A guide to the use of bioassays in exploration of natural resources

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Abstract:	Bioassay are the main tool to decipher bioactivities from natural resources thus their selection and quality are critical for optimal bioprospecting. They are used both in the early stages of compound isolation/purification/identification, and in later stages to evaluate their safety and efficacy. In this review, we provide a comprehensive overview of the most common bioassays used in the discovery and development of new bioactive compounds with a focus on marine bioresources. We provide a comprehensive list of practical considerations for selecting appropriate bioassays and discuss in detail the bioassays typically used to explore antimicrobial, antibiofilm, cytotoxic, antiviral, antioxidant, and anti-ageing potential. The concept of quality control and bioassay validation are introduced, followed by safety considerations, which are critical to advancing bioactive compounds to a higher stage of development. We conclude by providing an application-oriented view focused on the development of pharmaceuticals, food supplements, and cosmetics, the industrial pipelines where currently known marine natural products hold most potential. We highlight the importance of gaining reliable bioassay results, as these serve as a starting point for application-based development and further testing, as well as for consideration by regulatory authorities.
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Cover Letter

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Date: 7 July 2023

Dear Editor,

I am writing on behalf of 16 members of the COST action CA18238 Ocean4Biotech, who combine expertise in various areas of marine biotechnology. During our technical exchanges and discussions, we have found that understanding and conducting high quality bioassays is important for efficient use of natural resources such as the marine environment. Information on bioassays is abundant in the literature, but scattered, and reviews tend to focus on narrow fields or specific applications. Therefore, we decided to create an overview that covers all aspects of bioassays that are important in bringing a natural product from nature to the customer. We focused on marine natural products as they are an increasingly popular source, but the process is similar and applicable to all natural products. We believe that our proposed review entitled "**A guide to the use of bioassays in exploration of natural resources**" will be of interest and use to readers of Biotechnology Advances.

The aim of this review is to highlight the importance of selecting good bioassays and their properties, and to address aspects of the entire process from detection of bioactivity in natural extracts to bioactivity-guided purification and application. We raise important questions that the scientific community using bioassays should consider. We provide a comprehensive and critical overview of the bioassays most commonly used by the marine biodiscovery community, focusing on antimicrobial, cytotoxic, antiviral, antioxidant, and anti-ageing bioassays. We then introduce the concept of validation and quality control that ensures confidence in bioassay results. We then describe the importance of appropriate extraction methods and the steps involved in bioactivity-guided identification and purification. The review concludes with an application-oriented overview focusing on drug discovery, dietary supplements, and cosmetics, the industries most commonly supplied with marine-derived natural products. Safety and regulatory issues that are critical to the transition of substances to a higher stage of development are presented. In the conclusions, an outlook on trends and future developments is provided.

The review is extensive as it cites 273 references and includes 15,420 words, 9 figures, and 2 tables. In addition, a Supplementary table provides comprehensive information on commonly used bioassays with their advantages and limitations.

We believe that this topic will be of interest to a wide audience of researchers working with natural products, as bioassays are routinely used to characterise them. Such a guide would benefit novices in the field as well as those seeking to expand the potential of biodiscovery. The review focuses on marine natural products, but bioassays can be used universally for all natural products, so we believe the review will appeal to all communities that use bioassays in their research.

Yours sincerely,
Dr Jerica Sabotič, corresponding author

Author declaration

[Instructions: Please check all applicable boxes and provide additional information as requested.]

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Potential conflict of interest exists:

We wish to draw the attention of the Editor to the following facts, which may be considered as potential conflicts of interest, and to significant financial contributions to this work:

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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A guide to the use of bioassays in exploration of natural resources

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Highlights

Bioassay selection and quality is critical for optimal natural resource exploration.

Many variables should be considered when selecting or designing a bioassay.

Different types of bioassays are important for different phases of biodiscovery.

Validation of bioassays is important for more robust and reliable data generation.

Current marine biodiscovery mainly focuses on detection of antimicrobial activities.

A guide to the use of bioassays in exploration of natural resources

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Abstract

Bioassay are the main tool to decipher bioactivities from natural resources thus their selection and quality are critical for optimal bioprospecting. They are used both in the early stages of compound isolation/purification/identification, and in later stages to evaluate their safety and efficacy. In this review, we provide a comprehensive overview of the most common bioassays used in the discovery and development of new bioactive compounds with a focus on marine bioresources. We provide a comprehensive list of practical considerations for selecting appropriate bioassays and discuss in detail the bioassays typically used to explore antimicrobial, antibiofilm, cytotoxic, antiviral, antioxidant, and anti-ageing potential. The concept of quality control and bioassay validation are introduced, followed by safety considerations, which are critical to advancing bioactive compounds to a higher stage of development. We conclude by providing an application-oriented view focused on the development of pharmaceuticals, food supplements, and cosmetics, the industrial pipelines where currently known marine natural products hold most potential. We highlight the importance of gaining reliable bioassay results, as these serve as a starting point for application-based development and further testing, as well as for consideration by regulatory authorities.

Keywords

bioassay selection; bioactivity; **natural products**; **drug discovery**; **blue biotechnology; screening; bioactivity-guided purification; validation; preclinical trials; biodiscovery**

Abbreviations

ABTS/TEAC, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox®-Equivalent Antioxidant Capacity; ADMET, absorption, distribution, metabolism, excretion, toxicity; AFST, antifungal susceptibility testing; CADD, computer-aided drug design; CC50, 50 % cytotoxicity concentration; CLSI, Clinical and Laboratory Standards Institute; COST, European Cooperation in Science and Technology; CFU, colony forming unit; CPE, cytopathic effect; CTA, cell transformation assays; CUPRAC, CUPric Reducing Antioxidant Capacity; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; EC50, 50% effective concentration; EFSA, European Food Safety Authority; EMA, European Medicines Agency; ET, electron transfer; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FDA, United States Food and Drug Administration; FFA, focus-forming assay; FMCA, fluorometric microculture cytotoxicity assay; GI50, 50 % growth inhibition; GLP, good laboratory practice; HA, hemagglutinin; HAT, hydrogen atom transfer; HIA, hemagglutination inhibition assay; HTS, high-throughput screening; LLPS, liquid-liquid phase separation; LOD, limit of detection; LOQ, limit of quantitation; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; MIC, minimum inhibitory concentration; MOI, multiplication of infection; MBC, minimum bactericidal concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; a yellow tetrazole that is reduced to purple formazan in living cells; MS, mass spectrometry; MSPE, magnetic solid phase extraction; NAM, new approach methodology; OECD, Organisation for Economic Co-operation and Development; ORAC, oxygen radical absorbance capacity; PRA, plaque reduction assay; qPCR; quantitative real-time polymerase chain reaction; RBC, red blood cells; SI, selectivity index; SPE, solid phase extraction; VRA, virus yield reduction assay

Contents

1. Introduction

The most common approach to discovering new bioactive compounds is extensive screening of crude natural extracts using bioassay-guided protocols to determine their activity, followed by isolation and characterization of the active compounds, which are then used in a variety of biotechnological applications, including food, feed, agriculture, cosmetics, and veterinary and human medicine. The discovery of new marine natural products in the last five years has been driven primarily by marine fungi, but also by sponges, tunicates (ascidians), and molluscs/cyanobacteria, which are the source of most of the approved drugs in the marine pharmacology pipeline. In addition, marine viruses, bacteria, archaea, fungi, and phytoplankton or zooplankton, including cyanobacteria, green algae, thraustochytrids, and dinoflagellates, have long been studied as sources of natural bioactive products. To increase the chemical space and diversity of activities detected in bioassays, modifications of culture conditions or co-cultivation are used in the search for natural products from culturable microorganisms (e.g., (Lauritano et al., 2016; Marmann et al., 2014; Oh et al., 2005; Romano et al., 2018). Other sources of marine natural products include actinomycetes, brown, and red algae, cnidarians, bryozoans, and echinoderms (Barreca et al., 2020; Carroll et al., 2021; Jimenez et al., 2020; Rotter et al., 2021a).

The authors of this review are members of COST Action CA18238 Ocean4Biotech, a network of more than 150 blue biotechnology scientists and practitioners from 37 countries (Rotter et al., 2021b, 2020). Our goal is to provide a guide for decision making in the selection and use of bioassays to improve the efficiency of bioprospecting and discovery of bioactive marine compounds. A comprehensive overview of bioassays currently used in the marine bioprospecting community is provided, along with their strengths and weaknesses, followed by considerations for bioassay-guided identification and isolation. We also consider the importance of incorporating *in vitro*, *ex vivo*, and 3D human cell- or tissue-based bioassay protocols as important tools in the preclinical process to avoid drug failure in clinical trials, most often due to lack of clinical efficacy and/or unacceptable toxicity. We then present quality control procedures, including validation, that are required for further safety and efficacy testing, which will then pave the way for eventual regulatory approval for commercialization. The procedures and workflows described are general in nature and can be applied to a wide range of potential applications of bioactive compounds, from industrial enzymes to pharmaceuticals for human consumption. Therefore, we use the term bioactive compounds to refer to all structural variants of natural molecules, from small molecules to large polymers, including, for example, proteins and polysaccharides. Finally, we provide an application-oriented overview of the industrial pipelines most commonly supplied with marine-derived natural products, including those focused on the development of pharmaceuticals, dietary supplements, and cosmetics. By providing insight into the assays used to evaluate bioactivity and best practices in bioassays, this review aims to guide the natural products and blue biotechnology community in decision making for natural product discovery and development.

2. Bioassay types and their use in bioactive compound discovery

The biological relevance of natural extracts and pure compounds, whether natural or synthetic, is determined by the bioactivity assays or bioassays used (Weller, 2012). The term “bioactive” is defined as “having or causing an effect on living tissue” (Strömstedt et al., 2014). Different characteristics of bioassays such as throughput, complexity, speed, and cost are relevant to different stages of the biodiscovery process (Fig. 1). In the pre-screening and screening phase, the goal is to detect and

potentially quantify bioactivity potential. Therefore, bioassays should be performed in a high-throughput format screening format (HTS) that allows rapid and cost-effective testing of large number of samples or large libraries of extracts, extract fractions. or pure compounds. In the monitoring phase, bioassays are used to guide purification or fractionation processes to isolate and identify single pure bioactive compounds (bioactivity-guided approach), so they must be designed to have a high throughput capacity, be fast and easy to perform, and be cost-effective. Interestingly, innovative *in silico* approaches have recently been developed that do not require extract fractionation and are known as compound activity mapping (CAM) and are freely available (www.npanalyst.org) (Gaudêncio et al., 2023; Kurita et al., 2015; Lee et al., 2022; O'Rourke et al., 2020). Finally, in the secondary phase, bioassays are used to identify and characterize the biological mode of action of the bioactive compound, which typically requires a series of bioassays that must be highly specific and accurate and are usually time-consuming and expensive (Claeson and Bohlin, 1997; Strömstedt et al., 2014; Suffness and Pezzuto, 1991).

Bioassays can be performed *in silico*, *in vitro*, *ex vivo*, or *in vivo* at any of the levels described, and usually a combination of these methods is used to characterise a new compound or the bioactivity potential of a natural resource. When screening an extract for medicinal activity, *in silico* and *in vitro* assays are typically used to identify the bioactive compound and its mode of action, while *in silico* and *in vivo* assays (e.g., animal studies) provide information on pharmacological activity and toxicity (Mbah et al., 2012; Strömstedt et al., 2014).

3. Practical considerations in choosing bioassays to detect target bioactivity

The following paragraphs provide a list of questions and considerations, the answers to which provide information on what to consider when selecting or designing a bioassay (Table 1, Fig. 2).

At what stage of the discovery process and for what purpose will the bioassay be performed? Considering the target bioactivity of interest, appropriate bioassays can be selected and used to screen crude or fractionated extracts, to guide subsequent purification, or to explain underlying mechanisms of action, as described in the previous section. First and foremost, the target bioactivity should be selected. An overview of the most commonly used bioassays can be found in Supplementary Table S1.

Is there an interest in a specific or general activity? In general, bioassays can be divided into two distinct categories: "single-target bioassays" and "functional multi-target bioassays". Single-target bioassays are generally designed to detect the effect of the tested compounds on a particular target with a high degree of specificity and based on a distinct mechanism of action (Claeson and Bohlin, 1997). Examples include the analysis of specific enzymatic activities, such as the degradation of proteins or breakdown of plastics, or the inhibition of enzymatic activities, such as the inhibition of proteases and the blocking of target receptors. Another variation of single-target bioassays is "chemical-genetic profiling" in yeast. A panel of yeast strains with selective mutations that highlight sensitivity to specific drugs is used to screen known compounds with unknown modes of action or mixtures of compounds such as natural product extracts (Harvey, 2008). The second category, "functional multi-target bioassays", includes bioassays that use whole animals, organs or cells. These bioassays are non-specific in their outcome and measure phenotype change or a general biological effect, such as an antimicrobial or cytotoxic effect. The response to the bioactive compound tested

cannot necessarily be attributed to a specific mode of action. These are often referred to as the "phenotype-based approach" (Claeson and Bohlin, 1997; Swinney, 2013).

Which are the most common bioassays for determining target activity? The target bioactivity can be assessed using a variety of bioassays, but the scientific community may prefer certain assays for which troubleshooting, appropriate controls, and interpretation support are available (Supplementary Table S1).

Are resources available to perform bioassays (in terms of ease of execution or technical complexity)? Specialized equipment and/or trained personnel are required to perform certain bioassays. In terms of safety, it is also important to consider whether the bioassay uses hazardous chemicals or organisms that must be handled in safety chambers and comply with local regulations (e.g., consider the biosafety level (BSL) of the target organisms or the use of genetically modified organisms (GMOs)).

What are the associated costs for personnel, equipment, and materials? Will the bioassay be used as a routine method? A bioassay may be simple (e.g., an enzymatic reaction detected by a colour change) and performed by a technician, whereas some types of bioassays (e.g., bioassays using cell culture) require extensive training. Similarly, bioassays may be more or less labour-intensive and require specialised equipment or expensive consumables.

Is high throughput and full automation of the analytical process required? Bioassays often use a 96-well plate format. A common plate-related phenomenon is the so-called "edge effect", in which the response in peripheral wells differs from the response observed in the inner wells of a microplate. There are several approaches to avoid this problem, such as using only the inner wells, randomization in plate design, or replication (White et al., 2019). Recently, some manufacturers offer plates with a built-in moat surrounding the outer wells (or even both inner and outer wells), that is filled with water, and serving as an evaporation buffer during prolonged incubation. Depending on the desired throughput, robotic liquid handling systems can be used to fully automate almost any bioassay workflow, but the initial cost of such systems can be prohibitive for small laboratories.

Are standardized forms of bioassay available? Although standardization of bioassays facilitates interpretation and comparison of data between laboratories and allows better monitoring of bioassay performance, standardized bioassay protocols are available for only a limited number of bioassays. Inter-laboratory reproducibility or precision under the same operating conditions becomes more and more valuable in stages of higher levels of technology readiness (TRL).

What is required to interpret the results of the bioassay? What are the appropriate controls to distinguish true results from false positives or false negatives? Before beginning to interpret the results, it is assumed that the test performance was appropriate. This can be verified by including an external positive or negative control (or sometimes an internal standard) in the assays, such as organisms with a known phenotype, to ensure that the bioassay performance was optimal. The measurements obtained can be compared to positive and/or negative controls, as well as to blank measurements, to evaluate the effects of medium/buffer/background. Although method validation at the discovery level is not essential, evaluation of precision, i.e., the degree of scatter between a series of replicate measurements obtained from multiple samplings of the same homogeneous sample under the same conditions – expressed as coefficient of variation (CV) - makes the data more robust and reliable.

How are the results to be interpreted in a meaningful way? Is the extract/compound bioactive? Benchmarks and thresholds for bioactivity must be considered, as there are common thresholds below

which an extract is considered very active or moderately active, while above these thresholds it is considered of little interest for further development. Meaningful evaluation of the results in combination with chemical dereplication strategies (i.e., evaluating the presence of known compounds in the crude extracts) (Gaudêncio and Pereira, 2015) plays a very important role in prioritizing samples for further development and deciding which samples are worthwhile for further development investment.

What is the expected content of bioactive compounds in the extract? How complex is the crude extract and what is the level of background substances that would interfere with the measurement of bioactivity? Advanced dereplication methods are used for natural product profiling/fingerprinting of complex extracts (Gaudêncio and Pereira, 2015). An estimate of the expected content of bioactive target compounds helps in the selection of the bioassay to avoid false positives in terms of required sensitivity (high sensitivity for low-content compounds), selectivity (the extent to which the bioassay can differentiate and detect a target analyte without interference from concurrently present irrelevant compounds), and specificity, which is a measure of high selectivity (the ability to unambiguously detect the target analyte in the presence of other substances, including those with similar chemical structures). It also helps in the selection of appropriate controls and thus in the interpretation of data. For some compounds, spiking samples with a reference standard can be a solution for detection and quantitation, but a suitable standard must be available.

What is the desired level of quantitative response (qualitative, semi-quantitative, quantitative results)? Does the potency need to be accurately assessed? Measurements can be binary (activity present or absent), or quantitative information can be obtained by comparison with appropriate controls. Although only quantitative bioassays are suitable for unambiguous determination of potency, the need for such accurate information may be more important at later stages of discovery, purification, safety, and efficacy testing. Quantitative assays often use standard compounds (spiking, calibration curves), and it is worthwhile to check the availability of appropriate standards. In the context of interpretation of results, determination of the limit of detection (LOD) and limit of quantification (LOQ) provides better reliability of data. In addition, selection of bioassays with lower limits of detection and quantitation usually results in a higher degree of confidence in the final data.

It is useful to know what may affect the precision or repeatability of bioassays. Some metabolites show synergistic effects and bioactivity is lost after fractionation, or metabolites may act antagonistically and activity is detected only after fractionation. In addition, physical parameters of the extract (viscosity, pH, colour, etc.) can lead to false-positive and false-negative results. Potential interferences can arise from the material of the sample containers (usually polypropylene and polystyrene, treated or untreated, or glass), and these should be carefully selected based on the charge and polarity of the molecules to be tested, if known (Strömstedt et al., 2014).

What is the solubility and stability of the compound of interest? Is it a small molecule or a complex molecule? The solvent used for extraction must not be toxic or should not be used at a concentration that is toxic to the microorganisms, cells, tissues, organs, or organisms. When aqueous solutions are not used for extraction, extractions are usually performed with dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), methanol, or ethanol, which can be tolerated in microbial or cell-based assays only at low concentrations (e.g., up to 1 % DMSO) and whose presence may affect final results (Dyrda et al., 2019; Hipsher et al., 2021; Rekha et al., 2006). Compounds extracted with organic solvents can be vacuum dried to mitigate this issue. Nevertheless, the effect of extraction solvents can be evaluated by performing the bioassay with the solvent as a control. In addition, poor

water solubility can lead to misleading results. Bioassay optimization strategies are recommended to improve bioassay performance for poorly soluble compounds (Di and Kerns, 2006).

As mentioned earlier, the effect of extraction medium is evaluated by performing the bioassay with the extraction solution alone. If necessary, this control is performed each time the bioassay is conducted. Characteristics of the extraction medium such as thermostability, volatility, and complexity (sedimentation properties and migration) can also affect the design of the bioassay, while characteristics of the target substance such as thermostability, susceptibility to proteolytic degradation, and complexity that affect the temperature and timing of extraction can also affect the desired bioactivity. For example, enzymes are typically isolated at low temperatures because they can be sensitive to proteolytic degradation or thermal denaturation, which can lead to loss of bioactivity. In addition, natural products should be handled at temperatures below 40 °C to avoid degradation and loss of bioactivity. In general, it is preferable to work with compounds that are stable under various conditions, especially with regard to further development and for practical reasons with regard to the application and marketing of the final products.

Do seasonal and geographic differences or legal aspects of sampling affect samples used for bioactivity screening and thus affect biodiscovery? For many types of natural samples, re-sampling is limited due to large seasonal or geographic variations. In addition, issues of safety and sustainability should be considered. Legal issues can also limit transnational access to (marine) biological resources, but this obstacle can be effectively addressed under the Nagoya Protocol and by following well-regulated procedures (Schneider et al., 2022).

Is there a need and possibility to validate the bioassay? Validation of bioassays in the discovery phase is useful for evaluating efficacy of candidate bioactivities with high precision and accuracy. This is also important for planning safety and efficacy testing and clinical trials, establishing the basis for discussions with regulatory authorities during planning. At later stages, at the quality control level, bioassays should also reliably assess the quality across different product batches.

What are the relevant target organisms? In bioassays involving living organisms, e.g., microorganisms, cell lines, or animals, it is important to select appropriate target organisms with respect to their relevance and the particular requirements for handling these organisms. An important aspect to consider is the growth conditions, as different growth conditions may affect the outcome of the bioassay.

Do we have a clear idea of the intended application? If there is a clear idea of an application/use, the local regulatory authority should be approached early in biodiscovery, as it is beneficial to use those bioassays that are congruent with product development, as this can be very useful to expedite the process.

