Chapter 10
In Vitro Plant Cell Cultures: A Route to Production of Natural Molecules and Systematic In Vitro Assays for their Biological Properties

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10.1 Introduction

The connection between humans and their search of drugs from nature is boundless since ancient times. Written documents, preserved plant materials, and original plant medicines are some of the evidences that elucidate the use of plants as medicines by humans (Petrovska 2012a). After many experiences, enormous sufferings, and diseases, people attained the knowledge of using leaves, barks, fruits, and other parts of the plants for the treatment of ailments (Petrovska 2012b). The primeval document describing the use of medicinal plants for drug preparation was found on a Sumerian clay slab from Nagpur. It is about 5000 years old and encloses 12 drug preparation recipes using more than 250 plants (Petrovska 2012b). The Indian books like "Vedas" emphasize to utilize abundant plants, like clove and pepper for the benefit of mankind. Theophrastus (371–287 BC), "The Father of Botany," established botanical sciences and contributed books, namely, "De Causis Plantarum" and "Historia Plantarum," to the society. In his book, he classified more than 500 medicinal plants. Dioscorides, "The Father of Pharmcognosy," one of the most prominent writers on plant drugs in ancient history, described the utilization of parsley and oak bark for diuretic and gynecological problems (Petrovska 2012b). Galen (131 AD–200) compiled the whole list of medicinal plants and introduced some novel plant drugs, which were not described by Dioscorides, such as *Uvae ursi folium* which is used for uroantiseptic and mild diuretic ailments even today. In the seventh century AD Slavic people started using *Rosmarinus officinalis, Ocimum basilicum,* and *Mentha viridis* in cosmetics and *Cucumis sativus, Urtica dioica, Artemisia maritime* L., and *Lavandula officinalis* were used against various injurious insects, like moths, mosquitos, and spiders. In Middle Ages, Charles the Great

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(742 AD–814) used some medicinal plants for planting. Old people used plants in simple forms, like decoctions, maceration, and infusion, while sixteenth- and eighteenth-century people used compound drugs, which comprised medicinal plants with drugs of plant and animal origin. In the eighteenth century, alkaloids from poppy, quinine, pomegranate, and glycosides were isolated from various plants and in the nineteenth and twentieth centuries, regular use of plants for making medicines led to depletion of plant species. Also, many authors suggested that the healing property of the plant depends upon its mode of drying. The condition or environment while drying decides the efficacy of plant. Till today, medicinal plants are being used by people themselves or due to recommendation by doctor (Petrovska 2012a).

Even today, most of the pharmacologists prefer to use only plant resources as medicine due to its less toxic side effects. About 80% of world population depends upon herbal medicine to treat various kinds of ailments. Today it is known that the medicinal properties in plants lie in specific category of compounds, called secondary metabolites, produced by them (Wink 2015). Plants possess mainly two kinds of metabolites: primary and secondary. Primary metabolites are responsible for growth and reproduction of plants, for example nucleic acids, fats, and carbohydrates. On contrary to this, secondary metabolites have no role in any of the primary functions of the plant, like growth and reproduction, but are mostly produced by plants for their defense against herbivores, plants, and pathogens. Several research articles have proved that secondary metabolites possess a wide range of bioactivities; for example camptothecin and paclitaxel, isolated from *Camptotheca acuminate* and *Taxus brevifolia*, respectively, are widely used in tumor therapy (Wink 2015).

Different parts of the plants and in vitro callus cultures of *Baliospermum montanum* were tested for their phytochemical as well as antibacterial potential. Leaves and roots of naturally growing plant are used for in vivo studies while callus obtained from these explants is used for in vitro studies. During phytochemical studies the amount of steroids, terpenes, saponins, glycosides, alkaloids, flavonoids, phenols, tannins, and sugars was found to be higher in in vitro-derived callus cultures than their respective plant parts, i.e., leaves and roots. Also, the ethanol extract of in vitro leaf callus showed the maximum antimicrobial activity (Johnson et al. 2010). *Commiphora wightii* (guggul), a critically endangered plant, is well known for its oleo-gum-resin, which reduces cholesterol. The boon to save these plants was "plant tissue culture technique." It was observed that in vitro micropropagation using nodal cuttings was more effective than conventional propagation system using long cuttings as well as seedlings from mother plants which has limitations, like seasonal dependency, low success rate, and damage to mother plants.

This chapter focuses on the importance of in vitro plant cultures, ways for establishing in vitro callus cultures, and impact of in vitro cultures on metabolite production and bioactivity followed by in vitro assays to investigate the efficacy of in vitro cultures. In vitro assays help in understanding the mechanism of action of plant-based drugs (Sect. 10.2); it is either in crude form or in purified form. Moreover, in vitro assays also aid in assessing antioxidant, antifungal, antiulcer, antimalarial, and anticancer properties of the plants.

10.2 Significance of In Vitro Cultures

Plant-based drugs or plant extracts are drugs obtained from plants. Basically, these drugs are available in two forms: crude form and isolated or purified form. Crude form of drug is prepared by drying the plant or its parts, followed by extraction of its metabolites in specific solvent while purified form of drug is prepared in the same way as crude form but with an additional step of separation of active compounds using various analytical techniques. However, the usage of crude extracts and isolated metabolites from naturally growing plants has some limitations, like varying amounts of metabolite production associated with the environmental changes and regional variations (Sampaio et al. 2016). It causes difficulty in scale-up and downstream processing of metabolites (Hussain et al. 2012). Furthermore, consistent accessing of the medicinal plants from natural environment can lead the plants to their extinction. Thus, plant tissue culture technique is the finest solution to solve these problems. Haberlandt, "Father of Plant Tissue Culture," devised the concept of cell culture in 1902. In plant tissue culture technique, plant cells, tissues, and organs are grown in a controlled environment using appropriate media composition. In vitro cultures obtained via plant tissue culture technique provide consistent and optimum metabolite production throughout the year, irrespective of seasonal and regional variations. Furthermore, in vitro cultures can accumulate a wide variety of secondary metabolites ranging from simpler to complex, like terpenes, saponins, alkaloids, polyphenols, anthraquinones, tannin, and flavonoids (Anulika et al. 2016). Further, in vitro cultures also possess some additional metabolites as compared to their wild donor plants. For example, in vitro cultures of *Lantana camara* L. produce betulinic acid, which is usually absent in wild plants (Srivastava et al. 2010). The presence of additional metabolites along with the provision for their scale-up makes in vitro cultures more valuable.

10.3 Establishment of In Vitro Plant Cell Cultures

10.3.1 Micropropagation

The method of producing multiple copies of donor plants from any somatic tissues under in vitro conditions, using plant tissue culture technique, is known as micropropagation or more specifically clonal propagation to generate true-to-type plantations. Micropropagation involves two methods: meristem culture using apical or axillary buds and adventitiously from any somatic/vegetative tissues other than preexisting meristems. This is an alternative to conventional method of vegetative propagation by long stem cuttings (>30 cm) where success rate varies from season to season and region to region and is implemented in a few species only. On the other hand, micropropagation requires a small explant (<1 cm), such as nodal segments, shoot tips, and leaf discs, and hence favors large-scale propagation of

uniform plantations within a short duration, irrespective of the environmental fluctuations. Furthermore, it does not promote variations as happens with conventional methods by seed propagation where the seeds are the products of fertilization and resulting into variability. Thus, micropropagation perpetuates the parental characters to the progeny. Micropropagation by nodal segment cultures and adventitious shoot proliferation are two widely used methods for large-scale production.

