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Screening Methods in the Study of Fungicidal Property of Medicinal Plants

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1. Introduction

There is growing interest in the use of medicinal plants and herbal products for fungal infection related diseases control program because plant derived drugs are considered safe and free from adverse side effects. Plants generally produce many secondary metabolites which constitute an important source of antifungal drugs. Medicinal properties of plants are normally dependent on the presence of certain phytochemical principles such as alkaloids, anthraquinones, cardiac glycosides, saponins, tannins and polyphenols which are the bioactive bases responsible for the antimicrobial property (Ebana *et al*., 1993). It is difficult to discover antifungal agents against yeasts and filamentous fungi compared to bacteria caused diseases. Fungal cell wall is predominantly composed chitin and other polysaccharides such as β-glucans which become hindrance to the antibiotic activity on the cells. Hence appropriate screening methods to study the antifungal activity of natural resources play an important roles in the development of fungicide.

Plant drugs still remain the principal source of pharmaceutical agents used in orthodox medicine (Khaing, 2011). The ability of plant to produce various types of phytochemicals such as alkaloid, flavonoid and saponin attract the attention of natural products researcher. Although many plant or herbal products use for the treatment of various ailments in rural communities but there is a need for scientific verification of their activities against fungi. Currently, there is little scientific evidence on the antimicrobial properties of these medicinal plants under investigation against majority of fungi (Mann et al., 2008). *In vitro* and *in vivo* antifungal activities of medicinal plants have been studied widely in the present's days. A wide variety of methods can be applied to study anti-fungal activity. However, the outcome of these studies relies on appropriate and reliable methods use by the researchers.

In this chapter we analyze and compare the various reliable methods available for the study of antifungal activity. In this chapter, we describe and discuss plant sample extraction technique, antifungal screening with determination of minimum inhibition concentration, minimum fungicidal concentration and IC_{50} value on hyphal growth inhibition. Beside that the *in situ* antifungal study method by using various microscopy techniques such as confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and

transmission electron microscopy (TEM) also will be discussed. Fig. 1 shows the various steps involved in the evaluation of the medicinal plant's for fungicidal properties.

