Review

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Posted Date: 6 May 2024

doi: 10.20944/preprints202405.0208.v1

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Review

Reviving the Past for a Healthier Future: Ancient Molecules and Remedies as a Solution to the Antibiotic Crisis

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Abstract: Available options to combat bacterial infections are getting scarce. We require innovative and imaginative approaches to enhance the identification and discovery of effective antimicrobials capable of combating bacteria resistant to multiple or all available drugs. These methods should either directly eliminate the resistant bacteria or influence their viability indirectly by inhibiting their virulence or reducing their resistance to antibacterial agents. One interesting approach is to analyze ancient remedies presumably used to treat bacterial infections, try formulating them, and test them against modern microbes. The field was recently named ancientbiotics. This approach allows us to take advantage of centuries of accumulated empirical knowledge, from several traditional medicines of various ancient cultures across the world. The strategy has already resulted in promising formulations to combat the ESKAPE group of nosocomial pathogens. Moreover, molecular de-extinction consists of genome analysis of extinct species in the search for useful antimicrobials, such as peptides. The de-extinction of simple organisms such as bacteriophages may eventually become a resource for novel tools to combat multidrug-resistant bacteria. In this review, we summarize and discuss the reported antimicrobial effects of ancient remedies and de-extinct molecules known to modern science, and discuss possible new strategies to further harness the potential of past remedies and molecules to help us fight the rise of superbugs.

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Introduction

Antimicrobial resistance (AMR) is a growing global challenge that has resulted in 1.27 million direct deaths worldwide from infectious diseases resistant to available antibiotics, and 3.7 million more associated deaths in 2019 (Antimicrobial Resistance 2022). The situation is deeply concerning, with a projected 10 million deaths globally by 2050. AMR has been accelerated by several factors, including the excessive or inappropriate use of antibiotics and the contamination of water natural resources with antimicrobial agents. The COVID-19 pandemic has further exacerbated AMR, as

 doi:10.20944/preprints202405.0208.v1

heightened antibiotic use was observed in efforts to prevent bacterial co-infections among patients (Tang, Millar et al., 2023). Unfortunately, the development of new antibiotics is stagnating, and the scientific community's effort appears to be insufficient to address the growing threat of AMR (Widmer 2022). The current priority is the search for therapeutic agents that can offer new opportunities in the treatment of infections caused by bacteria resistant to antimicrobials, and that do not favor the development of new resistance in pathogens (Lopez-Jacome, Franco-Cendejas et al., 2019). Although a few interesting novel molecules are under development (Ling, Schneider et al., 2015), complementary approaches such as machine learning and other artificial intelligence algorithms have identified interesting antimicrobial candidates (Cesaro, Bagheri et al., 2023). The options for novel antimicrobials may still be limited, so drug repurposing is also useful (Quezada, Martinez-Vazquez et al., 2020). Furthermore, the development of powerful sequencing technologies and data analysis is intensifying, enabling the identification of sequences of extinct organisms of diverse origins. Algorithms designed to identify useful molecules with antimicrobial properties from these sequences are under development (Maasch, Torres et al., 2023), opening new avenues for the discovery of novel antimicrobials by looking into the past.

It is worth noting that traditional medicine has accumulated wisdom over centuries from various civilizations, and there are remarkable examples of ancient antimicrobials throughout history. For example, the ancient Egyptians used moldy bread to treat infections, which presumably had antibacterial properties due to the production of penicillin or other fungus-derived antimicrobials. This knowledge could be valuable in the search for new antimicrobials.

In summary, the situation of AMR is alarming, and the development of new antibiotics is stagnating. Thus, novel approaches such as machine learning, drug repurposing, and the identification of sequences from extinct organisms are being explored to discover new antimicrobials. Moreover, traditional medicine could provide valuable insights into the search for new antimicrobials.

Survey Methodology

To guarantee an extended analysis of literature, a database of relevant articles on the activity of ancientbiotics and the de-extinction of antimicrobial molecules was established by searching the following online databases: Medline (PubMed), Science Direct (http://sciencedirect.com) database, Web of Science, Scopus, and Google Scholar system. The following keywords were used: ancientbiotic, folk remedy antibacterial, anti-virulence remedy, antibacterial plants, bacterial infection, traditional medicine, as well as Boolean operators such as “AND” and “OR”.

Ancientbiotics and Ancient Antivirulence Compounds

Many modern societies with advanced science and technology tend to underestimate ancient civilizations’ knowledge and traditional medicine. However, it is worth noting that some past cultures developed complex and effective medications to treat several diseases through trial and error. These treatments were developed without a deep understanding of the mechanisms that made them work but were still potent remedies. Although we have advanced technology and abundant resources, recent pandemics caused by viruses with low mortality rates, such as Sars-Cov-2, have shown that our health services and economy are vulnerable. We could not contain the spread of the virus or provide adequate treatment to those in need globally (Nicola, Alsafi et al., 2020). This has led to a collapse of health services and has had a devastating impact on the economy. Furthermore, if the predictions regarding the consequences of antimicrobial resistance hold, bacterial infections are expected to become the leading cause of death by 2050 (O'Neill, 2016). This is a concerning prediction that could have catastrophic consequences for mankind if we do not take the necessary steps to prevent it.

Therefore, it is important to acknowledge and learn from the traditional medicine of ancient civilizations. It may offer insights that could help us in the development of new treatments and solutions to the problems we face today. We must also invest in our health services and support ongoing research to prevent future pandemics and tackle the issue of antimicrobial resistance.

The term ancientbiotics was coined in 2015 due to an interdisciplinary collaboration between microbiologists and historians in the UK (Connelly, Lee et al., 2022). The consortium´s first published research dealt with the identification of a remedy used for eye infections from the 10th century Saxon Bald's Leechbook. The formulation was relatively simple since it only included garlic (*Allium sativum*) wine, either onion (*Allium cepa*) or leek (*Allium porrum*), and oxgall, and was originally prepared in copper vessels and marinated for nine days. The recipe was replicated following the ancient instructions with slight modifications, such as adding brass sheet pieces instead of preparing the remedy in copper vessels and using plastic bottles in which the remedy was incubated for nine days at 4°C. Remarkably, the preparation killed planktonic and biofilm *Staphylococcus aureus* cells and worked well in an *in vitro* soft tissue infection model and *in vivo* against MRSA chronic wound infections in mice. Moreover, the remedy's potency was maximal when all ingredients, except brass, indicated synergy between the different components (Harrison, Roberts et al., 2015). Notably, Bald's eyesalve is not only effective but emphasizes the efficacy of the synergy between its components, whereas modern science currently tends to focus on single compounds in the development of new drugs, without considering that their combination with other substances could reveal novel mechanisms of action and potentially lead to the development of new types of antibacterial treatments.

