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Parimelazhagan Thangaraj

Pharmacological Assays of Plant-Based Natural Products

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Parimelazhagan Thangaraj

Pharmacological Assays of Plant-Based Natural Products

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Parimelazhagan Thangaraj
Department of Botany
Bharathiar University
Coimbatore, Tamil Nadu
India

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To Bioprospecting Research Team

Foreword

The health benefits and economic value of traditionally used medicinal plants are getting increasing attention in the past few decades. This is evident from the increase in the usage of herb-based drugs for the treatment of various diseases. Herbal medicines are prepared from live or dried plant resources and contain hundreds to thousands of interrelated active compounds. Science is beginning to demonstrate that the safety and effectiveness of herbs are often related to the synergy of its many constituents. The effectiveness thus relies on the multiple pathways of how a drug from plants would counteract on a particular disease. To a large extent, scientists and researchers all over the world have excelled in proving the exact mechanism behind the therapeutic property of herbal medicines and many are in the pipeline. This is the right time where this book would engage young researchers to have some novel approaches towards identifying cost-effective medicine from plants with prime concern to their conservation such that people who struggle for depending on costly synthetic drugs would gather a big relief in their search for healthy life.

It is indeed a great pleasure that Dr. T. Parimelazhagan is on the right track with his research responsibilities. He has taken a sincere effort to identify the needs of young researchers in the area of medicinal plant research. The way the protocols are sketched in the book will ease them to excel in their experiments to evaluate the usefulness of medicinal plants. This book will be great resources for the researchers to build up new steps to keep on climbing in their area of research.

I truly believe that the book will open new avenues for the students and researchers to focus into the new era of research in medicinal plants.

Mahavir B. Chougule
M. Pharm., Ph.D., Associate Professor
Department of Pharmaceutical Sciences
The Daniel K. Inouye College of Pharmacy
University of Hawaii
Hilo, USA

Preface

Plants are one of the best reservoirs of medicinal wealth among the natural medicines. The interest in harvesting them for the well-being of mankind has increased with increasing demand of drugs for diseases of major concerns and those emerging with modern lifestyles. But the challenge is in ensuring the safety and sustainability of such drugs over synthetic ones. Hence, it becomes necessary to validate the natural medicine from plants with various scientific experiments. At the same time, it also becomes necessary to built conservation strategies to resist mass destruction of plant life from earth. The prime aim of the book is to include all those scientific analyses of a plant that would promote it to a reliable medicine for human health. Here, an attempt has been made to combine the works done by the eminent researchers and scientists all over the world in such a manner that a quick glance would give hand full of information about how to initiate and move on research in phytomedicine. The book compiles the most relevant and recent trends of scientific information pertinent to ethnobotany, ethnopharmacology, phytochemistry, bioinformatics and biotechnology. The book also comprises experiments and protocols standardized by the research scholars of Bioprospecting Laboratory, Department of Botany, Bharathiar University. A researcher would find it very useful for the selection of plant, its use in various in vitro and in vivo studies, isolation and identification of active molecules, etc. This book contains 32 major assays with a special emphasis laid on screening of herbal drugs for antioxidant potential, pharmacological activity and phytochemistry involved in herbal research. The review processes of the articles have been carried out by the experts from various universities and research institutes. We hope the present compilation will be useful for the students, research scholars, academicians and industrialists, and people associated with herbal research. The author would like to convey sincere thanks to the Bharathiar University authorities and Prof. V. Narmatha Bai, head of the department, for their support and encouragement. The author would also like to thank his research team comprising of Dr. R. Senthil Kumar, Dr. Blassan P. George, Dr. M. Iniyavan, Dr. K. Arunachalam, Dr. S. Saravanan, Rahul Chandran, Sajeesh T., Murugan R., P. Revathi, Harini S., Dhivya S., Saikumar S., Kasipandi M. and Elizebeth George for their contribution in the

compilation of the assays in a well-designed manner. We would like to express our special appreciation for the publishers and their team for the sincere efforts in bringing out the book in time.

