METHODS FOR PRECLINICAL EVALUATION OF BIOACTIVE NATURAL PRODUCTS

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PREFACE

Natural products have always played a dominant role in the life of humans, influencing several aspects of biological processes and many of them are fundamental for their wellness. Taking into account, just to mention a few, the epigenetic role of natural compounds on metabolic pathways and, in the whole, on the development of the human race. Particularly, medicinal plants are an inexhaustible source of drug discovery and for the identification of scaffolds useful for the formulation of several pharmaceuticals. In this context, *in vitro* and *in vivo* methods for the assessment of preclinical activities of natural products are gaining importance. There are several methods that can be applied to assess the activities of medicinal plants and their secondary metabolites. There is a need for a detailed methodology (in terms of the procedure itself, the correct dose selection, preparation of test materials, utilization of the right solvent, specific evaluation of the solubility and compatibility properties, application particulars, as well as constructive critical evaluation of the obtained results) among the researchers who are carrying out bioactivity evaluation studies. Therefore, this book will cover every scientific aspect of preclinical scientific research on natural products. Natural product researchers, pharmacists, medical doctors, and students in pharmacognosy, chemistry and biology will be the target audience interested in the topics of this book.

The book offers an overview of preclinical methods throughout its ten chapters, focusing on the etiology of diseases, natural products as the materials for the bioassays, extract types, the concentration of the extracts/compounds for *in vitro* and *in vivo* assays, preparation of the test materials, application of the test materials, methods (step by step processes) and calculations.

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CHAPTER 1

Antioxidant Activity Methods

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Abstract: Antioxidants are groups of substances able to prevent and delay the oxidation of easily oxidizable molecules and avoid free radicals' formation. In living organisms, the main free radicals are reactive oxygen species and reactive nitrogen species. At low levels, they are involved in the regulation of diverse physiological processes, but an imbalance between free radicals and the ability of the body to eliminate them results in a pathological condition called oxidative/nitrosative stress. Oxidative/nitrosative stress causes damage to cellular structures such as lipids, nucleic acid, and proteins, compromising cellular health and viability and inducing the development of several diseases. Physiological systems are able to contrast the free radical excess, through the endogenous enzymatic materials (*e.g.*, uric acid, glutathione *etc.*), and *via* transcription factor activation. The uptake of natural antioxidants can contribute to prevent cellular damage and exert beneficial effects. Natural antioxidants are generally derived from plant sources and they play an important role by directly scavenging free radicals or increasing antioxidant defences. Natural antioxidants have gained remarkable interest and several methods have been developed for identifying their antioxidant capacity. This chapter reviews the major *in vitro* and *in vivo* assay procedures for the antioxidant activity estimation describing materials, extract types, extracts/pure compounds' concentrations, step by step processes and calculations for each assay. Advantages and limitations, as well as the molecular mechanisms of each method have been reported.

Keywords: Antioxidant activity, Electron transfer, Free-radicals, *In vitro* antioxidant assay, *In vivo* antioxidant methods, Natural antioxidants, Oxidative stress.

INTRODUCTION

Antioxidants are substances that, in low quantities, prevent or delay the oxidation of easily oxidizable substrates. In chemistry, oxidation reactions are well-known

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processes that lead to the removal of electrons from a compound, forming the free radicals [1].

Free radicals, usually referred to as reactive species of oxygen (ROS) or nitrogen (RNS), are highly unstable and reactive molecules that are missing one of two electrons in the outer orbital and are desperately looking for other molecules to attack, to complete the chemical structure. In this way, the free-radical gains or donates an electron, and the attacked molecule or atom becomes a free-radical itself, and so on, triggering a cascade reaction. Antioxidants can interrupt the chain reactions by destroying free-radical intermediates and blocking subsequent oxidation reactions by different mechanisms.

The main producers of endogenous free radicals are the mitochondria during aerobic respiration, peroxisomes and endoplasmic reticulum, due to high oxygen consumption [2].

The main free radicals include superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), and alkoxyl ($RO^{\bullet}$), or peroxyl ($ROO^{\bullet}$) radicals. Hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$)/peroxynitrous acid (ONOOH) do not contain unpaired electrons, but they belong to two-electron oxidants.

ROS and RNS have a double effect. Low levels of them are involved in the regulation of diverse physiological processes as the defence from infective agents or maintenance of the homeostasis status. The superoxide and nitric oxide production by neutrophils and macrophages contributes to destroy bacteria during the phagocytosis process. Nitric oxide promote the vascular smooth muscle relaxation causing vasodilation and increasing the blood flow [3].

Exogenous producers of harmful substances are solar radiation with ultraviolet rays, pollution, alcohol, tobacco smoke, heavy metals, industrial solvents, and pesticides. Also certain drugs contribute to increase the level of free radicals.

An excess of ROS/RNS leads to a pathological condition named oxidative/nitrosative stress. In biological systems, oxidative/nitrosative stress is characterized by an imbalance between free radicals and the ability of the body to eliminate these reactive species using endogenous and exogenous antioxidants. This is a harmful process that may generate serious damages to cellular structures. Free radicals can induce lipid peroxidation to the polyunsaturated membrane lipids with loss of fluidity and cell lysis, inactivation and denaturation of the proteins with loss of their biological functions, and modification of the nucleic acid bases, inducing carcinogenesis [4].

All these damages are implicated in the development of several diseases, including cancer, cardiovascular diseases, neurodegenerative disorders, liver diseases, ulcerative colitis, aging, and atherosclerosis [5].

Fortunately, nature has built-in-defence mechanisms against free-radicals. The human body and living organisms developed a complex system of physiological enzymatic and non-enzymatic antioxidant defences to counteract the harmful effects of free radicals and other oxidants.

Endogenous enzymatic defence system is equipped with superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes [6]. SODs are ubiquitous enzymes involved in the dismutation reaction of the superoxide anions in molecular oxygen and H_2O_2 (Eq. **1**). They represent the dominant detoxification system in living cells and the presence of cofactors (Zn, Cu) is required for their activity. Although H_2O_2 is not a free radical, it is the precursor of some radical species; it can diffuse a notable distance before its decomposition to give the reactive and dangerous hydroxyl radicals. The H_2O_2 is then converted by CAT or GPx in harmless molecules. CAT uses iron or manganese to catalyse the reduction of H_2O_2 into water and oxygen (Eq. **2**); it is mainly found in peroxisomes, and its main function is to eliminate the H_2O_2 generated during the oxidation of fatty acids. GPx, a selenoperoxidase, plays an important role in inhibiting the process of lipid peroxidation; it breaks down H_2O_2 in water and lipid peroxides in their corresponding alcohols mainly in the mitochondria and sometimes in the cytosol (Eq. **3**).

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \quad \textbf{(1)}$$

$$H_2O_2 \rightarrow 2H_2O + O_2 \quad \textbf{(2)}$$

$$H_2O_2\ (ROOH) + 2GSH \rightarrow GSSG + H_2O + ROH \quad \textbf{(3)}$$

Non-enzymatic endogenous antioxidants are molecules able to neutralize free radicals and oxidant agents [7]. Among endogenous antioxidants, uric acid and bilirubin fulfil an efficient defence antioxidant system in blood serum. Uric acid is the end-product of purine metabolism and provides an antioxidant defence in humans [8]. It is a donor of electrons and a selective peroxynitrite scavenger, requiring the presence of ascorbic acid and thiols to exert its action. Uric acid acts against hydroxyl radicals, singlet oxygen, and lipid peroxides by converting itself in urea and allantoin.

Bilirubin is derived from the enzymatic degradation of haemoglobin and other heme-proteins. In biological systems, bilirubin shows potent antioxidant properties, especially against peroxyl radicals [9].

