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Parimelazhagan Thangaraj

Pharmacological Assays of Plant-Based Natural Products

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Pharmacological Assays of Plant-Based Natural Products

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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.

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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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Materials

Test tubes, test tube stand, Whatman No. 1 filter paper, measuring cylinder, funnel and water bath.

Protocol

4.1 Detection of Alkaloids

Hager's test (Wagner et al. 1996)

1. Initially, take 50 mg of solvent-free extract, stir well with few mL of dilute hydrochloric acid and then filter it.
2. To a few mL of filtrate, add 1 or 2 mL of Hager's reagent (saturated aqueous solution of picric acid).
3. A prominent yellow precipitate indicates the test as positive.

4.2 Detection of Carbohydrates

Molish's test (Ramakrishnan et al. 1994)

1. Take 100 mg of extract and dissolve it in 5 mL of distilled water and then filter it.
2. To 2 mL filtrate, add two drops of alcoholic solution and shake well, then add 1 mL of concentrated sulphuric acid slowly along the sides of the test tube and allow to stand.
3. Formation of a violet ring indicates the presence of carbohydrates.

4.3 Detection of Glycosides

Borntrager's test (Evans 1997)

1. Take 50 mg of extract and hydrolyse with concentrated hydrochloric acid for 2 h on a water bath and filter it.
2. To 2 mL of filtrate, add 3 mL of chloroform and shake well, chloroform layer gets separated followed by it, and then add 10 % ammonia solution to it.
3. Pink colour indicates the presence of glycosides.

4.4 Detection of Saponins

Frothing test (Kokate 1999)

1. Dilute 50 mg of extract with distilled water and make up to 20 mL.
2. Shake the suspension in a graduated cylinder for 15 min.
3. A 2-cm layer of foam indicates the presence of saponins.

4.5 Detection of Protein

Biuret test (Gahan 1984)

1. Dissolve 100 mg extract in 10 mL of distilled water and filter it through Whatman No. 1 filter paper.
2. To 2 mL of filtrate, add one drop of 2 % copper sulphate solution. To this, add 1 mL of ethanol (95 %) followed by excess of potassium hydroxide pellets.
3. Pink colour in the ethanol layer indicates the presence of proteins.

4.6 Detection of Amino Acids

Ninhydrin test (Yasuma and Ichikawa 1953)

1. Add two drops of ninhydrin solution (10 mg of ninhydrin in 200 mL of acetone) to 2 mL of aqueous filtrate.
2. A characteristic purple colour indicates the presence of amino acids.

4.7 Detection of Phytosterols

Libermann–Burchard test (Finar 1986)

1. Dissolve 50 mg extract in 2 mL acetic anhydride.
2. To this, add one or two drops of concentrated sulphuric acid slowly along the sides of the test tube.
3. An array of colour changes shows the presence of phytosterols.

4.8 Detection of Fixed Oils and Fats

Saponification test (Kokate 1999)

1. Add a few drops of 0.5 N alcoholic potassium hydroxide solutions to a small quantity of extract along with a drop of phenolphthalein.
2. Then, heat the mixture on water bath for 2 h.
3. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

4.9 Detection of Phenolic Compounds

Lead acetate test

1. Dissolve 50 mg extract in distilled water, and to this, add 3 mL of 10 % lead acetate solution.
2. A bulky white precipitate indicates the presence of phenolic compounds.

4.10 Detection of Flavonoids

Alkaline reagent test (Raaman 2006)

1. Treat an aqueous solution of the extract with 10 % ammonium hydroxide solution.
2. Yellow fluorescence indicates the presence of flavonoids.

4.11 Detection of Flavonol Glycosides

Magnesium and hydrochloric acid reduction (Harborne 1998)

1. Dissolve 50 mg extract in 5 mL of alcohol and add few fragments of magnesium ribbon and concentrated hydrochloric acid (dropwise).
2. If pink to crimson colour develops, it indicates the presence of flavonol glycosides.

4.12 Detection of Gums and Mucilages (Whistler and BeMiller 1993)

1. Dissolve 100 mg extract in 10 mL of distilled water, and to this, add 25 mL of absolute alcohol with constant stirring.
2. White or cloudy precipitate indicates the presence of gums and mucilages.

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Chapter 5
Proximate Composition Analysis

Abstract The proximate composition of foods includes moisture, ash, lipid, protein and carbohydrate contents. These food components may be of interest in the food industry for product development, quality control (QC) or regulatory purposes. Analyses used may be rapid methods for QC or more accurate but time-consuming official methods. Sample collection and preparation must be considered carefully to ensure analysis of a homogeneous and representative sample, and to obtain accurate results. Estimation methods of moisture content, ash value, crude lipid, total carbohydrates, starch, total free amino acids and total proteins are put together in a lucid manner.

Introduction

Proximate analysis is used to estimate the relative amounts of protein, lipid, water, ash and carbohydrate in any sample. Proximate composition is the term usually used in the field of feed/food and means the components of moisture, crude protein, ether extract, crude fibre, crude ash and nitrogen-free extracts, which are expressed as the content (%) in the sample, respectively. Protein, lipid and carbohydrate each contribute to the total energy content of an organism, while water and ash only contribute mass.

P. Thangaraj, *Pharmacological Assays of Plant-Based Natural Products*,
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5.1 Moisture Content (Arunachalam and Parimelazhagan 2012)

Aim

To determine the percentage of water in a sample by drying the sample to a constant weight.

Principle

The water is evaporated under high temperature, and loss of water content is expressed as the percentage moisture content of the sample.

Protocol

1. Weigh fresh sample (plant part) immediately and record as 'wet weight of sample'.
2. Dry the sample to a constant weight, at a temperature not exceeding 239 °F (115 °C) using the suitable drying equipment.
3. Allow the sample to cool.
4. Weigh the cooled sample again and record as the 'dry weight of sample'.

Calculation

The moisture content of the sample is calculated using the following equation:

$$\%W = [(A - B)/A] \times 100$$

where
$\%W$ = Percentage of moisture in the sample,
A = Weight of fresh sample (grams) and B = Weight of dry sample (grams).

5.2 Ash Value (Arunachalam and Parimelazhagan 2012)

Aim

To determine the ash content of sample using muffle furnace.

Principle

The sample is incinerated at high temperature for longer duration to convert sample into ash. The ash content is expressed as the percentage of the dry sample.

Protocol

1. Place about 3 g of the ground material, accurately weighed, or the quantity specified in the monograph, in a suitable tared dish (e.g. of silica or platinum), previously ignited, cooled and weighed.
2. Incinerate the material by gradually increasing the heat, not exceeding 450 °C for 3 h in a muffle furnace, until free from carbon.
3. Cool and weigh the sample.
4. Calculate the content in mg of ash per g of air-dried material.

Calculation

The ash content of the sample is calculated using the following equation:

$$\text{Ash content}\,\% = (\text{A}/\text{B})\text{x}100$$

where A = Weight of ash (grams) and B = Weight of dry sample (grams).

5.3 Crude Lipid (Arunachalam et al. 2011)

Aim

To determine the crude lipid content of sample by Soxhlet method.

Principle

Crude fat is the term used to refer the crude mixture of fat-soluble material present in a sample. Crude fat also known as the ether extract or the free lipid content is the traditional measure of fat in food products. The lipid materials may include triglycerides, diglycerides, monoglycerides, phospholipids, steroids, free fatty acids, fat-soluble vitamins, carotene pigments and chlorophylls. The common approach for total crude fat determination is based on the solubility of lipids in non-polar organic solvents such as hexanes, petroleum ether or supercritical liquid carbon dioxide with or without a solvent modifier.

Protocol

1. Prepare a thimble with a known amount of dry sample.
2. Take a piece of cotton wool and place it in the top of the thimble.
3. Insert the thimble in a Soxhlet liquid/solid extractor.
4. Accurately weigh a clean, dry 500-mL-round bottom flask and pour about 300 mL of petroleum ether into the flask.
5. Assemble the extraction unit over an electric heating mantle.
6. Heat the solvent in the flask until it boils. Adjust the heat source so that solvent drips from the condenser into the sample chamber.
7. Continue the extraction for 6 h.

8. Remove the extraction unit from the heat source and detach the extractor and condenser.
9. Replace the flask on the heat source and redistill the solvent for recovery.
10. Place the flask in an oven at 102 °C and dry the contents until a constant weight is reached (1–2 h).
11. Cool the flask in a desiccator and weigh the flask and contents.

Calculation

Weight of empty flask (g) = $W1$,
Weight of flask and extracted fat (g) = $W2$ and
Weight of sample = S

$$\%\,\text{Crude fat} = [(W2 - W1)/S] \times 100$$

5.4 Total Carbohydrates (Chandran et al. 2013)

Aim

To estimate the amount of total carbohydrates present in sample by anthrone method.

Principle

Carbohydrates are important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone, a green-coloured product with an absorption maximum at 630 nm.

Materials and Equipments Required

Sample, boiling tubes, centrifuge tubes, test tubes, test-tube stand, pipettes, vortexer, water bath, centrifuge, spectrophotometer, etc.

Reagents Required

1. 2.5 N Hydrochloric acid.
2. Sodium carbonate.
3. Anthrone reagent
   Weigh 200 mg of anthrone and dissolve in 100 mL of ice cold 95 % (v/v) sulphuric acid, freshly before use.

4. Stock standard solution
   Dissolve 100 mg of glucose in distilled water and make up to 100 mL in a standard flask.
5. Working standard solution
   Dilute about 10 mL of stock standard solution to 100 mL with distilled water in a standard flask. Therefore, the concentration of glucose in the working standard solution is 100 μg/mL.

Protocol

(a) **Extraction of total carbohydrates from sample**

1. Weigh 100 mg of sample powder and transfer to a boiling tube.
2. Hydrolyse the sample by keeping it in a boiling water bath for 3 h with 5 mL of 2.5 N hydrochloric acid.
3. Then, cool the boiling tube with digested sample to room temperature and neutralize with solid sodium carbonate until the effervescence ceases.
4. Make up the content of the boiling to 100 mL with distilled water.
5. Transfer the homogenate to centrifuge tube and centrifuge at 3000× *g* for 10 min at room temperature.
6. Thereafter, collect the supernatant and use for estimation.

(b) **Estimation of total carbohydrates in sample**

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 0.5 and 1 mL of carbohydrate extract of sample into two other test tubes marked T_1 and T_2, respectively.
3. Make up the contents of all the test tubes to 1 mL with distilled water.
4. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
5. Then, add 4 mL of anthrone reagent to each test tube including the blank.
6. Vortex the test tubes well and keep in a boiling water bath for 8 min.
7. Cool the test tubes rapidly and read the absorbance of the green to dark green colour developed against the reagent blank at 630 nm using spectrophotometer.
8. Draw a standard graph by plotting concentration of glucose on *x*-axis and respective absorbance on *y*-axis.
9. Finally, the amount of total carbohydrates in the sample was calculated and expressed as g glucose equivalents/100 g sample.

5.5 Starch (Chandran et al. 2012)

Aim

To estimate the amount of starch present in sample by perchloric acid method.

Principle

Carbohydrates are important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The sample is treated with 80 % alcohol to remove sugars, and then, starch is extracted with perchloric acid. Starch, which is composed of several glucose molecules, is a mixture of two types of components, namely amylose and amylopectin. In hot acidic medium, starch is hydrolysed into glucose and is dehydrated to hydroxymethyl furfural. This compound forms with anthrone, a green-coloured product with an absorption maximum at 630 nm.

Materials and Equipments Required

Sample, mortar and pestle, centrifuge tubes, test tubes, test-tube stand, pipettes, vortexer, water bath, refrigerator, centrifuge, spectrophotometer, etc.

Reagents Required

1. 80 % ethanol.
2. 52 % (v/v) perchloric acid.
3. Anthrone reagent
   Weigh 200 mg of anthrone and dissolve in 100 mL of ice cold 95 % (v/v) sulphuric acid, freshly before use.
4. Stock standard solution
   Dissolve 100 mg of glucose in distilled water and make up to 100 mL in a standard flask.
5. Working standard solution
   Dilute about 10 mL of stock standard solution to 100 mL with distilled water in a standard flask. Therefore, the concentration of glucose in the working standard solution is 100 µg/mL.

Protocol

(a) **Extraction of starch from sample**

1. Weigh 100 mg of sample and grind well with 10 mL of hot 80 % ethanol using mortar and pestle.
2. Then, transfer the homogenate to centrifuge tube and centrifuge at 3000×*g* for 10 min at room temperature.
3. Retain the residue and discard the supernatant.
4. Wash the residue two times again with fresh 10 mL of hot 80 % ethanol to remove all the simple sugars.

5. Then, dry the residue well by keeping over a boiling water bath.
6. To the residue, add 5 mL of water and 6.5 mL of 52 % perchloric acid.
7. Extract the starch by keeping the tubes at 0 °C in a refrigerator for 20 min.
8. Then, centrifuge the tube at 3000 × *g* for 10 min at room temperature and collect the supernatant.
9. Repeat the starch extraction twice by adding fresh perchloric acid to the residue.
10. Pool the supernatants, make up to 100 mL with distilled water and use for the estimation.

(b) **Estimation of starch in sample**

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 0.5 and 1 mL of carbohydrate extract of sample into two other test tubes marked T_1 and T_2, respectively.
3. Make up the contents of all the test tubes to 1 mL with distilled water.
4. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
5. Then, add 4 mL of anthrone reagent to each test tube including the blank.
6. Vortex all the test tubes well and keep in a boiling water bath for 8 min.
7. Cool the test tubes rapidly and measure the absorbance of the green to dark green colour developed against the reagent blank at 630 nm using spectrophotometer.
8. Draw a standard graph by plotting the concentration of glucose on *x*-axis and respective absorbance on *y*-axis.
9. Calculate the amount of glucose content in the sample using the standard graph and multiply the value of glucose content by a factor 0.9 to reach the starch content.

5.6 Total Proteins (Krishna et al. 2014)

Aim

To estimate the amount of total proteins present in sample by Lowry's method.

Principle

The blue colour development takes place when reduction of phosphomolybdic–phosphotungstic components in the Folin–Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate is measured in the Lowry's method.

Materials and Equipments Required

Sample, mortar and pestle, centrifuge tubes, test tubes, test-tube stand, pipettes, vortexer, centrifuge, spectrophotometer, etc.

Reagents Required

1. 0.2 M phosphate buffer (pH-7.2)
   Solution A: 0.2 M monobasic sodium phosphate;
   Solution B: 0.2 M dibasic sodium phosphate.
   Prepare phosphate buffer by mixing 28 mL of solution A and 72 mL of solution B.
2. Reagent A
   2 % sodium carbonate in 0.1 N sodium hydroxide.
3. Reagent B
   0.5 % copper sulphate in 1 % potassium sodium tartrate.
4. Reagent C (alkaline copper solution)
   Mix 50 mL of reagent A and 1 mL of reagent B prior to use.
5. Reagent D
   Prepare 1 N Folin–Ciocalteu reagent by diluting 2 N Folin–Ciocalteu reagent with equal amount of water.
6. Stock standard solution
   Dissolve 50 mg of bovine serum albumin (BSA) in distilled water and make up to 50 mL in a standard flask.
7. Working standard solution
   Dilute about 10 mL of stock standard solution to 50 mL with distilled water in a standard flask. Therefore, the concentration of BSA in the working standard solution is 200 μg/mL.

Procedure

(a) **Extraction of protein from sample**

1. Weigh 100 mg of sample powder and grind well with 10 mL of 0.2 M phosphate buffer using mortar and pestle.
2. Then, transfer the homogenate to centrifuge tube and centrifuge at 3000×*g* for 10 min at room temperature.
3. Collect the supernatant and make up to 10 mL with distilled water.
4. Thereafter, dilute about 1 mL of the supernatant to 10 mL with distilled water and use for estimation.

(b) **Estimation of protein in sample**

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 0.5 and 1 mL of protein extract of sample into two other test tubes marked T_1 and T_2, respectively.

3. Make up the contents of all the test tubes to 1 mL with distilled water.
4. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
5. Then, add 5 mL of 'Reagent C' to each test tube including the blank.
6. Vortex all the test tubes well and allow to stand for 10 min at room temperature.
7. After that, add 0.5 mL of Folin–Ciocalteu reagent (reagent 'D') (1 N) to all the test tubes including the blank.
8. Vortex the test tubes again and incubate in the dark at room temperature for 30 min.
9. Measure the absorbance of the blue colour developed against the reagent blank at 660 nm using spectrophotometer.
10. Draw a standard graph by plotting the concentration of BSA on *x*-axis and the respective absorbance on *y*-axis.
11. Finally, calculate the amount of total proteins in the sample and express as g BSA equivalents/100 g sample.

5.7 Total Free Amino Acids (Sadasivam and Manikam 2008)

Aim

To estimate the amount of total free amino acids present in sample by Ninhydrin method.

Principle

The amino acids are colourless ionic compounds that form the basic building blocks of proteins. Apart from being bound as proteins, amino acids also exist in free form in many tissues and are known as free amino acids. Ninhydrin, a powerful oxidizing agent, decarboxylates α- amino acids and yields an intensely coloured bluish purple product which is colorimetrically measured at 570 nm.

$$\text{Ninhydrin} + \alpha - \text{amino acid} \rightarrow \text{Hydrindantin} + \text{Decarboxylated amino acid} + CO_2 + NH_3$$

$$\text{Hydrindantin} + \text{Ninhydrin} + NH_3 \rightarrow \text{Purple coloured product} + H_2O$$

Materials and Equipments Required

Sample, mortar and pestle, centrifuge tubes, test tubes, test-tube stand, pipettes, vortexer, water bath, centrifuge, spectrophotometer, etc.

Reagents Required

1. 80 % ethanol
2. 0.2 M citrate buffer (pH-5.0)
   Solution A: 0.2 M citric acid
   Solution B: 0.2 M Sodium citrate
   Prepare citrate buffer by mixing 20.5 mL of solution A and 29.5 mL of solution B.
3. Ninhydrin reagent
   Dissolve 0.2 g stannous chloride in 125 mL of 0.2 M citrate buffer (pH 5.0). Add this solution to 5 g of ninhydrin in 125 mL of methyl cellosolve (2-methoxyethanol).
4. Diluent solvent
   Mix equal volumes of water and n-propanol.
5. Stock standard solution
   Dissolve 50 mg of L-leucine in distilled water and make up to 50 mL in a standard flask.
6. Working standard solution
   Dilute about 10 mL of stock standard solution to 100 mL with distilled water in a standard flask. Therefore, the concentration of leucine in the working standard solution is 100 μg/mL.

Procedure

(a) **Extraction of free amino acids from sample**

1. Weigh 100 mg of sample powder and grind well with 10 mL of 80 % ethanol using mortar and pestle.
2. Then, transfer the homogenate to centrifuge tube and centrifuge at 3000× *g* for 10 min at room temperature.
3. Collect the supernatant, make up to 10 mL with distilled water and use for estimation.

(b) **Estimation of free amino acids in sample**

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 0.5 and 1 mL of amino acid extract of sample into two other test tubes marked T_1 and T_2, respectively.
3. Make up the contents of all the test tubes 1 mL with distilled water.
4. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
5. Then, add 1 mL of 'Ninhydrin reagent' to each test tube including the blank.
6. Vortex all the test tubes well and keep in a boiling water bath for 20 min.

7. After that, add 5 mL of diluent solvent to all the test tubes including the blank.
8. Vortex the test tubes again and allow to stand for 15 min at room temperature.
9. Measure the absorbance of the purple colour developed against the reagent blank at 570 nm using spectrophotometer.
10. Draw a standard graph by plotting the concentration of L-leucine on *x*-axis and respective absorbance on *y*-axis.
11. Finally, calculate the amount of total free amino acids in the sample and express as g leucine equivalents/100 g sample.

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Chapter 6
Mineral Quantification

Abstract Optimal intakes of elements, such as sodium, potassium, magnesium, calcium, manganese, copper, zinc and iodine, can reduce individual risk factors including those related to cardiovascular diseases among humans and animals. In order to meet the need for vitamins, major minerals, trace minerals, fatty acids and amino acids, it is necessary to include a full spectrum programme that can deliver all of the nutrients in the right ratio. Minerals are required for normal growth, activities of muscles, skeletal development (such as calcium), cellular activity, oxygen transport (copper and iron), chemical reactions in the body, intestinal absorption (magnesium), fluid balance and nerve transmission (sodium and potassium), as well as the regulation of the acid base balance (phosphorus). The chapter discusses the chemical and instrumentation techniques used for estimation of minerals such as N, P, Ca, Mg, K, Na, Fe, Cu, Zn, B and Mb.

Introduction

Minerals are required for normal growth, activities of muscles, skeletal development (such as calcium), cellular activity, oxygen transport (copper and iron), chemical reactions in the body, intestinal absorption (magnesium), fluid balance and nerve transmission (sodium and potassium), as well as the regulation of the acid base balance (phosphorus) (Ozcan 2003). In order to meet the need for vitamins, major minerals, trace minerals, fatty acids and amino acids, it is necessary to include a full spectrum programme that can deliver all of the nutrients in the right ratio.

The US government and the scientific community have grouped minerals into two categories. Those that are considered to be required in our diets in amounts greater than 100 mg per day are called major minerals. Those that are considered to be required in our diets in amounts of less than 100 mg per day are called trace minerals. There are seven major minerals: calcium, magnesium, potassium, phosphorus, sulphur, sodium and chlorine. Our bodies should contain significant amounts of each major mineral. Trace minerals, on the other hand, are present in the body in minute amounts. It is thought that each makes up less than one-hundredth of one per cent of our body weight. Studies originally showed that optimal intakes

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of elements, such as sodium, potassium, magnesium, calcium, manganese, copper, zinc and iodine, can reduce individual risk factors including those related to cardiovascular diseases among humans and animals (Sanchez-Castillo et al. 1998).

6.1 Estimation of Nitrogen

Aim

The aim was to estimate the nitrogen content of sample by Kjeldahl's method.

Principle

When organic or inorganic samples are treated with concentrated sulphuric acid, it results in conversion of nitrogen into ammonium sulphate. The resultant liquid is then treated with excess of alkali and the liberated ammonia gas is absorbed in boric acid solution. The amount of ammonia (and hence of nitrogen) is determined by finding the amount of boric acid solution neutralized by titration with standard acid.

$$\text{Sample} + H_2SO_4 \rightarrow (NH_4)_2SO_4 + H_2O + CO_2 + \text{by} - \text{products}$$
$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$
$$NH_3 + H_3BO_3 \rightarrow NH_4^+ + H_2BO_3^-$$
$$H_2BO_3^- + HCl \rightarrow H_3BO_3 + Cl^-$$

Materials Required

Sample, Kjeldahl apparatus, Kjeldahl flask, burette, conical flask, etc.

Reagents Required

1. Conc. sulphuric acid (H_2SO_4)
2. Copper sulphate and potassium sulphate (1:2)
3. Sodium hydroxide (NaOH) (15 % for digestion and 40 % for distillation)
4. Boric acid solution (pH: 6–7)

   480 mL of 2 % boric acid
   20 mL of 0.1 % bromocresol green (in 95 % ethanol)
   4 mL of 0.1 % methyl red solution

5. 0.01 N HCl.

Procedure

1. Weigh 500 mg of sample and transfer it into a special long-necked 'Kjeldahl flask'.
2. Add a little potassium sulphate and copper sulphate into the digestion tube.

(Potassium sulphate raises the boiling point of H_2SO_4 and thus ensures complete reaction, while copper sulphate acts as a catalyst.)

3. Add 30 mL of conc. H_2SO_4 to the digestion tube.
4. Digest the solution at 400 °C for 3 h till the brown colour of the liquid first produced disappears leaving the contents clear or apple green.
5. After cooling the digest material, transfer the flask to the distillation unit and dilute it with a known volume of ammonia-free water.
6. Place a conical flask with 25 mL of boric acid solution at the collection side of the distillation unit.
7. Add a known volume of 40 % sodium hydroxide solution to the digested test sample.
8. Distil the test solution till the colour changes from pale green to brownish and the boric acid from dark green to blue.
9. Collect the boric acid solution and titrate it against 0.01 N HCl.
10. End the titration process when the boric acid solution turns back to dark green.
11. Do the same procedure for standard sample also.
12. Run a reagent blank with an equal volume of distilled water and subtract the titration volume from that of the sample titre volume.
13. Express results in g/Kg of sample.

Calculation

$$\mathrm{N(g/Kg)} = \frac{\text{Mol. Wt.} \times \text{Normality of acid} \times \text{titre value}}{\text{Sample (g)}}$$

$$\mathrm{N(g/Kg)} = \frac{14.01 \times \text{ Normality of acid} \times (\text{titre value of sample} - \text{titre value of blank})}{\text{Weight of sample in grams}}$$

6.2 Estimation of Phosphorous

Aim

The aim was to estimate the quantity of phosphorous in the sample.

Principle

Treating sample with double acid and Burton's reagent, the phosphorous forms complex which results in yellow colour formation.

Chemicals and Reagents Required

1. Double acid—nitric acid and per chloric acid (3:1)
2. Burton's reagent

Procedure

1. Take 0.3 g of dry sample in a conical flask with 5 mL of double acid and perform wet digestion
2. Then, wash the residue with water and filter through ordinary filter paper and made up to 100 mL
3. Take 10 mL from 100 mL and add 2.5 mL of Burton's reagent and allow it to stand for half an hour. Then, the volume is made to 25 mL
4. The colour intensity thus obtained was spectrophotometrically read at 420 nm.

Calculation

$$P\% = (1\,OD - Bl) \times F \times 100/wt \times 100/10^6$$

where F = factor (100 = how much ppm).

6.3 Estimation of Calcium and Magnesium

Aim

The aim was to estimate the quantity of calcium and magnesium in the sample.

Principle

Calcium forms complex with EDTA at definite pH.
Magnesium in solution can be titrated with 0.01 N versanate (EDTA) using Solochrome Black T-dye as an indicator at pH 10 in the presence of ammonium chloride and ammonium hydroxide buffer. The end point is the change in colour from wine red to green.

