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Review

Evaluation of Biological Activity of Natural Compounds: Current Trends and Methods

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Abstract: Natural compounds have diverse structures and are present in different forms of life. Metabolites such as tannins, anthocyanins, and alkaloids, among others, serve as a defense mechanism in live organisms and are undoubtedly compounds of interest for the food, cosmetic and pharmaceutical industries. Plants, bacteria, and insects represent a source of biomolecules with diverse activities, poorly studied in many cases. To use these molecules for different applications, it is essential to know their structure, concentrations, and biological activity potential. *In vitro* techniques that evaluate the biological activity of the molecules of interest have been developed since the 1950s. Currently, different methodologies have emerged to overcome some of the limitations of these traditional techniques, mainly the reduction of time and costs. However, emerging technologies continue to appear due to the urgent need to expand the analysis capacity of a growing number of reported biomolecules and the lack of therapeutic options to treat various diseases. This review presents an updated summary of the conventional and current methods to evaluate natural compounds' biological activity, including a diagram that summarizes the minimum techniques essential for correctly assessing molecules with biological potential.

Keywords: Natural product; bioactive compounds; antimicrobial; antioxidant

1. Introduction

According to the WHO, 80% of the world population uses medicinal plant-based medicine to alleviate or cure diseases [1]. Currently, new molecules from natural resources with potential bioactivity are reported every day; however, only a few of these molecules are evaluated for their suitability for use as drugs [2]. Identifying bioactive compounds (hits or leads) is the initial step for drug discovery. For this, it is necessary to select suitable bioassays to evaluate both the activity against the disease and its potency. For this purpose, target-based screening is mainly used to identify compounds that modulate the activity of a target that is involved in some human disease. This screening involves different *in vitro* biological assays designed to measure primary activities, selectivity, cellular toxicity, and physiologically relevant activity. The initial phases of a target-based screening cascade typically employ a range of *in vitro* assays, especially high-throughput screening (HTS); however, the study is more expensive and time-consuming [3]. In the first instance, the selection of the assays can be made considering that structurally similar compounds do have similar biological activity [4]; however, this cannot always be carried out, especially when working with natural extracts. Therefore, it is essential to have several assays at hand to discard or confirm activities. Within this context, the current review presents the most common *in vitro* assays currently used that will allow the identification of bioac-

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tive compounds and the preliminary identification of the target. Finally, the authors provide a recommended workflow to begin screening these biological activities, guiding the reader through the initial steps of discovering potential drug candidates.

2. Cytotoxicity activity in cultured mammalian cells

In the early stages of drug development, extensive toxicity screening is essential [5–7]. Animal studies involve high costs and are often restricted by differential responses due to physiological differences between species and limitations in test feasibility. Alternatively, *in vitro* cytotoxicity assays are advantageous in preclinical studies based on eligibility, cost-effectiveness, and reproducibility. Natural compounds have become particularly relevant in identifying safer and more effective treatments [8].

To evaluate cytotoxicity in mammalian cells, it is critical to select the proper cell line for each particular experiment considering: relevant species, specific organs, and chosen route of administration. Multiple cell lines are available for *in vitro* testing, including immortalized cell lines, primary cultures, and stem cells. Each cell line has its requirements that need to be defined before the experiment to ensure a proper understanding of the potential mechanisms of toxicity [9–11].

A wide range of *in vitro* assays are currently available for cytotoxicity testing [12,13]. While direct cytotoxicity assays focus on detecting loss of membrane integrity associated with cell death, cell viability assays are developed to measure the activity related to cellular maintenance and survival. Additionally, other assays allow for the direct and indirect quantification of changes in the population at specific phases of the cell cycle and provide information on the mechanism of cell death [14,15]. Different approaches are employed in parallel to understand better the cytotoxicity mechanisms, such as the TUNEL assay and the comet assay for the analysis of DNA fragmentation [16]. The determination of the activation of apoptosis-related caspases [17,18] or the detection of the relative level of telomerase activity (TRAP, telomerase repeat amplification protocol) [19,20]. Determining cytotoxicity against tumor cells, detecting cell the rate and regulation of cell migration, and analyzing anchorage-independent proliferation, chemotaxis, and invasion are essential factors usually evaluated through the colony-forming assays in soft agar scratch assay using dual-chamber systems [21–23].

In short, each assay has its limitations. Therefore, before selecting an appropriate assay, it should also consider cost, reliability, timing, user-friendliness, and equipment requirements [24]. Table 1 presents the advantages and limitations of these assays.

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Table 1. Most common methods for the determination of cytotoxicity in cultured mammalian cells.

H: hemocytometer, M: microscopy, PR: plate reader, FC: flow cytometry, LI: light imager, T: thermocycler, GE: gel electrophoresis, PI: Propidium iodide, 7-AAD: 7-aminoactinomycin D, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT:

Method	Principle	Assay Detection	Advantages	Disadvantages	Ref.
Cell viability and Proliferation					
Dye exclusion	Detection of plasma membrane integrity	Trypan blue colorimetric / H + M	– Simple – Low-cost – Immediate readout – Widely-available – Not affected by enzyme changes/activity	– Not suited for large numbers of samples – Limited sensitivity: dead cells vs. live damaged cells – It may not detect cell injury – Dye uptake estimate can be subjective – Toxic to mammalian cells – Possible counting errors (~10%): poor dispersion/dilution of cells, cell loss during cell dispersion, air bubbles, etc.	[25–28]
		Crystal violet colorimetric / M, PR	– Simple – Rapid – Reliable – Sensitive – Economical – Can be used under different conditions – "Additive or synergistic" interactions (Metabolism-independent)	– Does not detect changes in cell metabolic activity – Not suitable for analyses featuring affected cell metabolism compounds. – Not suited to determine cell growth rate	[12,29]
Metabolic activity	Detection of mitochondrial dehydrogenase and	Tetrazolium salts (MTT, XTT, MTS, WST)	– Easy to use – Sensitive – Safe	– Hours to readout	[25,29–32]

	oxidoreductase activity	colorimetric / PR	– High reproducibility – Economical – Used for large samples	– High background (interference with reagent/media) – Additional control experiments needed to reduce false-positives/-negatives – Reduction is affected by metabolic and other factors – Incubation time, concentration, metabolic activity, can affect the final reading	
		Resazurin fluorescent / PR	– Inexpensive – More sensitive than tetrazolium assays – Can be multiplexed with other techniques	– Possible high background (interference with reagent/media). – Hours to readout – Fluorescent interference – Close cell-cell interactions affect uptake – Direct toxic effects on the cells	[33–35]
Energy metabolism	Correlation between a bioluminescent reaction and the ability to synthesize ATP	Luciferase and luciferin luminescent / PR	– Immediate readout – Sensitive – Deficient background – Stable luminescent signal – Useful to detect cellular death in a mixed cell culture models – Does not need an incubation step	– Limited repeatability – Endpoint – Needs cell engineering – Decrease in luminescent signal with increased cell death – Difficult to distinguish small changes in the number of dead cells	[13,25]

(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide-2*H*-tetrazolium), MTS: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2*H*-tetrazolium, WST: Water-soluble tetrazolium salts, EC: electric conductivity.

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Enzyme release-based	Determination of plasma membrane integrity (LDH release)	Lactate + tetrazolium salts/fluorescent probe colorimetric, fluorescent / PR	– Reliable – Quick – Simple – Non-destructive measurement – The culture medium can be used for analysis	– High background – Limited to serum-free or low-serum conditions – Low EC – Difficult to detect low cytotoxic effects – High variability	[25,30,36]
Colony formation	Determination of clonogenic growth	Low-adherence plates / M	– Low interference – Colonies can be counted without being stained	– Time-consuming and labor-intensive – High intra-individual variability – Limited to adherent cells – Restricted by low cell density conditions and growth factors – Stress may affect cellular repair – Colonies may be lost during washing and staining – Overestimate cell damage/death – Overly sensitive threshold – Low sensitivity range	[37–40]
Cell cycle and apoptosis					
Cell cycle arrest	Distribution of cell population in each cell cycle phase	PI, 7-AAD fluorescent / FC	– Simple to use – Single-cell quantification of stained DNA – Bright fluorescent signal	– Endpoint (fixed-permeabilized cells) – Does not detect floating cells	[41,42]
Apoptosis/ necrosis	Detection of membrane integrity	PI/7-AAD, annexin V fluorescent / FC	– Simple protocols – Short incubation time – Economical – Stable	– Difficult to differentiate between living and dead fixed cells – Cells are continuously dying in the sample	[43–45]

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		– Confirmation by other methods needed to avoid false-negative bias

Although two-dimensional mammalian monocultures stemming from specific cell types are widely used based on their reproducible and rapid growth, high productivity levels, ease of data interpretation, and value, the artificial nature of the culture environment presents limitations in the process of drug safety and efficacy evaluation [46,47]. In this context, co-culture systems, human organoids, and other sophisticated three-dimensional culture models collect more physiologically relevant data and represent methods that could better connect traditional cell culture and *in vivo* models [48–50] (Figure 1).

