help to better understand their antimicrobial mechanisms of action and to develop more effective therapies [220]. In turn, this point also has a positive impact on synergy studies

between antibiotics and phytochemicals such as those described in the previous sections, and they are currently showing such good results.

4. Conclusions and Future Perspectives

In conclusion, most NPs do not have sufficient therapeutic power to perform monotherapies based on them against antibiotic resistant bacteria, however, their joint application in combination therapy with traditional antibiotics could contribute to enhance their effect, reduce their dosage, side effects and improve its pharmacokinetics and pharmacodynamics properties. Natural antimicrobial products offer a promising avenue of study in the field of antibiotic development thanks to their unique properties, natural availability and enormous chemical diversity. The prospects in the discovery of new NPs with antibiotic activity are very positive. There is a tendency to revise the traditional sources of NPs that offered such good results during the "Golden Age" [221]. The use of new technologies and applications of non-existent knowledge during that age opens the door to the second era of massive discovery of molecules with remarkable and novel biological activity against AMR bacteria.

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5. DISCUSIÓN

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A continuación, se abordan las cuestiones más relevantes surgidas en la línea de investigación de la presente Tesis Doctoral con la finalidad de esclarecer el rol de los compuestos antimicrobianos de origen natural en las terapias frente a infecciones resistentes a antibióticos.

¿Poseen capacidad antimicrobiana los compuestos de origen natural?

Desde la Antigüedad, los seres humanos han empleado sustancias naturales en forma de ungüentos, cataplasmas o infusiones para el tratamiento de diversas enfermedades, incluidas las infecciones [62-66]. El descubrimiento de la penicilina durante la primera mitad del siglo XX marcó un hito en la historia de la medicina [3], al cual siguieron los descubrimientos de múltiples familias de antibióticos clásicos como las sulfamidas [6], los aminoglicósidos [10], las teraciclinas [12], los macrólidos [13] o los glicopéptidos [14], entre otros. A pesar del gran periodo de esplendor y efectividad del que gozaron estos antibióticos durante décadas, la eventual aparición de bacterias resistentes a ellos ha supuesto una gran amenaza para la eficacia de terapias antibióticas. Esta amenaza ha propiciado el estudio de terapias alternativas o complementarias, como aquellas basadas en antimicrobianos de distintos orígenes naturales.

Los estudios bibliográficos y experimentales llevados a cabo en la presente Tesis Doctoral corroboran la capacidad antimicrobiana de multitud de compuestos de origen natural tanto de origen vegetal, animal, fúngico como bacteriano. Los extractos vegetales complejos como los extractos de té verde [189] o plantas del género *Cistus* [80, 180] y los polifenoles puros descritos en el Capítulo 1 de la presente Tesis Doctoral como la punicalagina [131], la quercetina [190], el resveratrol [191] o el kaempferol [143] o compuestos de origen natural como la vejovina [192], la mucroporina [193], la polimixina [194] o la albomicina [195] entre muchos otros descritos en el Capítulo 3, han demostrado poseer capacidad antimicrobiana frente a diferentes especies bacterianas de gran interés clínico como *S. aureus, E. coli, P. aeruginosa* o *K. pneumoniae*, incluyendo cepas resistentes a antibióticos. La actividad antimicrobiana de estos compuestos es muy variada y abarca rangos de valores de CMI desde las pocas decenas de µg/mL para los más potentes, hasta los mg/mL correspondientes a los compuestos menos activos. Estos resultados están en consonancia con los obtenidos por otros investigadores. De esta manera, se pone de manifiesto que la literatura sobre la capacidad antimicrobiana de compuestos naturales es muy abundante y genera un gran interés científico.

La tendencia observada en los repositorios virtuales de artículos científicos apunta a que el número de investigaciones relacionadas con los productos antimicrobianos naturales va a seguir creciendo en el futuro próximo.

¿Cuáles son los mecanismos de acción de los compuestos antimicrobianos naturales?

La gran variedad química estructural de los compuestos naturales es la causa de que estos presenten mecanismos de acción muy diversos, los cuales se describen en los Capítulos 1 y 3 de la presente Tesis Doctoral. Los mecanismos de acción antimicrobiana más comúnmente hallados en la presente investigación están relacionados con la inhibición de la biosíntesis de proteínas [196-201] y de la pared celular bacteriana [202-207]. No obstante, también se han descrito diversos mecanismos relacionados con múltiples alteraciones estructurales de la membrana citoplasmática, resultando en formación de poros [203], deslocalización de proteínas [208] u otro tipo de daños [193, 194, 209]. Otros mecanismos moleculares a través de los cuales los compuestos antimicrobianos de origen natural ejercen su acción son la producción de especies reactivas de oxígeno en el interior de las bacterias [210, 211], la inhibición de girasas de ADN [212, 213], la inhibición de la biosíntesis de ADN y ARN [214, 215], la inhibición de la adhesión a tejidos [216], la activación de la autolisis [217, 218], la inhibición de la peptidasa de señal tipo I [219] o de la ribonucleasa P [220]. Otras dianas moleculares potenciales para los polifenoles pueden ser las proteínas implicadas en la biosíntesis de peptidoglucanos como las familias de proteínas PBP, Mra o Mur. A este respecto, los resultados de las técnicas de cribado virtual realizadas en el Capítulo 1 de la presente Tesis Doctoral para el acoplamiento de los 931 polifenoles de la base de datos Phenol-Explorer 3.6 contra nueve enzimas implicadas en la biosíntesis de peptidoglucano de seis especies bacterianas distintas revelan que ciertas teaflavinas, proantocianidinas y catequinas son inhibidores más potentes que los usados experimentalmente en la actualidad. Esto es un ejemplo del poder del cribado molecular *in silico*, como técnica complementaria para acelerar el descubrimiento de nuevos agentes antimicrobianos eficaces.