Chemicals and Reagents required

1. Ammonium chloride–ammonium hydroxide buffer (pH 10).
2. Potassium cyanide: 3.5 %.
3. Solochrome Black 1 indicator.

Procedure

1. Pipette out 5 mL of sample solution into a beaker.
2. Add 5 mL of KCN solution and 10 mL of ammonium chloride–ammonium hydroxide buffer, and follow by adding 2 drops of Solochrome Black 1 indicator titrate against EDTA.
3. The end point result is that the wine red colour of the solution changes to pure blue colour.
4. Blank solution of about 5 mL is also titrate as above.

Calculation

The first three values is a direct measure of calcium present in 5 mL aliquot. The amount of Mg present is obtained from the difference between the first- and the second-titrate values.

$$\mathrm{Ca}\% = \mathrm{V} \times \mathrm{N} \times 100/\mathrm{wt} \times 100/5 \times 20/1000$$
$$\mathrm{Mg}\% = \mathrm{V} \times \mathrm{N} \times 100/\mathrm{wt} \times 100/5 \times 12/1000$$

6.4 Estimation of Potassium

Aim

The aim was to estimate potassium in the sample.

Principle

When an alkali metal salt drawn into a non-luminous flame will ionize, absorb energy from the flame and then emit light of a characteristic wavelength as the excited atoms decay to the unexcited ground state. The intensity of emission is proportional to the concentration of the element in the solution. While sprinkle KCl into a gas flame, it glows in purple colour. This is the basic principle of flame photometry. A photocell detects the emitted light and converts it to a voltage, which can be recorded. Since K^+ emits light of different wavelengths (colours), by using appropriate coloured filters, the emission due to K^+ (and hence their concentrations) can be specifically measured in the same sample.

Chemicals and Reagents required

1. Double acid—nitric acid and perchloric acid (3:1)

Procedure

1. Weigh 0.3 g of dry sample in a conical flask.
2. Then, add 5 mL of double acid to it and digest the mixture until solution become clear.
3. Wash the residue with water and filter through ordinary filter paper and made to 100 mL. This solution was taken as A.
4. Take 5 mL from solution A and made to 50 mL using distilled water. This solution is B and used K estimation.
5. Then, check the compressor and gas in flame photometer before working.

6. After 5 min, switch on the galvanometer and allow distilled water to run for ten minutes. Distilled water reading should come to '0'.
7. After standardizing, blank and samples are allowed to run. If the reading goes above 50, the samples are diluted.

Calculation

$$K\% = GR \times F \times 100/wt \times 50/5 \times (\text{dilution factor}) \times 100/10^6$$

where F = factor (100 = how much ppm)

$$ppm = GR \times F \times 100/wt \times 50/5$$

6.5 Estimation of Sodium

Aim

The aim was to estimate the quantity of sodium in the sample.

Principle

While sprinkle table salt (NaCl) into a gas flame, it glows in bright orange colour. This is the basic principle of flame photometry. A photocell detects the emitted light and converts it to a voltage, which can be recorded. Since Na^+ emits light of different wavelengths (colours), by using appropriate coloured filters, the emission due to Na^+ (and hence their concentrations) can be specifically measured in the same sample.

Chemicals and Reagents required

1. Sodium chloride solution 1000 ppm or 1000 mg/L

Procedure

1. Weigh 0.3 gm of dry sample in a conical flask and then add 5 mL of double acid to digest until solution become clear.
2. Wash residue with water and filter through ordinary filter paper and made to 100 mL to estimate sodium.
3. Take 5 mL from solution A and made to 50 mL using distilled water. This solution is B and used K estimation.
4. Then, check the compressor and gas in flame photometer before working.

5. After 5 min, switch on galvanometer and allow distilled water to run for ten minutes. Distilled water reading should come to '0'.
6. After standardizing, blank and samples are allowed to run. If the reading goes above 50, the samples are diluted.

Calculation

$$\text{Na}\% = \text{OD} \times \text{F}(100 = \text{how much ppm of Na}) \times 100/\text{wt} \times 50/5 \times 100/10^6$$

6.6 Estimation of Iron, Copper, Zinc and Boron

Aim

The aim was to estimate the quantity of iron, copper, zinc and boron in the sample.

Principle

When an alkali metal salt or a calcium, barium salt is heated strongly in the Bunsen flame, a characteristic flame colour is observed (Na—yellow, Li—crimson, Ca—brick red, Sr—crimson, Ba—green).

Chemicals and Reagents required

1. Hydrochloric acid (12 M HCl), concentrated reagent grade
2. Nitric acid (16 N HNO_3), concentrated reagent grade
3. Distilled water

Standards

$$\text{Iron(Fe)},\ \text{Copper(Cu)},\ \text{Zinc(Zn)}\ \text{and}\ \text{Boron(B)}$$

Procedure

1. Perform either a dry ash or wet acid digestion on a known dry weight (usually about 1 g) of tissue, the obtained ash or digest is wetted with a small amount of distilled water and then brought into solution using 2 mL concentrated HC1.
2. The final dilution with distilled water should be based on the predicted concentration of the element to be determined, ensuring that the final concentration is neither at or below the method detection limits nor above the normal operation range.
3. For determination of the elements such as K, Ca, Mg, Al and Na, a 100 mL final volume should provide concentrations sufficiently above the detection limit for a 1.0 g sample of most plant materials.
4. For determination of the elements such as Mn, Fe, Cu and Zn, final volumes between 10 and 50 mL are required.

5. For Si estimation, follow AOAC (1990).
6. The wavelength setting, concentration range and sensitivity for the elements are as follows:

Element	Wavelength (nm)
Copper (Cu)	324.7
Iron (Fe)	248.3
Magnesium (Mg)	285.2
Zinc (Zn)	213
Boron (B)	249.7

6.7 Estimation of Molybdenum

Aim

The aim was to estimate the quantity of molybdenum in the sample using Molybdenum Dithiol Colorimetric Method.

Principle

Thiocynate technique for determination of Mo is based on the principle of reduction of Mo in acidic solution from oxidation state (VI) to reduced state (V) using stannous chloride. Reduced 'Mo' complexes with thiocyanate to form dominantly a reddish complex, Mo (SCN).

Chemicals and Reagents required

1. Deionized Type 1 water
2. Sulphuric acid (H_2SO_4), concentrated
3. Hydrogen peroxide (H_2O_2), 30 %
4. Ferrous ammonium sulphate ($FeSO_4(NH_4)2SO_4$), 9.1 %
5. Potassium iodide (KI), 50 % w/v
6. Ascorbic acid, 5 % w/v
7. Tartaric acid, 50 % w/v
8. Thiourea, 10 % w/v
9. Amyl acetate (B.P. 136 to 142OC)
10. Sodium hydroxide (NaOH), analytical grade
11. Toluene-3,4-Zinc Dithiol Derivative
12. Molybdenum standard solutions

Procedure

1. Weigh 2.0 g dried ground (2 mm sieve) plant material into a silica crucible.
2. Then place the crucibles in a cold furnace and ash at 550 Â° for 4 h.
3. Then remove crucible from the muffle furnace, cooled, and then add 4 mL concentrated H_2SO_4 to the crucible.

4. When the reaction with acid ceases, add 1.5 mL of 30 % H_2O_2 and swirled.
5. Now, place crucibles on a hot plate for 30 min and reheated with additional 1.5 mL H_2O_2 if the digest remains black, indicating incomplete destruction of organic matter.
6. After cooling, transfer contents to a 50-mL volumetric flask and a known volume is made with deionized water.
7. Filter the contents using a Whatman No. 40 filter paper and use it for Mo analysis.

Analysis

1. Transfer all the filtered aliquot into a 125-mL separatory funnel and then add 0.25 mL of 9.1 % $FeSO_4$-$(NH_4)_2SO_4$, 0.25 mL of 50 % KI and mix it well and allow it to stand for 15 min.
2. Add 0.25 mL of 5 % ascorbic acid and shake until the colour disappears.
3. Add 0.25 mL of 50 % tartaric acid and 2 mL of 10 % thiourea and mix it thoroughly.
4. Add 4 mL of 0.2 % toluene-3,4-zinc dithiol derivative solution, shake it for 20 min and allow the contents to stand for 30 min.
5. Add 10 mL of amyl acetate, shake vigorously for 2 min and allow it to stand for 1 h for complete separation.
6. Then draw off aqueous phase and discard it.
7. Drain off the organic phase and centrifuge it for 15 min at 2000 rpm.
8. Measure the colour on a spectrophotometer at 680 nm using a red filter and sample blank as reference.
9. Prepare calibration curve and determine the amount of Mo in the digest.

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Chapter 7
Anti-nutritional Factors

Abstract Anti-nutritional factors such as trypsin inhibitor, phytic acid and cyanogen are as important as nutritional content of any edible plant part. The anti-nutritional factors can be defined as those substances generated in natural food substances by the normal metabolism of species and by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process or metabolic utilization of feed) which exert effects contrary to optimum nutrition. Hence, trypsin inhibitor, phytic acid and cyanogens present in edibles with the methods in the chapter would be helpful.

7.1 Introduction

The anti-nutritional factors can be defined as those substances generated in natural food substances by the normal metabolism of species and by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process or metabolic utilization of feed) which exert effects contrary to optimum nutrition.

Most of the anti-nutritional factors or enzyme inhibitors are found in the seeds of various plants, but are not necessarily restricted to the seeds alone.

7.2 Trypsin Inhibitor

Aim

To find out the anti-nutritional content by estimating the trypsin-inhibiting activity in the plant samples.

Principle

The trypsin inhibitor activity is measured by inhibiting the activity of trypsin. A synthetic substrate (BAPNA) is subjected to hydrolysis by trypsin to produce yellow-coloured p-nitroanilide. The degree of inhibition by the extract of the yellow colour production was measured at 410 nm using spectrophotometer (Sadasivam and Manickam 1991).

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Reagent preparations

1. 30 % glacial acetic acid (v/v).
2. Trypsin
   Dissolve 6.25 mg of trypsin in 25 mL of 0.001 M HCl. Dilute 2 mL of this solution in 25 mL for assay.
3. Tris-HCl buffer (pH-8.2)
   Weigh 6.05 g Tris and 2.94 g $CaCl_2 \cdot H_2O$ and dissolve in 900 mL water, adjust pH 8.2 with HCl and make up to 1000 mL water.
4. BAPNA (N-α-Benzoyl-DL-Arginine-paranitroanilide)
   Dissolve 40 mg of BAPNA in 0.5 mL of DMSO and then make up to 100 mL with Tris-HCl buffer (pH-8.2).
5. Preparation of plant samples
   a. Weigh 500 mg of sample in 25 mL water by grinding in a prechilled mortar and pestle.
   b. Extract the ground sample in a refrigerator for 2–3 h with mechanical shaker.
   c. Centrifuge the homogenate at 4000 × *g* for 20 min at 4 °C.
   d. Dilute 1 mL of the supernatant to 10 mL with water and used for estimation.

Protocol

1. Pipette out different aliquots of plant samples in a series of test tubes.
2. Make up the volume to 1 mL with Tris-HCl buffer including the negative control.
3. Add 1 mL of trypsin to each test tube.
4. Incubate all the test tubes in a water bath at 80 °C.
5. Then, add 2.5 mL of substrate BAPNA to each test tube.
6. Allow the reaction to proceed for 10–60 min at 80 °C.
7. Stop the reaction by adding 0.5 mL of 30 % glacial acetic acid.
8. 1 mL Tris-HCl buffer serves as blank.
9. Read the absorbance at 410 nm in a spectrophotometer.
10. Determine the protein content in the extract by the Lowry's method.

Calculation

1. Plot the absorbance against the volume of extract.
2. Determine the aliquot size of the extract require to inhibit 50 % of the trypsin activity. That aliquot size is considered to be one unit of trypsin inhibitor.
3. One unit of activity corresponds to the amount of trypsin inhibitor in μg protein which gives 50 % inhibition of enzyme activity under experimental conditions. The trypsin inhibitor activity is expressed as trypsin inhibitor units (TIUs) per gram sample or per mg protein.

7.3 Phytic Acid

Aim

To find out the anti-nutritional content by estimating the phytic acid content in the plant sample.

Principle

The phytate is extracted with trichloro acetic acid and precipitated as ferric salt. The iron content of the precipitate is determined colorimetrically, and the phytate phosphorous content is calculated from this value, assuming a constant 4Fe:6P molecular ratio in the precipitate (Sadasivam and Manickam 1991).

Reagent preparations

1. 3 % trichloro acetic acid,
2. 3 % sodium sulphate in 3 % trichloro acetic acid,
3. 1.5 N NaOH,
4. 3.2 N HNO_3,
5. $FeCl_3$ solution (dissolve 583 mg in 3 % trichloro acetic acid) and
6. 1.5 M potassium thiocyante (KSCN).

Preparation of standard

1. Weigh 433 mg of $Fe(NO_3)_3$ in 100 mL of water.
2. Dilute 2.5 mL of the above stock solution to 250 mL with distilled water.

Protocol

1. Weigh 500 mg of sample and grind with 50 mL of 3 % trichloro acetic acid using prechilled mortar and pestle.
2. Keep the ground the sample for 30 min in mechanical shaker.
3. Centrifuge the suspension and transfer a 10 mL aliquot of the supernatant to a conical flask.
4. Add 4 mL of $FeCl_3$ solution to the conical flask rapidly.
5. Heat the contents in a boiling water bath (80 °C) for 45 min. If the supernatant is not clear after 30 min, add one or two drops of 3 % sodium sulphate.
6. Centrifuge the content 200 × *g* for 10 min.
7. Wash the precipitate twice by dispersing well in 20 mL of 3 % trichloro acetic acid, heat in boiling water bath (80 °C) for 10 min and centrifuge.
8. Again, wash the precipitate twice with water.
9. Disperse the precipitate in a few mL of water and add 3 mL 1.5 N NaOH with mixing.
10. Bring the volume up to 30 mL with water and heat in boiling water bath (80 °C) for 30 min and centrifuge.
11. Filter the content by Whatman No. 1 filter paper.
12. Wash the precipitate with 60 mL hot water and discard the filtrate.

13. Dissolve the precipitate from the paper with 40 mL hot 3.2 N HNO_3
14. Wash paper with several portions of water, collecting the washing in the same flask.
15. Cool the contents to room temperature and dilute to 70 mL water.
16. Add 20 mL of 1.5 M potassium thiocyante (KSCN) dilute to volume and read colour immediately within 1 min at 480 nm.

Calculation

Find out the μg iron present in the test from the standard curve, and calculate the phytate P as mg/100 g sample.

7.4 Cyanogen

Aim

To find out the anti-nutritional content by estimating the cyanogen content in the plant sample.

Principle

Hydrocyanic acid which is evolved from the sample forms a red-coloured compound with sodium picrate, and the intensity is measured at 625 nm.

Reagents

1. Chloroform:
2. Take Whatman filter paper No. 1 and saturate them with alkaline picrate solution.
3. Alkaline picrate solution:
   Dissolve 25 g sodium carbonate and 5 g picric acid in 1 L of water.
4. Standard hydrogen cyanide solution:
   Dissolve 0.241 g of KCN/litre of water. This gives a KCN solution containing 100 μg/mL.

Protocol

1. Homogenize 1 g of the sample in 25 mL water.
2. Add few drops of chloroform.
3. Place the homogenate in 500 mL conical flask.
4. Place the saturated filter paper in the hanging position with the help of a cork stopper inside the conical flask.
5. Incubate the mixture at room temperature at 20 °C for 24 h.
6. The sodium picrate present in the filter paper is reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved.
7. Elute the colour by placing the paper in a clean test tube containing 10 mL water and compare it with standards at 625 nm.

Preparation of standard curve

1. Place 5 mL of the alkaline picrate solution and 5 mL of the potassium cyanide solution in a test tube.
2. Heat for 5 min in boiling water bath.
3. Take the different concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mL.
4. Bring the volume of each test tube to 10 mL with water and cooled to room temperature.
5. Measure the absorbance at 625 nm.

Calculation

Calculate the hydrogen cyanide content of the sample from the standard graph.

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Chapter 8
Quantification of Secondary Metabolites

Abstract Plants are a rich source of secondary metabolites that have medicinal and aromatic properties. Secondary metabolites such as alkaloids, iridoids and phenolics generally produced by plants for their defence mechanisms have been implicated in the therapeutic properties of most medicinal plants. Hence, quantification of these metabolites will aid to discover new and effective drugs from plant sources and also to scientifically validate the existing traditional practices. Quantification of large group of phytochemicals such as phenolics and flavonoids is quantified in this context.

Introduction

According to some estimates, at least ~100,000 such secondary metabolites are now known to occur in 50,000 plant species and ~4000 new secondary metabolites are being discovered every year from a variety of plant species. For thousands of years, these natural plant products have been utilized for human healthcare in the form of drugs, antioxidants, flavours, fragrances, dyes, insecticides and pheromones. Hence, the quantification of secondary metabolites is essential for discovery of lead and potent drug from the plant source and also for scientific validation of formulated drug.

8.1 Total Phenolic Content

Aim

To estimate the amount of total phenolics present in sample by Folin-Ciocalteu method described by Makkar (2003).

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Principle

Phenolics, the aromatic compounds with hydroxyl groups, are widespread in plant kingdom. Their estimation involves the reduction of phenols by phosphomolybdic–phosphotungstic components in the Folin-Ciocalteu reagent. Phenolic compounds are oxidized in a basic medium resulting in the formation of superoxide ion, which in turn react with molybdate to form molybdenum oxide. The blue-coloured complex thus developed has a very intensive absorbance at 725 nm.

Materials and Equipments Required

Sample, mortar and pestle, centrifuge tubes, test tubes, test-tube stand, pipettes, vortexer, centrifuge, spectrophotometer, etc.

Reagents Required

1. 80 % ethanol
2. 1 N Folin-Ciocalteu reagent
   Prepare 1 N Folin-Ciocalteu reagent by diluting 2 N Folin-Ciocalteu reagent with equal volume of water.
3. 5 % sodium carbonate
4. Stock standard solution
   Dissolve 50 mg of Gallic acid (GA) in methanol and make up to 50 mL in a standard flask.
5. Working standard solution
   Dilute 5 mL of stock standard solution to 100 mL with distilled water in a standard flask. Therefore, the concentration of gallic acid in the working standard solution is 50 μg/mL.

Protocol

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 50 μL of phenolics extract of sample into series of test tubes.
3. Perform the analyses in triplicates.
4. Make up the contents of all the test tubes to 1 mL with distilled water.
5. Another test tube marked ‘B’ with 1 mL of distilled water serves as the blank.
6. Then, add 0.5 mL of Folin-Ciocalteu reagent (1 N) to each test tube including the blank.
7. Vortex all the test tubes well and allow to stand for 5 min at room temperature.
8. After that add 2.5 mL of 5 % sodium carbonate to all the test tubes including the blank.
9. Vortex the test tubes again and incubate in the dark at room temperature for 40 min.
10. Measure the absorbance of the blue colour developed against the reagent blank at 725 nm using spectrophotometer.

11. Draw a standard graph by plotting concentration of gallic acid on *x*-axis and respective absorbance on *y*-axis.
12. Finally, calculate the amount of total phenolics in the sample and express as mg gallic acid equivalents/g sample.

8.2 Tannin Content

Aim

To estimate the amount of tannins present in sample by Folin-Ciocalteu method described by Makkar (2003).

Principle

The total phenolics contain both tannin and non-tannin phenolics. The amount of tannins is calculated by subtracting the total non-tannin phenolics from total phenolics. The incubation of extracts with PVPP under cool condition precipitates the tannins. Therefore, the supernatant contains only the non-tannin phenolics which can be determined by the same method described for the quantification of total phenolics.

Materials and Equipments Required

Sample, Eppendorf tubes, test tubes, test-tube stand, pipettes, vortexer, centrifuge, spectrophotometer, etc.

Reagents Required

1. 1 N Folin-Ciocalteu reagent
   Prepare 1 N Folin-Ciocalteu reagent by diluting 2 N Folin-Ciocalteu reagent with equal volume of water.
2. 5 % sodium carbonate
3. Stock standard solution
   Dissolve 50 mg of tannic acid (TA) in methanol and make up to 50 mL in a standard flask.
4. Working standard solution
   Dilute 5 mL of stock standard solution to 100 mL with distilled water in a standard flask. Therefore, the concentration of tannic acid in the working standard solution is 50 µg/mL.

Protocol

1. Weigh 100 mg of polyvinyl polypyrrolidone (PVPP) in 2-mL Eppendorf tubes.
2. Add 500 µL of plant sample and 500 µL of distilled water.
3. Incubate the tubes for 4 h at 4 °C.

4. After incubation, centrifuge the Eppendorf tubes at 3000 rpm for 10 min at 4 °C. The supernatant contains only the non-tannin phenolics.
5. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
6. Then, take 100 μL of non-tannin phenolics extract of sample into series of test tubes.
7. Perform the analyses in triplicates.
8. Make up the contents of all the test tubes to 1 mL with distilled water.
9. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
10. Then, add 0.5 mL of Folin-Ciocalteu reagent (1 N) to each test tube including the blank.
11. Vortex all the test tubes well and allow to stand for 5 min at room temperature.
12. After that add 2.5 mL of 5 % sodium carbonate to all the test tubes including the blank.
13. Vortex the test tubes again and incubate in the dark at room temperature for 40 min.
14. Measure the absorbance of the blue colour developed against the reagent blank at 725 nm using spectrophotometer.
15. Draw a standard graph by plotting concentration of tannic acid on *x*-axis and respective absorbance on *y*-axis.
16. Finally, calculate the amount of non-tannin phenolics and total phenolics in the sample as mg tannic acid equivalents/g sample.
17. Then, calculate tannins in the sample by the following equation: Tannins (g) = Total phenolics (g) − Non-tannin phenolics (g).

8.3 Flavonoid Content

Aim

To estimate the amount of flavonoids present in sample by aluminium chloride method described by Zhishen et al. (1999).

Principle

The basic principle of colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, respectively. In addition, it also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids resulting in pink colour formation, and it is measured at 510 nm.

Materials and Equipments Required

Sample, test tubes, test-tube stand, pipettes, vortexer, spectrophotometer, etc.

Reagents Required

1. 5 % sodium nitrite,
2. 10 % aluminium chloride and
3. 4 % sodium hydroxide.
4. Stock standard solution
   Dissolve 50 mg of rutin in methanol and make up to 50 mL in a standard flask.
5. Working standard solution
   Dilute 10 mL of stock standard solution to 50 mL with distilled water in a standard flask. Therefore, the concentration of rutin in the working standard solution is 200 μg/mL.

Protocol

1. Initially, pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL aliquots of working standard solution into the series of test tubes marked S_1, S_2, S_3, S_4 and S_5, respectively.
2. Then, take 500 μL of extract of sample into series of test tubes.
3. Perform the analyses in triplicates.
4. Make up the contents of all the test tubes to 1 mL with distilled water.
5. Another test tube marked 'B' with 1 mL of distilled water serves as the blank.
6. Then, add 150 μL of 5 % sodium nitrite to each test tube including the blank.
7. Vortex all the test tubes well and incubate at room temperature for 5 min.
8. After that add 150 μL of 10 % aluminium chloride to all the test tubes including the blank.
9. Vortex the test tubes again and incubate at room temperature for 6 min.
10. Add 2 mL of 4 % sodium hydroxide to all the test tubes.
11. Make up the contents of test tubes to 5 mL using distilled water.
12. Vortex the test tubes well and allow to stand for 15 min at room temperature.
13. Measure the absorbance of the pink colour developed due to the presence of flavonoids against the reagent blank at 510 nm using spectrophotometer.
14. Draw a standard graph by plotting concentration of rutin on *x*-axis and respective absorbance on *y*-axis.
15. Finally, calculate the amount of flavonoids in the sample and express as mg rutin equivalents/g sample.

8.4 Flavonol Content

Aim

To determine the flavonol content of plant extract.

Principle

The flavonol containing sample results in green colour formation when react with aluminium chloride and sodium acetate, and the samples are read at 440 nm in UV-Vis spectrophotometer.

Materials

1. Test tubes, test-tube stand, pipette, measuring cylinders, vortexer and UV-Vis spectrophotometer.
2. Aluminium chloride (20 gm/L).
3. Sodium acetate (50 gm/L).
4. Rutin (1 mg/mL).

Protocol

1. Estimate total flavonol in rutin equivalents and expressed as mg of rutin per gram.
2. Take the aliquots of rutin (0.2, 0.4, 0.6, 0.8 and 1 mL) and then add 2 mL aluminium chloride and 6 mL sodium acetate.
3. Then, incubate the mixture at 20 °C for 2.5 h.
4. Then, read the samples at 440 nm using UV-Vis spectrophotometer.
5. Repeat the same procedure to the triplicates samples (Miliauskas et al. 2004).

8.5 Vitamin E Content

Aim

To determine the vitamin E content of plant extract.

Principle

In the presence of vitamin E, the reduction of molybdenum takes place at 37 °C results green colour formation and read the samples at 695 nm using UV-Vis spectrophotometer.

Protocol

1. Take the aliquots (0.2, 0.4, 0.6, 0.8 and 1 mL) of standard α-tocopherol.
2. Add 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubate at 37 °C for 90 min with vigorous shaking.
3. After this, cool the samples at room temperature and then measure absorbance of the mixture at 695 nm against the reagent blank.
4. Repeat the same procedure for triplicate samples.

5. Typical blank solutions contain 3 mL of reagent solution and incubate it under the same conditions, and values are expressed as milligrams of α-tocopherol equivalents per gram extract (Prieto et al. 1999).

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Chapter 9
In Vitro Antioxidant Assays

Abstract Various chemical in vitro assays have been developed to measure antioxidant capacities of plant products. Despite the recent popularity in the antioxidant research, the lack of standardized assays to compare research results from different research groups has been a major challenge. The examination of various antioxidant assays is required for the development of standard methods that are broadly applicable by researchers and industry. However, due to the complex nature of biological systems, there is no single universal method for measuring antioxidant capacity. Antioxidant methods such as $DPPH^{\cdot}$, $ABTS^{\cdot+}$, nitric oxide, super oxide, metal chelating confirming the free radical scavenging property of the plants with widely used methods are simplified in this chapter.