From the array of cell cultures available for *in vitro* testing that offers diverse degrees of intricacy and similarity to the *in vivo* setting, organotypic cultures are tissue slices that maintain cell interactions and extracellular matrix composition of the original tissue and tissue function [51–54]. Yet, this system lacks intercommunication with the circulatory and immune systems and is inadequate for medium to high throughput analysis [10]. Three-dimensional spheroids and organoids self-organize into organ-specific structures that accurately replicate paracrine and direct intercellular interactions [55–58]. While spheroids are usually made from cell lines and offer lower complexity, organoids are derived from stem cells of different origins and resemble the original tissue in structure, histologically and genetically [59,60]. Particularly relevant are tumor organoids since these systems provide suitable platforms to recapitulate the complex tumor microenvironment and heterogeneity, allowing the study of chemical and metabolic gradients and mechanisms of resistance [61,62].

Advanced culture systems are increasing interest, particularly microfluidic devices [63]. Compared to static conditions, microfluidic systems can reproduce specific flow, temperature, pressure, and chemical gradients *in vivo* systems [64–66]. Thus, it reconstructs the continuous renewal of nutrients and gasses and removes toxic wastes, migration, and microcirculation. This system also supports longer culture times and drug treatments that are more pharmacologically significant [67].

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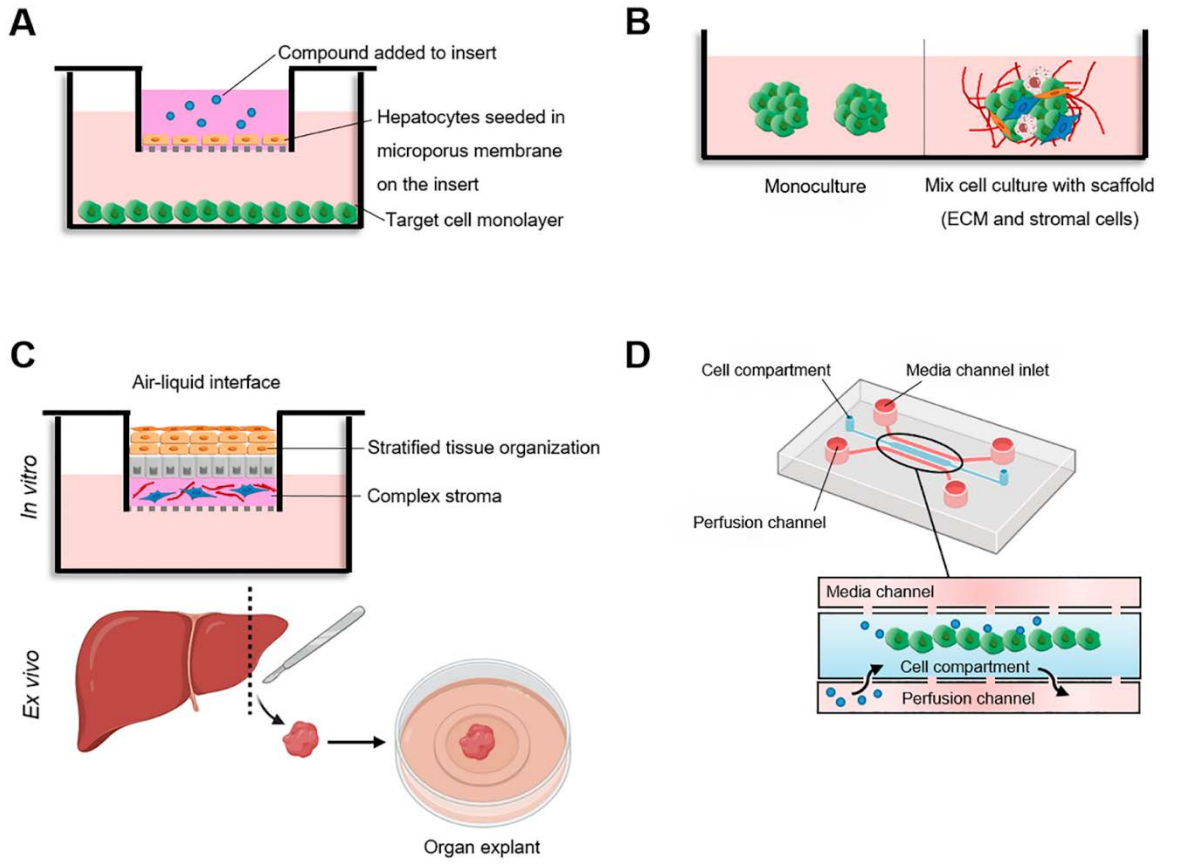

Figure 1. High-tech *in vitro* models to assess cytotoxicity in cultured mammalian cells. A) Dual chamber, taste compound, and metabolites diffuse through the microporous barrier toward target cells; B) Three-dimensional cellular models based on multicellular spheroids/organoids consisting of target cells or the co-cultivation of several types of cells on extracellular matrix; C) Organotypic cultures; cells, organ slices or whole organs are cultured on a tissue culture insert that is either submerged in medium or maintained at an air-liquid interface to ensure sufficient oxygen supply; D) Microfluidic system based on a mixture of cells and matrix collected in the central channel and medium flowing from the lateral channels that keeps particles in homogenous suspension.

3. Antihyperglycemic activity

Diabetes is a global health disease affecting 422 million people worldwide. [68] This disease is characterized by elevated blood glucose levels, which, if untreated, leads to severe multi-organ failure and 1.5 million deaths each year [68]. Antihyperglycemic agents are a heterogeneous group of molecules obtained by chemical synthesis or isolation from natural sources that lower glucose concentration in the blood or prevent its increase [69].

The methods to evaluate the *in vitro* antidiabetic properties of natural compounds are summarized in Table 2 and can be classified into two major groups: i) assays based on the inhibition of isolated enzymes involved in the regulation of blood glucose levels and ii) assays to measure major cellular processes that directly alter glucose levels, mainly glucose uptake and insulin secretion. [70]. The first group includes enzymes that catalyze the breakdown of poly- and oligosaccharides such as α-amylase and α-glucosidase, respectively (Table 2). The inhibition of the mentioned enzymes and others with a similar role

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in carbohydrate digestion is considered antidiabetic. The reduction in glucose concentration available to absorb in the intestine prevents a further increase in blood glucose [71]. The assays of inhibition of α-amylase and α-glucosidase are reactions of commercially available enzymes in the optimal conditions (buffer, pH, cofactors) and substrates allowing the detection of the reaction product(s).

The most common substrate used to measure α-amylase is starch. The method is based on the reaction of starch with dinitrosalicylic acid (DNS), which reacts with reducing sugars producing a compound measured spectrophotometrically at 540 nm. Most α-glucosidase assays rely on substrates that can be detected spectrophotometrically after hydrolysis, such as p-nitrophenyl-α-D-glucopyranoside (pNPG), which can be measured at 400 nm or by fluorescent product. Dipeptidyl peptidase IV (DPP4) and tyrosine phosphatase 1B (TP1B) are involved in the indirect regulation of glucose levels by modulating insulin secretion (DPP4) [72] and signaling (TP1B) [73,74], respectively (Table 2). DPP4 is a serine exopeptidase that cleaves different peptides, including GLP-1, a major regulator of insulin secretion in response to glucose [75]. Thus, inhibition of DPP-4 *in vivo* increases the availability of GLP-1 and secretion of insulin, reducing blood glucose [76]. TP1B negatively regulates insulin and leptin signaling by dephosphorylating the insulin receptor (IR) and its downstream signaling components [77,78]. Inhibition of TP1B releases insulin signaling from TP1B-mediated dephosphorylation and allows insulin downstream signaling [74,77].

The second group includes assays designed to measure the potential inhibitory effects of different molecules on relevant cellular processes controlling blood glucose levels, such as glucose uptake and insulin secretion (Table 2). The glucose uptake assay is based on the internalization of a labeled glucose analog that cannot be fully utilized because of its modification, and it accumulates inside the cells, facilitating its detection. Output generated by the accumulation of labeled analogs is proportional to the glucose uptake and can be detected and quantified using standard equipment such as fluorescence/bioluminescence readers [79–81] or FACS [82]. These assays can be performed in mammalian cell lines, given the importance of measuring physiologically relevant effects. Still, some studies report using yeast cells as an alternative to mammalian cell lines [83,84]. For example, a recent study described a label-free method to measure glucose uptake in yeast cells using pHluorin, a genetically encoded pH-sensitive green fluorescent protein [84]. In general, insulin secretion assays are performed using ß-cells isolated from pancreatic or islet cell cultures. Cells are stimulated by glucose and incubated with the compound /plant extract to measure the insulin secretion modulation effect [85]. After being released from cells, insulin can be measured by radioimmunoassay [86,87] or ELISA. Recently a luminescent alternative to detect insulin has been described [88,89].