10.3.2 Callus Cultures

The undifferentiated mass of cells obtained after inoculating an explant on a particular media in sterile conditions is known as callus culture. The explant could be any part of the plant showing totipotency. Some of the important benefits of callus cultures are their fast growth, availability of single or small cluster of cells, easy scale-up of cell biomass, and high metabolite content. The initiation and multiplication of callus from leaf disc cultures of *Lantana camara* (Fig. 10.1). Fresh and young leaves were collected, and washed with Tween-20, followed by surface sterilization with $HgCl_2$. Under aseptic conditions, leaf discs were prepared from

Fig. 10.1 Establishment of in vitro callus cultures of *Lantana camara* L. using leaf disc as an explant: (**a**) expansion and browning of the leaf disc began after 1 day of inoculation in media; (**b**) induction of callus from the incised ends of leaf disc occurred after 15 days; (**c**) same, after 28 days, develops compact brown callus with initiation of cream calli; (**d**) proliferation of fresh, friable, and green-color callus from compact brown callus occurred after another 30 days; (**e**) massively grown fresh, friable, cream-color callus proliferated after subsequent subcultures, every 2 weeks

sterilized leaves using cork borer and explants were inoculated on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP; 5 μM), 1-naphthaleneacetic acid (NAA; 1 μM), and 2,4-dichlorophenoxyacetic acid (2,4-D; 1 μM) (Srivastava et al. 2010).

10.3.3 Cell Suspension Culture

The method to culture cells and tissues in liquid nutrient media is known as cell suspension cultures. In suspension cultures, each individual cell is in direct contact with the medium nutrients, resulting in faster cell growth compared to that in semi-solid media. Cell suspension cultures are basically of two types: batch and continuous culture. In batch culture, also called as closed system, the cell inoculum is added to the media in the beginning and is allowed to grow without subsequent addition of fresh media. With the time, the nutrient is used up by the growing cells and, hence, the cells pass through the four distinct stages, lag phase, log phase or exponential phase, stationary phase, and death phase. At the end of the process, the cells are harvested for product isolation. On the other hand, in continuous culture system, media is added after certain intervals and spent media is replaced with the fresh media at the same rate while keeping the rest of the conditions at optimum. Due to continual exchange of nutrient medium, it is considered as an open system and aims to maintain the cells at exponential phase. The remarkable benefit of cell suspension cultures is that they can serve as inoculum for large-scale production of biomass and metabolites in batch or continuous systems.

10.4 Plant Secondary Metabolites

10.4.1 Chemical Classes of Secondary Metabolites

10.4.1.1 Terpenes

Terpenes or terpenoids are the largest class of secondary metabolites. Majority of the compounds of this class are commonly water insoluble. They are made up of five carbon atoms with branched carbon skeleton of isopentane. Saponins and carotenoids are two important derivatives of triterpenes. These are mainly known for their defensive role in plants. Some of the plants, like basil, lemon, sage, and tobacco, are known to have terpenes. Terpenoids perform several functions in plants (Table 10.1), primarily plant pollination and defense against herbivores. For example, the eugenol, isolated from *Ocimum gratissimum*, serves as fragrance to attract insects for pollination and the *Nicotiana attenuata* plant secretes trans-β-ocimene, cis-α-bergamotene, and trans-β-farnesene terpenoids after herbivore attack (Anulika et al. 2016; Kessler and Baldwin 2001)

10.4.1.2 Phenolic Compounds

Phenolic compounds from the plant sources are the secondary metabolites containing hydroxyl group on aromatic ring. Plant phenols are highly heterogeneous as some dissolves only in organic solvents and a few in water and others remain as insoluble polymers. Anthocyanin, tannins, lignin, flavonoids, and isoflavones are few derivatives of phenols (Table 10.1). Flavonoids are one of the largest classes of phenols and their basic function is pigmentation and defense (Anulika et al. 2016). Apple, blackberries, tea, and woody plants possess tannins in them (Lazar 2003). A broad range of phenolic compounds from plants impart organoleptic properties (i.e., taste and odor) to the food products (O'Connell and Fox 2001).

10.4.1.3 Nitrogenous Compounds

Nitrogenous compounds include secondary metabolites containing nitrogen in their structures like alkaloids and glucosides (Lazar 2003) (Table 10.1). Morphine is the first medically applicable alkaloid, isolated from *Papaver somniferum* (Thummel 1979).

Table 10.1 Types of secondary metabolites occur in plants and their uses (Karban and Baldwin 1997, Bidlack 2000, Rosenthal and Berenbaum 1991)

Class	Compounds	Sources	Effects and uses
Nitrogen containing			
Alkaloids	Nicotine	*Nicotiana tabacum*	Interfere with neurotransmission
Nitrogen and sulfur containing			
Glucosinolates	Sinigrin	Cabbage relatives	–
Terpenoids			
Monoterpenes	Menthol	Mint and relatives	Interfere with neurotransmission, anesthetic
Sesquiterpenes	Parthenolide	Parthenium and relatives	Contact dermatitis
Diterpenes	Gossypol	Cotton	Block phosphorylation; toxic
Triterpenes	Betulinic acid, oleanolic and ursolic acid	*Lantana camara*	Cytotoxic effect on HeLa cancer cells
Tetraterpenoids	Carotene	Several plants	Antioxidant; orange coloring
Sterols	Spinasterol	Spinach	Interfere with animal hormone action
Phenolics			
Phenolic acids	Caffeic, chlorogenic	All plants	Oxidative damage, browning in fruits and wine
Coumarins	Umbelliferone	Carrots, parsnip	Cross-link DNA, block cell division
Flavonoids	Anthocyanin, catechin	Almost all plants	Flower, leaf color; inhibit enzymes, antioxidants
Phenols	Hydroxytectoquinone	*Rubia cordifolia*	Anticancer
Lignin	Lignin	All land plants	Toughness, structure, fiber

Many alkaloids provide defense mechanism, especially against mammals due to their toxicity (Hartmann 1999).

10.4.2 Quantification of Secondary Metabolites

Continuous monitoring of secondary metabolites is necessary for the successful establishment of production technology. Analytical techniques, like high-performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS), and spectrophotometry, are used for quantification of metabolites from in vitro plant cultures (Matkowski 2008). To prepare plant sample for quantification, the plant materials are dried and then soaked in a suitable solvent to extract metabolites. Of the various extraction methods, the most commonly used ones are Soxhlet extraction, microwave-assisted extraction, sonication, etc. (Kim and Verpoorte 2010).

10.5 Strategies to Enhance Secondary Metabolite Production

The metabolite content from in vitro-grown cultures can further be enhanced by several ways such as optimizing media and culture conditions, selecting high metabolite-producing cell lines, precursor feeding, elicitation, and biotransformation.