Parimelazhagan Thangaraj

Contents

Chapter 1
Ethnobotanical Study

Abstract Ethnobotany is the study of interrelationships between human cultures and plants, animals, and other organisms in their environment. It also creates an awareness of the link between biodiversity and cultural diversity. From the beginning of civilization, people have been using plants for various purposes like food, shelter, medicines, etc. Ethnobotanists play a key role in exploring these kinds of information from indigenous people which creates a gateway for formulating a novel drug. The content in this chapter deals with these aspects in an approachable manner.

Introduction

Ethnobotany, the largest subdiscipline of ethnobiology, is generally defined as the 'science of people's interaction with plants' (Turner 1995). Ethnobotany stands at a crossroads between social and biological sciences; ethnobotanists have the responsibility to address the importance of wild medicinal plants, and documentation of indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources (Muthu et al. 2006). Therefore, establishment of local names and documentation of the indigenous uses of plants have significant potential societal benefits (Bağci 2000). It helps to understand the relationship between plants and human beings and to conserve heritage sites. It could create an awareness of each species and their benefits; this knowledge can be exploited for prospecting novel drugs.

Aim

The aim is to document the traditional knowledge from people and evaluation through various ethnobotanical tools about the utilization of plants.

Principle

The tools in this study help in measuring particular plant species abundantly used by people for various ailments. The documentation of traditional knowledge play a

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key role in bioprospecting of novel drug from the medicinal plants and also in situ conservation of medicinally valuable plants.

Materials required

Research diary, camera, plant press with all the required materials such as scissors, plant clippers, field data sheet, collection tag (collection tag can be prepared by using thread and chart paper), collection bag—plastic and paper bags.

Protocol

1. Select the traditionally enriched area or tribal community.
2. Approach the people with the help of a familiar person of an area or need to become familiar by frequent visit and to know the knowledgeable persons in the area.
3. Collect the information of plants from different people through interviews by using an uncomplicated questionnaire like following:
   - Does the person know the plant?
   - Can the person recall a name for the plant?
   - Can the person recall any uses for the plant?

   (Similar to the method described by Martin (1995) with some modifications.)
4. Cross-check the acquired data from other local informants either by showing the plant specimen or notifying the local names of plants.
5. Initially identify the plants by their vernacular names through consultation with the local people. The scientific identification of plants could be done with the help of taxonomists.

Data analysis tools

Use value (UV)

The relative importance of each plant species known locally to be used as herbal remedy is termed as UV, and it was calculated using the following formula (Barnert and Messmann 2008).

$$\mathrm{UV} = \frac{\sum U}{n}$$

where UV is the use value of a species, U is the number of use-reports cited by each informant for a given plant species, and n is the total number of informants interviewed for a given plant. The UV is helpful in determining the plants with the highest use (most frequently indicated) in the treatment of an ailment. UVs are high when there are many use-reports for a plant and low when there are few reports related to its use.

Fidelity level (FL)

FL is used to determine the most frequently used plant species for treating a particular ailment category by the informants of the study area. The FL is calculated using the following formula (Martin 1995).

$$\mathrm{FL}\,(\%) = \frac{\mathrm{Np}}{N} \times 100$$

where Np is the number of use-reports cited for a given species for a particular ailment category and N is the total number of use-reports cited for any given species. Generally, high FLs are obtained for plants for which almost all use-reports refer to the same way of using it, whereas low FLs are obtained for plants that are used for many different purposes (Heinrich et al. 1998).