Table 2. Antihyperglycemic activity

Method name	Type of assay	Description	Detection (output)	Advantages	Disadvantages	Ref.

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α-amylase inhibition	Isolated enzymes	Measurement of the ability of novel molecules to inhibit the activity of α-amylase.	Colorimetric and fluorometric assays.	– Rapid – Simple – Cheap – Easy to escalate/automate	– Specialized lab equipment. – Required the isolated enzymes	[90]
α-glucosidase inhibition	Isolated enzymes	Measurement of the ability of novel molecules to inhibit the activity of α-glucosidase	Colorimetric, fluorometric and bioluminescent assays.	– Rapid – Simple – Easy to escalate/automate	– Specialized lab equipment. required – Required the isolated enzymes	[90,91]
Dipeptidyl peptidase IV inhibition	Isolated enzymes	Measurement of the ability of molecules to inhibit the activity of dipeptidyl peptidase IV	Colorimetric, fluorometric, immunoassay	– Rapid – Simple – Easy to escalate/automate	– Specialized lab equipment required – Required the isolated enzymes	[90] [91]
Tyrosine phosphatase 1B inhibition	Isolated enzymes	Measurement of the ability of molecules to inhibit the activity of tyrosine phosphatase 1B.	Colorimetric, fluorometric	– Rapid – Simple – Easy to escalate/automate	– Specialized lab equipment required – Required the isolated enzymes	[77,92]

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Glucose uptake	Cell-based	Measurement of the ability of molecules to modify glucose uptake into cells	Colorimetric, fluorometric, bioluminescent assays	– Physiologically meaningfull – Various cell models – Easy to escalate/automate	– Specialized lab equipment required – Highly trained personnel required	[80,93]
Insulin secretion	Cell-based	Measurement of the ability of molecules to modulate insulin secretion.	Bioluminiscent assay, immuno/radioimmunoassay	– Physiologically meaningfull – Easy to escalate/automate	– Highly trained personnel required – Specialized lab equipment required	[88,89]

Despite continuous improvements in measuring glucose and glucose-associated processes, a relevant challenge is fully understanding the physiological and pathological role of glucose blood levels and their impact on health and disease conditions. *In vitro* methods play a critical role in discovering natural compounds with antidiabetic activity. Methods to simplify the detection and increase the throughput of measurements can be the next step for bioprospecting novel active principles from natural sources

4. Anti-inflammatory activity

Inflammation is a protective response of a given organism's immune system against harmful external agents, such as pathogens, toxins, or irritants [94]. This mechanism aids the recovery from infections, disease, and tissue damage, therefore favoring the healing process [95]. Inflammatory responses include activation of macrophages by pro-inflammatory mediators such as lipopolysaccharide (LPS), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and the nuclear factor kappa B (NF-κB) which triggers the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) production primarily [96–98]. All of these pro-inflammatory mediators are studied to identify the anti-inflammatory properties of natural molecules *in vitro*.

In terms of anti-inflammatory treatments, nonsteroidal drugs have shown to block the production of arachidonic acid and, therefore, reduce prostaglandin levels, which contributes to less pain and inflammation [99]. However, their use is associated with multiple side effects, including stomach ulcers, indigestion, headaches, allergic reactions, and increased cardiovascular conditions [99]. Fortunately, the incredible variety of phytoconstituents present in plants includes flavonoids, alkaloids, saponins, coumarins, anthraquinones, saccharides, glucosinolates, tannins, phenolic acids, and nitrile glycosides, etc. are

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an excellent source for drug development due to their vast biological activities [100]. Specifically, flavonoids [101] [102] and anthocyanins [103] have shown anti-inflammatory properties. Similarly, secondary metabolites, such as atranorin from lichens inhibit the inflammatory process [104]. These few examples highlight the incredible potential of natural extracts for developing anti-inflammatory pharmaceuticals.

A simple way of measuring inflammation is through the overproduction of NO, which is associated with tissue toxicity, several inflammatory conditions, and carcinomas [57]. An easy, cheap, and rapid quantification of nitric oxide levels directly from a given compound or from a cell culture subjected to an inflammatory stimulus is possible through the Griess assay (Figure 2) [96,105–107]. Thus, a test compound with low NO levels suggests an anti-inflammatory potential. However, this method has drawbacks, including its variable sensitivity, low detection levels, and interference of some compounds with the Griess Reaction assay [108,109]. Alternatives to the Griess assay for NO detection are covered by Bryan and Grisham, 2007 [110].

Other approaches, such as enzyme-linked immunosorbent assay (ELISA) and qRT-PCR, have been extensively employed to determine the decrease of pro-inflammatory enzyme levels and their change in expression, respectively (Figure 2) [95,106,111]. The advantages of ELISA are its simplicity, high specificity, and sensitivity. However, on the other hand, it is a time-consuming process and involves high costs associated with antibodies [112]. In the case of the qRT-PCR, advantages include high sensitivity and relatively high throughput. In contrast, disadvantages are associated with increased complexity, variable reproducibility, and the high cost of the equipment and reagents [113]. Also, qRT-PCR measures the mRNA levels but does not provide information about the overall enzyme levels. Alternative methods to measure inflammation include nuclear factor kappa B (NF-κB) luciferase assay [106], immunofluorescence staining of NF-κB p65, and inducible nitric oxide synthase (iNOS) [97], ferrous oxidation−xylenol orange (FOX) assay for determination of lipid hydroperoxides [114]. However, they are less common during the early stages of anti-inflammatory activity identification.

It is essential to identify the best method for the investigation based on its advantages and limitations. Currently, researchers employ a combination of methods (e.g., NO inhibition assay and pro-inflammatory enzyme quantification) to obtain more information about the potential anti-inflammatory properties of different compounds. Figure 2 details the most common strategies used to establish the anti-inflammatory potential of natural and synthetic molecules *in vitro*

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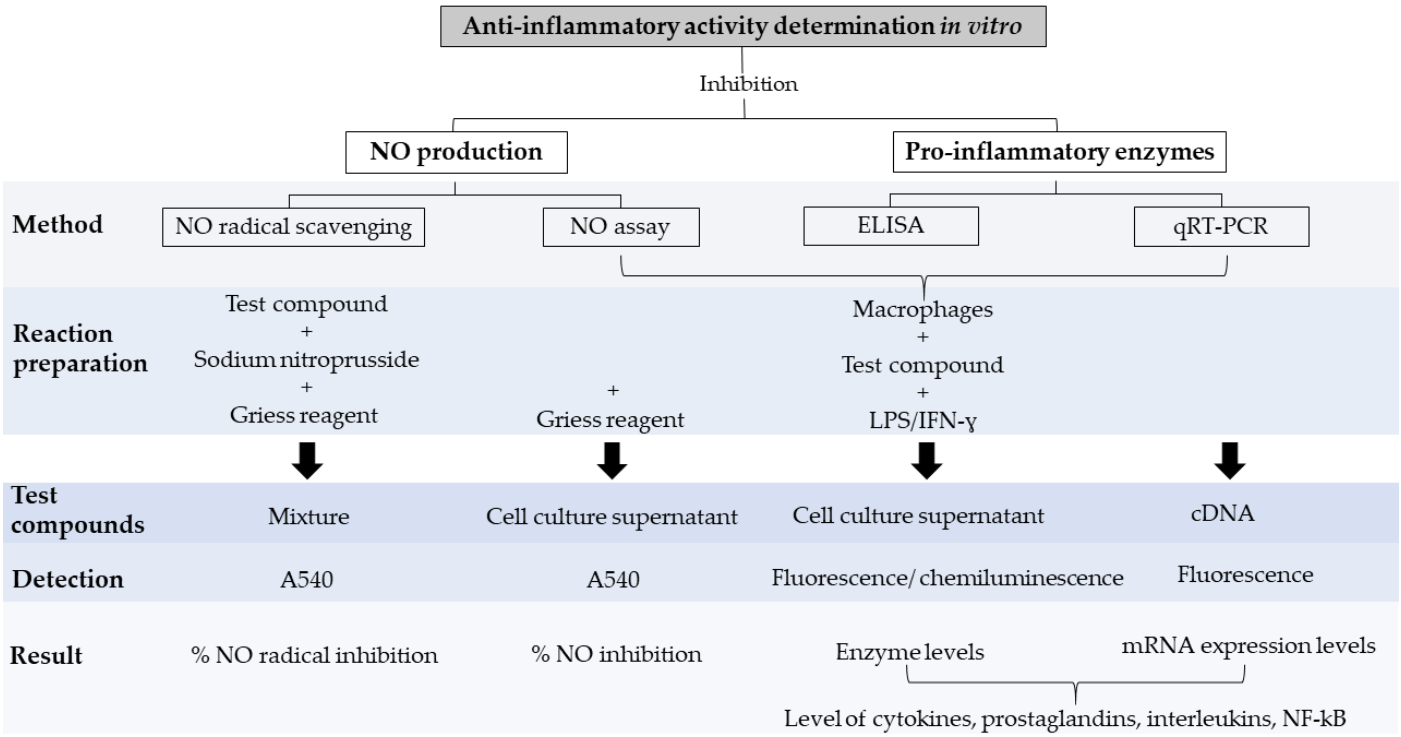

Figure 2. Strategies employed to assess anti-inflammatory activity *in vitro*. Nitric oxide (NO), lipopolysaccharide (LPS), interferon-gamma (IFN-Ɣ), nuclear factor kappa B (NF-κB).