Introduction

Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easy oxidizable substrates. When antioxidants react with ROS or RNS, the antioxidant is itself often transformed into an 'antioxidant radical'. Although the resulting radical has a reduced ability to react with vital cellular targets, it can still cause damage (Buettner 1993). The 'antioxidant radical' needs to react with another antioxidant to bring the reduction potential and the reactivity further down. These antioxidant reactions can continue in a stepwise fashion, involving a large number of antioxidant molecules, until the 'antioxidant radical' is no longer a threat to the cell, simply because it has been reduced to a product which does not contain enough reduction potential to react with lipids, protein, DNA and other important cellular molecules.

Based on reaction mechanisms involved, antioxidant capacity assays can be divided into two major groups: those based on hydrogen transfer (HAT) reactions and others involving single electron transfer (SET) reactions (Huang et al. 2005; Prior et al. 2005). Since hydrogen atom transfer is a key step in the radical chain, HAT-based methods are more relevant to radical chain-breaking antioxidant capacity. In contrast, SET-based assays involve one redox reaction in which the oxidant is also the probe for monitoring the reaction. Single-electron transfer-based

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assays involve two components in the reaction, i.e. the antioxidant and oxidant (also the probe) and follow the relationship (Huang et al. 2005):

$$\text{Probe (oxidant)} + \text{e (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidised antioxidant}$$

9.1 DPPH Scavenging Activity (Blois 1958a, b)

Aim

To determine the DPPH radical scavenging activity of plant extracts.

Principle

The hydrogen donating or radical scavenging ability of the extract reduces 2,2-diphenyl-1-picrylhydrazyl radical to 2,2-diphenyl-1-picrylhydrazine which results in a pale yellow solution.

Chemicals Required

2,2-diphenyl-1-picrylhydrazyl, methanol.

Reagent Preparation

Prepare 0.1 mM DPPH solution in methanol.

Protocol

1. Take different concentrations of the extracts and make the volume to 100 μL with methanol.
2. Add 5 mL of 0.1 mM methanolic solution of $DPPH^{\cdot}$ and incubate for 20 min at 27 °C.
3. Measure the absorbance of the solution at 517 nm.
4. Methanol alone will serve as blank.
5. A test tube with 100 μL of methanol and 5 mL of DPPH solution serves as negative control.
6. The mixture of methanol, DPPH and standard (BHT, BHA, quercetin and α-tocopherol) will serve as positive control.
7. The reduction in purple colour of the DPPH solution to pale yellow will give the percentage of inhibition.
8. Calculate the percentage inhibition from the absorbance of sample and negative control using the equation,

$$\%\,\text{Inhibition} = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

8. Calculate the IC_{50} (Inhibitory Concentration 50 %) from the concentration and percentage inhibition graph.

9.2 $ABTS^{\cdot+}$ Scavenging Activity (Re et al. 1999)

Aim

To estimate the total antioxidant activity of plant extracts by assay of radical cation 2,2′-azinobis (3-ethylebenzothiozoline-6-sulphonic acid) ($ABTS^{\cdot+}$).

Principle

The generation of ABTS radical involves the direct production of the blue/green ABTS chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the preformed radical cation reduces it to ABTS (decolorized solution), to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction.

Chemicals Required

Potassium persulfate (2.45 mM), 2,2-azinobis (3-ethylbenzothiozoline-6-sulphonic acid) disodium salt (ABTS) (7 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (2.5 mM) and absolute ethanol.

Reagent Preparation

1. $ABTS^{\cdot+}$ was produced by reacting 7 mM $ABTS^{\cdot+}$ aqueous solution with 2.45 mM potassium persulphate in the dark for 12–16 h at room temperature.
2. Dilute the reagent solution in ethanol (about 1:89 v/v) and equilibrate at 30 °C to give an absorbance at 734 nm of 0.7 ± 0.02.

Protocol

1. Add 1 mL of diluted ABTS solution to different concentration of sample. The concentration of the samples should be taken in triplicates.
2. Add the same volume of diluted ABTS solution to trolox standards (final concentration 0–15 μM) in ethanol.
3. Take ethanol as blank and ethanolic solution of ABTS as negative control.
4. Measure the absorbance exactly 30 min after initial mixing at 734 nm in UV-Vis spectrophotometer.
5. The unit of total antioxidant activity (TAA) of extracts is measured as the concentration of trolox having equivalent antioxidant activity expressed as μM/g extract from the standard curve graph.

9.3 Phosphomolybdenum Assay (Prieto et al. 1999)

Aim

To estimate the antioxidant property of plant extract using phosphomolybdenum assay.

Principle

In the reaction mixture, Mo (IV) gets reduced to Mo (V) and forms a green phosphomolybdenum complex.

Chemicals Required

Sulphuric acid, sodium m-phosphate, ammonium molybdate.

Reagent Preparation

1. 0.6 M sulphuric acid
2. 28 mM sodium phosphate
3. 4 mM ammonium molybdate
4. Add sodium phosphate and ammonium molybdate in the desired volume of 0.6 M H_2SO_4.

Protocol

1. Add a standardized concentration of sample solution in triplicate in test tubes.
2. Add 1 mL of the reagent solution.
3. A reaction mixture without sample or standard will serve as blank.
4. Incubate the reaction mixture in a water bath at 95 °C for 90 min.
5. Cool at room temperature and measure the absorbance of the mixture at 765 nm against a blank.
6. Express the results in ascorbic acid equivalents per gram extract (AEAC).

9.4 Ferric Reducing Antioxidant Power (FRAP) Assay (Pulido et al. 2000)

Aim

To analyse the ferric reducing antioxidant power of plant extract.

Principle

This is a method of determining the reduction of a ferric-tripyridyl-s-triazine complex to its ferrous, thereby forming violet-coloured solution.

Chemicals Required

Ferric chloride, 2,4,6-tripyridyl-s-triazine (TPTZ), hydrochloric acid, acetic acid, sodium acetate.

Reagent Preparation

FRAP reagent

a. 20 mM TPTZ in 40 mM HCl
b. 20 mM $FeCl_3$
c. 25 mL of 0.2 M acetate buffer (pH 3.6) using 0.2 M acetic acid and 0.2 M sodium acetate

1. Mix the solution in order of c, a, b.
2. Incubate the reagent at 37 °C for 30 min.
3. Take 5 mM of $FeSO_4 \cdot 7H_2O$ as standard.

Protocol

1. Take the sample in triplicate determination and add distilled water to make equal volume of solution in each test tube.
2. Add 900 µL of FRAP reagent, vortex well and incubate at 37 °C for 30 min.
3. Read the absorbance of the chromophore developed, immediately after incubation at 593 nm.
4. The FRAP value is expressed as mmol Fe (II) equivalent/mg extract.

9.5 Metal Chelating Activity (Dinis et al. 1994)

Aim

To determine the metal chelating activity of plant extract.

Principle

The ligation of extract components and ferrous chloride will be characteristic for antioxidant activity or prevention of such high-energy-free radicals.

Chemicals Required

Ferrozine, ferrous chloride, deionized water, EDTA.

Reagent Preparation

1. $FeCl_2$—2 mM
2. Ferrozine—5 mM.

Protocol

1. Add 50 µL of 2 mM $FeCl_2$ to the extracts in test tubes of triplicate determaination.
2. Initiate the reaction by adding 0.2 mL of 5 mM ferrozine solution.

3. A test tube with deionized water alone will act as blank and reaction mixture without standard or sample will be negative control.
4. Make the solution to a volume of 1–3 mL.
5. Shake the reaction mixture vigorously and leave to stand at room temperature for 10 min.
6. Measure the absorbance of the solution at 562 nm.
7. The metal chelating activity will be determined in EDTA equivalence comparing the sample with the standard curve graph.

9.6 Nitric Oxide Scavenging Activity (Sreejayan and Rao 1997)

Aim
To determine the nitric oxide radical scavenging activity of plant extract.

Principle
The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.

Materials and Equipments Required
Plant extract, test tubes, test-tube stand, pipettes, vortexer, spectrophotometer, etc.

Reagents Required

1. Phosphate-buffered saline (0.2 M, pH-7.4).
2. Sodium nitroprusside (10 mM) in phosphate-buffered saline.
3. Griess reagent.

The reagent mixture is prepared to contain 1 % sulphanilamide, 2 % H_3PO_4 and 0.1 % N- (1-naphthyl) ethylene diamine dihydrochloride.

Protocol

1. Take 100 μL of plant extract and standards (BHT and rutin) in triplicates.
2. Add 3 mL of sodium nitroprusside (10 mM) to the extracts in test tubes.
3. Incubate all the test tubes at room temperature for 150 min.
4. Add 3 mL of Griess reagent (1 % sulphanilamide, 2 % H_3PO_4 and 0.1 % N-(1-naphthyl) ethylene diamine dihydrochloride) to all the test tubes.
5. The same reaction mixture without the sample is the negative control.
6. A test tube with phosphate-buffered saline alone will act as blank.

7. Read the absorbance of the chromophore formed at 546 nm against the blank.
8. Calculate the scavenging activity (%) using the following equation:

$$\text{Scavenging activity}\,(\%) = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

9.7 Superoxide Radical Scavenging Activity (Beauchamp and Fridovich 1971)

Aim

To determine the superoxide radical scavenging activity of plant extract.

Principle

The assay is based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin–light–NBT system.

Materials and Equipments Required

Plant extract, test tubes, test-tube stand, pipettes, vortexer, spectrophotometer, etc.

Reagents required

1. Sodium phosphate buffer (50 mM, pH-7.6)
2. Riboflavin
3. EDTA
4. Nitroblue tetrazolium salt
5. Reagent mixture.

The reagent mixture is prepared such that 3 mL of reaction mixture contains 20 μg riboflavin, 12 mM EDTA and 0.1 mg NBT in 50-mM sodium phosphate buffer (pH-7.6).

Protocol

1. Take 100 μL of plant extract and standards (BHT and rutin) in triplicates.
2. Add 3 mL of reaction mixture containing 50-mM sodium phosphate buffer (pH-7.6), 20 μg riboflavin, 12 mM EDTA and 0.1 mg NBT to the extracts in test tubes.
3. Start the reaction by illuminating the reaction mixture with samples for 90 s.
4. The illuminated reaction mixture without sample is the negative control.
5. The unilluminated reaction mixture without plant sample serves as the blank.
6. Immediately after illumination, measure the absorbance at 590 nm against the blank.

7. Calculate the scavenging activity (%) of superoxide anion generation using the following equation:

$$\text{Scavenging activity}\,(\%) = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

9.8 Reducing Power Ability (Oyaizu 1986)

Aim
To determine the reducing power ability of plant extract.

Principle
The reducing power of plant extracts or standards can be detected based on the ability to reduce ferric ions in the reaction mixture to ferrous ions. The reduction will favour the production of green colour complex where the intensity is dependent on the concentration of redutants.

Materials and Equipments Required
Plant extract, test tubes, centrifuge tubes, test-tube stand, pipettes, vortexer, centrifuge, spectrophotometer, etc.

Reagents Required

1. Phosphate buffer (0.2 M, pH-6.6)
2. Potassium ferricyanide (1 %)
3. Trichloroacetic acid (10 %)
4. Ferric chloride (0.1 %).

Protocol

1. Take different aliquots of extracts (50–250 µg) and standards (BHT and rutin) into a series of test tubes in triplicates.
2. Make up the volume to 1 mL with methanol in all the test tubes.
3. A test tube with 1 mL of methanol serves as the blank.
4. Add 2.5 mL of phosphate buffer (0.2 M, pH-6.6) and 2.5 mL of potassium ferricyanide (1 %) sequentially to the test tubes including the blank.
5. Incubate the mixture at 50 °C for 20 min.
6. Then, add 2.5 mL of trichloroacetic acid (10 %) to the mixture.
7. Centrifuge the contents at 650 × *g* for 10 min at room temperature.
8. Mix the upper layer of solution (2.5 mL) with distilled water (2.5 mL) and add 0.5 mL of ferric chloride (0.1 %).
9. Read the absorbance of the green colour formed at 700 nm.
10. Increased absorbance of the reaction mixture indicates increased reducing power.

9.9 Hydroxyl Radical Scavenging Activity (Klein et al. 1991)

Aim

To determine the hydroxyl radical scavenging activity of plant extract.

Principle

The hydroxyl radicals generated in the reaction mixture produce formaldehyde by oxidizing dimethyl sulphoxide. Iron-EDTA has been shown to stimulate the production of OH by the xanthine oxidase reaction. H_2O_2 is produced during the autoxidation of Fe^{2+} or ascorbate (ascorbate system) or during the xanthine oxidase reaction. A Fenton reaction between the H_2O_2 and the Fe^{2+} results in the production of OH. In the ascorbate system, ferric iron is reduced by ascorbate to the ferrous state and catalyses the production of formaldehyde during the metabolism of dimethyl sulphoxide. The compounds in plant extract scavenge the hydroxyl radical until the reaction is terminated by ice-cold TCA. The extent of formaldehyde production or scavenging of hydroxyl radicals can be assayed through the reaction with Nash reagent.

Materials and Equipments Required

Plant extract, test tubes, test-tube stand, pipettes, vortexer, spectrophotometer, etc.

Reagents Required

1. Iron-EDTA solution
   Prepare the solution containing 0.13 % ferrous ammonium sulphate and 0.26 % EDTA.
2. EDTA solution (0.018 %)
3. Phosphate buffer (0.1 M, pH-7.4)
4. Dimethyl sulphoxide (0.85 % v/v in 0.1-M phosphate buffer, pH-7.4)
5. Ascorbic acid (0.22 %)
6. Ice-cold trichloroacetic acid (17.5 % w/v)
7. Nash reagent.

Mix 75.0 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone and raise to 1 L with distilled water.

Protocol

1. Take 100 μL of plant extract and standards (BHT and rutin) in triplicates.
2. Add 1 mL of iron-EDTA solution (0.13 % ferrous ammonium sulphate and 0.26 % EDTA) to all the test tubes.
3. Then, add 0.5 mL of EDTA solution (0.018 %) and 1 mL of DMSO (0.85 % v/v in 0.1-M phosphate buffer, pH-7.4) sequentially.
4. Initiate the reaction by adding 0.5 mL of ascorbic acid (0.22 %) to the reaction mixture.
5. Incubate at 80–90 °C for 15 min in a water bath.

6. Terminate the reaction by adding 1 mL of ice-cold TCA (17.5 % w/v).
7. Add 3 mL of Nash reagent to all the tubes.
8. Incubate at room temperature for 15 min.
9. The reaction mixture without sample serves as control.
10. Measure the intensity of the colour formed at 412 nm against the phosphate buffer blank.
11. The percentage hydroxyl radical scavenging activity is calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

9.10 Hydrogen Peroxide Scavenging Activity (Ruch et al. 1989)

Aim
To determine the hydrogen peroxide scavenging activity of plant extract.

Principle
The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2 by phytocompounds.

Materials and Equipments Required
Plant extract, test tubes, test-tube stand, pipettes, vortexer, spectrophotometer, etc.

Reagents Required

1. Phosphate buffer (0.2 M, pH-7.4)
2. Hydrogen peroxide (2 mM).

Prepare in 0.2-M phosphate buffer (pH-7.4) and determine its concentration spectrophotometrically from absorption at 230 nm with molar absorptivity 81 M^{-1}/cm.

Protocol

1. Take 100 μL of plant extract and standards (BHT and rutin) in triplicates.
2. Add 600 μL of hydrogen peroxide solution to the plant sample.
3. Make up the volume to 4 mL with phosphate buffer in all the test tubes.
4. The identical reaction mixture without the sample serves as negative control.
5. Incubate all the test tubes for 10 min at room temperature.
6. Measure the absorbance of hydrogen peroxide at 230 nm against the blank (phosphate buffer).

7. The hydrogen peroxide scavenging activity is calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

9.11 Lipid Peroxidation (Ruberto et al. 2000)

Aim
To determine the inhibition of lipid peroxidation by plant extract.

Principle
A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm.

Materials and Equipments Required
Plant extract, test tubes, test-tube stand, centrifuge tubes, pipettes, vortexer, centrifuge, spectrophotometer, etc.

Reagents Required

1. Phosphate-buffered saline (pH 7.4)
2. $FeSO_4$ (0.075 M)
3. L-ascorbic acid (0.1 M)
4. EDTA (0.1 M)
5. Thiobarbituric acid (TBA) reagent.

Dissolve 3 g TBA, 120 g TCA and 10.4 mL 70 % $HClO_4$ in 800 mL of distilled water.

Protocol

1. Take 300 μL of plant extract and standards (BHT and rutin) in triplicates.
2. Add 500 μL egg homogenate (10 % v/v in phosphate-buffered saline pH 7.4) to all the test tubes and make up to 1.0 mL with distilled water.
3. Then, sequentially add 50 μL of $FeSO_4$ (0.075 M) and 20 μL of L-ascorbic acid (0.1 M) to the tubes.
4. Incubate for 1 h at 37 °C to induce lipid peroxidation.
5. Add 0.2 mL of EDTA (0.1 M) followed by 1.5 mL of TBA reagent in each sample.
6. Heat the tubes for 15 min at 100 °C.
7. After that, cool the test tubes and centrifuge for 10 min at 3000 × *g*.
8. Measure the absorbance of supernatant at 532 nm against the blank.

9. All the reagent mixtures except plant sample serve as control test tube.
10. The inhibition (%) of lipid peroxidation is calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = [(\text{Control OD} - \text{Sample OD})/\text{Control OD}] \times 100$$

9.12 DNA Protection Assay (Rajan et al. 2014)

(A) Isolation of Plasmid DNA

Aim

To isolate the plasmid DNA from a given bacterial culture using Quiagen mini kit.

Principle

Bacteria contain an extra-chromosomal circular DNA called plasmid DNA. This plasmid is widely used for various experimental analyses and needs to be isolated from the bacterial cell. The cell has to be lysed to obtain the cellular contents, then neutralized and finally eluted. The plasmid is then precipitated, washed, dried and dissolved in sterile water before storage.

Materials Required

Quiagen mini kit, nutrient broth, bacterial culture, isopropanol, 70 % ethanol, DEPC-treated water, ultracentrifuge, sterile Eppendorf tubes, test tubes, cotton plug, micropipettes, shaker, etc.

Protocol

1. Prepare 10 mL nutrient broth in a test tube and sterilize it by autoclaving.
2. In sterile conditions, transfer the bacterial inoculum (100 μL) to the nutrient broth and keep the culture for overnight incubation in a shaker and maintain it at 37 °C. (If the bacterial culture is not a recombinant, then add suitable antibiotic at a concentration of about 50 μg/mL of broth.)
3. Transfer the contents of the culture into an Eppendorf tube and centrifuge it for 5 min in 1000 × *g*. After removing the supernatant, store the pellet in −20 °C for further use.
4. This pellet from the culture is used to extract plasmid using Quiagen mini kit 25.
5. The resuspension buffer is prepared according to the instructions given in the kit by mixing Buffer P1 and RNase provided within the kit.
6. Dissolve the pellet from 10-mL culture in 0.6-mL resuspension buffer and mix it properly.
7. Add 0.6-μL lysis buffer (Buffer P2) to the above mixture and mix thoroughly.
8. Add 0.6 μL lyse blue solution (1:1000) to it and slowly mix the contents well to get a uniform blue colour which shows proper lysis.

9. Add 0.6-μL-prechilled neutralization buffer (P3 buffer) and incubate it for 5 min.
10. Centrifuge at 4 °C for 10 min at 12,000 × *g* and take out the supernatant.
11. Equilibrate the Quiagen tip by applying 1-mL QBT buffer. Allow the buffer to move through the tip by gravity.
12. Transfer the centrifuged contents into the Quiagen tip and centrifuge for 2 min at 4 °C with 5000 × *g*. Buffers flow through the tip to the larger tube while plasmid retains in the tip.
13. Wash the Quiagen tip with 1-mL QC buffer. Allow the QC buffer to move through the Quiagen tip by gravity flow. Discard the solvent in the tube.
14. In a fresh tube, elute the DNA with 0.8 mL QF buffer and transfer the eluted DNA into a sterile Eppendorf tube.
15. Add equal volume of isopropanol (0.8 mL) and incubate it for 30 min. After the incubation, centrifuge at 12,000 × *g* for 30 min (4 °C) to get the plasmid DNA precipitated. Remove the supernatant.
16. Then, wash the precipitated DNA pellet twice with 70 % ethanol and after discarding the ethanol, dry the pellet.
17. After drying the ethanol from the pellet, dissolve the DNA in DEPC water in required concentration. Store the DNA plasmid in −20 °C.

Inference

The plasmid DNA can be successfully isolated from the given bacterial culture.

(B) Determination of Plasmid DNA Breakage by Hydroxyl Radicals

Aim

To determine the plasmid DNA breakage by hydroxyl radicals and the protection of this damage by antioxidant-rich extracts.

Principle

Hydroxyl radicals are one of the chief factors for the generation of ROS causing the oxidation of DNA. The ability of certain antioxidant compounds in protecting the cell against this free radical-induced damage needs to be analysed. In this assay, H_2O_2–UV-induced damage is used to study the DNA protecting ability of the test samples (Phanikumar et al. 2013). H_2O_2 in presence of UV is converted to free radicals and causes plasmid breakage. The test sample present in the system will prevent the severe damage. Moreover, the dark incubation for 6–12 h will stabilize the effects. The degree of fragmentation of the DNA is then analysed by agarose gel electrophoresis. Damaged DNA is seen as a streak while the one without damage will show an intact band of DNA.

Materials Required

Plasmid DNA, H_2O_2 (50 % solution), test sample, agarose, ethidium bromide, Na_2EDTA, boric acid, tris buffer, PCR tubes, conical flask, magnetic stirrer, gel electrophoresis system, gel doc system with UV transilluminator, microwave oven, etc.

Preparation of Reagents

1. **TBE Buffer (10X)**

   (a) **To prepare 1 L of 10X TBE stock solution**:

   1. 1.Dissolve 108 g Tris and 55 g boric acid in 800 mL of distilled water.
   2. 2.Add 40 mL of 0.5 M Na_2EDTA (pH 8.0).
   3. 3.Adjust the volume to 1 L.

   (b) **To prepare 1 L of 0.5X TBE running buffer**:
   Take 50 mL of 10X TBE stock solution and 950 mL of sterile water and mix them properly.

2. **Agarose gel**

   1. Weigh out the required amount of agarose into a conical flask (0.8–1 % gel).
   2. Add 0.5X running buffer for the required quantity and heat it till the agarose melts. Occasionally swirl the contents to mix it well.
   3. After a few minutes, add ethidium bromide at a concentration of 0.5 μg/mL and mix the molten gel properly.
   4. Place an appropriate comb in the mould. After cooling, pour the gel into the gel mould.
   5. Allow the gel to solidify and then use it for loading the samples after removing the comb and placing in the gel box.

Protocol

1. In different PCR tubes, take 2 μL of plasmid and different concentrations of the test samples (50–200 μg).
2. To this sample, add 20 μL of 50 % H_2O_2 and irradiate with UV light for 10 min.
3. In another tube, add the plasmid and H_2O_2 and perform the same with UV radiation as above. This can serve as the negative control.
4. Take a separate sample with a standard antioxidant, plasmid, H_2O_2, and give the UV irradiation.
5. Take plasmid alone in another sample without any H_2O_2 and UV treatment.
6. Incubate all these samples for 6–12 h in room temperature in dark.
7. After incubation, load the samples into a precasted agarose gel (0.8 or 1 % gel) and keep it in the electrophoresis apparatus for separation of the DNA fragments.
8. Use the 0.5X TBE buffer as the gel running buffer and pour it into the tank to dip the gel in the buffer.
9. Mix all the samples with the loading dye (3 μL) and load into the well carefully. Load an appropriate DNA size marker along with the samples. Run the gel until the dye migrates to an appropriate length. View the gel in the gel documentation system.

Observation

The DNA bands can be observed in the gel. The sample with plasmid alone will show a clear single band which shows the intact plasmid DNA. The sample with plasmid DNA treated with H_2O_2 and UV will show a streak of DNA signifying the fragments of DNA generated by the free radical damage. The antioxidant samples added to the plasmid before the treatment with H_2O_2 and UV will not show much streaking as the antioxidants prevent or lessen the damage caused by the hydroxyl radicals. If the test samples are showing retrieval of the DNA band more than the fragmentation, then they can be considered as effective against hydroxyl radical damage.

Inference

The samples which shows streak of DNA patch are having the fragments of DNA and the ones with band can be considered as having intact or with lesser DNA fragmentation.

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Chapter 10
In Vitro Antihaemolytic Activity

Abstract In this assay, the rat erythrocytes are induced with hydroxyl radical damage by the addition of H_2O_2. This causes the rupture of the RBC and the contents leak out giving the red colour of the haemoglobin to the medium. Hence, anti-haemolytic activity has been presented here in a brief and easy manner using the rat erythrocytes.

Aim

To analyse the in vitro antihaemolytic capacity of a given test sample.

Principle

Red blood cells are important targets for the free radical damages caused in our body leading to the leakage of the cell. The radical scavenging effects of many compounds can effectively relieve the blood cell damage. In this assay (Magalhaes et al. 2009; Nabavi et al. 2010; 2012), the rat erythrocytes are induced with hydroxyl radical damage by the addition of H_2O_2. This causes the rupture of the RBC and the contents leak out giving the red colour of the haemoglobin to the medium. The test sample added to the reaction mixture is expected to protect the RBC membrane. The reactions are carried out in physiological pH and temperature. Here, reaction frequency is analysed by the measurement of the absorbance for each of the samples. Vitamin C can serve as a standard.

Materials Required

Blood from Wistar albino rat, test sample, vitamin C, dextrose, sodium citrate, citric acid, sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, hydrogen peroxide, ultra centrifuge, spectrophotometer, test tubes, conical flasks, etc.

Reagent Preparation

1. Alsever's solution
   Dissolve 2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid, 0.42 % sodium chloride in the required amount of water. Sterilize this solution in an autoclave.