Informant consensus factor (Fic)

Fic is used to see whether there is an agreement in the use of plants in the ailment categories between the plant users in the study area. The Fic was calculated using the following formula (Bağci 2000):

$$\mathrm{Fic} = \frac{\mathrm{Nur} - \mathrm{Nt}}{\mathrm{Nur} - 1}$$

where Nur refers to the number of use-reports for a particular ailment category and Nt refers to the number of taxa used for a particular ailment category by all informants. The product of this factor ranges from 0 to 1. A high value (close to 1.0) indicates that relatively few taxa are used by a large proportion of informants. A low value indicates that the informant's disagree on the taxa to be used in the treatment within a category of illness. This method is used to check the homogeneity of information among the users. Fic values will be low (close to 0 value) if plants are chosen randomly or if informants do not exchange information about their use and values will be high (close to 1 value) if there is a well-defined selection criterion in the particular community or if information is transmitted between the informants (Kaya 2006).

Field data sheet preparation

The following information is necessary in field data sheet:

General information (collection number, date, locality, recorded by, interpreter), geographical information (latitude, longitude, altitude, temperature, rainfall, soil, topography, vegetation), social information (community, population size, informant name, age, gender, occupation, linguistic, religion) and botanical information (plant local name, botanical name, family, habit, habitat, parts used, ingredients, mode of preparation/processing, mode of administration, medicinal use, other uses).

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Chapter 2
Pharmacognostical Studies

Abstract The chapter deals with tools and techniques employed in pharmacognosy. Pharmacognostic evaluation helps to screen the commercial varieties, substitutes, adulterants and any other quality control of the drugs. It is a simple and reliable tool, helps to obtain information about biochemical and physical properties of crude drug. Methods such as macroscopic and microscopic analysis, maceration, histochemical colour reaction, photomicrography, organoleptic character of plant powder and extracts, fluorescence analysis of plant powder with different chemical reagents, determination of pH of plant powder, water solubility index (WSI) and water absorption index (WAI) and acid value are discussed.

Introduction

Pharmacognosy is the study of medicinal material derived from natural source. It is the study of the physical, chemical, biochemical and biological properties of drug found in nature as well as the search of new drug from natural origin. Pharmacognostic evaluation helps to screen the commercial varieties, substitutes, adulterants and any other quality control of the drugs. It is a simple and reliable tool, by which the complete information of the crude drugs can be obtained (WHO 1998).

Aim

To find out the macroscopic, microscopic, histochemical and physicochemical characteristic features of the plant sample.

Principle

When the sample is treated with particular chemical agent, it forms specific colour or it predicts specific substances or cells through which it aids for the quality control of drug.

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Materials

1. Test tubes, Whatman No. 1 filter paper, measuring cylinder, funnel, water bath, embryo cup with lid, slides and cover slip.
2. Formalin (mix formalin, acetic acid and 70 % ethanol in ratio of 1:1:12).
3. Safranin (mix 1 g of safranin in 10 mL of 95 % ethanol).
4. Fast Green (take ethanol and methyl salicylate in the ratio of 1:1 and then mix with 14 mg of Fast Green).
5. Clearing solution (mix methyl salicylate, absolute alcohol and xylene in the ratio of 2:1:1).
6. Jeffrey's maceration solution (1:1 of 10 % nitric acid and 10 % chromic acid).

Protocol

2.1 Macroscopic Analysis

The morphological characters (Trease and Evans 1983; Wallis 1985) of the plant sample being observed are as follows:

1. Shape;
2. Surface;
3. Colour;
4. Size.

2.2 Microscopic Analysis

1. Initially, pile up the sections of plant parts in formalin for fixation.
2. Then stain the sections with safranin for 5 min and then wash it with water.
3. Treat the sections with 30, 70, 90 and 100 % ethanol for 5, 1, 1 and 1 min, respectively, for dehydration.
4. After that, stain the sections with Fast Green for 15 s.
5. Wash the sections with absolute alcohol for 1 min and with clearing solution for 2 min.
6. Then treat the sections with xylene for less than 10 s.
7. Finally, mount the sections on the slide using D.P.X. liquid mountant (Pandey 2005).