5. Analgesic activity

Pain is a symptom of many diseases that require analgesic treatment [115]. An analgesic is a drug used to achieve pain relief without loss of consciousness; analgesics can act on the central or peripheral nervous system [115]. There are two groups of drugs to reduce pain; these are opiate analgesics which are highly effective because they reduce but do not block the perception of the central nervous system and even induce sleep transforming pain into a non-bothersome sensation [116]. There are several types of opioid receptors: μ receptors, when stimulated, the typical effects of narcotic analgesics are produced; κ receptors are responsible for spinal analgesia. When the drug acts on these receptors, it does not have addiction power but causes unpleasant hallucinations; δ receptors are responsible for cardiovascular manifestations [116,117].

Radioligand binding assays are used to assess the affinity and selectivity of the new molecules for specific receptors to evaluate the analgesic activity of new compounds. There are three experimental types of radioligand binding assays: saturation, competitive, and kinetic. The most widely used is the competitive assay, which studies equilibrium binding at a fixed radioligand concentration and different concentrations of an unlabeled competitor [118]. In Table 3, the *in vitro* methods for analgesic activity are described [119].

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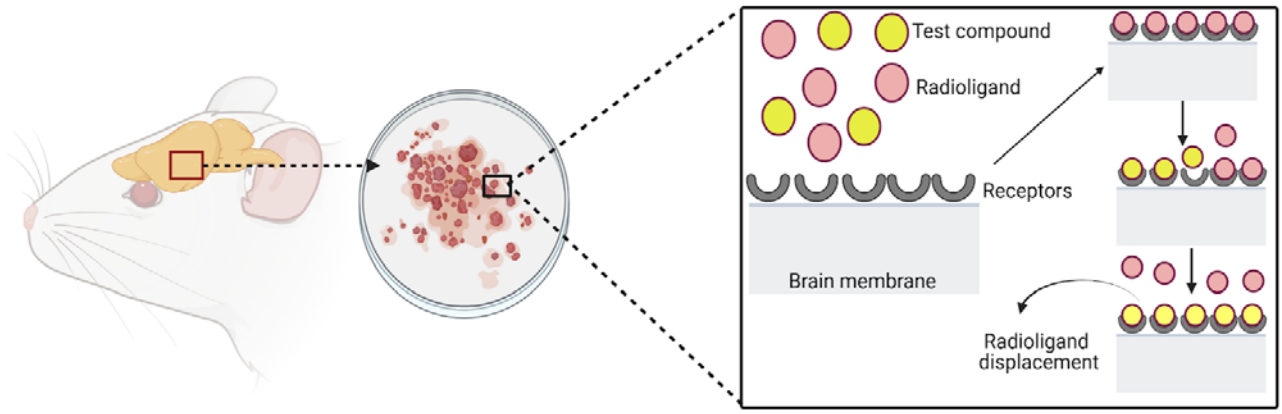

Figure 3. Sites in the rat brain tissue, both opiate antagonists and agonists compete for the same receptors, opiate potencies, and antagonists in displacing 3H-naloxone binding parallel their pharmacological potencies [120].

There is a good correlation between *in vivo* pharmacological potency tests of opiate agonists and antagonists to evaluate the ability to displace radiolabeled compounds such as Naloxone, Bremazocine, Dihydromorphine, substances that bind to receptors of opiates and nociceptin [98]. The correlation mentioned above can be exploited to evaluate new compounds. As shown in Figure 3, these experiments are performed on animal brain membranes, which have a high density of the receptors of interest; in the assay, different concentrations of the test compound are evaluated against a fixed concentration of specific radioligands [99]. These assays help identify compounds that recognize the same binding site as a known radiolabeled ligand. The results can be quantified and expressed as a percentage displacement of the radium compound and IC50 [100].

Table 3. Methods for determination of the analgesic activity of molecules based on the displacement of radioligands.

Method	Assay type	Description	Advantages	Disadvantages	Ref
Displacement of radioligands at receptors	^{3}H-N binding assay	Radiolabeled ligand: ^{3}H-N; Type of receptor: opiate; Tissue culture: brain rat	– Sensitive method – Good robustness,	– Other multiple opioid receptors are not considered.	[116,118,121]

^{3}H-DHM binding assay	Radiolabeled ligand: ^{3}H-DHM Type of receptor: μ-opiate; Tissue culture: brain rat	–	Precise determination of ligand binding sites and affinity.	– High-cost – Hazards of handling high levels of radioactivity – Requires a certain level of expertise	[117] [116]
^{3}H-B binding assay	Radiolabeled ligand: ^{3}H-B Type of receptor: κ opiate; Tissue culture: guinea pig cerebellum				[121]
^{3}H-NCNH$_2$ binding assay	Radiolabeled ligand: ^{3}H-NCNH$_2$ assay Type of receptor: nociceptin; Tissue culture: brain rat				[122] [121]
^{35}S-GT binding assay	Radiolabeled ligand: ^{35}S-GT Type of receptor: cannabinoid Tissue culture: human brain				[123] [124]
^{3}H-R binding assay	Radiolabeled ligand: ^{3}H-R; Type of receptor: vanilloid; Tissue culture: brain rat				[122] [125]

^{3}H-N: ^{3}H-naloxone; ^{3}H-DHM: ^{3}H-dihydromorphine; ^{3}H-B: ^{3}H-bremazocine; ^{3}H-NCNH$_2$: ^{3}H-nociceptin amide; ^{3}H-R: ^{3}H-resiniferatoxin; ^{35}S-GT: ^{35}S-guanosine-5′-*O*-(3-thio) triphosphate.

Enzyme assays are also used to determine anticoagulant activity (Figure 4). Inhibition of enkephalinases has been investigated to have antinociceptive properties [126]. The determination of the inhibition is carried out by fluorescence, for which the DAGNPG

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molecule is used as a selective enzymatic substrate, and the enkephalinase breaks the Gly-Phe(pNO2) DAGNPG peptide bond, which causes an increase in fluorescence [121].

Table 4. Enzyme inhibition method for determination of the analgesic activity of molecules.

Method type	Detection mechanism	Description	Advantages	Disadvantages	Ref
Enzyme inhibition	Fluorometric detection using fluorogenic peptide DANGPG	– Detect the inhibition of the degradation of enkephalinase. This enzyme uses DANGPG as substrate and cleaves peptide bond of DANGPG leading to a fluorescence increase. – Enkephalinase induces inactivation of ANF. The protection of endogenous ANF against inactivation may result in analgesic applications.	– light-sensitive, quantitative data – Rapid	– High-cost – DANGPG is light-sensitive – Susceptible to different fluorescence interferences	[126] [119] [127]

DANGPG: dansyl-*D*-Ala-Gly-Phe(pNO_2)-Gly; ANF: atrial natriuretic factor

These assays allow screening of the analgesic activity in new drugs and encourage the isolation of the main compounds present in nature and relate them to the biological responses. Furthermore, knowing the chemical structure of the isolated compounds would allow an understanding of the possible biomolecular targets and their mechanism of action to mitigate pain. Future research is focused on developing enzymatic inhibition tests to monitor protein kinetics in real-time, which could be employed for rapid and specific quantification shortening the evaluation time [118]. In addition, multiple fluorescent ligands are being developed for studies with other types of specific binding to σ and ε, bradykinin B1 receptors, as well as develop novel fluorogenic peptide, dansyl-Gly-(p-NO2) Phe-beta Ala (DGNPA), which improves the affinity selectivity, and sensitivity of enzymatic inhibition test.