La gran variedad de dianas moleculares de los compuestos antimicrobianos de origen natural supone uno de los principales atractivos para su investigación en el desarrollo terapéutico. Su versatilidad y promiscuidad molecular los hace ideales para el desarrollo de terapias combinatorias que ataquen simultáneamente múltiples dianas moleculares bacterianas para maximizar el efecto antimicrobiano y disminuir la capacidad de respuesta bacteriana. Este

nuevo paradigma polifarmacológico o de *network pharmacology* se aleja de la visión clásica de "un compuesto, una diana" y postula que la interacción entre ligando y diana es más compleja, pudiendo un ligando interactuar con múltiples dianas y una diana ser objetivo de múltiples ligandos [221]. Esta nueva aproximación permite reposicionar compuestos ya conocidos y estudiar su eficacia frente a dianas de nuevas patologías o frente a nuevas dianas de patologías anteriormente estudiadas, aumentando exponencialmente el potencial terapéutico de la vasta diversidad de compuestos de origen natural existentes. Los avances en biología computacional para el modelado polifarmacológico serán claves para el desarrollo de estas nuevas estrategias [222-224].

¿Para qué tipo de infecciones bacterianas podrían usarse los compuestos naturales antimicrobianos?

Los resultados obtenidos en la presente Tesis Doctoral indican que los compuestos antimicrobianos de origen natural poseen actividad antimicrobiana frente a un amplísimo abanico de especies bacterianas patogénicas. Además, poseen niveles de actividad distintos en función de las propiedades y características de las bacterias diana. Por ejemplo, ciertos compuestos como la tobramicina, la kanamicina o la cecropina son más eficaces frente a infecciones causadas por bacterias gram-negativas [225-227], mientras que, compuestos como la daptomicina, la pristinamicina o la royalisina muestran mayor eficacia frente a bacterias gram-positivas [214, 228, 229]. Asimismo, basándonos en los resultados obtenidos en el Capítulo 2, se observa que los extractos vegetales antimicrobianos de *C. salviifolius* y *P. granatum* poseen niveles de actividad antimicrobiana diferentes en función del perfil de resistencias a antibióticos de las bacterias tratadas, incluso dentro de la misma especie bacteriana. Este estudio indica que el extracto de *C. salviifolius* es más eficaz contra aislados clínicos de *S. aureus* resistentes a antibióticos betalactámicos, mientras que el extracto de *P. granatum* muestra mayor eficacia frente a aislados sensibles a quinolonas. Estos hechos demuestran que existen diversos parámetros, como la estructura molecular del compuesto o de sus componentes, su diana molecular o su mecanismo de acción, que influyen directamente en la efectividad de los agentes antimicrobianos de origen natural. Conociendo dichos parámetros, podrían desarrollarse terapias antibióticas personalizadas basadas en las características de la infección bacteriana de cada paciente para así obtener unos resultados óptimos para su salud.

¿Por qué es crucial hallar estrategias alternativas o complementarias al uso de antibióticos?

Debido al progresivo aumento en la resistencia a antibióticos por parte de ciertas bacterias, los antibióticos actuales han ido perdiendo eficacia paulatinamente. Además de la reducción de la efectividad de los antibióticos, actualmente existe un pronunciado declive en el descubrimiento y producción de nuevos antibióticos por parte de la industria farmacéutica [41]. La investigación en antibióticos se ha convertido en una inversión arriesgada y poco atractiva para la industria farmacéutica debido a la amenaza de la aparición de resistencias que inactiven sus nuevos antibióticos en poco tiempo sin poder rentabilizar la cuantiosa inversión necesaria para el desarrollo de nuevos fármacos [44, 45]. Para intentar obtener resultados terapéuticos adecuados frente a bacterias resistentes, se ha tenido que recurrir a aumentar las dosis de antibióticos o incluso a usar combinaciones de varios antibióticos [230, 231]. Este incremento en la administración de antibióticos conlleva un aumento proporcional en las consecuencias negativas para los pacientes, los conocidos como efectos secundarios. Por ejemplo, el uso combinado de vancomicina y piperacilina/tazobactam, un tratamiento administrado comúnmente para tratar infecciones severas en hospital, ha sido asociado con lesiones renales agudas [232]. Entre los efectos secundarios más comunes derivados del uso de antibióticos se encuentran el sarpullido, los mareos, las náuseas, la diarrea, las infecciones por hongos y las reacciones alérgicas. Estas complicaciones pueden ser graves y potencialmente mortales en ciertos casos [233]. Además, las infecciones nosocomiales multirresistentes a antibióticos suponen una grave amenaza inmediata para la salud de la población mundial [234, 235], ya que pueden causar infecciones fatales a pacientes ingresados en recintos hospitalarios y sus genes de resistencia a antibióticos pueden extenderse rápidamente hacia otras poblaciones bacterianas [236]. Por todo ello, la búsqueda de tratamientos alternativos para el tratamiento de este tipo de infecciones es un elemento prioritario para la salud humana a nivel global.

¿Podrían los antibióticos ser reemplazados por compuestos naturales antimicrobianos?

En la actualidad es inevitable el uso de antibióticos, ya que a menudo suponen la herramienta terapéutica más potente y efectiva disponible frente a las infecciones bacterianas. Estrategias como las propuestas en la presente Tesis Doctoral, basadas en los antimicrobianos naturales, se encauzan hacia evitar el sobreuso de antibióticos y aumentar la seguridad de los tratamientos frente a infecciones resistentes con el menor número de efectos secundarios asociados. La

hipótesis más plausible actualmente radica en la aplicación conjunta de antibióticos tradicionales con otros compuestos adyuvantes que potencien su actividad o reviertan las resistencias bacterianas sin daños adicionales para los pacientes. En la actualidad existen evidencias de que algunos compuestos naturales como el ácido clavulánico, los péptidos antimicrobianos y múltiples fitoquímicos contribuyen a mejorar o recuperar la efectividad de diversos antibióticos sin efectos adversos para los pacientes [237-239], convirtiéndolos en potenciales herramientas terapéuticas de gran valor. Los compuestos naturales antimicrobianos pueden beneficiar a la terapia antibiótica mediante la inhibición de los mecanismos de resistencia bacterianos, como es el caso del ácido clavulánico inhibiendo las betalactamasas [240]; facilitando su entrada en la bacteria mediante la formación de poros o disrupción de las membranas bacterianas, como en el caso de la mucroporina [193] o la polimixina [194]; o bien reforzando la capacidad antimicrobiana por adición de más compuestos antimicrobianos sin incurrir en más efectos secundarios o incluso aportando otros beneficios sanitarios que puedan contribuir a una mejor recuperación del paciente.