CHAPTER 2

Antidiabetic Activity Methods

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Abstract: Diabetes mellitus (DM) is a metabolic disease characterized by the destruction of pancreatic β cells or reduced insulin secretion and action, and is one of the most common health problems worldwide. Its incidence is increasing at a high rate, resulting in enormous social costs. Various drugs show their effectiveness by improving insulin sensitivity, and reducing glucose production in the liver or other tissues. Several preclinical studies on diabetes-induced in animals using surgical, pharmacological or genetic methods demonstrated the effectiveness of these drugs. The anti-diabetic activity of plants has been attributed to the occurrence of primary and secondary metabolites characterized by many beneficial effects with advantages over chemical treatments. A number of studies have demonstrated the potential health benefits of phytocomponents in treating DM by acting on multiple molecular targets. Therefore, it is important to test *in vitro* assays. This review includes methods for the evaluation of preclinical anti-diabetic activities and summarizes the potential of natural resources to prevent and/or treat diabetes. In addition, the database contains information including the plant name, useful plant parts, active compounds, and their mechanisms of action, in which *in vitro* and *in vivo* methods were studied.

Keywords: Anti-diabetic Activity, *In vitro* Assays, *In vivo* Assays, Natural Products, Preclinical Investigations, Mechanism.

INTRODUCTION

Diabetes is a chronic disease characterized by complex etiology and elevated blood glucose levels as a result of insulin deficiency from the pancreas or impaired use of produced insulin. Under normal conditions, carbohydrates are broken down into glucose after food ingestion, leading to elevated blood glucose levels. Subsequently, insulin is secreted by the β cells of the pancreatic islets of Langerhans to enable cellular glucose uptake from the bloodstream by reducing its circulating levels [1, 2]. Three major types of diabetes are documented; type 1, type 2, and gestational, where type 2 is the most commonly seen diabetes, coun-

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ting for up to 90% of overall diabetes cases worldwide [3]. Type 1 (accounts for 5–10%) diabetes is a cellular-mediated autoimmune disorder characterized by the destruction of insulin-producing pancreatic β cells and absolute deficiency of insulin secretion. Type 2 diabetes is caused by insulin resistance leading to impaired insulin secretion by β cell dysfunction [4, 5]. Diabetes is highly associated with macrovascular complications, including cardiovascular diseases and microvascular complications like neuropathy, nephropathy, and retinopathy, leading to organ and tissue damages [6]. According to the International Diabetes Federation, the number of people with diabetes, which is 463 million in 2019, is estimated to reach 700 million by 2045, and the mean annual health cost for people with four or more diabetes complications is 20 times higher than those without complications. This means expenditures are considerably related to the number of complications [3] that clearly demonstrates treatment's importance before the disease progresses. Various chemicals or drugs show their efficacy by improving insulin sensitivity, reducing glucose production in the liver or other tissues (glycogenolysis, gluconeogenesis), and also slowing the intestinal uptake of carbohydrates [7 - 9].

Diabetes can be created by surgical, pharmacological or genetic methods in various animal species. Experiments are carried out on smaller "rodent" models as well as larger species. The first classical experimental model of diabetes was created by removing 90% of the pancreas (pancreatectomy) in dogs. In this way, glucose uptake in different tissues is studied by both hyperglycemic and euglycemic clamp methods. Among the pharmacological methods, streptozotocin (STZ; 69%) and alloxan (31%) are the most widely used diabetogenic agents and are good models for studying many aspects of the disease. When STZ and alloxan were administered parenterally, intravenously, intraperitoneally or subcutaneously, the buffer solution given and its pH are important. According to the given doses of these drugs, syndromes similar to type-1 and type-2 or glucose intolerance are seen in experimental animals, and these protocols are widely used.

Today, the anti-diabetic activity of a drug can be understood by measuring many parameters in experimental animal models after acute or chronic drug treatment (such as oral glucose tolerance test, serum lipid levels, serum insulin level, glycated haemoglobin level (HbA1 c) *etc.*). In the studies conducted on diabetic animal models generally created with chemical agents, the amount of decrease in blood glucose is reported usually after the fasting period afterwards acute or chronic treatment with drugs or medicinal plants. Comparative studies are conducted in groups of non-diabetic and/or diabetic animals treated with anti-diabetic drugs. Glucose measurement is carried out by the standard glucose oxidase and dehydrogenase method, with commercially available glucometers. Insulin determination is made in experimental animals with different methods

(radioimmunoassay [RAI], immunometric determinations). Glycosylated haemoglobin (A1c) can be measured in chronic assays [7 - 9].

Today, biguanide metformin, sulfonylureas, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors, thiazolidinediones, sodium-glucose cotransporter-2 (SGLT2) inhibitors, α-glucosidase inhibitors, dopamine receptor agonist bromocriptine, bile acid sequestrant colesevelam, GLP-1 (glucagon-like peptide-1) receptor agonists, insulin and amylin analog pramlintide acetate have been approved for blood glucose-lowering treatment [10].

Most of these medications have adverse effects [11], encouraging researchers to search for new therapeutic metabolites that originated from natural sources [12 - 14]. This section contains some bioactivity methods that could give an idea for those interested in this research area in identifying anti-diabetic natural products.

PRECLINICAL ACTIVITY ASSESSMENT OF MEDICINAL PLANTS

In vitro and *in vivo* assay methods, mechanisms of actions of medicinal plants as well as their constituents are summarized in (Table **1**).

Table 1. Plants having anti-diabetic activities [15 - 19].

Plant name	Parts used/Extract	Assay method	Mechanism of action	Constituents
Abies pindrow (Royle ex D.Don) Royle (Pinaceae)	Whole plant	*In vitro*/INS-1 cells	Insulin secretagogue activity	Volatile oil
Abroma augusta (L.) L.f. (Malvaceae)	Roots and Leaves/ Aqueous extract	*In vivo*/diabetic rats	Lowering blood sugar	Fixed oil, Alkaloid
Acacia arabica (Lam.) Willd. (Fabaceae)	Bark and Seed/ Chloroform extract	*In vivo/* normal and alloxan-induced diabetic rabbits	Initiate the release of insulin	Arabin
Achyranthes aspera L. (Amaranthaceae)	Whole plant	*In vivo/* normal and alloxan-induced diabetic rabbits	Decrease blood sugar	-

CHAPTER 3

Anti-inflammatory Activity Methods

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Abstract: The inflammatory process can be unleashed by a wide variety of biological, chemical, and physical factors, and arises to counteract these dangerous enemies. In case of failure by the organism to defeat these exogenous stimuli, a chronic inflammatory status occurs, hence potentially leading to several diseases. Therefore, anti-inflammatory drugs, from both synthetic and natural sources, represent valuable allies to fight the phlogistic process. The search for novel candidate drugs is never ceasing, also from the plant kingdom, known to provide products with generally lesser or more tolerable side effects than synthetic drugs. Researchers can take advantage of a wide plethora of *in vitro* and *in vivo* methods in order to investigate the anti-inflammatory potential of unknown natural products. Cell cultures (*i.e.*, stimulated macrophages) offer a fast and highly reproducible first-line screening, while animal models, thanks to their complexity, grant to achieve a broader and multifactorial view of the inflammatory process. The underlying mechanism of action of candidate drugs can also be explored by several cell-free assays, which are crucial to assess the activity of key enzymes involved in the inflammatory cascade. Here, we report the most widely employed models for the assessment of the anti-inflammatory potential of natural products, discussing the overall procedure, and also providing examples of plant drug screening. Together, these approaches represent the basis for a thorough and proper investigation of the anti-inflammatory activity of novel candidates.

Keywords: Inflammation, Cell Models, Animal Models, Methods, *In vitro*, *In vivo*, Cell-free, Carrageenan, Lipopolysaccharide, Cytokines, Natural Products, Polyphenols, Extracts, Flavonoids, IBD, Arthritis, Periodontitis, Pleurisy.

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INTRODUCTION

Inflammation is a biological response of the immune system, triggered by several factors, including pathogens, toxic compounds, damaged cells or irradiation [1, 2], in order to remove injurious stimuli and commence the healing process [3]. The key features of inflammation are redness, warmth, swelling, pain, and loss of functionality.