6. Anticoagulant activity

Anticoagulants make it difficult for the blood to coagulate, thus preventing the formation of clots or their growth and favoring their disappearance [128]. When an injury occurs to a blood vessel or tissue within the body and bleeding occurs, the body initiates a process of clot formation at the site of the damage to help stop the bleeding; this mech-

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anism is known as hemostasis [128]. During this process, platelets stick together and become activated at the injury site. In parallel, the coagulation cascade is initiated, and coagulation factors, including fibrinogen, are also activated. Fibrinogen is converted by thrombin into insoluble fibrin strands that cross each other, forming a fibrin network that adheres to the main focus of the lesion. Thus, a stable clot prevents further blood loss with platelets and remains in place until the wound heals [129].

The coagulation cascade is a series of enzymatic reactions, each of these participating compounds in the coagulation cascade is called "Factor," commonly designated by a Roman numeral chosen according to the order in which they were discovered and with an "a" lowercase to indicate the active form [129]. In these reactions, a zymogen, that is, an inactive enzyme precursor, and its glycoprotein cofactor are activated to become active components that then catalyze the subsequent reaction in the cascade; an active enzyme cleaves a portion of the next inactive protein in the cascade, activating it; ending in the formation of cross-linked fibrin, as shown in Figure 4 [128].

When new compounds with anticoagulant potential are evaluated, the ability to prevent the action of coagulation factors is determined, thus blocking the cascade almost from its inception [130]. Each component must function correctly and sufficiently in the homeostatic process to ensure clot formation occurs. If one or more coagulation factors are deficient, or if one or more of them are not working correctly, the clot may not form, and bleeding may not be controlled [128].

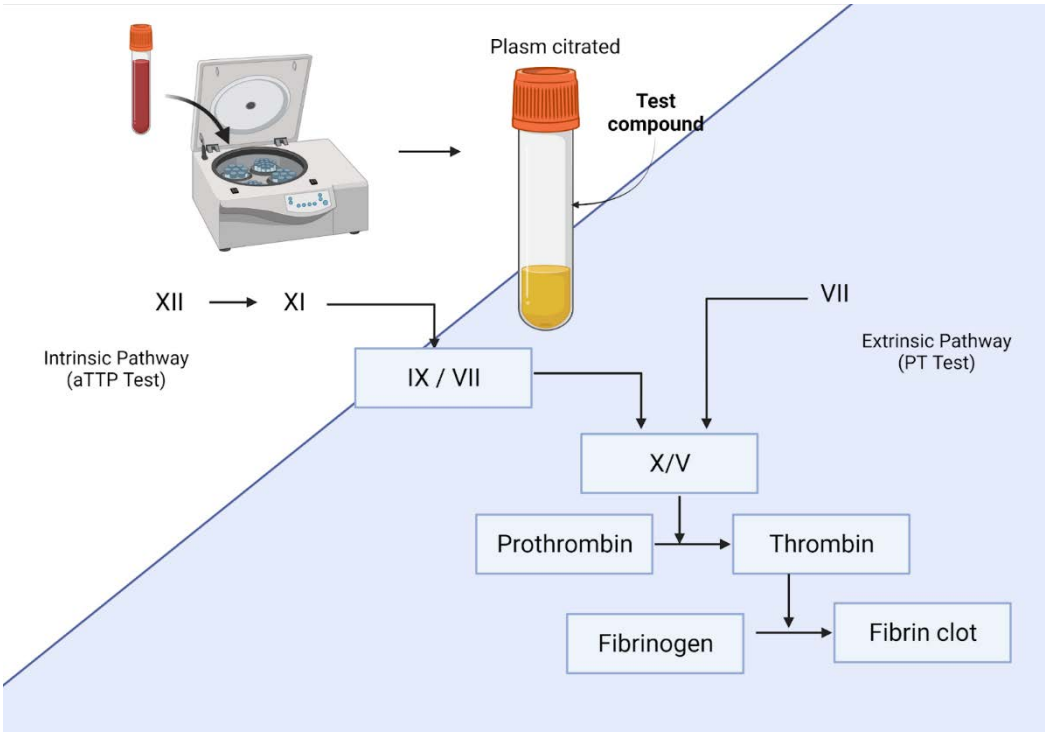

Figure 4. Activation of coagulation factors *in vitro* for clot formation by adding the test compound as a possible therapeutic agent.

Coagulation tests such as prothrombin time (PT) and activated partial thromboplastin (aPTT) are used *in vitro* to assess the influence of compounds on blood plasma coagulation [130]. PT, aPTT, and thrombin time (TT) tests can inhibit blood coagulation through the intrinsic, extrinsic, and common pathways of the thrombin cascade and allow assessment of coagulation, respectively [131]. The intrinsic and extrinsic pathways are two separate pathways that lead to the formation of a blood clot. The main difference between the

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intrinsic and extrinsic pathways in blood coagulation is that the former pathway is activated by trauma within the vascular system. In contrast, the second pathway is activated by external trauma [128] [129].

As shown in Figure 5, these three tests are mainly used for discovering drugs with anticoagulant properties. First, the test compound is incubated with human platelet-rich plasma using a specific reagent for each type of test. Then fibrin formation is evaluated through clots, and the test result is determined through an analyzer and expressed in coagulation time (seconds) [130]. To perform anticoagulant activity tests, platelet-rich plasma (PRP) must be obtained from healthy people because contamination by anticoagulant agents and liver diseases can result in false-positive tests with the compounds of interest. For anticoagulant evaluation, it is recommended to use in parallel an intrinsic and extrinsic pathway test (aPTT, PT) to determine the anticoagulant properties of the compound of interest [130]. The available tests to evaluate the anticoagulant capacity of new molecules offer a prospect of rapid and low-cost identification.

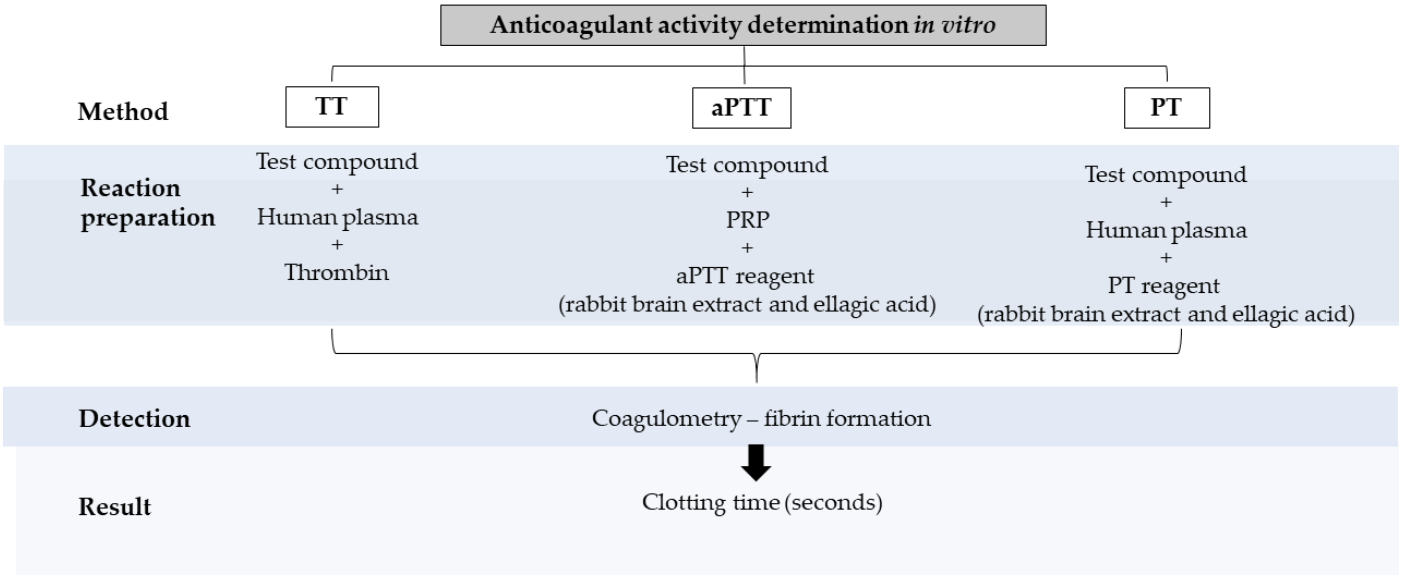

Figure 5. Strategies employed to assess analgesic activity *in vitro. TT: thrombin time; aPTT: activated partial thromboplastin; PT: prothrombin time; PRP: platelet-rich plasma.*

7. Antihypertensive activity

Hypertension or high blood pressure is a complex, multifactorial disease and contributes to morbidity and mortality in industrialized countries [132]. Although numerous preventive and therapeutic pharmacological interventions exist, as of 2021, approximately 700 million people worldwide still suffer from poorly controlled hypertension [133]. Therefore, there is growing interest in finding novel natural or synthetic molecules helpful in preventing and treating hypertension.