Later, the same research group demonstrated that the antibacterial effect was even wider since it was also seen against several other Gram-positive and Gram-negative species, including *Acinetobacter baumannii, Stenotrophomonas maltophilia, S. aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes*) (Furner-Pardoe, Anonye et al., 2020).

Bald's original eyesalve recipe includes ingredients with known antibacterial properties, such as garlic or onion; and the alcohol contained in wine can act as an extraction agent that facilitates the release of active compounds from the other ingredients. Bile salts could play a disruptive role in bacterial cell membranes or biofilms. In a recent work, Bald's eyesalve was fractionated to identify its main antibacterial agents. This study identified allicin as the principal antibacterial compound, suggesting that combining wine and bile salts in Bald's eyesalve can promote the extraction of allicin from minced garlic. Previous reports indicated that allicin with other compounds exhibits antagonistic antibacterial activity when used against *S. aureus*, whereas it displays synergistic activity against *Pseudomonas aeruginosa* (Fuchs, Weaver et al. 2018). The authors then suggest that the efficacy of Bald's eyesalve against these resistant bacteria is due to the unique combination of ingredients, which work synergistically to produce potent antibacterial effects. However, further studies are needed to unravel the action mechanism of the remedy.

Previously, the medieval remedy Bald's eyesalve has demonstrated *in vitro* antimicrobial activity against multidrug-resistant *S. aureus*, *P. aeruginosa* and *Neisseria gonorrhoeae*; however, it is crucial to establish its safety profile before carrying on clinical trials for its utilization against infections. Results obtained in *in vivo* models, such as bovine corneal permeability and opacity (BCOP), slug mucosal irritation (SMI) assay, and wound models in mice showed that Bald's eyesalve exhibits low levels of cytotoxicity and irritation and does not inhibit skin wound closure, suggesting that this ancient remedy may be a good candidate for topical use in humans and use as a future treatment for bacterial infections, especially in conditions such as diabetic ulcers and neonatal conjunctivitis (Anonye, Nweke et al., 2020).

A step forward for the application of novel antimicrobials based on ancient remedies is to test that they are safe in humans by the implementation of phase 1 clinical trials. In 2022, Harrison and her group evaluated Bald's eyesalve in 109 healthy volunteers by application on the upper arm of a patch containing the ingredients of the remedy (garlic, onion, white wine, and Ox gall), sterilized by filtration, and the remedy was covered with a dressing. It was left for 48 hours, and skin-related adverse effects were recorded. As expected, no significant adverse events were identified. Only around 13% presented minor symptoms such as mild irritation, itchiness, and garlic odor, hence validating that the crude remedy is safe (Bruce, Oyedemi et al., 2022) and paving the way for further trials evaluating its antibacterial properties in treating skin infections.

Because obtaining practical ancient recipes of antimicrobial mixtures may be laborious, a systematic automatization aimed to identify ingredients with putative or known antimicrobial properties that tend to be present in co-occurrence in several remedies may identify useful combinations whose effectiveness can be tested *in vitro* and *in vivo*. To this effect, Conelly and collaborators created an electronic database with the ingredients present in the recipes of putative antimicrobial remedies used to treat cutaneous and mouth infections in the 15th-century, *Lylye of Medicynes* book (Connelly, Del Genio et al. 2020). Notably, out of 360 recipes for treating 124 diseases, 62 may have been used for treating some infections, of which the authors identified around 41. The complexity of the task was evidenced by the identification of nearly 3600 different ingredients. Their network analysis algorithm allowed them to identify four core ingredients and three core combinations of ingredients; some of the core ingredients, such as acetic acid, and one recipe, were selected for the study of antibacterial properties. The chosen recipe was a gargle intended to alleviate mouth and throat infections. It was made of ox gall, rind, and pomegranate blossoms, mastic (resin from *Pistacia lentiscus*), frankincense, honey, and vinegar. The remedy and its components were tested against two Gram-positive and two Gram-negative bacteria that infect soft tissue, *S. aureus, Enterococcus faecalis, P. aeruginosa* and *Escherichia coli.* Remarkably, the reconstituted remedy killed 100% of all bacteria, as did the individual ingredient frankincense. Interestingly, some combinations, such as ox gall with honey or frankincense-sumac with honey, exhibited synergistic effects in killing *E. faecalis*, while other combinations were antagonistic. Noteworthy, a similar approach could be used for analyzing several ancient texts in search of antimicrobial ingredients or combinations not yet discovered.

Vinegar and honey were among the ingredients more frequently combined for the treatment of infections in medieval Europe. Both have validated antimicrobial properties and are used individually for the treatment of infected wounds. Even their combination has its own name: *oxymel*.

Recently, Harrison and coworkers found that these ingredients are mentioned in about 36% of 418 recipes for treating skin, eyes, mouth and throat infections, and infected wounds in 4 medieval manuscripts between the 9th and the 15th centuries. Moreover, they tested different kinds of vinegar to see if they had antibacterial effects against the Gram-negative *P. aeruginosa* and the Gram-positive *S. aureus* ESKAPE bacteria in both planktonic and biofilm cultures, beyond the effects of their respective content of acetic acid. They also analyzed the metabolic profile of the different vinegars by HPLC. Based on HPLC, two groups of vinegar with similar chromatography patterns were identified. One of them represented vinegar produced from wine, and the other vinegar produced from pomegranate. Not surprisingly, they found that some types of vinegar had lesser antimicrobial effects than their respective content of acetic acid for either planktonic or biofilm cultures of the tested bacteria, while others had stronger effects, indicating complex interactions between the metabolites present in the vinegar, and showing that some of their ingredients had synergic effects as well. Moreover, they also demonstrated that specific vinegars also had synergistic effects with medical-grade honey against biofilm cultures, hence validating the ancient use of *oxymel* (Harrison, Blower et al., 2023). One limitation of this study was that it used only reference bacterial strains, so the effects of vinegar or honey alone or in combination against clinical strains of the bacteria remain to be tested.