2.3 Maceration

1. Macerate the plant samples with Jeffrey's maceration solution.
2. Decant remaining acid and then wash the bleached powder fragments with water repeatedly.
3. Add a few drops of ammonium hydroxide for neutralization.
4. Stain the macerated plant samples with safranin and mount using glycerine (Pandey 2005).

2.4 Histochemical Colour Reaction

The histochemical colour reactions of plant samples are performed separately in order to identify major cell components by chemical reagents. The following table represents the procedure of histochemical reaction (Khandelwel et al. 1996).

S. No.	Reagents used	Test	Colour formation	Histochemical zone
1	T.S. of plant parts + iodine solution	Starch	Blue	Spongy parenchyma
2	T.S. of plant parts + iodine solution + H_2SO_4	Cellulose	Bright yellow	Chlorenchyma
3	T.S. of plant parts + safranin	Lignin	Red	Vascular zone
4	T.S. of plant parts + methylene blue	Mucilage	Deep violet	Spongy parenchyma
5	T.S. of plant parts + amido black	Protein	Green	Cambium

2.5 Photomicrography

Photographs of microscopic section of different magnifications are taken with Olympus BX51 light microscopic unit. Descriptive terms of the anatomical features are as given in the standard anatomy book (Esau 1965).

2.6 Organoleptic Character of Plant Powder and Extracts

The organoleptic parameters (Trease and Evans 1983) of plant powder and the extracts are as follows:

1. Colour;
2. Texture;
3. Odour.

2.7 Fluorescence Analysis of Plant Powder with Different Chemical Reagents

1. Take a pinch of plant powder and treat it with different chemicals separately.
2. Use the chemicals such as sodium nitroprusside, lead acetate solution, potassium hydroxide, 1 N NaOH, 1.5 N HCl, conc. H_2SO_4, HNO_3, 50 % H_2SO_4 and 0 % HNO_3.
3. Then allow the mixture to stand at room temperature for 5 min.
4. Then filter it using Whatman No. 1 filter paper.
5. After this process, observe the colour changing behaviour of the plant powders under daylight and UV light (Kokoshi et al. 1958).

2.8 Determination of PH of Plant Powder

1. Take 1 g of plant powder in the conical flask.
2. Add 10 mL of distilled water to the conical flask and blend it.
3. Then allow it to stand for 5 min at room temperature.
4. Measure the pH of sample using pH meter (Indian Pharmacopoeia 2010).

2.9 Water Solubility Index (WSI) and Water Absorption Index (WAI)

1. Take 2.5 g of plant powder in a 50-mL centrifuge tube and add 30 mL of distilled water to it at 30 °C and stir intermittently for 30 min.
2. Then centrifuge for 10 min at 5100 × *g*.
3. Pour the supernatant carefully into a Petri dish and then allow both supernatant and pellet to dry overnight (Gomez 1984).

Calculation

WSI = Amount of solid in the dried supernatant/weight of plant powder
WAI = Weight of dry solid/weight of plant powder

2.10 Acid Value

1. Take 1 g plant sample and then dissolve it in 50 mL of equal volume of ethanol (95 %) and petroleum ether.
2. Then filter the sample using Whatman No. 1 filter paper.
3. Then add few drops of phenolphthalein and then titrate it with 0.1 M potassium hydroxide until it remained faintly pink after shaking for 30 min.

Calculation
Acid value is calculated by the formula:

$$\text{Acid Value} = 5.61\ n/W,$$

where n = number of mL of 0.1 M potassium hydroxide required and W = weight in grams of substance (Morkhade et al. 2006; Ohwoavworhua and Adelakun 2005).

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Chapter 3
Extraction of Bioactive Compounds

Abstract A bioactive compound influences the health of living organisms and it has extranutritional constituents that typically occur in low quantities in foods, which helps to enhance or boost the immune system. Plants and their products possess bioactive compounds, i.e., secondary metabolites. Here, extraction is an important process to isolate the bioactive compounds. Biological activities of the extract show a significant variation depending on the extraction methods and this also opens a gateway for selecting suitable extraction methods. Hence, different extraction methods have been discussed in this section, which influences the extraction of phytochemicals.