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2. 0.02 M Phosphate-buffered saline (pH 7.4)
   To prepare 100 mL of buffer, mix 19 mL of 0.02 M monobasic sodium phosphate solution and 81 mL of 0.02 M dibasic sodium phosphate solution. Adjust the pH to 7.4. Now add 0.9 g of NaCl and mix properly for a homogenized mixture of phosphate-buffered saline.
3. H_2O_2 solution
   Add 1 M of H_2O_2 in PBS maintained in the pH 7.4 for the required quantity.

Protocol

(a) **Preparation of rat erythrocytes**

1. Collect blood from the retina of Wistar albino rats. Make sure that the blood does not clot. (Preferably use heparin coated tube for blood collection).
2. Mix the blood with equal volume of sterilised Alsever's solution.
3. Centrifuge the mixture at 3000 × *g* for 10 min.
4. Wash the packed cells with isosaline till a clear solution is obtained.
5. Make a suspension of blood in 5 % (v/v) isosaline.

(b) **Activity of the sample against haemolysis**

1. Take 300 μL of 5 % (v/v) suspension of erythrocytes in PBS in different test tubes.
2. Add aliquots of test sample (200–1000 μL) in different test tubes.
3. To this mixture, add 200 μL of H_2O_2 (l M in PBS, pH 7.4).
4. Incubate the reaction mixture at 37 °C for 3 h. Gently shake the contents while incubating.
5. After incubation, dilute the mixture with 4 mL of PBS and centrifuge at 2000 × *g* for 10 min.
6. Measure the absorbance of the resulting supernatant at 540 nm using spectrophotometer.

Calculation

$$\% \text{ inhibition} = \frac{(A_n - A_s)}{A_n} \times 100$$

where
A_n is the absorbance of negative control
A_s is the absorbance of test sample/standard
Calculate IC_{50} using the percentage inhibition.

Inference

The antihaemolytic activity of the given test samples is analysed and compared to the efficiency of the standard compound.

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Chapter 11
Antimicrobial Activity

Abstract Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with conventional antimicrobials. A method using scanning electron microscope (SEM) to study the morphology of the bacterial and fungal microbes and thus determining antimicrobial activity is presented in the chapter.

Determination of antimicrobial activity by scanning electron microscope

Aim

To image the morphological alterations of the micro-organisms by scanning electron microscope (SEM).

Principle

The SEM analysis of the micro-organisms clearly put in evidence on morphological changes by the plant sample, and it is very valuable in the assessment of antimicrobial activity.

Chemicals and reagents

1. Nutrient broth;
2. Czapek-Dox broth;
3. Potato dextrose agar;
4. 70–100 % ethanol;
5. 2.5 % glutaraldehyde.

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Protocol

11.1 Determination of Effect on Bacterial Morphology

1. Prepare about 5 mL of overnight inoculums with nutrient broth and individually treat with different concentrations of plant sample.
2. Keep positive and negative controls separately.
3. Harvest the bacterial cells after the treatment with the plant sample as above described, by centrifugation for 10 min at 5000 × *g*.
4. Swab the bacterial cells on the autoclaved glass slides and fix the cells by 2.5 % glutaraldehyde for 2 min.
5. Then, wash the glass slides gradually with 70–100 % ethanol and keep for air-dry.
6. Finally, sputter-coat the slides with gold palladium under vacuum and examine the slides under SEM (Shi et al. 1996).

11.2 Determination of Effect on Fungal Morphology

1. Prepare mother culture using Czapek-Dox broth.
2. Take the spores from mother culture, grow on glass slides containing Potato dextrose agar.
3. Incubate the glass slides with plant sample.
4. Directly sputter-coat the slides with gold palladium under vacuum and take images in ESEM mode by changing the detector (Shi et al. 1996).

Results

1. To analyse the activity of plant sample and to draw the size in both control and treated images.
2. Examine the minor changes in cell morphology of both fungal and bacterial strains.
3. Typically, distorted cells with small surface depressions, bleb formations and cell fragments.

Reference

Shi, J., Ross, C. R., Chengappa, M. M., Sylte, M. J., McVey, D. S., & Blecha, F. (1996). Antibacterial activity of a synthetic peptide (PR-26) derived from PR-39, a proline-arginine-rich neutrophil antimicrobial peptide. *Antimicrobial Agents and Chemotherapy, 40*(1), 115–121.

Chapter 12
In Vitro Anthelmintic Assay

Abstract Nematodes are believed to have originated 1000 million years ago as free living anaerobic benthic organisms. Modern anthelmintic drugs have been in use but the worms have created resistance to most of the broad spectrum anthelmintics such as benzimidazoles, levamisole, avermectins, etc. However, these drugs cause side effects. More recently, plant extracts have been reported as having developed resistance against anthelmintics. A study using earthworms to determine the activity has been developed and explained in a well-defined manner in this chapter.

Introduction

Helminthiasis is one of the 'Neglected Tropical Diseases (NTDs)' which are a diverse group of diseases that thrive mainly among the poorest populations. Helminth infections are caused by a group of parasitic agents and are easily spread by factors such as unhygienic lifestyle, poor sanitation, poverty, unsafe water, malnutrition. Generally, helminth infections do not cause direct effects, but the host's immune system gets affected and becomes susceptible to many microbial infections such as tuberculosis, pneumonia, anaemia, eosinophilia. Anthelmintics or antihelminthics are drugs that expel helminths (parasitic worms) from body (Gastrointestinal/GI tract) by stunning (vermifuge) or killing (vermicides) them. A majority of ethnoveterinary medicine surveys and validation studies indicate effective and wider use of plants as anthelmintics than for curing other diseases.

Aim

To determine the in vitro anthelmintic activity of plant samples.

Principle

The earthworms resembled intestinal parasitic roundworms of human beings both anatomically and physiologically and hence can be used for in vitro anthelmintic assay (Thorn et al. 1977; Vigar 1984). The activity can be measured based on the time taken for the death of earthworms which can be compared with the standard and control groups.

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Materials and Reagents

Adult earthworms, samples, standard drug—albendazole, Petri dishes, etc.

Protocol

1. Collect adult Indian earthworms (*Pheretima posthuma*) of uniform size (Ajaiyeoba et al. 2001).
2. They are washed to remove the bounded soil particles and other wastes.
3. The worms are divided into groups, each containing 6 worms.
4. The plant extracts/standards (10 mg/mL) are poured in Petri dishes and the earthworms are released.
5. Control group used distilled water in place of plant extracts.
6. All the solutions were prepared freshly before starting the assay.
7. Monitor the movement of the worms. The changes include rapid movement, release of body exudates, segment breakage and decolorization.
8. Note the time taken for the death of worms. Confirm the death with immobility and fading of body colour of the worms.
9. Sometimes paralysis may be witnessed which can be identified by dipping the non-motile worms in hot water that makes it to resurrect. In this case, the time taken for paralysis can also be noted.

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Chapter 13
Toxicity Studies

Abstract Toxicity studies in the animal models are done to determine the dose level recommended for the treatment of disease as drug. This guideline enables the characterization of adverse effects following repeated daily inhalation exposure to a test. This chapter includes oral and dermal toxicity studies which are discussed as per OECD guidelines. Both acute and subacute toxicity studies are given special emphasis.

Introduction

OECD guidelines for the testing of chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The original Guideline 420 was adopted in July 1992 as the first alternative to the conventional acute toxicity test, described in Test Guideline 401. Based on the recommendations of several expert meetings, revision was considered timely because: (i) international agreement had been reached on harmonized LD_{50} cut-off values for the classification of chemical substances, which differ from the cut-offs recommended in the 1992 version of the Guideline, and (ii) testing in one sex (usually females) is now considered sufficient.
Guidance on the selection of the most appropriate test method for a given purpose can be found in the Guidance Document on Acute Oral Toxicity Testing. This Guidance Document also contains additional information on the conduct and interpretation of Guideline 420 (Van den Heuvel et al. 1987, 1990; British Toxicology Society Working Party on Toxicity 1984; Whitehead and Curnow 1992; Stallard and Whitehead 1995; Stallard et al. 2002; OECD 2000a, b, c).

13.1 Acute Oral Toxicity Study

Aim

To identify the toxicity and reversibility of toxicity and to identify parameters in the plant bioactive compounds for clinical monitoring.

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Principle

Groups of animals of a single sex are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2000 mg/kg (exceptionally an additional fixed dose of 5000 mg/kg may be considered). The initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Clinical signs and conditions associated with pain, suffering and impending death are described in detail in a separate OECD Guidance Document (OECD 2000a, b, c). Further groups of animals may be dosed at higher or lower fixed doses, depending on the presence or absence of signs of toxicity or mortality. This procedure continues until the dose-causing evident toxicity or no more than one death is identified, or when no effects are seen at the highest dose or when deaths occur at the lowest dose.

Protocol

1. Perform the acute oral toxicity study according to organization for economic co-operation for development (OECD) guidelines for the testing of chemicals, Test No. 423 (OECD guidelines 2001).
2. Group the mice such that each group contains 6 male Swiss albino mice.
3. Keep the animals overnight with access to water but not food, after the plant extracts administered orally at a dose level of 500, 1000 and 2000 mg/kg body weight.
4. Observe the mice behaviour up to 24 h.
5. Observe the morbidity continuously for the first 2 h and mortality up to 24 h.
6. Fifty per cent of mortality among the animals indicates the toxicity concentration of the substance.
7. Observe the parameters such as skin and fur, eyes, mucous membranes, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma and also respiratory, circulatory, autonomic and central nervous systems and somatomotor activity and behaviour pattern.

13.2 Acute Dermal Toxicity Study

Introduction

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from a short-term exposure by the dermal route. Data from an acute dermal toxicity study may serve as a basis for classification and labelling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route.

Aim

To determine the dermal toxicity of the plant extracts in albino rats.

Principle

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test, the surviving animals are sacrificed and necropsied. Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

Protocol

1. Perform the acute dermal toxicity study in rats as per the OECD Guidelines 402 (1987).
2. Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a test substance.
3. It is an initial step in establishing a dosage regimen and may provide information on dermal absorption and mode of toxic action of a substance by this route.
4. It is done by applying the ointment-containing plant extracts or phytoconstituents at the high concentrations of 5 % (w/w) on the shaved back of the rats and observe any sort of allergic or toxic signs in the rats.
5. Observe the rat's behaviour up to 24 h.
6. However, the duration of observation should not be fixed rigidly.

13.3 Subacute Toxicity

Introduction

OECD guidelines are periodically reviewed in the light of scientific progress, animal welfare considerations and changing regulatory needs. The original subacute inhalation Test Guideline 412 (TG 412) was adopted in 1981. TG 412 has been revised to reflect the state of the science and to meet current and future regulatory needs. This guideline enables the characterization of adverse effects following repeated daily inhalation exposure to a test article for 28 days. The data derived from 28-day subacute inhalation toxicity studies can be used for quantitative risk assessments (if not followed by a 90-day subchronic inhalation toxicity study (TG 413)). This test guideline is not specifically intended for the testing of nanomaterials. Definitions used in the context of this guideline can be found in the Guidance Document 39.

Aim

To determine the subacute toxicity of the plant extracts in albino mice.

Principle

The subacute toxicity test generally involves daily or frequent exposure to the compound over a period up to 14 days. It provides information on the major toxic effects of the test compound and the affected target organs. The latency of development of the effect as related to dose, the relationship of the blood and tissue levels of the compound to the development of lesions, and the reversibility of the effects may also be studied. Data derived from these studies are used for designing chronic toxicity tests, in which animals are exposed to the chemical for longer periods of time.

Protocol

Group the mice such that each group contains 6 male Swiss albino mice.
Fast overnight and treat with plant extracts to the animals.

Group I:	Untreated
Group II:	Control (0.1 % CMC)
Group III and IV:	Plant extracts

1. Administer the plant extracts orally for 14 days.
2. Terminate the drug administration on the 14th day, after the rats are fasted for 24 h.
3. On the 15th day, weigh the mice in each group and anesthetize with diethyl ether.
4. Collect the blood samples for biochemical and haematological analyses.
5. Perform histopathological analysis through necropsy after euthanizing.

Collection of Blood for Biochemical Parameters

1. Collect the whole blood for haematogram in bottle-containing anticoagulant, ethylene diamine tetra-acetic acid (EDTA).
2. Separate the serum by centrifugation at 10,000 rpm for 10 min.
3. Store the sera in the −20 °C freezer and analyse for various biochemical parameters.
4. Perform the liver function test using serum biomarkers such as plasma concentrations of alkaline phosphatase (ALP), Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and bilirubin.
5. Determine the biochemical parameters such as creatinine and serum urea for kidney function test.

Haematological Profile

Analyse the haematological parameters such as haemoglobin count using haematology analyser (Model ABX-Micro-S-60).

Measure the total white blood cells (WBC) after diluting the blood in Turk's fluid and counting using a haemocytometer.

Histopathological Analysis

Perform the histopathological analysis of liver, spleen, kidney and heart by cutting 2-mm sections of the tissues using a microtome and fixing in 10 % formalin and staining with haematoxylin and eosin.

13.4 Calculation

(A) Vehicle of Choice, Drugs Dissolution and Volume Selection Rationale

A vehicle is any substance that acts as a medium in which a drug is administered. Vehicle, which is an essential consideration in all animal research should be biologically inert, has no toxic effects on the animals and not also influence the results obtained for the compound under investigation. Example of suitable vehicles for animal research includes water, normal saline (0.9 % sodium chloride), 50 % polyethylene glycol, 5–10 % Tween 80, 0.25 % methylcellulose or carboxymethylcellulose (Karl-Heinz et al. 2001; Nebendahl 2000).

In most researches involving experimental animals, dosages are usually calculated from stock solution of the test drugs dissolved in appropriate volume of solvent (vehicle). According to the OECD (Organization for Economic Corporation and Development)'s guidelines, dosage of drug (mg) should be constituted in an appropriate volume not usually exceeding 10 mL/kg (1 mL/100 g) body weight of experimental animals (mice and rats) for non-aqueous solvent in oral route of administration. However, in the case of aqueous solvents, 20 mL/kg (2 mM/100 g) body weight can be considered (OECD 2000b). Large dose volumes (40 mL/kg body weight) can cause unnecessary stress to animals and can also overload the stomach capacity and pass immediately into the small bowel or can result in passive reflux in the stomach, aspiration pneumonia, pharyngeal, oesophageal and gastric irritation or injury with stricture formation, oesophageal and gastric rupture and stress (Bonnichsen et al. 2005). Lower volume (5 mL/kg) can be considered to dissolve highly soluble solute drugs. Such low volume would ease the administration of drug in solution. However, highly viscous drug solution should be diluted, whenever possible, for ease of administration. However, final dilution volume should not exceed 20 mL/kg. Based on 10 mL/kg volume selection, required dose volume for a 100 g rat can be calculated as follows:

$$\frac{100\,\text{g}}{1000\,\text{g}} \times 10\,\text{mL} = 1\,\text{mL}$$

NB: 1 kg = 1000 g.

Based on 20 mL/kg volume selection, required dose volume for a 100 g rat can be calculated as follows:

$$\frac{100\,\text{g}}{1000\,\text{g}} \times 20\,\text{mL} = 2\,\text{mL}$$

(B) Dosage Calculation and Preparation of Stock Solution of Crude Plant Extract for Experimental Animals

Stock solutions and doses of a plant extract (with selected doses, 200 and 400 mg/kg) for a rat weighing 120 g are calculated as follows:

Step 1: Dosage calculation

Body weight of animal = 120 g

$$\text{Dosage in mg} = \frac{\text{Body weight of the animal(g)}}{1000\,\text{g}} \times \text{dose(mg)}$$

$$\text{Dosage in mg} = \frac{120\,\text{g}}{1000\,\text{g}} \times 200\,(\text{mg}) = 24\,\text{mg}.$$

Step 2: Dissolution of dose in a suitable vehicle for oral administration

From the OECD's guidelines, 120 g rat requires 24 mg of the crude plant extract which should be constituted in not more than 1.2 ml of normal saline according to the OECD guideline.

In a nut shell, 120 g ≡ 24 mg ≡ 1.2 ml of normal saline.

Bulk volume of the stock solution required for large number of animals (40) can be calculated by multiplying both sides by a constant value as follows:

$$24\,\text{mg} = 1.2\,\text{mL}$$
$$40 \times 24\ \text{mg} = 40 \times 1.2\,\text{mL}$$

960 mg of crude plant extract will be dissolved in 48 mL of normal saline = $\frac{960\,\text{mg}}{48\,\text{mL}} = 20\,\text{mg/mL}$.

This example shows that 1 mL of dissolved plant extract from a given stock solution (960 mg/48 mL = 20 mg/mL) is the required dose (from selected dose of 200 mg/kg) for a rat weighing 100 g. However, 1.2 mL from the same stock solution is the required volume for a rat weighing 120 g (which is meant to receive 24 mg of the plant extract).

Having successfully prepared a stock solution (960 mg/48 mL = 20 mg/mL) for a selected dose of 200 mg/kg, stock solution of the same plant extract with a higher selected dose (400 mg/kg) can be easily prepared by dissolving 960 mg of plant

extract with half the volume (24 mL) used in the previous stock (960 mg/48 mL), thereby yielding a higher concentration (960 mg/24 mL = 40 mg/mL) which is twice the concentration of the formal stock. In this case, animals with similar body weight from two different selected dose categories (200 and 400 mg/kg, respectively) will receive the same volume, but different concentrations.

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Chapter 14
In Vivo Antioxidant Assays

Abstract Oxidative stress and antioxidant deficiency have been implicated in the pathophysiology of a wide range of diseases and conditions. Consequently, over recent years many different supplementation trials have been implemented, aimed at improving clinical outcomes by boosting antioxidant levels. These trials included supplementation with individual antioxidants, antioxidant combinations, and antioxidant-rich foods such as fruit and vegetable juices and other plant extracts. To ensure that data from these trials are interpreted correctly, it is essential that suitable biomarkers are used to assess changes in in vivo antioxidant activity resulting from supplementation. Therefore, the measurement of antioxidant systems, such as superoxide dismutase, catalase, glutathione reductase, and status of other molecules in biological fluids with their quantification methods are simplified in this chapter.

Introduction

Oxidative stress is associated with a variety of chronic degenerative diseases, including cancer, diabetes, cardiovascular diseases and Alzheimer's disease. An imbalance of oxidants and antioxidants within the human body, in which either oxidants are high or antioxidant protection is low, will lead to a state of oxidative stress. Therefore, the measurement of the antioxidant status of biological fluids could be used as an early warning sign of possible disease onset.

Aim

To estimate the antioxidant activity of plant extracts.

Protocol

1. Group the rats such that each group contains 6 male Swiss albino rats.
2. Treat the groups with different doses of plant extracts

   Group I: Control (0.6 % CMC),
   Group II: Glibenclamide (10 mg/kg),
   Group III: Plant extract: dose 1 (100 mg/kg) and
   Group IV: Plant extract: dose 2 (200 mg/kg).

3. Kill the animals and collect the blood by cardiac puncture.

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4. Wash the liver with ice-cold Tris–HCl buffer (0.1 M, pH 7.4).
5. Rinse the liver with ice-cold 0.15 M potassium chloride and homogenize using 0.05 % potassium dihydrogen phosphate buffer (pH 7.5) in 0.5 mM EDTA.
6. Centrifuge the cytosolic sample of liver homogenate at 10,000 rpm for 10 min at 4 °C.
7. Remove the upper lipid layer carefully and centrifuge the supernatant at 5000 rpm for 10 min at 4 °C.
8. Estimate the in vivo antioxidant activity of the obtained supernatant.
9. Estimate the parameters such as total protein, SOD, catalase, glutathione peroxidase, lipid peroxidation inhibition, reduced glutathione, glutathione S-transferase and glutathione reductase by standard procedures in both blood and liver to assess the oxidative stress.

14.1 Determination of Total Protein

Aim

To determine the protein content in the tissue homogenate by the Lowry et al. (1951) method.

Principle

The tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of Folin–Ciocalteau reagent in an alkaline medium to give a bluish purple colour with absorbance at 660 nm.

Reagents

1. Sodium carbonate (2 % in 0.1 N NaOH);
2. Copper sulphate (0.5 in 1 % potassium sodium tartrate);
3. Alkaline copper solution.

   (i) Mix the 0.5 % $CuSO_4$ in 1 % sodium potassium tartrate and 2 % Na_2CO_3 in 0.1 N NaOH in the ratio 1:50);

4. Folin–Ciocaltaeu reagent (1 N);
5. Standard (100 μg BSA/mL);
6. Tris buffer (0.1 M, pH 7.5).

Procedure

1. Mix the 10 μL of the homogenate with 990 μL of distilled water.
2. Add 5 mL of alkaline $CuSO_4$ and keep the reaction for 10 min at room temperature.
3. Add 0.5 mL of 1 N Folin–Ciocalteau reagent and measure the absorbance at 660 nm after 30 min against the reagent blank.
4. Calculate the protein content from the standard Bovine serum albumin (BSA).

14.2 Determination of Superoxide Dismutase (SOD)

Aim

To determine the SOD level in the blood and tissue homogenate by the Mccord and Fridovich (1999) method.

Principle

The photoillumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then reoxidizes and simultaneously reduces oxygen to O_2-, which is allowed to react with a detector molecule NBT. Upon reaction, NBT is reduced to a formazan blue. The SOD in the sample inhibits the formazan production.

Reagents

1. Sodium pyrophosphate buffer (0.025 M, pH 8.3),
2. Phenazine methosulphate (PMS) (186 μM),
3. Nitroblue tetrazolium (NBT) (300 μM),
4. NADH (780 μM),
5. Glacial acetic acid,
6. n-Butanol and
7. Potassium phosphate buffer (50 mM, pH 6.4).

Procedure

1. Centrifuge the heparinized blood at 2500 rpm to remove the plasma.
2. Add the normal saline to the packed RBCs and again centrifuge the solution and remove the supernatant.
3. Use the remaining packed RBCs for the experiment and determines the haemoglobin concentration.
4. Then, haemolyse the 100 μL of the packed RBCs by 900 μL of cold water.
5. Treat the haemolysate sample with 250 μL of $CHCl_3$ and 500 μL of ethanol with vigorous mixing to remove the haemoglobin.
6. Centrifuge the mixture at 15,000 rpm for 60 min at 4 °C.
7. Then, 100 μL of the clear supernatant containing haemoglobin is used for the SOD estimation.
8. Mix the 100 μL of supernatant with 200 μL of 0.1 M EDTA (containing 0.0015 % NaCN).
9. Add 100 μL of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.95 mL.
10. Measure the absorbance at 560 nm after adding 0.05 mL of riboflavin against distilled water.
11. The tubes were then uniformly illuminated with an incandescent lamp for 15 min and absorbance was taken again at 560 nm.

Calculation

1. Same method was followed to estimate SOD level in tissue homogenate.
2. Calculate the percentage of inhibition by comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity before illumination).
3. The volume of the sample required to scavenge 50 % of the generated superoxide anion is considered as 1 unit of SOD activity and expressed as U/g Hb in the case of blood and as U/mg protein for tissue.

14.3 Determination of Catalase Activity in Blood and Tissue Homogenate

Aim

To evaluate the catalase activity in the blood by the method of Aebi (1974) and tissue catalase was determined by the method of Beers and Sizer (1952).

Principle

The catalase activity was assayed by measuring the decomposition of H_2O_2. The H_2O_2 has absorption maxima at 240 nm and absorption decreases with the decomposition of H_2O_2. The difference in extinction per unit time is a measure of the catalase activity.

Reagents

1. Phosphate buffer: 0.067 M (pH 7.0) and
2. Hydrogen peroxide (2 mM) in phosphate buffer.

Procedure

(A) Catalase in blood

1. Prepare the lysate-packed RBCs in ice-cold water containing 5 g Hb/dL.
2. Prepare 1:500 dilution of haemolysate with sodium–potassium phosphate buffer (0.05 M, pH 7).
3. Reference cuvette contained 1 mL of buffer and 2 mL of haemolysate and test cuvette contained 2 mL diluted haemolysate.
4. Then, add 1 mL of H_2O_2 (30 mM in the buffer, fresh every time) to start the reaction.
5. Measure the decrease in extinction at 240 nm at 1 min with an interval of 15 s.

Calculation

1. Calculate the catalase activity by the following formula:

$$\text{Catalase}(\text{k/g Hb}) = \frac{2.303 \times (\log A_1 - \log A_2) \times \text{dil. Factor}}{15 \times g\,\text{Hb/mL of blood}}$$

   where A_1 is A_{240} at $t = 0$ and A_2 is A_{240} at $t = 15$ s
2. The results are expressed as k/g Hb, where k is a rate constant of first-order reaction.

Catalase in Tissue

1. Mix the 0.1 mL of the tissue homogenate (approximately 0.1 mg protein) with 1.9 mL of phosphate buffer.
2. Add 1 mL of H_2O_2 solution in buffer.
3. Measure the decrease in extinction at 240 nm at 1 min with an interval of 3 min.
4. Place the sample control in the reference cuvette containing 0.1 mL of tissue homogenate and 2.9 mL of the buffer.

Calculation

1. Calculate the activity of catalase by the molar extinction coefficient at 43.6.
2. Define the specific activity at 25 °C in terms of millimoles of H_2O_2 consumed/min/mg of protein sample.

$$\text{mM } H_2O_2 \text{ decomposed/min/mg protein (U/mg protein)} = \frac{\text{DA/min} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}$$

14.4 Determination of Glutathione Peroxidase (GPx) Activity

Aim

To find out the glutathione peroxidase activity in the tissue homogenate by method of Hafeman et al. (1974).

Principle

The GPx enzyme degrades the H_2O_2 in the presence of GSH by the following reaction:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Measure the remaining GSH by the addition of DTNB.

Reagents

1. Phosphate buffer (0.12 M, pH 7.2),
2. EDTA (15 mM),
3. Sodium azide (10 mM),
4. Oxidized glutathione (6.3 mM) and
5. NADPH (9.6 mM).

Procedure

1. Treat the 100 μL of tissue homogenate with 100 μL of 5 mM GSH, 100 μL of 1.2 mM H_2O_2, 100 μL of 25 mM NaN_3 and phosphate buffer (1 M, pH 7.0) in a total volume of 2.5 mL at 37 °C for 6 min.
2. Stop the reaction by addition of 2.0 mL of 1.65 % H_3PO_4.
3. Centrifuge the reaction mixture at 3000 rpm for 10 min.
4. Mix the supernatant (2.0 mL) with 2.0 mL of 0.4 M Na_2HPO_4 and 1 mL of 1 mM DTNB (in buffer).
5. Measure the absorbance of the yellow colour complex at 412 nm after incubation for 10 min at 37 °C.