Inflammation can be distinguished as either acute or chronic, depending on the type, intensity, and length of stimulus, as well as the effectiveness of the inflammatory process resolution. Acute inflammation is the primary response of the body to injurious stimuli and implies a local vascular involvement and an immune response. When the immune system successfully eliminates harmful agents during acute inflammation, the process resolves; whereas, if it fails to eliminate them, a chronic phase occurs. If uncontrolled, inflammation can lead to the onset of several diseases, among which rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis and other immune-inflammatory ailments [3 - 5]. Moreover, a chronic inflammatory status may contribute to the development of the perfect environment for tumorigenesis, hence being a known risk factor for the occurrence of different types of cancer [6 - 8]. Furthermore, many chronic diseases manifest due to the presence of low-grade sustained inflammation [9].

Several classes of drugs, among which corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), along with biologics, are used to manage inflammatory ailments. However, they are not free from side effects and the biological ones are expensive to be employed. In order to limit these drawbacks, over the past decades, the use of herbal medicines, nutraceuticals and food supplements has increased greatly as an alternative and/or complementary medicine to treat several diseases, such as inflammation [10]. This is because, even if natural products are not risk-free, commonly they are safer than both synthetic or biologic drugs. In this frame, many *in vitro* and *in vivo* models have been developed for the evaluation of drug anti-inflammatory properties. The *in vitro* studies help to investigate the cellular response in a closed system, where the experimental conditions are maintained [11]. Together with abiotic assays aimed at assessing the activity of key enzymes involved in the inflammatory process, *in vitro* models are useful for providing a clear insight into the mechanism of the anti-inflammatory activity of drug candidates. On the other hand, the choice of an appropriate animal model is crucial to establish the efficacy and mechanism of action of drugs in a complex environment, in order to be translated as a potential therapeutic effect in humans. Similar to *cell-free* and *in vitro* experimental models, an improper selection of the animal ones can lead to false positive or false

negative results, and may limit the discovery of promising novel candidates [12]. Therefore, the use of a rigorous approach for the anti-inflammatory screening of natural products or their constituents is highly advisable. Notably, when dealing with phytocomplexes, it is crucial to investigate their quali-quantitative composition if it is intended to evaluate the major players [13].

This chapter comprehensively discusses the different *in vitro* and *in vivo* models used for the evaluation of anti-inflammatory potential of drugs from natural sources, along with their mechanisms of action.

ASSAYS FOR IDENTIFYING THE MECHANISM OF ACTION OF ANTI-INFLAMMATORY DRUGS

The start of the inflammatory machinery arises by the recognition of extracellular receptors of noxious signals, defined as either pathogen- or danger-associated molecular patterns (PAMPs and DAMPs, respectively) [14]. Among the most relevant receptors linked to inflammation, there are toll-like ones (TLRs). Moreover, PAMPs and DAMPs activate immune cells to release cytokines, such as interleukin 6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α), which, along with their specific receptors, interact with TLRs, leading to the activation of signaling cascades, that in turn promote the translocation of several transcriptional factors (see below) [14]. Cytokines are considered the most important markers of inflammation and hence represent one of the main targets investigated in the field of inflammation. Their levels can be appreciated by enzyme-linked immunosorbent assay (ELISA), both *in vitro* and *in vivo*. Given the high sensitivity of this type of assay, concentrations of the order of picograms per milliliter can be easily detected by ELISA assay. Otherwise, concentrating samples can be helpful to reach the optimal detectable concentration. Their gene expression can be investigated by Real-time polymerase chain reaction (qPCR), while their protein level by Western Blot (WB) analyses [15].

The most important signaling pathway activated by TLRs is that of NF-κB, which comprises five different transcriptional factors [16]. The onset of translocation of NF-κB starts from the activation of IκB kinase (IKK), which promotes the phosphorylation of IκB, a cellular inhibitor of NF-κB, and hence its degradation by the proteasome. This allows the subunits RelA/p65 to translocate into the nucleus and activate the transcription of several inflammatory, survival, and pro-angiogenetic factors [17]. Protein expression of these factors can be assessed by WB analyses, whereas their gene expression is analysed by qPCR. Moreover, the electrophoretic mobility shift assay (EMSA) allows to quantitatively investigate transcriptional factors, such as NF-κB [18].

CHAPTER 4

Antimicrobial Activity Methods

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Abstract: Plant extracts are widely utilized for their antimicrobial effect, alone or in combination with traditional drugs. However, the current global lack of harmonization on the assays used to investigate their biological effect makes the result comparison very challenging. In the present chapter, we highlight the variables used for the antimicrobial testing, which may affect the end result. The *in vitro* antimicrobial methods, including diffusion methods, dilution methods and thin-layer chromatography-bioautography are reported, together with a range of assays aimed at evaluating the mechanisms of action of the extract. Furthermore, *in vivo* test procedures are evaluated, with a focus on the impact of the differences in the testing animal and the procedure used.

Keywords: Antimicrobial methods, Plant extracts, *In vitro* testing, Diffusion methods, Dilution methods, Bioautography, *In vivo* antimicrobial assays, Synergy testing.

INTRODUCTION

The increased rates of drug resistance pose a serious threat worldwide, with a considerable impact on treatment failures associated with multidrug-resistant pathogens. Therefore, global effort is currently focused on searching for novel therapeutics with the antimicrobial and antiviral potential to be used alone or in combination with existing drugs. Natural products represent an important source of bioactive compounds, and polyphenols play a key role in providing a wide range of beneficial health effects. We have recently assessed the antimicrobial and antiviral activity of *Olea europaea* L. (*Oleaceae*) leaf extracts against *Herpes symplex* 1 (HSV-1) and Gram-positive bacteria [1]. The biological activities of two extracts from a non-psychotropic *Cannabis sativa* L. cannabidiol (CBD)-chemotype and a Chinese accession of non-psychotropic *Cannabis sativa* L. have

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also been investigated [2, 3]. Tree nuts have been explored as sources of natural antioxidants with antimicrobial potential [4, 5]: almond skin polyphenols exhibited antimicrobial (MIC values of 0.31-1.25 mg/mL) and antiviral (decrease in the viral titer ** $p < 0.01$, and viral DNA accumulation * $p < 0.05$) activity against *Staphylococcus aureus* and HSV-1, respectively [6] whereas polyphenols-rich extracts from natural pistachios and roasted pistachios were active against *S. aureus* 6538P and clinical isolates of *Staph.* spp [7]. Amongst *Citrus* (*Rutaceae*) polyphenols, the antioxidant, anti-inflammatory, antiviral, antimicrobial, and anticancer activities of flavones and flavonols have been recently described [8, 9]. The effectiveness of *Citrus* fruits on *Helicobacter pylori* has also been explored [10]. Information on the nature of the inhibitory effect, bactericidal or bacteriostatic, time-dependent or concentration-dependent, cell damage inflicted is also relevant for the evaluation of the antimicrobial potential of plant extracts.

On the other hand, when reviewing the literature on the antimicrobial effect of plant extracts, the comparison across the presented data is often difficult. This could be due to the employment of non-standardized approaches in relation to the inoculum preparation and size, growth medium and incubation conditions, as well as endpoints determination. Therefore, it is important to better understand the antimicrobial techniques available to assess the bioactivity of plant extracts. A comparative study was performed to test the ability of the common qualitative agar diffusion methods and a quantitative broth dilution assay for the antimicrobial activity of plant extracts and a variety of phenolic compounds [11].

Here, we will explore the commonly used techniques to evaluate the antimicrobial activity of plant extracts, analyzing both the *in vitro* and *in vivo* assay procedures and detailing the assays for the mechanisms of action.

IN VITRO ANTIMICROBIAL ASSAYS

A number of methods are employed to evaluate the antimicrobial potential of a plant extract or an isolated compound *in vitro*. Nevertheless, standardized and reliable methods are necessary to investigate the potential antimicrobial properties of plant extracts. The extraction process, the use of standardized inocula and the techniques for the preparation of test samples are examples of factors influencing the variability of the obtained results. The Clinical and Laboratory Standards Institute (CLSI) has collected some accepted methods for the evaluation of the antimicrobial potential [12], which are, however, designed for pure compounds rather than plant extracts [13] (Table **1**). Although various authorities, such as EUCAST and CLSI provide antimicrobial susceptibility testing guidelines for antibiotics [14 - 17] in terms of clinical breakpoints and result interpretation, no international consensus and harmonization are reached for plant-derived

compounds. A breakpoint is the minimum concentration of the antimicrobial expected to be clinically effective against a microorganism.