Table 1

What to consider when selecting a bioassay to search for a selected bioactivity

Purpose
Is it aimed at general or specific bioactivity?
How selective should it be?
Are quantitative or qualitative results needed?
How sensitive should it be (what is the requirement for the minimal amount of compound)?
Cost
Time requirement

Labour intensiveness
Cost of material
Requirement of special equipment (different modes of detection)
Effect of the extraction procedure on bioactivity
Selection of source material (amount available, possibility to reacquire)
Availability of source material (seasonal, geographic, legal)
Organic solvent or water-based
Temperature of extraction
Length of extraction
Homogenization steps
Cultivation steps
Stability of bioactive compound
Interference with materials used for extraction (e.g., plastic, solvent components)
Feasibility
Errors caused by the colour or viscosity of extracts
Reproducibility
High-throughput capacity or automation possibility
Ease of results interpretation
Other
Availability of standards
Bioactivity threshold
Capability of dereplication
Regulatory requirements (e.g., use of BSL2 or GMO organisms)

3.1. Specifics of marine samples

When working with marine extracts or marine microorganisms in bioassays, special considerations should be made and adapted to the presence of salt, poorly hydrophilic, often highly coloured or autofluorescent, and chemically complex materials. Moreover, when working with higher organisms as a source of bioactivity, it should be verified whether the bioactivity originates from the macroorganism or from the associated microbiota (Beutler, 2009; De La Calle, 2017; Macedo et al., 2021). Geographic or seasonal variations in the production of bioactive metabolites, which have been demonstrated for different marine organisms (El-Wahidi et al., 2011; Heavisides et al., 2018; Hellio et al., 2004; Henrikson and Pawlik, 1998), are another important issue.

4. Prevalent bioassays in marine biodiscovery

Using a literature search of the PubMed database, we analysed research efforts on marine natural product discovery between 2000 and 2022 (Fig. 3). There is a panoply of bioassays that can be used to screen natural resources for their bioactive properties. We have compiled the most common of these in Supplementary Table S1 and provided a critical overview of their advantages and disadvantages.

Here, we provide an overview of antimicrobial, antifungal, antiviral, and cytotoxicity bioassays, as well as those that investigate the antioxidant and anti-ageing potential of marine extracts. These include both phenotype-based and single-target bioassays to varying degrees, e.g., antimicrobial assays are mostly phenotype-based, whereas both phenotype-based and single-target bioassays can be used to assess cytotoxicity.

4.1. Antimicrobial bioassays

The strongest research efforts in the field of bioactivity of natural marine sources have been dedicated to the detection of antimicrobial activities using phenotypic assays (Fig. 3). The term antimicrobial activity encompasses both antibacterial and antifungal activities, but is often used in studies that work only with bacteria, whereas other studies claiming antimicrobial activities examine both bacteria and fungi. In addition, there are studies that focus on one group of organisms and investigate either antibacterial or antifungal bioactivity. The increased efforts are mainly due to the worldwide decline in the development of antibiotics, while the increasing emergence of microorganisms resistant to antimicrobials is becoming a global health threat (Dadgostar, 2019). The problem is of particular concern for the Gram-positive and Gram-negative bacterial pathogens that belong to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.),* and some fungal pathogens (*Candida auris, Candida glabrata, Aspergillus fumigatus, Cryptococcus neoformans*), for which an increasing number of multidrug-resistant strains have been identified worldwide (Arendrup and Patterson, 2017; Liu et al., 2019; Minarini et al., 2020). The ecological diversity of the marine environment and (micro)organisms in this habitat, combined with the large genetic diversity, represents a unique and rich source of compounds that can be exploited by the pharmaceutical industry and potentially provide solutions to the increasing number of drug-resistant infectious diseases (Hughes and Fenical, 2010; Liu et al., 2019).

The most commonly used bioassay to investigate the antimicrobial activity of marine natural products is the determination of minimum inhibitory concentration (MIC) in the form of broth microdilution, macrodilution, and agar dilution, followed by the disc diffusion/Kirby–Bauer method (Fig. 4, Supplementary Table S1). These bioassays determine the lowest concentration of an antimicrobial agent that prevents visible or measurable growth of a microorganism. Two organizations develop standardized reference methods for antimicrobial susceptibility testing: the Clinical & Laboratory Standards Institute (CLSI) (https://clsi.org/) and the European Committee on Antimicrobial Susceptibility Testing (https://www.eucast.org/). Although some guidelines from standardized protocols should also apply to bioassays performed on marine samples, noncompliance with these guidelines is relatively common. Items whose standardization has a critical impact on the repeatability and reliability of results include the selection of microbial species and strains, the size and age of the *inoculum*, the type of culture medium, and the duration of incubation. To ensure the quality of the bioassay performed, a positive control of a standard antibiotic should be tested against authenticated microbial strains, preferably from a type culture collection such as national type cultures collections (e.g., National Collection of Type Cultures (NCTC) in the United Kingdom; German Collection of Microorganisms and Cell Cultures DSMZ; American Type Culture Collection - ATCC). Reagent sterility controls and negative controls (e.g., influence of solvents) should also be included in each bioassay. When working with complex samples such as natural extracts, the presence of other metabolites in the extract can potentially serve as a carbon source for the microorganism used, which can mask the

effect. Both technical and biological replicates should be performed to increase measurement accuracy.

The main advantages of dilution methods are the ability to obtain quantitative MIC values (minimum concentration that inhibits microbial growth) and MBC values (minimum bactericidal concentration, lowest concentration at which 99.9% of bacteria are killed). Published MIC values for marine extracts vary from μg/mL to even mg/mL and are generally below 100 μg/mL for pure compounds (Choudhary et al., 2017). There are common thresholds at which the extract is considered very active (<10 μg/mL), moderately active (10-250 μg/mL), and with little or no activity (> 250 μg/mL) (Fajarningsih et al., 2018; Nweze et al., 2020; Pech-Puch et al., 2020). The optimal MIC and IC_{50} (concentration at which 50 % of growth inhibition is achieved) for a pure substance should be below 1 μg/mL, while concentrations above 10 μg/mL are considered of little interest for further research (Cushnie et al., 2020). In the diffusion-based method, there is no quantitative result or only a limited one. However, both types of bioassays can be useful to analyse the difference in antimicrobial activity of individual natural products observed in different strains of a given species (e.g., resistant and non-resistant mutants). *In vitro* assays are characterised by simplicity of design and performance. They are traditionally time-consuming but can be automated. However, the results are usually not available within a day and do not provide information on the mechanism of action.

Gram-positive bacteria are more sensitive to the effects of many known agents than Gram-negative ones, which increases the likelihood of hits in screening studies (Cos et al., 2006). For this reason, microorganisms from different groups should be included in the screening process. For each microorganism tested, the optimal growth medium and inoculum size should be determined to avoid underestimation or masking of antimicrobial activity (Wiegand et al., 2008). Many published studies have used Lysogeny Broth (LB) media for antibacterial testing, but their use should be avoided due to the imbalanced composition of carbohydrates, low availability of divalent cations, and occasional contamination with bile salts (Nikaido, 2009; Sezonov et al., 2007).

The type of extracts (the type of solvent used) should be considered when choosing one of the above methods. For example, lipophilic compounds do not diffuse well into solid culture media, whereas strongly charged molecules may undergo ion exchange processes in agar. Therefore, the agar diffusion method is more suitable for the analysis of single metabolites with known polarity and not for complex extracts.

To further investigate the antimicrobial activity of natural molecules, time-kill assays and flow cytometry methods can be used to provide information on the nature of the inhibitory effect and the cellular damage inflicted on the test microorganism (Balouiri et al., 2016). This bioassay is used in a second phase of testing to determine the dynamics of microbial inhibition kinetics (Dinarvand et al., 2020). Most antimicrobial bioassays are performed *in vitro*, but secondary screening for highly potent compounds may also include *in vivo* assays, (e.g., in murine models), to gain better insight into their preclinical potential (Martín et al., 2013). *In vivo* bioassays are generally not performed with extracts because of the difficulty of interpreting effects based on an unknown mixture of compounds. However, in some examples, *in vivo* testing is recommended early in the development timeline because potential systemic side effects may be antagonistic or synergistic (Sabotič et al., 2020).

4.1.1. Antibiofilm assays

In recent years, the control of microbial biofilms has gained significant attention as it is increasingly recognized that biofilms are responsible for microbial persistence. New strategies for

combating microorganisms focus on the one hand on preventing biofilm formation by inhibiting adhesion or quorum sensing and on the other hand on eliminating biofilms by dispersion. Antibiofilm agents are therefore considered as an alternative to fight microbial resistance to antibiotics, since microorganisms do not need to develop resistance to adapt, as their population is not decimated, but merely prevented from persisting in the selected environment. To date, there is only one standardized assay for antibiofilm activity, namely the single-tube method (ASTM E2871), which is supported by a standard practice for biofilm growth in a CDC biofilm reactor (ASTM E3161) optimized for biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (ASTM E2871-21, 2021; ASTM E3161-21, 2022; Lozano et al., 2020). Biofilm formation is usually monitored by crystal violet staining, which is used to stain the biomass of the biofilm. Other commonly used methods include measuring the metabolic activities of biofilm cells with tetrazolium salts, culturing biofilm cells after sonication to determine the number of CFUs (colony forming units) in the biofilm, or microscopy, which can be either scanning electron microscopy or confocal laser scanning microscopy (Bridier et al., 2010; Haney et al., 2021; Kırmusaoğlu, 2019; Klančnik et al., 2017; Peeters et al., 2008). Antibiofilm activity is often expressed as minimum biofilm inhibitory concentration (MBIC) or CFU log reduction. A microplate format can be adapted for high-throughput screening evaluation of antibiofilm efficacy, typically screening individual compounds at concentrations of up to 100 µM and identifying active hits as those that inhibit biofilm formation by ≥ 80% while simultaneously inhibiting bacterial growth by ≤ 40% (Kwasny and Opperman, 2010). Inhibiting biofilm formation without affecting bacterial growth is preferable because there is less pressure on survival and consequently on the development of resistance (Sterniša et al., 2022).

Bacterial cell-to-cell communication, which senses the density of bacterial cells and is referred to as quorum sensing, is an important component of the biofilm formation process and bacterial virulence. To identify antibiofilm agents, inhibition of quorum sensing is usually tested using quorum sensing reporter strains. However, this approach has some limitations, including negative effects on reporter strain growth, so appropriate control experiments are essential to obtain reliable results (Defoirdt, 2018; Defoirdt et al., 2013; Taga and Xavier, 2011; Zhao et al., 2020). Alternative methods have been developed that provide a better approximation of real biofilm conditions but require specialized equipment, such as delicate microfluidic systems (Goeres et al., 2005; Millar et al., 2001; Tremblay et al., 2015), the Calgary Biofilm Device (Ceri et al., 1999) or the BioFilm Ring Test (Olivares et al., 2016). Simultaneous detection of antimicrobial and antibiofilm activity against important pathogenic bacteria is also possible by studying their growth kinetics with a microplate reader and using a growth curve analysis (Sterniša et al., 2022). Determination of both antibiofilm (i.e., inhibition of biofilm formation or promotion of biofilm dispersion) and antimicrobial (i.e., inhibition of growth and/or survival) activity is important to understand whether the compounds tested affect biofilm formation directly or indirectly.

4.1.2. Special consideration for antifungal bioassays

The nature of filamentous fungal growth requires the use of adapted bioassays to test the antifungal activities of metabolites and molecules. The prevalence of fungal infections (both invasive and opportunistic fungal infections) is rising due to the increase in the ageing population and immunocompromised patients (Webb et al., 2018). In addition, acquired resistance has emerged in clinically relevant fungi such as *Candida* spp. and *Aspergillus* spp. Therefore, antifungal susceptibility testing (AFST) is of increasing importance in clinical microbiology laboratories, both for selection of appropriate therapy and to provide information on resistance rates at local and global levels in

epidemiological studies. The same tests are also used for screening natural products and guiding the discovery of new antifungal agents. Many factors can influence the outcome of *in vitro* AFST tests, including the definition of the endpoint, the inoculum size of the studied fungus, the incubation period, the temperature, and the culture media used for the test (Berkow et al., 2020). For this reason, AFST is not recommended for every fungal pathogen detected in a sample and is performed in clinical microbiology laboratories primarily for yeasts.

Broth microdilution bioassays are routinely used for fungi, and there are two standard methods for broth microdilution testing of yeasts in clinical laboratories and two others for molds: those established by the Clinical and Laboratory Standards Institute (CLSI) and those established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Arendrup et al., 2008; Clinical and Laboratory Standards Institute, 2017a, 2017b; Rodriguez-Tudela et al., 2008). The four standards use the same criteria to define the test endpoint and use similar criteria to develop clinical breakpoints and thus interpret antifungal resistance and/or susceptibility. However, they differ in several aspects regarding media composition, test microorganism preparation (including inoculum size), measurement methods, and positive controls. Standardized protocols based on disk diffusion are available for both yeasts (Clinical and Laboratory Standards Institute, 2009) and filamentous fungi (Clinical and Laboratory Standards Institute, 2010). Although the qualitative results of the disk diffusion method are suitable for routine use in the clinical laboratory, the quantitative MIC data are more relevant for the treatment of invasive infections. Agar-based antifungal screening or "poisoned food assays", in which fungal growth on a standard agar containing antifungal agents is evaluated. Commercial kits are available for antifungal screening of *Candida* and *Aspergillus* spp..

Alternative methods for determining antifungal activity using specialized equipment have also been developed. These techniques include flow cytometry, in which changes in fluorescence are interpreted as changes in cell viability and fungal damage (Chaturvedi et al., 2004). With MALDI-TOF, changes in the proteome compared to a drug-free control are interpreted as indicators of antifungal activity (Sanguinetti and Posteraro, 2016). Isothermal microcalorimetry is used to determine changes in metabolic heat flow of cultured fungi in response to an antifungal agent and indirectly assess its activity (Furustrand Tafin et al., 2013).

4.2. Cytotoxicity bioassays

Cytotoxic activity is the second most studied bioactivity for marine natural products in the last twenty years (Fig. 9, Fig. 3, Fig. 5). Cytotoxicity is often studied in terms of possible anticancer activity. There are several types of bioassays to analyse the cytotoxic properties of natural products, which include phenotypic and single-target bioassays. They are based either on the selective penetration of dyes into dead and living cells or on the detection of markers leaking from the cytoplasm of dead cells. Cytotoxicity bioassays based on selective dye penetration can be divided according to the nature of their endpoints into colorimetric assays (e.g., tetrazolium salts such as MTT, MTS, XTT, or WST, trypan blue, sulforhodamine B (SRB), neutral red uptake (NRU), crystal violet), fluorometric assays (Alamar Blue (AB), 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM), carboxyfluorescein succinimidyl ester (CFSE), propidium iodide (PI), Hoechst-33342, protease viability using glycylphenylalanyl-aminofluorumarin (GF-AFC) as substrate, and luminometric assays (ATP-based and real-time viability) as reviewed elsewhere (Aslantürk, 2018; Riss et al., 2019). The most commonly used bioassays based on markers leaking from dead cells measure the activity of lactate dehydrogenase (LDH), adenylate kinase (AK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or

aminopeptidase. Another option is to preload cells with a measurable marker such as calcein-AM or radioactive ^{51}Cr, which is typically used for mixed cell assays in immunology (Aslantürk, 2018; Riss et al., 2019). Assays are usually performed either in microplate format or flow cytometrically.

Testing different cell types is essential, especially in the context of cancer research, as each cell type may respond differently to treatment (Niepel et al., 2017). The screening of 60 human tumour cell lines for anticancer drugs (NCI60) by the US National Cancer Institute (NCI) was developed in the late 1980s as a tool for *in vitro* drug discovery and then expanded into a service screening to support cancer research. In 2018, the NCI established a Program for Natural Product Development (NPNPD) to develop a publicly accessible HTS-amenable library of more than 1,000,000 fractions from 125,000 marine, microbial, and plant extracts gathered from around the world to advance HTS efforts and accelerate NP drug development. By 2019, 384-well plates containing over 326,000 fractions were made available for free screening against any disease target (Gaudêncio et al., 2023; Thornburg et al., 2018). Regarding the evaluation criteria for cytotoxic activity, it was suggested that crude extracts showing 50 % growth inhibition (GI_{50}) at concentrations below 100 µg/ml should be considered cytotoxic, while those holding promise for further investigation should have a GI_{50} below 30 µg/ml (Suffness and Pezzuto, 1991). Although cytotoxicity screening aims to identify compounds with growth inhibitory or toxic effects on specific tumour types (disease-oriented approach), the patterns of relative drug sensitivity and resistance generated with standard anticancer drugs can also help to determine the mechanisms of action of the compounds tested. The information-rich nature of the screening data thus provides additional insight into cytotoxic effects (Shoemaker, 2006). The pattern recognition algorithm COMPARE assigns a biological response pattern to the 60-cell line dose-response data for a compound and evaluates whether the response is unique or resembles a known or prototypical compound to assign a putative mechanism of action to a tested compound. As more data are collected on the characterization of different cellular molecular targets of the compounds tested, the compounds most likely to interact with a particular molecular target can be selected (Park et al., 2010; Zaharevitz et al., 2002). The accuracy of cytotoxic bioassays is strongly influenced by cell type, seeding density, and medium composition. Therefore, it is important to include appropriate controls such as background control (no cells), negative control (untreated cells), and positive control (all cells dead) and to test different cell types (Aslantürk, 2018; Carlsen et al., 2020; Cox et al., 2021; Riss and Moravec, 2004).

An important aspect to consider when selecting an appropriate bioassay is understanding the mechanism of cell death and the resulting kinetics. In this context, apoptosis-specific (e.g., Annexin-V binding or addition of a caspase inhibitor) or necrosis-specific assays (e.g., detection of the released High mobility group box 1 (HMGB1) protein or addition of specific inhibitors) can be used (Raucci et al., 2007; Riss and Moravec, 2004; Shounan et al., 1998). Preferably, cytotoxicity assays should be performed to cover multiple endpoints and determine multiple parameters from the same cell sample that can reveal the actual cause of cell death (Aslantürk, 2018; Santacroce et al., 2015). Another aspect to consider is whether the effect is cytotoxic or cytostatic (Anttila et al., 2019; Mervin et al., 2016). Understanding the mode of action and molecular mechanisms targeted by cytotoxic compounds is important for rational decision making about their use in specific cancer types, and for assessing the risk of potential cross-reactivity with other treatments, and side effects.

4.3. Antiviral Bioassays

Viral infections are a major cause of disease in the world because of their complexity, diversity, and rapid spread, which is often accelerated by urbanization, increased migration, and globalization (Drexler, 2011). The 21st century is characterized by major viral epidemics and pandemics, such as influenza A (H1N1) pdm/09, Ebola, Zika, severe acute respiratory syndrome (SARS), Middle Eastern respiratory syndrome (MERS) and SARS-CoV-2 (Ong et al., 2020). In light of these emerging viruses, as well as endemic viruses and the emergence of viral resistance, attention has focused on natural products as sources of new antiviral drugs, including those from the marine environment (Bhadury et al., 2006; da Silva et al., 2006; Dias et al., 2018; Linnakoski et al., 2018; Tziveleka et al., 2003).

The very first step before an antiviral assay is to determine the potential toxicity of the compounds or extracts to host cells (Fig. 6). This is essential to rule out the possibility that the antiviral properties observed *in vitro* are not due to cytotoxicity. For cytotoxicity screening, any of the methods described in the previous section can be used. Although the MTT assay has been widely used in the past, the ATP-based assay has proven to be the gold standard for measuring cell viability to date. It is more sensitive than conventional biochemical methods because it detects cell death by a general rather than a specific biological mechanism (Herzog et al., 2007; Ponti et al., 2006). However, assays based on cell metabolism are not suitable for metabolically inactive cells, for which the fluorometric microculture cytotoxicity assay (FMCA) is becoming increasingly popular. The FMCA assay is based on the hydrolysis of the fluorescein diacetate (FDA) probe by the cytosolic esterases of intact cells (Burman et al., 2011; Lindhagen et al., 2008; Strömstedt et al., 2014), and cell survival is reported as an index of survival after treatment. If the results of the cytotoxicity assays indicate no effect on cell line fitness, the compounds can then be tested with primary antiviral assays (Supplementary Table S1, Fig. 6)(Gomes et al., 2016).