Calculation

$$\text{The GPx activity (U/mg protein)} = \frac{\text{OD of blank} - \text{OD of sample} \times 1}{0.001 \times g\,\text{Hb or mg protein} \times 10}$$

Define the one unit of enzyme activity by the decrease in log GSH by 0.001/min/mg protein after subtraction of the decrease in log GSH per min for the non-enzymatic reaction.

14.5 Estimation of Lipid Peroxidation

Aim

To estimate the lipid peroxidation (LPO) inhibition by Hogberg et al. (1974) method.

Principle

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (thiobarbituric acid) to give a pink-coloured product having absorption maxima at 535 nm.

Reagents

1. Isotonic KCl (1.15 %),
2. Hypotonic KCl (0.5 %),
3. Tris-buffered saline (TBS) (10 mM Tris, 0.15 M NaCl, pH 7.4),
4. Ferrous sulphate (10 μM),
5. Thiobarbituric acid (TBA) (1 %),
6. Ascorbic acid (0.06 mM),
7. Ethanol (70 %) and
8. Acetone.

Procedure

1. The reaction mixture contains 0.03 M Tris–HCl buffer (pH 7.4), 0.2 mM sodium pyrophosphate and 0.2 mL of tissue extract in a total volume of 2 mL.
2. Incubate the reaction mixture at 37 °C for 20 min.
3. Stop the reaction by the addition of 1 mL of 10 % TCA.
4. Shake well, add 1.5 mL of TBA and then heat the reaction mixture in a boiling water bath for 20 min.
5. Centrifuge the tubes and measure the colour formation at 532 nm.

14.6 Determination of Reduced Glutathione (GSH) Content in Blood and Tissue Homogenate

Aim

To determine the reduced glutathione in the blood and tissue based on the method of Moron et al. (1979).

Principle

Reduced glutathione forms a yellow-coloured complex with DTNB with an absorbance at 412 nm.

Reagents

1. TCA (5 %),
2. Phosphate buffer (0.2 M, pH 8.0),
3. DTNB (0.6 mM in 0.2 M phosphate buffer) and
4. Standard GSH (10 nM/mL of 5 % TCA).

Procedure

1. Prepare the haemolysate from heparinized blood in distilled water.
2. Mix the haemolysate or tissue homogenate (500 μL) with 125 μL of 25 % TCA and cooled on ice for 5 min followed by further dilution of the mixture with 600 μL of 5 % TCA.
3. Centrifuge at 3000 rpm for 5 min to settle down the precipitate.
4. Mix, 150 μL of the supernatant with 350 μL of sodium phosphate buffer (0.2 M, pH 8.0) and 1.0 mL of DTNB (0.6 mM in 0.2 M, pH 8.0 phosphate buffer).
5. Measure the yellow colour reaction mixture at 412 nm against a blank

Calculation

1. Plot a standard graph with different concentrations (10–50 nM) of GSH.
2. Calculate the GSH content of the sample from the standard graph and expressed as nM/mL for blood and nM/mg protein for tissue.

14.7 Assay of Glutathione S-Transferase (GST)

Aim

To evaluate the glutathione S-transferase enzyme in the blood and tissue homogenate by the Habig et al. (1974) method.

Principle

The enzyme is assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340 nm.

Reagents

1. Glutathione (1 mM),
2. 1-Chloro-2,4-dinitrobenzene (CDNB) (1 mM in ethanol) and
3. Phosphate buffer (0.1 M, pH 6.5).

Procedure

1. Homogenize the samples (0.5 g) with 5.0 mL of phosphate buffer.
2. Centrifuge the homogenates at 5000 rpm for 10 min and collect the supernatants.
3. Determine the activity of the enzyme by observing the changes in absorbance at 340 nm.

4. The reaction mixture contains 0.1 mL of GSH, 0.1 mL of CDNB and phosphate buffer in a total volume of 2.9 mL.
5. Initiate the reaction by the addition of 0.1 mL of the enzyme extract.
6. Record the readings at every 15 s at 340 nm against distilled water blank in a spectrophotometer.
7. The assay mixtures without the extract serve as a control to monitor non-specific binding of the substrates.
8. Calculate the GST activity by the extinction coefficient of the product formed (9.6 mM^{-1} cm^{-1}).
9. The results are expressed as nmoles of CDNB conjugated/minute.

14.8 Assay of Glutathione Reductase (GR)

Aim

To estimate the glutathione reductase in the homogenate by the David and Richard (1983) method.

Principle

Glutathione reductase catalyses the conversion of oxidized glutathione to reduced glutathione employing NADPH as substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Reagents

1. Phosphate buffer (0.12 M, pH 7.2),
2. EDTA (15 mM),
3. Sodium azide (10 mM),
4. Oxidized glutathione (6.3 mM) and
5. NADPH (9.6 mM).

Procedure

1. The assay system contains 1 mL of phosphate buffer, 0.1 mL of EDTA, 0.1 mL of sodium azide, 0.1 mL of oxidized glutathione and 0.1 mL of enzyme source, and the volume is made up to 2 mL with distilled water.
2. Incubate the tubes for 3 min and add 0.1 mL of NADPH.
3. Read the absorbance at 340 nm in a spectrophotometer at every 15-s interval of 2–3 min.
4. The enzyme activity is expressed as μmoles of NADPH oxidized/minute/g liver tissue.

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Chapter 15
Analgesic Activity

Abstract Analgesics are agents which selectively relieve pain by acting in the CNS and peripheral pain mediators without changing consciousness. Analgesics may be narcotic or non-narcotic. The study of pain in animals raises ethical, philosophical, and technical problems. Both peripheral and central pain models are included to make the test more evident for the analgesic property of the plant. This chapter highlights methods such as hot plate and formalin and acetic acid-induced pain models to check the analgesic activity of medicinal plants.

Introduction

Analgesics refer to a group of drugs used to temporarily relieve pain. They are sometimes known as painkillers. They block pain signals by changing how the brain interprets the signals and slowing down the central nervous system. Combining analgesics with alcohol, prescription or illegal drugs can create dangerous and unpredictable effects. Even low doses can impair driving ability. Therefore, a new drug from medicinal plants is needed to replace the synthetic drugs with low toxicity.

15.1 Hot Plate Method

Aim

To find out the central analgesic activity of plant extracts by hot plate method.

Principle

The hot plate test is normally used to evaluate the centrally acting analgesics including sedatives and narcotic analgesics. The centrally acting analgesic exerts their effect via. supraspinal and spinal receptors. The plant extracts inhibit the pain mediators such as histamine and serotonin which are produced during the arachidonic acid pathway.

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The analgesic activity of the plant extracts may be due to the presence of tannin contents in the plants.

Protocol

1. The study is based on the method described by Eddy and Leimbach (1953) and Sharma et al. (2003).
2. Select the animals based on the mice body weight (20–25 g).
3. Group the mice such that each group contains 6 male Swiss albino mice and administer the standards and extracts in the following order:

   Group I: Control (0.6 % CMC);
   Group II: Pentazocine (5 mg/kg);
   Group III: Plant extracts: doses (two or more).

4. Place the animal on a hot plate (analgesiometer), and temperature is maintained at a 55 ± 1 °C.
5. Observe and note the paw licking or jumping and withdrawal of the paws.
6. Perform the measurement of animal's reaction at 0, 30, 60, 90, 120, and 240 min after the first thermal stimulus.

Calculation

Calculate the percentage of inhibition per every 30 min by the following formula:

$$\text{Percentage of inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

15.2 Acetic Acid-Induced Abdominal Writhes

Aim

To find out the peripheral analgesic activity of plant extracts by acetic acid-induced test.

Principle

The acetic acid writhing test in mice is commonly used to study the peripheral analgesics of drugs. Ribeiro et al. (1997) reported that the abdominal constriction evacuated by acetic acid is dependent on the production and release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-1α (1L-1α) and IL-α, from resident peritoneal macrophages and mast cells. The plant extracts may involve in the anti-inflammatory substances and reduce the peripheral analgesic activity.

Protocol

1. The study is based on the method described by Koster et al. (1959).
2. Pain is produced by the injection of acetic acid into peritoneal cavity of mice.
3. Select the animals based on the mice body weight (20–25 g).

4. Group the mice such that each group contains 6 male Swiss albino mice.

   Group I: Control (0.6 % CMC);
   Group II: Aspirin (150 mg/kg);
   Group III: Plant extract: doses (two or more).

5. The animals react with characteristic movements such as extension of hind limb, abdominal constriction, and trunk twisting movement called writhing.
6. After 30 min of drug administration, inject the 0.1 mL/10 g b.w. acetic acid solution intraperitoneally (i.p.) to all the groups of animals to induce writhing.
7. Record the number of writhings which occur between 0 and 30 min after the acetic acid injection.

Calculation

Calculate the percentage of inhibition per every 30 min by the following formula:

$$\text{Percentage of inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

15.3 Formalin-Induced Nociceptive Behaviour

Aim

To find out the peripheral analgesic activity of plant extracts by formalin-induced test.

Principle

The formalin test produces a distinct biphasic response, and different analgesics act differently in the early and late phases of this test. The first phase of nociception (neurogenic nociceptive response), which occurs on the first 5th min after the formalin injection, is characterized by the direct stimulation of nociceptors present on afferent C and, in part, Aδ fibres (glutamate nociceptors and substance P release). The second phase of nociception (inflammatory nociceptive response), which occurs between the 15th and 30th min after formalin injection, is related to the release of pro-inflammatory mediators such as adenosine, bradykinin, histamine, prostaglandin and serotonin (Reeve and Dickenson 1995). The plant extracts may involve in the anti-inflammatory substances and reduce the peripheral analgesic activity.

Protocol

1. The study is based on Hunskaar and Hole's (1987) method.
2. Nociceptive behaviour is produced by the injection of acetic acid in the intraplantar region of right hind paw of the mice.

3. Select the animals based on the mice body weight (20–25 g).
4. Group the mice such that each group contains 6 male Swiss albino mice.

Group I: Control (0.6 % CMC);
Group II: Indomethacin (10 mg/kg);
Group III: Plant extract: doses (two or more).

5. Inject the 1 % (20 μL) of formalin solution after one hour of drug administration at right hind paw to all the groups of animals to induce nociception.
6. Measure the time that the animal spent licking or biting its paw during the first phase (0–5 min) and the second phase (15–30 min).
7. Compare the extract-treated groups with control and standards.

Calculation

Calculate the percentage of inhibition by the following formula and compare it with the first phase and second phase.

$$\text{Percentage of inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

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Chapter 16
Anti-inflammatory Activity

Abstract Inflammation is the body's first response to infection or injury and is critical for both innate and adaptive immunity. It can be considered as part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. The search for natural compounds and phytoconstituents that are able to interfere with these mechanisms by preventing a prolonged inflammation could be useful for human health. Here, the anti-inflammatory properties of plant-based drugs are put together with both in vitro and acute (carrageenan, egg albumin and croton oil) and chronic (cotton pellet) in vivo models.

Introduction

Inflammation is the body's reaction to an injury such as invasion by microorganism or mechanical or chemical damage and is characterized five cardinal signs: heat (calor), pain (dolor), redness (erythema rubor), swelling (tumour) and loss of function. Inflammation may have beneficial effects, such as the destruction of invading microorganisms and the walling of an abscess cavity, thus preventing spread of infection. The inflammatory process involves the concerted action of the immune, kinin, fibrinolytic and clotting systems which interact to maintain the integrity of the vascular system and to limit the spread of infection damage.

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16.1 In Vitro Anti-inflammatory Activity by Membrane Stabilization Method

Aim

To determine the in vitro anti-inflammatory activity by membrane stabilization method (Shinde et al. 1999).

Principle

Anti-inflammatory activity can be studied in vitro using human red blood cell (HRBC) membrane as it is analogous to the lysosomal membrane and could be extrapolated to the stabilization of lysosomal membrane. Its stabilization (hypotonicity-induced membrane lysis) by standard drug/samples can be taken as a measure for in vitro anti-inflammatory activity.

Materials and Reagents

Alsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid, 0.42 % sodium chloride), human/rat blood, isosaline (0.9 %, pH-7.2), reaction mixture (phosphate buffer (pH-7.4), hyposaline (0.45 %), RBC (red blood cells) suspension, sample/standard), standard—diclofenac sodium, autoclave, centrifuge, etc.

Protocol

1. Prepare the Alsever's solution by dissolving 2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid and 0.42 % sodium chloride in distilled water and sterilize the solution.
2. Collect the blood from retina of Wistar albino rats.
3. Mix the collected blood with equal volume of sterilized Alsever's solution and centrifuge at 3000 rpm for 10 min.
4. Wash the packed cells three times with isosaline (0.9 %, pH-7.2) and make a suspension in 10 %(v/v) isosaline.
5. Prepare the reaction mixture (4.5 mL) by mixing 1 mL phosphate buffer (pH-7.4), 2 mL hyposaline (0.45 %), 1 mL sample/standard (1 mg/mL) and 0.5 mL RBC suspension.
6. Use the reaction mixture without plant sample as control and phosphate buffer as blank.
7. Incubate the assay mixtures at 37 °C for 30 min and centrifuge again.
8. Estimate the haemoglobin content in the supernatant solution spectrophotometrically at 560 nm.

Calculation

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

16.2 Carrageenan and Egg-Albumin-Induced Acute Paw Oedema in Rats

Aim

To find out the acute anti-inflammatory activity of plant extracts by egg-albumin- and carrageenan-induced method.

Principle

The bioactive compounds from plant extracts inhibit the egg-albumin-induced oedema by blocking the release of histamine and serotonin.

Carrageenan involves cell migration and plasma exudation, and it is characterized by a biphasic response with marked oedema formation resulting from the rapid production of several inflammatory mediators such as histamine, serotonin, and bradykinin (first phase), which is subsequently sustained by the release of prostaglandins and nitric oxide (second phase) with peak at 3 h, produced by inducible isoforms of COX and nitric oxide synthase (iNOS), respectively. The non-steroidal anti-inflammatory drugs and plant extracts inhibit the carrageenan-induced oedema by blocking the production of mediators in the first and second phases.

Protocol

1. The study of egg-albumin- and carrageenan-induced acute paw oedema is based on the method described by Okokon and Nwafor (2010) and Winter et al. (1962), respectively.
2. Select the animals based on the Swiss albino mice body weight (100–150 g).
3. Acute inflammation is produced by injection of egg-albumin and carrageenan plantar region of left hind paw.
4. Note the paw thickness by Vernier caliper.
5. Group the rat such that each group contains six male Swiss albino rats.

   Group I: Control (0.6 % CMC),
   Group II: Indomethacin (10 mg/kg) and
   Group III: Plant extract: doses (two or more).

6. After one hour, inject the 0.1 mL of 1 % (w/v) egg albumin and carrageenan (suspended in normal saline) in the planter region of the left paw of control and plant-extract-treated groups.
7. Note the paw volume of left leg of control and plant-extract-treated rats every 1 h after the administration carrageenan.
8. Observe the paw thickness up to 7 h.

Calculation

Calculate the percentage of inhibition by the following formula and compared with control.

$$\text{Percentage of inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

16.3 Croton Oil-Induced Ear Oedema Model

Aim

To find out the inhibitory activity of plant extracts against acute inflammation, croton oil-induced mice ear oedema (George et al. 2013) is performed according to the method of Wang et al. (2008) modified by Lin et al. (2008).

Principle

Croton oil-induced ear oedema method is used to detect the inflammatory/anti-inflammatory potential effects of histamine ligands in a model of acute skin inflammation induced by local application of croton oil. Croton oil contains 12-*o*-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. TPA is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen-activated protein kinases (MAPK) and phospholipase A2 (PLA2), leading to release of platelet activation factor (PAF) and AA. This cascade of events stimulates vascular permeability, vasodilation, polymorphonuclear leucocytes migration, release of histamine and serotonin and moderate synthesis of inflammatory eicosanoids by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes (Patel et al. 2012).

Protocol

1. Apply 10 μL acetone solutions containing 5 % croton oil topically to the right ear of mice.
2. Apply equal volume of acetone to the left ear.
3. Administrate the plant extracts orally at different doses (mg/kg) about 60 min before the croton oil treatment.
4. As a reference, the non-steroidal anti-inflammatory drug (NSAID), indomethacin (10 mg/kg), can be used.
5. Six hours later, kill the mice and remove both ears uniformly by a sharp scissors and individually weighed on a sensitive balance.
6. Measure the oedematous response as the weight difference between the two plugs and calculate the percentage inhibition.

Calculations

$$\text{Percentage inhibition} = \frac{\text{Weight of right ear} - \text{weight of left ear}}{\text{Weight of right ear}} \times 100$$

16.4 Histamine-Induced Acute Paw Oedema in Rats

Aim

To find out the anti-inflammatory activity of plant extracts by histamine-induced method.

Principle

Histamine is an important inflammation mediator, potent vasodilator substance and also increases the vascular permeability. When histamine is subcutaneously injected into a rat, it forms a wheal around the injected place, due to increase of vascular permeability of the host capillary venules in the skin. Substances that antagonize the activity of histamine receptors reduce the area of the wheal formed.

Protocol

1. To study the anti-inflammatory property of extract on histamine-induced acute paw oedema in rats is performed based on the Kulkarni and Dandiya (1975) method.
2. Group the rats such that each group contains six male Swiss albino rats.
   Group I: Oral feeding of saline,
   Group II: Injection of Indomethacin intraperitoneally and
   Group III: Plant extract: doses (two or more).
3. After 30 min, inject the 0.1 mL of 1 % (w/v) histamine in the planter region of the right paw of control and plant-extract-treated groups.
4. Note the paw volume of left leg of control and plant-extract-treated rats every 1 h after the administration carrageenan.
5. Observe the paw thickness up to 7 h.
6. Note the paw thickness by Vernier caliper.

Calculation

Calculate the percentage of inhibition by the following formula and compared with control.

$$\text{Percentage inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

16.5 Neutrophil Migration into the Peritoneal Cavity

Aim

To find out the anti-inflammatory activity of plant extracts by Neutrophil migration into the peritoneal cavity test.

Principle

Plant extracts and non-steroidal anti-inflammatory drugs were able to reduce the neutrophil rolling, adhesion (selectin and lectin) and migration which was occurred at inflammation site by inhibiting the inflammatory mediators into the peritoneal cavity after carrageenan injection (Murugan and Parimelazhagan 2013).

Protocol

1. Study the anti-inflammatory property of extract on neutrophils migration into the peritoneal cavity in rat model as described by Bastos et al. (2001).
2. Neutrophil migration in the peritoneal cavity is induced by the injection of carrageenan.
3. Group the rats such that each group contains six male Swiss albino rat.

Group I: Control (0.6 % CMC),
Group II: Dexamethasone (Subcutaneously) (2 mg/kg),
Group III: Plant extract: dose 1 (100 mg/kg) and
Group IV Plant extract: dose 2 (200 mg/kg).

5. Inject carrageenan (500 µg/cavity, i.p., 500 µg, 1 mL) into the peritoneal cavity of rats to induce Neutrophil migration after 1 h administration extract and dexamethasone.
6. Anaesthetize the animals with diethyl ether and euthanized by cervical dislocation after 4 h of carrageenan injection.
7. Shortly after, inject the phosphate-buffered saline (pH-7.4) containing EDTA (1 mg/mL, i.p., 10 mL).
8. Collect the fluid from the peritoneal cavity and centrifuge the fluid at 1000 × *g* for
9. Five minutes at room temperature.
10. Dispose the supernatant and resuspend the precipitate with 1 mL of saline.
11. Dissolve the 10 µL aliquots in a 200 µL of Turk's solution.
12. Count the total cells in a Neubauer chamber under optic microscopy.

Calculation

1. Express the results by number of neutrophils/mL in the different doses of plants and standards.

2. Calculate the percentage of inhibition by the following formula:

$$\text{The percentage of the neutrophil migration inhibition} = (1 - \text{T/C}) \times 100,$$

where
T represents the treated groups leucocyte counts and
C represents the control group leucocyte counts.

16.6 Cotton Pellet-Induced Granuloma Model

Aim

To find out the chronic anti-inflammatory activity of plant extracts by cotton pellet-implanted granuloma method.

Principle

The subcutaneous implantation of a cotton pellet directly triggers an acute inflammatory response. When the acute response is insufficient to eliminate the pro-inflammatory agents, it leads to a chronic inflammatory reaction. Chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and mononuclear cells (Swingle and Shideman 1972). A few days after pellet implantation, the amount of newly formed tissue can be measured, after its removal, by weighing the dried pellets. The increased weight of the dried cotton pellet has been shown to correlate with the amount of granulomatous tissue formed (Bailey et al. 1982).

This procedure induces an inflammatory angiogenic and proliferative response that reproduces many features of the healing occurring after mechanical and natural injuries such as ballon angioplasty, atherosclerosis, inflamed synovium and surgical wounds. Many mediators seem to be involved in the formation of the fibrovascular tissue, including cytokines, chemokines and eicosanoids. Plant extracts inhibit the formation of collagen and fibrovascular tissues by blocking the inflammatory mediators.

Protocol

1. Study the chronic anti-inflammatory property of extract based on Bastos et al. (2001) method.
2. Granuloma tissue is produced by implantation of cotton pellets (subcutaneously) in the rats.
3. Group the rats such that each group contains six male Swiss albino rats.
   Group I: Control (0.6 % CMC),
   Group II: Indomethacin (10 mg/kg) and
   Group III: Plant extract: doses (two or more).
4. Anaesthetize the animals after shaving the fur.

5. Implant the sterile preweighed cotton pellets (50 ± 1 mg) in the axilla region of each rat through a single needle incision.
6. Give the plant extracts and standards to rats by orally for seven consecutive days from the day of cotton pellet implantation.
7. On the eighth day, anaesthetize the animals again.
8. Remove the cotton pellets surgically and make free from extraneous tissues.
9. Incubate the pellets at 37 °C for 24 h and dried at 60 °C to constant weight.

Calculation

1. Calculate the weight of the granulomatous tissue by the difference between the initial and the final dry weight of the cotton pellets.
2. Calculate the percentage of inhibition by the following formula:

$$\text{Percentage of inhibition} = [(\text{Control value} - \text{sample value})/\text{Control value}] \times 100$$

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Chapter 17
Antipyretic Activity

Abstract The concept regarding the pathogenesis of pyrexia is almost exclusively concentrated on humoral mediatory mechanism. As per these views, exogenous pyrogens first persuade the formation of pyrogenic cytokines and these endogenous pyrogens order to increase the synthesis of prostaglandin E in different structures of the central nervous system (CNS). The subsequent alterations of CNS functions are reflected in the modifications of peripheral thermoregulatory effector functions, which hoist body temperature during the development of fever and maintain it during the whole fever course. Brewer's yeast-induced pyrexia model has been furnished in the chapter.

Introduction

Fever is a secondary effect of infection, tissue damage, inflammation, malignancy, graft rejection and other inflammatory disease conditions. The higher level of prostaglandin (PG) synthesis increases the body temperature and leads to fever or pyrexia. The concept regarding the pathogenesis of pyrexia is almost exclusively concentrated on humoral mediatory mechanism. As per these views, exogenous pyrogens first persuade the formation of pyrogenic cytokines and these endogenous pyrogens order to increase the synthesis of prostaglandin E in different structures of the central nervous system (CNS). The subsequent alterations of CNS functions are reflected in the modifications of peripheral thermoregulatory effector functions, which hoist body temperature during the development of fever and maintain it during the whole fever course (Szikely and Romanovsky, 1998).

17.1 Brewer's Yeast-Induced Pyrexia

Aim

The aim is to study the antipyretic activity by the Brewer's yeast-induced pyrexia model in animals according to the method of Adams et al. (1968).

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Principle

Normally, the infected or damaged tissue initiates the enhanced formation of pro-inflammatory mediators (cytokines, such as interleukin-1ß, interleukin-α, interleukin-ß, and TNF-α), which increase the synthesis of prostaglandin E2 (PgE2) near hypothalamic area and thereby trigger the hypothalamus to elevate the body temperature. Brewer's yeast induces both TNF-α and prostaglandin synthesis. Hence, the inhibition of higher level of prostaglandin E2 synthesis by the test drug reduces the elevated body temperature and maintains it as constant body temperature level.

Materials

Wistar albino male/female rats (150–250 g), cages and husk, digital thermometer, Brewer's yeast, carboxymethyl cellulose (CMC), oral gavages, surgical hand gloves, experimental drugs and standard drug (paracetamol).

Protocol

1. Maintain the animals for 7 days prior to the experiment and select the animals showing approximately constant rectal temperature for the study.
2. Induce the pyrexia through subcutaneous injection of 15 % (w/v) Brewer's yeast suspension (10 mL/kg) into the animal's dorsum region.
3. Measure the rectal temperature of each rat after 18 h of injection using digital thermometer and select the rats which shows an increase in temperature of 0.5–1°C for further experimental studies.
4. Group the selected rats as control, standard and test groups with six animals each.
    a. Control group—distilled water + yeast suspension
    b. Standard group—paracetamol (150 mg/kg) + yeast suspension
    c. Test groups—test samples + yeast suspension
5. Measure the rectal temperatures of the rats at 1st, 2nd, 3rd, 4th and 5th h after the standard or test drug administrations (George et al. 2013).
6. Compare the results with the control group.

Calculations

(i) 15 % (w/v) Brewer's yeast suspension preparation
15 g of Brewer's yeast dissolved in 100 mL of 0.9 % saline water

(ii) Injection volume of Brewer's yeast suspension
10 mL/kg of animals
For example, if average weight of animal is 170 g, the injection volume = 10/1000 × 170 = 1.70 mL.

(iii) Temperature measurement (Sajeesh et al. 2011)
18th h temperature (increased body temperature) − 5th h temperature (after the standard or test drug administration).

For example, 18th h temperature = 37.05 °C; 5th h temperature is 36.01 °C. The temperature reduction by the standard or test drug treatment = 37.05 − 36.01 = 1.04 °C.