Other studies performed by the same research consortium demonstrated that, in contrast with vinegar and honey, other natural ingredients commonly used in medieval recipes for the treatment of topical infections, like Nettle, do not present any significant antibacterial properties themselves (Harrison, Furner-Pardoe et al., 2022), but were probably included in the remedies for performing other functions such as serving as an absorbent media, allowing the permanence of the actual antibacterial and emollient substances at the site of the infection.

Given the current crisis caused by the increase in the appearance of strains with multi-resistance to modern antimicrobials, the study of ancientbiotics is a useful concept (Connelly, Del Genio et al. 2020). Moreover, recently, ancestral formulations or folk remedies with antivirulence capacity ("ancient antivirulence") have also begun to be investigated (Dettweiler, Lyles et al., 2019, Gomez-Salgado, Beltran-Gomez et al., 2024).

Antivirulence-type antimicrobials do not directly interfere with microorganism growth; their effects are based on blocking the factors that allow them to establish themselves and cause damage (Castillo-Juarez, López-Jácome et al. 2017). Various antivirulence targets have been described, the most studied being biofilms, quorum sensing (QS), and bacterial secretion systems (Diaz-Nunez, Garcia-Contreras et al. 2021).

An ancient Chinese herbal mixture, Xiao Cheng Qi (XCQ), has been utilized for many years to treat slow-transit constipation (STC) by incorporating such anti-virulence therapies. Recently, research has hinted at a connection between constipation and gut microbiota, particularly through the impact of microbial metabolites such as short-chain fatty acids (SCFAs) on serotonin (5-HT) production by activating GPR43 receptors to maintain metabolic homeostasis. To assess XCQ's effectiveness, a study induced slow-transit constipation in mice using loperamide, and administered varying doses of the ancient herbal concoction. Results revealed Xiao Cheng Qi's capability to relieve constipation symptoms in mice, accompanied by significant alterations in gut microbiota composition, higher SCFA levels, increased plasma 5-HT, and enhanced expression of GPR43 and 5-HT4 receptors in the colon. These findings suggest that XCQ's ability to alleviate constipation may stem from its ability to influence gut microbiota, stimulate SCFA production, elevate plasma 5-HT, and boost colonic receptor expression. Thus, Xiao Cheng Qi emerged as a promising natural remedy for managing constipation, adding to the growing list of potential ancientbiotics (Tuohongerbieke, Wang et al., 2024).

Sometimes, the process by which the ancientbiotic is consumed is also important. Ayurveda is an ancient system of medicine originating in India, focusing on achieving balance and harmony within the body through diet, herbal remedies, yoga, and lifestyle practices. It emphasizes individualized treatments tailored to a person's unique constitution, known as doshas, to promote overall health and prevent illness. A common theme throughout the Ayurvedic practice is the consumption of fermented products, such as Panchagavya (PG), a multicomponent formulation containing cow-origin substances: urine, dung, milk, curd, and ghee (Gajera, Funde et al., 2024). The fermented dishes were also commonly served in copper vessels, another ancientbiotic practice in Ayurveda. Research studies analyzed Panchagavya's potential by infecting the model organism, *Caenorhabditis elegans*, with *S. aureus*, *Chromobacterium violaceum*, *Serratia marcescens*, *P. aeruginosa*, *S. pyogenes* and *E. coli*. These pathogens were also highly drug-resistant bacterial strains, proving effective in testing the strength of the fermented Ayurvedic concoction. Panchagavya was also administered in varying resting levels of copper exposure. Fermented samples with a rest period of 30-60 minutes in copper vessels proved successful in significantly combating the bacterial infections, which may be due to increasing Planctomycetes, Gammaproteobacteria, and Verrumicrobiota in the gut microbiome (Gajera, Funde et al., 2024). These gut bacteria have proven beneficial against various pathogens for their free metal absorption, secondary metabolite production, and T immune cell regulation. In combination with the holistic Ayurvedic approaches, Panchagavya is a powerful ancientbiotic with multiple applications in infectious diseases.

It should be noted that, among natural products, those of microbial origin have stood out for their bactericidal properties (Schneider, 2021). At the same time, in recent years, plants have positioned themselves as a relevant source of anti-virulence products (Diaz-Nunez, Garcia-Contreras et al., 2021). Likewise, the antibacterial and anti-pathogenic activity of formulations or folk remedies that contain plant species with anti-virulence capacity has been documented (Dettweiler, Lyles et al., 2019, Gomez-Salgado, Beltran-Gomez et al., 2024).

Native American cultures developed in an area with a great diversity of plant species and other elements of nature, with which they designed various remedies to treat diseases (Górniak, Bartoszewski et al., 2019). Most of this knowledge was lost with the European colonization of America; however, in some historical texts and within the original ancestral communities, information about ancestral medical practices can still be obtained.

During the American Civil War (1861-1865), many soldiers died from complications resulting from microbial infections. Because this armed conflict occurred a century before the development of modern antibiotics, Confederate doctors had to turn to medicinal plants used by North American natives to save lives (Dettweiler, Lyles et al., 2019). The mission was entrusted to botanist Francis Porcher, who documented some plants that Native Americans used to treat wounds and infections (Porcher 2020). This work served as the basis for Confederate doctor Samuel Moore to prepare a manual detailing the collection, preparation and use of medicinal plants (Office and Moore 1863).

Some of the plant species documented by Porcher (*Liriodendron tulipifera*, *Aralia spinosa* and *Quercus alba*) have been analyzed and have been shown to inhibit the growth of multidrug-resistant bacteria associated with wounds, such as *S. aureus*, *Klebsiella pneumoniae* and *A. baumannii* (Dettweiler, Lyles et al., 2019). Likewise, they exhibit anti-virulence activity by inhibiting the formation of *S. aureus* biofilms, while *A. spinosa* also reduces the transcription of *agr* gene operons necessary for QS (Dettweiler, Lyles et al., 2019). The authors propose that the effectiveness of these plants in controlling infections can be attributed to an adjuvant effect of anti-virulence metabolites with bactericides (Dettweiler, Lyles et al., 2019). On the other hand, none of the plant extracts showed bactericidal activity against *P. aeruginosa* AH7, although it is worth mentioning that their anti-virulence properties were not analyzed.

The World Health Organization lists *P. aeruginosa* as a "critical priority" for developing new antimicrobials due to the increase in the emergence of resistant strains (Tacconelli, Carrara et al., 2018). This opportunistic pathogen causes hospital-acquired infections, mainly in immunocompromised patients, with mechanical respiratory assistance or major burns (Reig, Le Gouellec et al., 2022).