Para revelar el verdadero potencial de los compuestos naturales antimicrobianos y poder emplearlos de forma segura y eficaz en terapia, es necesario el desarrollo de ensayos preclínicos y clínicos, lo cual supone un largo camino que aún queda por recorrer. No obstante, las expectativas en este ámbito son positivas y existen indicios que apuntan a que los compuestos antimicrobianos naturales podrían constituir una herramienta terapéutica útil en el futuro próximo.

¿Tienen los compuestos naturales antimicrobianos la misma potencia que los antibióticos?

Aunque existen cientos de miles de compuestos naturales antimicrobianos diferentes, la mayoría de los compuestos descritos en el presente trabajo no poseen suficiente potencia terapéutica por sí mismos para realizar monoterapias basadas en ellos contra bacterias resistentes a los antibióticos. Los antibióticos son una herramienta poderosísima para tratar las enfermedades infecciosas causadas por bacterias patógenas, ejerciendo su acción a concentraciones muy bajas con un rango de valores de CMI de unos pocos µg/mL o incluso menores. Por este motivo, los compuestos antimicrobianos naturales deberían emplearse en terapias combinatorias con antibióticos u otros compuestos naturales con el fin de alcanzar rangos terapéuticos adecuados. No obstante, existen estudios que describen compuestos naturales con una potencia antimicrobiana excepcionalmente elevada frente a ciertas especies

bacterianas como la quercetina, el kaempferol [143] o el neolignano eupomatenoide-5 [241], que exhiben valores de CMI tan bajos como 1,95 µg/mL, 7,8 µg/mL y 4 µg/mL frente a *S. aureus*, respectivamente.

Por otro lado, los compuestos naturales de mayor potencia pueden erigirse como cabezas de serie para la búsqueda de nuevos derivados basados en ellos que presenten una mayor potencia, un mejor índice terapéutico, menores efectos secundarios y/o coste más reducido para el sistema sanitario. Para ello, las técnicas de cribado HTS e *in silico* se erigen como herramientas de futuro imprescindibles para este campo de investigación.

¿Poseen los compuestos naturales de origen natural otras propiedades distintas a la antimicrobiana potencialmente beneficiosas en el ámbito sanitario?

Los resultados obtenidos apuntan a que los compuestos antimicrobianos naturales podrían aportar otras propiedades adicionales a la terapia antibiótica más allá del efecto antimicrobiano. Muchos de los compuestos antimicrobianos naturales que se estudian en la actualidad son moléculas que forman parte de la dieta humana común, como son los polifenoles presentes en frutas, verduras e infusiones o los péptidos antimicrobianos presentes en la miel de abeja. En la mayoría de los casos, el metabolismo del ser humano es capaz de asimilar y tolerar dichas moléculas en su organismo, abriendo la puerta a posibles combinaciones de diversos productos naturales para potenciar su actividad antimicrobiana sin resultar en efectos tóxicos. Estas combinaciones de compuestos naturales pueden actuar de forma sinérgica sobre distintas dianas moleculares para potenciar su capacidad antimicrobiana y dificultar el posible desarrollo de resistencias bacterianas [242-244]. Además del beneficio que aportan a la terapia antibiótica, múltiples compuestos naturales, especialmente fitoquímicos como la curcumina [245], la quercetina [246] o el resveratrol [247], pueden impactar de forma positiva sobre la salud del paciente que los recibe. De esta manera, a algunos compuestos naturales se les atribuyen propiedades beneficiosas para la salud humana como la actividad antioxidante, actividad protectora cardiovascular, actividad antiinflamatoria, actividad anticancerígena o capacidad para prevenir las patologías asociadas al envejecimiento, entre otras [248-255]. Esta actividad multifactorial beneficiosa para la salud humana unida a su promiscuidad molecular antimicrobiana [77, 131] convierten a los compuestos antimicrobianos naturales en excelentes candidatos para el estudio de terapias antibióticas basadas en su uso.

¿Tienen los compuestos naturales antimicrobianos aplicaciones fuera del ámbito clínico?

La lucha contra las bacterias resistentes a antibióticos se libra en distintos frentes, siendo el ámbito clínico solamente uno de ellos. Los compuestos antimicrobianos de origen natural pueden ser empleados en sectores como la industria alimentaria, la cosmética, la agricultura y la ganadería para minimizar el uso de antibióticos y el paso masivo de éstos a la biosfera. Reducir la cantidad de antibióticos xenobióticos en el entorno es una estrategia fundamental para disminuir la cantidad de potenciales bacterias multirresistentes a fármacos y ralentizar la propagación de los genes de resistencia entre poblaciones [256].

La industria alimentaria es un claro ejemplo de la aplicación de antimicrobianos naturales, sobre todo de origen vegetal con gran contenido en polifenoles, que actúan como conservantes y/o antioxidantes. Algunos de los compuestos más usados para preservar los productos alimentarios son los aceites esenciales extraídos de cebolla [257], ajo [258], romero [259] o semilla de uva [260], entre otros [261]. Una situación similar se vive en la industria cosmética, en donde los ingredientes de origen natural cuentan con una mayor aceptación que los de origen químico [262]. La agricultura y la ganadería también pueden beneficiarse del uso de compuestos antimicrobianos naturales, ya que existen estudios que apuntan a que el uso de compuestos naturales antimicrobianos y antioxidantes son capaces de extender la preservación de productos tanto cárnicos como vegetales [263].