Introduction

Natural products or plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. Extraction is an important step involved in the discovery of bioactive components from medicinal plants. If the plant was selected on the basis of traditional uses, then it is needed to be prepared as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. Different extraction methods have been used to extract polyphenolic compounds from plant materials. Biological activities of plant extracts showed significant differences depending upon the different extraction methods, emphasizing the importance of selecting the suitable extraction method.

Aim

To extract the bioactive compounds from the plants by different extraction methods.

Preparation of plant extracts

The basic steps include prewashing, drying of plant materials or freeze drying, and grinding the plant sample to obtain a homogenous sample.

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3.1 Maceration (Chandran et al. 2012)

1. Weigh 10 g of plant material.
2. Extract the plant material with organic solvents such as n-hexane, ethyl acetate, methanol and ethanol (100 mL) in a mechanical shaker with temperature control (room temperature) at constant stirring rate at 200 rpm.
3. Leave the sample for 24 h and filter it using Whatman No. 1 filter paper.
4. Repeat the extraction for three times.

3.2 Soxhlet Extraction (Sajeesh et al. 2011 and Arunachalam et al. 2011)

Soxhlet extraction can be done in two ways:

(a) Direct—Extraction using single solvent
(b) Successive—Extraction using solvents successively in the order of polarity; generally low polar to high polar.

1. Weigh 100 g of plant material and prepare thimble by packing the plant material using a Whatman No. 1 filter paper.
2. Place the thimble in a Soxhlet extractor.
3. Extract the plant material with organic solvents (300 mL) such as n-hexane, ethyl acetate, methanol.
4. Collect the crude extracts after redistilling the solvent.
5. Concentrate by rotary vacuum evaporator and then air dry.

3.3 Fractionation (Murugan and Parimelazhagan 2014)

1. Weigh 50 g of plant material and prepare thimble by packing the plant material using Whatman No. 1 filter paper.
2. Place the thimble in a Soxhlet apparatus.
3. Extract the plant material with high-polar organic solvents (acetone, ethanol, methanol, etc).
4. Collect the crude extracts from the sample.
5. Concentrate the extract by removing the solvent using rotary vacuum evaporator.
6. Again, extract the methanol crude extract packed in thimble successively by organic solvents using Soxhlet apparatus.
7. Collect the fractions of crude extract and concentrate by rotary vacuum evaporator and then air dry.

Extract yield percentage
Calculate the extract yield by the following formula:

$$\text{Extract Recovery Percent} = \frac{[\text{Container with extract (g)} - \text{Empty container (g)}]}{\text{Amount of plant sample (g)}} \times 100$$

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Chapter 4
Preliminary Phytochemical Studies

Abstract Plants are the natural producers of medicinal agents like alkaloids, flavonoids, tannins, and phenolics. These phytocompounds alone or in combination act as a therapeutic agent in various disease complications. Various chemical reagents are used to determine the major phytochemicals present in plant parts. Protocols involved in screening of alkaloids, carbohydrates, glycosides, saponins, phytosterols, fixed oils, and fats are shown in this chapter.

Introduction

Plants are the sources of traditional medicines containing a wide range of ingredients such as bioactive compounds (alkaloids, flavonoids, tannins and phenolics) that can be used to treat chronic as well as infectious diseases. These are responsible for the medicinal value of plants that produce a definite physiological action on the body. Many plant-derived substances collectively termed "phytonutrients" or "phytochemicals" are becoming increasingly known for their antioxidant activity.

Aim

To identify the presence of phytochemicals such as alkaloids, carbohydrates, glycosides, saponins, phenolic compounds and tannins.

Principle

When the sample is treated with particular reagent, it indicates the presence of phytochemicals with the formation of a complex with the relevant colour formation.

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