A characteristic of *P. aeruginosa* infection in burn patients is its ability to establish itself in the lesion, spread to the bloodstream, and cause septicemia (Bahemia, Muganza et al., 2015). Therefore, preventive treatments in the first hours after the burn occurs are essential to avoid the death of the patients (DeLeon, Balldin et al., 2009).

Recently, in a thermal injury model in mice, the anti-pathogenic activity of a pre-Hispanic remedy with cuachalalate stem bark (*Amphipterygium adstringens*) was validated to treat lesions infected with *P. aeruginosa* (Gomez-Salgado, Beltran-Gomez et al., 2024). Previously, it had been documented those organic extracts from the bark of the tree lacked bactericidal capacity against *P. aeruginosa* but that they reduced its virulence (Castillo-Juarez, Garcia-Contreras et al., 2013). Specifically, non-polar extracts such as hexane and one of its major constituents, anacardic acids, reduced the production of phenotypes regulated by QS and the phospholipase ExoU secreted by the type 3 secretion system (T3SS) (Castillo-Juarez, Garcia-Contreras et al., 2013). However, although *in vitro* results were promising, topical application of the hexane extract or individual constituents were inactive in counteracting *P. aeruginosa* infection. It failed to prevent the death of the animals (Gomez-Salgado, Beltran-Gomez et al., 2024). Remarkably, the result was different when it was applied following a folk method, which consists of washing the wounds with the aqueous extract of the bark and then applying it as a powder (Gomez-Salgado, Beltran-Gomez et al., 2024).

The folk method reduced animal mortality by avoiding systemic dispersion and septicemia. In addition, it stimulated the formation of granulation tissue and blood vessels, favoring the restoration of damaged tissue (Gomez-Salgado, Beltran-Gomez et al., 2024). Analysis of the chemical composition of the aqueous extract of the bark revealed the presence of glycosylated flavonoids and catechins. Furthermore, the lyophilized aqueous extract exhibited antivirulence capacity by inhibiting caseinolytic activity and pyocyanin production (Gomez-Salgado, Beltran-Gomez et al., 2024).

It is relevant to highlight that the effectiveness of this ancient method was superior to that of silver sulfadiazine, which is a broad-spectrum synthetic sulfonamide used in burn patients to prevent infections (Abul Barkat, Abul Barkat et al., 2023).

Applying the folk method with cuachalalate correlates with the information described in some historical texts. In the work *History of the Plants of New Spain* from the 16th century, it is mentioned that the bark of "chalalactli" should be applied to "reduce tumors" (Sotelo-Barrera, Cilia-Garcia et al., 2022). In the essay on Mexican materia medica of 1832, the bark of "cuauchalalá" must be cooked in water to cure sores on the skin of animals (De la Cal 1832). The Mexican Pharmacopeia, compiled and published by the Pharmaceutical Academy of the Capital of the Republic in 1846, indicates that

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to heal wounds, the bark of "cuanchalalate" or "cuanchalalá" must be applied (Sotelo-Barrera, Cilia-Garcia et al., 2022). Although some complementary studies are required, recent results suggest that the antimicrobial effectiveness of cuachalate bark depends on the application form, the synergistic action of several antivirulence-type compounds, and mechanisms of actions that contribute to tissue healing.

The Mayan civilization is one of the most important pre-Hispanic cultures, and its direct descendants protect ancestral knowledge, traditions, and beliefs (Vargas and Andrade-Cetto 2018). In a pioneering study in Mayan communities of the Yucatan Peninsula in Mexico, anti-virulence properties of various plants traditionally used to treat infectious diseases were identified (Munoz-Cazares, Pena-Gonzalez et al., 2023). The anti-biofilm property of *Ceiba aesculifolia* and *Colubrina yucatanensis* stands out, as well as its ability to reduce *P. aeruginosa* pyocyanin production and the protease activity of the plants *Bonellia flammea*, *Capraria biflora*, *Cissampelos pareira* and *Bursera simaruba* (Munoz-Cazares, Pena-Gonzalez et al., 2023).

In a subsequent investigation, Castillo-Juarez and colleagues tested three popular formulations used by residents of the Yucatan peninsula to treat injuries and infections. These formulations were made with various plant species from the area (Espíndola-Rodríguez, Muñoz-Cázares, *et al*; 2024). Fresh plant structures were used for preparation, supervised by traditional Mayan doctors.

In the thermal damage injury model in mice, the antipathogenic activity of the formulation called "herbal soap" (HS) was identified. It consists of incorporating the aqueous extract obtained from seven plant species (*Astronium graveolens*, *Parthenium hysterophorus*, *Hamelia patens*, *Momordica charantia*, *Psidium guajava*, *Tradescantia spathacea*, and *Kalanchoe laciniata*) into a bar of commercial soap for local use.

Washing the burns with HS twenty-four hours before inoculation with *P. aeruginosa* and its subsequent application for ten days reduced the establishment and dispersion of the bacteria into the bloodstream. It prevented the animals' death (Espíndola-Rodríguez, Muñoz-Cázares, *et al*; 2024). The herbal component of HS was analyzed, and anti-virulence properties related to swarming inhibition and phospholipase ExoU secretion were identified. These results can be correlated with previous studies, in which swarming and T3SS (especially ExoU) are determinants of pathogenicity in murine infection models and are targets with great potential for developing new antibacterials (Foulkes, McLean et al., 2019).

Zuccagnia punctata is a plant used in Argentine traditional medicines. The plant's infusions and decoctions in water, as well as extracts obtained by maceration in ethanol have been shown to have antiseptic activity (Ratera and Ratera 1980, Toursarkissian 1980, Ortega, María et al., 2000). Hydroethanolic extract of *Z. punctata* aerial parts was active against several Gram-negative bacteria isolated from human lesions, such as *E. coli*, *K. pneumoniae*, *S. marcescens*, *A. baumannii*, *P. aeruginosa*, among others (Zampini, Vattuone et al., 2005). Three major compounds isolated from *Z. punctata* extract were: 7-hydroxyflavanone (HF), 2,4-dihydroxychalcone (DHC) and 3,7-dihydroxyflavone (DHF). Antibacterial effects of *Z. punctata* extract, DHC, 3,7-DHF and 7-HF were assessed using a mouse model infected with *S. pneumoniae*. After infecting the mice with *S. pneumoniae*, the products were orally administered the next day. Treatment with *Z. punctata* extract and 7-HF significantly decreased the number of viable *S. pneumoniae* in the lungs, whereas DHC and 3,7-DHF showed no discernible effect *in vivo* (Zampini, Villena et al., 2012). *Z. punctata* has also been tested against growth and virulence factors of *Candida* species (Gabriela, Rosa et al. 2014). The *Z. punctata* extract showed inhibitory activity against planktonic cells of all assayed *Candida* species. The main active compounds identified in the extract were chalcones (2′,4′-dihydroxy-3′-methoxychalcone, 2′,4′-dihydroxychalcone), flavones (galangin, 3,7-dihydroxyflavone and chrysin) and flavanones (naringenin, 7-hydroxyflavanone and pinocembrine).