In cytotoxicity evaluation, the value of the 50% cytotoxicity concentration (CC_{50}), defined as the concentration of a compound that produces a 50% cytotoxic effect (Hu and Hsiung, 1989), is determined and used together with the value of the 50% effective concentration (EC_{50}, i.e., the concentration of a compound that produces a 50% inhibition of viral replication) to evaluate the efficacy of an antiviral candidate. This relative efficacy of a compound in inhibiting viral replication with respect to inducing cell death is defined as the therapeutic or selectivity index (SI). Theoretically, a high SI ratio corresponds to a safer and more effective compound that is cytotoxic only at very high concentrations and exhibits antiviral activity at very low concentrations (Naesens et al., 2006; Reymen et al., 1995). The antiviral activity is considered effective/useful when the CC_{50} value is 20 times higher than the EC_{50} value (Cao et al., 2015). Since the CC_{50} and EC_{50} values for a given compound depend on the assays used, the SI value varies from laboratory to laboratory. Nevertheless, the SI value is a widely accepted parameter of a compound that expresses its *in vitro* efficacy in inhibiting viral replication (Naesens et al., 2006; Reymen et al., 1995).

Several different assays can be used to determine antiviral activity (Supplementary Table S1)(De Clercq et al., 1980; Sauer et al., 1984; Sidwell, 1986; WHO Scientific Group, 1987). At this point, it is necessary to determine the cell system(s) best suited for virus replication on which to test new antiviral agents. Depending on the cell type used, the replication capacity of the virus and its actual effect on cells varies considerably (i.e., some viruses may cause a cytopathic effect (CPE), while others may form plaques or induce specific functions such as hemagglutination (e.g., orthomixyxovirus and paramixovirus) or hemadsorption.

A cytopathic effect (CPE) test is based on the observation of morphological changes that occur in host cells as a result of viral infection and replication. The CPE-based assay was the first assay developed to evaluate whether a compound is antivirally effective, and it can also be scaled up for high-throughput screening (Maddox et al., 2008; Severson et al., 2007). Because viral replication leads to cell death, cell viability assays can be considered a substitute for CPE assessment because they are more accurate, automatable, and objective compared to visual assessment by an operator. Since CPE is an indirect measure of viral load, the result regarding the protective effect of drugs against a virus may also vary and be lower than other tests that measure viral load directly (PRA, VRA, see below) (Gorshkov et al., 2021). Although the CPE assay was one of the first antiviral assays developed, commercial kits (e.g., Viral ToxGlo Assay) that measure cellular ATP as an indicator of host cell survival have allowed standardization of the procedure in many laboratories, and ATP depletion can be correlated with viral load.

A widely used quantitative biological titration method is the plaque reduction assay (PRA). This method is based on counting plaques formed by lysis of infected cells in a monolayer. The plaques are visible to the naked eye or under a light microscope after staining with neutral red or crystal violet. The plaque assay is the preferred method of viral titration because it is economical and technically simple, but it can be tedious because visible viral plaques can take from 24 hours to several weeks to form (El Sayed, 2000). Conflicting results may be obtained due to various limitations (see Supplementary Table S1). Therefore, in addition to PRA, the virus yield reduction assay (VRA) is recommended to determine the EC_{50} value by assessing viral progeny production in a growth experiment. The assay conditions must be optimized, especially the multiplication of infection (MOI, i.e., the ratio of virus to cell number), because this single parameter can significantly affect the evaluation of antiviral activity and (a high MOI reduces the sensitivity of the virus to an antiviral agent (Collins and Bauer, 1977; Sauer et al., 1984). Therefore, it is advisable to perform VRA at both low MOI (multicycle viral replication) and high MOI (single-cycle replication), to compare the resulting EC_{50} values, and to evaluate the range of action of the antiviral molecule as accurately as possible (Yang et al., 1989).

For viruses that do not cause cytopathic effects, the focus-forming assay (FFA), an indirect method for virus measurement, can be used. This is a variant of the plaque assay that relies on immunohistochemical techniques, as it uses chemically or fluorescently labelled antibodies specific for a viral antigen to detect infected cells (Flint S.J et al., 2009). For example, quantification of infectious viral particles for α- (hCoV229-E) and β- (hCoV-OC43) coronaviruses relies on an enzymatic antigen detection method that uses horseradish peroxidase (HRP) to label antigen-antibody complexes (Lambert et al., 2008). Alternatively, if the viruses express hemagglutinin (HA), an envelope glycoprotein (e.g., influenza virus, respiratory syncytial virus), the hemagglutination inhibition assay (HIA) can be used. The method is based on measuring the ability of virions to adsorb to and agglutinate red blood cells (RBCs) by binding to glycans (e.g., sialic acid) on the surface of red blood cells (usually from rabbits, horses, chickens or guinea pigs). In practice, the hemagglutination assay is used to determine the viral concentration that agglutinates an exact (standard) number of erythrocytes, making it extremely accurate, although it is only applicable to certain viruses (Joklik, 1988). Standardization of the HIA assay has been described (Kaufmann et al., 2017). In particular, before performing the assay, the following should be considered: (i) although HIA assays provide consistent results across multiple plates, the same amount of virus particles must be used in each plate; (ii) according to WHO, the standard amount of HA used in the HIA assay is 4 units per 25 µL [HA unit is the

amount of virus required to agglutinate an equal volume of standardized RBC suspension]; (iii) the RBCs used depend on the type of influenza virus in the assay; and (iv) for different types of 96-well microtiter plates (V- or U-bottom), the incubation time and the occurrence of nonagglutinated cells are different (Kaufmann et al., 2017).

After a certain type of antiviral activity is detected, it is necessary to further investigate this activity using several specialized secondary bioassays for screening and/or monitoring purposes. These *in vitro* or *in vivo* assays are time-consuming, more expensive, and more challenging than the primary screening bioassays and require the expertise of biochemists or pharmacologists. Therefore, they can only be performed by a multidisciplinary team. Such secondary assays are necessary/mandatory to select potential candidates to be tested in human clinical trials (Gomes et al., 2016; Öberg and Vrang, 1990).

Meanwhile, new modern assays such as flow cytometry, tunable resistive pulse sensing (TRPS), and quantitative real-time PCR (qPCR) are also increasingly being developed to determine antiviral activity. In particular, qPCR was widely used during the SARS-CoV-2 pandemic because it allowed testing of antiviral activity of many molecules against SARS-CoV-2 in a short time. However, it is important to emphasize that during viral replication, the ratio of whole virions to nucleic acid copies is rarely 1:1 and that the viral assembly process can produce complete virions, empty capsids, and/or an excess of free viral genomes. Therefore, positive qPCR results may also be due to the presence of residual viral nucleic acid (i.e., noninfectious virus) rather than infectious virus (Tandon and Mocarski, 2012). For this reason, many molecules with true antiviral activity might be rejected *a priori* simply because they are unable to reduce viral genome copy number in a solution, even if the viruses present are no longer active or infectious. Therefore, it is better to use qPCR-based methods for routine laboratory testing and to confirm the results obtained with the classical methods described above when necessary.

4.4. Bioassays for cosmetics and cosmeceuticals with a focus on antioxidant and anti-ageing effects

A variety of specialized bioassays have been developed and routinely used to evaluate the overall cosmetic activity of a marine extract (Fig. 7). The majority of these bioassays are single-target bioassays, but phenotypic bioassays are also available. In the primary screening and secondary testing phases for potential cosmetics and cosmeceuticals, bioassays are mostly based on *in vitro* assays for cytotoxicity, antioxidant and anti-inflammatory activities, using either biochemical cell-free assays or immortalized cell lines (e.g., THP-1 and HaCaT cells). Once selected, the extracts or compounds are tested for safety, activity, and mode of action in preclinical assays using primary cells (e.g., keratinocytes) and/or *ex vivo* skin tissue models (Brancaccio et al., 2022), with the option to perform final testing in clinical trials (Fig. 9). The anti-inflammatory activity of extracts or pure compounds can be assessed by TNF-α or IL-1β production measured in LPS-stimulated THP-1 activated human macrophage cells (Lauritano et al., 2016). For example, *in vitro* bioassays are used to investigate the antioxidant capacity of extracts by mimicking the damage caused by radicals in the skin and by assessing the efficacy of natural extracts in combating this damage (Thring et al., 2009). Depending on the mechanism by which radicals are scavenged, antioxidant capacity assays are broadly divided into two categories: electron transfer (ET) and hydrogen atom transfer (HAT) based assays (Apak et al., 2007). Compared to HAT-based assays, the ET reaction is relatively slow, and its actual rate depends greatly on laboratory conditions, such as solvent and pH (Apak et al., 2007; Huang et al., 2005). ET

assays widely used in cosmetics include the DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS/TEAC (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox®-Equivalent Antioxidant Capacity), CUPRAC (CUPric Reducing Antioxidant Capacity), and Folin-Ciocalteu methods, each of which uses a different chromogenic reagent with different redox potential (Ratz-Lyko et al., 2012), as shown in Supplementary Table S1. Although the actual reducing capacity of an extract or compound is not directly related to its ability to scavenge radicals, these biochemical assays are useful for initial screening procedures (Amorati and Valgimigli, 2015; Apak et al., 2007). Most HAT−based assays are kinetic and rely on a competitive reaction scheme in which the antioxidants of a natural extract and an oxidizable probe compete for peroxyl radicals, the latter being thermally generated in a solution by the decomposition of azo compounds (Apak et al., 2007; Huang et al., 2005). This is the case with the oxygen radical absorbance capacity (ORAC) assay, which is widely used to measure the antioxidant capacity of natural products with anti-ageing and cosmetic potential (Baldisserotto et al., 2012; Dávalos et al., 2004; Dudonné et al., 2011; Ky and Teissedre, 2015; Le Lann et al., 2016). However, it must be emphasized that most HAT and ET assays are sensitive to either hydrophilic or hydrophobic antioxidants and therefore may underestimate the total activity of an extract (Fraga et al., 2014; Ratz-Lyko et al., 2012). Thus, a combination of these biochemical methods may be required to obtain reliable results (Ratz-Lyko et al., 2012).

Another set of *in vitro* assays commonly used in the screening of cosmetics and cosmeceuticals investigates the anti-ageing effects of the extracts, which include antioxidant and anti-inflammatory activities (Brancaccio et al., 2022), but may also be related to their specific ability to block enzymes involved in the breakdown of skin firmness (Thring et al., 2009). These include matrix metalloproteinases (e.g., collagenase), serine proteases (e.g., elastase), and endoglycosidases (e.g., mucopolysaccharide hyaluronidase), which degrade the major components of the extracellular matrix (ECM) of the skin: collagen, elastin, and hyaluronic acid (Li et al., 2019; Rittie and Fisher, 2002). Maintaining high levels of these components is critical for skin elasticity, firmness, and hydration, and thus inhibitors of these hydrolytic enzymes are being sought (Madan and Nanda, 2018). In addition, there is particular interest in the regulation of melanin levels in the skin (i.e., changes in skin pigmentation), the overproduction of which leads to aesthetic problems such as pigmentation spots (Lall and Kishore, 2014; Saghaie et al., 2013) as well as other skin conditions such as discoloration, freckles, and skin cancer (An et al., 2005). Specific assays are available to study the inhibitory properties of extracts on the activity of the enzyme tyrosinase, which catalyses the first rate-limiting steps of the melanin biosynthetic pathway in melanocytes (Parvez et al., 2006). Typically, L-DOPA (an intermediate in melanogenesis) is used as a substrate and its enzymatic oxidation to the red-colored dopachrome is monitored spectrophotometrically to assess inhibition of tyrosinase. Despite the widespread use of (bio)chemical antioxidant assays, they are usually performed under non-physiological conditions without taking into account the cellular uptake of compounds and their mode of action at the subcellular level, which inherently limits their ability to predict the true antioxidant effect in living systems.

In vitro phenotypic assays usually investigate the regenerative properties of extracts on specific skin cell lines (e.g., fibroblasts) by monitoring their stimulatory effects on the production of ECM components (Adil et al., 2010; Boonpisuttinant et al., 2014; Pastorino et al., 2017; Roh et al., 2013; Yodkeeree et al., 2018), as well as their photoprotective effects in terms of cell viability (Moon et al., 2008). The protective role of extracts against photooxidative skin damage can also be evaluated by *ex vivo* approaches. Specifically, a cosmetic formulation is applied to the skin of human volunteers and

after a short period of time, strips of the outermost skin layers are removed, exposed to UV radiation, and lipid peroxidation is assessed by measuring the losses of unsaturated fatty acids and the amounts of primary, secondary, or end products of the reaction (Alonso et al., 2009). Cell line-based bioassays are also used to estimate safety parameters by assessing skin irritation by evaluating direct cytotoxicity or other types of damage to the epithelial barrier of the skin by measuring the permeability of fluorescein through epithelial cell monolayers (OECD test no. 460). In addition, mutagenicity and carcinogenicity (OECD test no. 451) are assessed using cell cultures, e.g., the *in vitro* micronucleus test (OECD test no. 487) to detect chromosomal aberrations and the bacterial reverse mutation test (OECD test no. 471) to detect gene mutations. An alternative to animal models for carcinogenicity testing is cell transformation assays (CTA), which are used in combination with other approaches to evaluate carcinogenic potential (Creton et al., 2012; Mascolo et al., 2018; Organisation for Economic Co-operation and Development - OECD, 2022; Scientific Committee on Consumer Safety - SCCS, 2021).

The biochemical and cell-based *in vitro* methods described are suitable for the screening and monitoring phases of the drug discovery pipeline (Fig. 9) because of their greater simplicity, speed, throughput, and cost-effectiveness, although they may not adequately reflect the actual biological processes in skin cells. Therefore, *ex vivo* bioassays using skin tissues have been developed for toxicological studies, such as the reconstructed human epidermis (RhE) test methods (OECD test no. 439, 431), using four validated commercial human skin models, viz. i.e., EpiSkin™, EpiDerm™, SkinEthic™, and EpiCS®, which use reconstructed human epidermis equivalents to evaluate cell viability and are used to assess skin corrosion or irritation potential. Bioassays for the assessment of ocular damage include organotypic assay methods using tissues from slaughterhouses, such as bovine corneas (OECD test no. 437) or chicken eyes (OECD test no. 438), or *in vitro* assays using corneal epithelial cell lines to assess irritation by measuring direct cytotoxicity on rabbit corneal cell lines (OECD test no. 491) or human cornea-like epithelium (OECD test no. 492) (e.g., EpiOcular™). For assessment of genotoxicity or reproductive toxicity, new alternative approach methodologies to animal testing are being implemented worldwide, including *in vitro* methods using the whole embryo culture test (WEC) to evaluate developmental toxicity in rodent embryos maintained in culture during the early stages of organ formation, the MicroMass Test (MM), which uses embryonic limb mesenchyme or central nervous system cells from chickens, mice, or rats to evaluate effects on cell differentiation into chondrocytes and neurons as an indication of potential teratogenicity, and the embryonic stem cell assay (EST), which is based on permanent cell lines to predict embryotoxicity by evaluating effects on cell differentiation (Organisation for Economic Co-operation and Development - OECD, 2022; Scientific Committee on Consumer Safety - SCCS, 2021; Seiler and Spielmann, 2011).

5. Quality control and bioassay validation

5.1. The concept of validation

The concept of validation can be defined as a systematic approach to collecting and analyzing a sufficient amount of data under specified conditions and based on documented evidence (validation report) and scientific judgment, to provide reasonable assurance that the process of interest will reliably and consistently reproduce results within predetermined specifications when operated within specified parameters (Haider, 2006).

The main objective of the validation process is to produce reliable and consistent data (quality data). In addition, four critical components of data quality are identified, including analytical

instrument qualification, analytical method validation, system stability testing, and quality control sampling (United States Pharmacopeial Convention, 2018), with each of these components contributing to overall quality:

- Analytical instrument qualification (AIQ) is the collection of documented evidence that an instrument is fit for its intended purpose and that its use provides confidence in the validity of the data produced. It includes (i) design qualification (DQ), which is performed by the manufacturer prior to purchase to ensure the technical characteristics required by the user; (ii) installation qualification (IQ), which is performed prior to and at the time of installation; (iii) operational qualification (OQ), which is performed after installation and major repairs; and (iv) performance qualification (PQ), which is performed periodically to ensure continued satisfactory performance during routine operation and includes preventive maintenance, recalibration, and performance testing (Bansal et al., 2004; Kaminski et al., 2010; Valigra, 2010)
- Analytical method validation is the collection of documented evidence that demonstrates that an analytical method is fit for its intended purpose and provides assurance that its use with qualified analytical instruments will generate accurate data of acceptable quality (Haider, 2006).
- System suitability tests (SSTs) are used to verify that the system meets predefined criteria. They are performed in conjunction with sample analyses to ensure that the system is functioning properly at the time of testing.
- Quality control (QC) samples help to ensure the quality of analytical results by being included immediately prior to or during sample analysis.

5.2. Validation of the analytical method

The concept of bioassay validation is often associated with compounds that are classified as drugs by regulatory authorities, because the development, production and testing of these products are strictly regulated. Consequently, bioassay validation is an integral part of the quality control system. This may not be the case for cosmetic preparations or dietary supplements, where product characteristics and claims dictate testing or trial requirements, however, in practice many cosmetic preparations claiming bioactivity are also subject to rigorous testing. For biodiscovery and research, it is not usually necessary to meet quality control requirements, but it is good to keep the concepts of validation in mind and apply them wherever possible. This can facilitate the transition from research to industrial development, as well as communication with regulatory agencies, regardless of the type of application.

It is important that the operator performing the validation of the analytical procedure has the scientific and technical understanding, process knowledge, and/or risk assessment capability to adequately perform the quality functions of analytical method validation (Chan, 2011). The parameters to be evaluated for validation depend on the type of method, and the measures used to describe the performance of the analytical method are typically: accuracy (trueness), precision (repeatability), limit of detection (LOD), limit of quantitation (LOQ), linearity (calibration curve), range, selectivity, specificity, and robustness. All of these parameters must be determined for validation of a quantitative analytical method, whereas specificity and limit of detection may be sufficient for a qualitative method. There are numerous guidelines (more than 30) published by regulatory organizations; some of them are summarized in Table 2. These guidelines can be used as a frame of reference for the validation process. Unlike instrument qualification, the type of analytical method (e.g., sample matrix, analytical equipment) determines the parameters to be evaluated, so it is important to select an appropriate

guidance document as a frame of reference. It is important to note that the terminology used in different guidelines varies. For example, selectivity, specificity, or diagnostic specificity are defined differently in different guidelines (Borman and Elder, 2017; Chan, 2011; Kadian et al., 2016).

Table 2

The summary of selected validation guidelines and corresponding organizations

Organization	Abbreviation	Sample Guideline(s)	Area of Interest	Remarks and References
European Medicines Agency	EMA	Guideline on bioanalytical method validation (EMEA/CHMP/EWP/192217/ 2009)	Bioanalytical assays for drug development studies (with all clinical trials)	Biological matrices such as blood, urine, tissues etc. (European Medicines Agency, 2011)
European Network of Forensic Science Institutes	ENFSI	Guidelines for the single laboratory Validation of Instrumental and Human Based Methods in Forensic Science	Forensic	Biological matrices such as blood, urine, tissues etc. (De Baere et al., 2014)
International Council for Harmonisation	ICH	Validation Of Analytical Procedures: Text And Methodology Q2(R1)	Pharmaceutical QC analyses	Pharmaceutical samples such as; Active Pharmaceutical Ingredient (API), finished drug samples (ICH Expert Working Group, 2005)
		Bioanalytical method validation and study sample analysis (M10)	Bioanalytical assays for drug development studies	Biological matrices such as blood, urine, tissues etc., Draft document (European Medicines Agency, 2019)
United States Food and Drug Administration	USFDA	Bioanalytical Method Validation-Guidance for Industry	Bioanalytical assays for drug development studies (with all clinical trials) and for veterinary drug development as well	Biological matrices such as blood, urine, tissues etc.(USFDA, 2018)
Association of Analytical Communities	AOAC	Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals	Food & Feed Quality	Food and feed stuffs (Harnly et al., 2012)
International Union of Pure &Applied Chemistry	IUPAC	Harmonized Guidelines for Single laboratory Validation of Methods of Analysis	General terminology on analytical method characteristics	Sample matrices are not specified (Thompson et al., 2002)
European Directorate for the Quality of Medicines & HealthCare-The Directorate-General for Health and Food safety	EDQM/DG-SANTE	Analytical Quality Control and Method Validation; Procedures for Pesticide Residues Analysis in Food and Feed (SANTE/12682/2019)	Food & Feed Quality	Specified on the pesticide analysis in food and feed samples (Philström et al., 2019)
EURACHEM	n/a	The Fitness for Purpose of Analytical Methods- A Laboratory Guide to Method Validation and Related Topics	General terminology on analytical method performance characteristics	Sample matrices is not specified (Barwick et al., 2014)
European Commission Joint Research Centre Institute for Health and Consumer Protection	ECJRC-IHCP	Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials (EUR 24105 EN - 1st edition/2009)	Food Quality	Migration analysis (from the food contacting part of the packing materials) (Bratinova et al., 2009)

Organization	Abbreviation	Sample Guideline(s)	Area of Interest	Remarks and References
United States Pharmacopeia	USP	General Chapter <1225> Validation of Compendial Procedures	Pharmaceutical QC analyses	Pharmaceutical samples such as Active Pharmaceutical Ingredient (API) and finished drug samples (USP 40, 2017)
United States Environmental Protection Agency	USEPA	Guidance for Methods Development and Methods Validation for the RCRA Program	Environmental analysis	Test Methods for Evaluating Solid Waste (SW-846) Methods (EPA Office of Solid Waste, 1992)

Validation of analytical methods is a progressive, dynamic, and time-consuming process, so it is recommended that a validation schedule (or protocol) be established (EURL, 2022; Shabir, 2003). In addition, there are fundamental differences in validation parameters between different types of assays (e.g., chromatography-based or ligand-binding assays), and this issue is addressed differently by different regulatory agencies, either by providing separate validation guidelines (e.g., ICH, EMA) or by specifying certain aspects in a guideline (e.g., FDA) (Borman and Elder, 2017; EMA Committee for human Medicinal Products, 2011; USFDA, 2018).