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Chapter 18
Inflammatory Bowel Disease

Abstract Inflammation response plays an important role in host survival, and it also leads to acute and chronic inflammatory diseases such as rheumatoid arthritis, bowel diseases, allergic rhinitis, asthma, atopic dermatitis and various neurodegenerative diseases. During the course of inflammation, the ROS level increases. In addition to ROS, several inflammatory mediators produced at the site lead to numerous cell-mediated damages. Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a chronic intestinal disorder resulting from a dysfunctional epithelial, innate and adaptive immune response to intestinal microorganisms. The methods involving indomethacin-induced enterocolitis in rats with macroscopic changes of IBD, myeloperoxidase assay, microscopic (histologic) characters and biochemical parameters are discussed.

Introduction

Inflammation is a complex protective reaction caused by endogenous and exogenous stimulations. However, inflammation response has important role for host survival, and it also leads to acute and chronic inflammatory diseases such as rheumatoid arthritis, bowel diseases, allergic rhinitis, asthma, atopic dermatitis and various neurodegenerative diseases (Mainardi et al. 2009). During the course of inflammation, the ROS level increases. In addition to ROS, several inflammatory mediators produced at the site lead to numerous cell-mediated damages (Kang et al. 2008). Previous literatures reported that overproduction of ROS and an inequality of important antioxidants were observed in the intestine of patients receiving continual doses of NSAIDs, leading to oxidative damage (Nagano et al. 2005). Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a chronic intestinal disorder resulting from a dysfunctional epithelial, innate and adaptive immune response to intestinal microorganisms. Crohn's disease is first described as a disease in the distal ileum; however, it may be found anywhere in the GIT. Ulcerative colitis (UC) invariably affects the rectum and may extend proximally in a confluent pattern to involve a part of or the entire colon.

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18.1 Indomethacin-Induced Enterocolitis in Rats

Aim

The aim is to investigate the indomethacin-induced enterocolitis (inflammation bowel disease) in animal models (Elson et al. 1995).

Principle

The nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat several inflammatory disorders; however, the regular use of NSAIDs causes severe adversative effects including ulcers, erosions, bleeding, perforation and strictures in the gastrointestinal (GI) region. Inflammatory bowel disease (IBD) is one of the most severe chronic inflammatory disorders in the GI tract. Indomethacin is one of the NSAIDs for the treatment of several inflammation- and pain-related diseases. The continual usage will produce severe chronic inflammation in bowel tissues. The test substance may have the capability to reduce the inflammation in the gastrointestinal tissues which can be observed by the analysis of the myeloperoxidase and colonic lipid peroxide levels.

Materials

Wistar albino male/female rats (150–250 g), cages and husk, oral gavages and surgical hand gloves, indomethacin, experimental drugs and standard drug (prednisolone), dissection table, scissors, normal saline, formalin, etc.

Protocol

1. Maintain the Wistar albino male rats (150–250 g) in groups for a minimum of 7 days at 22 ± 2 °C with 12-h light/12-h dark cycle prior to the experiment.
2. Provide the animals with commercial food pellets and clean drinking water.
3. Group the animals into control, standard and test groups with six animals in each as follows:

   (a) Control group: distilled water + 10 mg/kg indomethacin (two consecutive days subcutaneously).
   (b) Standard group: prednisolone (2 mg/kg, p.o.) for 4 days + indomethacin (10 mg/kg, s.c., for 2 days).
   (c) Test group: particular test samples (7 days) + indomethacin on 8th and 9th day.

4. Continue the treatment of test groups till 11th day.
5. On 11th day, kill the animals by cervical dislocation and dissect open to remove gastrointestinal tract which ranges from stomach to anus.

6. Flush the GIT gently with saline and isolate 5 cm of proximal duodenum, 10 cm of distal jejunum, 10 cm of proximal ileum, whole caecum and 5 cm of proximal colon from all the animals for further evaluations.

18.2 Evaluation Based on Macroscopic Characters

Weight of the Tissues

1. Clean the intestinal tissues (5 cm of proximal duodenum, 10 cm of distal jejunum, 10 cm of proximal ileum, whole caecum and 5 cm of proximal colon) using saline.
2. Weigh the tissues after drying them.
3. Compare the mean weight of the intestinal tissues of standard drug and plant-sample-treated groups with untreated control group.

Scoring for Rat Gastrointestinal Parts

1. Observe the pieces of rat gastrointestinal tract parts macroscopically and score them according to the following scoring pattern:

18.3 Macroscopic Changes of IBD

Score	Macroscopic changes
0	No visible change
1	Hyperaemia at sites
2	Lesions having diameter l mm or less
3	Lesions having diameter 2 mm or less (number <5)
4	Lesions having diameter 2 mm or less (number 5–10)
5	Lesions having diameter 2 mm or less (number >10)
6	Lesions having diameter more than 2 mm (number <5)
7	Lesions having diameter more than 2 mm (number 5–10)
8	Lesions having diameter more than 2 mm (number >10)

2. Measure the damaged area in rat gastrointestinal tract parts using Vernier calliper and observe them macroscopically.

3. Score them using the following scoring pattern:

Scoring Pattern of IBD

Score	Percentage area affected
0	0
1	1–5
2	5–10
3	10–25
4	25–50
5	50–75
>6	75–100

4. Calculate the score for an individual rat as a combined score of ileum, duodenum, distal jejunum, colon and caecum.

18.4 Myeloperoxidase Assay for Quantification of Inflammation

1. Rinse the pieces of inflamed tissues with ice-cold saline, blot to dryness and weigh them.
2. Mince the tissues and homogenize in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4), using Remi tissue homogenizer (RQ-127A).
3. Centrifuge the homogenate at 3500 rpm for 30 min at 4 °C (Remi centrifuge C23).
4. Discard the supernatant and add 10 mL of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 % hexadecyl trimethyl ammonium bromide (HETAB) and 10 mM EDTA to the pellet.
5. Then, subject it to one cycle of freezing and thawing and a brief period of sonication (15 s).
6. After sonication, centrifuge the solution again at 15,000 rpm for 20 min and store the supernatant.
7. Myeloperoxidase (MPO) activity in the supernatant is determined by adding 100 µL of supernatant to 1.9 mL of 50 mM phosphate buffer (pH 6.0) and 1 mL of 1.5 M 0.167 mg/mL O-dianisidine hydrochloride containing 0.0005 % H_2O_2.
8. Record the change in absorbance at 460 nm of each sample for 3 min using spectrophotometer (Shimadzu UV 1 60A UV–Vis spectrophotometer).
9. Express the MPO activity of the tissues as µmol/min/mg tissue (Elson et al. 1995).

Calculation of MPO activity is as follows:

$$\text{MPO activity (U/g)} = \frac{\text{X}}{\text{Weight of the piece of tissue taken}}$$

where

$$\text{X} = \frac{10 \times \text{ change in absorbance per minute}}{\text{Volume of supernatant taken in the final reaction}}$$

18.5 Measurement of Colonic Lipid Peroxide Concentration

Measure the lipid peroxidation (LPO) in colon tissue by estimating the formation of thiobarbituric acid reactive substances (TBARSs) (Niehius and Samuelsson, 1968).

1. To the tubes containing 1 mL of homogenate of colon tissue, add 2 mL of reagent mixture (15 % TCA, w/v; 0.375 TBA, w/v; 0.25 N HCl).
2. Boil the tubes for 5 min and cool them down to room temperature.
3. Centrifuge the contents at 1000 rpm for 20 min and collect the supernatant.
4. Measure the absorbance of the supernatant at 535 nm against reagent blank.
5. Express TBARS as μM of malondialdehyde (MDA) released/mg protein.

18.6 Determination of Colonic GSH Contents

Reduced glutathione in blood and tissue can be determined by the formation of yellow-coloured complexes with DTNB with an absorbance at 412 nm (Moron et al. 1979).

1. Mix the tissue homogenate (500 μL) with 125 μL of 25 % TCA and cool on ice for 5 min.
2. Further, dilute the mixture with 600 μL of 5 % TCA and centrifuge at 3000 × *g* for 5 min to settle down the precipitate.
3. 150 μL of the supernatant was mixed with 350 μL of sodium phosphate buffer (0.2 M, pH 8.0) and 1 mL of DTNB (0.6 mM in 0.2 M, pH 8.0 phosphate buffer).
4. Measure the yellow colour obtained at 412 nm against a blank containing 5 % TCA in place of the supernatant.
5. Prepare a standard graph using different concentrations (10–50 nmoles) of GSH.

6. Calculate the GSH content of the sample from the standard graph and express the results as nmol/g Hb for blood and nmol/g protein for tissue homogenate.

18.7 Evaluation Based on Microscopic (Histologic) Characters

1. Wash the freshly excised proximal duodenum, distal jejunum, proximal ileum, caecum and proximal colon from each group with saline and preserve it in 10 % formaldehyde solution for histopathological studies.
2. Fix the tissues for 12 h using isopropyl alcohol and xylene and embed them in paraffin for light microscopic study.
3. Make 5-μm sections of paraffin-embedded tissues and stain them after deparaffinization using haematoxylin and eosin stain.
4. Examine the stained sections for any inflammatory changes such as infiltration of the cells, necrotic foci, damage to tissue structures like Peyer's patches and damage to nucleus from the photomicrographs captured at a magnification of 40×.

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Chapter 19
In Vitro Anti-arthritic Activity

Abstract Formaldehyde-induced arthritis and complete Freund's adjuvant (CFA)-induced arthritis in rats are well-known models to determine the anti-arthritic activity. Arthritis is a symptom of chronic inflammation. It is a chronic progressive disorder, developing over months or years and involving essentially the synovial joints of the body. Hence, these two models become the basis of this chapter to analyse the anti-arthritic activity.

Introduction

Arthritis is developed as a symptom of chronic inflammation especially in the body joints. Rheumatoid arthritis is one of the most important rheumatic diseases. It is relatively common, and its serious forms can cause severe disability.

19.1 In Vitro Anti-arthritic Activity by the Inhibition of Protein Denaturation

Aim

To determine the in vitro anti-arthritic activity by the inhibition of protein denaturation method by Williams et al. (2008).

Principle

Protein denaturation is a process in which the proteins lose their secondary and tertiary structures by the application of external stress or compounds, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Denaturation of proteins is a well-documented cause of inflammation in conditions such as rheumatoid arthritis, and the protection against such denaturation is the main mechanism behind the anti-rheumatic non-steroidal anti-inflammatory drugs (NSAIDs).

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Materials and Reagents

2 % Bovine serum albumin, phosphate-buffered saline (pH 7.4), standard drug—diclofenac sodium, spectrophotometer, etc.

Protocol

1. Prepare the reaction mixture (0.5 mL) by dissolving 0.4 mL of Bovine serum albumin (2 % aqueous solution), 0.05 mL distilled water and 0.05 mL of sample.
2. Incubate the samples at 37 °C for 30 min and then heat at 57 °C for 10 min.
3. After cooling, add 2.5 mL of phosphate-buffered saline (pH 7.4) in each test tube.
4. Measure the turbidity spectrophotometrically at 600 nm.
5. For control, use 0.05 mL distilled water instead of standard/sample.

Calculation

Calculate the percentage inhibition of protein denaturation as follows:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

19.2 Formaldehyde-Induced Arthritis in Rats

Aim

The aim is to study the anti-arthritic activity by formaldehyde-induced arthritic model in animals according to the method of Singh and Majumdar (1996).

Principle

Inhibition of formaldehyde-induced paw oedema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis.

Materials

Wistar albino male/female rats (150–250 g), cages and husk, oral gavages, surgical hand gloves, plethysmometer, formaldehyde, experimental drugs and standard drug (diclofenac sodium).

Protocol

1. Divide the healthy Wistar rats of either sex (150–200 g) into groups of six animals each.
   (a) Control group—distilled water;
   (b) Arthritic control group—0.1 mL of 2 % (v/v) formaldehyde;
   (c) Standard group—diclofenac sodium (DCS) (13.5 mg/kg p.o.);
   (d) Test group—test samples of various concentrations p.o.
2. Inject 0.1 mL of 2 % (v/v) formaldehyde solution in the plantar surface of the left foot, on the first and the third day of the test.
3. Start the drug treatment from the initial day, i.e. from the day of formaldehyde injection (0th day) till 10th day.
4. Record the rat paw volume daily by using plethysmometer (Ugo Basile, Italy 7140).

19.3 Complete Freund's Adjuvant (CFA)-Induced Arthritis in Rats

Aim

The aim of the study is to evaluate the anti-arthritic activity by complete Freund's adjuvant (CFA)-induced arthritic model.

Principle

Complete Freund's adjuvant (CFA) contains heat-killed *Mycobacteria* in a water-in-oil emulsion. After subcutaneous injection, CFA induces adjuvant arthritis that can serve as a model to test the anti-arthritic and anti-inflammatory effects of investigational substances. The effect observed in this model seems to be parallel to that observed in human disease.

Materials

Wistar albino male/female rats (150–250 g), cages and husk, oral gavages, surgical hand gloves, plethysmometer, formaldehyde, experimental drugs and standard drug (diclofenac sodium).

Protocol

1. Maintain the Wistar albino male rats (150–250 g) in groups for a minimum of 7 days at 22 ± 2 °C with 12-h light/12-h dark cycle prior to the experiment.
2. Provide the animals with commercial food pellets and clean drinking water.
3. Divide the healthy Wistar rats of either sex into groups of six animals each.
   (a) Control group—distilled water;
   (b) Arthritic control group—0.1 mL of 2 % (v/v) formaldehyde;
   (c) Standard group—diclofenac sodium (DCS) (13.5 mg/kg p.o.);

(d) Test group—test samples of various concentrations p.o.

4. Induce arthritis by injecting CFA (Kamalutheen et al. 2009). In brief, on the 0th day, inject 100 μL of CFA containing killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC- 25177) into the paw of the left hind limb of each rat. (Complete Freund's adjuvant is available in pharmacies).

Evaluation of Severity of Arthritis

1. Measure the paw volume using mercury plethysmometer before the injection of the adjuvant and on 4th, 8th, 14th, and 21st days.
2. Also, measure the body weight every 3rd day after the adjuvant injection (Patil and Suryavanshi, 2007).

19.4 Haematological Parameters

Haematological parameters such as red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb) value and erythrocyte sedimentation rate (ESR) are evaluated by routine laboratory method (Patil et al. 2009) using a haemocytometer.

(i) **Red blood cell count**

1. Determine the erythrocyte count using haemocytometer.
2. Collect the blood samples in an EDTA bottle from each animal in the groups.
3. Add 0.02 mL of the blood sample to 4 mL of red blood cell–diluting fluid in a clean test tube to make a 1:200 dilution of the blood sample.
4. Load the diluted blood sample onto a Neubauer counting chamber and count all red blood cells in the five groups of 16 small squares in the central area of the Neubauer chamber using a light microscope (Leica Inc, USA) at 40× objective.
5. Multiply the number of cells enumerated for each sample by 10,000 to obtain the total red blood cell count per microlitre of blood.

(ii) **Total white blood cell count**

1. Determine the total leucocytes using haemocytometer.
2. Collect 0.02 mL of blood in a small test tube containing 0.38 mL of white blood cell–diluting fluid to make a 1:20 dilution of the blood sample.
3. Load the diluted sample onto the Neubauer counting chamber and count all the cells on the four corner squares using a light microscope at 10× objective.

4. Multiply the number of cells counted for each blood sample by 50 to obtain the total white blood cell count per microlitre of blood.

(iii) **Haemoglobin concentration (Hb)**

1. Determine the haemoglobin concentration of the blood samples by cyan-methemoglobin method.
2. Place 5 mL of Drabkin's haemoglobin reagent in five test tubes.
3. Then, add 0.02 mL of the blood sample from individual rat in the different groups to the reagent in four of the test tubes and mix properly.
4. Allow the mixture to react for 20 min, transfer into cuvettes and read the absorbance at 540 nm against a reagent blank in the 5th test tube using spectrophotometer.
5. Also, prepare the standards and read as stated above.
6. Multiply the absorbance of the sample with a calibration factor (×36.8) derived from the absorbance and concentration of the standard to obtain the haemoglobin concentration of the blood samples.

(iv) **Erythrocyte sedimentation rate (ESR)**

1. ESR can be determined by Westergren's method.
2. Take the anticoagulant blood in the ESR tubes exactly up to the "0" mark and place the tubes vertically (upright position) in the ESR stand.
3. Take the reading after 5 min as "zero"-hour reading and further at one-hour intervals twice (1 and 2 h) subsequently.
4. ESR normal scale: 0 mm at 1 h and 2–3 mm at 24 h. If the value is high at 24 h, it indicates some pathological defects.

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Chapter 20
Anti-diarrhoeal Activity

Abstract Diarrhoea is characterized by an increase in the frequency of bowel movements, wet stool and abdominal pains. It is the world's third highest killer disease, contributing substantially to paediatric morbidity and mortality, especially in the malnourished. Antibiotics used as anti-diarrhoeal drugs sometimes provoke adverse effects and microorganisms tend to develop resistance towards them. Therefore, the search for safe and more effective agents from plant origin using standard protocols of charcoal meal-induced diarrhoea has been defined in this chapter.

Introduction

Diarrhoea is characterized by an increase in the frequency of bowel movements, wet stool and abdominal pains. It is the world's third highest killer disease, contributing substantially to paediatric morbidity and mortality, especially in the malnourished. Antibiotics used as anti-diarrhoeal drugs sometimes provoke adverse effects and microorganisms tend to develop resistance towards them. Therefore, the search for safe and more effective agents from plant origin has continued to be an important area of active research.

20.1 Charcoal Meal-Induced Diarrhoea

Aim

The study is aimed to evaluate the anti-diarrhoeal activity by charcoal meal-induced diarrhoeal model according to the method of Rouf et al. (2003).

Principle

Activated charcoal prevents the absorption of drugs and chemicals into the system by avidly adsorbing them on the surfaces of the charcoal particles. For the evaluation of gastrointestinal transit, atropine is used as standard drug which is known to inhibit gut motility probably due to its anti-muscarinic effect. The activated charcoal

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model is used in the gastrointestinal motility test to find out the effects of plant extracts on the peristaltic movement.

Materials

Wistar albino male/female rats (150–250 g), cages and husk, sodium carboxymethyl cellulose (CMC), oral gavages, surgical hand gloves, experimental drugs and standard drug (atropine).

Protocol

1. Fast the Wistar rats for 18 h.
2. Divide the rats into groups of six animals each
   (a) Control group—0.5 % (w/v) sodium carboxymethyl cellulose in distilled water (oral),
   (b) Standard group—atropine (5 mg/kg, i.p.),
   (c) Test groups—test samples of different concentrations.
3. After 1 h, administer charcoal meal 0.25 mL (10 % charcoal in 0.5 % (w/v) sodium carboxymethyl cellulose) to each animal orally.
4. Sacrifice the animals after 30 min.
5. Isolate the total small intestine from pylorus to caecum and measure the total length and the length travelled by the charcoal meal.

Calculation

$$\% \text{ Inhibition} = \frac{\text{Distance travelled by charcoal meal in control group} - \text{treated group}}{\text{Distance travelled by charcoal meal in control group}} \times 100$$

Reference

Rouf, A. S., Islam, M. S., & Rahman, M. T. (2003). Evaluation of antidiarrhoeal activity of *Rumex maritimus* root. *Journal of Ethnopharmacology, 84*, 307–310.

Chapter 21
Anti-ulcer Activity

Abstract This chapter explains the procedure of ethanol-induced ulcer to check the protective effect of drugs over induced ulcer in rats. Ulcer is defined as the erosion in the lining of the stomach or duodenum and is caused by the disruptions of the gastric mucosal defence and repair systems. Ulceration of stomach is called gastric ulcer and that of duodenum is called duodenal ulcer and together peptic ulcer. In clinical practice, peptic ulcer is one of the most prevalent gastrointestinal disorders, which commonly occurs in developed countries.

Introduction

Ulcer is defined as the erosion in the lining of the stomach or duodenum and is caused by the disruptions of the gastric mucosal defence and repair systems. Ulceration of stomach is called gastric ulcer and that of duodenum is called duodenal ulcer and together peptic ulcer. In clinical practice, peptic ulcer is one of the most prevalent gastrointestinal disorders, which commonly occurs in developed countries.

21.1 Ethanol-Induced Ulcer

Aim

The study is aimed to evaluate the anti-ulcer activity by ethanol-induced ulcer model according to the method of Abdulla et al. (2010).

Principle

Ethanol has been shown to produce gastric damage by impairing gastric defensive factors, such as mucus and mucosa circulation by releasing vasoactive factors, by producing oxygen radicals and causing lipid peroxidation. While ethanol damages the superficial epithelial layers, inhibits the release of mucosal prostaglandins and

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depresses the gastric defensive mechanisms, the secondary metabolites of plants appear to enhance the gastric mucosal defence indicating its cytoprotective potentials.

21.2 Materials

Wistar albino male/female rats, cages and husk, sodium carboxymethyl cellulose (CMC), oral gavages, surgical hand gloves, ethanol, experimental drugs and standard drug (ranitidine).

Protocol

1. Fast all the animals for 24 h before the experiment but provide with clean drinking water up to 2 h before the experiment.
2. Divide the animals into groups of six each such as
   a. Ulcer control group—vehicle CMC, 0.25 % (w/v) p.o.
   b. Standard group—ranitidine (20 mg/kg) p.o.
   c. Test groups—test samples of different concentrations
3. Induce gastric ulcer in rats by administration of 80 % ethanol (1 mL/100 g) orally after 45 min of standard/sample treatment.
4. After 60 min euthanize, the rats by cervical dislocation (Paiva et al. 1998) under overdose of diethyl ether anaesthesia and excise the stomach of rats immediately.
5. Open the stomach along the greater curvature, rinse with saline to eliminate gastric contents and blood clots and examine by a 10× magnifier lens to measure the formation of ulcers.
6. The numbers of ulcers were counted. The following table indicates ulcer score and descriptive observation (Kulkarni 2002).

Ulcer score	Observation
0	Normal coloured stomach
0.5	Red colouration
1	Spot ulceration
1.5	Haemorrhagic streak
2	Ulcers ≥ 3 but ≤5 mm
3	Ulcers > 5 mm

7. Mean ulcer score for each animal will be expressed as ulcer index (UI).

Calculation

$$\text{Percentage of Inhibition} = [(\text{UIC} - \text{UIT})/\text{UIC}] \times 100$$

where

UIC—Ulcer index of control group
UIT—Ulcer index of treatment group
UI = Ulcer index was calculated using the formula

$$\text{UI} = U_S + U_N + U_P \times 10^{-1}$$

where

U_S = Mean severity of ulcer score
U_N = Average numbers of ulcer per animal
U_P = Percentage of animals with ulcer incidence

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Chapter 22
Hepatoprotective Activity

Abstract The liver performs a vital role in metabolism, secretion, storage, and detoxification of endogenous and exogenous substances. Oxidative stress and free radicals enhance the severity of hepatic damage, which can be overcome by the antioxidant mechanism. Plant extracts can be the best source of such antioxidants and mediate hepatoprotective activity. In this chapter, high-dose paracetamol-induced hepatotoxicity in rat model is discussed with explanations of biochemical and histopathological studies.

Introduction

Liver is one of the most important organs in the human body system because of its high rate of metabolic activity. It is the vital organ that functions as a collective centre of nutrient metabolism, digestion, storage and excretion of the products (Ghosh et al. 2011). Liver serves as an integral part of drug metabolism and removal of xenobiotics from the body, thus protecting against foreign substances by detoxifying and eliminating them. Liver ailments or hepatic syndrome remains one of the serious health problems (Baranisrinivasan et al. 2009) in the current treatment system. Various chemotherapeutic agents such as carbon tetrachloride, thioacetamide, paracetamol and during their metabolism inside the liver cause severe damages to hepatocytes. Oxidative stress is also attributed to either an increase in reactive oxygen species (ROS) generation or decreases the antioxidant defence mechanisms which lead to many degenerative diseases including a variety of hepatopathies (Hensley et al. 2000).

22.1 Paracetamol-Induced Liver Damage

Aim

The aim of the study is to investigate the paracetamol-induced hepatotoxicity in animal models (Sreedevi et al. 2009).

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Principle

Paracetamol is widely used analgesic and antipyretic drug, but produces acute and chronic liver damages at higher dose. The hepatotoxicity of paracetamol has been attributed to the formation of highly reactive toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) in liver cells which causes severe liver damages. The active test drugs have the ability to overhaul the liver damages and to eliminate the ROS in the process of the metabolic reaction.

Materials

Wistar albino male/female rats (150–250 g), oral gavages, surgical hand gloves, paracetamol, experimental drugs and standard drug (Silymarin), dissection table, scissors, normal saline, formalin for tissue storage, etc.

Protocol

1. Maintain the Wistar albino male rats in groups for a minimum of 7 days at 22 ± 2 °C with 12-h light or 12-h dark cycle prior to the experiment.
2. Feed the animals with commercial food pellets and clean drinking water.
3. Group the animals into control, standard and test groups with six animals in each

   (a) Control group: distilled water + paracetamol
   (b) Standard group: silymarin (25 mg/kg p.o.) + paracetamol
   (c) Test groups: particular test samples + paracetamol

4. Administer 2 g/kg, p.o. of paracetamol for 14 days for all the groups.
5. On 14th day, collect the blood samples from all animals by puncturing the retro-orbital plexus under mild ether anaesthesia and store it in bottles containing the anticoagulant and ethylene diamine tetra acetic acid (EDTA).
6. Then, sacrifice the animals, excise their liver and rinse them in ice-cold normal saline.
7. Analyse the blood samples for hepatic injury using biochemical markers.
8. Liver antioxidants are estimated using tissue samples and are subjected for histopathological studies.

Haematological Studies

1. Analyse the haematological parameters of the collected blood such as

   (a) Haemoglobin count using haematology analyser (Model ABX-Micro-S-60)
   (b) Total white blood cells (WBCs)
   (c) Red blood cells (RBCs) and
   (d) Platelets count using haemocytometer

2. Allow the collected blood to clot and separate the serum by centrifuging at 600 × *g* for 15 min and analyse the following biochemical parameters using Span diagnostics Limited Kit, India.