Table 1. Antimicrobial susceptibility testing guidelines for the different microorganisms as recommended by CLSI.

MICROORGANISM	***ANTIMICROBIAL SUSCEPTIBILITY TESTING DOCUMENTS***		
	DISK DIFFUSION METHODS	DILUTION METHODS	TIME KILL TEST
BACTERIA	Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, 13th ed., CLSI standard M02.* Wayne (PA): CLSI; 2018 [16]	Clinical and Laboratory Standards Institute (CLSI). *Methods for DilutionAntimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved standard, 11th ed., CLSI document M07-A9.* Wayne, (PA): CLSI; 2018 [17]	Clinical and Laboratory Standards Institute (CLSI). *Methods for Determining Bactericidal Activity of Antimicrobial Agents. Approved Guideline, CLSI document M26-A.*, Wayne, (PA): CLSI; 1998 [19].
YEASTS	Clinical and Laboratory Standards Institute (CLSI). *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts, Approved Guideline. CLSI document M44-A.* Wayne (PA): CLSI; 2004. [20]	Clinical and Laboratory Standards Institute (CLSI). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard, 4th ed., NCCLS document M27.* Wayne (PA): CLSI; 2017 [21]	-
MOULDS	Clinical and Laboratory Standards Institute (CLSI). *Method for Antifungal Disk Diffusion Susceptibility Testing of Non-dermatophyte Filamentous Fungi, Approved guideline, CLSI document M51-A.* Wayne (PA): CLSI; 2010 [22].	Clinical and Laboratory Standards Institute (CLSI). *Reference Method for Broth Dilution Antifungal Susceptibility Testing Filamentous Fungi, Approved Standard, 2nd ed., CLSI document M38* Wayne (PA): CLSI; 2017 [23].	-

Othman *et al.* compared four different antimicrobial assays to screen plant extracts using a standardized inoculum [18]. The authors concluded that both agar- and broth-based assays are needed for assessing the antimicrobial activity of plant extracts *in vitro*.

The principles and variables influencing the antimicrobial potential of plant extracts, together with the evaluation of diffusion and dilution methods, will be examined as follows.

CHAPTER 5

Cytotoxic Activity Methods

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Abstract: Natural products have formed the basis of traditional medicine systems throughout human history. Today, drug discovery studies from natural origins continue rapidly and efficiently with modern methods. Among the many activities, cytotoxic activity is related to the behaviour of test material on cell viability and cellular growth. Cytotoxicity methods, used as a screening test or initial test for guiding other activities, provide useful information for biocompatibility studies for medical devices or materials, drug discovery and development processes, toxicity evaluation of cosmetics, research of disease mechanisms and treatments, and determination of chemopreventive agents. *In vitro* cytotoxicity analyses have emerged as an alternative to *in vivo* studies and have become preferable due to their ease of application, standardization, rapid, low cost, and compatibility with data from *in vivo* studies. With cell-based cytotoxicity studies, basic information about the cytostatic and cytotoxic effects of the tested substance is obtained. In studies dealing with natural products, the most appropriate cytotoxic method should be selected according to the properties and chemical structures of natural compounds, the ultimate goal of the study, cell types, *etc*. Although there are many cytotoxicity methods, this chapter is an introductory overview of the most commonly used assay methods to estimate the cytotoxic activity in natural products.

Keywords: Cytotoxicity, Natural products, Phytochemicals, Colorimetric Methods, Metabolic cell proliferation assays, Dye exclusion assays.

INTRODUCTION

'Cytotoxicity' is defined as the toxicity of substances on living cells *via* cell damage or death. When a cytotoxic compound is administered to a living cell, uncontrolled death may be observed due to the cell's loss of membrane integrity and fragmentation, cessation of cell growth and proliferation, or programmed cell death. To date, several mechanisms related to death have been described, including necrosis, apoptosis, senescence, and autophagy [1]. While necrosis is

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associated with irreversible cell injury and eventual cell death due to pathological processes, apoptosis is characterized by the activation of caspases and loss of membrane integrity. Senescent cells continue to exist viable but have morphological changes and come to an end synthesizing DNA, however, autophagy has appeared as vacuolization and LC3 lipidation [2]. Moreover, it should also be kept in mind that Galluzzi *et al.* classified the types of cell death in more detail by focusing on the molecular and basic aspects of death after numerous studies on cell death [3]. Although cell death in multicellular organisms is critical for various physiological processes, including a regulatory role in maintaining cellular integrity, embryological development, *etc.*, substances-induced toxicity is generally an unwanted situation. Many proteins have controlled these processes, and the underlying mechanisms of cell death have been extensively researched, particularly in understanding disease treatment and toxicity mechanisms [4].

Cytotoxicity tests are considered screening assays and provide basic information about the behaviour of a compound on the cell viability and ability for cellular growth. Biocompatibility studies for medical devices or materials, drug discovery and development processes, toxicity evaluation of cosmetics, and research of disease mechanisms and treatments have covered cytotoxic activity methods. Cytotoxic activity can also be used as an initial test to guide testing concentrations of other assays, such as antifungal and antiviral activities. In addition, agents that can prevent cell death are of considerable interest and these tests can be used to determine them. The agents could treat conditions in which there is unwanted cell death, like a heart attack or neurodegenerative disorders [5]. Cytotoxicity is almost by default an unwanted characteristic in drug discovery since cytotoxic compounds could have serious adverse effects. On the other hand, cytotoxic agents still have remarkable potential as anti-cancer agents in several advanced malignancies. Cancer is one of the most common life-threatening diseases recognized by abnormal signaling processes that lead to aberrant cellular growth. It is obvious that new strategies or novel compounds still need to be discovered. The development of specific or highly selective anti-cancer agents, the designs of improved treatments to prevent resistance to an individual or multiple drugs or combinations for increasing effectiveness are dependent on more scientific efforts [6]. Natural products have been recognized as prominent mediators of the critical pathways involved in the development and progression of cancer. Thus, their chemopreventive and chemotherapeutic anti-cancer activities are scrutinized thoroughly.

It is stated that one of the situations that causes the greatest loss of time, cost, and labor in new drug discovery and development studies is that the candidate compounds are seen to have toxicity in the late stages of the studies. In that

situation, all the studies done up to that stage are thrown aside. For this reason, the pharmaceutical industry and regulatory agencies mention that *in vitro* rapid screening tests to be applied in the early stages of drug discovery and development studies and investigation of the cytotoxicity of substances will partially prevent these losses. There is increasing interest in using *in vitro* cytotoxicity tests as alternatives to *in vivo* test systems. Especially, alternative tests which have been dealt with by the scientific world are methods based on the principles of the 3Rs (Replacement, Reduction, and Refinement) that were developed over 50 years ago, providing a framework for conducting more humane research. Today, many alternatives to biological activity tests have been developed, and *in vitro* studies have emerged as an alternative to animal experiments. These tests have become frequently preferred in laboratories due to their ease of application, lowered costs, shortened testing time, reduced numbers of experimental animals as well as small amounts of chemicals needed for testing [7]. On the other hand, nowadays, the fourth "R" has appeared and is defined as responsibility. This principle, which includes the ethical treatment of animals, aims to increase the awareness of researchers on animal welfare [8]. Since raising awareness of alternative approaches, many international organizations have been pioneers in developing new testing methods. However, an important step in the acceptance of alternative methods in regulatory steps is their validation. Therefore, international efforts continue to support the harmonization of processes and principles, the ultimate goal of which is to promote the harmonization of international acceptance and the recognition of alternative methods. In this context, the organizations also transformed their approaches by developing and implementing new *in vitro* concepts. One of the most known initiatives is that the US National Cancer Institute (NCI) established a program to evaluate extracts, fractions, and isolated compounds of natural products in a panel of 60 human tumor cell lines representing various histologies beginning in 1990 instead of their routine *in vivo* approach. In the subsequent 5 years, more than 100,000 materials from natural products were tested with an effective tool for discovering clinically useful new antitumor drugs [9]. Until now, there have been a huge number of studies on cytostatic and cytotoxic effects for many test compounds using cell-based studies in the literature. With compatibility data obtained from *in vivo* studies, these studies supply information for future animal or clinical trials [10].