5.3. Data integrity and documentation

The term data integrity refers to the degree of a data-generating system in which the acquisition and storage of data is undivided, coherent, reliable, and accurate. This does not depend on whether the data are in paper or electronic form (Wingate, 2004). The critical issue in ensuring the quality of analytical procedures and data integrity is the documentation of all steps. Good documentation practices (GDocP) is a term used in the pharmaceutical industry to describe the guidelines, standards, and regulations for creating, maintaining, and archiving documents. These apply to all parties involved in a process and to all activities. GDocP-based records have the following characteristics: they are complete, truthful, clear, permanent, accurate, consistent, legible, and concise (Davani, 2017).

5.4. Good Laboratory Practice (GLP)

It is recommended that the principles of good laboratory practice (GLP) are followed at all times when performing bioassays. GLP is a quality assurance system that addresses the organizational process and conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived, and reported (OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring, https://www.oecd.org/chemicalsafety/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm, accessed 4 May 2022).

6. Bioactivity-guided fractionation and/or purification

With the desired bioactivity in mind, a series of fractionation and analytical steps can be applied to natural resources to isolate and/or purify specific compounds that exhibit the bioactivity of interest. The path from a natural extract exhibiting a specific bioactivity to a dereplicated, purified, identified, and characterised compound exhibiting that bioactivity is often quite long and labour intensive.

A significant portion of the labour and operating costs in a biochemical and analytical laboratory is devoted to the preparation (extraction) of samples for subsequent analytical separation.

During the extraction process, the target compound is pre-concentrated and converted into a form suitable for subsequent instrumental analysis and chromatographic or electrophoretic separations, and the complexity of the matrix is reduced. Depending on the solvents and procedures used for extraction, we expect to isolate either small molecules such as polyketides, alkaloids, and terpenoids or complex polymers such as proteins and polysaccharides, and the purification steps are then designed accordingly (Fig. 8).

Extraction is the first important step in the screening process, and the selection of extraction method and solvent(s) is critical for successful downstream processing. For example, bioactive compounds may be present in both a highly polar/aqueous extract and a moderately nonpolar/organic extract. In addition, the physicochemical properties of the starting material determine the steps in the extraction process. For example, microalgae have a rigid cell wall that acts as a natural barrier to prevent solvent molecules from diffusing into the cells and must be broken by mechanical and/or physical techniques such as high-pressure homogenization, shear mixing (high-speed homogenization), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) prior to or simultaneously with chemical extraction (Benbelkhir and Medjekal, 2022; Tian et al., 2022). It is important to consider all available alternatives of the extraction procedure to optimize the extraction time and avoid possible structural or conformational changes of the extracted molecules that can alter their bioactivity. Such changes are more likely to occur in large molecules (e.g., polysaccharides, oligosaccharides). Switching from slow extraction methods (e.g., hydrothermal extraction), which require longer processing time, to faster technologies such as UAE, MAE, or UMAE can help shorten the extraction process and increase the likelihood that the molecule will remain intact (Guo et al., 2022; Qiu et al., 2022). However, chemical reactions can also occur when microwaves and/or ultrasound are used for extraction.

Since a natural extract contains a mixture of molecules, the concept of bioactivity-guided purification is based on the sequential application of different types of fractionations that separate molecules from a mixture and the concurrent application of the selected bioassay to identify fractions containing the bioactive compounds until a satisfactory level of purity is achieved. In each purification step, the individual fractions are tested with the bioassay to select the fractions with the highest bioactivity for further purification. Since numerous fractions usually need to be tested, it is optimal to use a rapid and inexpensive bioassay with low volume requirements. A quantitative bioassay is sufficient to guide the purification.

Purification is usually performed by either liquid-liquid phase separation (LLPS) or the currently predominant solid phase extraction (SPE). SPE has become a standard analytical procedure for the enrichment of target analytes by partitioning and/or adsorption onto a solid stationary phase. SPE is currently the most widely used method for the extraction, concentration, purification, and fractionation of organic compounds from a variety of samples, as well as for solvent exchange; in addition, SPE is also used efficiently for the desalting of proteins and glycan samples. SPE offers several advantages over liquid-liquid extraction, including higher recoveries, avoidance of emulsion formation, lower organic solvent consumption, simpler operation and automation capability, improved selectivity and reproducibility, and shorter sample preparation time. The standard SPE procedure begins with the application of an analysed solution to a solid phase (sorbent), usually in a cartridge, in which the target analytes are eluted with a suitable solvent and collected (Andrade-Eiroa et al., 2016; Faraji et al., 2019)

There are numerous adsorbents for the extraction of different types of molecules. Various SPE mechanisms can be applied to separate target molecules using specific sorbent materials, such as

adsorption (e.g., using silica gel, alumina, florisil, or graphitic carbon-based packing), normal separation (e.g., cyanogen-, diol-, or amino-based silica), reversed phase separation (e.g., octadecyl-, octyl-, butyl-, or phenyl-bonded silica), ion exchange (various cation or anion exchangers), size exclusion (e.g., macropore silica or organic gels), affinity separation (carriers with immobilized affinity ligands), and immunoaffinity separation (carriers with immobilized specific antibodies); often two separation mechanisms can be used simultaneously (e.g., ion exchange and reverse phase separation) (Andrade-Eiroa et al., 2016).

Efficient SPE can also be performed with magnetically responsive adsorbents. Magnetic SPE (MSPE) is becoming increasingly popular due to its ease of use, high extraction efficiency, and straightforward automation (Jiang et al., 2019; Pena-Pereira et al., 2021; Šafaříková and Šafařík, 1999; Vasconcelos and Fernandes, 2017). MSPE uses various types of magnetically responsive adsorbents based on ferrimagnetic iron oxides (magnetite, maghemite) or ferrites to which specific affinity ligands are immobilized. A popular variation of MSPE is immunomagnetic separation (IMS), which uses magnetic nano/microbeads with immobilized specific antibodies (monoclonal, polyclonal, or engineered) to capture target analytes or cells via antigen-antibody interactions (De Meyer et al., 2014; He et al., 2018; Safarik et al., 2012; Šafaříková and Šafařík, 1999). Magnetically responsive materials can also be used to separate and purify various biologically active compounds on a larger scale (Franzreb et al., 2006; Safarik and Safarikova, 2014, 2004). Stir-bar sorptive extraction (SBSE) is based on the use of a magnetic stir bar covered with a suitable sorbent (usually polydimethylsiloxane or ethylene glycol-modified silicone material) into which the analytes are extracted. The technique has been successfully used for the analysis of samples of varying complexity and for the detection, concentration or removal of marine toxins in crude extracts (Chen et al., 2019; González-Jartín et al., 2020; Pena-Pereira et al., 2021; X. Wang et al., 2017).

Various SPE mechanisms are used to separate compounds from the extracts, which can be performed in a column chromatography format. These are used to fractionate either by size (e.g., size exclusion chromatography), charge (e.g., ion exchange chromatography), hydrophobicity (e.g., hydrophobic interaction chromatography), polarity (e.g., reversed-phase vs. normal phase chromatography), or other specific binding interactions (e.g., affinity chromatography). These chromatographic stationary phases can be used in a variety of platforms/equipments, such as fast protein liquid chromatography (FPLC), generally used for proteins or nucleic acids, or high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC), used for both proteins and small molecules. In addition to column mode, other SPE formats can be used such as extraction disks and membranes, which are usually composed of glass fibers forming a matrix on which particles of pure or modified silica gel are anchored (Andrade-Eiroa et al., 2016). Supercritical fluid adsorption (SFA) or supercritical fluid chromatography (SFC) are another option, especially for nonpolar volatile compounds. SFA can also be used for polar compounds that are poorly soluble in supercritical CO_2 by using a suitable co-solvent such as ethanol (Dinarvand et al., 2020). Various types of chromatography used for isolation, purification, and characterization of natural products have been reviewed (e.g., (Bucar et al., 2013; Nehete et al., 2013; Saini et al., 2021; Sarker and Nahar, 2012; Yang et al., 2020). Alternatively, variants of preparative polyacrylamide gel electrophoresis (PAGE) (e.g., native PAGE, isoelectric focusing, 2D PAGE) can be used to separate mixtures of compounds from extracts. Miniaturized analytical techniques can also be used for sample processing. Pipette tip or in-syringe SPE is a miniaturized version of standard SPE in which the absorbent material is packed in plastic micropipette tips or in the needle of syringes; analytes are extracted by repeated aspiration and

desorption of the sample. SPME can also be used for *in vivo* analyses, such as fish tissue sampling, due to its low invasiveness. Headspace SPME allows selective extraction of volatile and semi-volatile compounds from samples. Thin film microextraction (TFME) increases the volume of the extraction phase and the surface-to-volume ratio, allowing higher extraction efficiency and rapid analysis (Faraji et al., 2019; Pena-Pereira et al., 2021).

An important step in the isolation process is dereplication (Gaudêncio and Pereira, 2015), which is usually performed using tandem mass spectrometry (MS/MS), which determines the presence of known compounds. The bioactive extracts containing unknown compounds are usually selected for further fractionation. Alternatively, known compounds can be tested for new types of bioactivities using other types of bioassays, a process known as repurposing (Dinarvand et al., 2020; Houssen and Jaspars, 2012; Nothias et al., 2018; Pereira et al., 2020; Pushpakom et al., 2019; Veerapandian et al., 2020).

Information about the properties of the bioactive compound can be derived from the purification process, and separation into specific fractions provides information about their characteristics. The number of purification steps required to purify compounds varies from case to case and usually ranges from two to eight. Finally, the structures of compounds are elucidated using 1D and 2D nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HR-MS), X-ray diffraction (for crystalline compounds), and other techniques to determine the absolute configuration (for non-crystalline compounds) (Gaudêncio et al., 2023). It is important to note that the use of low-resolution tandem mass spectrometers (e.g., triple quadrupole mass spectrometers) may be sufficient for targeted analysis of known compounds, but for untargeted analysis of unknown compounds, the use of a high-resolution mass spectrometer (HR-MS) in tandem mode (e.g., quadrupole time-of-flight, Orbitrap) is essential for accurate measurement of both molecular and fragment ions (Berlinck et al., 2022; Guo et al., 2022).

7. Application-oriented development

Given the enormous richness of the marine environment in terms of global biodiversity, almost unlimited resources of bioactive compounds are available for various applications (Atanasov et al., 2021; Newman and Cragg, 2020; Rotter et al., 2021a). Over 38 000 compounds of marine origin are listed in the Dictionary of Marine Natural Products (https://dmnp.chemnetbase.com), the MarinLit database (http://pubs.rsc.org/marinlit/), and the Comprehensive Marine Natural Products Database CMNPD (https://www.cmnpd.org/) (Lyu et al., 2021). Currently, around 1500 new marine compounds are reported annually (Carroll et al., 2021), a substantial increase from the annual average of 1200 compounds reported nearly a decade ago (Kiuru et al., 2014). However, marine natural product discovery faces several challenges. Despite support from research funding organisations in the EU and worldwide, access to the marine environment and sampling of aquatic organisms remain very challenging, while several technical issues, including supply of active compounds and sustainable production, can hinder the biodiscovery process (Schneider et al., 2023, 2022). Furthermore, extracts derived from marine organisms are very complex, and the potentially bioactive components are usually present at low concentrations or are characterised by high structural novelty/complexity, making their identification and isolation in sufficient quantities for extensive biological testing difficult.

By overcoming the above-mentioned challenges, a limited number of promising bioactive compounds are eventually isolated in quantities large enough to enable bioactivity studies and to

support the different stages of natural product development. There are no universal sets of bioassays that should be used for specific research applications, while different types of bioassays are important for different phases of biodiscovery and product development. Much practical information on selecting a bioassay has been discussed in Section 3, but it is prudent to keep in mind the potential uses and regulatory requirements associated with the various intended applications from early discovery on. To illustrate this point, we consider the development pipeline (Fig. 9) of a general natural source value chain and focus on marine products intended for specific target markets, namely the pharmaceutical industry (medicines), the cosmetics industry, and the food industry (dietary supplements and/or ingredients for food or feed).

7.1. Pharmaceutical drug discovery

The entire process to approval of a new drug can take 12-15 years for the pharmaceutical industry and costs up to $2.8 billion (Wouters et al., 2020). In particular, drug discovery based on natural products has proven to be an extraordinary laborious, costly, and time-consuming process. Nevertheless, this is the most effective approach to new drug development, and the number of natural-product-inspired drugs is much higher than synthetic drugs, as over 67% of modern drugs are based on natural products or their derivatives. Many pharmaceutical companies have turned to combinatorial chemistry for drug structure discovery and optimization; however, only three new chemical drugs have been approved based on this methodology (Jimenez et al., 2020; Newman and Cragg, 2020). To date, 14 approved marine drugs are in clinical use, including 11 anticancer drugs, and 38 marine natural products are in clinical trials (18 in Phase I, 14 in Phase II, and 6 in Phase III). The vast majority of the latter (i.e., 33 of 38) are being tested as anticancer drugs, whereas two are being investigated for viral diseases, one for Alzheimer's disease, one for chronic pain and one for relapsed or refractory systemic amyloidosis (https://www.marinepharmacology.org/approved). The drug development process involves five major steps: (i) discovery and development; (ii) preclinical research; (iii) clinical development; (iv) review by a health authority (e.g., FDA or EMA); and (v) postmarketing surveillance, including numerous phases and stages within each of these steps. Bioassays are primarily used during the first two steps of (i) discovery, including screening and bioactivity-guided purification, and during (ii) preclinical research, which serves as the decision-making basis for the next step of clinical trials (Fig. 9). For pharmaceutical and nutraceutical products, both of which promise health benefits and are subject to the same regulatory requirements, preclinical testing is followed by (iii) the clinical development phase, which includes a sequence of clinical trial phases. Phase I clinical trials focus on testing safety, dose and side effects in a small group of healthy volunteers. Phase II then enrols a medium-sized group of patients with the target disease or condition and treats them for several months to two years, comparing them to a placebo control group or an approved standard drug to obtain efficacy and additional safety data. Phase III studies are larger and of longer duration (1-4 years) and include approximately 300-3000 patients who are treated and compared to a control group. Data collected in phase III provide information on long-term and rare side effects compared to the last two phases. After the drug has been approved (iv) by the regulatory authorities, i.e., the European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) in Europe and the Food and Drug Administration (FDA) in the U.S.A., (iv) post-marketing surveillance (Phase IV) is conducted to obtain additional information on the benefits and risks of using a particular drug.

The screening phase relies on *in silico* and *in vitro* biochemical assays to identify bioactive extracts, fractions, or lead compounds, with high-throughput screening playing a central role. However, in recent decades, interest from the pharmaceutical industry in conducting HTS programmes, particularly for natural products, has tended to decline (Harvey et al., 2015). This is

primarily due to a number of bottlenecks associated with the complexity of biological extracts that can affect the accuracy of targeted molecular screening (e.g., the effects of active compounds can be masked by other components in the crude extract), associated costly efforts to reduce matrix complexity, and the limited success of large HTS campaigns previously conducted by companies. Nonetheless, interest in HTS natural products for drug discovery remains a hot research topic in academia. Laboratory-scale studies have reported the application of HTS techniques to a repertoire of natural products to identify potential therapeutic agents for tumour metastasis (Gallardo et al., 2015), cancer and necroptosis (Li et al., 2016), cell stress and cytotoxicity (Judson et al., 2016), metabolic and age-related disorders (C. Wang et al., 2017), and, more recently, COVID -19 (Chen et al., 2021; Coelho et al., 2020; Gaudêncio et al., 2023). Other studies have investigated natural product-like small molecules for their antimalarial activity (Kato et al., 2016) and their suitability for genome engineering technologies (e.g., inhibition of CRISPR-Cas9 (Maji et al., 2019)).

In addition to experimental efforts, complementary dry-lab approaches (e.g., virtual screening) have emerged under increasing pressure to reduce costs and improve the speed and simplicity of the biodiscovery process (David et al., 2015). These efforts primarily involve the use of structure-assisted drug design in conjunction with virtual HTS. With respect to natural products, this approach has been applied in a substantial number of studies to accelerate the discovery of antiviral agents against coronaviruses (Jin et al., 2020; Naik et al., 2020), while others have focused on identifying molecular entities with inhibitory activity against typical disease-related enzymes (e.g., cancer, diabetes, and neurodegenerative disorders) (Jhong et al., 2015; Khan et al., 2019; Mohammad et al., 2019).

Natural products that have been evaluated for pharmacological or biological activity and have the potential to be therapeutically useful can be considered drug hits. However, in the early stages of drug development, a hit-to-lead (H2L) process is used that includes mechanism-of-action studies to identify the pharmacological targets of potent hits and a limited optimization of their chemical structure to reduce potential side effects, increase affinity and selectivity, improve efficacy, potency, metabolic stability (half-life) and oral bioavailability. A lead-optimization (LO) process is then performed to synthesize, evaluate, and modify the bioactive compounds using medicinal chemistry approaches to form new chemical entities (NCEs) that improve efficacy and reduce side effects. Lead optimization also involves experimental *in vitro* and *in vivo* testing in a variety of efficacy studies, pharmacokinetic studies, and toxicological assessments, as well as ADMET (absorption, distribution, metabolism, excretion, toxicity) assessments through the use of *in silico* models and animal testing to develop therapeutically effective drugs. For this reason, the preclinical phase is typically more time-consuming, more expensive, and requires less testing capacity than the preceding screening phases, and may require more qualified personnel working according to the principles of good laboratory practice (Andrade et al., 2016; Claeson and Bohlin, 1997; Collins et al., 2020).

Before the bioactive compound (lead structure) enters a new phase of development for a specific application, its toxicity to humans, animals, and the environment must be determined. The conclusions drawn from the safety and toxicity tests are highly dependent on the results of the bioassays used. Bioactivity must be quantified at this stage to determine dose (exposure) and derive potency. Different types of bioassays may be required for these steps, but often only validated versions of the quantitative bioassays already used in the discovery phase are used. The pure compounds (lead compounds) are tested *in vitro* on primary cell lines or *ex vivo* tissue models, or combinations thereof, specifically designed for the application of interest. The lack of adequate human disease models has been described as a major limitation in preclinical drug development (Khanna, 2012). Recently, however, several preclinical human disease models have been developed for several common chronic

inflammatory diseases (e.g., osteoarthritis, cardiovascular disease, chronic lung disease, psoriasis, atopic dermatitis) and various cancer types, using two-dimensional (2D) cell culture methods, *ex vivo* and co-culture models and three-dimensional (3D) organoid structures. These disease models serve as immediate *in vivo* testing platforms to evaluate the efficacy and safety of drug candidates prior to entering clinical phases (Araújo et al., 2020; Ho et al., 2018; Jessica E Neil et al., 2022; Muenzebrock et al., 2022; Veldhuizen et al., 2019). Results from disease models form the basis for designing and planning potential clinical trials or conducting other safety and efficacy testing required by regulatory authorities for a particular application (e.g., pharmaceutical, nutraceutical or cosmetic). It should be emphasized that the safety evaluation of pharmaceutical, food, and cosmetic ingredients is more stringent than that of well-characterised non-food substances, such as industrial chemicals or pesticides (Śliwka et al., 2016). Moreover, cosmetics and dietary supplements are not required to be approved for sale by the FDA or EMA. Nevertheless, the cosmetics industry has recently become interested in incorporating marine bioactive compounds into cosmetic products (e.g., creams and lotions) that have medicinal or drug-like effects. In this context, the term "cosmeceuticals" has been coined to describe the combination of cosmetics and pharmaceuticals, but it does not yet have any legal meaning under current regulations.