10.5.1 Optimization of Metabolite Synthesis by Culture Conditions

Nutrients and environment highly influence the metabolic pathway of secondary metabolites for example light-induced anthocyanin pigments. The anthocyanidin profile is different in both leaves and fruits; for example, cyanidin-3-rutoside occurs dominantly in leaves. Media optimization is needed for higher biomass as well as metabolite production. Auxins and cytokinins are two important plant growth regulators, responsible for the stimulation of metabolic pathways. In anthocyanin-producing *Glehnia littoralis* callus cultures, NAA (1 mg/L) was preferred as auxin over IAA (indole-3-acetic acid; 1 mg/L) and 2,4-D (1 mg/L) and further addition of kinetin (0.01 mg/L) enhanced the cell growth and pigment biosynthesis (Miura et al. 1998). In some reports two-stage system is used to optimize the production of secondary metabolites. The first stage is optimized to achieve maximum cell proliferation and faster biomass growth while second stage aims to accumulate more products. For example, when two-stage system was applied on *Crocus sativus*, crocin accumulation increased to 430 mg/L, by using IAA (2 mg/L) and BAP (0.5 mg/L) instead of NAA (2 mg/L) and BAP (1 mg/L) (Chen et al. 2003).

10.5.2 Selection of High Metabolite-Yielding Tissues

The production of metabolites depends on the type of tissues they are present. For example, in *Salvia officinalis* (Grzegorczyk et al. 2007) and *Rosmarinus officinalis* (Caruso et al. 2000) in vitro cultures, carnosic acid (a diterpene) is found only in shoot cultures but not in callus, suspension, or hairy roots, while higher phenols are accumulated by undifferentiated cell suspensions. The active cell lines chosen for metabolic production should produce metabolites higher than the other cultures and the normal producing cell lines. The efficient cell lines can be selected on the basis of the amount of their compounds or by selecting the agents supporting the process of production of those compounds. The cell line selected for the study should exhibit both the levels of production obtained in unselected cultures and the natural biosynthetic productivity of an intact organism. When a phenylalanine analogue is added to the culture medium, it will damage majority of the cells except those ones expressing high PAL (phenylalanine ammonia lysate) activity. The selected cells exhibiting overexpression of PAL produce more phenylpropanoid compounds (Matkowski 2008).

10.5.3 Precursor Feeding and Biotransformation

In a few in vitro-grown medicinal plants, the intact plant forms various valuable metabolites through its biosynthetic pathway whereas the dedifferentiated form is unable to complete the biosynthesis. This could be due to lack of environmental stimuli, insufficient expression of related genes, or improper enzymatic machinery. If the enzymes responsible for production of a particular valuable metabolite are expressed in one portion of the metabolic pathway, then precursor applications are recommended to overcome such type of problem. For example, strawberry cultures were producing negligible amount of anthocyanin but feeding phenylalanine (precursor) increased its production (Edahiro et al. 2005). Likewise curcumin is a yellow-colored potent antioxidant compound obtained from the roots of *Cucuma longa* commonly known as turmeric but due to its insolubility in water it is not absorbed by intestine and, thus, exhibits no pharmaceutical importance (Sharma et al. 2007). Later, in *Catharanthus roseus* cell suspension cultures, the supplied curcumins were modified into peculiar glycosides that are much more soluble in water. The water solubility of these curcumin-modified glycosides (curcumin-4′,4″-*O*-β-D-digentiobioside) was 20 million folds higher than that of curcumin (Kaminaga et al. 2003).

10.5.4 Elicitation and Stress-Induced Production

Many medicinal plants when exposed to stress exhibit increased production of secondary metabolites (Verpoorte et al. 2002). In vitro cultures can be elicited by using stress-related growth regulators, such as jasmonic acid or its esters, biotic elicitors

(for example, bacteria and fungus), and abiotic factors (for example, metals and radiation). Methyl jasmonate (MeJa), a stress-related growth regulator, is widely used in inducing the metabolite biosynthesis including anticancer alkaloids (Verpoorte et al. 2002; Mulabagal Vanisree et al. 2004). Yeast extract, chitin, or chitosan (biotic elicitors) was independently tested for production of silymarin where only yeast extract was found to be effective and its efficacy was increased when supplemented along with MeJa (Sanchez-Sampedro et al. 2005). Chitin intensified the production of flavonoids in *Cephalocereus senilis* (cactus) cell cultures (Qin et al. 1993). Vanadium salts and rare elements like La^{3+} and Ce^{3+} have shown to be increasing the accumulation of rosmarinic acid (Georgiev et al. 2007) and crocin in in vitro cell cultures (Chen et al. 2003). In certain plant cells, UV irradiation was found to increase the production of antioxidants.

10.5.5 Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation is one of the highly preferred methods to increase the secondary metabolite production. The plant species to be transformed are co-cultivated with *Agrobacterium rhizogenes* to induce hairy-root development. Using the method, rosmarinic acid (RA) production and its antioxidant activity were increased to much higher level in *Salvia officinalis* hairy roots as compared to that in untransformed organs (Grzegorczyk et al. 2006, 2007).

10.5.6 Scale-Up in Bioreactor

The ultimate aim of raising cell biomass and cell suspension cultures from medicinal plants, using plant tissue culture techniques, is to increase the availability of cell biomass as a raw material and also to increase the production of valuable metabolites at industrial scale. This could be possible when the callus and cell suspension cultures grown successfully in small culture vials and shake flasks, respectively, are to be grown at the same rate in large-size bioreactors. As the volume of the cultures increases in bioreactors, several parameters, like rotational speed, medium pH, cell concentration, temperature, and illumination, are to be optimized. However, the plant cell cultures are not as commercially utilized as microbial cell cultures due to the increased size of plant cells making it more sensitive to shear stress. The other major limitations are lack of availability of data; mostly the published work are dealt with plant cells at lab scale due to their comparatively large size and rigid cell wall than animal cells (Griffits 1985). Till 2001, the best examples of success were shikonin, paclitaxel, and ginseng production, where the industries employ plant cell culture at pilot scale (Zhong 2001). Bioprocess parameters like engineering considerations, optimization of process parameters, and process strategies can be applied to increase the production of secondary metabolites from plant cell suspension

cultures (Chattopadhyay et al. 2002). Stirred tank reactor with setric impeller was used to optimize podophyllotoxin production in suspension cultures of *Podophyllum hexandrum*. Stirred tank reactor with setric and turbine impeller was used to optimize azadirachtin production in cell cultures of *Azadirachta indica*. In this case, less shear and better mass transfer were observed. Biomass and azadirachtin yield with setric and turbine impeller was obtained as 18.7 and 15.5 and 0.071 and 0.05 g/L, respectively, at 125 agitation and 0.2 vvm aeration rates (Prakash and Srivastava 2006). Cell cultures of *Scrophularia striata* Boiss were grown in shake flasks and in bioreactor and analyzed for increase in biomass and phenylethanoid glycoside (PeG) production (Falahi et al. 2017). Cell biomass and PeG content were found higher in bioreactor than shake flasks, i.e., 15.64 g/L DW and 1404.20 μg/g and 14.16 g/L DW and 459.71 μg/g (Pavlov et al. 2007).