CYTOTOXIC ACTIVITY POTENTIAL OF NATURAL RESOURCES

Natural resources are of great importance in treating various diseases, including cancer. Over 60% of current drugs used in cancer treatment originated from natural sources [11]. Nature is an important source of biologically active compounds, and few of the isolated natural compounds are directly converted into clinically effective drugs, while the majority serve as models for synthesizing

CHAPTER 6

Activity Methods for Endometriosis

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Abstract: Endometriosis is an estrogen-dependent disease that causes pelvic pain and reduces fertility by developing inflammatory lesions outside the uterine. Although many studies have been performed to investigate the etiopathogenesis of endometriosis, there is still no direct evidence. Endometriosis, a common gynecological disease, often recurs even if treated. *In vivo* studies are used to investigate its etiopathogenesis and potential treatment methods. Hormonal therapy is generally used for endometriosis cases. This conventional therapy aims to lower estrogen levels in the body, however, it can be inadequate for the treatment and has numerous negative effects. The urgency of finding novel and cheap long-term safety therapies for endometriosis is highlighted by the need to manage it as a chronic disease. Medicinal plants and natural plant-derived compounds are interesting options for this purpose. Indeed, there is an increasing interest in using herbal therapy to treat endometriosis. Several studies have been conducted on natural products to find a drug candidate for the management of the mentioned problem. Many of these drugs have a pleiotropic action profile, meaning they block multiple processes involved in endometriosis pathogenesis, including proliferation, inflammation, reactive oxygen species (ROS) production, and angiogenesis. As a result, including them in multimodal treatment approaches may help to improve therapeutic efficiency and reduce adverse effects in future endometriosis treatments. Several methods have been described for the assessment of the potential effectiveness of the bioactive agents against endometriosis. In the present chapter, we aimed to give general information regarding endometriosis, the use of plant products in the treatment of this disease, and the methods that have been used for the activity investigation.

Keywords: Anti-inflammatory, Antioxidant, Bioactivity, Cell line, Endometriosis, Extract, Gynecological disease, *In vivo*, *In vitro*, Medicinal plant, Mouse, Natural products, Rat, Secondary metabolite.

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INTRODUCTION

Endometriosis

The uterus consists of three layers: perimetrium (outer, thin layer), myometrium (thick muscular layer), and endometrium (inner mucosal layer) [1]. Endometriosis, a chronic inflammatory disease, is characterized by the abnormal growth of endometrial cells in the endometrium outside the uterine cavity. Generally, endometrial cells grow within the myometrium, fallopian tube, intestine, abdominal muscle, and umbilicus in endometriosis cases. When the endometrial cells migrate to these ectopic sites, progesterone (P4) resistance with estrogen (E2) dominance can be observed. Therefore, it is also an estrogen-dependent disease that can affect the pelvic organs. Cell proliferation, increased inflammation, and angiogenesis are seen due to E2 dominance [2 - 5].

Etiopathogenesis of the Disease

The etiopathogenesis of endometriosis is still not fully elucidated, although it was first diagnosed in 1860. Many factors, such as immunological, hormonal, genetic, environmental, and psychological, are known to play important roles in the pathogenesis of endometriosis. There are some theories based on the etiopathogenesis of this disorder. One of the most accepted theories is the "retrograde menstruation hypothesis". An amount of the menstrual flow, including eutopic endometrial cells, growth factors, and cytokines, goes backward through the fallopian tubes into the abdominal cavity [3, 5 - 7]. These factors are able to penetrate and proliferate by avoiding apoptosis in some tissues, such as the intestine, abdominal muscle, and umbilicus. It is also known that the growth cells of the endometrium lay in the inner layer of the myometrium. In addition, endometrial implants can involve the fallopian tubes and cause adhesions in the salpinges. Fallopian adhesions can lead to infertility, a secondary cause of endometriosis [3].

In endometriosis, ectopic tissues respond to immunomodulators and inflammatory mediators, eventually initiating the oxidative process. Therefore, related to the pathogenesis of the disease, pelvic inflammation can arise due to an increase in inflammatory cells and cytokines, including peritoneal macrophages, prostaglandins, proteolytic enzymes, complement fragments, interleukins (ILs), tumor necrosis factor-alpha (TNF-α), and growth factors in the ectopic endometrial sites. As a result, these molecules have been detected in the peritoneal fluid of patients with endometriosis.

Apoptosis and angiogenesis also play a fundamental role in the pathogenesis of endometriosis. In other words, reduced apoptosis or enhanced cellular

proliferation in response to appropriate stimuli could result in endometriosis [8, 9]. New blood supply and angiogenesis are required for the survival of endometriosis implants and the growth of endometriosis. The most important angiogenic agent is the vascular endothelial growth factor (VEGF), which is released by activated peritoneal neutrophils and macrophages [10]. Angiogenesis is also aided by platelet-derived growth factor (PDGF), angiogenin, and some growth factors and cytokines such as transforming growth factor beta (TGF-β), IL-8, erythropoietin, hepatocyte growth factor (HGF), neutrophil-activating factor, TNF-*α*, and macrophage migration inhibitory factor [11]. An increase in the synthesis of cellular adhesion molecules such as integrins, cadherins, and intercellular adhesion molecule (ICAM)-1, as well as matrix metallopeptidases (MMPs) like MMP-1, MMP-2, MMP-3, MMP-9, and their inhibitors, was reported. MMPs are zinc-dependent endopeptidases that play important roles in extracellular matrix remodeling [12]. The B cell lymphoma-2 (Bcl-2) family, the Fas/FasL system, and cysteine-aspartic proteases (caspases) all play essential roles in the regulation of apoptosis, according to several studies. When compared to endometrium from healthy women, eutopic endometrium from women with endometriosis shows a lower expression of pro-apoptotic factors (*e.g.*, Bax- *BCL-2 Associated X*) and higher expression of anti-apoptotic factors (*e.g.*, Bcl-2). Wnt signaling pathways are a group of signal transduction pathways that transfer signals into a cell through protein binding to cell surface receptors. Wnt signaling regulates the early and late stages of apoptosis. This signaling pathway enables the cell to proliferate and regenerate. Wnt signaling is abnormally activated due to an imbalance in estrogen and progesterone levels in cases of endometriosis. Estrogen leads to the induction of the Wnt ligand expression. This process causes the transcription of MMP9 and VEGF genes and endometrial cells to migrate to other organs. Like Wnt signaling, Hh and Notch [13, 14] signaling pathway is also associated with estrogen and progesterone hormones. These signaling pathways also mediate cell proliferation, migration, differentiation, adhesion, and death. Ectopic endometrial tissue can cause pain through neuronal sprouting of nerve fibers in blood vessels. It has been shown that nerve growth factor (NGF) is found in ectopic endometrial tissue [15, 16]. As a result, endometriosis confirmation necessitates extracellular matrix modification and cellular invasion [17].