The potential toxicity of compounds is determined based on their chemical structure and mechanism of action to characterize concentration-dependent effects, long-term effects, and effects of exposure at low concentrations. Animal testing can provide valuable information on toxicity and pharmacological activity, including pharmacokinetics (ADME) and pharmacodynamics (interaction with the organism), but interspecies differences in drug toxicity and efficacy can become an important issue. Despite the recognized limitations and benefits, there are ongoing efforts to reduce the use of animals for testing. Indeed, *in vivo* testing in animals and humans is subject to strict ethical constraints, is costly, and therefore is generally performed only in the final stages of development (Ferdowsian and Beck, 2011). Current regulatory approaches to toxicity testing and evaluation continue to rely primarily on a checklist of *in vivo* tests that follow standardized test guidelines or protocols. The Interagency Coordinating Committee on the Validation of Alternative Methods ICCVAM, along with other organizations, is promoting the development of non-animal alternatives to current *in vivo* acute systemic toxicity tests (Clippinger et al., 2018; Hamm et al., 2017; Kleinstreuer et al., 2018). There is a trend toward increased use of new technologies such as high-throughput screening (HTS), tissue chips, and computational modelling to better predict human, animal, and environmental responses to a wide range of substances relevant to new product development. The International Cooperation on Alternative Test Methods (ICATM) partnership was created to establish international cooperation in validation studies and the development of harmonized recommendations to ensure global acceptance of alternative methods and strategies (https://ntp.niehs.nih.gov/whatwestudy/niceatm/iccvam/international-partnerships/icatm/index.html).

Significant efforts are being made to develop *in vitro* tests that cover endpoints and target organs/tissues that are most relevant to humans (Bal-Price et al., 2015). However, in some cases, animal models may still be needed to address specific developmental toxicity questions (Clippinger et al., 2018; Leist et al., 2013; Wambaugh et al., 2018). In this context, zebrafish-based bioassays offer an interesting combination of an *in vivo* model and the possibility of high-throughput screening with low compound consumption. For example, zebrafish embryos have been established as an *in vivo* model for the analysis of angiogenesis and vascular development and can be further developed for other specific high-throughput screening (Crawford et al., 2011). Another alternative to these assays is the

use of the whole-animal *Caenorhabditis elegans* (e.g. (Durai et al., 2013; Palacios-Gorba et al., 2020). In addition, phenotype-based bioassays are also used to retarget known compounds to unknown and novel targets (Pushpakom et al., 2019).

In recent years, computer-assisted methods have been used to predict or model the ADMET properties of lead compounds, enabling drug design and identification of potentially problematic structures in the early stages of drug discovery to avoid late-stage failures (Ortega et al., 2012). Computer-aided drug design (CADD) is increasingly being used in drug discovery. Existing tools for predicting and visualising ADME/toxicity data include: i) predictors of ADME parameters, ii) predictors of metabolic fate, iii) predictors of metabolic stability, iv) predictors of cytochrome P450 substrates, and v) software for physiology-based pharmacokinetic (PBPK) modelling (Romano and Tatonetti, 2019; Wishart, 2009, 2007). These enable pharmacophore modelling (PM), molecular docking (MD), inverse docking, chemical similarity search (CS), development of quantitative structure-activity relationships (QSAR) (Pereira et al., 2015, 2014), virtual screening (VS) (Cruz et al., 2018; Dias et al., 2018; Gaudêncio and Pereira, 2020) and molecular dynamics simulations (MDS), which effectively predict the therapeutic outcome of lead structures and drug candidates and accelerate the discovery process. The importance of predictive models for clinical pharmacology is recognized by regulatory agencies, and this approach is being used for various applications. These models combine different types of data and parameters to estimate pharmacological activities and are commonly referred to as physiologically based pharmacokinetic (PBPK) models. By linking the properties of individual lead molecules to physiological properties, PBPK models also provide a rational approach to predicting drug similarity (Benjamin et al., 2010; Deepika and Kumar, 2023; Karnati et al., 2023; Mbah et al., 2012; Strömstedt et al., 2014).

By exploring structural and other data about the target (enzyme/receptor) and ligands, CADD approaches have identified compounds that can treat disease. Examples of approved drugs that have been supported by CADD include dorzolamide, saquinavir, ritonavir, indinavir, captopril, and tirofiban (Dar et al., 2019). Given the success of this approach, the development of ”go/no-go” selection criteria and optimization strategies for drug candidate development should include the use of advanced CADD for drug metabolism and pharmacokinetics (DMPK) profiling in the development of safe and effective drugs.

7.2. Cosmetics

Cosmetic products are intended to be applied to the external parts of the human body, including the teeth and oral mucous membranes, to cleanse, protect, change their appearance, improve their odour or keep them in good condition. Their use is regulated in the EU by the EU Cosmetics Directive (Directive 1223/2009) and in the US by the Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Fair Packaging and Labelling Act (FPLA). In the EU, all cosmetic products are registered with the EU Cosmetic Products Notification Portal (CPNP) and must undergo a safety assessment, have a product information file, and report serious undesirable effects. Manufacturing must be in accordance with good manufacturing practice (GMP), must not involve animal testing, and labelling is subject to strict rules (Regulation EC 1233/2009). In the U.S., registration under the FDA’s Voluntary Cosmetic Registration Program (VCRP) is not required but it is encouraged, the use of animals for testing is not prohibited, and truthful labelling is also regulated. It is also important to distinguish between pharmaceuticals and cosmetics, as pharmaceuticals require FDA approval and include products that claim, for example, hair restoration, pain relief, anti-ageing effects, relief of eczema, dandruff or acne,

sun protection, etc. Therefore, the path of regulation may vary depending on the product's intended use. Similarly, if a product corrects or alters physiological functions by exerting a pharmacological, immunological or metabolic effect, it should be classified as a medicinal product in the EU (Regulation EC 1233/2009, FDA Cosmetics Laws & Regulations https://www.fda.gov/cosmetics/cosmetics-guidance-regulation/cosmetics-laws-regulations, accessed May 6, 2023).

The ingredients of cosmetic products must not be harmful or toxic and must comply with the lists of prohibited and restricted substances. Only approved colorants, preservatives, and UV filters may be included in cosmetic products. The International Nomenclature Committee (INC) manages internationally recognized systematic names for cosmetic ingredients such as plant extracts, oils and chemicals with the abbreviation INCI (International Nomenclature Cosmetic Ingredient), which are used in the European Commission's database for information on cosmetic substances and ingredients CosIng (https://ec.europa.eu/growth/tools-databases/cosing/index.cfm, accessed May 6, 2023), but inclusion in the database does not imply approval for use. INCI names are primarily used for cosmetic product labelling to avoid confusion, as an ingredient may have different chemical names (e.g., common names, CAS or IUPAC names) in different countries.

Typical safety assessment procedures for cosmetic ingredients include the following elements: (i) hazard identification to identify the intrinsic toxicological properties of the substance using New Approach Methodology; (ii) exposure assessment calculated based on the declared functions and uses of a substance as a cosmetic ingredient, the amount present in each cosmetic product category, and the frequency of its use; (iii) dose-response assessment; and (iv) risk characterization, which usually focuses on systemic effects. The ban on animal testing and the requirement to use only validated replacement alternative methods in Europe ensure that the New Approach Methodology (NAM) is followed, which includes *in vitro*, *ex vivo*, *in chemico*, and *in silico* approaches, read-across, and combinations thereof, to support regulatory decision-making by providing information for hazard and risk assessment (Scientific Committee on Consumer Safety - SCCS, 2021).

Marine resources offer an interesting repertoire of bioactive ingredients with cosmetic potential. Extracts from seaweed, algae, soft corals, or other marine life are rich in proteins, amino acids, exopolysaccharides, carbohydrates, vitamins (A, B and C), fatty acids, and trace elements that contribute to hydration, firming, slimming, shine, and protection of human skin, as well as bioactive compounds with, for example, antioxidant and anti-inflammatory properties that protect the skin from ageing and photooxidation (Guillerme et al., 2017). Therefore, beauty products with marine ingredients are becoming increasingly widespread.

7.3. Food and feed supplements

Food supplements are foods whose purpose is to supplement the normal diet and consist of concentrated sources of nutrients (e.g., vitamins, amino acids, and minerals) or other substances with nutritional or physiological effects. Their use is regulated by the establishment of substance lists that are positively evaluated by a food safety authority, such as the European Food Safety Authority (EFSA) or United States Food and Drug Administration (FDA) for safety of ingestion and bioavailability (i.e., the effectiveness with which the substance is released into the body). These agencies also provide guidance on the type and extent of information that should be submitted to demonstrate bioavailability and toxicological data. Special regulations apply to foods for infants and young children and to foods for special medical purposes (Younes et al., 2021)(https://www.fda.gov/food/guidance-regulation-food-and-dietary-supplements, accessed May 6, 2023).

Safety testing evaluates safety based on biological, physical, and chemical parameters. Physical tests check for the presence of foreign objects. Biological safety tests ensure the absence of pathogens and toxins, and chemical tests detect trace elements or contaminants such as food additives, flavourings, contaminants such as heavy metals, nitrates, disinfectants, pesticides, dioxins, residues of veterinary drugs including antibiotics, and components of food contact materials (EU Food safety 2022, https://ec.europa.eu/food/safety_en, accessed May 6, 2023).

There is a growing interest in functional food ingredients and dietary supplements for which the marine environment is an important resource. Numerous compounds such as enzymes, proteins, peptides, polysaccharides, polyunsaturated ω-3 fatty acids (PUFA), phenols, pigments, and other secondary metabolites have already found use in the food industry (Boziaris, 2014; Šimat et al., 2020). In addition to routine identification of known toxins or contaminants using analytical chemistry methods, bioassays for detection of potentially unknown or unexpected toxic components are important for food and feed safety. Apart from animal testing, bioassays are the only way to identify novel risks in food or feed ingredients, especially when new and alternative resources are introduced. This will become especially important with the advent of the circular economy and green waste plans, which will increase the input of waste streams into the food chain (Gerssen et al., 2019).

8. Conclusions

Many new and repurposed biologically active natural products from microorganisms and macroorganisms from the marine environment have been detected and characterized using *in vivo*, *in vitro*, and *in silico* bioassays. The choice of bioassays used in biodiscovery is critical to the successful path from extract to marketed product. Therefore, it is important to realise that each extract contains many bioactivities and that when pursuing a bioactive compound using a series of bioassays to isolate and purify the targeted bioactive compound, the other components of the extract should not be discarded as inactive. Additional valuable bioactivities may be revealed by other bioassays. Conversely, a bioactive compound targeted for a particular application can be reassessed for other types of bioactivities as part of the repurposing process. Only when all these aspects are taken into account is it possible to optimize the potential and make the best use of the various natural resources and, in particular, the marine environment, which is now being increasingly explored.

A careful inspection of the literature reveals many questions regarding the performance of bioassays used for screening and identification of bioactivity. Some of these issues relate to possible artifacts in assay results, variations in activity within different methods, differences in solubility, synergy of compounds in the tested extract, proper use of controls, storage conditions of extracts, etc. For many bioassays routinely used in research laboratories, there are no standardized assay procedures, so it is often very difficult to compare results reported by different laboratories. To improve the potential for standardization of bioassays, fundamental properties such as robustness, reproducibility, relevance, sensitivity, cost-effectiveness, automation, accuracy, and selectivity should be considered in the development and selection of bioassays to be used. A practical aspect is the use of validated protocols, appropriate controls, and biologically relevant concentrations in bioassays. In this way, it can be assessed at an early stage of biodiscovery whether the selected bioactivity has realistic potential, for example, for pharmacological or cosmetic applications, or whether it is merely an interesting but descriptive discovery.

It is important to note that computational approaches should be widely incorporated into biodiscovery screenings for two reasons: (i) these approaches are data-driven, so their inclusion in screening protocols will provide large amounts of data that can be examined for valuable patterns for further discovery; and (ii) large amounts of data are already available for analysis, so systematic analysis of data should become routine, including genome sequences, gene expression, chemical structures analytical data, genotype or proteome data, human microbiome, or electronic health records. These analyses, performed using computational tools, can save time through dereplication, prediction of new targets for already known compounds, and information on modes of action. Understanding the molecular mode of action of bioactive compounds is particularly important because this knowledge helps in the development of new ways to elicit the same effect when the original bioactive compound proves toxic or immunogenic, cannot be synthesised, and/or is not available in sufficient quantity or is lost from natural resources.

Finally, scientific research must be supported by innovation. The search for products for human and environmental health and well-being, including the development of new bioassays, must consider the principles of ethics, responsible research and innovation (RRI) (Schneider et al., 2022), good laboratory practices, and respect for natural ecosystems and habitats.

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Figure legends

Fig. 1. Characteristics of bioassays used at different stages of biodiscovery.

Fig. 2. Characteristics of a good bioassay.

Fig. 3. Distribution of research efforts to assess bioactivity of marine natural products from 2000 to 2022 based on the PubMed database. For each bioactivity, the number of publications without reviews is indicated. The last two years are highlighted, with the number of publications (without reviews) next to the columns.

Fig. 4. Distribution of research methods and target microorganisms for antimicrobial bioactivity of marine natural products from 2000 to 2022 based on PubMed database. The number of publications (excluding reviews) is shown for each method or microorganism used. A, research effort by bioassay method; B, research effort by microbial species. The last two years are highlighted, with the number of publications (excluding reviews) next to the columns. MIC, minimum inhibitory concentration assay determines the lowest concentration of a substance that inhibits the visible growth of a microorganism.

Fig. 5. Distribution of research methods used between 2000 and 2022 to assess cytotoxic activity of marine natural products (based on PubMed database). The total number of publications (excluding reviews) is shown for each method used, with the last two years highlighted in light blue and the number next to each column. NRU, neutral red uptake cytotoxicity assay; alamar blue is a metabolic dye used to quantify proliferation; calcein assay measures cell viability by following conversion of calcein-AM to fluorescent calcein in living cells; LDH measures the activity of lactate dehydrogenase released from damaged cells; SRB, sulforhodamine B is a fluorescent dye used to quantify cellular proteins; PI, propidium iodide is a fluorescent dye that can pass freely through the cell membranes of dead cells and is excluded from viable cells; ATP, adenosine triphosphate assay measures cell viability based on the presence of ATP; Annexin-V is a protein that binds to phosphatidylserine on the plasma membrane and is used to detect apoptosis; MTT, MTS, XTT are tetrazolium salts that are reduced to formazan in living cells, where MTS and XTT yield a water-soluble formazan dye that is detected spectrophotometrically.

Fig. 6. Screening for antiviral activity begins with determining the potential toxicity of the compounds or extracts to cell lines that allow viral replication using bioassays such as tetrazolium salts or ATP-based assays or fluorometric microculture cytotoxicity assays (FMCA). It is then necessary to determine which cell system(s) is best suited for virus replication to test for antiviral activity. The ability of the cell line to support viral replication varies and can be measured by cytopathic effect (CPE), focus-forming assay (FFA), plaque quantification (PRA, VRA), or hemagglutination inhibition (HI). Once specific antiviral activity has been established, it needs to be verified in more complex systems and using *in vivo* models.

Fig. 7. Distribution of research methods for antioxidant and anti-ageing activities of marine natural products from 2000 to 2022 based on PubMed database. The number of publications (excluding

reviews) is shown for each method. The last two years are highlighted, with the number of publications (excluding reviews) next to the columns. CUPRAC, CUPric Reducing Antioxidant Capacity; ORAC, oxygen radical absorbance capacity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox®-equivalent Antioxidant Capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

Fig. 8. The approach for the discovery of new bioactive compounds from marine extracts, with the methodology for small (left) and large (right) biomolecules indicated separately. After extraction, bioassays are performed to determine the potential bioactivities of the extract, and several purification steps are performed to fractionate the extract for analysis and prioritise the purified compounds according to their novelty, for which the dereplication step is crucial. Several purification and analysis runs are required to narrow down the selection of bioactive compounds. Finally, a purification procedure is applied to obtain larger amounts of bioactive compounds that can be further used for compound identification and structure elucidation. The general approach for the discovery of new bioactive compounds is the same for each type of molecule, but the analysis and separation methodology differs depending on the properties.

Fig. 9. Overview of the different stages of drug discovery in the early discovery and preclinical phases of natural product development. Examples are shown of various bioactivity and safety assays that can be used specifically at each stage.