¿Son las bacterias capaces de desarrollar mecanismos de resistencia frente a los compuestos naturales antimicrobianos tal y como ha ocurrido con los antibióticos?

Esta es una cuestión fundamental a la hora de estimar el potencial terapéutico de los compuestos antimicrobianos naturales. Históricamente, las bacterias han logrado desarrollar resistencia en mayor o menor medida contra la mayoría de los agentes antimicrobianos utilizados en medicina. Sin embargo, la capacidad de las bacterias para desarrollar mecanismos de resistencia contra productos naturales no está bien documentada y la literatura sobre el tema es escasa [264]. Los bajos niveles de resistencia a los compuestos de origen natural a menudo se atribuyen a la gran diversidad química y estructural entre los productos antimicrobianos de origen natural, su promiscuidad molecular, la polifarmacología intrínseca y a su evolución constante al formar parte de organismos vivos que compiten por su supervivencia y que han tenido que hacer frente a las infecciones bacterianas durante su

evolución [265, 266]. Además, es posible que los compuestos antimicrobianos naturales, que por lo general poseen una potencia antimicrobiana menor que los antibióticos sintéticos que se diseñan frente a una sola diana terapéutica, hayan gozado de una mayor permisividad evolutiva en cuanto al desarrollo de mecanismos de resistencia frente a ellos al no ejercer una presión selectiva tan intensa como los antibióticos.

Gracias al carácter multifactorial de los compuestos antimicrobianos de origen natural, que está relacionado con su promiscuidad molecular de, las bacterias sufren dificultades para cambiar varias dianas moleculares simultáneamente [131]. Múltiples cambios moleculares simultáneos en una bacteria para sobreponerse a la acción de un agente antimicrobiano multifactorial afectarían catastróficamente al metabolismo bacteriano, resultando en un decrecimiento de su capacidad de replicarse y sobrevivir en un entorno competitivo. Las bacterias expuestas a compuestos antimicrobianos con actividad multi-diana tendrían grandes dificultades para desarrollar mecanismos de resistencia frente a estos ya que su desarrollo supondría un elevado *fitness cost* (repercusión sobre la capacidad para replicarse y sobrevivir en un entorno competitivo) potencialmente inasumible para su desarrollo ulterior. Asimismo, las mutaciones que conllevan un alto *fitness cost* son menos propensas a persistir en las poblaciones bacterianas una vez que la presión selectiva desaparece [267]. Este coste sería mayor si las dianas moleculares del antimicrobiano fueran moléculas o rutas muy conservadas evolutivamente, ya que resultarían más difíciles de cambiar manteniendo la eficiencia metabólica necesaria para la supervivencia y la competición con otros seres vivos. Además, existen estudios que afirman que muchos de los compuestos antimicrobianos naturales atacan estructuras macromoleculares como la membrana o la pared bacteriana y que este hecho podría dificultar la aparición de resistencias, dado que son dianas muy difíciles de variar en su conjunto [242, 243].

Los resultados presentados en el Capítulo 2 de la presente Tesis Doctoral apuntan a que los niveles de resistencia de diferentes cepas de *S. aureus* frente a distintos antibióticos de uso clínico están relacionados con la susceptibilidad de las cepas frente a los extractos vegetales de *C. salviifolius* y *P. granatum*. En general, el extracto de *C. salviifolius* presenta mayor eficacia frente a bacterias resistentes a antibióticos betalactámicos, mientras que el extracto de *P. granatum* posee mayor eficacia frente a bacterias sensibles a quinolonas y oxacilina. El análisis estadístico MCA sugirió un mecanismo de acción de los extractos relacionado con la alteración de la membrana plasmática o la pared celular. Este resultado es consistente con lo reportado

previamente en la bibliografía y recogido en otros capítulos de la presente Tesis Doctoral. Estos datos anteriores postulan a estos mecanismos como la principal diana molecular de polifenoles como los taninos hidrolizables, componentes principales en ambos extractos. Se postula que la mayor actividad del extracto de *C. salviifolius* frente a cepas resistentes a antibióticos podría atribuirse a su contenido en flavonoides con actividad específica anti-SARM debido a su estructura química [268] o a la sinergia con los taninos hidrolizables presentes en el extracto previamente descrita por el grupo de investigación [131]. Estos resultados son consistentes con los obtenidos por otros autores como Atef M. *et al.*, en los que se correlacionó la actividad antimicrobiana diferencial de extractos vegetales frente a varias cepas de *P. aeruginosa* con sus diferentes perfiles de resistencia a antibióticos [269]. Estas observaciones abren la puerta a realización de futuros estudios centrados en la relación entre la resistencia bacteriana a ciertas clases de antibióticos y moléculas naturales con una actividad antimicrobiana mejorada. Los resultados obtenidos sobre el poder y mecanismo antimicrobiano del extracto de *C. salviifolius* lo convierten en un candidato prometedor para protagonizar próximas líneas de investigación.

Por otro lado, existen algunos estudios recientes que sugieren que las bacterias pueden desarrollar ciertos niveles de resistencia contra los compuestos vegetales, especialmente las bacterias entéricas [270]. Los mecanismos de resistencia detrás de estas observaciones siguen siendo desconocidos y la literatura sobre el tema es escasa, por lo que es necesario un permanente y cuidado seguimiento de este aspecto para apoyar los nuevos desarrollos basados en compuestos de origen natural.

¿Qué podemos hacer para evitar o frenar la aparición de bacterias resistentes a los antibióticos y antimicrobianos naturales?