The Classification of Endometriosis

Endometriosis is categorized as superficial (peritoneal), deep (>5 mm in depth), and ovarian endometriosis. In *superficial endometriosis*, the endometrial cells are implanted and proliferated into the surface of the peritoneum. Vascularization occurs through the subperitoneal blood vessels, and the inflammatory process begins then. Superficial or peritoneal endometriosis can be seen after cesarean

CHAPTER 7

Activity Methods for Polycystic Ovary Syndrome

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Abstract: Polycystic ovary syndrome (PCOS) is one of the most common gynecological disorders among women of reproductive age. The secretion rate and metabolism of estrogens and androgens are disrupted in patients with PCOS. Patients have systemic inflammation, hyperinsulinemia, hyperandrogenism, and polycystic ovaries. Several experimental models have been developed and studied for the investigation of this disease. These studies aim to explore the pathophysiology of PCOS and new treatment approaches. Some plant extracts and phytochemicals obtained from them are shown to be effective against PCOS in literature. In this chapter, general information about PCOS, plant-based treatment approaches, and activity methods are explained in detail.

Keywords: Animal models, activity methods, gynecological disease, medicinal plants, natural products, polycystic ovarian syndrome.

INTRODUCTION

Ovarian cysts are among the most important endocrine disorders affecting women's health. Follicular growth, ovulation, and regression of the corpus luteum are important processes for the continuity of the estrus cycle. The endocrine imbalance occurring in the hypothalamus-pituitary-gonadal axis leads to the formation of cystic structures in the ovaries, and therefore, the estrus cycle is disrupted [1, 2].

Polycystic ovary syndrome (PCOS) is the most common ovarian problem among women of reproductive age. It is defined as polycystic ovary syndrome because it consists of many small fluid-filled cysts in the ovaries. This syndrome is a condit-

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ion that occurs due to abnormal androgen production in the ovaries. Thus, it is characterized by hirsutism, disruption of the menstrual cycle, obesity, and infertility. Since PCOS is a heterogeneous disease, its etiology has not been fully explained yet. PCOS affects 1 in 16 young women and causes anovulatory infertility [3, 4].

PCOS can be considered a *reproductive disorder* as well as a *metabolic disorder. Reproductive effects* include anovulation which leads to oligomenorrhea, amenorrhea, infertility, and androgen increase. The outcomes caused by PCOS, including insulin resistance, obesity, dyslipidemia, and hypertension are classified as *metabolic disorders*. Indeed, PCOS patients also take various medications to treat different aspects of the disease due to the increased risk of cardiovascular disease, insulin resistance, and dyslipidemia [5, 6]. Basically, medical treatment of PCOS is aimed at symptom relief. Oral contraceptives are used to regulate menstrual cycles. However, androgen receptor blockers (*i.e.*, spironolactone and flutamide) are preferred to prevent the production of active forms of testosterone. Metformin, known as insulin sensitization, is prescribed to improve insulin sensitivity, lose weight and decrease lipid levels [5]. Furthermore, it helps to improve the success rate of ovulation inducers (*i.e.*, clomiphene) [7]. Besides, medicinal plants and natural compounds derived from them can be effective in PCOS treatment. Especially, phytoestrogens and polyphenols can improve PCOS symptoms *via* affecting serum levels of sex hormones, insulin resistance, and ovulation. There are several methods to evaluate efficacy against this disease. In light of the literature data, this chapter aimed to give general information about PCOS and activity methods used for the investigation of bioactive plant extracts and natural compounds in the treatment of PCOS.

PCOS

Etiopathogenesis

PCOS is observed due to the defect, especially in the theca cells of the ovary. This situation causes abnormal androgen synthesis, which reflects the clinical and biochemical findings of the disease. It has been reported that genetic factors are involved in the etiology of PCOS. However, a high ratio of luteinizing hormone (LH) to follicle stimulating hormone (FSH) is one of the main causes. The enhancement of gonadotropin-releasing hormone (GnRH) pulse frequency has also been suggested as the cause of PCOS. In addition, weight loss reduces the clinical symptoms of PCOS, while obesity increases menstrual irregularities and hyperandrogenism. Factors that cause an increase in adrenal androgen production can also stimulate PCOS by causing changes in peripheral cortisol metabolism [8, 9].

Genetic Factors

The role of genetic factors had been concluded on the basis of obtained evidence from family relationships. Mothers and fathers of women with PCOS are more likely to have cardiovascular disease than those without PCOS. It has been stated that transcription factor 7-like 2 (TCF7L2) single-nucleotide polymorphism (SNP) is associated with the development of type 2 diabetes mellitus and weight gain, and genes such as insulin-induced gene 2 (INSIG2) and melanocortin 4 receptor (MC4R) are effective in increasing insulin resistance in women with PCOS [6].

Anovulation

Anovulation is a condition in which the ovaries do not ovulate in a normal cycle process. The absence of ovulation within the cycle is usually noticed in women who cannot conceive. Ovulation failure occurs due to endocrine abnormalities in the hypothalamo-pituitary-gonad axis, which controls egg release through the ovarian cycle. Ovulation cannot occur as a result of failure of the dominant follicle selection in anovulatory infertility. In women with PCOS, many follicles begin to mature, however, there is a failure to select the dominant follicle and the formation of multiple small cysts is characterized. Another cause of anovulation is the enlargement of the stroma of the ovaries and an increase in the number of theca cells that produce testosterone. Follicle immaturity and increased testosterone level are seen due to a disruption in the hypothalamo-pituitary system (GnRH pulsation) that causes excess LH concentration and insufficient FSH concentration. It is known that FSH provides follicular maturation and the production of estrogen in the granulosa cell. Increased LH to FSH ratio promotes androgen synthesis and causes normal follicle maturation and ovulation. Insulin acts synergistically with LH to increase the production of theca cells and inhibits hepatic synthesis of sex hormone-binding globulin (SHBG). Therefore, it is important to reduce the level of circulating insulin for the improvement of ovarian function, ovulation and fertility rates in the metabolic treatment of PCOS [7, 10].

Androgen Excess/Hirsutism

Aromatase is an enzyme responsible for the biosynthesis of estrogens. This enzyme particularly provides the aromatization of androgens into estrogens. It transforms androstenedione to estrone and testosterone to estradiol. The hypo-expression of aromatase can lead to PCOS. The ovaries produce excessive testosterone, and SHBG is decreased in PCOS, as described above. The increased testosterone is converted to the more active form of dihydro-testosterone after binding to androgen receptors in the hair follicles. Excess androgens increase facial and body hair and cause scalp hair loss [6, 11].

CHAPTER 8

Methods for Studying the Activity of Natural Extracts on Renal Transporters İnvolved in the Regulation of Blood Pressure

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Abstract: Hypertension is one of the leading causes of chronic kidney disease (CKD) due to the deleterious effects that increased blood pressure (BP) has on kidney vasculature. Patients with CKD and hypertension often require a combination of antihypertensive medications to target BP. Several classes of antihypertensive/diuretic agents may have a role in the treatment of CKD and hypertension. Besides these conventional therapies, it is extremely useful to identify new natural sources (herbs and botanicals) of diuretics and their targets in the kidney that can be used alone or in combination with traditional synthetic drugs. The use of herbal medicine and natural products to contrast hypertension has increased many-fold over the past decade driven by documented experimental evidence. However, many nephrologists are unaware of these potential therapeutic benefits in treating hypertension, mainly for the lack of knowledge of molecular mechanisms underlying their effects. Indeed, rigorous preclinical assays are necessary to scientifically validate the utilization of medicinal plants for the treatment of hypertension. This chapter provides detailed methods to study the effects of two representative herbal extracts on renal transporters involved in blood pressure regulation by *in vitro*, *in vivo,* and *ex vivo* approaches. Of note, these methods can be applied to any herbal extracts.

Keywords: Nephron, Herbal extract, Aquaporins, Ion channels, Membrane transporters.