In addition, several articles have reported the study of different plant species abundant in the Argentinian Puna as possible antimicrobials. Puna is a highland region of the central Andes that extends from southern-central Peru to northern Argentina and Chile. This region is characterized by high altitude (3000–5000 masl), intense exposure to ultraviolet radiation, low oxygen levels, significant daily temperature fluctuations, and mean annual rainfall ranging from 100 to 200 mm, or

 doi:10.20944/preprints202405.0208.v1

even less. Plants within these ecosystems have developed adaptations to environmental stress, including the synthesis of secondary metabolites with notable pharmacological properties (Kleier and Lambrinos, 2005).

Thus, Zampini et al. (2009) tested the antimicrobial activity of 11 plants from this region used in traditional medicine to treat skin and soft tissue infections in humans and animals. They found that ethanolic extracts of aerial parts of *Baccharis boliviensis, Fabiana bryoides, Fabiana densa, Fabiana punensis, Fabiana triandra, Parastrephia lucida, Parastrephia lepidophylla* and *Parastrephia phyliciformis* had antibacterial activity on methicillin, oxacillin and gentamicin resistant *Staphylococcus*. Furthermore, plants such as *Chuquiraga atacamensis* had potent antibacterial activity on multi-resistant Gram-negative strains. *Parastrephia* species showed activity against *Enterobacter cloacae, P. aeruginos*a and *Proteus mirabilis*. They also showed that ethanolic extracts were more effective than aqueous extracts against sensitive and multi-resistant clinical isolates (Zampini, Cuello et al. 2009).

Later, D'Almeida et al. (2012) identified the main antimicrobial constituents from the Argentinean Puna plant *P. lucida*, explaining, at least in part, the traditional use of the species in the Andes highlands medicine. The main antimicrobial constituents identified were phenylpropanoid esters yielding prenyl or phenethyl alcohol. The antimicrobial activity was effective against *E. faecalis, S. aureus* and *C. albicans* (D'Almeida, Alberto et al. 2012).

Otero et al., (2022), reviewed the effect of 11 plants native to Argentina and Chile (Otero, Fuentes et al. 2022). Although in many cases they have not been scientifically examined, some plants are commonly consumed by native indigenous or rural populations due to their widely known benefitial effects on human health. Endemic species, such as *Peumus boldus* and *Quillaja saponaria*, have been extensively studied in terms of their components and the biological properties of their extracts and purified metabolites, but have been less scientifically examined (Salehi, Sharifi-Rad et al. 2021). *P. boldus* is endemic to central and south-central Chile (Rodriguez, Marticorena et al. 2018). Its leaves are traditionally used in infusions for gastrointestinal and liver problems (Bruce, Oyedemi et al.). Phenolic compounds such as catechin, epicatechin and rutin were identified as their main components. The extracts were evaluated against different Gram negative and Gram positive pathonic bacteria, including members of the ESKAPE group.

The aporphine alkaloid boldine, abundant in the leaves of the plant, was evaluated in a murine macrophage model, showing its ability to reduce *Leishmania amazonensis* infection. Tietjen et al., (2015) further demonstrated that boldine was able to inhibit the replication of both the human deficiency virus (HIV-1NL4-3) and the hepatitis C virus (HCV) (Tietjen, Ntie-Kang et al. 2015). *Quillaja saponaria* has high content of saponins, where the triterpenic aglycone quillaic acid is the prevalent molecule (Reichert, Salminen et al. 2019). The commercial aqueous bark extract, containing saponins, polyphenols, and tannins, has demonstrated antimicrobial activity against Enterohemorragic *E. coli*, causing severe membrane damage after short term exposition to the extracts (Sewlikar and D'Souza 2017). Furthermore, a saponin-rich extract was effective against *S. aureus* ATCC 49525, *S. Typhimurium* NCIM 2719, and *E. coli* ATCC 933 (Hassan, Byrd et al. 2010).

Other regions of the world, such as Asia Minor and Asia Central, also possess a great collection of ancient knowledge suitable for utilization in search of antimicrobials, since the endemic medicinal plants and minerals were at the core of traditional medicine in those regions during the Middle Ages, as evidenced in the ancient manuscripts or collections written since the 5th century. The names of thousands of plants are deposited in pharmacognostic encyclopedias in Armenia, and more than 14,000 valuable manuscripts are deposited in a "Mesrop Mashtots Matenadaran," available for further analysis. In Georgia, scientists are developing the National Formulary of Georgian Medicine project, based on 500 ethnomedicine-related manuscripts (Elizbarashvili, Gurgenidze et al. 2023). The Uzbek Academic Collection has in its possession 181 manuscripts from the 13th and later centuries, which describe local medicinal herbs in "The Canon of Medicine" (Sina 1700, Shterenshis 2000). Endemic plants from other countries in Central Asia, such as Kazakhstan, Kyrgyz Republic, Tajikistan, and Turkmenistan, might have gaps in the documentation of traditional knowledge but are now extensively studied for modern medicine and pharmacology purposes (Sina 1700, Sharopov, Braun et al. 2015, Pawera, Verner et al., 2016, Sharopov and Setzer 2018). Leaves, fruits, berries, rhizomes, seeds, barks, etc., of medicinal plants were used to attend to leprosy, wound healing, respiratory and intestinal infections, etc.

A widely dispersed *Hypericum perforatum***,** Hypericaceae, known also as a St. Jon´s Wort, is rich in lipophilic phloroglucinol (a hyperforin), hypericin and flavonoids (Kitanov 2001). Hyperforin inhibits the growth of Gram-positive bacteria, especially MRSA and penicillin-resistant *S. aureus*, but not of Gram-negative bacteria (Bystrov, Chernov et al. 1975). Hypericin (a naphthodianthrone) is the main active component of yellow flowers of *Hypericum spp*. and was shown to prevent the replication of encapsulated viruses such as SARS and VIH (Diwu 1995). These and other components, such as pseudohypericin, quercetin, kempferol, and 22 essential oils, were probably important in ancient medicine due to their antiseptic and antimicrobial properties (Kitanov 2001, Sharopov, Braun et al., 2015).