Angiotensin-converting enzyme (ACE) is one of the primary regulators of blood pressure. It acts by two central mechanisms: i) converting the decapeptide angiotensin I (angI) into the potent vasoconstrictor (hypertensive) octapeptide angiotensin II (angII) and ii)

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catalyzing the degradation of the antihypertensive peptide bradykinin [134]. Angiotensin II increases blood pressure by stimulating a GPCR-activated pathway [135], resulting in the release of Ca2+, activation of protein kinase C (PKC), and subsequent vasoconstriction by inactivation of the myosin-light-chain kinase (MLCK) [132] (Figure 6). Hypertension or high blood pressure is a complex, multifactorial disease and contributes to morbidity and mortality in industrialized countries [132]. Although numerous preventive and therapeutic pharmacological interventions exist, as of 2021, approximately 700 million people worldwide still suffer from poorly controlled hypertension [133]. Therefore, there is growing interest in finding novel natural or synthetic molecules helpful in preventing and treating hypertension.

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ACE is a dicarboxypeptidyl peptidase, which cleaves off the two terminal amino acids of angI, to form angII. Unlike other peptidases, ACE is not specific to a single substrate. Instead, it cleaves several natural peptides such as bradykinin, substance P, and tetrapeptide N-acetyl-Ser-Asp-Lys-Pro (Ac-SDKP) [136] several synthetic substrates. Therefore, the ability of molecules to inhibit the production of angII by ACE is an effective strategy for measuring the antihypertensive activity of isolated plant extracts *in vitro* (Figure 6).

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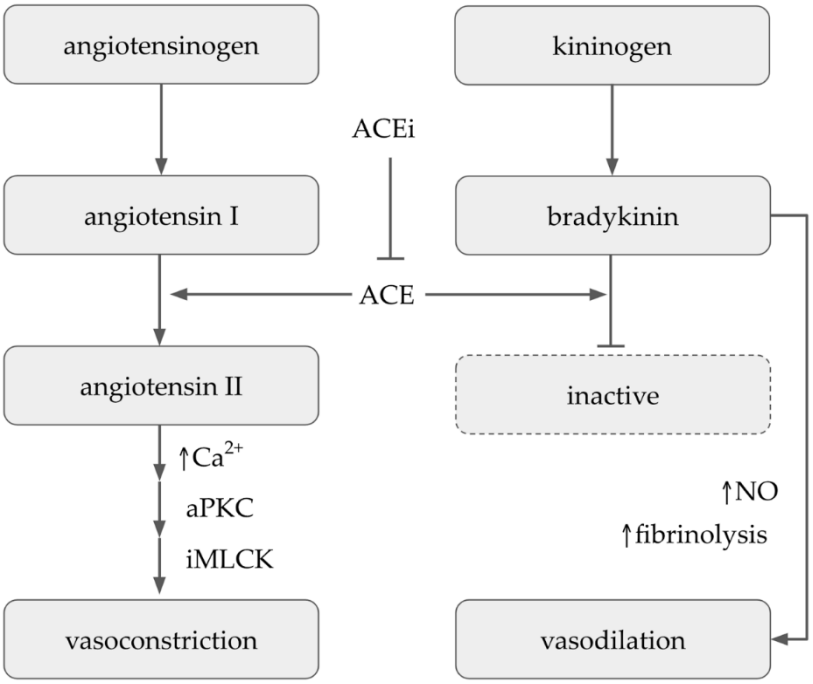

Figure 6. Inhibition of angiotensin-converting enzyme: Calcium (Ca^{2+}), angiotensin-converting enzyme (ACE), angiotensin-converting enzyme inhibitors (ACEi), atypical protein kinase C (aPKC), myosin-light-chain kinase (MLCK), nitric oxide (NO)

Most of the methods for measuring ACE activity *in vitro* rely on incubation of a reaction mix containing purified ACE and synthetic substrates such as furanacryloyl (FA-PGG) [137], 3-hydroxybutyryl-Gly-Gly-Gly (3HB-GGG) [138], hippuryl-histidyl-leucine (HHL) [139], dansyltriglycyne [140], or benzoyl-[l1-14C] glycyl-L-histidyl-L-leucine [141]. Each technique uses the same principle, only differing in the output (colorimetric, fluorometric, radioactive, etc.). ACE activity -or inhibition- is determined by adding the substrate and the potential inhibitor compound and comparing the activity to a sample without the inhibitor molecule/extract. The major advantages of using ACE activity tests for evaluating the activity of potential antihypertensive agents are physiologically relevant. Given the central role of ACE in controlling blood pressure *in vivo*, finding novel ACE inhibitors can directly impact the development of antihypertensive agents. ii) Versatility. Because it has been used since the 1960s and different substrates and outputs have been described, the assay can be adapted to evaluate the activity of a vast, diverse array of isolated molecules or mixtures.

Many of the original ACE tests require several intermediate reactions and laborious sample preparation steps (purification, extraction, etc.) to finally read the test result, making the assay hard to automate or increase throughput. Improvements have been made in the last decades, and current methods are more straightforward, cheaper, and faster, allowing the development of some high-throughput ACE assays [142,143]. Despite these efforts, given the enormous burden of hypertension globally, there is still room for improvements and simple readout, high-performance, low-cost ACE activity tests.

8. Antioxidant activity

Antioxidant activity can be described as the property of a given compound that, in low concentrations, can inhibit or decrease the oxidation of its substrate [144]. An antioxidant acts on free radicals that negatively affect biological systems by neutralizing them [145]. This inhibition or neutralization can be measured through chemical (e.g., ABTS,

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DPPH, FRAP, ORAC, CUPRAC) and biochemical methods (e.g., oxidation of low-density lipoprotein (LDL) assay, thiobarbituric acid reactive substances (TBARS) assay*) in vitro*, nevertheless, the first ones are preferred over the second ones due to their simplicity, speed, and low costs. Therefore, this section is focused on the most frequently used chemical-based methods to evaluate the antioxidant activity of natural molecules *in vitro* (Table 5). For a complete review of antioxidant methods, refer to [146] [145] [147] [148].

Several metabolites and phytoconstituents have shown antioxidant properties because of their scavenging ability; these include phenolic compounds, flavonoids [149,150], anthocyanins [151], and polysaccharides [152]. Many of these molecules are commonly present in natural sources, making the study of antioxidant properties a must in almost every research about biological activities.

Chemical-based assays will fall into two categories: hydrogen atom transfer (HAT) reaction-based and single electron transfer (ET) reaction-based assays. However, some combine the two mechanisms (i.e., ABTS, DPPH). Notably, several methods (i.e., ABTS, DPPH, FRAP) have limited biological relevance because they measure inhibition of radicals that do not exist in biological systems (e.g., DPPH•, ABTS•+) or the reducing capacity based upon one specific ion (Fe3+). The exception to this is the oxygen radical absorbance capacity assay (ORAC), which monitors the inhibition of the biologically relevant peroxyl radical (ROO•), and chemiluminescence, which can detect the quenching capacity of different oxygen species/reactive nitrogen species [153]. Overall, the advantages and disadvantages of the chemical methods are described in Table 5 and should be considered when assessing antioxidant activity. Overall, the selection of a specific antioxidant method is mainly based on its simplicity, low cost, and type of sample; nevertheless, currently, coupling of many chemical assays to high-throughput or automated systems (i.e., HPLC) facilitates the analysis and saves time [157] [155], which makes the parallel use of more than one method possible. It is recommended that antioxidant activity be evaluated using assays of biological relevance or complementary assays (e.g., chemical and biochemical) to eliminate possible over-or under-estimations due to the absence of a biological system and its components. However, the use of methods of biological irrelevance could provide insights into antioxidant properties and could guide the research towards more appropriate quantification methods, including *in vivo* analysis. Future research is focused on developing electrochemical sensors and biosensors, which could be employed for rapid quantification of complex samples in small amounts and could provide additional information about kinetics and mechanisms involved in the antioxidant activity [172]

Table 5. Common chemical methods for determining the antioxidant activity of natural molecules.