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INTRODUCTION

Preclinical Activity Assessment of Natural Product on Renal Transporters

The first step for designing a preclinical assay is the identification of a valid target. In the mammalian kidney, the loop of Henle plays a crucial role in the urine concentrating mechanism [1] and consequently in blood pressure regulation [2]. The descending part of the loop reabsorbs water while the ascending part, the thin and thick segments, mainly reabsorb salt. The thin ascending limb (tAL) exhibits a large Cl^- permeability in both the apical as well as basolateral membrane, which is mediated by the renal specific ClC-Ka channel in association with its accessory subunit barttin [3]. The resulting positive transepithelial voltage provides a driving force for paracellular Na^+ reabsorption. The role of this segment of the nephron on the regulation of the blood pression is often underestimated. Of note, mice lacking the ClC-Ka homologue, ClC-K1 KO mice, exhibit a phenotype resembling Nephrogenic Diabetes Insipidus (NDI), characterized by dramatic polyuria associated with low urine osmolality [4], thus suggesting ClC-Ka as a suitable target for diuretics. In the thick ascending limb (TAL), the functional cooperation between the apical $Na^+/2Cl^-/K^+$ cotransporter (NKCC2) and the inwardly rectifying potassium channel (ROMK1) with the basolateral complex of ClC-Kb/barttin results in a transcellular and electrogenic NaCl reabsorption. This salt movement toward the *interstitium* generates the cortico-medullary osmotic gradient needed for water reabsorption in the water-permeable tracts of the nephron. For this reason, NKCC2 is a known target for diuretics (loop diuretics) and a suitable target for new and natural diuretics. In the collecting ducts, the vasopressin-dependent aquaporin 2 (AQP2) translocation on the luminal membrane of collecting duct cells renders collecting ducts permeable to water, thus making this nephron segment responsible for the concentrating urine mechanism. This segment is indeed a suitable target for the so called 'aquaretics', which unlike the other diuretics discussed previously, enhance the excretion of electrolyte-free water by blocking either arginine vasopressin binding to vasopressin receptors (V2Rs) or AQP2 translocation in collecting ducts.

Indeed, this orchestrated plethora of membrane transporters regulating urine concentration and blood volume provides a number of possible targets for antihypertensive therapies with herbal extracts. In the following, we will describe functional assays based on the activation mechanisms of these transporters. NKCC2 may serve its function in the TAL upon its phosphorylation on regulatory threonines on the N-terminal tail [5]. Indeed, a natural extract able to inhibit NKCC2 phosphorylation either in renal cells or in isolated kidneys can be considered a possible 'loop diuretic'. On the other hand, ClC-Ka can be functionally expressed in cells only in combination with its cooperative barttin

subunit and the rate of its activation can be calculated as the amount of Cl^- current flowing through the channel by patch-clamp electrophysiology [6]. Indeed, a natural extract with diuretic activity targeting ClC-Ka channel should be able to decrease ClC-Ka dependent Cl^- currents.

Then, AQP2 translocates at the apical membrane of collecting ducts upon the increase in the levels of intracellular cAMP resulting from the activation of adenylate cyclase-coupled receptors such as V2Rs and Beta 3 adrenergic receptors [7, 8]. This event can be monitored by immunofluorescence laser confocal imaging in polarized AQP2-expressing cells. A natural extract acting as a diuretic should be able to inhibit the translocation of AQP2 toward the apical membrane.

Finally, when administered *in vivo* a natural extract with a diuretic effect should be able to increase urine volume output as well and Na^+ and Cl^- urinary excretion [9].

In vitro Assay Procedures

Experimental Models for the Bioassays

- To study NKCC2 activation "*in vitro*", transfect HEK293 cells with full-length NKCC2 [10] using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and select stable clones in culture medium supplemented with 1 mg/ml geneticin.
- To study ClC-Ka activity, transfect HEK293 cells with two pcDNA3.1 plasmids encoding for ClC-Ka tagged with GFP at the N-terminal tail and for barttin tagged with m-Cherry at the C-terminal tail, respectively [6].
- To study AQP2 translocation, stably transfect M-1 cells with a cDNA that encodes for human AQP2 as previously described [4, 9]. The stable AQP2-expressing M1 cells that we used in our functional experiments are reported as MCD4 cells.
- Maintain HEK293 cells in Dulbecco's modified Eagle's medium high glucose, 2 mM l-glutamine, 10% foetal bovine serum, penicillin (50 U/mL) and streptomycin (50 U/mL) at 37°C, 5% CO_2 in a humidified incubator.
- Maintain mouse cortical collecting-duct M1 cells in DMEM/F12 1:1 supplemented with 5% foetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 5 μM dexamethasone at 37°C, 5% CO_2 in a humidified incubator.

CHAPTER 9

Activity Methods for Cardiovascular System Diseases

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Abstract: This chapter provides a collection of methods generally used in experimental pharmacology to test compounds with different activities in the cardiovascular system. There are many models *in vitro* and *in vivo* and we have highlighted those used in the field of natural compounds research. For the *in vitro* procedures, the focus was on the inhibition of enzymes, such as an angiotensin-converting enzyme or nitric oxide synthase, which plays a pivotal role in maintaining vascular control and overall blood pressure. Moreover, test procedures on isolated vessels were also considered for compounds whose activity is exerted on specific targets expressed in the vasculature. Regarding the *in vivo* methods, heart failure and hypertension models were highlighted for their high incidence of overall deaths cause. In particular, we have analyzed models of heart failure in rats and mice, animals generally used in preclinical studies. In addition, it also evaluated useful models of hypertension involving mainly rats; however, in many cases, the procedure could be translated to different animal species.

Keywords: Cardiovascular system, Hypertension, Endothelium, Cardiac failure, Vascular function.

INTRODUCTION

The diseases associated with cardiovascular system failure represent the major cause of morbidity and mortality throughout the world. Thus, it is consistent that current scientific biomedical research is focused on discovering the underlying mechanisms in pathologies and directed towards identifying novel or more effective treatments that could change and/or modify the actual situation. In addition, nowadays, natural compounds have gained significant attention for the cost-effectiveness balance since they are often less expensive than chemicals used in the pharmaceutical industries. Another important key issue is the possibility of

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finding beneficial properties in compounds that are, or could be, part of the daily diet. Natural medicaments have been largely used for centuries to treat several affections; however, it has always been difficult to dissect the actual compound/s responsible for therapeutic activity. Therefore, the possibility of testing natural compounds for their effects on the cardiovascular system and uncovering the underlying mechanism of action could open wide new perspectives in the therapeutic scenario and preventive "medicine". This chapter provides several approaches that are useful to unmask possible effects derived from natural compounds that could be, in turn, considered novel drugs in natural medicine.

Extract, Doses and Administration in Experimental Methods

All fruits, nuts and vegetables were used as whole or separated into the skin, "pulp" or "meat". Therefore were weighed and minced in 2 volumes of deionized water. Therefore, pestle grinding was carried out for at least 5 minutes.

Commercial herbs and spice powders were used in water (10 mg/ml) and mashes were allowed to sit for a time of 5 to 10 minutes at room temperature (normally ranging between 21°C and 23°C) in the case of fruits, nuts, herbs, and spices.

For vegetables, a heating procedure in boiling water was used, followed by filtration.

For initial screening, extracts were used in a volume of 100 µL, and this was changed according to the relative activity (range 50 µL-500 µL). For *in vitro* experiments, the volume was added directly into the isolated organ system; for *in vivo* approach, the extract was given per oral gavage or intraperitoneal injection.

In vitro Assay Procedures

Inhibition of the Angiotensin-Converting Enzyme

Angiotensin-converting enzyme (ACE) is a crucial modulator of vascular function and blood pressure. This protein is part of the Renin Angiontensin Aldosterone system, known as RAAS, and it is able to convert angiotensin-I into Angiotensin II, one of the most powerful contractile agents produced in the human body. Inhibition of ACE is a potent therapeutic strategy to decrease blood pressure in hypertensive patients. Therefore, it is important to find novel compounds able to interfere with ACE activity to regulate blood pressure.

An *in vitro* system can be used to screen potential angiotensin-converting enzyme inhibitors. Fluorescence generated by an artificial substrate in the presence or absence of the inhibitor is measured to detect inhibitory activity.

Reagents

1. 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl

2. 10 mM potassium phosphate buffer, pH 8.3

3. Substrate: O-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline

a) Stock solution: 10 mg substrate in 10mL 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl

b) Working solution: 2 mL stock solution is added to 18 mL 50 mM Tris-HCl buffer, pH 8.0+100 mM NaCl; the final concentration in the assay is 170.2 μM.