10.6 In Vitro Methods for Assessment of Biological Properties

10.6.1 Antibacterial Activity

Today, most of the antibiotics like tetracycline, cephalosporin, and aminoglycosides are synthesized chemically. The existence of these essential compounds is at risk due to developing resistance in microbes, against these antibiotics. As a result, drugs become inactive and infections persist and spread. Hence, these multidrug-resistant microbes are a threat to human health (Balouiri et al. 2016; Mayers et al. 2009). Thus, the discovery of new antibiotics is a crucial issue among many researchers. The production of antibiotics via in vitro plant tissue culture technique is a biological route of synthesis as well as sustainability of cultures. In order to measure the antibacterial potential of plant-based drugs, following assays are generally performed (Guschin et al. 2015).

10.6.1.1 Diffusion Methods

Diffusion methods are based on the principle of mass transfer due to diffusion. In diffusion, the "movement of molecules takes place from higher concentration to lower concentration." Most of the lifesaving molecules, including water, are transported by diffusion. Using the similar phenomena, the antibacterial activity of plant-based drugs is measured using the assays described in the following lines. The efficacy of the active compound is quantified in terms of zone of inhibition (ZOI), followed by minimum inhibitory concentration (MIC). The clear zone around the filter disc containing antibiotic defines the ZOI, where there is no visible growth of bacteria. Higher ZOI represents higher potency of the drugs. The activity of the compounds is compared with that of positive and negative controls for all the experiments. Positive control is any antibiotic known to kill the test bacteria while the

negative control is usually the solvent in which the test organism is suspended without any antibiotic. As the name suggests, positive control surely produces ZOI while negative control gives no ZOI (Balouiri et al. 2016). The minimum concentration of the antibacterial test compound (i.e., compound to be tested for antibacterial activity) is called minimum inhibitory concentration (MIC), which is the minimum concentration required to kill the visible population of test bacteria. It is generally expressed as μg/mL or mg/mL.

Agar disc diffusion method is one of the most commonly used methods in many clinical laboratories for testing antibacterial susceptibility of various drug candidates. It offers many advantages over other methods like simplicity of procedure, low cost, facility to test multiple test bacteria (i.e., bacteria used for the study), and antibacterial compounds with the ease to interpret results. The procedure to test the antibacterial potency of in vitro plant extract (IPE) through disc diffusion method is represented in Fig. 10.2. Nutrient agar (NA) plates are prepared by pouring 20 mL of NA media in 90 mm petri plates inside the laminar hood and once the media is solidified the plates are ready for testing the antimicrobial activity. Thereafter, the bacterial cultures ($\approx 1 \times 10^6$ cells) are spread over NA plates with the help of spreader. Afterwards, known concentrations of IPE are added to filter paper discs (6 mm dia). After drying these filter discs, these are placed over the petri plates inoculated with test bacteria (Choma and Grzelak 2011). Antibacterial compounds of IPE diffuse into the agar and form a ZOI, which is measured in millimeter (mm). This method is not suitable for MIC determination as the quantification of antibacterial compounds diffused into the agar is very difficult (Balouiri et al. 2016).

Agar well diffusion method is similar to the disc diffusion method except that the wells are made in the agar plate, instead of using the filter discs. After spreading the test bacteria on agar plates, wells (of dia 6–8mm) are made aseptically.

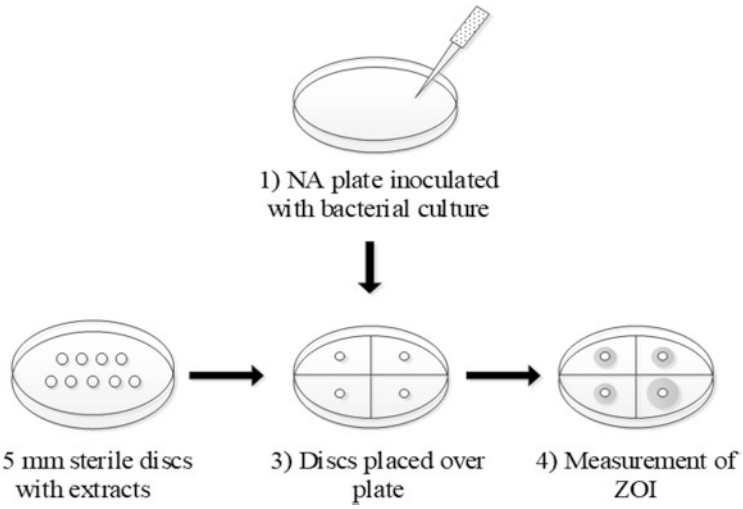

Fig. 10.2 Protocol for agar disc diffusion method

Subsequently, 20–100 μL of IPE is added and plates are incubated at optimum growth conditions of test bacteria. The diffusion of IPE occurs through agar and produces ZOI, which in turn gives the information about antimicrobial potential of the IPE. The more the ZOI (in mm), the more the antimicrobial potential of the IPE (Balouiri et al. 2016).

10.6.1.2 Dilution Methods

Dilution method is the most applicable technique for MIC determination as it gives the quantified amount of antimicrobial drug (Choma and Grzelak 2011). The broth and agar dilution methods are widely used methods for quantitative measurement of the antimicrobial activity against bacteria and fungus.

Broth dilution method is a basic method to test antibacterial activity. It is of two types: broth macro- or micro-dilution methods. If the twofold dilution (i.e., 1, 2, 4, 8, and 32 μg/mL) of test antimicrobial compound is performed in 2 mL tube it is called as macro-dilution and if it is performed in lower volumes in 96-well plate it is known as micro-dilution (Balouiri et al. 2016). Bacterial inoculum preparation is one of the most important factors influencing the MIC value. The EUCAST guidelines, standardized for broth dilution for testing antimicrobial activity by broth dilution method, are described in the following lines. As per the EUCAST guidelines if the inoculum is fungus (conidium and spores), the inoculum to be used for activity test will be adjusted to 0.4×10^4–5.0×10^4 CFU/mL by adding phosphate-buffered saline (PBS) to the inoculum. The dilution of inoculum, in order to get the required number of cells, can be achieved in two ways: by preparing 0.5 McFarland solution and comparing the inoculum with 0.5 McFarland scale (reference) or by measuring absorbance by spectrophotometer (Fig. 10.3). After inoculum preparation, the test antimicrobial compounds (twofold diluted, i.e., 1, 2, 4, 8, and 32 μg/mL) were added to inoculum (in tubes or in 96-well plates) and incubated (mostly without agitation) at optimum growth conditions of the test bacteria (Balouiri et al. 2016; Choma and Grzelak 2011) and their absorbance is measured using spectrophotometer at 625 nm (Fig. 10.4).

Agar dilution method is used particularly when IPE color inhibits the detection of bacterial growth. It involves the addition of antimicrobial agent to molten agar medium, using twofold serial dilution method, followed by addition of bacterial inoculum, measurement of absorbance, and MIC determination (Balouiri et al. 2016, Choma and Grzelak 2011).