Además de las propiedades intrínsecas de los compuestos antimicrobianos empleados, existen otros factores dependientes del comportamiento de los seres humanos que repercuten directamente en la capacidad de las bacterias para desarrollar y propagar las resistencias adquiridas. Estos factores son conocidos como la resistencia debida a las actividades antropogénicas [30]. Un ejemplo es el uso indiscriminado y abusivo de antibióticos en la sociedad, tanto en el ámbito clínico como en el industrial, que ha acelerado el proceso de adquisición de resistencias y ha potenciado su diseminación al generar una presión selectiva en el entorno que favorece el desarrollo de las bacterias resistentes. Se estima que la gran

mayoría de resistencias a antibióticos no surgen de forma espontánea en la naturaleza, sino que es impulsada por los antibióticos presentes en la biosfera resultado de la actividad humana [271]. Muestra de estos procesos que generan gran cantidad de antibióticos xenobióticos en la naturaleza son los vertidos de ciprofloxacino en ríos en cantidades superiores a 50 kg por día por la industria farmacéutica en Hyderabad, en la India [272]. La regulación por parte de las autoridades pertinentes es fundamental para evitar estos hechos nocivos para la salud humana a nivel global.

La falta de higiene y saneamiento también es un factor decisivo a la hora del surgimiento de bacterias resistentes y su propagación. La concienciación de la sociedad es una herramienta crucial para potenciar un uso más responsable de los antibióticos y antimicrobianos, independientemente de cuál sea su origen. Se deben potenciar comportamientos que incluyen la finalización de los tratamientos antibióticos prescritos por los profesionales sanitarios, evitar la automedicación, tomar medidas de prevención y protección personal cuando haya riesgo de infección y mantener una buena higiene personal y del entorno.

¿Quedan compuestos de origen natural con capacidad antimicrobiana por descubrir o identificar en el futuro?

El número de compuestos naturales con capacidad antimicrobiana descubiertos en la actualidad es enorme. Sin embargo, se estima que el número de compuestos aún por descubrir es abrumadoramente mayor [273, 274]. Las perspectivas apuntan a que en los próximos años su número podría aumentar de manera exponencial, como afirman los estudios de Harvey *et al.* [275] o Lewis K. [276]. Existe también una tendencia a revisar las fuentes tradicionales de compuestos naturales que ofrecieron tan buenos resultados durante la "Edad de Oro" del descubrimiento de antibióticos [277]. El uso de nuevas tecnologías y la aplicación de conocimientos inexistentes durante la "Edad de Oro" abren la puerta a una segunda era de descubrimiento masivo de moléculas con una actividad biológica notable y novedosa [275]. Por ejemplo, hoy se sabe que los genomas de bacterias como los actinomicetos son mucho más complejos de lo que se pensaba a mediados del siglo XX y que existen múltiples grupos de genes de metabolitos secundarios (SMGC) que podrían producir nuevos compuestos naturales. Se estima que, bajo las condiciones de los estudios de fermentación clásicos para el aislamiento de compuestos naturales, menos del 10 % de los SMGC están activos. Éstos podrían ser activados utilizando técnicas genéticas y variando las

condiciones de cultivo para revelar nuevos compuestos naturales potenciales ocultos dentro de la "materia biosintética oscura" [261]. Al combinar el abaratamiento progresivo de la secuenciación masiva de genomas bacterianos y el avance del software de análisis y predicción, será posible identificar nuevos SMGC y sus productos [278, 279]. El descubrimiento y la profundización del conocimiento de las macroenzimas modulares productoras de compuestos naturales, como las sintetasas peptídicas no ribosómicas y las sintetasas de poliquétidos abren la puerta a nuevas estrategias de producción de compuestos naturales basadas en la biosíntesis combinatoria [280]. Por otro lado, los científicos ahora tienen un mayor acceso a muestras de suelo y otras fuentes potenciales de compuestos naturales, lo que aumenta significativamente la probabilidad de encontrar nuevos compuestos. Un claro ejemplo es la existencia de la iniciativa internacional *Small World*, en la cual estudiantes de todo el mundo aíslan bacterias del suelo de su entorno local con el fin de descubrir nuevos antibióticos [281]. El uso de técnicas metagenómicas no dependientes de laboratorio y la expresión heteróloga de ADN extraído directamente de muestras complejas permitirá la identificación y producción de nuevos compuestos naturales hasta ahora desconocidos o imposibles de producir [282]. Otras nuevas tecnologías, como las simulaciones por ordenador y el cribado virtual abren la puerta al descubrimiento efectivo de nuevos compuestos antimicrobianos naturales [283] y al reposicionamiento de compuestos naturales ya conocidos que pueden volver cribarse frente a las estructuras de nuevas dianas bacterianas con potencial farmacológico [222].

¿Cómo podrían administrarse los compuestos antimicrobianos de origen natural al ámbito clínico?

Una de las potenciales limitaciones para la aplicación clínica de algunos compuestos de origen natural podría ser su vía de administración clínica para alcanzar rangos de concentración terapéuticos. En la actualidad existen algunos estudios de farmacocinética, farmacodinámica y biodisponibilidad de compuestos naturales como la quercetina, el resveratrol o el pteroestilbeno, no obstante, dichos estudios muestran resultados contradictorios o inconsistentes, lo que se atribuye al empleo de técnicas y métodos poco optimizados o más bien poco validados como técnicas de referencia a nivel internacional [284, 285]. Se necesitan más estudios para probar los parámetros farmacocinéticos y farmacodinámicos reales que presentarían los compuestos naturales ingeridos o inyectados y conocer qué transformaciones

sufrirían por parte de las enzimas o incluso la microbiota humana hasta llegar a su diana molecular. Los novedosos sistemas de administración dirigida de fármacos o administración inteligente podrían ser clave para lograr rangos terapéuticos eficaces en pacientes [286].