(a) Alkaline phosphatase (ALP),
(b) Serum glutamic oxaloacetate transaminase (SGOT),
(c) Serum glutamic pyruvic transaminase (SGPT),
(d) Creatinine and bilirubin and lipid profile such as total cholesterol (TC) and
(e) Triglycerides (TG).

Histopathological Studies

1. Examine the organs grossly and store it in 10 % formalin.
2. Fix the slices of the excised organs in Bouin's solution for 12 h, process them and embed using paraffin.
3. Stain the sections of 5 mm thick with slum haematoxylin and eosin and observe them under microscope for histological changes.

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Chapter 23
Anti-diabetic Activity

Abstract The hyperglycaemia continues to be a major health problem in India and other developing countries. This imbalance of blood glucose causes serious health problems such as damages to the blood vessel, poor healing of wounds, retinal damage, renal damage—kidney failure. The in vitro enzyme models and evaluation of hypoglycaemic effect of sample on normal and glucose-loaded rats has been used as a prediction experiment in this chapter before going for anti-diabetic experiment using animal models.

Introduction

Diabetes is a global metabolic epidemic disease which is fast becoming a leading cause of morbidity, mortality and disability across the world (Gupta et al. 2008). It is becoming the third killer of humankind, after cancer and cardiovascular disease, because of its high prevalence (Li et al. 2004). The hyperglycaemia continues to be a major health problem in India and other developing countries. This imbalance of blood glucose causes serious health problems such as damages to the blood vessel, poor healing of wounds, retinal damage, renal damage—kidney failure.

The evaluation of hypoglycaemic effect of sample on normal and glucose-loaded rats has been used as a prediction experiment before doing the anti-diabetic experiment with induced animals. The oral glucose tolerance test (OGTT) is a widely used procedure that was originally developed to classify carbohydrate tolerance (WHO).

23.1 In Vitro Anti-diabetic Activity

Introduction

In humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of polymeric substrates into shorter oligomers. Later on in the gut, these are further hydrolysed by pancreatic

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α-amylases into maltose, maltotriose and small malto-oligosaccharides. The digestive enzyme (α-amylase) is responsible for hydrolysing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of α-amylase can lead to reduction in post prandial hyperglycaemia in diabetic condition.

Alpha-glucosidase is a membrane-bound enzyme located on the epithelium of the small intestine, catalysing the cleavage of disaccharides to form glucose. Inhibitors can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycaemia. Therefore, inhibition of α-glucosidase could be one of the most effective approaches to control diabetes (Sheikh et al. 2008). Glucosidases are not only essential to carbohydrate digestion, but also vital for the processing of glycoprotein and glycolipids. This enzyme is a target for antiviral agents that interfere with the formation of essential glycoproteins required in viral assembly, secretion and infection. Glucosidases are also involved in a variety of metabolic disorders and carcinogenesis (Dennis et al. 1987).

23.1.1 α-Amylase Inhibitory Activity

Aim

To find out to the α-amylase inhibitory activity by DNS method.

Principle

α-Amylase activity can be measured in vitro by hydrolysis of starch in presence of α-amylase enzyme. This process was quantified by using iodine, which gives blue colour with starch. The reduced intensity of blue colour indicates the enzyme-induced hydrolysis of starch into monosaccharides. If the substance/extract possesses α-amylase inhibitory activity, the intensity of blue colour will be more. In other words, the intensity of blue colour in test sample is directly proportional to α-amylase inhibitory activity.

Protocol

1. Premix the α-amylase with extract at various concentrations (50–200 μg/mL).
2. Add starch as a substrate at 0.5 % to start the reaction.
3. Incubate the reaction at 37 °C for 5 min and terminate by the addition of 2 mL of DNS (3, 5-dinitrosalicylic acid) reagent.
4. The reaction mixture was heated for 15 min at 100 °C and dilute with 10 mL of distilled water in an ice bath (Miller 1959).
5. Determine the α-amylase activity by measuring spectrum at 540 nm.

Calculation

Calculate the alpha-amylase inhibitory activity by the following formula:

$$\% \text{ Inhibition} = [(\text{Abs Control} - \text{Abs Samples})/\text{Abs Control}] \times 100$$

23.1.2 α-Glucosidase Inhibitory Activity

Aim

To find out α-glucosidase inhibitory potential of plant extract.

Principle

The α-glucosidase activity is determined by a reaction in which α-glucosidase hydrolyses *p*-nitrophenyl-α-D-glucopyranoside resulting in the formation of a colorimetric (405 nm) product, proportional to the α-glucosidase activity present. One unit of α-glucosidase is the amount of enzyme that catalyses the hydrolysis of 1.0 μ mole substrate per minute at pH 7.0.

Protocol

1. Premix α-glucosidase (0.075 units) with the extract at various concentrations (50–200 μg/mL).
2. Add 3 mM *p*-nitrophenyl glucopyranoside (pNPG) as a substrate to the reaction mixture to start the reaction (Miller 1959).
3. Incubate the reaction mixture at 37 °C for 30 min and stop by adding 2 mL of Na_2CO_3.
4. Determine α-glucosidase activity by measuring the *p*-nitrophenol release from pNPG at 400 nm.

Calculation

Calculate the α-glucosidase inhibitory activity by the following formula:

$$\% \text{ Inhibition} = [(\text{Abs Control} - \text{Abs Samples})/\text{Abs Control}] \times 100$$

23.2 Evaluation on Normal Healthy Rats and Oral Glucose Tolerance Test

Aim

The aim is to determine the hypoglycaemic nature of given sample.

Principle

The instant glucose directly elevates the blood glucose level and the substance may have capability to reduce the blood glucose level in different mechanisms depending upon their chemical nature. Monitoring blood glucose level in fasting and glucose-loaded metabolism helps to determine the hypoglycaemic ability of the substance.

Materials

1. Wistar albino rats (adult Wistar albino rats weighing between 150 and 200 g of either sex studies)
2. Cages and husk
3. Glucometer
4. Glucose (2 g/5 mL)
5. Vehicle—carboxymethyl cellulose (CMC)
6. Standard drug (glibenclamide—commercial hypoglycaemic drug)

Preparation of Sample and Standard

1. Blend the samples and standard with 0.6 % CMC.
2. Prepare the dose.
3. Dissolve 500 mg of sample in 5 mL of CMC, one ml will have 100 mg of sample. Hence, according to the body weight, administer the samples.

Procedure

Evaluation on Normal Healthy Rats

1. Group the animals into normal control, positive control and test groups (6 animals of each group).
2. Maintain the animals in fasting condition.
3. Administrate the fasted animals with sample as fixed dose which should be blend with permissible vehicle.
4. Then, monitor the blood glucose levels at 30 and 60, 120, 180 and 240 min (4 h) for observe the hypoglycaemic nature of the sample.

Evaluation of Oral Glucose Tolerance Test

1. The OGTT has to be performed on glucose-loaded rats.
2. Load the rats with glucose (2 g/kg) orally and then observe of blood glucose levels continuously to determine the hypoglycaemic effect of extracts like previous at 30, 60, 120,180 and 240 h.

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Chapter 24
Evaluation of Anti-diabetic Property on Streptozotocin-Induced Diabetic Rats

Abstract The diabetes-inducing agent streptozotocin (STZ) is a glucosamine–nitrosourea compound produced by *Streptomyces achromogenes*, which specifically induces DNA strand breakage in β-cells causing diabetes mellitus. The destruction of pancreatic β-cells by STZ is associated with a huge release of insulin in their first phase, and then in the second phase, the destruction has lead to deficiency of insulin causes hyperglycaemia. A detailed method on selection of diabetic animals, investigation of blood glucose levels, body weight, biochemical and histopathological parameters is presented in this chapter.

Introduction

Cardiovascular diseases are due to the hyperlipidaemia which is also being one of the main diabetic complications. The risk factors like high levels of cholesterol particularly total cholesterol (TC), triglycerides (TG) and low density lipoprotein (LDL) and electrolyte imbalance are emphasized. Though hyperglycaemia is being a complex metabolic disorder, it evokes the damages in various tissues, which in turn, deregulates the cellular functions (Chander et al. 2003).

Type II diabetes is a complex and heterogeneous disorder presently affecting more than 100 million people worldwide and causing serious socio-economic problems. Appropriate experimental models are essential tools for understanding the pathogenesis, complications, and genetic or environmental influences that increase the risks of type II diabetes and testing of various therapeutic agents. The animal models of type II diabetes can be obtained either spontaneously or induced by chemicals or dietary or surgical manipulations and/or by combination thereof: selective loss of pancreatic beta-cells (alloxan/STZ) leaving other pancreatic alpha- and delta-cells intact; residual insulin secretion makes the animals live long without insulin treatment; ketosis and resulting mortality is relatively less; comparatively cheaper, easier to develop and maintain (Srinivasan and Ramarao 2007).

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Streptozotocin (STZ) is a preferred agent to induce experimental diabetes since it has some advantages over alloxan, such as relatively longer half-life (15 min), sustained hyperglycaemia for longer duration and the development of well characterized diabetic complications with fewer incidences of ketosis as well as mortality (Ozturk et al. 1996).

The diabetes-inducing agent streptozotocin (STZ) is a glucosamine–nitrosourea compound produced by *Streptomyces achromogenes*, which specifically induces DNA strand breakage in β-cells causing diabetes mellitus. The destruction of pancreatic β-cells by STZ is associated with a huge release of insulin in their first phase, and then in the second phase, the destruction has lead to deficiency of insulin causes hyperglycaemia.

Aim

The aim is to finding out the anti-diabetic potential of sample on STZ-induced diabetic rats.

Principle

STZ is a compound that has a preferential toxicity towards pancreatic β-cells. The selectivity for β-cells is associated with preferential accumulation of the chemical in β-cells after entry through the GLUT2 glucose transporter receptor: chemical structural similarity with glucose allows STZ to bind to this receptor. At high doses, typically given singly, STZ targets β-cells by its alkylating property corresponding to that of cytotoxic nitrosourea compounds (Dufrane et al. 2006). At low doses, generally given in multiple exposures, STZ elicits an immune and inflammatory reaction, presumably related with the release of glutamic acid decarboxylase autoantigens. Under this condition, the destruction of β-cells and induction of the hyperglycaemic state is associated with inflammatory infiltrates including lymphocytes in the pancreatic islets (Paik et al. 1980). STZ has well-known adverse side effects, which include hepatotoxicity and nephrotoxicity (Dufrane et al. 2006; Palm et al. 2004).

The substance/drugs are helped to repair the pancreatic cells and induced the beta-cells to secrete insulin to maintain the blood glucose level as desired. Hypoglycaemic agents balancing the glucose level either by increasing the insulin output or inhibit the intestinal absorption of glucose or by facilitating insulin metabolites and dependent process.

Materials

1. Adult Wistar albino rats (150 and 200 g of either sex),
2. Water,
3. Streptozocin (STZ),
4. 0.1 M citrate buffer (pH 4.5) and
5. Carboxymethyl cellosolve (CMC).

Preparation of Inducing Drug

1. Blend STZ with 0.1 M citrate buffer (pH-4.5).
2. Dissolve 500 mg STZ in 10 mL of buffer; Induce diabetes by intraperitoneal (IP) injection. Since 1 mL contains 50 mg of STZ, 0.22 mL has to be administered for 200 g rat.

Preparation of Standard

1. Blend the glibenclamide with 0.6 % CMC.
2. Administrate orally as 5 mg/kg.
3. Blend 10 mg glibenclamide with 5 mL of CMC. (Since 1 mL contains 2 mg glibenclamide, 0.5 mL has to be given for 200 g rat.)

Preparation of Sample

1. Blend the sample with 0.6 % CMC.
2. Administrate orally as per fixed dose (e.g. 400 mg/kg).
3. If 500 mg of sample is dissolved in 5 mL of CMC, 1 mL contains 100 mg of sample. Hence, administer the samples according to the body weight.
4. 400 mg/kg = 100 mg/250 g; One mL of prepared sample has to be administered for 250 g rat.

Protocol

1. Initially maintain the adult Wistar albino rats in the laboratory for two days before commencement of the experiment and provided with standard laboratory chow diet and free access of water, 12 h day/dark cycle and room temperature is maintained 27 °C. The night before the commencement of the experiment food is withdrawn but free access of water is provided (Kameshwara Rao et al. 2003).
2. Then inject streptozocin (STZ) via intraperitoneal at the dose of 55 mg/kg to 16-h fasted rats (Sezik et al. 2005).
3. The destruction of pancreatic β-cells by the inducing drug is associated with a huge release of insulin which makes animals more susceptible to severe hypoglycaemia that may be lethal. Thus, following treatment with STZ, in order to overcome the hypoglycaemia which occurred during the first 24 h, animals are needed to feed with glucose solution (5 %) for 12–24 h (Frode and Medeiros 2008).
4. After 5 days measure fasting blood glucose levels, animal with blood glucose concentration level above 250 mg/dL was considered to be with diabetes.

5. The diabetic animals are divided into four groups, each containing six animals and one group of normal (non-diabetic) animals. The samples have administered orally for a period of 21 days as a suspension dissolved in CMC to different groups of diabetic animals.
   Group I: Normal animals received only vehicle (each 0.5 mL),
   Group II: Diabetic animals received vehicle (each 0.5 mL),
   Group III: Diabetic animals received standard anti-diabetic drug glibenclamide (5 mg/kg, p.o.) and
   Group IV: Diabetic animals received doses (two or more).
6. At the end of the experimental period, the animals are fasted overnight.
7. Take blood from the retino orbital plexus under mild ether anaesthesia, and then separate out serum and blood sugar level have to evaluated by the method of glucose oxidase–peroxides method using span diagnostic kits.
8. Examine the lipids such as TG, TC, high-density lipoprotein (HDL), LDL and very-low-density lipoprotein (VLDL).
9. Observe blood serum electrolytes, urea, creatinine, protein and enzymes such as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT).
10. The pancreas, liver and kidney are collected for further histopathological observation.

Calculation

Monitor the animals' blood glucose levels at every week up to 21st day. Analyse lipids, blood serum enzymes and proteins with animal blood collected on 21st day. Compare the values of test groups and control groups and distinguish significance with SPSS—one-way ANOVA Duncan's t-test in suitable probability.

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Chapter 25
In Vivo Wound Healing Studies

Abstract Wound healing has emerged as a major treatment issue which has provoked the development of drugs that can improve the healing process. Studies using plant drugs have revealed many interesting results about existing commercial drugs. Effective wound healing leads to the restoration of tissue integrity and occurs through a highly organized multistage. Use of plant-derived medicines against excision, incision, and dead space models accelerates the wound healing process, which is briefly discussed in a manner to be followed easily during experimental sessions.

Introduction

Healing wounds and their treatment represent a major medical and economic problem. Basic research helps to understand the stimulation and inhibition of wound healing and provide the basis for introduction of novel treatment methods. Effective wound healing leads to the restoration of tissue integrity and occurs through a highly organized multistage. Although the healing process is continuous, it can be arbitrarily divided into four main phases: haemostasis, inflammation, proliferation and remodelling, and these phases rely on complex biological and molecular events involving cell migration and proliferation, extracellular matrix deposition and angiogenesis and are regulated by growth factors.

Preparation of Ointment

Preparation of ointment is as per British Pharmacopeia (Anonymous 2009).

1. Melt the hard paraffin (0.5 g) and cetostearyl alcohol (0.5 g), then add wool fat (0.5 g), yellow soft paraffin (8.5 g) to it and stir all the ingredients continuously until melt.
2. Remove the foreign particles by decantation, and the mixture is stirred thoroughly until cold.
3. Then, add the extract (250 and 500 mg) with the above simple ointment base; it served as 2.5 and 5 %, respectively.

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Experimental Animals

1. Wistar rats of both sex (150–200 g) are used for the study. The animals are maintain in polypropylene cages with paddy husk bedding at a temperature of 24 ± 2 °C and relative humidity of 30–70 %.
2. All animals are allow to free access to water and feed with standard commercial pellet rat chow and maintain in a 12:12 (light: day) cycle.

25.1 Excision Wound Models (Manjunatha et al. 2006)

Aim

To determine the wound healing property of plant extract by using excision wound model.

Principle

The strength of the repaired wound tissue is a result of the remodelling of collagen and the formation of stable intra- and inter-molecular cross-linking to form fibres. Thus, the treatment with extract may contribute to stimulation of cellular proliferation and enhancement of collagen synthesis, thereby facilitating the changes in wound contraction, period of epithelization and breaking strength.

Material Required

Reagent Preparation

Saline
Dissolve 0.9 g of sodium chloride in 100 ml of distilled water.

Protocol

1. In excision wound model, the animals are divided into 5 groups of 6 animals each such as:
   Group I: Untreated control,
   Group II: Control treated with simple ointment base,
   Group III: Neomycin (w/w) standard,
   Group IV: 2.5 % (w/w) extract ointment and
   Group V: 5 % (w/w) extract ointment (dose % can be fixed from dermal toxicity study).
2. Anaesthetize all the animals with diethyl ether.
3. Create an excision wound of 1.5 cm in diameter and 0.2 cm depth on the shaved dorsal side.
4. The wound left open and treat with prepared ointment topically once in a day till the complete epithelialization.
5. Every 3 days once take photo for observing healing process till complete healing.

6. Healing property is evaluated by wound contraction percentage and closure time. The area of wound is calculated using a graph sheet in every third day till complete healing.
7. Wash the granuloma tissue excised from the wound site in saline and use the tissue for the estimation of hydroxyproline, and a small portion is fixed in 10 % formalin and sectioned for histopathological analysis.

Calculation

$$\text{Percentage of wound contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

25.2 Incision Model (Saringat and Wasim 1995)

Aim

To determine the wound healing property of plant extract by using incision wound model.

Principle

Cutting of the skin or other tissue with a sharp blade results in rapid disruption of tissue integrity with minimal collateral damage. There is rapid extravasations of plasma and blood cells into the new tissue space and, depending on the extent and rapidity of haemostasis, the formation of a fibrin clot that bridges the injury margins. Wounds close by mechanical means heal rapidly with minimal scar tissue formation. Whether it is bandaged, sutured, stapled or clipped, the principle is always to reduce the tissue gap to a minimum to allow rapid and efficient bridging of the wounded edges by granulation tissue and new epithelium. As a consequence, this type of wound is excellent for biomechanical analysis of wound strength.

Materials

Surgical thread, curved needle, tensiometer.

Protocol

1. In incision wound model, the animals are divided into 5 groups of 6 animals each such as:

   Group I: Untreated control,
   Group II: Control treated with simple ointment base,
   Group III: Neomycin (w/w) standard,

Group IV: 2.5 % (w/w) extract and
Group V: 5 % (w/w) extract (dose % can be fixed from dermal toxicity study).

2. Anaesthetize all the animals with diethyl ether.
3. Create a long incision of 6 cm through the full thickness of the skin.
4. Close the wounds with sutures of 1 cm apart using surgical thread and curved needle.
5. Apply the drugs topically once daily for 10 days.
6. Remove all the sutures on the last day (10th day) and measure tensile strength of previously wounded and treated skin using tensiometer.
7. Tensile strength increment indicates improved wound healing.

Calculation

$$\text{Tensile strength} = \frac{\text{Breaking force } (N)}{\text{Area } (\text{cm}^2)}$$

$$\text{Area} = \text{thickness} \times \text{width}$$

25.3 Dead Space Wound Model

Aim

To determine the wound healing property of plant extract by using dead space wound model.

Principle

Connective tissue formation can be isolated from other events of tissue repair, such as epithelialization and contraction, by employing porous, subcutaneous implants. Though they differ somewhat in design, all such models function by creating an artificial tissue space into which plasma infuses. This leads to development of a fibrin clot and subsequent formation of granulation tissue. Depending on the implant material, further maturation into scar may occur, and a connective tissue capsule comprised of several collagenous fascias usually surrounds the implant. These models are ideal for biochemical assessment because of the well-defined volume enclosed, and many of the implant materials are soft enough to be suitable for embedding in paraffin and sectioning. These implants generally have a symmetrical organization, with the least mature portion at the core, and tissue organization may be assessed simply by the histological progression of granulation tissue into the centre of the implant.

Protocol

1. In dead space wound model, the animals are divided into 5 groups of 6 animals each such as:
   Group I: Untreated control,
   Group II: Control treated with simple ointment base,
   Group III: Neomycin (w/w) standard,
   Group IV: 2.5 % (w/w) extract and
   Group V: 5 % (w/w) extract (dose % can be fixed from dermal toxicity study).
2. Initially, incise the skin of the animals with sterile surgical blades and implant the sterile cotton pellets (10 mg weight each) subcutaneously in the lumbar region of each mouse.
3. Apply the ointment topically until the 10th post-wounding day.
4. Harvest the wet granulation tissue from each implanted cotton pellets, and their weights are noted.
5. The tissue is then dried in oven at 60 °C for 12 h, and the dry weight is again noted. The tissue is processed to determine the hydroxyproline content.
6. Increased weight of implanted material indicates formation of high granulation tissue.

25.4 Estimation of Hydroxyproline Content (Leach 1960)

Aim

To estimate the hydroxyproline content of tissue collected from wounded rats.

Principle

A significant increase in the hydroxyproline content of the granulation tissue indicates increased collagen formation. Collagen is a major protein of the extracellular matrix and is the major component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of the hydroxyproline could be used as an index for collagen turnover.

Material Required

Reagent Preparation

1. Hydrochloric acid (6 N),
2. Copper sulphate solution (0.01 M),
3. Sodium hydroxide (2.5 N),
4. Hydrogen peroxide solution (6 %),
5. Sulphuric acid,

6. p-dimethylaminobenzaldehyde solution in propanal (5 %) and
7. Standard solution: To prepare 1 mg/ml of L-hydroxyproline solution.

Protocol

(A) Preparation of hydrolysate of tissue

1. Determine hydroxyproline content of the tissue using the method described by Leach (1960).
2. Treat the animals with the formulation for 10 days of the circular wound created in the excision model and granulation tissue in dead space wound model.
3. Kill each animal from the respective group on the 10th day using a high dose of anaesthesia.
4. Excise the wound tissue, record its weight and store in a refrigerator using 10 % formalin.
5. On the day of the experiment, hydrolyse the tissue (Y mg) with 10 mL 6 N hydrochloric acid for 24 h at 110 °C in sealed glass tubes.
6. Neutralize the hydrolysate to pH 7.
7. Take 1 mL of the supernatant solution from each of the acid hydrolysate and measure hydroxyproline content.

(B) Estimation of hydroxyproline

1. Pipette out aliquots of standard solution into the series of test tubes.
2. Then, add 1 mL of hydrolysate of sample into other test tube.
3. Make up the contents of all the test tubes to 1 mL with distilled water.
4. Another test tube marked 'B' with 1 mL of distil water serves as the blank.
5. Into each test tube, pipette in succession 1 mL of each 0.01 M copper sulphate solution, 2.5 N sodium hydroxide and 6 % hydrogen peroxide solution.
6. Mix solutions and shake thoroughly for 5 min and then incubate for 5 min at 80 °C with regular shaking.
7. Cool the tubes and add 4 mL of 3 N sulphuric acid with agitation.
8. Then, add 2 mL of 5 % p-dimethylaminobenzaldehyde solution and incubate for 16 min at 70 °C.
9. Finally, cool all the test tubes and measure their absorbance at a wavelength of 572 nm.
10. Calculate the hydroxyproline content in each sample solution by using the equation which is obtained from the calibration curve.

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Chapter 26
Determination of Cytotoxicity

Abstract Cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Nowadays, various reagents are used for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production and nucleotide uptake activity. Many have established methods such as colony formation method, crystal violet method, tritium-labelled thymidine uptake method, MTT and WST methods, which are used for counting the number of live cells. Moreover, trypan blue is a widely used assay for staining dead cells. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments. This chapter is a collection of all these methods to be followed by researchers in a sequential manner.

Introduction

Cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Nowadays, various reagents are used for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production and nucleotide uptake activity. Many have established methods such as colony formation method, crystal violet method, tritium-labelled thymidine uptake method, MTT and WST methods, which are used for counting the number of live cells. Moreover, trypan blue is a widely used assay for staining dead cells. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments.

26.1 Trypan Blue Dye Exclusion Method

Aim

To screen the cytotoxic properties of the plant extracts using trypan blue dye exclusion method as described by Talwar (1974).

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Principle

Trypan blue is a blue acid dye that has two azo chromophores group. Trypan blue is a vital stain used to estimate the proportion of viable cells in a population. Since the chromophore of the dye is negatively charged and does not react with cells unless there is damage to the membrane, viable (live) cells do not take up the dye and so remain clear, whereas non-viable (dead) cells do take up the dye and so stain blue. Trypan blue is not permeable in live cells due its intact cell membrane. When the cells are dead, due to the cell membrane damage, they will take up the dye. The method is an index of the dead cells in a cell population.

Protocol

1. Bring cells into suspension and resuspend in serum-free media/PBS/HBSS.
2. Make different dilutions and adjust the cell number to 1×10^6 cells/mL using a hemocytometer.
3. Set up the experiment by incubating different concentrations of plant extract (e.g. 50–200 μg/mL) with 1×10^6 cells.
4. Make up the final volume of the assay mixture to 1 mL using PBS and incubate at 37 °C for 3 h.
5. After incubation, mix 0.1 mL of cell suspension with 0.1 mL of 0.4 % trypan blue, keep for 2–3 min and load on a haemocytometer (dilution factor of 2).
6. Count the number of stained (dead cells) and unstained (live) cells separately.
7. Calculate the percentage of viability using the following equation.

$$\% \text{ Viability} = \frac{\text{Number of viable cells}}{\text{Number of total cells}} \times 100$$

26.2 MTT Cell Viability Assay

Aim

To find out the long-term cytotoxicity of the plant extracts on various cancer cell lines using the colorimetric MTT assay.

Principle

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes in metabolically active cells. Dead or non-viable cells do not cause this change. This water insoluble formazan can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye (Mosmann

1983; Wilson 2000). MTT is converted by the succinate dehydrogenase enzyme in the cell, into an insoluble coloured formazan product. It is solubilized in DMSO and the cell viability depends on its absorbance value. The absorbance of untreated cells was taken as the 100 % viable cells.