10.6.1.3 Antimicrobial Gradient Method (E-test)

This method combines the principle of dilution and diffusion methods. An inert and nonporous plastic reagent stripe comprising increasing concentration of IPE is introduced to the agar medium (Choma and Grzelak 2011). To detect the synergy between two drugs, stripe containing the first drug is placed on pre-inoculated agar

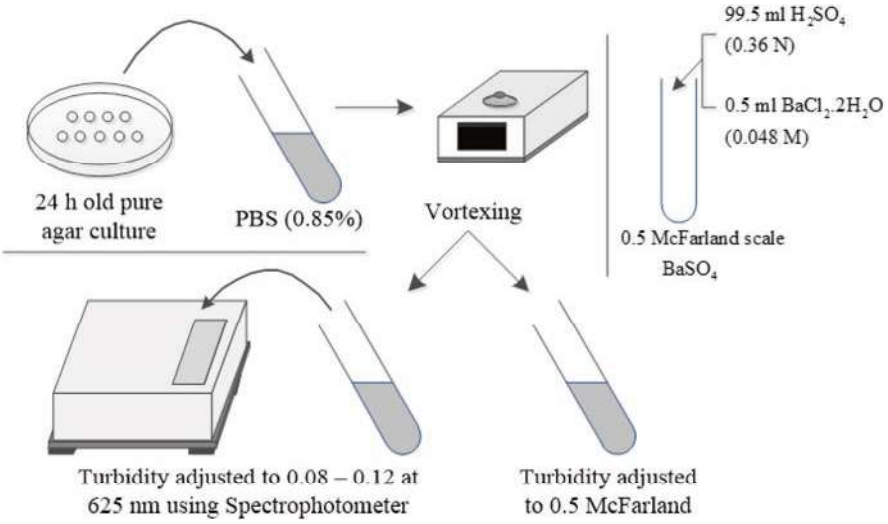

Fig. 10.3 Protocol for inoculum preparation for antimicrobial activity

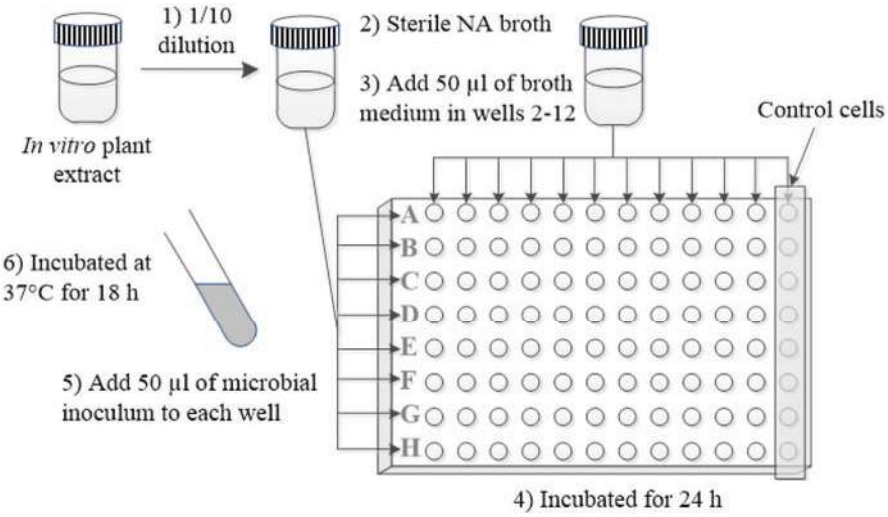

Fig. 10.4 Broth microdilution method for determination of MIC of test compound

media followed by another stripe containing the second drug and the MIC is calculated. The drugs were considered to be synergistic in action, when the MIC value of both the drugs in combination is at least more than two dilutions as compared to the most active drug tested alone (Balouiri et al. 2016). Two drugs are considered to be synergistic if their combined effect is more than the effect of individual drugs individually (Fig. 10.5).

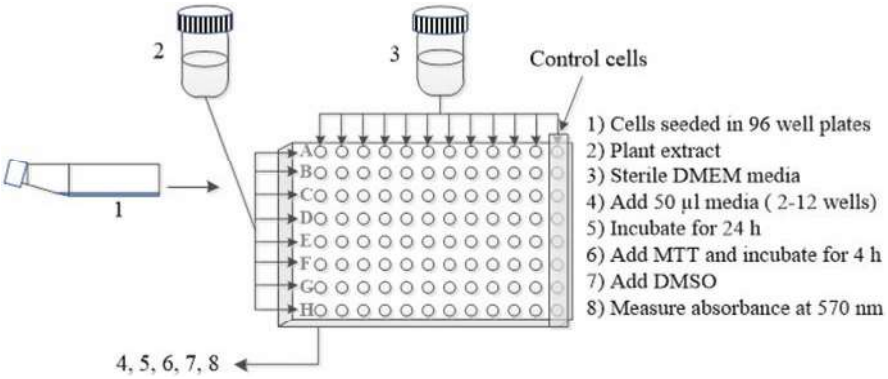

Fig. 10.5 MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay for determination of cytotoxicity of test compounds

10.6.1.4 TLC Bioautography

Bioautography is a microbe detection technique grouped with planar chromatography. Bioautography technique coupled with thin-layer chromatography (TLC) is known as TLC bioautography. The protocol for this assay is identical to agar diffusion methods except that the diffusion of test compound occurs from chromatographic layer (i.e., adsorbent or paper) to the agar plate. This method is of three types: agar diffusion or agar contact method, direct bioautography, and agar overlay bioassay (Balouiri et al. 2016, Choma and Grzelak 2011).

10.6.1.5 Time Kill Test

Time kill test or time kill curve is one of the important techniques to determine bactericidal or fungicidal activity. To perform this, 1×10^5 CFU/mL bacterial cultures were taken in three tubes, followed by the addition of test compound at concentrations of 0.25 × MIC and 1 × MIC in two tubes and control (without test compound) in third tube. All the tubes were incubated for a period of 0, 4, 8, 12, and 24 h. Thereafter, percentage of dead cell is calculated by comparing with the number of living cells in control at every stage of respective time interval (Balouiri et al. 2016).

10.6.1.6 ATP Bioluminescence Assay

ATP is a chemical form of energy universally present in all the living cells. This assay calculates the ATP generated by bacteria or fungi, which in turn gives the amount of microbe in a sample. However, the sample is treated with a potent antimicrobial agent. In the presence of ATP, D-luciferin gets converted into oxyluciferin which gives illumination. It is measured by a luminometer and is expressed as a relative light unit (RLU). Based on the RLU values, the linear relationship between

cell viability and luminescence is established for measuring the bioactivity of a particular antimicrobial agent (Balouiri et al. 2016).

10.6.1.7 Flow Cytofluorometric Method

Flow cytometry is used to quantify the damaged cells by using appropriate dyes that stain specific organelles of the cell. Many studies reported the use of flow cytometric method in antibacterial assays. For example, propidium iodide (PI) is used for membrane disruption study and carboxyfluorescein diacetate (cFDA) is used to detect esterase activity. Apart from lysed cells, these methods also differentiate live, dead, and injured cells (Balouiri et al. 2016).