¿Qué beneficios puede reportar la investigación de los antimicrobianos de origen natural para los retos de la sociedad establecidos en las estrategias nacionales e internacionales de investigación?

El objetivo último y fundamental del presente trabajo de investigación científica es hallar soluciones a problemas presentes en la sociedad para contribuir a su desarrollo. La presente Tesis Doctoral se alinea pues con los siguientes Objetivos de Desarrollo Sostenible (ODS) de las Naciones Unidas:

ODS 3 (salud y bienestar): el desarrollo de nuevas terapias basadas en agentes antimicrobianos naturales para combatir las enfermedades infecciosas resistentes a antibióticos tiene un impacto directo en la salud humana a nivel global. El hallazgo de herramientas terapéuticas más eficaces y/o seguras que las existentes en la actualidad mejora las probabilidades de éxito de los tratamientos frente a infecciones.

ODS 6 (agua limpia y saneamiento): el uso de terapias basadas en antimicrobianos de origen natural supone la reducción en el uso y producción de antibióticos tradicionales. La industria productora de antibióticos es altamente contaminante que produce grandes cantidades de vertidos de xenobióticos a las aguas, convirtiéndolas en perjudiciales para la salud y no aptas para el consumo humano.

ODS 9 (industria, innovación e infraestructuras): la investigación en el descubrimiento y uso de antimicrobianos naturales supone una estrategia innovadora a nivel global. Las materias y tecnologías empleadas en la obtención de los compuestos antimicrobianos de origen natural resultan más limpias, sostenibles y seguras que las involucradas en la producción de antibióticos de síntesis química tradicional.

ODS 15 (vida de ecosistemas terrestres): el uso masivo de antibióticos en industrias como la ganadera produce el paso de ingentes cantidades de xenobióticos al suelo, produciendo contaminación y promocionando la aparición y propagación de bacterias multirresistentes a antibióticos. La minimización del uso de antibióticos gracias al uso alternativo o complementario de compuestos antimicrobianos de origen natural reduciría la contaminación de los ecosistemas terrestres.

En paralelo a los ODS anteriormente mencionados, la presente investigación se alinea con los desafíos sociales de investigación y desarrollo marcados por el Programa Horizonte 2020 de la Unión Europea:

- **Desafío social número 1 (salud, cambio demográfico y bienestar):** el hallazgo de nuevas terapias basadas en compuestos antimicrobianos naturales más eficaces e inocuas repercute directamente en la salud y bienestar del conjunto de la sociedad. Además, existen apartados dentro de este desafío exclusivamente para combatir el aumento de la resistencia bacteriana a antibióticos, que es otro de los objetivos principales del presente trabajo.

- **Desafío social número 2 (seguridad alimentaria, agricultura y silvicultura sostenibles, investigación marina y marítima y de aguas continentales y bioeconomía):** la reducción en el uso de antibióticos de forma masiva en la industria alimentaria, agricultura y acuicultura promovida por el uso de compuestos antimicrobianos de origen natural resultaría en un beneficio para la salud de la población. La producción de los compuestos antimicrobianos empleados como herramienta terapéutica o conservantes puede promover la creación de empresas de base biológica, cumpliendo otro de los subapartados de este desafío social.

Al mismo tiempo, la presente Tesis Doctoral también se alinea con los objetivos generales de los Ejes de Desarrollo fijados por la Estrategia de Especialización Inteligente en Investigación e Innovación de la Comunidad Valenciana (RIS3-CV):

- **Eje de Desarrollo 1A (posicionar a la Comunidad Valenciana como referente global en la producción de alimentos y cosmética saludables y de calidad, orientados a las necesidades de las personas):** la disminución o reemplazo de los conservantes

químicos por antimicrobianos de origen natural más seguros, inocuos o mejor valorados por los consumidores constituye una mejora sustancial en la calidad de los productos producidos.

- **Eje de Desarrollo 1C (ser un referente en la producción sostenible de alimentos, cosmética y productos del hogar teniendo en cuenta factores económicos, medioambientales y un uso adecuado de los recursos naturales):** en consonancia con el objetivo anterior, el reemplazo de productos químicos por otros de origen natural más seguros para la salud humana y el medioambiente contribuye a la sostenibilidad del sistema productivo valenciano.

- **Eje de Desarrollo 1D (impulsar la gestión personalizada de la salud, la prevención y el diagnóstico):** el desarrollo de terapias personalizadas es uno de los objetivos de la investigación llevada a cabo en este trabajo. Los resultados obtenidos en el Capítulo 2 abren la puerta al diseño de terapias personalizadas empleando diferentes extractos vegetales en base al perfil de resistencia a antibióticos de las cepas bacterias que causan una infección concreta en cada paciente.

- **Eje de Desarrollo 1F (lograr productos y servicios sanitarios más eficientes y orientados a mercado):** el hallazgo de métodos más eficientes para hora de tratar las infecciones resistentes a antibióticos es el objetivo principal de la presente Tesis Doctoral.

Finalmente, es necesario abordar la cuestión que dio origen a la presente Tesis Doctoral:

¿Suponen los compuestos antimicrobianos de origen natural una verdadera oportunidad para el tratamiento de enfermedades infecciosas resistentes a antibióticos?

Los resultados expuestos en la presente Tesis Doctoral afirman que el uso de ciertos compuestos naturales y sus combinaciones sí constituyen estrategias prometedoras para el tratamiento de las infecciones resistentes a antibióticos. No obstante, se requieren más esfuerzos para profundizar en la investigación, el desarrollo y la innovación en compuestos antimicrobianos de origen natural para obtener las terapias seguras, eficaces y duraderas que el mundo necesita.

6. CONCLUSIONES

CONCLUSIONES

1. Existen numerosas evidencias de compuestos de origen natural que poseen capacidad antibacteriana probada, siendo algunos eficaces frente a bacterias resistentes a antibióticos especialmente aquellas responsables de infecciones nosocomiales.