Endemic plants used in antiquity for wound healing and as antiseptics in Tadjikistan (Sharopov and Setzer 2018), Kyrgyzstan, and bordering Turkestan (Sharopov, Braun et al., 2015, Pawera, Verner et al., 2016) showed that essential oils obtained from local *Achillea filipendulina and A. arabica* L.: Asteraceae, *Galagania fragrantissima,* and *Origanum tyttanthum* exhibit high antimicrobial and antioxidant activity against MRSA.

Flowers and berries from *Sambucus nigra* L.: Adoxaceae were used in medieval medicine in Europe and Armenia (with their own endemic species: *Sambucus tigranii*) (Асатрян 2021). *Sambucus* spp. rich in polyphenols, quercetin, anthocyanins kaempferol, and other compounds and is extensively reviewed in Mlynarczyk et al. (Mlynarczyk, Walkowiak-Tomczak et al., 2018). Ethanol extract from berries and elder flowers inhibits S*taphylococcus*, *Salmonella*, *Pseudomonas* and other nosocomial pathogens (Hearst, McCollum et al., 2010). An aqueous extract from leaves moderates the growth of Enterobacteria *S. marcescens, S. pyogenes, Streptococcus* Group C and G, *and Moraxella catarrhalis* (Krawitz, Mraheil et al., 2011).

Several possibly imported medicinal species are mentioned in the old manuscripts as well: *Phyllanthus emblica (Emoica officinalis Gaerth L.),* known today for its antioxidant, antiviral and antibiotic properties against Gram-negative bacteria, was used in ancient gerontology in Armenia, as well as in Ayurvedic medicine (Saeed and Tariq 2007). Manuscripts in the Matenadaran repository also indicate a *Laserpitium L.* Apiaceae for ulcers and abscesses; physicians from Asiatic countries treated gastrointestinal disorders with *Laserpitium* as well (Pawera, Verner et al., 2016). Ethanolic and water extracts of essential oils (very rich in terpenes) from *Laserpitium ochridanum* were able to interrupt QS activity of *P. aeruginosa*, inhibiting pigment production and reducing twitching and swimming motility (López-Carreras, Miguel et al., 2012).

Many other ethnomedicinal plants are mentioned in the manuscripts or in folk medicine (also orally transmitted): *Plantago psyllium* L., *P. mayor* L. and *Inula helenium* for antiseptic purposes and treatment of intestinal and respiratory infections, as well as a *Brionia dioica*, *Nigella sativa*, *Ficus caruca L.*, *Allium* sp., *Apiaceae* and several others, waiting to be indexed, can perhaps become helpful in today's fight against multidrug-resistant microorganisms.

Japan boasts a rich culinary tradition that includes raw foods consumption like sushi and sashimi. Consequently, the risk of foodborne illnesses is notably high. To mitigate this risk, wasabi (Japanese horseradish, *Eutrema japonicum*) has long been utilized as a preventive measure against food poisoning. Historically, wasabi holds a significant place in Japan, as evidenced by the discovery of the word "wasabi" on excavated artifacts dating back approximately 1,400 years ago (around the 7th century). Moreover, culinary texts from the 12th to 14th centuries mention its uses in cooking. Even in modern times, wasabi continues to be incorporated into Japanese households, sometimes discreetly kneaded into kagami mochi—a traditional New Year's decoration. Its traditional role as a preservative has been passed down through generations. Wasabi is endemic to Japan and is the sole cultivated species in the *Eutrema* genus (Yamane, Sugiyama et al., 2016, Haga, Kobayashi et al., 2019). However, cultivating Japanese wasabi is challenging. As a result, a wasabi-like paste made from horseradish (*Armoracia rusticana*) is commonly sold in Japan. While this paste shares similarities with Japanese wasabi in composition, it differs significantly in its botanical classification. The primary pungent component of wasabi is allyl isothiocyanate (Yamane, Yamada-Kato et al., 2023), and its

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aromatic, pungent taste and tear-inducing smell play a crucial role in defense against pathogens (Rask, Andréasson et al., 2000). Recently, it has been discovered that the 60% ethanol extract of Japanese wasabi exerts the strongest anti-inflammatory, antibacterial, and cytotoxic activity (Dos Santos Szewczyk, Skowronska et al., 2023). However, numerous mysteries still surround Japanese wasabi. Thanks to recent advancements in sequencing technology and bioinformatics, a high-quality reference genome was finally identified last year (Tanaka, Hori et al., 2023). It is hoped that this will lead to further advancement in wasabi research, not only in terms of understanding its ingredients and antibacterial activity, but also in breeding efforts.

Around five thousand medicinal plants are currently being used on the African continent, and African traditional medicine is probably the most diversified and one of the oldest. Nevertheless, it is considered that there is still a considerable lack of knowledge of all endemic plants promising medical use, and it is worthwhile to make a concerted effort to obtain proper documentation of the medicinal uses given to the various plants and their products (Mahomoodally 2013). African plants also provide substances that fight against bacteria. Tannins and saponins present in barks from *Acacia senegal L.: Mimosoideae* (also known as Arabic gum), which grow on the Northern and Western coast of Africa, possess efficient antimicrobial properties against *E. coli*, *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, *Salmonella typhi*, *Shigella dysenteriae*, *S. pyogenes*, *P. aeruginosa*, and *Proteus vulgaris* (Okoro, Kawo et al., 2012). The root extract from endemic in South Africa *Pelargonium sidoides* or Geraniaceae (also known as Umckaloabo RM), endemic to South Africa, is one of the most widely used remedies against respiratory infections. The main phytochemical component in roots of *P. sidoides* is umkalicin, which is absent in other ornamental Pelargonium cultivars. However, isolated catechin and gallic acid from all these *Pelargonium spp*. together, can inhibit biofilm formation, and have quorum-quenching activity in *P. aeruginosa* (Kayser 1997, Abdel Bar, Alossaimi et al., 2022). The native African plants *Lippia adoensis*, *Polysphaeria aethiopica*, *Rumex abyssinicus* and *Cirsium englerianum* (Ethiopia and Madagascar); *Cucumis pustulatus* (Mali and Arabian Peninsula); *Discopodium penninervium* and *Euphorbia depauperate* (Tropical Africa) were used to extract their phytochemicals for use in aqueous or methanol solutions; they showed appreciable activity against MRSA, *S. pyogenes*, MDR *E. coli* and *K. pneumoniae* (Kebede, Gadisa et al., 2021). Isolated anthraquinones from *Aloe pulcherrima* of the Asphodelaceae family, one of 15 endemic Aloe species from Ethiopia, inhibited the growth of *P. aeruginosa*. On the other hand, the endemic South African plant *Aloe ferox* Mill has 130 medicinal agents (Grace, Simmonds et al., 2008, Mahomoodally 2013), but what is interesting to note is that *A. ferox* grows in association with endophytic actinobacterium adapted to the interior leaf pulp. An extract from the endosymbiotic *Streptomyces olivaceus* had no anthraquinones but exerted bactericidal action against Gram-positive bacteria (Maliehe, Mbambo et al., 2022). Otherwise, plant endosymbionts are part of a novel field of discovery called bioprospection, which, together with ancientbiotics, may provide safe and effective insights for modern medicine.