10.6.2 Antidiabetic Activity

10.6.2.1 α-Amylase Inhibition Assay

α-Amylase inhibition assay is performed by 3,5-dinitrosalicylic acid (DNSA) method. Leaf extract of *Adenanthera pavonina* was initially dissolved in a minimum amount of 10% dimethyl sulfoxide (DMSO), followed by its further dissolution in *reagent* 1 to prepare concentrations ranging from 10 to 1000 μg/mL. Thereafter, 200 μL of *reagent* 2 is added to 200 μL of extract, followed by incubation at 30 °C for 10 min. The measured volume of 1 mL of *reagent* 3 is added. After 3 min of incubation, *reagent* 4 is added to stop the reaction and this reaction mixture is boiled in water bath for 10 min at 85–90 °C. Thereafter, the reaction mixture is cooled to room temperature, followed by its dilution with 5 mL of distilled water. The absorbance is taken at 540 nm via spectrophotometer with the help of Eq. (10.1). Plant extract and acarbose (2–100 μg/mL) were used as blank and positive controls, respectively (Wickramaratne et al. 2016). The detailed list of reagents required is provided in Table 10.2:

$$\%\text{Inhibition} = \frac{A_{540\text{CONTROL}} - A_{540\text{EXTRACT}}}{A_{540\text{CONTROL}}} \times 100 \tag{10.1}$$

10.6.2.2 Glucose Diffusion Inhibitory Assay

This assay describes the protocol of with some minor modifications. *Reagent* 5 (2 mL) was laden into a dialysis tube containing IPE (50 mg/mL) and then the dialysis tube was tied from both its ends. It is then placed in a centrifuge tube containing 45 mL solution of 0.15 M NaCl. Thereafter, the centrifuge tube was placed in an orbital shaker at room temperature. Diffusion of glucose into the external solution was monitored after every 1 h by measuring the concentration of glucose in the

external solution (Gray and Flatt 1997). The control consists of 1 mL of 0.15 M NaCl containing *reagent* 5 and 1 mL distilled water (Vijayalakshmi et al. 2014).

10.6.3 Antioxidant Activity

Antioxidants are compounds that delay or inhibit the oxidation of a substrate even if it is in lower concentration than substrate (Halliwell and Gutteridge 1995). It investigates the potential of natural antioxidants in both forms, as pure compounds and plant extracts. Due to high sensitivity, these methods have become popular (Salazar et al. 2008). Some of the methods are described below.

10.6.3.1 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

One of the widely used methods to measure the radical scavenging activity (RSA) of antioxidant compounds is DPPH assay (Blois 1958; Mensor et al. 2001; Singh et al. 2014). It involves the reduction of DPPH to form DPPH-H in methanol when a hydrogen-donating antioxidant is present. The assay is performed in author's laboratory where 2 mL of extract (stock solutions of 10 mg/mL in methanol) is mixed with 500 mL of *reagent* 6. After a duration of 30 min, absorbance is recorded at 517 nm by using control having same amount of DPPH. Decrease in absorbance shows higher RSA. It is calculated using Eq. (10.2)

$$\%\text{Inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \tag{10.2}$$

where A_0 and A_1 are absorbance of control and test sample, respectively. Positive controls can be gallic acid, ascorbic acid (Blois 1958), and α-tocopherol (Shimada et al. 1992).

10.6.3.2 Superoxide Anion Radical Scavenging (SO) Assay

Superoxide anion is known to be a weak oxidant but it generates hazardous hydroxyl radicals and singlet oxygen (Meyer and Isaksen 1995). Many other biological reactions also generate highly toxic superoxide radicals. This method is performed using the protocol of Robak and Gryglewski (1988). To generate superoxide anion radicals, 0.5 mL of *reagent* 8, 0.5 mL of *reagent* 9, 1.0 mL of extract, and 0.5 mL of *reagent* 7 are mixed. Thereafter, 0.5 mL of r*eagent* 10 is added to start the reaction. After addition of antioxidants, depletion of superoxide anion in the reaction mixture causes decrease in absorbance which is measured at 560 nm using spectrophotometer. Positive controls such as gallic acid (Robak and Gryglewski 1988), curcumin, BHA, ascorbic acid, and α-tocopherol can be used (Nishikimi et al. 1972).

Table 10.2 Reagents required to test particular bioactivity

Activity	In vitro assays	Reagents required
Antidiabetic activity	DNSA activity	1. Sodium phosphate buffer (0.02 M) with NaCl (0.006 M) pH 6.9 2. α-amylase solution (0.5 mg/mL) 3. Starch solution (1% in water (w/v)) 4. DNSA color reagent
	Glucose diffusion inhibitory assay	5. Glucose solution (0.22 mM in 0.15 M sodium chloride)
Antioxidant activity	DPPH assay	6. DPPH in methanol (0.5 mg/mL)
	SO assay	7. Tris–HCl buffer (16 mM, pH 8.0) 8. Nitro-blue tetrazolium dye, (NBT; 0.3 mM) 9. β-Nicotinamide adenine dinucleotide reduced sodium salt, (NADH solution; 0.936 mM) 10. PMS solution (0.12 mM)
	XO method	11. Phosphate buffer (0.05 M, pH 7.5) 12. Xanthine oxidase solution (0.2 units/mL) 13. Xanthine substrate solution (0.15 M)
	H_2O_2 assay	14. H_2O_2 (40 mM) 15. Phosphate buffer (50 mM; pH 7.4)
	NO assay	16. Sodium nitroprusside in phosphate buffer (10 mM) 17. Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO3)
	HO assay	18. 2-Deoxy-D-ribose (28 mM) in KH_2PO_4-KOH buffer (20 mM); pH 7.4 19. Ethylenediaminetetraacetic acid (EDTA; 1.04 mM) 20. $FeCl_3$ (1:1 v/v) 21. H_2O_2 (1.0 mM) 22. Ascorbic acid (1.0 mM) 23. Thiobarbituric acid (1%) 24. Trichloroacetic acid (2.8%)
	ORAC assay	25. 2, 2′- Azo-bis, 2-amidinopropane dihydrochloride (AAPH)
	FRAP assay	26. 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) 27. FRAP reagent (10 parts of 300 mM sodium acetate buffer; pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. $6H_2O$ solution)
Anticancer activity	MTT assay	28. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
	XTT assay	29. 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H* tetrazolium-5-carboxyanilide inner salt (XTT)
	MTS assay	30. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)
	Trypan blue dye exclusion assay	31. Hanks' buffered salt solution (HBSS) 32. Trypan blue (0.4%)
	Microscopic observations of cell morphology	33. Acridine orange or ethidium bromide (AO/EB)
	Resazurin cell growth inhibition assay	34. Trypsin (0.025%) 35. EDTA (0.25 mM) 36. Resazurin (0.01% w/v)

10.6.3.3 Xanthine Oxidase (XO) Method

This method is performed using the protocol of Noro et al. (1983). Allopurinol of 100 μg/mL in methanol and 500 μL (0.1 mg/mL) of extract were mixed with 1.3 mL and 0.2 mL of *reagent*s 11 and 12, respectively. This mixture is incubated for 30 min at 25°C, followed by addition of 1.5 mL of *reagent* 13 reaction mixture. It is again incubated for 30 min at 25°C and absorbance is recorded at 293 nm by spectrophotometer. The solution of 1.3 mL of *reagent* 11, 0.2 mL of *reagent* 12, and 0.5 mL of methanol is used as blank. The mixture of 1.3 mL of *reagent* 11, 0.2 mL of *reagent* 12, 1.5 mL of *reagent* 13, and 0.5 mL of methanol is used as control. It is calculated using Eq. (10.3)

$$\%\text{Inhibition} = \left(1 - \frac{A_s}{A_c}\right) \times 100 \tag{10.3}$$

where A_s and A_c are the absorbance values of test sample and control, respectively. Catechin can be used as positive control (Schmeda-Hirschmann et al. 1996).