2. Dentro de los compuestos naturales, los polifenoles de plantas suponen un arsenal antimicrobiano aún por explorar, tanto de forma aislada como en combinación con antibióticos.

3. Por lo general, los polifenoles poseen una mayor actividad antimicrobiana frente a bacterias gram-positivas que frente a gram-negativas.

4. Los mecanismos de acción de los polifenoles son variados y multifactoriales: perturbación de la pared celular y/o de la membrana plasmática, alteración o modulación de receptores de membrana o canales iónicos, inhibición de metabolitos bacterianos o de la formación de biofilm. Se postula que la perturbación de la estructura o de la síntesis de los componentes de la pared celular y/o la membrana lipídica es el mecanismo predominante para la mayoría de los polifenoles de plantas.

5. Dado su carácter multifactorial y promiscuidad molecular, el estudio de los compuestos naturales como antimicrobianos debe abordarse a través de la polifarmacología, la farmacología de sistemas y el cribado virtual computacional. Esto permitirá aumentar la eficacia de compuestos nuevos, reposicionar compuestos ya conocidos y reducir los posibles efectos secundarios.

6. Los extractos vegetales ricos en polifenoles de *C. salviifolius* (rico en taninos hidrolizables y flavonoles) y *P. granatum,* (rico en taninos hidrolizables)*,* han demostrado poseer actividad antibacteriana frente a aislados clínicos de *S. aureus*, incluyendo cepas SARM.

7. La susceptibilidad de los aislados clínicos de *S. aureus* frente a los extractos vegetales de *C. salviifolius* y *P. granatum* está relacionada con el perfil de resistencia a antibióticos de cada aislado. El extracto de *C. salviifolius* es más eficaz frente a aislados clínicos de *S.*

aureus resistentes a antibióticos betalactámicos, mientras que el extracto de *P. granatum* es más eficaz frente a aislados sensibles a quinolonas y oxacilina.

8. Existe una actividad sinérgica entre distintas clases de polifenoles y entre ciertos polifenoles y antibióticos de uso clínico. Las combinaciones entre flavonoides y antibióticos betalactámicos han mostrado niveles de sinergia especialmente elevados.

9. El uso de terapias antibacterianas combinadas, empleando extractos vegetales o polifenoles junto con antibióticos posee potenciales ventajas como la minimización del riesgo de desarrollo de resistencias y la disminución de la aparición de efectos secundarios para los pacientes. Según los resultados obtenidos en la presente tesis, el conocimiento de la composición de los extractos/mezclas estudiadas y su acción antimicrobiana permitiría el desarrollo de terapias individualizadas según el perfil de resistencia a antibióticos de la cepa bacteriana a tratar.

CONCLUSIONS

1. There is a lot of evidence of natural compounds that have proven antibacterial capacity, some being effective against bacteria resistant to antibiotics, especially those responsible for nosocomial infections.

2. Among natural compounds, plant polyphenols represent an antimicrobial therapeutic arsenal that hasn't been fully explored yet, either in isolated form or in combination with antibiotics.

3. In general, polyphenols have greater antimicrobial activity against gram-positive bacteria than against gram-negative bacteria.

4. The mechanisms of action of polyphenols are varied and multifactorial: disturbance of the cell wall and/or the plasma membrane, alteration or modulation of membrane receptors or ion channels, inhibition of bacterial metabolites or biofilm formation. It is postulated that the perturbation of the structure or of the synthesis of cell wall components and/or the lipid membrane is the predominant mechanism for most of plant polyphenols.

5. Given their multifactorial nature and molecular promiscuity, the study of natural compounds as antimicrobials must be approached through polypharmacology, network pharmacology, and computational virtual screening. This will increase the effectiveness of new compounds, reposition already known compounds, and reduce possible side effects.

6. Plant extracts rich in polyphenols from *C. salviifolius* (rich in hydrolyzable tannins and flavonols) and *P. granatum*, (rich in hydrolyzable tannins), have been shown to possess antibacterial activity against clinical isolates of *S. aureus*, including MRSA strains.

7. The susceptibility of the clinical isolates of *S. aureus* to the plant extracts of *C. salviifolius* and *P. granatum* is related to the antibiotic resistance profile of each isolate. The *C. salviifolius* extract is more effective against clinical isolates of *S. aureus* resistant to beta-lactam antibiotics, while the *P. granatum* extract is more effective against quinolones and oxacillin sensitive isolates.

8. There is a synergistic activity between different classes of polyphenols and between certain polyphenols and clinical use antibiotics. Combinations between flavonoids and beta-lactam antibiotics have shown particularly high levels of synergy.

9. The use of combined antibacterial therapies using plant extracts or polyphenols together with antibiotics show potential advantages, such as minimizing the risk of resistance development and a lesser occurrence of side effects for patients. According to the results obtained in the present thesis, the knowledge of the composition of the studied extracts/mixtures and their antimicrobial action would allow the development of individualized therapies according to the antibiotic resistance profile of the bacterial strain to be treated.

7. PROYECCIÓN FUTURA

PROYECCIÓN FUTURA

Uno de los principales estudios que se están llevando a cabo como parte de la presente línea de investigación es el estudio del efecto a nivel evolutivo que pueden tener los extractos al ser aplicados sobre diversas poblaciones bacterianas. Se estudian las poblaciones bacterianas a nivel genético y evolutivo para determinar si la exposición continuada a extractos vegetales antimicrobianos es susceptible de generar procesos de resistencia en las bacterias diana o si, por el contrario, las bacterias no logran adaptarse eficazmente a estos agentes vegetales. Gran parte de estos ensayos genéticos se han desarrollado en la Universidad de Carleton (Ottawa, Canadá) bajo la tutela del doctor Alex Wong durante una de las estancias de investigación realizadas durante el periodo de la Tesis Doctoral. Los resultados preliminares obtenidos hasta el momento apuntan a que *S. aureus* es capaz de adaptarse y adquirir ciertos niveles de resistencia frente al extracto de *P. granatum* empleado. Sin embargo, ninguna de las cepas de SARM empleadas hasta el momento han logrado desarrollar resistencia frente al extracto de *C. salviifolius*, siendo este un resultado alentador de cara a desarrollar terapias que incluyan este agente activo como componente principal o como adyuvante.