Protocol

1. Plate about 5×10^3 cells in 96-well flat bottom titre plate in appropriate volume of the medium supplemented with FCS and antibiotics.
2. Allow to adhere for 24 h at 37 °C.
3. Add plant extracts (100 µL) or drugs 48 h after the plating of the cells and continue incubation.
4. About 4 h before (44th h) the completion of incubation, add 20 µL of MTT (5 mg/mL PBS) to each well and incubate for further reaction.
5. Centrifuge the plates and remove the supernatant.
6. Add DMSO (100 µL/well) to all wells to solubilize the formazan crystals and incubate for 15 min at room temperature.
7. Take the optical density in an ELISA reader at 570 nm with reference at 630 nm.
8. Calculate the percentage dead cells using the following equation.

$$\%\,\text{Dead cells} = 1 - \frac{\text{OD of plant extract/drug treated well}}{\text{OD of the control well}} \times 100$$

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Chapter 27
Induction of Apoptosis

Abstract The apoptotic activity of plants is checked to confirm its anti-tumour and anti-cancer activity. Apoptosis is a specific process that leads to intrinsic programmed cell death which is essential in the homeostasis of normal tissues of the body and occurs in various physiological and pathological situations. Method to check apoptosis in EAC cells and DNA analysis are featured in this chapter as a preliminary test manner.

Introduction

Apoptosis is a specific process that leads to intrinsic programmed cell death which is essential in the homeostasis of normal tissues of the body and occurs in various physiological and pathological situations (Hengartner 2000).

Aim

To evaluate whether the cytotoxic effect of plant extracts on various cancer cell lines was due to the induction of apoptosis.

Principle

Apoptosis or programmed cell death is a genetically regulated cellular suicide mechanism that plays a crucial role in the development and defence of homeostasis. Apoptosis is associated with a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane. Early in apoptosis, the cells round up, losing contact with their neighbours, and shrink. In the cytoplasm, the endoplasmic reticulum dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, chromatin condenses and aggregates into dense compact masses and is fragmented internucleosomally by endonucleases, which can be analysed by the typical "DNA ladder" formation in apoptosis.

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Protocol

1. Treat EAC cells with different concentrations of plant extract (5, 20 and 50 μg/mL) for 12 h at 37 °C in DMEM medium.
2. Harvest the cells after the treatment and wash with PBS.
3. Isolate DNA following the phenol–chloroform extraction procedure (Herrmann et al. 1994).
4. Dissolve the DNA in TE buffer.
5. Run in 1.5 % agarose gel to observe the ladder formation.
6. Analyse the EAC cells treated with different concentrations of plant extracts for the morphological changes.
7. Smear the cells on the dry glass slide, dehydrate using 5 % formaldehyde and stain using Leishman stain.
8. Observe the slides through confocal microscope.

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Chapter 28
Anti-tumour Activity

Abstract Experimental tumours have great importance for the purposes of modelling where Dalton's ascites lymphoma (DAL) and Ehrlich ascites carcinoma (EAC) are the commonest. It appeared firstly as a spontaneous breast cancer in a female mouse and then Ehrlich and Apolant (Berlin KlinWshr 28:871–874, 1905) used it as an experimental tumour by transplanting tumour tissues subcutaneously from mouse to mouse. In 1932, Loewenthal and Jahn obtained the liquid form in the peritoneum of the mouse and named it as 'Ehrlich ascites carcinoma' due to the ascites liquid, together with the carcinoma cells. In this chapter, two most common tumour cells (EAC and DAL) are chosen and protocol has been framed to determine the antitumor activity.

Introduction

Experimental tumours have great importance for the purposes of modelling where Dalton's ascites lymphoma (DAL) and Ehrlich ascites carcinoma (EAC) are the commonest. It appeared firstly as a spontaneous breast cancer in a female mouse and then Ehrlich and Apolant (1905) used it as an experimental tumour by transplanting tumour tissues subcutaneously from mouse to mouse. In 1932, Loewenthal and Jahn obtained the liquid form in the peritoneum of the mouse and named it as 'Ehrlich ascites carcinoma' due to the ascites liquid, together with the carcinoma cells.

28.1 Solid Tumour Development

Aim

To find out the anti-tumour activity of plant extracts using DAL cell line–induced solid tumour model.

Principle

Lymphoma is a disease of the lymphocytes (a type of white blood cell involved in immune responses) and the lymphatic system, which includes the spleen, thymus, and liver, as well as other lymphatic tissues. DAL is transplantable, poorly

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differentiated malignant tumour which appeared originally as lymphocytes in a mouse. One of the requisites of cancer chemopreventive agent is elimination of damaged or malignant cell through cell cycle inhibition or induction of apoptosis with less or no toxicity to normal cells.

Protocol

1. Group the mice such that each group contains 6 male Swiss albino mice (Rajeshkumar et al. 2002; George et al. 2014).
   Group I and II: DAL cells alone (tumour control for solid),
   Group III and IV: DAL cells + cyclophosphamide (10 mg/kg b.wt.) and
   Group V to Group X: DAL cells + 100, 200 and 400 mg/kg b.wt. of plant extracts.
2. Inject DAL cell lines (10^6 cells/animal) subcutaneously to the right hind limb of Swiss albino mice for solid tumour development.
3. After 24 h of DAL cells inoculation, treat the animals with different concentrations of extract (100, 200 and 400 mg/kg b.wt.) and cyclophosphamide (10 mg/kg b.wt.) for consecutive 10 days.
4. Note the initial diameter of the right hind limb using digital vernier caliper.
5. Measure the tumour volume every third day and record up to one month for solid tumour model.

Calculation

Calculate the tumour volume by the following formula:
$V = 4/3(\pi\ r_1^2\ r_2)$, where r_1 and r_2 are the radii of tumours at two different planes.

28.2 Ascites Tumour Development

Aim

To find out the antitumor activity of plant extracts using Ehrlich ascites carcinoma (EAC) cell-induced ascites tumour model.

Principle

EAC is referred to as an undifferentiated carcinoma and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100 % malignancy and also does not have tumour-specific transplantation antigen (Kaleoglu and Isli 1977). EAC can be used as ascites when injected intraperitoneal (i.p.) while when given subcutaneously (s.c.) it forms a solid tumour. EAC cells grow in suspension in the peritoneal cavity of mice and they do not adhere to the synthetic surface in vitro. Following the inoculation into the peritoneal cavity of mice, EAC cells grow in two phases. These two phases are a proliferating phase, in which the number of cells increases exponentially, and a plateau phase followed by a resting phase, in which a number of cells stay almost constant (Song et al. 1993).

Protocol

1. Group the mice such that each group contains 6 male Swiss albino (Kuttan et al. 1985; George et al. 2014).
   Group I and II: EAC cells alone (tumour control for ascites),
   Group III and IV: EAC cells + cyclophosphamide (10 mg/kg b.wt.) and
   Group V to Group X: EAC cells + 100, 200 and 400 mg/kg b.wt. of plant extracts.
2. Inject EAC cell lines (10^6 cells/animal) intraperitoneally for solid tumour development.
3. After 24 h of EAC cells inoculation, treat the animals with different concentrations of extract (100, 200 and 400 mg/kg b.wt.) and cyclophosphamide (10 mg/kg b.wt.) for consecutive 10 days.
4. Note the death pattern of the animals due to tumour burden every day.
5. Calculate the percentage of increase in lifespan (ILS) for ascites tumour model.

Calculation

The increase in lifespan will be calculated using the formula:

$$\text{ILS} = (T - C)/C \times 100$$

where 'T' and 'C' are the number of days that treated and control animals survived, respectively.

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Chapter 29
Protein Bioavailability in Animal Model

Abstract All the nutrients in food are not absorbed by our system. The relative amount of nutrition from an administered dosage through which it enters the system of circulation and the rate at which the level of nutrition appears in the systemic circulation is called 'bioavailability'. Although plant foods have high nutrient contents our digestion system cannot absorb it fully. Hence, it becomes necessary to determine the bioavailability of the administered sample to check the overall effect on the living system. Animal food preparation, protein bioavailability in animal model, food efficiency, apparent digestibility, protein digestibility tests, etc., are mentioned in well explained methodologies.

Introduction

The bioavailability of orally administered drugs or food depends on the processes of absorption which can be affected by the presence of certain dietary components in the gastrointestinal tract. In pharmacology, bioavailability is a subcategory of absorption and is the fraction of an administered dose of drug that reaches the systemic circulation. When a medication is administered intravenously, its bioavailability is 100 % (Griffin 1997). However, when a medication is administered via other routes (such as orally), its bioavailability generally decreases (due to incomplete absorption and first-pass metabolism) or may vary from person to person. For dietary supplements, herbs and other nutrients in which the route of administration is nearly always oral, bioavailability generally designates simply the quantity or fraction of the ingested dose that is absorbed (Welling 1977; Heaney 2001).

Principle

Wistar albino rats are excellent living model to estimate the bioavailability of food or drug because the metabolic pathway of rats was already well defined in several scientific studies. The rats administered with different types of diets continuously for 21 days normally show variation in body weight, food consumption and food absorption. The estimation of nitrogen content in faecal matters collected from experimental animals is derived from the protein absorption and food efficacy of sample protein. The variation between normal diet-treated animals and sample

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diet-treated animals gives a clear view about the bioavailability percentage of sample protein.

Aim

To quantify the bioavailability of given protein sample by using animal model (Wistar albino rats).

Materials Required Starch, corn oil, non-nutritive cellulose, salt mixture and vitamin mixture.

Protocol

Animals and Maintenance

1. The Wistar albino male rats weighing 40–80 g are desirable to carryout bioavailability study.
2. House the animals in a clean polypropylene cage and maintain under standard laboratory conditions (temperature 25 ± 3 °C with dark/light cycle 12/12 h; 35–60 % humidity).
3. Feed animals with standard pellet diet and water ad libitum.
4. Acclimatize the animals to laboratory conditions for one week prior to the experiment.

Animal food preparation (prepared as per Indian Standards I.S. 7481; 1974)

1. Carry out all the preparation under sterile condition. Otherwise, food pellets may be contaminated by microbes.
   Group 1

   The basal diet consisted of corn starch (80 %), corn oil (10 %), non-nutritive cellulose (5 %), salt mixture (4 %) and vitamin mixture (1 %).
   Group 2

   The casein diet consisted of starch (80 %), corn oil (10 %), non-nutritive cellulose (5 %), salt mixture (4 %), vitamin mixture (1 %) and casein (10 mg/kg).
   Group 3

   The test diet 1 consisted of starch (80 %), corn oil (10 %), non-nutritive cellulose (5 %), salt mixture (4 %), and vitamin mixture (1 %) and sample (10 %).
   Group 4

   The test diet 2 consisted of starch (80 %), corn oil (10 %), non-nutritive cellulose (5 %), salt mixture (4 %), vitamin mixture (1 %) and sample (20 %).
2. Prepare the mixtures in separate containers in powdered condition.
3. Then, add sufficient amount of water (normal tap water) with it to make pellets. Pellets can be preparing with help of hands or using 2 cm × 5 cm PVC pipes.
4. Dry the pellets in hot air oven at 21 °C and store in air tight containers.

Study the protein bioavailability in animal model

1. Divide the animals into four groups consisting of five animals each and house in different plastic cages maintained at room temperature, photoperiod of 12 h and frequent air changes. Free access to food and water ad libitum.
2. Feed the rats with 4 different diets for 21 days (3 weeks).

   Group 1: basal diet (protein-free diet) (control);
   Group 2: casein diet (standard);
   Group 3: basal diets with sample (10 %)—Test Diet 1;
   Group 4: basal diets with sample (20 %)—Test Diet 2.

3. Record the body weight of the each animal daily (0–21 days).
4. Note the food consumption, variation in food consumption and behavioural changes of each group separately every fifth day.
5. Collect faecal samples of individual groups, weigh, dry and mill to a size above 50 mm (faecal with moisture content cannot be used for nitrogen estimation in Kjeldahl method).

Calculation

Food efficiency

Calculate the food efficiency ratio by the relationship between weight gained by the animal and food consumption:

$$\text{Food efficiency} = \text{Weight gain (g)}/\text{Food intake (g)}$$

Weight gain = Average weight gain of each group in 21 days.
Food intake = Average food intake of each group in 21 days.

Estimation of nitrogen in faecal matters

Estimate the nitrogen content by micro-Kjeldahl method.

Crude protein

Multiply total nitrogen value with 6.25 to arrive the crude protein content.

Apparent digestibility (AP)

Proportion between the food intake and food absorption is calculated as follows:

$$\%\,\text{AP} = (\text{Food intake (g)} - \text{fecal excretion (g)}/\text{Food intake (g)}) * 100$$

Food intake = Average amount of food intake in 21 days (each group).
Faecal excretion = Average amount of faecal excretion in 21 days (each group).

Protein digestibility (PD)

Proportion of the protein intake to that of absorbed by the body is the percentage of protein digestibility. It is calculated by the following formula:

$$\%\,\mathrm{PD} = \frac{\text{Total protein in sample} - \text{total protein in pellet}}{\text{Total protein in sample}} \times 100$$

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Chapter 30
Detection of Phenolic and Flavonoid Compounds Using High Performance Thin Layer Chromatography (HPTLC)

Abstract High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layerchromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation,hyphenation, etc., enable it to be a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic andbiomolecules which are characterized in this chapter.

Introduction

A wide variety of active phytochemicals,including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, phthalides, tannins, gallic acid, quercetin, phytosterols, alcohols, aldehydes have been identified from medicinal plants [3].These phtochemicals are estimated by a variety of techniques such as spectroscopy and chromatography. High performance thin layer chromatography (HPTLC) chromatographic fingerprints can be applied for this kind of certification.

Aim

To identify the phenolics and flavonoid contents in plant extract by HPTLC (Srivastava 2011).

Principle

The separation of the components of a mixture is due to their different affinities for a stationary phase such as a solid or a liquid and their differential solubility in a moving phase such as a liquid or gas.

Materials Required

1. Silica gel $60F_{254}$
2. TLC plate
3. Hamilton syringe
4. LINOMAT 5 instrument
5. Photo-documentation chamber

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6. Folin cio-calteu reagent
7. Ethanolic aluminium chloride (1 %)
8. Ethyl acetate and methanol

Protocol

Sample Preparation

1. Dissolve 50 mg of plant extract in 1 mL acetone and centrifuge.
2. Use the supernatant as test solution for HPTLC analysis.

Sample Loading

Load 3 μL of the above solution in the 5 × 10 silica gel $60F_{254}$ TLC plate (LINOMAT 5 instrument) using Hamilton syringe.

Spot Development

1. Load the sample in a TLC plate and keep in TLC twin trough developing chamber with respective mobile phase for phenolic compounds and flavonoids for chamber saturation up to 20 min.
2. Develop the plate in the chamber using respective mobile phase up to 80 mm distance.

Photo-Documentation

1. Develop the plate and dry in hot air to evaporate solvents from the plate.
2. Keep the plate in photo-documentation chamber and capture the images in a white light, UV 254 nm and UV 366 nm.

Derivatization

1. Spray the respective spray reagents in a plate and dry at 120 °C in a hot air oven for 5 min.
2. Photo-document the plate in a white light for phenolic compounds and UV 366 nm for flavonoids using photo-documentation chamber.

Scanning

1. Fix the plate in a scanner stage and scan the plate at 500 nm for phenolic compound and 366 nm for flavonoids.
2. Note the peak table and densitogram.
3. Mobile phase (for phenolic compounds and flavonoids).
4. Ethyl acetate–methanol–water (10:1.65:1.35)

Spray Reagent

For phenolic compounds

1. Apply the 20 % aqueous sodium carbonate solution over the plate followed with 25 % aqueous Folin cio-calteu reagent after brisk dry.
2. Dry the plate at 120 °C for 5 min.

For flavonoids

1. Apply the 1 % ethanolic aluminium chloride solution over the plate and dry at 120 °C for 5 min.

Retardation Factor

1. Retardation factor (R_f) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula.
2. It depends on time of development and velocity coefficient or solvent front velocity.

$$R_f = \frac{\text{Migration distance of the substance}}{\text{Migration distance of the solvent front from origin}}$$

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Chapter 31
Isolation of Compounds

Abstract Plants are the storehouse of many chemical compounds that possess various biological activities. Identification of these compounds becomes critical in understanding the exact mechanism behind the therapeutic potential of these plants. Screening and isolation of compounds from plants important to human health involves various methods that need careful handling and attention. A detailed method of isolation using thin layer chromatography (TLC) and column is explained.

Introduction

Plants possess vast range of compounds which can be biologically active in one way or other. The process of screening to identification of these compounds and understanding their importance in human health has geared-up a serious concern. A careful follow of standardized protocols could help in utilizing these phyto-compounds for nutrition and medicine.

Selection of Extracts for Isolation and Purification of Active Compounds

Selection of the extracts can be based on the following criteria:

(a) Based on the objective of the study;
(b) Based on the in vitro and in vivo activity of the extracts;
(c) Based on the previous reports on the sample or plants; and
(d) Random isolation if the isolation is done for the first time from the plant (genus or species).

Preparation of Extracts

1. Take the extract to be used for the isolation and purification.
2. Dissolve the extract completely in the respective solvent.

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Silica Gel

1. For the thin layer chromatography (TLC) use silica gel GF_{254} and for column chromatography silica gel 60-120.

31.1 Thin Layer Chromatography (TLC)

Aim

To separate the phytocompounds through TLC technique.

Principle

The principle of TLC is the distribution of a compound between a solid fixed phase applied to a glass or plastic plate and a liquid mobile phase, which is moving over the solid phase.

Chemicals Required

Silica gel GF_{245}

Plate Preparation

1. Prepare the plate by mixing the silica gel with small amount water.
2. Spread as thick slurry on an unreactive carrier sheet, usually glass, thick aluminium foil or plastic.
3. Dry and activate by heating in an oven for 3 h at 121 °C.
4. The thickness of the adsorbent layer can be around 0.1–0.25 mm for analytical purposes and around 0.5–2.0 mm for preparative TLC.

Loading and Running of Sample

1. Apply a small amount of a prepared extract to a starting point just above the bottom of TLC plate.
2. Develop the plate in the developing chamber that has a shallow pool of solvent just below the level at which the sample was applied.
3. The solvent is drawn up through the particles on the plate through the capillary action, and as the solvent moves over the mixture each compound will either remain with the solid phase or dissolve in the solvent and move up the plate. Whether the compound moves up the plate or stays behind depends on the physical properties of that individual compound and thus depends on its molecular structure, especially functional groups.
4. For a random isolation of compounds, the TLC with maximum number of spots or bands with a solvent system should be standardized before going for column chromatography.

5. Calculate the R_f value for each spot.

$$R_f = \frac{\text{Distance travelled by the component}}{\text{Distance travelled by the solvent}}$$

31.2 Column Chromatography

1. Selection of the column should be determined by the amount of sample to be loaded to the column.
2. Activate the silica at 121 °C for 3 h.

Packing of Column

1. Adjust the column with a cotton or sintered disc filter and add the silica gel wet in solvent.
2. Allow the silica to settle down completely.
3. For a tight packing of silica, a high pressure is blown from the top of the column or column allowed to run with the added solvent alone for 48 h.
4. Sample packing can be done in two ways:
   (a) Wet packing: Wet the sample in solvent and pour into the column
   (b) Dry packing: Mix the completely dissolved extract in the solvent with few grams of activated silica. Homogenate thoroughly till the extract get completely blend with the silica. Stir till the mixture reaches to dryness in the form of a free flow powder.
5. Add the sample above the packed silica in column with desired solvent mode.

Collection of Elution and Isolation

1. Based on the TLC profiles, solvents and their combinations are decided.
2. Packing with low polar solvent is advisable (toluene will give a clear column).
3. Collect the elution dropwise so that the separation of compounds from the column could be more effective.
4. Collect separate elution for each concentration of solvent or solvent combinations.
5. Based on the affinity, solubility and molecular weight, the compounds get separated from the column into the elution.
6. Spot and develop each elution collected from the column on TLC.
7. Pool the elution based on the similarity in R_f values. The similarity indicates similar form of compounds in the elution.
8. Finally from the pooled elution, isolate the compounds by preparative TLC technique.

9. In preparative TLC, make a band of elution on the plate with a thin capillary tube and run.
10. A clear band if develops, scrap the band separately and repeat the technique further.
11. Dissolve the scrapped portion of the silica with band in HPLC grade solvents (methanol or chloroform).
12. Centrifuge the mixture for 20 min at 6000 rpm.
13. Collect the supernatant and pour it into a watch glass.
14. Let the poured portion dry and check for specific characters of phytocompounds (e.g. texture).

Isolation by Fractionation of Elution

1. Add HPLC grade solvents from low to high polar (try with Chloroform, ethyl acetate and methanol) to the elution and shake/vortex thoroughly. (Sometimes combination can also be used.)
2. Pour the dissolved portion alone to the watch glass.
3. In both the cases, the compounds in watch glass can be identified with characteristic physical properties such as crystalline, amorphous solid, and white to brown powder.

Chapter 32
Molecular Docking of Bioactive Compounds Against BRCA and COX Proteins

Abstract The focus of molecular docking is to computationally simulate the molecular recognition process. A binding interaction between a small molecule ligand and protein may result in activation or inhibition of the protein. The docking method using BRCA1 or BRCA2 genes and COX proteins is carefully texted in this chapter to check docking of the best inhibitor molecule.

Introduction

Molecular docking is a method to predict the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Hereditary breast and ovarian cancer (HBOC) is a syndrome resulting in an increased lifetime risk for developing breast and/or ovarian cancer. The genetic basis of HBOC is usually an inherited germ line mutation in one of the alleles of BRCA1 or BRCA2 genes and subsequent loss of heterozygosity in somatic tissues. In the presence of a BRCA1 mutation, women have a 70–80 % lifetime risk of developing breast cancer and a 50 % risk of developing ovarian cancer. Women carrying a BRCA2 mutation have a 50–60 % lifetime risk of developing breast cancer and a 30 % risk of developing ovarian cancer (Roy et al. 2012). COX, originally called prostaglandin H synthase (PGHS), is the major enzyme responsible for the oxidation of AA to PGG2 and PGH2. The COX-1 and COX-2 isoforms both catalyse a cyclooxygenase reaction in which the substrate AA and two molecules of molecular O_2 are converted to PGG_2 and a peroxidase reaction in which PGG_2 is reduced to PGH2 by a two-electron reduction.

Aim

To study how molecular docking technique is used to predict whether a bioactive drug candidate (inhibitory molecules) will bind to its target receptor (proteins).

Principle

Computer programs are used to predict or simulate the possible reactions/interactions between two molecules based on their 3-dimensional structures. There are two main types of molecular docking in practice: small molecule-protein (ligand-protein docking) and protein–protein docking. The theory behind molecular

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docking lies behind the enzyme–substrate recognition process. In this problem, the orientation of the ligand (small molecule or substrate protein) will be "fitted" to the receptor of interest using either of two approaches: matching technique and simulation processes. A compound (drug) which binds to a biological macromolecule (protein) may inhibit its function and thus acts as drug (Brem and Dill 1999; Murray et al. 1999). This research covered a broad range on the areas of pharmacology, medicinal chemistry and computational biology, for the identification of new plant-based anti-inflammatory and anticancer agents.

Protocol

Protein retrieval and preparation

1. Obtain three-dimensional structures of BRCA1, BRCA2, COX1 and COX2 proteins from PDB database (PDB id: 1T15, 3EU7, 1EQH, 3LN1) (PDB www.rcsb.org).
2. Prepare the retrieved protein for docking using protein preparation wizard (Sastry et al. 2013).
3. The preparation of proteins involves a number of steps. The procedure assumes that the initial protein structure is in a PDB-format file, includes a cocrystallized ligand and does not include explicit hydrogens.
4. Import three-dimensional structures of the proteins into Maestro (Maestro, version 9.4, Schrodinger, LLC, New York, NY, 2013).
5. Delete water molecules and adjust metal ions and cofactors.
6. Fix charges, bond orders, formal changes of cofactors and orientation.
7. After that, examine the prepared structures.
8. Refine the protein structure for correct formal charges and protonation states and make the final adjustments as needed.

Grid generation

1. Grid files represent physical properties of a volume of the receptor that are searched when attempting to dock a ligand.
2. The complex for this exercise is actually in two files: one containing the receptor and one containing the ligand.
3. Display the prepared protein in the workspace.
4. Then display the protein structure in ribbon representation.
5. Display the import panel and import options dialogue box opened.
6. Take the receptor structures used for grid generation from the workspace, so that the ligand atoms will be excluded from consideration as part of the receptor.
7. Generate the grid for target proteins BRCA and COX.

Inhibitory molecules retrieval and preparation

1. Retrieve inhibitory molecules from PubChem database (PubChem http://www.ncbi.nlm.nih.gov/pccompound) in 3D SDF format.
2. The compounds such as cyclophosphamide, letrozole, doxorubicin, paclitaxel and tamoxifen (PubChem id: CID 2907, CID 3902, CID 31703, CID 36314,

CID 2733526) are the commercially available standard drugs for the treatment of cancer.
3. The compounds diclofenac, indomethacin, paracetamol, aspirin and morphine (PubChem id: CID 3033, CID 3715, CID 1983, CID 2244, CID 5288826) are the NSAIDS used commonly for the treatment of inflammation-related diseases.
4. Screen the bioactive compounds isolated from plant extract for their inhibitory activity against BRCA and COX proteins.
5. Prepare retrieved molecules for docking using LigPrep module (LigPrep, version 2.6, Schrodinger, LLC, New York, NY, 2013.).
6. Finally, use the prepared compounds for docking with BRCA and COX proteins.

Molecular docking of target proteins with inhibitory molecules

1. Perform the docking using glide module (Friesner et al. 2006; Halgren et al. 2004), through blind docking approach, in which commercially available drugs and newly isolated compounds will be docked against BRCA and COX proteins (Plackal George et al. 2014).
2. Examine docked complex with an emphasis on visual rather than numerical appraisal.
3. For this purpose, they can be visualised using XP visualiser.

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