It is essential to recognize that different historical cultures developed and utilized distinct antibiotics due to various factors, including geographical location, available resources, cultural practices, and medical knowledge. Geographical diversity led to the availability of different plants, minerals, and animals, influencing the selection of materials used for medicinal purposes. These factors may have contributed to differences in the physiological components of microflora, affecting the medicine's potency throughout civilizations. Cultural practices and beliefs have additionally shaped the development and transmission of medical knowledge, resulting in unique approaches to healthcare and the use of specific remedies. Furthermore, varying levels of scientific understanding and technological advancements in different civilizations influenced their ability to isolate and identify effective antimicrobial agents. Ancient cultures developed diverse ancientbiotic treatments based on their respective contexts, resources, and understanding of disease. Regardless of their individual components, these ancientbiotics converge in their effectiveness in treating several infections. Figure 1 shows the global distribution of the ancientbiotics and ancient anti-virulence preparations discussed in the text.

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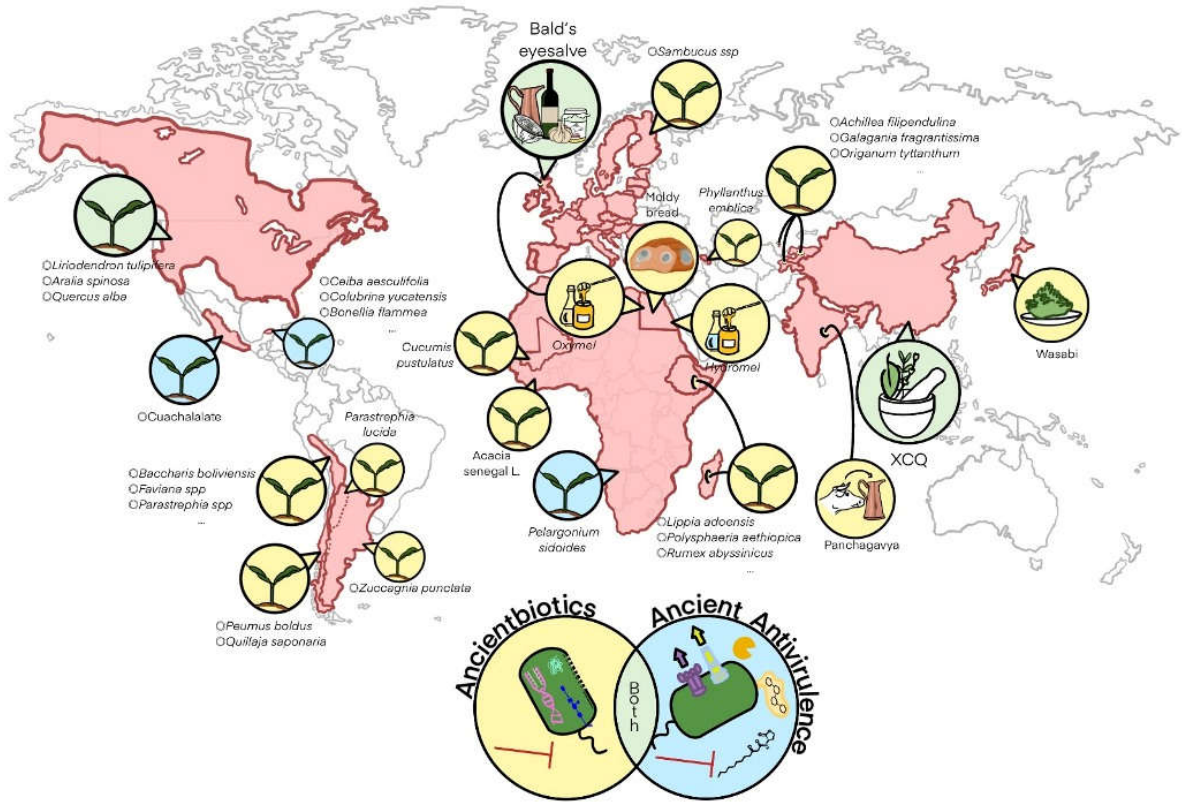

De-Extinction of Molecules and Bacteriophages

It is estimated that more than 90% of the species that had once existed are now extinct; hence, the repertoire of molecules and metabolites with antibacterial properties could be largely enriched if we could have access to extinct species, although bringing back to life those species is very complicated at present and may raise ethical concerns. We had access to genomic data regarding several ancient organisms and to computational methods suitable for the identification of potential antibacterial molecules. In this regard, de la Fuente and colleagues surveyed the inferred proteomes of Neanderthals and Denisovans and, using a machine learning approach, were able to identify several potential antimicrobial peptides absent in the *Homo sapiens* genome (Maasch, Torres et al., 2023). In addition to its well-established antibacterial properties by mechanisms such as membrane disruption and promotion of the production of active oxygen species, antimicrobial peptides also have immunomodulatory properties (Martell, González-Garcia et al., 2021). Some peptides can combat biofilm formation and the production of virulence factors (Castillo-Juárez, Blancas-Luciano et al., 2022), hence molecular de-extinction of peptides may also identify those with immunostimulant and antivirulence properties. Beyond molecule de-extinction, perhaps more complex entities such as bacteriophages may be resurrected and used as antimicrobials. Bacteriophages are viruses that infect and kill bacteria and are the most abundant biological entity on earth. They were discovered in the early 20th century and studied by Felix d'Herelle, who also contributed the idea of using them as antibacterial agents, thus creating phage therapy; later, bacteriophage therapy was developed in some countries of the former Soviet Union, such as Georgia, but it was forgotten in Western medicine due the rise and success of antibiotics (Strathdee, Hatfull et al., 2023). Nevertheless, phage therapy is currently becoming a therapeutic option for recalcitrant infections untreatable with antibiotics, and it has often been able to cure infections and save patients' lives (Uyttebroek, Chen et al., 2022); moreover, the first promising trials for curing a large number of patients with standardized phages alone or in combination (phage cocktails) are under development (Pirnay, Djebara et al., 2023). Importantly, phages can also be used to treat animal infections and to protect plants against bacterial disease; hence, they may be an eco-friendly alternative to antibiotics in farming and agriculture, and