Otra rama de la línea de investigación se centra en el análisis de la capacidad antibacteriana de los extractos vegetales frente a diversos aislados bacterianos patógenos de relevancia clínica. Estos ensayos se están llevando a cabo en colaboración con el Hospital General Universitario de Alicante. Por el momento, se ha hallado una interesante actividad del extracto de *C. salviifolius* frente a aislados clínicos del patógeno *Stenotrophomonas maltophilia*, cuyos resultados están siendo redactados para ser publicados.

En paralelo a las líneas de investigación anteriormente citadas, se están llevando a cabo ensayos de dinámica de membranas por simulación computacional en colaboración con el Dr. José Villalaín Boullón. El objetivo de estos ensayos *in silico* es predecir y visualizar las interacciones entre los polifenoles más abundantes presentes en los extractos de *C. salviifolius* y *P. granatum* y las membranas biológicas de SARM y SASM para profundizar en el conocimiento de los mecanismos de acción antimicrobiana de los polifenoles y sus combinaciones.

Como proyecto a medio plazo se propone la puesta a punto de un modelo animal de infección para probar la actividad *in vivo* de los extractos vegetales antimicrobianos seleccionados. El animal modelo propuesto es el ratón y como procedimiento la punción de ligadura cecal, que emula las respuestas sistémicas producidas durante las sepsis humanas. Los resultados

obtenidos de este primer ensayo *in vivo* serán clave para determinar la ruta de ensayos futuros de la línea de investigación, siendo los ensayos clínicos en humanos el horizonte a largo plazo.

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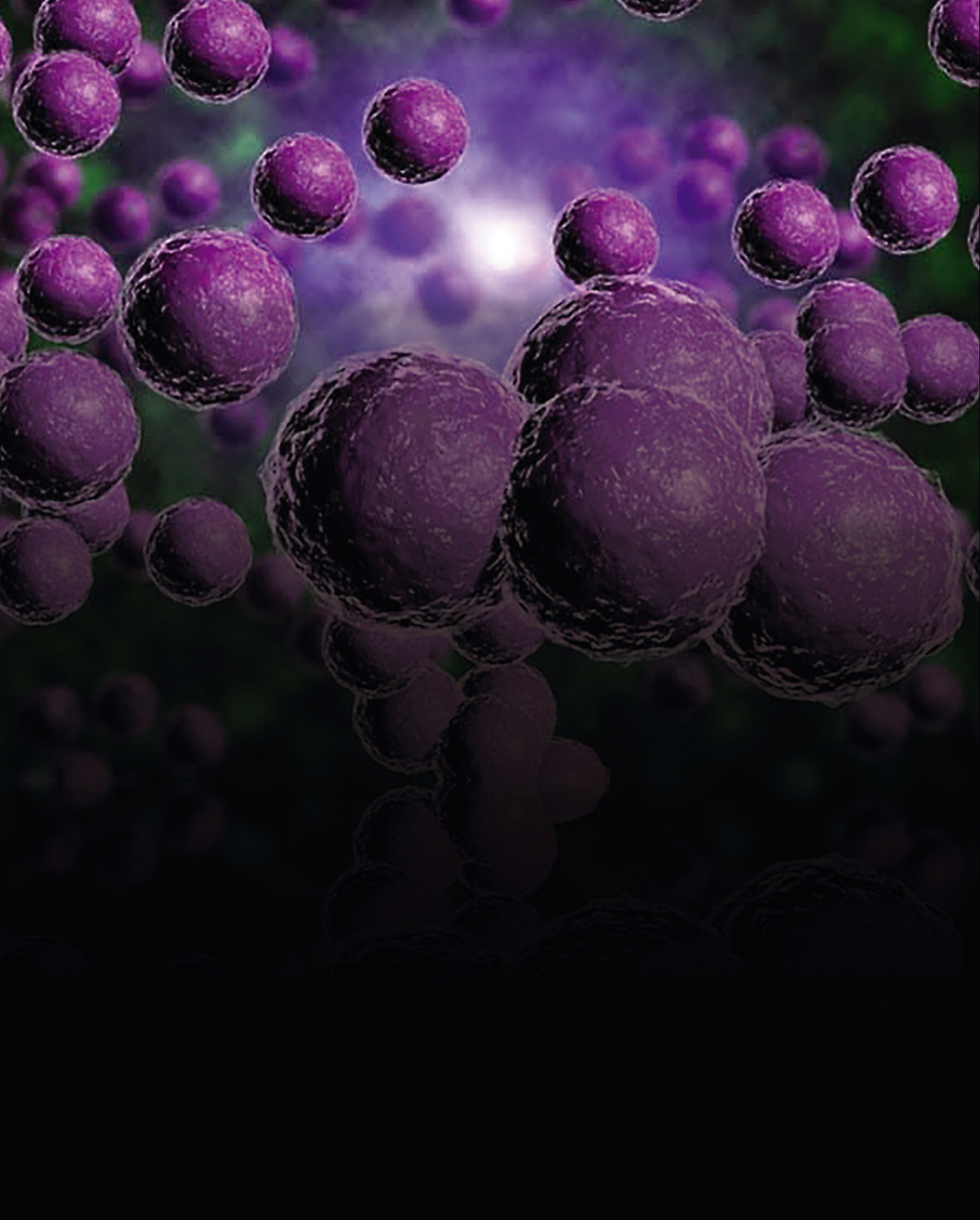
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