Figure 1

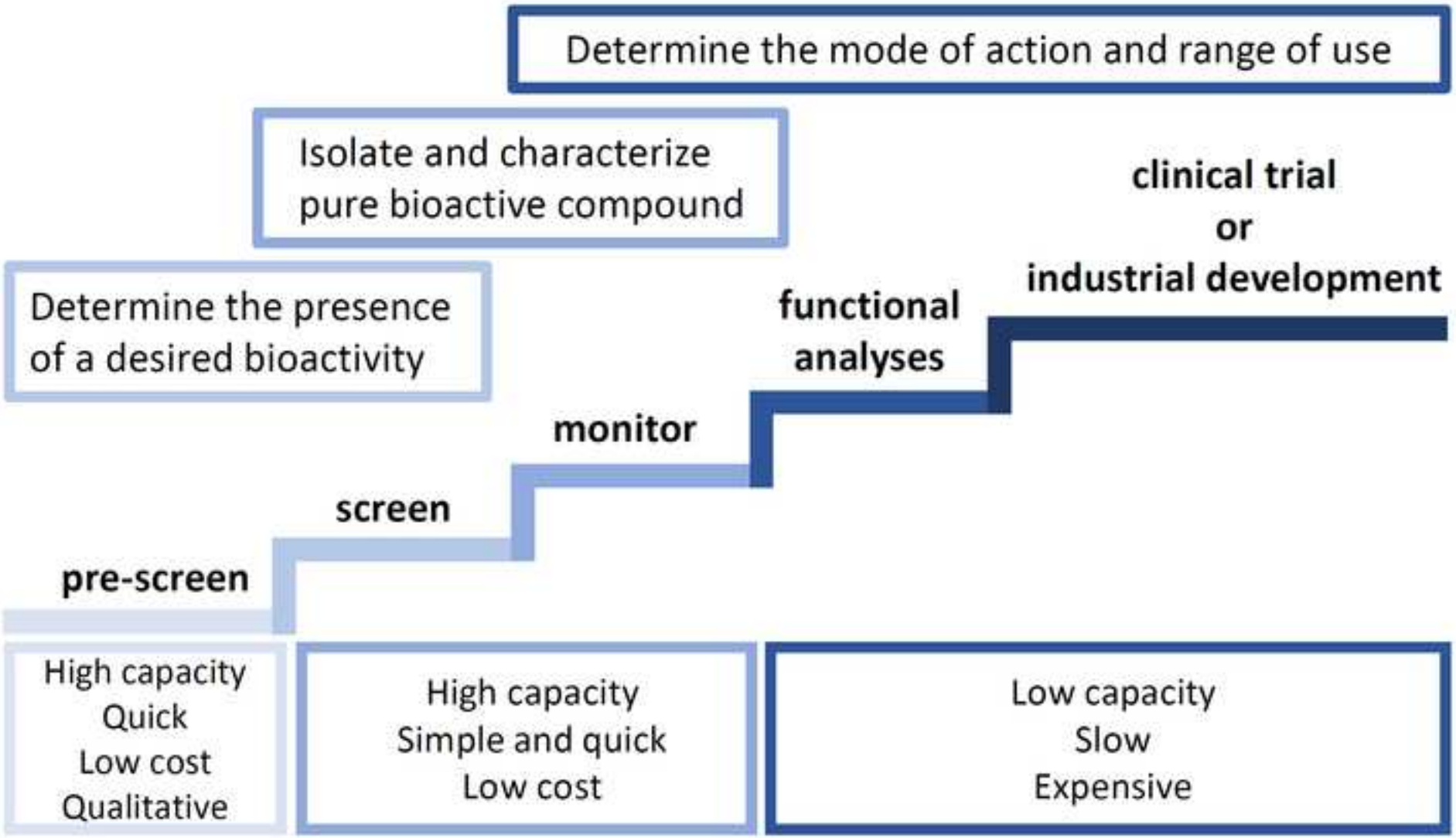


Figure 2

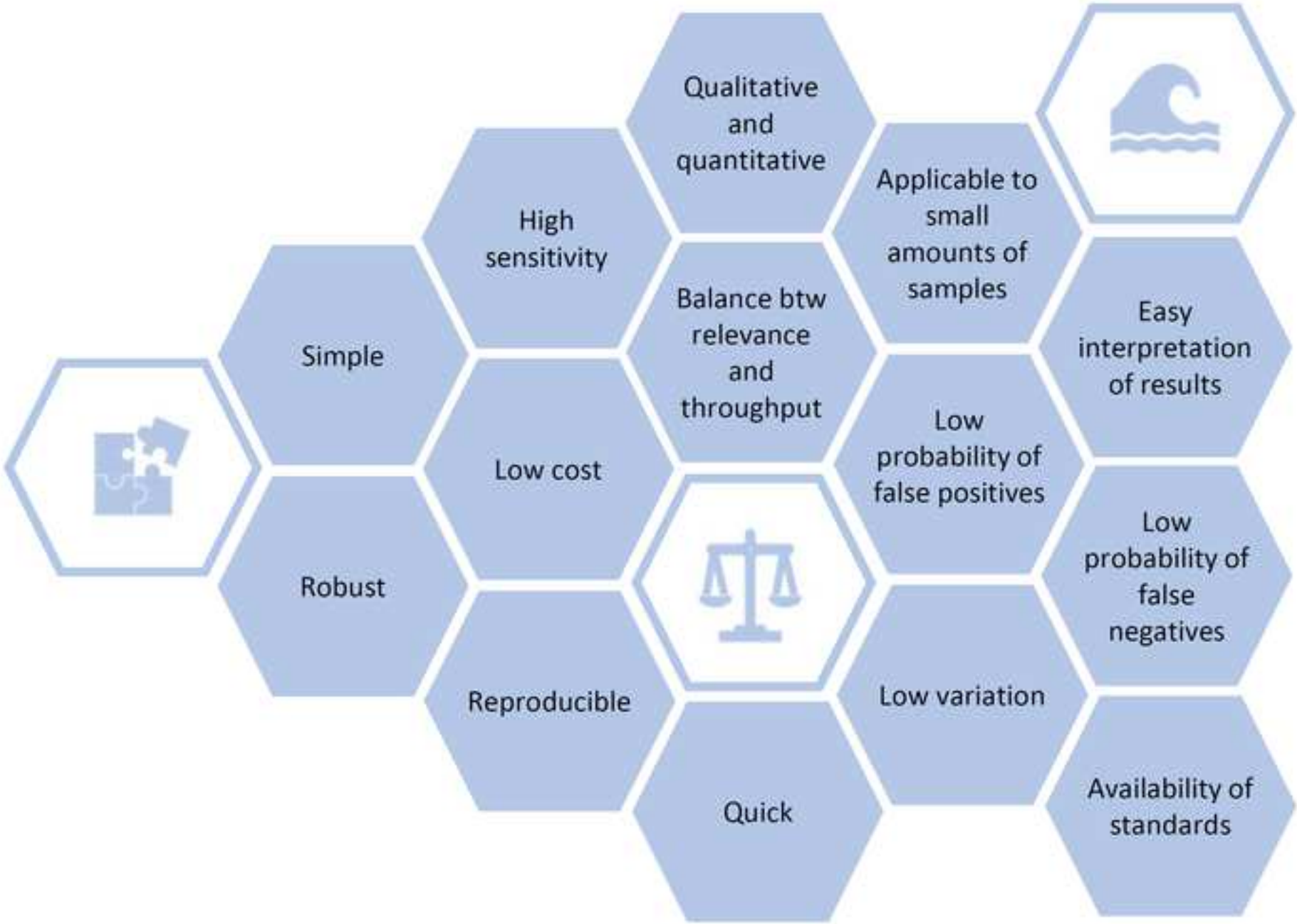


Figure 3

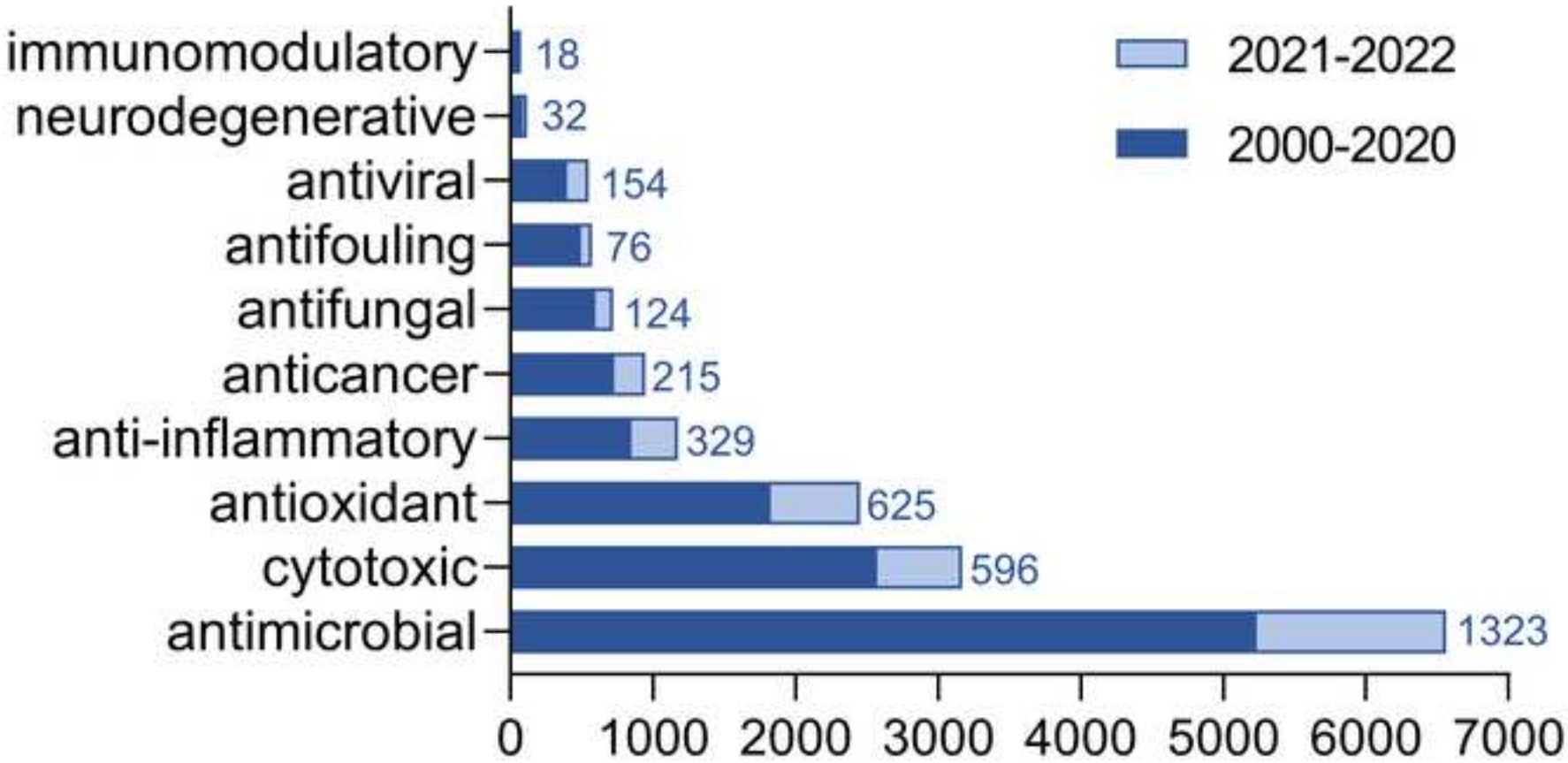


Figure 4

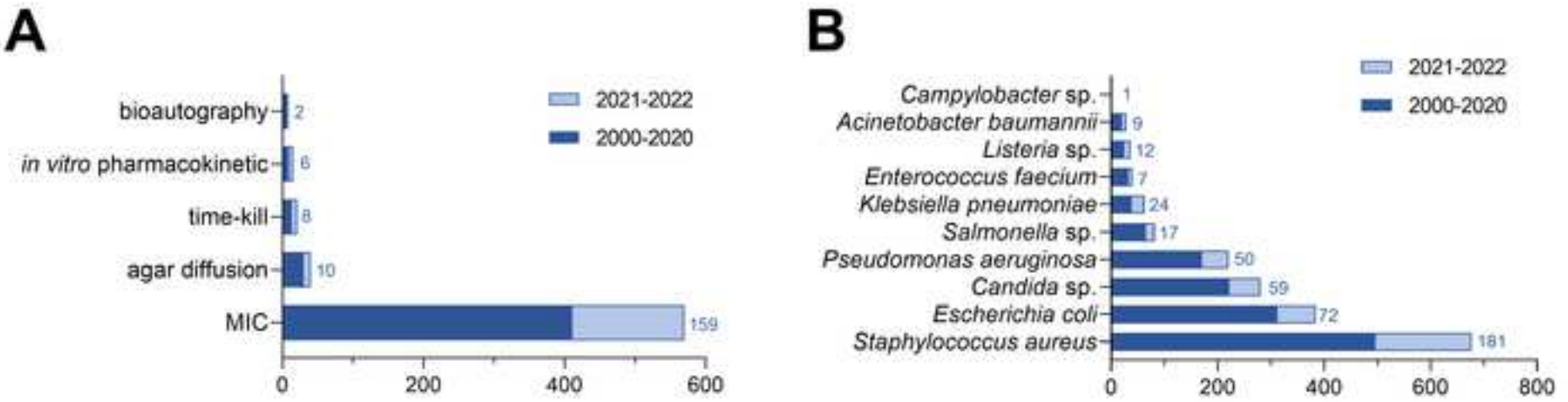


Figure 5

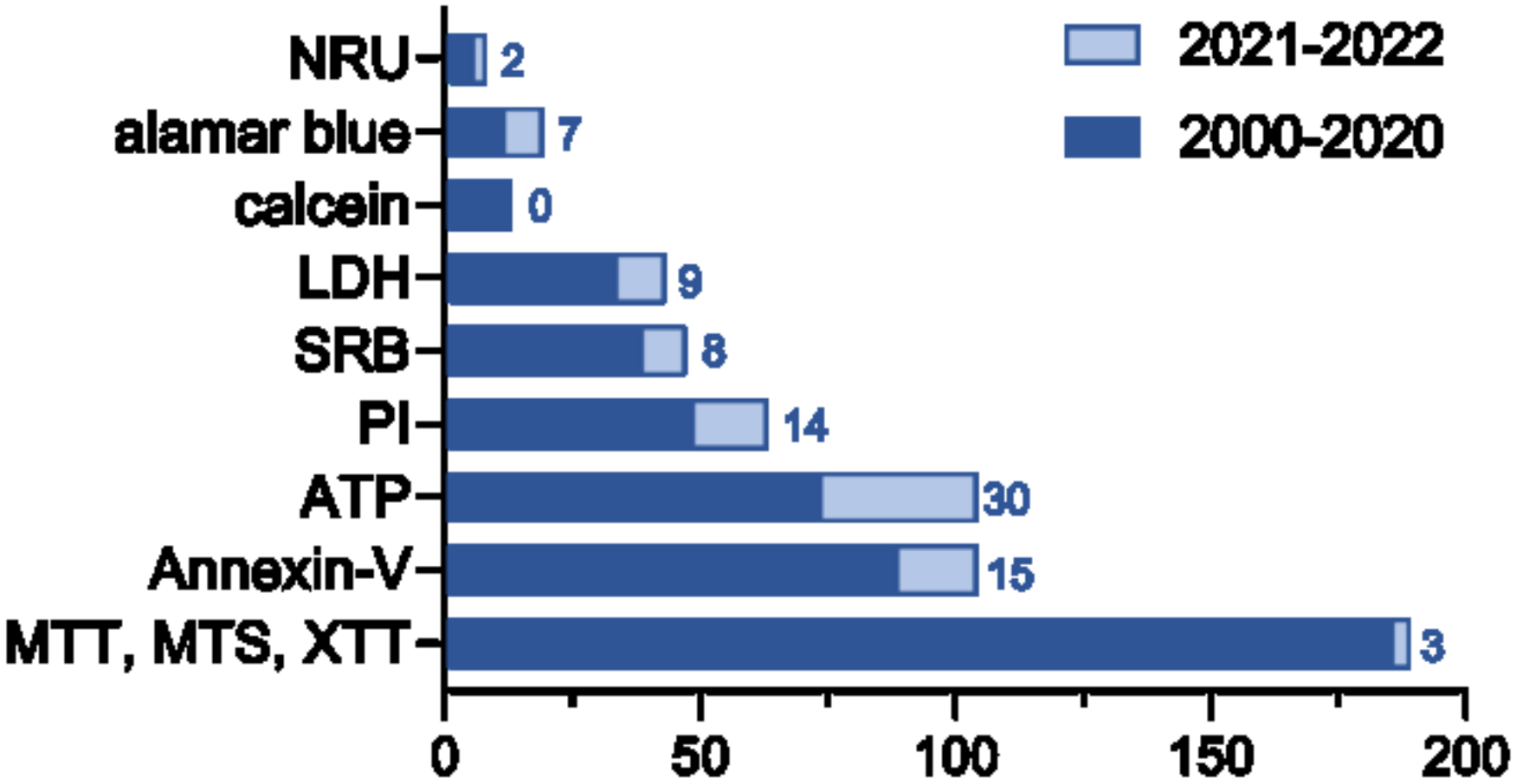


Figure 6

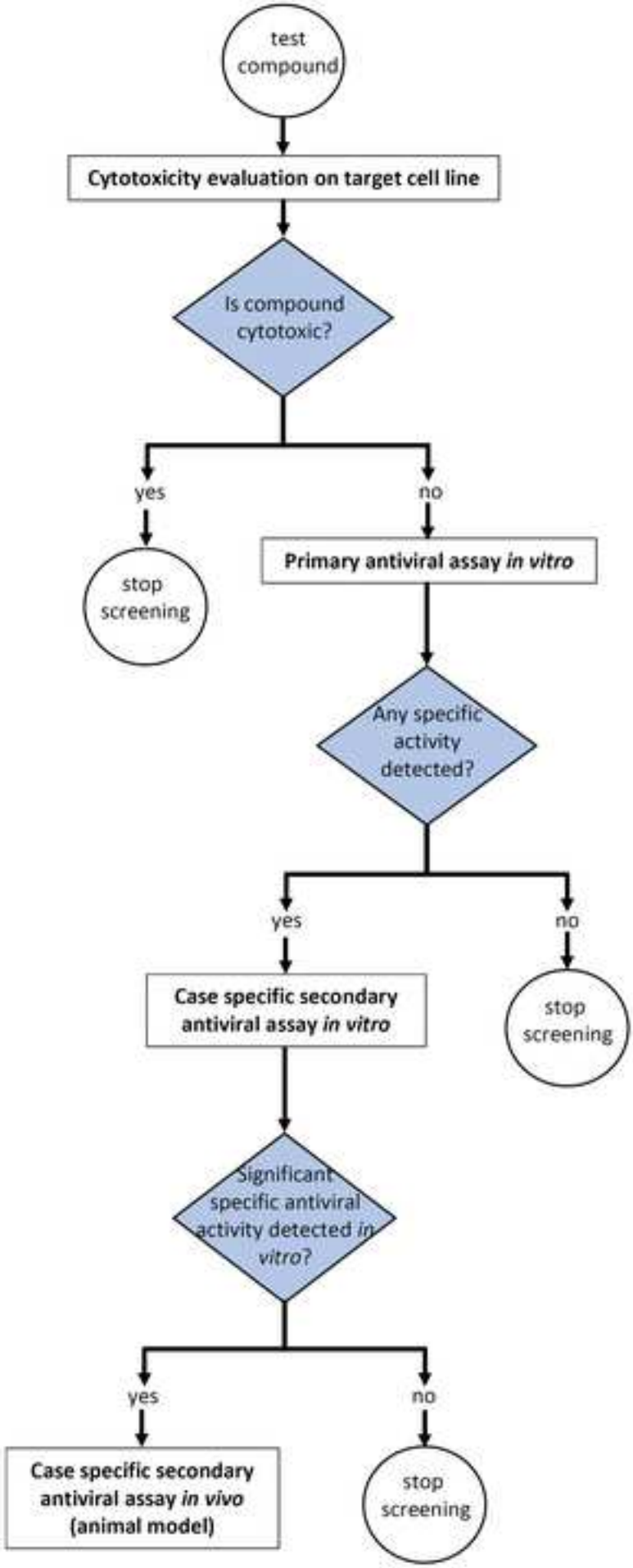


Figure 7

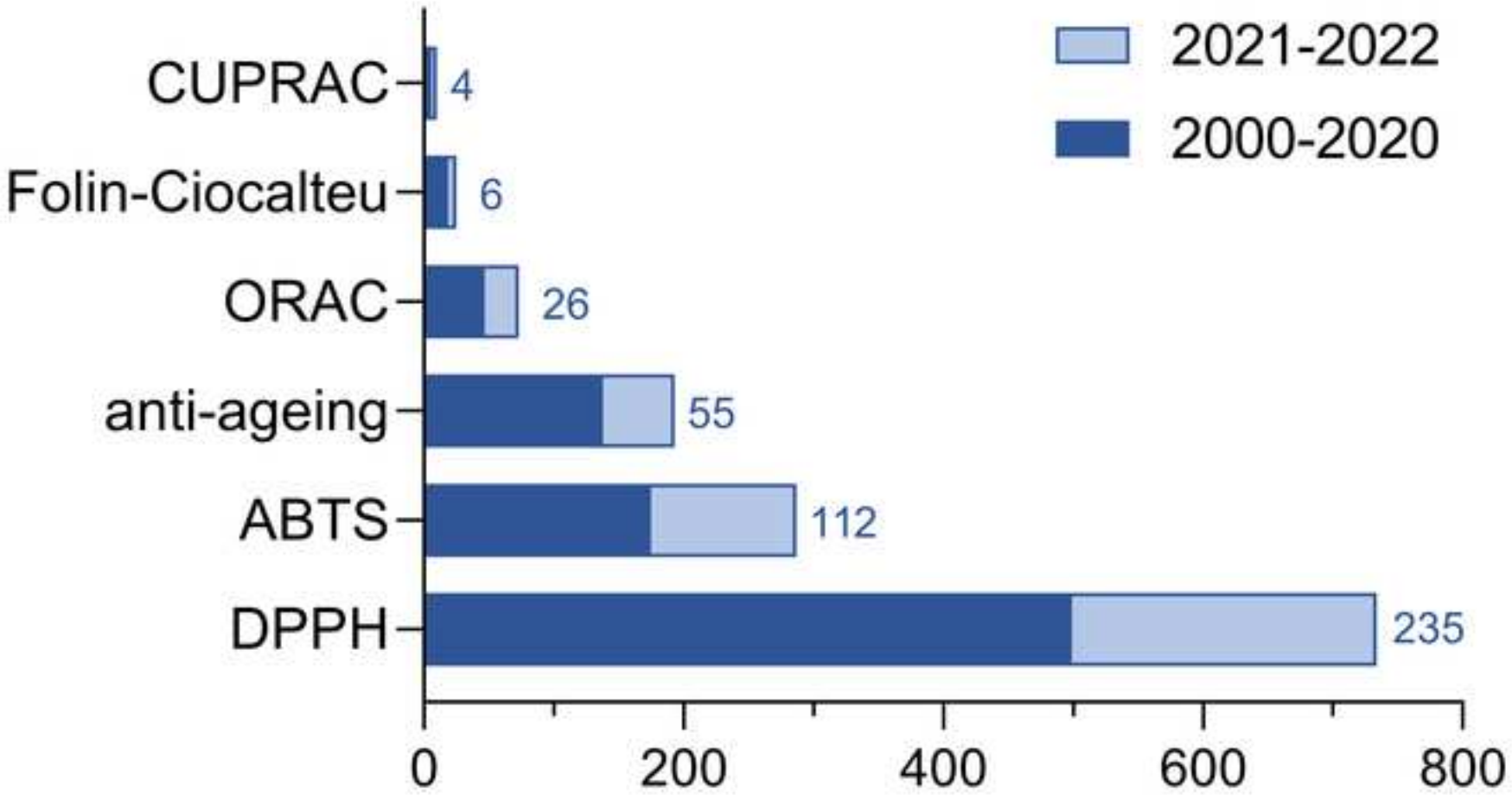


Figure 8

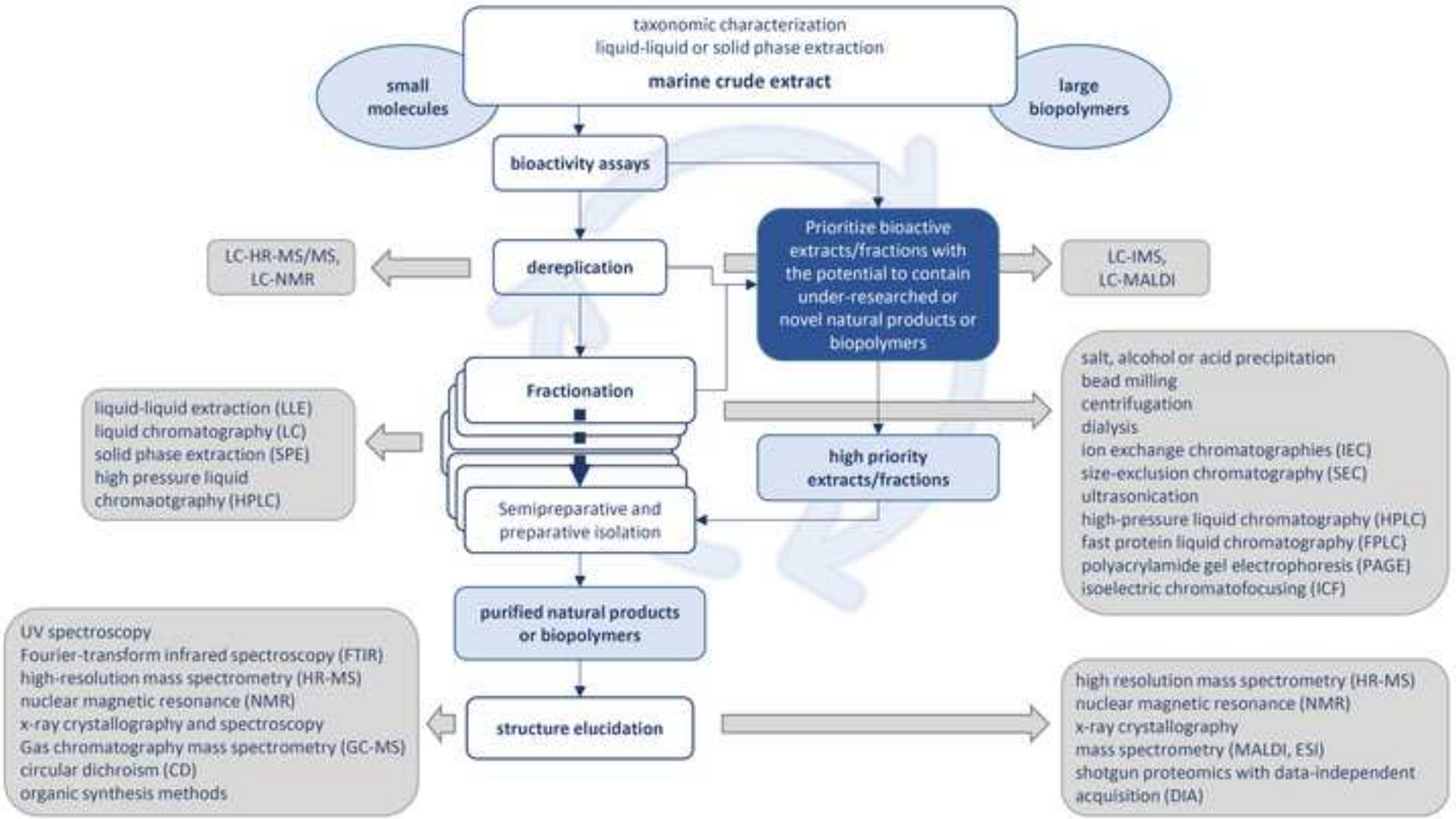
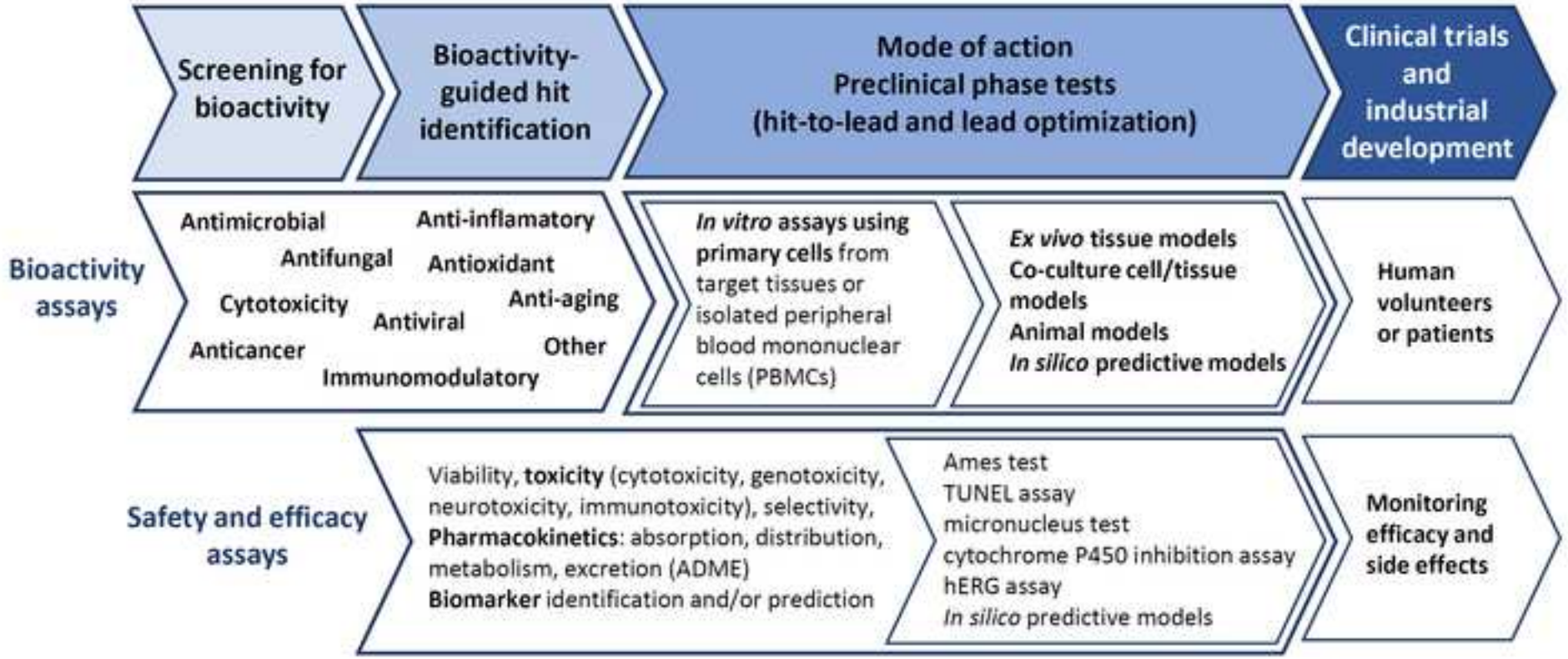


Figure 9



Supplementary Table S1: Principles and characteristics of popular bioassays used in pre-screening and screening of bioactivities

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Antimicrobial bioassays				
Disc diffusion = Agar disc diffusion method = Kirby-Bauer test = Disc-diffusion antibiotic susceptibility test	*In vitro* detection of effects on microbial growth or survival on solid media. A microbial inoculum suspension (e.g., 1-2 10^8CFU/mL for bacteria) is spread on agar plates and the test extract/compound is applied on impregnated paper discs. After 12-24 h incubation (bacteria) or 24 – 48 h incubation (fungi) in suitable growth conditions for the tested microbial strain inhibition zone diameters are read at the point where no growth is observed. Variations are available for yeasts and molds.	- Simple - Standardized protocols available for bacteria and yeast (CLSI, EUCAST) - Versatile (suitable for majority of bacterial pathogens) - Controls for bioassay performance available in form of antibiotics and characterized typing strains with known phenotype and antibiogram - No special equipment, only basic microbiological utilities required - Easily used in routine - Reproducible and accurate if standard protocols are followed - Inexpensive - Easy to interpret - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not appropriate for all bacterial pathogens - Diffusion of the extract/compound can be non-homogeneous and affect accuracy - Not appropriate for large molecules, amphiphilic molecules - Importance of the inoculum size and preparation - Importance of growth medium used - Not quantitative – cannot determine MIC value - Qualitative categorization into susceptible, intermediate or resistant is possible based on standardized MIC breakpoints - Cannot distinguish between bactericidal and bacteriostatic effect - Few interpretative criteria are available - Not adapted for filamentous fungi as breakpoints for standard antibiotics are not defined	(Alastruey-Izquierdo et al., 2015; Balouiri et al., 2016; Matuschek et al., 2014; Strömstedt et al., 2014)
Antimicrobial gradient method = Epsilometer testing (commercial version Etest®)	*In vitro* detection of effects on microbial growth or survival on solid media. Variant of agar diffusion method that combines the principle of dilution and diffusion methods to determine MIC. Exponential gradient of substance applied on a plastic or nitrocellulose strip (marked with concentration scale) and placed on a previously inoculated agar surface. After 12-24 h incubation (bacteria) or 24 – 48 h incubation (fungi) in suitable conditions ellipse-shaped zone of inhibition indicates the MIC that can be read off the strip.	- Simple - Used for antibiotics, also antimycobacterials - High sensitivity (can detect trace amount of beta-lactamase (ESBL) - Quantitative (provides MIC value) - Can be used to test interaction of two antimicrobials - Cost-effective - Useful also for yeast and filamentous fungi	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not appropriate for all bacterial pathogens - Subjective interpretation - Diffusion of the extract/compound can be non-homogeneous and affect accuracy - Not appropriate for large molecules, amphiphilic molecules - Cannot distinguish between bactericidal and bacteriostatic effect - Not used for MNPs (problematic preparation of gradient strip)	(Idelevich et al., 2018)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- No special equipment, only basic microbiological utilities required - Easy to interpret - Commercial kits available that can be used as controls		
Agar plate assay = Poisoned food method for filamentous fungi	*In vitro* evaluation of antifungal effect against filamentous fungi. The substance or extract is incorporated homogeneously into the molten agar and mycelia disc are inoculated at the center of plate. After incubation under suitable growth conditions the diameters of growth inhibition are read and compared with the unexposed control.	- Simple - Standardized protocols available (CLSI, EUCAST) - Easy to interpret - Relatively sensitive - Low cost - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - There are some commercial kits that combine identification-susceptibility testing assay for *Candida* and *Aspergillus* spp. - Resources for work with fungi - Not quantitative - Possible interference with growth medium components - Not appropriate for heat labile compounds - Requires large amounts of compounds - Time consuming	(Chadwick et al., 2013)
Broth (micro)dilution for determination of MIC (Minimum Inhibitory Concentration)	*In vitro* detection of microbial growth inhibition in liquid culture containing a known concentration of drug. Two-fold dilutions of antimicrobial agent or extract are mixed with the inoculum in liquid medium and after suitable growth time period of incubation (12 – 24 h) MIC value is determined by detecting the lowest concentration that inhibited visible microbial growth. Usually performed in 96-well plates (microdilution). Detection of growth is by naked eye or colorimetric assays using tetrazolium salts, resazurin, or ATP can be used to detect metabolically active cells. Different procedures are adapted for yeasts and molds including longer incubation time (24 – 72 h).	- Standard protocols are available (CLSI, EUCAST) - Gold standard in clinical microbiology - High-capacity bioassay - Versatile - Accurate and reproducible - Applicable to both yeasts and molds - Economic if plates are produced in the laboratory - Can be used for any new discovered antimicrobials - Low sample volume required - Cost-effective - Adequate for primary screening - Appropriate for high-throughput screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Solubility of organic extract in broth medium can be challenging - Not suitable for large polycationic, amphiphilic molecules - Plastic interference of 96 well plates for peptide antimicrobial assessment - Importance of the inoculum size and preparation - Importance of growth medium used - Subjective interpretation by CLSI methodology alleviated using EUCAST protocol - Labor-intensive - Technical training requirement high - Risk of error with dilution preparation - Edge effect	(Arendrup et al., 2008; Balouiri et al., 2016; Ezra et al., 2004; Rodriguez-Tudela et al., 2008; Strömstedt et al., 2014)
MBC (Minimum bactericidal	Common estimation of bactericidal or fungicidal activity determined after broth dilution by	- Simple - Quantitative	- BSL2 and BSL3 level microorganisms require work in suitable facility	(Balouiri et al., 2016)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
concentration), or MFC (minimum fungicidal concentration), or MLC (minimum lethal concentration)	subculturing samples from wells with incubation time from 24 h to 72 h. It is the lowest concentration of antimicrobial agent needed to kill 99.9 % of the final inoculum after 24 h incubation in standardized conditions.	- Cost-effective - Adequate for primary screening	- Labor intensive - Importance of growth medium used - Only culturable cells are detected	
Time-kill assay = Time-kill curve = Growth curve analysis	*In vitro* test to measure the kinetics of dynamic interaction between the compound and the microbial strain to reveal a time-dependent or a concentration dependent antimicrobial effect. The log CFU/mL of microbial/antimicrobial solution is determined on time scale depending on the bacteria strain and the media used. Alternatively, growth is followed in a microplate reader measuring optical density at 600 nm. Typically used in secondary testing.	- Existing standard guidelines CLSI and ASTM - Growth curve analysis offers many variables that may indicate mode of action: growth rate, growth dynamics - Can be used to study synergy/antagonism between substances	- BSL2 and BSL3 level microorganisms require work in suitable facility - Special software needed for growth curve analysis - Labor intensive - Specialized equipment needed - Inoculum size, growth phase, growth medium affect outcome - Possible interference with growth vessels, medium components and method of growth detection	(Balouiri et al., 2016)
Bioautography	*In vitro* direct detection of antibacterial compounds on TLC (Thin Layer Chromatography) plate based on incubation (12 – 24 h) and visualization of microbial growth using vital stains or metabolic stains or dehydrogenase-activity-detecting reagent to reveal zones of inhibition. A variation is possible using bioluminescent bacteria as reporters. Particularly adequate for monitoring.	- Simple - Rapid - Results easily visualized - Inexpensive - Applicable to both bacteria and fungi - Can be utilized for spore-producing fungi - Little amount of extract/compound required	- BSL2 and BSL3 level microorganisms require work in suitable facility - Volume of agar or broth has to be well defined otherwise resulting in poorly defined inhibition zones or irregular bacterial growth - Not quantitative - Difficult to standardize	(Balouiri et al., 2016; Choma and Grzelak, 2011; Dewanjee et al., 2015; Klöppel et al., 2008; Patil et al., 2017)