10.6.3.4 Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay

Hydrogen peroxide is present at low levels of concentration in plants, human body, microorganisms, air, and water. In human body, it can enter while breathing or by contact with skin and eye. After entering inside, H_2O_2 decomposes into water and oxygen. It can also form hydroxyl radicals ($OH^{\cdot}$), which can even damage DNA. The H_2O_2 scavenging potential of plant extracts is analyzed by method of Ruch et al. (1989). *Reagent* 14 is mixed with *reagent* 15. Extract (20–60 μg/mL) is prepared in distilled water and added to H_2O_2. Absorbance is measured at 230 nm by spectrophotometer. *Reagent* 15 alone is used as blank. It is measured by Eq. (10.4):

$$\%\text{Scavenged}\left(H_2O_2\right) = \left(A_0 - \frac{A_1}{A_0}\right) \times 100 \tag{10.4}$$

where A_0 and A_1 are absorbance of control and test sample. Positive controls can be α-tocopherol (Gülçın et al. 2003), ascorbic acid, and BHA (Jayaprakasha et al. 2004).

10.6.3.5 Nitric Oxide (NO) Assay

Nitric oxide is produced in solution when *reagent* 16 reacts with oxygen to produce nitrite ions at physiological pH which is observed by *reagent* 17 (Green et al. 1982). A measured volume of 3 mL of *reagent* 16 is added to 2 mL of extract and reference compound, in various concentrations ranging from 20 to 100 μg/mL, followed by

incubation at 25 °C for 60 min. Methanol is used as control. An amount of 5 mL each, from incubated sample and *reagent* 17, is taken and mixed. Absorbance is measured at 540 nm. The percentage inhibition is calculated by comparing the absorbance values of test and control samples. Positive controls used are α-tocopherol (Garrat 1964), ascorbic acid, BHA (Jayaprakasha et al. 2004), or caffeic acid, sodium nitrite, and curcumin (Sreejayan and Rao 1997).

10.6.3.6 Hydroxyl Radical Scavenging (HO) Assay

Hydroxyl radicals are one of the most effective reactive oxygen species (ROS) in the biological system. The method measures the scavenging activity (Kunchandy and Rao 1990). An amount of 1 mL of reaction mixture is prepared by adding 100 μL of *reagent* 18, 500 μL of extract, 200 μL of *reagent* 19, 200 μM of *reagent*-20, 100 μL of *reagent* 21, and 100 μL of *reagent* 22. Then, it is kept for incubation at 37 °C for 1 h. The mixture is heated for 15 min at 95 °C and 1.0 mL of both *reagents* 23 and 24 is added to it. After 20 min of incubation at 100 °C, it is cooled and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant is recorded at 532 nm. Positive controls are taken as vitamin E (Halliwell et al. 1987), ascorbic acid, or rutin (Jayaprakasha et al. 2004).

10.6.3.7 Oxygen Radical Absorbance Capacity (ORAC) Assay

Investigation of the peroxyl radical scavenging potential of the compounds, produced by spontaneous decomposition of *reagent* 25, is done by ORAC assay (Prior et al. 2005). For the estimation, reaction mixture is prepared by adding 0.5 mL of extract in phosphate buffer (75 mM, pH 7.2) with 3.0 mL of fluorescein solution, followed by preincubation for 10 min at 37 °C. After the addition of 0.5 mL of *reagent* 25, decrease in fluorescence (FL) is observed and difference in area under the FL decay curves is calculated for both sample and control. O.

XYGEN-RADICAL ABSORBANCE CAP

10.6.3.8 Ferric Reducing Antioxidant Power (FRAP) Assay

The potential of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of reagent 26, where a blue complex Fe^{2+} TPTZ is formed with an absorption maximum at 593 nm, is measured by FRAP assay. The antioxidant potential is proportional to a decrease in absorbance (Benzie and Strain 1996). For reaction mixture, 0.2 mL of extract and 3.8 mL of *reagent* 27 are mixed, followed by incubation at 37 °C for 30 min. Absorbance is taken at 593 nm. Positive controls used are BHA, BHT, ascorbic acid, quercetin, or catechin (Benzie and Strain 1996).

10.6.4 Anticancer Activity

A number of methods are available to investigate the anticancer potential of plants such as 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H tetrazolium-5-carboxyanilide inner salt (XTT) assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay, lactic acid dehydrogenase (LDH) assay, sulforhodamine B (SRB) assay, Resazurin cell growth inhibition assay, and trypan blue dye exclusion assay. The description of reagents is provided in Table 10.2.

10.6.4.1 MTT (3-[4,5-Dimethylthiazole-2-yl]-2,5-Diphenyltetrazolium Bromide) Assay

One of the preliminary assays to test toxic effects of a compound or drug on various cell lines is MTT assay. It is an extremely fast, reliable, convenient, and economical method (McCauley et al. 2013) to determine the number of viable cells. Anticancer potential of callus extract of *Lantana camara* was tested on cervical cancer cell line (HeLa) cells using normal fibroblast cell lines (BHK-21) as control in the author's laboratory. The HeLa and BHK-21 cells were grown on T-25 culture flasks in 10% Dulbecco's modified Eagle medium (DMEM). The cells were harvested by trypsinization, followed by seeding in 96-well plates (density $\approx 10^4$ cells/mL) and incubated for 24 h. Thereafter, cells seeded in 96-well plate were washed with phosphate-buffered saline (PBS) followed by addition of different concentrations of callus extract (2.5–200 μg/well) and curcumin (positive control; 1–10 μg/well). Plates were incubated for various time intervals like 24, 36, 48, 60, and 70 h, followed by addition of *reagent* 28 and 4-h incubation. Thereafter, addition of dimethyl sulfoxide (DMSO) dissolves the formazan granules formed inside the living cells and absorbance is measured at 570 nm via multi-plate reader (Srivastava et al. 2010).

10.6.4.2 XTT (2,3-Bis[2-Methoxy-4-Nitro-5-Sulfophenyl]-2*H* Tetrazolium-5-Carboxyanilide Inner Salt) Assay

This assay depends on the splitting of yellow tetrazolium salt (XTT) for the formation of an orange formazan dye by mitochondrial enzyme, formazan dehydrogenase, in living cells. It measures the amount of viable cells by spectrophotometry. Cells were grown in medium supplemented with 10% fetal bovine serum (FBS) in 96-well plates until 70–80% confluency followed by drug treatment and 24-h incubation. Each well is supplied with 50 mL of XTT followed by 4-h incubation at 37 °C. Thereafter, formazan dye is solubilized using aqueous solutions and optical density is measured at 450 nm and is compared with control wells by a multi-well spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader.