Method name	Description	Detection method	Advantages	Disadvantages	Ref.
Radical/ROS-based scavenging assays					

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2,2′-azinobis(3-ethylbenzo-thiazoline 6- sulphonate) (ABTS)/Trolox equivalent antioxidant capacity (TEAC) test	– HAT/ET – Antioxidant reaction with an organic cation radical – ABTS is converted to its radical cation by addition of sodium or potassium persulfate – The ABTS•+ radical loses absorption at 734 nm if reduced by an antioxidant	Spectrophotometry (A_{734})	– Rapid – Cheap – Simple – Can be used over a wide range of pH values – Can be coupled with online HPLC – Used for hydrophilic and lipophilic antioxidants – –	– Limited relevance to biological systems – Difficulties with the formation and stability of colored radicals – Phenolic compounds with low redox potentials can react with ABTS•+	[154];[145,155,156]
N,N-diphenyl-*N*′-picrylhydrazyl (DPPH) free radical	– HAT/ET – Antioxidant reaction with an organic radical – The DPPH• free radical loses absorption at 515-517 nm if reduced by an antioxidant or a free radical species	– Spectrophotometry (A_{515}) – EPR – Amperometric detection	– Rapid – Cheap – Simple – Stable at room temperature – Used for hydrophobic antioxidants – Can be coupled with online HPLC	– Limited relevance to biological systems – Difficulties with the formation and stability of colored radicals – Not recommended for samples with anthocyanin leads	[156]; [157]

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				– Could be interfered by borate presence	
Oxygen radical absorbance capacity (ORAC)	– HAT – Monitors the inhibition of peroxyl radical-induced oxidation – Requires peroxyl radical generators – The peroxyl radical reacts with a fluorescent probe resulting in the loss of fluorescence	Fluorometry	– Considered to be of biological relevance – High-throughput assay possible	– Peroxyl radical formation is thermosensitive – Could be interfered by hydroxyl radical scavengers and metallic ions – B-phycoerythrin (fluorescent probe), may show inconsistency from lot to lot and photoinstability	[158–161]

Chemiluminescence	– HAT – Consists of a chemiluminescent species, an oxidant (hydrogen peroxide) in the presence or absence of a metal or enzymatic catalyst, and an antioxidant or extract – Decreases of chemiluminescence intensity as a result of the antioxidant	Fluorometry	– Rapid – Cheap – Sensitive – Robust – Stable – It has been automated – Can be coupled with online HPLC	– Limitations with luminol-based antioxidant assays – Requires pH > 8.5	[161];[162–164]; [165]
Non-radical redox potential-based assays					
Ferric reducing antioxidant power (FRAP)	– ET – Measures the reduction (Fe^{3+})–ligand complex to (Fe^{2+})-complex by antioxidants. – Ligand used to facilitate detection: TPTZ – Antioxidant activity is determined as an increase of absorbance at 593 nm	– Spectrophotometry (A_{593}) – Electrochemical (coulometric titrants)	– Rapid – Cheap – Simple – Modified to allow the measurement of diverse sample types – Used for hydrophilic antioxidants – FRAP test by coulometric titration is	– Limited relevance to biological systems – Redox chemistry of ferric ion involves slower kinetics than the copper ones – Not sensitive toward thiol-	[166];[155,162,163,167,168]

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			extremely sensitive and reliable – Can be coupled with online HPLC	type oxidants – Requires acidic pH (pH 3.6)	
Cupric reducing antioxidant capacity (CUPRAC)	– ET – Measures the reduction Cu^{2+} to Cu^{+} by antioxidants. – Ligand used to facilitate detection: neocuproine – Reduction of Cu^{2+}–neocuproine complex to Cu^{+}–neocuproine has an absorption peak at 450 nm.	Spectrophotometry (A_{450})	– Simple – Stable – Sensitive – Favorable redox potential – Favorable reaction pH (pH 7.4) – Used for hydrophilic and lipophilic antioxidants – Can be coupled with online HPLC – High-throughput use possible	– Takes longer time to measure complex mixtures compared to other methods – Resulting product is more unstable than in other methods – Possible interference of absorption spectra between the oxidizing agent and the studied compound	[169,170];[145,169,171] [147] [148]

HAT (Hydrogen atom transfer), ET (Single electron transfer), EPR (Electron paramagnetic resonance), HPLC (High-performance liquid chromatography), TPTZ (tripyridyltriazine)

9. Antibacterial activity

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The usefulness of plant extracts for antimicrobial therapy has been promising since ancient times [173]. However, over the last few years, about 250,000 higher plant species were described, 17% of them have been evaluated according to biological aspects, only 4 to 10% have been assessed for antimicrobial activity, and several classes of secondary metabolites (alkaloids, flavonoids, saponins, etc.,) [174].

Antimicrobial resistance (AMR) is a significant threat to human health worldwide. In 2019, an estimated 4.95 million deaths were associated with AMR. The six leading pathogens for fatalities related to antibiotic resistance are Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa [175]. This number could rise to 10 million by 2050, according to estimates by the World Health Organization (WHO [176]). The synergistic effect of antibiotics with plant extracts against resistant bacteria may lead to new options in treating infectious diseases when the antibiotic is no longer effective during treatment.

Although new technologies have emerged to assess bacterial susceptibility to antimicrobial agents in recent years, traditional technologies are the most widely used. For example, *in vitro* antimicrobial evaluation methods emerged in the 1960s and responded to rapid, simple tests that evaluate various plant extracts or pure compounds at a low cost. These methods involve the diffusion of the potential antimicrobial compound through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms Figure 6 [177]. The reference methods are disk-diffusion and broth or agar dilution assays; however, in the last decades, new evaluation methods emerged to overcome some of the disadvantages of traditional methods, such as response time, low sensitivity, reproducibility, etc. [178,179]. An overview of commonly used susceptibility testing methods is shown in Table 6

Tabla 6. Traditional and emerging methodologies to assess the antibacterial activity of natural products.

Method	Description	Advantages	Disadvantages	References
Agar Diffusion Method	The antimicrobial agent diffuses from disks or strips into the solid culture medium that has been seeded with a pure culture	– Low-cost – Rapid and time-saving – Ability to test enormous numbers of	– Does not work on fastidious bacteria – Qualitative – Does not distinguish bactericidal and bacteriostatic activity	[180] [178] [181]
Well diffusion method	Diffusion of a liquid antimicrobial agent placed in a well punched into the solid culture medium that has been seeded with a pure culture			

Diffusion Methods			– microorganisms and antimicrobial agents – Ease to interpret results provided	– Disc/well preparation is time-consuming – No automation available	
	Agar plug diffusion method	The first bacterium is grown on agar plates, here it will secrete molecules that diffuse in the agar which then is cut and placed on another agar plate inoculated with a different bacteria	– Low cost – Simplicity – Highlights the antagonism between microorganisms	– Time-consuming – No automation available	[182]
	Antimicrobial gradient method (Etest)	Based on creating a concentration gradient of the antimicrobial agent tested in the agar medium where it is exposed to the selected microorganism.	– Quantitative – Used for MIC determination – Simplicity	– Expensive when compared to other diffusion methods	[183]
	Cross streak method	The first microbial strain is streaked in the center of the agar plate and incubated then in the same plate is seeded the second microorganism by a single streak perpendicular to the central streak.	– Simplicity – Rapid screen – Identifies antagonism between microorganisms	– No quantitative – Margins of the zone of inhibition are usually very fuzzy.	[184]

Thin-layer chromatography (TLC)–bioautography methods	Bioautographic method direct	The anti-microbial activity is assessed directly onto the TLC plates where the extracts are separated by chromatography across a TLC plate, then the microorganisms are also applied by spray identifying the localization of the fraction with antimicrobial potential.	– Works for fungi and bacteria – Consistent with spore-producing fungi – Fast and cheap – Simple	– Difficulties in obtaining complete contact between the agar and the plate	[185] [186]
	Agar overlay bioassay	The TLC plate is covered with agar seeded with the test microbe and the antimicrobial compounds are diffused onto the agar medium.	– Provides well-defined growth inhibition zones – Not sensitive to contamination –	– Time-consuming – Low sensitivity	[187] [188]
	Agar diffusion	The antimicrobial agent is transferred from a TLC to an agar plate previously inoculated with the test microorganism.	– Fast and cheap – Sensitive – Works with bacteria and fungi	– Agar is prone to adhere to silica gel due to the prominent adsorption between them – Compounds will be lost during the transfer from the thin-layer plate to the culture medium	[188] [189]