Volatile antibiotics bioassays	All versions of these bioassays use the same principle to detect volatile organic compound (VOC) activity. The source of the volatile (a living organism or chemical) is placed on one side of a chamber without direct contact with the target organism, while the target is grown or located on another side or compartment of the chamber. The effect of the volatile on the growth (inhibition) or survival of the target organism is compared to a control using the same container and conditions without the volatiles.	- Easy to perform and interpret - Low cost - Sensitive - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Not quantitative - Special equipment or material required (sealed chambers)	(Ezra, 2004; Liarzi et al., 2016; Tomsheck et al., 2010)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Antibiofilm bioassays				
Crystal violet	Gold standard for biofilm quantification in microtiter plates. Inoculum in liquid medium incubated for 24 – 72 h at selected temperature under static conditions. Washing steps and short incubation times in crystal violet, are followed by the colorimetric detection of the stained biomass.	- Adapted protocols available for different bacterial species - Different surfaces can be assayed using coupons - Versatile: both for G+ and G- - Qualitative or quantitative, but characterized control strains need to be incorporated for interpretation - Low cost - Can be used to monitor biofilm growth and biofilm eradication - High-throughput (96-well plates) - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Non-specific binding to anionic proteins and other negatively charged molecules, like capsules, lipopolysaccharides, and DNA/nucleic acids, leading to an inability to distinguish between live and dead bacterial populations and/or exopolysaccharides - Large variability between samples leading to possibly complicated interpretation - Medium composition important - Culture conditions important - Strain to strain variability is high, need to know primary biofilm phenotype - Interference of the stain with experimental setup possible	(Haney et al., 2021; O'Toole, 2011)
CFU (Colony Forming Units)	Biofilm is sonicated to dislodge adhered biomass and serial dilutions of homogenized bacterial suspension is plated onto agar plates, incubated 24 – 48 h to count the colony forming units (CFUs).	- Simple - Low cost - Adequate for primary screening	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment - Sonication parameters important (can reduce viability of recovered CFUs), - Sonication parameters are different for different bacterial species - aggregation can affect CFU count - Labor intensive - Only culturable cells are detected	(Haney et al., 2021)
The BioFilm Ring Test	Mobility measurement of magnetic microbeads mixed with bacterial suspension in a polystyrene microplate. Without biofilm growth beads gather together in a visible central spot under magnetic action, while no spot indicates bead immobilization by biofilm formation.	- Simple - Rapid - No dyes or stains - No washing steps - Low sample volume required - High-throughput (96- well plates)	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment - Interpretation may be challenging - Qualitative	(Olivares et al., 2016)
The Calgary Biofilm device	Two-part reaction vessel containing a lid with 96 pegs that sit in channels of the reaction vessel that	- Standardized protocols available - High-throughput (96-well plates) - Quantitative	- BSL2 and BSL3 level microorganisms require work in suitable facility - Need for specialized equipment	(Haney et al., 2021;

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	allows flow of medium across pegs to create consistent shear force.		- Use of multiple sterile microplates for treatment and washing steps - Relies on viable cell counting for experimental validation	Kırmusaoğlu, 2019)
MBEC (Minimum biofilm eradication concentration) Assay®	High-throughput screening of antibiofilm activity. Plastic lid with 96 pegs on which biofilms establish under batch conditions and the lid with pegs is transferred to a new 96 well for testing, biofilm is dislodged by sonication and CFUs are determined.	- Standardized method for *Pseudomonas aeruginosa* (ASTM E2799-17)	- BSL2 level microorganisms require work in suitable facility - Aggregation can affect CFU count - Labor intensive - Only culturable cells are detected	(ASTM, 2022; Parker et al., 2014)
SIMBA – simultaneous detection of antimicrobial and antibiofilm activity	The SIMultaneous detection of antiMicrobial and anti-Biofilm Activity (SIMBA) method combines the testing of antimicrobial and antibiofilm activity against bacteria with the evaluation of the 20-hour growth curve of the *Salmonella* Infantis ŽM9 strain determined with absorbance measurements at 600 nm in a 96-well plate.	- Simple - Rapid - No dyes or stains - Cost-effective - Information on both antimicrobial and antibiofilm activity in one assay - Low sample volume required - High-throughput (96-well plates) - Possibility of automation	- Optimized for one *Salmonella* strain - Not suitable for dark colored samples - Need for specialized equipment - (spectrophotometer with temperature control and shaking capabilities)	(Sterniša et al., 2023, 2022)
Cytotoxicity bioassays				
MTT (also MTS, XTT, WST)	*In vitro* colorimetric assay usually performed in 96-well plates to evaluate cellular metabolic activity - glycolytic production of NADH. Based on tetrazolium salts (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; WST, water-soluble tetrazolium salts) – difference between them is the tetrazolium salt used and the solubility and/or absorption spectrum of the formazan product. Eukaryotic cells are treated for 24 - 48 hours with different concentrations of compounds to determine the concentration of the tested	- Commercial kits with standardized protocols available - Cost-effective - Relatively simple - Assay for whole cells - Linearity between absorbance and cell count - Versatile: suitable - for both adherent and suspended cell cultures - One-step procedure variants using water soluble tetrazolium salts include XTT, MTS, WST - Possibility of automation - Appropriate for high-throughput screening	- BSL2 and BSL3 level cell lines require appropriate facility - Lengthy two-step procedure - Highly variable results depending on: the number of cells per well, and the high pH of the culture medium - Requires optimization of cell density (untreated cells have absorbance values that fall within the linear portion of the growth curve (conditions not too close to saturation) - Requires optimized incubation time - Not suitable for reducing compounds - Not for metabolically poor cells, i.e. thymocytes and splenocytes	(Balbaied and Moore, 2020; Jo HY et al., 2015; Mccauley et al., 2013; Riss et al., 2019)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	compounds, which produces 50% of cytotoxicity (CC_{50}). Tetrazolium salt (e.g., MTT) is then added to the cells for 2 hours at 37°C. MTT is reduced by a cellular mitochondrial enzyme (succinate dehydrogenase) to violet formazan precipitates, which are subsequently solubilized by organic solvents before absorbance is read. Alternatively, water-soluble tetrazolium salts can be used, omitting the final solubilization step.		- Linearity between absorbance and cell count is lost when cells are confluent and cellular metabolism slows down - The result can be variable because metabolic activity depends not only on the number of cells per well but also on several other factors	
Sulforhodamine B (SRB) assay	Used for cell density determination, based on the measurement of cellular protein content. Toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader.	- Simple - Cost-effective - Results linear over a 20-fold range of cell numbers - Sensitivity comparable to those of fluorometric methods - Appropriate for high-throughput screening	- Requires microplate reader (absorbance)	(Vichai and Kirtikara, 2006)
ATP-based test	Gold standard luminescence test. See MTT for the procedure. Quantification of released intracellular ATP by enzymatic reaction between the enzyme luciferase and its substrate, luciferin, to produce luminescence. There is a linear relationship between the intensity of the light signal and the ATP concentration or cell number. It is one of the most sensitive endpoints for measuring cell viability.	- One-step procedure - Faster than MTT and MTS - Reduction of artifacts - Sensitive measure of intracellular ATP rather a specific biological effect - More sensitive than conventional biochemical methods - Sensitive compared to other cytotoxicity tests - Interferences minimal - Commercial kits available - Possibility of being automated	- BSL2 and BSL3 level cell lines require suitable facility - More expensive than MTT and MTS and fluorescent methods - The ATP assay sensitivity is usually limited by reproducibility of pipetting - Replicate samples rather than a result of the assay chemistry - Need for specialized equipment (luminescence detection)	(Aslantürk, 2018; Herzog et al., 2007; Ponti et al., 2006)
Automated fluorometric microculture	Based on the measurement of fluorescence generated from cellular hydrolysis of fluorescein diacetate (FDA) to fluorescein by viable cells with	- Highly standardized and reproducible one-step procedure - Possibility of being automated	- BSL2 and BSL3 level cell lines require suitable facility - Need for specialized equipment	(Burman et al., 2011;