10.6.4.3 MTS (3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2*H*-Tetrazolium) Assay

Like MTT, it is also a reliable, convenient, and economical method for cytotoxicity determination. This method is performed in similar way like MTT but *reagent* 30 is used to perform this assay (McCauley et al. 2013). Some of the routine in vitro assays which are performed to investigate the bioactivity of in vitro cultures are provided in Table 10.3.

10.6.4.4 Trypan Blue Dye Exclusion Assay

It is the most commonly performed test to assess the viability of cells. For this assay, cells are washed with *reagent* 31 and centrifuged for 10–15 min at 10,000 rpm and this entire step is repeated thrice. Cells are added to *reagent* 31 and cell count is adjusted to 1×10^6 cells/mL. These cells were transferred into Eppendorf tubes (2×10^5 cells in 0.1 mL), followed by addition of plant extracts and incubation for 3 h at 37 °C. Thereafter, these treated cells were mixed with *reagent* 32, incubated for 1 min, and assessed for their viability using hemocytometer. The growth inhibition percentage is calculated by Eq. (10.5):

$$\%\text{Growth inhibition} = \left(\frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}}\right) \times 100 \tag{10.5}$$

10.6.4.5 Resazurin Cell Growth Inhibition Assay

Resazurin cell growth inhibition assay or Alamar blue assay measure the cellular viability as well as function of mitochondria. Cells harvested from tissue culture flasks were treated with *reagents* 34 and 35 for 5 min. Subsequently, cells were washed with PBS, counted, seeded in 96-well plate containing 5×10^3 cells/well, and incubated for overnight growth. Then, cells were treated with samples and kept for 48-h incubation, followed by addition of 20 μL of *reagent* 36 and further incubation for 1–2 h at 37 °C. Fluorescence of 96-well plate is measured by multi-plate reader at excitation and emission wavelength of 540 and 590 nm, respectively, and inhibitory concentration (IC_{50}) values are calculated. The IC_{50} value is the amount of anticancer drug (here, plant extract) needed to inhibit 50% of cell proliferation (Kuete et al. 2011).

10.6.5 Anthelmintic Activity

This assay is performed in author's laboratory using the protocol described by Singh et al. (2014). Live fluke worms, commonly parasitizing inside the rumen of cattle livestock, were collected in 0.9% phosphate-buffered saline (PBS; 8 g NaCl, 1.21 g

Table 10.3 In vitro assays to investigate the bioactive potential of in vitro cell cultures

S. No.	In vitro plant/ part/callus	In vitro assay	Inference	References
1	In vitro leaf callus extract of *Spilanthes acmella* Murr.	Anthelmintic (time of paralysis)	Aqueous extract of in vitro callus showed stronger anthelmintic activity than extract of field-grown plant	Singh et al. (2014)
2	In vitro leaf callus extract of *Spilanthes acmella* Murr.	Antioxidant (1,1-diphenyl-2-picrylhydrazyl: DPPH)	Methanol extract of *Spilanthes acmella* possesses antioxidant activity with IC_{50} = 1342.9 µg/mL	Singh et al. (2014)
3	In vitro flower callus extract of *Spilanthes acmella* Murr.	Schizonticidal	In vitro flower callus extract showed significant antimalarial activity	Rajendran et al. (2017)
4	In vitro leaf callus extract of *Lantana camara* L.	Anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	Aqueous fraction showed anticancer activity at 36 h (100 µg/mL) to 72 h (25 µg/mL) whereas ethyl acetate fraction at 1500–3000 µg/mL	Srivastava et al. (2009, 2010)
5	In vitro leaf extracts of 14 plants	Anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	Extract showed activity against NCI-H292 (lung cancer) cell line	Gomes de Melo et al. (2010)
6	In vitro callus of *Decalepis*	Antioxidant ferric reducing antioxidant power (FRAP)	In vitro callus of *Decalepis* showed significant antioxidant activity with IC_{50} = 20 ± 1.54 µg/mL	Umesh (2014)
7	In vitro leaf, stem and root callus extract with wild leaf, stem and root of *Tephrosia tinctoria*	Antioxidant (1,1-diphenyl-2-picrylhydrazyl: DPPH) and anticancer (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide: MTT)	More amount of anticancer compound (phenol, 2,4-bis (1,1-dimethylethyl)) is obtained in stem callus extract and root callus extract showed significant inhibition of cell growth of HepG2 cells (IC_{50} = 20 µg/mL) after 72-h treatment	Rajaram et al. (2013)
8	In vivo and in vitro leaf callus cultures of *Baliospermum montanum*	Antibacterial (well-diffusion method)	Ethanol extract of in vitro leaf callus showed the maximum antimicrobial activity than in vivo leaf	Johnson et al. (2010)

K_2HPO_4, and 0.34 g KH_2PO_4, pH 7 ± 0.3). Thereafter, the worms were incubated at 37 ± 1 °C in PBS supplemented with 1% dimethyl sulfoxide (DMSO) with extract concentrations of 10, 20, and 30 mg/mL or without extract (control) and time taken for paralysis and death of worm is recorded. Distocide (drug) is used as reference.

10.6.6 Schizonticidal Activity

Plasmodium falciparum is a protozoan parasite which causes malaria in humans. It is the most fatal strain of genus plasmodium (Rich et al. 2009). Malaria is transferred by biting of female anopheles mosquito. Wild natural flowers and in vitro flower callus were utilized for extract preparation followed by isolation of two important metabolites, i.e., spilanthol and UDA from these extracts. *P. falciparum* 3D7 parasites were initially maintained in red blood corpuscles (RBCs) and later transferred to RPMI 1640 media (25 mM HEPES, 0.4% glucose, 0.2% sodium bicarbonate, 0.5% albumax, 50 mg/L hypoxanthine, 40 μg/mL gentamycin, and 25 μg/mL amphotericin B, at 37 °C). The synchronous development of erythrocytic stages of these parasites is observed on transferring to 5% D-sorbitol. These parasitic cultures were then incubated with different concentrations (0–50 μg/mL) of extract as well as isolated metabolites followed by 36-h incubation (Parveen et al. 2013; Rajendran et al. 2017). A thin blood smear is prepared and stained with Giemsa and number of schizont-containing RBCs was counted under microscope (Lambros and Vanderberg 1979; Zhang et al. 2015).

10.7 Conclusion and Future Prospects

Plants are an outstanding source of medicine. As mentioned in Sect. 10.2, the major benefit of using plants as drugs is that they rarely show any lethal or toxic effects to cells which is a major issue with most of the synthetic drugs. Therefore, establishment of in vitro cultures and investigating their potential by using various in vitro assays is a route to conquer many life-threatening diseases. To conclude, if explored suitably, they can even replace the allopathic drugs.

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