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contribute to decreasing the selective pressure for the development and maintenance of antibiotic resistance in the environment (Garcia-Cruz, Huelgas-Mendez et al., 2023). In addition, phages may also contribute to reducing antibiotic resistance and virulence, since bacteria often acquire bacteriophage resistance by losing virulence factors or proteins directly involved in antibiotic tolerance and resistance, such as lipopolysaccharide, pily and components of antibiotic efflux pumps (Gordillo Altamirano, Forsyth et al., 2021; Fujiki, Nakamura et al., 2023; Garcia-Cruz, Rebollar-Juarez et al., 2023). Moreover, other phages directly interfere with the horizontal transfer of genetic information, including plasmids encoding antibiotic resistance genes, by blocking bacterial conjugation (Lin, Jimenez et al., 2011).

Interestingly, some authors suggest that phages may have been used to treat bacterial infections like cholera. Before their discovery, for example, traditional Indian folklore stated that the waters of some rivers, such as the Ganges, had curative effects against this disease; moreover, the antibacterial activity of those waters was confirmed by investigations in the late nineteenth century. Nevertheless, some researchers argue that it is unlikely that phages were the antibacterial agents since high phage densities may have been needed and, more importantly, since the activity of the waters remains after heating in sealed containers, a treatment that presumably would inactivate the phages (Abedon, Thomas-Abedon et al., 2011). However, some phages have been shown to resist temperatures near the boiling point of water for prolonged periods (Capra, Neve et al., 2013).

Although our current phage repertoire seems to be enough to construct a robust collection and design cocktails suitable to combat several multidrug-resistant bacteria, it is conceivable that in the future bacterial resistance against phages may limit our available phage arsenal, which has already happened with antibiotics (Amabile-Cuevas 2022). Therefore, a diversified approach consists in analyzing metagenomes of ancient samples in order to unravel ancient extinct bacteriophages whose genomes could be synthesized *de novo*, or by modifying modern related phages and testing their potential against the bacteria of interest. Remarkably, several cases of ancient eukaryotic viruses have been resurrected, for several reasons, including the implementation of better gene delivery platforms (Zinn, Pacouret et al., 2015). Regarding phages, recently, the genomes of around 300 ancient bacteriophages have been recovered from palaeofaeces samples collected in Europe and North America, ranging in age from 150 to 5300 years. Interestingly, around half had no similarity to modern-day phages (Rozwalak, Barylski et al., 2024), being, therefore, potential candidates for de-extinction. Another potential source for the recovery of ancient dormant bacteriophages is permafrost. Already, several eukaryotic viruses trapped there have been resuscitated (Legendre, Bartoli et al., 2014) (Alempic, Lartigue et al., 2023). Besides yielding bacteriophages, permafrost can also be used as a source of ancient microorganisms which can produce antimicrobial compounds and/or predate pathogenic bacteria. Some researchers have postulated that permafrost may constitute a risk for releasing ancient pathogenic organisms (Wu, Trubl et al., 2022), samples should be handled carefully. Figure 2 illustrates the de-extinction approach for the identification of suitable antimicrobials.

Conclusions

The discovery and testing of ancient recipes with antibacterial properties present several challenges, such as the assembly of interdisciplinary teams containing experts in both humanities like history and ancient languages, and in microbiology. Collaborations of this kind have proven very useful for the identification of several promising remedies that outperform modern antibiotics (Connelly, Lee et al., 2022). Due to the current alarming situation regarding bacterial multi-resistance, it is foreseeable that eventually, modern formulations based on ancient remedies will be used in clinical practice. Another complication inherent to some of the ancient remedies is that the mixture of ingredients may be complex, and their interaction may have either synergistic or antagonistic tendencies. Hence, the identification of their main antimicrobial components and the elucidation of the effects of their interaction with other components is advisable. Moreover, some of the ingredients or their properties may differ from those presented in the ancient versions of the remedies. Since they are based on natural components like plants, variability also depends on many factors, such as the plants' location, age, environmental conditions, coexistence with other species, etc. However, the complex nature of the remedies may also be an advantage, since the nature of the antibacterial action mechanisms is likely multifactorial, probably reducing the resistance to these remedies, making it lower than conventional antibiotics, although this possibility remains to be explored.

The analysis of bactericidal or anti-virulence properties in ancestral formulations is based on the synergistic action of their constituents; therefore, the objective of the analysis is not the isolation of compounds that act on unique targets. Likewise, in the anti-pathogenic capacity of the formulations, the mechanisms that contribute to the healing of damaged tissue and the effect on the immune system must also be considered. Thus, one of the critical challenges in using complex ancestral formulations is to identify the bioactive molecules and interpret the synergies involved. Currently, technologies that allow analyzing and interpreting the results of the interactions of several compounds that act on various targets have yet to be fully developed. Overcoming these challenges and others will be essential in designing effective and safe antimicrobial formulations.

Also, searching for antimicrobial molecules using sequences of extinct organisms has its complications, such as getting samples with enough quality to recover intact gene sequences that allow searching for peptides or other kind of molecules with antimicrobial properties and the development of robust and accurate algorithms to maximize their identification. However, they may strengthen the potential of expanding the repertoire of antibacterial molecules that may be helpful in our fight against superbugs. Therefore, we encourage further research in these fields and the search for other innovative strategies for the identification of beneficial antibacterials that could be implemented in the near future.

Acknowledgements: MD-G was supported by a postdoctoral fellowship from Dirección General de Asuntos del Personal Académico, UNAM. R G-C research is supported by DGAPA PAPIIT grant IN200224.

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 doi:10.20944/preprints202405.0208.v1