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
cytotoxicity assay (FMCA)	intact plasma membranes after a 48 – 72 hour culture period in microtiter plates. See MTT for procedure.		(fluorescence detection)	Lindhagen et al., 2008)
Dye exclusion method	The membrane integrity of cell is determined by its permeability to several dyes (eosin, Trypan blue, erythrosine B, Congo red assays). Trypan blue has been used the most extensively to assess the percentage of viable cells in suspension culture.	- Simple - Rapid - Small numbers of cells needed - Can be applied in non dividing cell populations	- BSL2 and BSL3 level cell lines require suitable facility - Can be challenging to process a large number of samples simultaneously, particularly when the exact timing of progressive cytotoxic effects is taken into consideration - Careful interpretation needed for living cells with metabolic activity loss (trypan blue) - Its toxic side effect of some dyes on mammalian cells (trypan blue) - Not suitable for adherent monolayer cell cultures - Labor intensive	(Aslantürk, 2018)
LDH (lactate dehydrogenase) cytotoxicity assay	LDH is a cytosolic enzyme present in many different cell types that is released upon damage to the plasma membrane. The assay quantitatively measures the activity of stable, cytosolic LDH released from damaged cells. It is a colorimetric assay.	- Suitable for both adherent and suspended cell cultures - Commercial kits available - Detects low level damage to cell membranes which cannot be detected using other methods	- BSL2 and BSL3 level cell lines require suitable facility - LDH assay is limited to serum-free or low-serum culture conditions to avoid high background readings. - Interference with serum components	(Kocherova et al., 2020)
Clonogenic cell survival assay	Determines the ability of a cell to proliferate indefinitely, retaining its reproductive ability to form a colony or a clone. These cells are considered clonogenic. Cells are seeded at low density and growth of colonies/clones is analysed after a week by staining and counting. The gold standard for measuring cellular reproductivity.	- Simple - Cost-effective - Gold standard	- BSL2 and BSL3 level cell lines require suitable facility - Suitable only for adherent cells - Not suitable for all adherent cell lines (not all cells are able to form colonies in vitro – cell-to-cell contacts and self-produced growth factors are limited at low cell density)	(Munshi et al., 2005)
DNA synthesis assay 3H-labeled thymidine (3HT)	The process of DNA synthesis is relatively specific for cell division and can therefore be considered a marker of cell proliferation activity. Nucleoside	- This assay is commonly regarded as reliable and accurate.	- BSL2 and BSL3 level cell lines require suitable facility - Potential use of radioisotopes	(Romar et al., 2016)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	analogue incorporation assays are based on the introduction of chemically or radio-labelled nucleosides that are subsequently incorporated into DNA strands synthesised during S phase. A scintillation beta counter is used to measure radioactivity in DNA recovered from cells to determine the extent of cell division that has occurred in response to a test agent. The nucleoside analogue 5-bromo-2'-deoxyuridine (BrdU) is used to avoid the use of radioisotopes and is detected with monoclonal antibodies. Alternatively, thymidine analogues are available that do not require antibody detection.	- Suitable for immunohistochemistry or immunocytochemistry, - In-cell ELISA, flow cytometry - It can be performed in experiments *in vitro* and *ex vivo*, but not *in vivo* - Not suitable for screening, used for mechanistic studies - Commercial kits available - Allows quantitative assessment of proliferation levels - Direct measures of proliferation - Appropriate for high-throughput screening	- It is an endpoint assay because of the DNA extraction step, and so no further studies can be performed with the treated cells. - synthetic analogues such as 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), are usually preferred (can be used not only *in vitro* or *ex vivo* but also *in vivo)* - Both assays cannot identify cells that have undergone numerous divisions - Need for specialized equipment	
Antiviral bioassays				
Flow cytometry cell count assay (FACS)	Cytotoxicity-based antiviral assay based on the detection of intact and damaged cells using a flow cytometer and dyes to stain the cells (e.g., propidium iodide, carboxyfluorescein diacetate).	- Three populations discriminated (dead, viable, injured) - Reproducible - Rapid (2-6 h to results)	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Need for specialized equipment: flow cytometry equipment - Need for trained personnel - Not easy to interpret - Specific cell lines known to be susceptible to and allowing viral infection with the virus of interest.	(Balouiri et al., 2016; Zamora and Aguilar, 2018)
Cytopathic effect assay (CPE)	Suitable for primary *in vitro* antiviral screening. In this assay, cells permissive for a virus are infected with the same virus at serial dilutions. Cells are observed daily until a cytopathic effect is detected. The virus concentration is expressed as infectious tissue culture dose ($TCID_{50}$), which is the multiple of dilutions that result in CPE in 50% of wells.	- Commercial kit available allowing standardization and automated procedures - For viruses that do or do not form viral plaques - Cell fixation and staining not required - Cost-effective - Operator independent - Technically simple - Reduced reading time	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Only for viruses that cause morphological changes in infected cells - Lengthy: the time required for the cytopathic effect to become apparent - Indirect measure of viral load - Works only with specific cell lines known to be susceptible and permissible to viral infection with the virus of interest.	(El Sayed, 2000; Suchman and Blair, 2007)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Appropriate for high-throughput screening	- Equipment required to work with viruses and specialized virology trained personnel	
Plaque reduction assay (PRA)	Primary *in vitro* antiviral screening for the detection of infectious viral particles A viral inoculum of approximately 50-70 viral plaques/well is adsorbed onto permissive cells in the presence of the test substance. After viral adsorption, the unbound virus is removed and the culture is covered with a semi-solid medium (agar, Avicel, methylcellulose). After an incubation period equal to the duration of the replication cycle of the virus, the cells are fixed and stained to count the viral plaques microscopically. Titers are expressed as the number of plaque-forming units (PFU) per milliliter (PFU/ml).	- Validation with a positive control, such as a commercial compound with known antiviral activity - Commonly used - No special equipment is required in addition to a cell culture laboratory - Results are easily visualized under a microscope or with the naked eye - Cost-effective - Sensitive - Protocols vary from laboratory to laboratory and depend on the type of cells used - Appropriate for high-throughput screening	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Only for viruses that form plaques - Labor intensive - Sometimes lengthy - Results not reproducible: depends on cell density, CPE and plaque size - Counting of plaques can be subjective - Specific cell lines known to be susceptible and permissible for viral infection with the virus of interest - Protocol must be adapted for each host-virus combination	(El Sayed, 2000)
Virus reduction yield assay (VRA)	Primary *in vitro* antiviral screening to detect infectious viral particles. Permissive cell cultures are infected with a specific amount of virus, and after virus adsorption (usually 2 hours at 37°C or 33°C for temperature-sensitive viruses), the unbound virus is removed, and different concentrations of the same compound are added. After an incubation period that allows virus replication, the total viral yield is titrated and determined.	- Less operator-dependent than the PRA - Cost-effective - Sensitive	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Time/material-intensive - Not-automatable - Not reproducible: results depend on harvesting time - Specific cell lines known to be susceptible and permissible to viral infection of the specific virus in focus	(Collins and Bauer, 1977; Hu and Hsiung, 1989)
Focus Forming assay (FFA)	Primary *in vitro* antiviral screening for viruses that do not induce CPE. Procedure identical to PRA. FFA doses are expressed as concentration units per milliliter (FFU/mL).	- Faster than PRA or $TCID_{50}$ - Reading time varies depending on the replication cycle of the virus - Sensitive	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Indirect method - Expensive - Specific reagents and equipment required	(Flint et al., 2009)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
			- Specific cell lines that are known to be susceptible and permissible to infection with the virus of interest - Reading time of foci depends on the size of the area the operator is counting. A larger area will take longer, but may provide a more accurate representation of the sample.	
Hemagglutination inhibition assay (HIA)	Primary *in vitro* antiviral screening to detect infectious and noninfectious viral particles for viruses that do not form plaques or cause CPE. For HIA, viral samples are first mixed with dilutions of compounds that take time to bind the virus. Then red blood cells (RBCs) are added to the mixture. Antiviral activity: means that there are no free virus particles and the RBCs fall to the bottom of the well by gravity, creating a distinct red spot in a conical well. No antiviral activity: the erythrocytes clump together, resulting in a lattice-like structure.	- Simple - Does not require special equipment - Fast evaluation of virus particles - Standardized protocols available - Validation of a modified HAI: more sensitive, easy to analyse, required only a single source of erythrocytes and allowed utilisation of virus strains which are difficult to handle by the standard HAI (e.g., H3N2, H5N1 and H1N1pdm09)	- BSL2 and BSL3 level cell lines and/or viruses require suitable facility - Less sensitive than other methods - Only for hemagglutinating viruses - The red blood cells used depend on the type of influenza virus in the test - Required source of suitable red blood cells (horse, rabbit, chicken, guinea pig) - Optimization of the type and concentration of red blood cells used is necessary to obtain reliable results. - Requires skilled personnel - Manual evaluation may lead to misinterpretation of results - Non-specific inhibition of hemagglutination possible - Low sensitivity - Semiquantitative data	(Joklik, 1988; Morokutti et al., 2013)
Quantitative polymerase chain reaction (qPCR)	PCR involves amplifying short stretches of longer genomic molecules in a thermocycler, a device that exposes the reaction to a series of different temperatures for a specified time (1 amplification cycle). With each PCR cycle, the amount of target sequence (amplicon) in the reaction theoretically doubles. In quantitative polymerase chain reaction, the amplification rate is monitored in real time during PCR using nonspecific intercalating	- Rapid (1-4h response) - Sensitive - High specificity - Possible to validate - Quantitative or semi-quantitative - Protocol needs to be adapted for each virus, but the general guidelines are the same	- Cell lines and/or viruses of BSL2 and BSL3 levels require a suitable facility - More complex compared to PRA - Need for specialized equipment: flow cytometry equipment - Need for trained personnel - Positive detection does not equate to viable (or infectious) virus, therefore not recommended for initial screening - Expensive	(Engstrom-Melnyk et al., 2015; Kralik and Ricchi, 2017)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	fluorescent dyes or fluorescently labeled sequence-specific DNA probes.			
Antioxidant assays				
DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) assay	Based on the reaction of the tested antioxidant with the stable synthetic radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), accompanied by a color shift of the latter. Aliquots of the extracts are mixed with a methanolic solution containing DPPH radicals, and the mixture is incubated in the dark for 30 min. Absorbance is measured with a spectrophotometer at 517 nm. Usually, quercetin is used as a reference standard, and DPPH results are expressed as quercetin equivalents (QE) in µmol per 100 mL.	- Commercial kits available - Simple - Cost-effective - Good repeatability - Quantitative - Adequate for primary screening - Appropriate for high-throughput screening	- Applicable only for compounds soluble in organic solvents - Radical strongly affected by light, oxygen, pH and type of solvent - Steric hindrance effects for bulky antioxidants - Narrow linear range - Limited relevance to biological systems - Need for specialized equipment (spectrophotometer, multiplate reader)	(Apak et al., 2006; Awika et al., 2003; Molyneux P, 2004)
ABTS/TEAC (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox equivalent antioxidant capacity	With the help of an oxidizing agent, the colorless ABTS salt is converted into its radical cation with characteristic blue-green color, which is then reduced back to its original colorless ABTS form by reaction with the tested antioxidant. Antioxidant activity is defined as the amount of ABTS·+ quenched after a given time (usually 5 minutes) and is expressed in Trolox (6-hydroxy-2,5,7,8-tetramethylochroman-2-carboxylic acid) equivalents as TEAC (Trolox Equivalent Antioxidant Capacity).	- Rapid - Simple - Sensitive - Reproducible - More sensitive than DPPH assay, high response to antioxidants - Can be performed in a 96-well microplate. - Diverse, flexible usage in multiple media (pH, solvents) - Applicable to both lipophilic and hydrophilic anti-oxidants - Commercial kits available - Quantitative - Adequate for primary screening	- Limited relevance to biological systems - Difficulties in the formation of the colored radical and limited stability - Steric hindrance effects for bulky antioxidants - Specialized equipment required (spectrophotometer, multiplate reader)	(Apak et al., 2007; Awika et al., 2003; Erel, 2004; Lee et al., 2015; Re et al., 1999)
Cupric ion (Cu^{2+}) reducing assay (CUPRAC)	*In vitro* assay for measurement of the absorbance of the colored Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction between the chromogenic oxidizing CUPRAC reagent (i.e., Cu(II)-Nc) and the chain-breaking antioxidant under study. Trolox is used as the standard.	- Applicable to both lipophilic and hydrophilic antioxidants - Selective detection of antioxidants - Simulates antioxidant action under nearly physiological conditions	- Unable to react with compounds having isolated hydrocarbon double bonds or alternating double and single bonds (e.g., ferulic acid, β-carotene) - An incubation at elevated temperature may be required for slow-reacting compounds (e.g., naringin and naringenin)	(Apak et al., 2007, 2006; Gulcin, 2020; Özyürek et al., 2011)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
		- Ffavorable redox potential - High stability of reagents - No steric hindrance effects - Commercial kits available - Quantitative - Adequate for primary screening - Appropriate for high-throughput screening	- Need for specialized equipment (spectrophotometer, multiplate reader)	
Folin-Ciocalteu	The Folin-Ciocalteu phenolic reagent is used to obtain a rough estimate of the total amount of phenolic compounds present in an extract. Specifically, the phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphomolybdic acids present in the reaction mixture, yielding a blue color proportional to the amount of phenols. The assay can be performed in a 96-well microplate. The absorbance is read at 760 nm and quantification is based on a calibration curve generated using gallic acid standards (GA).	- Adequate for primary screening - Simple - Reproducible - Excellent correlation between measured “antioxidant capacity” and “total phenolic content” - Quantitative - Commercial kits available - Adequate for primary screening	- Non-specific to phenolics (it reacts with many non-phenolic compounds) - not applicable to lipophilic components - Need for specialized equipment (spectrophotometer, multiplate reader)	(Apak et al., 2007; Bravo et al., 2016; Singleton et al., 1999)
Oxygen radical absorbance capacity (ORAC)	This method is based on the ability of antioxidants to protect fluorescein, a highly fluorescent protein, from oxidative damage caused by peroxyl radicals. The experimental procedure of ORAC involves the addition of the extract under study and a free radical, usually AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), which forms a moiety together with fluorescein, followed by heating in a phosphate buffer. Thermal decomposition produces free radicals that react with antioxidant compounds, resulting in loss of fluorescence due to decrease in radical concentration. The test can be performed in a 96-well microplate.	- Easily automated and largely standardized - adaptable for numerous sample matrices - High biological relevance - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- It is based on fluorescence detection and it requires more expensive instrumentation - Need for specialized equipment (fluorescence detection, multiplate reader)	(Awika et al., 2003; Bravo et al., 2016; Ou et al., 2001)
Anti-aging enzyme-based assays				
Anti-elastase	This *in vitro* assay is performed in Tris-HCl buffer and at room temperature using porcine pancreatic	- Rapid - Simple	- High cost and limited lifetime of enzymes used	(Pastorino et al., 2017;

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
	elastase (PPE; E.C.3.4.21.36) and N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Suc-Ala3-*p*NA) as substrate. Inhibition of PPE by natural extracts is determined spectrophotometrically by monitoring the release of p-nitroaniline from Suc-Ala3-pNA at 410 nm. Can be performed in a 96-well microplate. Epigallocathechin-3-gallate (EGCG) is commonly used as a positive control.	- Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	Thring et al., 2009)
Anti-collagenase	The ability of the extracts to inhibit collagenase activity is evaluated by a spectrophotometric method based on hydrolysis of the synthetic substrate N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) using collagenase from *Clostridium histolyticum* (ChC – EC.3.4.23.3). Can be performed in a 96-well microplate. EGCG is usually used as positive control.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	(Thring et al., 2009; Van Wart and Steinbrink, 1981)
Anti-hyaluronidase	*In vitro* assay that determines activity indirectly by measuring the amount of undegraded hyaluronic acid (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37°C.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Standardized protocol - Commercial kits available - Quantitative	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and *in vivo* conditions - Need for specialized equipment (turbidimeter)	(Bailey and Levine, 1993; Kim et al., 1995)
Anti-tyrosinase	The ability of the extracts to inhibit the catalytic action of tyrosinase in the oxidation of L- DOPA, a precursor of melanin biosynthesis, is usually determined by an enzymatic procedure using the substrate L- DOPA and fungal tyrosinase followed by incubation in a phosphate buffer. The absorbance of the final solutions is measured at 492 nm using a microplate reader. Kojic acid (500 mM) is usually used as a reference inhibitor.	- Rapid - Simple - Provide effective approaches to evaluate inhibitory effects of unknown samples against skin-aging enzymes - Quantitative - Commercial kits available - Appropriate for high-throughput screening	- High cost and limited lifetime of enzymes used - Considerable consumption of tested compounds/samples - Do not closely mimic cellular processes and in vivo conditions - Need for specialized equipment (absorbance detection with spectrophotometer or microplate reader)	(Momtaz et al., 2008)

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Anti-aging Fibroblast-based assays				
Cytotoxicity/cytoprotection	Cultured human fibroblast cell lines are pretreated with the samples and subjected to UV irradiation. Cell viability is measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The amount of formazan is measured by recording the absorbance changes at 570 nm with a spectrophotometer.	- Rapid - Precise - Avoids manipulation of radioactive isotopes - Constitutes a vital cellular setting and a real-life model for simulating oxidative damages and assessing the protective role of natural extracts/compounds	- Handling and preservation of human fibroblast cell lines can be cumbersome - Results should be interpreted with caution as the biological effect is evaluated against a specific type of cells (the interaction of the tested substance with other cell types are not taken into account) - Need for specialized equipment (cell culture, absorbance detection)	(Mosmann, 1983; Ramata-Stunda A et al., 2013; Ratz-Lyko et al., 2012; Riss et al., 2019, 2004)
Regenerative potential	This assay involves exposure of seeded human fibroblast cells to extracts followed by washing with chemical reagents and measurement of procollagen type I or hyaluronic acid content in cell-free supernatants by enzyme-linked immunosorbent assay (ELISA).	- Constitutes a vital cellular setting and a real-life model for simulating oxidative damages and assessing the protective role of natural extracts/compounds	- Expensive - Results should be interpreted with caution as the biological effect is evaluated against a specific type of cells (the interaction of the tested substance with other cell types are not taken into account) - Need for specialized equipment	(Koudan et al., 2022)
Pesticidal bioassays				
Feeding bioassay = poisoned food assay	Compound is incorporated into food (mixing in an artificial diet or producing a genetically modified plant) or spread/sprayed over food. Different parameters can be followed after exposure depending on the pest – e.g., survival, weight gain, size gain, offspring count, food consumption or a specific trait	- Simple - Easy interpretation - Qualitative or quantitative – depending on the set up	- Live animals (e.g., arthropods, gastropods) are used so a rearing facility is required - Dependent on test insect availability – laboratory cultures or seasonal collection - Time-consuming - Development of artificial diet or GM food can be challenging	(Burgess et al., 2020; Phan et al., 2020; Portilla, 2020; Razinger et al., 2014; Sanané et al., 2021; Šmid et al., 2015)
Volatile organic compounds (VOCs) Anti-insect activity test	The bioactivity of metabolites can be based on different mechanisms, two of which that are most often studied are to repel or to kill the insect.	- Simple - Easy interpretation - Qualitative or quantitative – depending on the set up	- Live animals (e.g., arthropods, gastropods) are used so a rearing facility is required - Dependent on test insect availability – laboratory cultures or seasonal collection - Time-consuming - Need for specialized equipment	(Daisy et al., 2002; Sternberg et al., 2014)
Other				

Bioassay designation	Principle and general characteristics	Advantages	Limitations	References
Enzymatic activity or inhibition of enzymatic activity	To determine enzymatic activity, the sample is incubated with the substrate in an appropriate buffer and at an appropriate temperature, and the reaction is followed by measuring absorbance or fluorescence change (depending on the substrate used). For inhibition of enzymatic activity, the sample is added to an enzyme in a suitable buffer, and after pre-incubation period of 10 to 60 min the substrate is added and the reaction is followed with a spectrophotometer or fluorimeter kinetically or at a selected endpoint (incubation time).	- For some enzymes SOPs (Standard Operating Procedures) available - Simple - Versatile - Quantitative or qualitative - Mechanism of action can be determined - Commercial kits available for selected enzymes - High-throughput	- High cost and limited lifetime of enzymes used - Can be time-consuming - Optimization of conditions (buffer, pH, temperature, cofactors, incubation time) needed for each enzyme - Prone to false positive and false negative results - Enzyme inhibitors in the extracts may affect activity - Specific for each enzyme-substrate pair	(Brooks et al., 2012; Mohan et al., 2018; Pohanka, 2019; Sabotič et al., 2009; Sepčić et al., 2019)
In-gel detection of enzymatic activity	Sample is resolved in polycrylamide gel under nondenaturing conditions and gel is then incubated in a series of solutions until colored or fluorescent bands appear.	- Additional info on size of enzyme - Can be simple one-step but also multiple step staining - Qualitative, can be semiquantitative	- Not all enzymes withstand the conditions of in-gel separation - Optimization of each enzymatic reaction required with many variables - Can take variable time for signal development (e.g. from minutes to days)	(Covian et al., 2012; Rivoal et al., 2002; Sabotič et al., 2007; Sepčić et al., 2019; Sims, 1965; Žun et al., 2017)

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