4. Test compounds

Compounds are made up to a concentration of 1 mM in 50 mM Tris-HCl buffer, pH 8.0 + 100 mM NaCl, or 10% methanol in Tris/NaCl if insoluble in aqueous buffer alone. This will give a final concentration in the assay of 0.1 mM. If inhibition is seen, further dilution in Tris/NaCl should be made. If the compound concentration is not determinable, methanol/ethanol extract could be used and tested to determine the exact range of activity.

Enzyme Preparation

Lung tissue from 10 rats is diced and homogenized in a blender with 3 pulses of 15 s each. The homogenate is centrifuged at 5000 g for 10 min. The pellet is discarded, while the supernatant is dialyzed by using 1.0 liter of 10 mM potassium phosphate buffer, pH 8.3, overnight at 4°C for three times. Therefore, centrifugation (40,000g for 20 min) is performed as the final step. The pellet is discarded, 390 mg $(NH_4)_2SO_4$ is added for each ml of supernatant. This will give 60% saturation. The solution is stirred on ice for 15 min. The pellet formed is dissolved in 15 mL potassium phosphate buffer, pH 8.3, and dialyzed against the same buffer overnight at 4°C three times. Some proteins will precipitate during dialysis. The suspension is centrifuged at 40,000 g for 20 min and the supernatant is discarded. The final solubilized enzyme preparation can be aliquoted and stored at –20°C for at least 6 months.

Enzyme Inhibition Studies

1. Enzyme activity is measured using a Fluorescence Spectrophotometer (excitation wavelength: 357 nm; emission wavelength: 424 nm).

2. Enzyme assay: 50 μL vehicle or inhibitor solution and 40 μL enzyme are

CHAPTER 10

Activity Methods For Animal Pharmacokinetic and Pharmacodynamic Studies

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Abstract: This chapter provides an overview of the main features that need to be assessed with respect to the general pharmacology (pharmacokinetics, PK; pharmacodynamics, PD) of all molecules, including natural compounds. Here, the attention has been focused on bioavailability, the volume of distribution, and clearance to describe the physiological processes of absorption/distribution and elimination of drugs from the body. It is worth pointing out that PD studies always require an *in vitro* preliminary approach, not issued here, that needs to be assessed and confirmed *in vivo*. Indeed, PD studies aim to the individuation of specific molecular targets, responsible for an observed pharmacological effect. Thus, the use of animal models as a complex approach does not allow to dissect the molecular mechanisms of natural compounds but can confirm their efficacy *in vivo*. Therefore, specific PD approaches to determine a specific action by a compound are addressed in the relevant chapters within this book.

Keywords: Pharmacokinetics, Bioavailability, Distribution, Elimination, Half-life.

INTRODUCTION

Much debate surrounds the issue of which animal species most closely resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans [1]. Due to availability issues (mainly compound) and animal care laws, small rodents, such as rats or mice, are usually used for primary *in vivo* evaluation of novel agents' pharmacokinetics and, therefore, for natural product investigations. However, there is a great reservation about moving a compound into clinical trials based on oral bioavailability data derived only from rat experi-

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ments. For this reason, larger animals such as dogs or non-human primates, which have similar gastrointestinal morphology to humans, are the next step in evaluating the pharmacokinetics of new agents. Studying the pharmacokinetic profile of novel agents in a number of species is, indeed, a well-established approach used to aid in identifying compounds for advancement to human testing [2]. Namely, acceptable bioavailability assessed in different species increases the probability that the selected compound is also bioavailable in humans. Therefore, preclinical *in vivo* pharmacokinetics investigations made on different animal species are important to assess the pharmacokinetic parameters of natural compounds and their appropriate route of administration. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the efficacy of test compounds because the ultimate proof-of-concept experiment is to demonstrate the efficacy by the intended route of administration.

Extract, Doses, and Administration in Experimental Methods Described

To investigate the pharmacokinetics of active molecules from different natural resources, it is important to prepare the vegetal drug or the extract. This step can be pursued in different manners, according to species and types and are subject to variations [3]. For example, when fruits, nuts, or vegetables are studied, they can be used as a whole or separated into the skin, "pulp" or "meat" [4]. The selected section of the natural drug is weighed and minced in 2 volumes of deionized water. Therefore, pestle grinding is carried out for at least 5 minutes.

In the case of commercial herbs, fruit and spice powders can be used in water (10 mg/mL) and mashes are allowed to sit for a time of 5 to 10 minutes at room temperature (normally ranging between 21°C and 23 °C). A heating procedure in boiling water is normally used for vegetables, followed by filtration.

For initial screening, extracts could be used in a volume of 100µL and this can be changed according to the relative activity (range 50 µL–500 µL). For *in vitro* experiments, the volume is added directly into the isolated organ system; for *in vivo* approach, the extract can be given per oral gavage or by intraperitoneal injection.

Pharmacokinetic Studies

Bioavailability Determination

The bioavailability of a drug is the correspondence of a free available amount of an active compound within the bloodstream related to the dose administered. Usually, the maximum bioavailability (100%) is obtained by intravenous (iv) injection of the test compound. The ratio between the area under the curve (AUC)

obtained for a compound following a specific route of administration and that achieved after iv injection represents the bioavailability [5 - 7].

For example, if the same dose of a test compound has an AUC of 432 and 567 after oral and iv administration, respectively (Fig. **1**), therefore, its oral bioavailability is (432/563) x 100 = 77%. If the same compound gives an AUC of 236 after subcutaneous administration, its bioavailability is (236/563) x 100 = 42%.

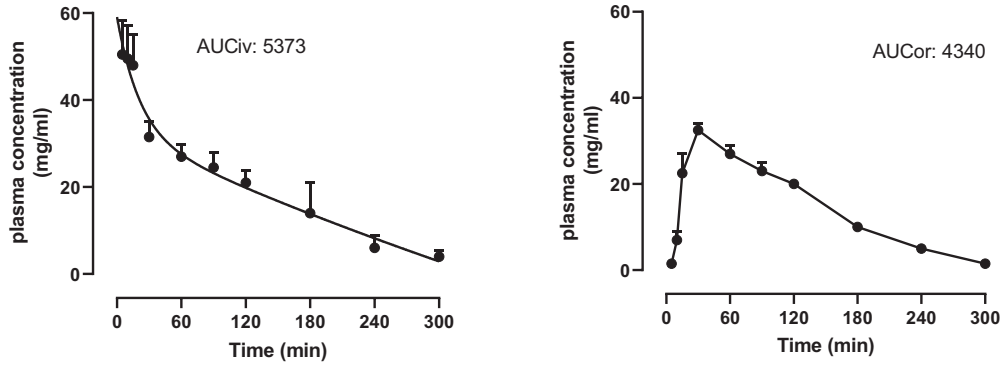

Fig. (1). Example of drug plasma concentration trend following two different administration routes (intravenous, iv; oral, or). AUC determination has been assessed by using statistics software.

The determination of the AUC for each route of administration or compound tested is performed by taking blood from different veins, depending on the animal species considered. For instance, in mice, a caudal or cannulated jugular is used to collect blood samples to quantify the test compound. However, it is noteworthy that quantification also depends on the nature of the compound and the analysis method available in each research laboratory [8](Birer-Williams *et al.*, 2020).

Procedure and Calculations

The tested compound is given intravenously, and blood samples are collected at 5, 10, 15, 30 minutes, 1h, 1h 30min, 2h, 3h, 4h, 5h, 6h, 12h, 24h, 48h, or at a longer time if needed. The concentration of the compound within the blood samples is performed by colorimetric, fluorometric, mass spectrometry and HPLC apparatus, then the curve is obtained by using appropriate statistical software. The same procedure is followed for an alternative route of administration (*e.g.*, oral). Indeed, the example reported in (Fig. **1**) displays the determination of AUC after iv injection that is compared to that achieved following oral drug dosing. The calculation is reported below:

AUC iv (4340) / AUC or (5373) x 100 = 81%.

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