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DIlution Methods	Broth dilution method Minimum inhibitory concentration (MIC) determination	Uses tubes or microdilution plates to measures the lowest concentration of antimicrobial agent that completely inhibits the growth of the bacteria	– Quantitative – Good reproducibility	– Manual task of preparing the antibiotic solutions for each test	[190]
	Agar dilution method	Similarly to the procedure used in the disk-diffusion method, a desired concentration of the antimicrobial agent is placed into an agar medium.	– Suitable for both antibacterial and antifungal susceptibility testing	– If not automated, very laborious	[191] [192]
	Time-kill test (time-kill curve)	Based on a time/concentration-dependent analysis of antimicrobial effects. Several tubes containing varying concentrations of the antimicrobial agent are seeded with the bacteria and the percentage of dead cells is determined along with the assay.	– Can be used to determine synergism or antagonism between drugs – Suitable to identify bacteriostatic and bactericide effects	– Time-consuming	[193]
ATP bioluminescence assay	Bioluminiscnece ATP based	Based on the capacity to measure adenosine triphosphate (ATP) produced by bacteria or fungi	– Rapid and easy – Quantitative – In situ evaluation	– Expensive – Difficult to differentiate the microbial ATP from other organic debris – Adapted only for solid surfaces	[194] [195]
Flow cytofluorometric method	Flow cytofluorometric	Based on the capacity of damaged cells to emit a positive signal that is detected by flow cytometry analysis.	– Three subpopulations (dead, viable, and injured	– More expensive	[196] [197]

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	Bacteria are exposed to antimicrobial agents and then stained with the intercalating agent propidium iodide.	cells) can be clearly discriminated – High throughput screening	– Flow cytometry equipment is required

ATP: adenosine triphosphate

Research into natural compounds has shown significant progress in discovering new molecules with antimicrobial activity. The primary natural compounds with valuable antimicrobial activity are medicinal plants and microorganisms. However, given the great diversity of compounds with antimicrobial potential, it is necessary to have adequate tools to facilitate screening, reduce costs, and obtain rapid quality results [198].

The current tools to assess antimicrobial activity are considered the gold standard and are highly reliable systems being used for decades in the clinical microbiology labs (Figure 6). However, they mostly rely on detecting qualitative changes based on bacterial metabolism and require pure cultural isolates. In addition, the emergence of new antimicrobial resistance mechanisms requires that the performance of susceptibility devices be constantly reassessed and updated periodically, along with new automated instruments that could provide faster results, save money, and reduce labor requirements [183,199]. Hence, further improvement in the currently used and novel AST methods and instruments is mandatory for speeding up the determination of antimicrobial efficacy in clinical and research microbiology laboratories in the foreseeable future.

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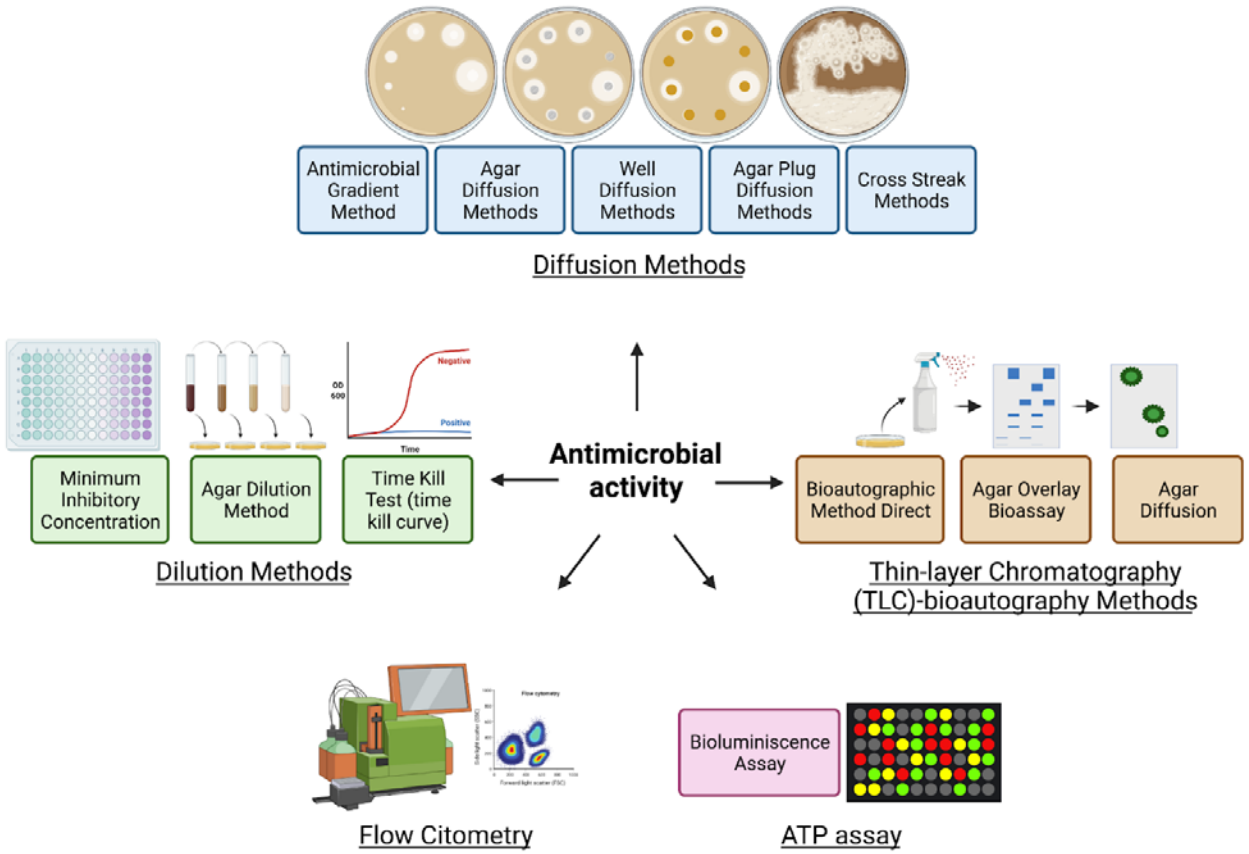

Figure 7. Summary of the most relevant antimicrobial activity methods.

10. Conclusion

The high structural and physicochemical diversity of natural molecules produced by plants, microorganisms, fungi, insects, and all organisms makes them a virtually inexhaustible source with potential for discovery and development of novel bioactive compounds with applications in the pharmaceutical, food, or biotechnological industries, to name only a few [200,201]. Given the immense variety of biological molecules and the high array of promising activities and evaluation methods, it is crucial to establish the most relevant tests for the initial screening [7].

The first step in the biological evaluation of natural molecules commonly involves evaluating the compound in a range of *in vitro* biochemical and pharmacological assays. These experiments aim to establish a solid grounding regarding the compound properties and understand its mechanism of action. This information is then used to select the most suitable compounds and evaluate their activity in more complex *in vitro* and *in vivo* assays [202]. (Figure 7). Future trends in assessing the biological activity of natural molecules are focused on establishing simplified and automated protocols that enable high-throughput screening, facilitating [203] subsequent analysis, shortening the evaluation time, and allowing parallel use of multiple methods [204]. Moreover, different systems are being developed to support more physiologically relevant data collection and enable real-time monitoring. Lastly, advances in computational chemistry, the use of public databases, and

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the implementation of in silico models to assess efficacy are tools that assist in initial screenings of multiple compounds [205–207]

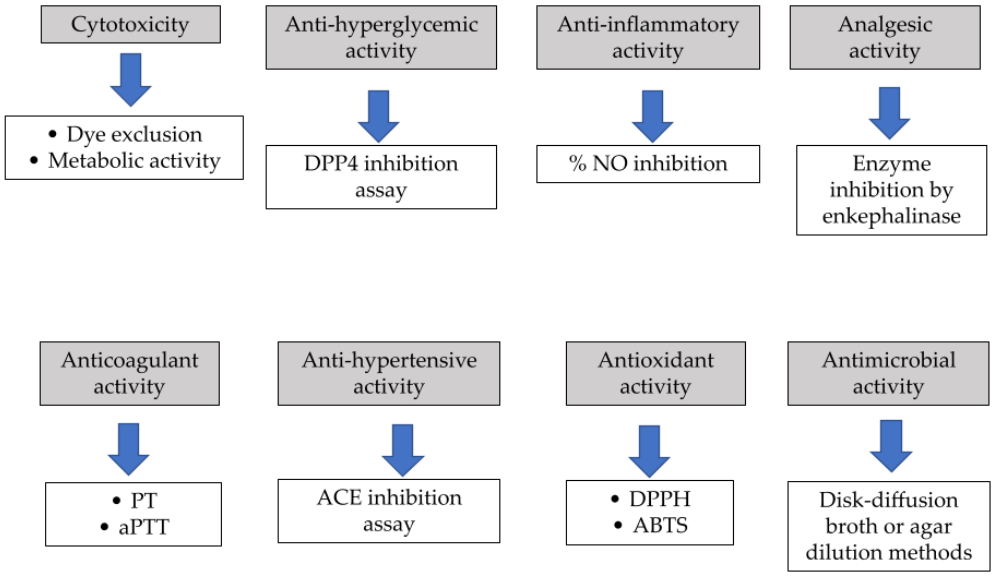

Figure 7. Minimal *in vitro* biochemical and chemical assays for determination of biological activities

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