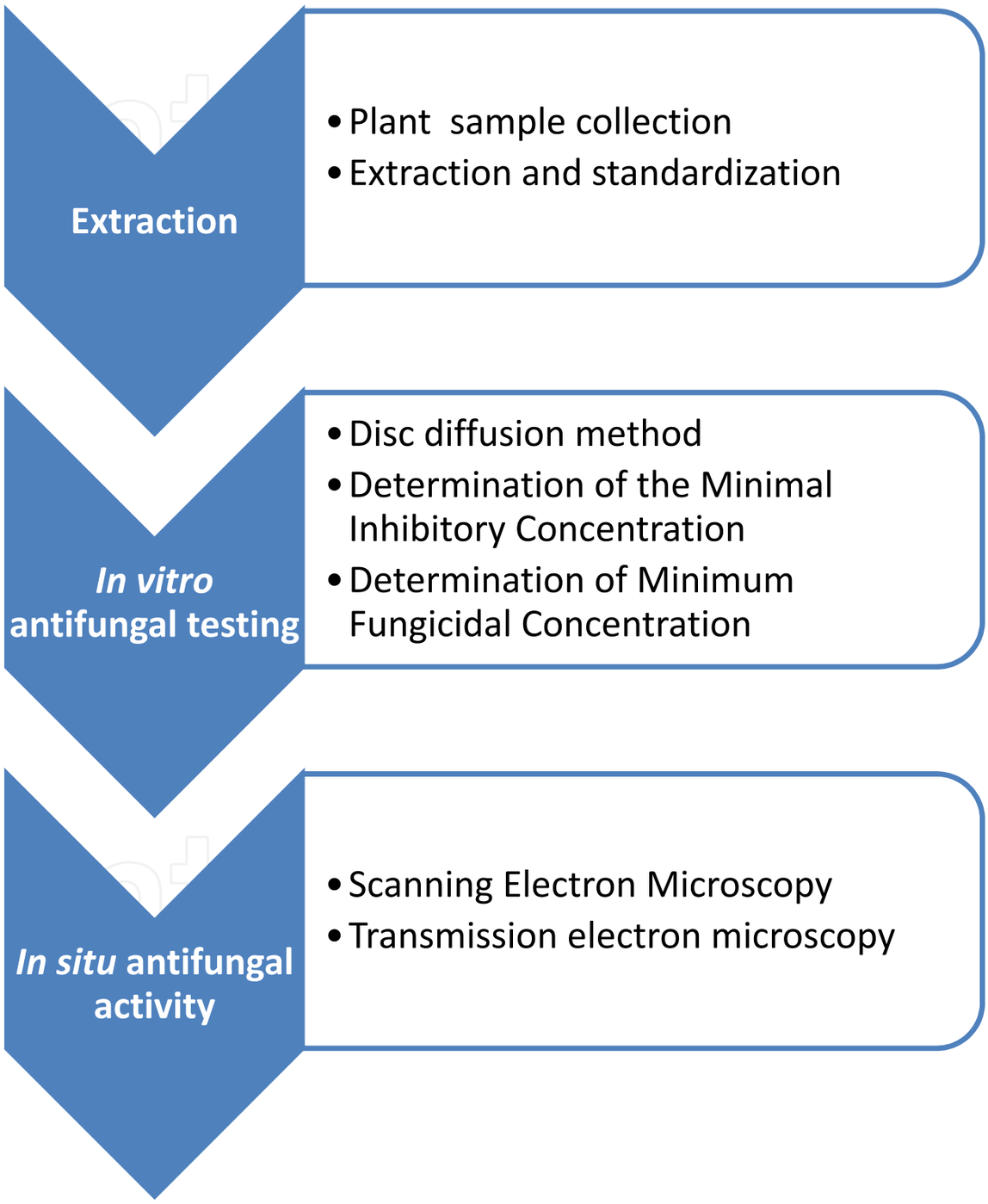

Fig. 1. Various steps involved in the development and evaluation of fungicidal property of medicinal plants

2. Extraction

First steps in the process of screening medicinal plants for antifungal activity is extraction. Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts (Sukhdev et al., 2008). The basic operations of extraction include steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples.

The general techniques (Fig. 2) of medicinal plant extraction include maceration (Fig. 3), infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet). Recently, modern extraction methods have been developed which includes microwave-assisted extraction (MAE), ultrasound extraction (sonication) and supercritical fluid extraction (SFE) (Table 1).

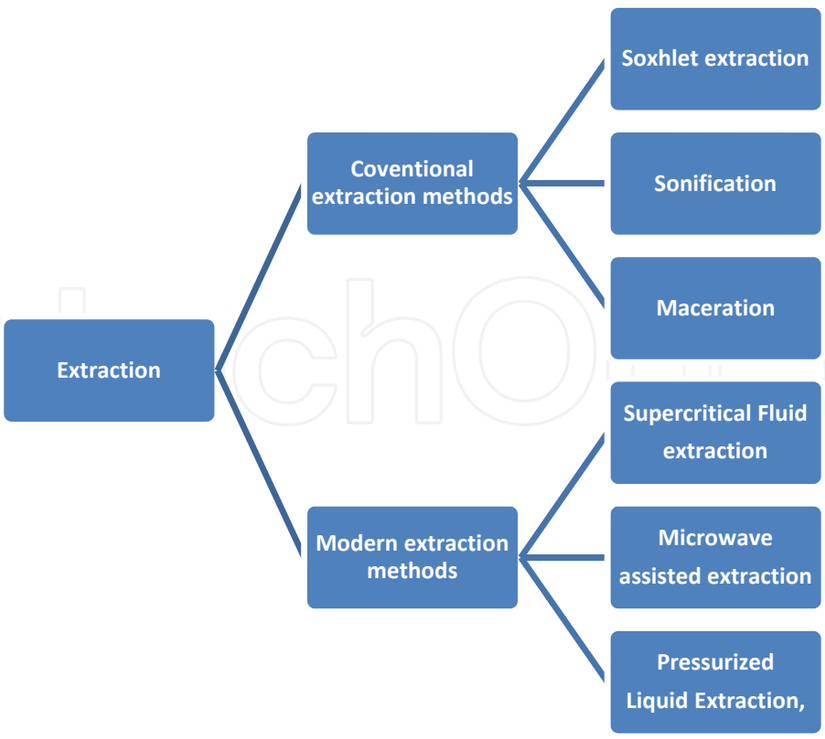

Fig. 2. Conventional and modern extraction methods

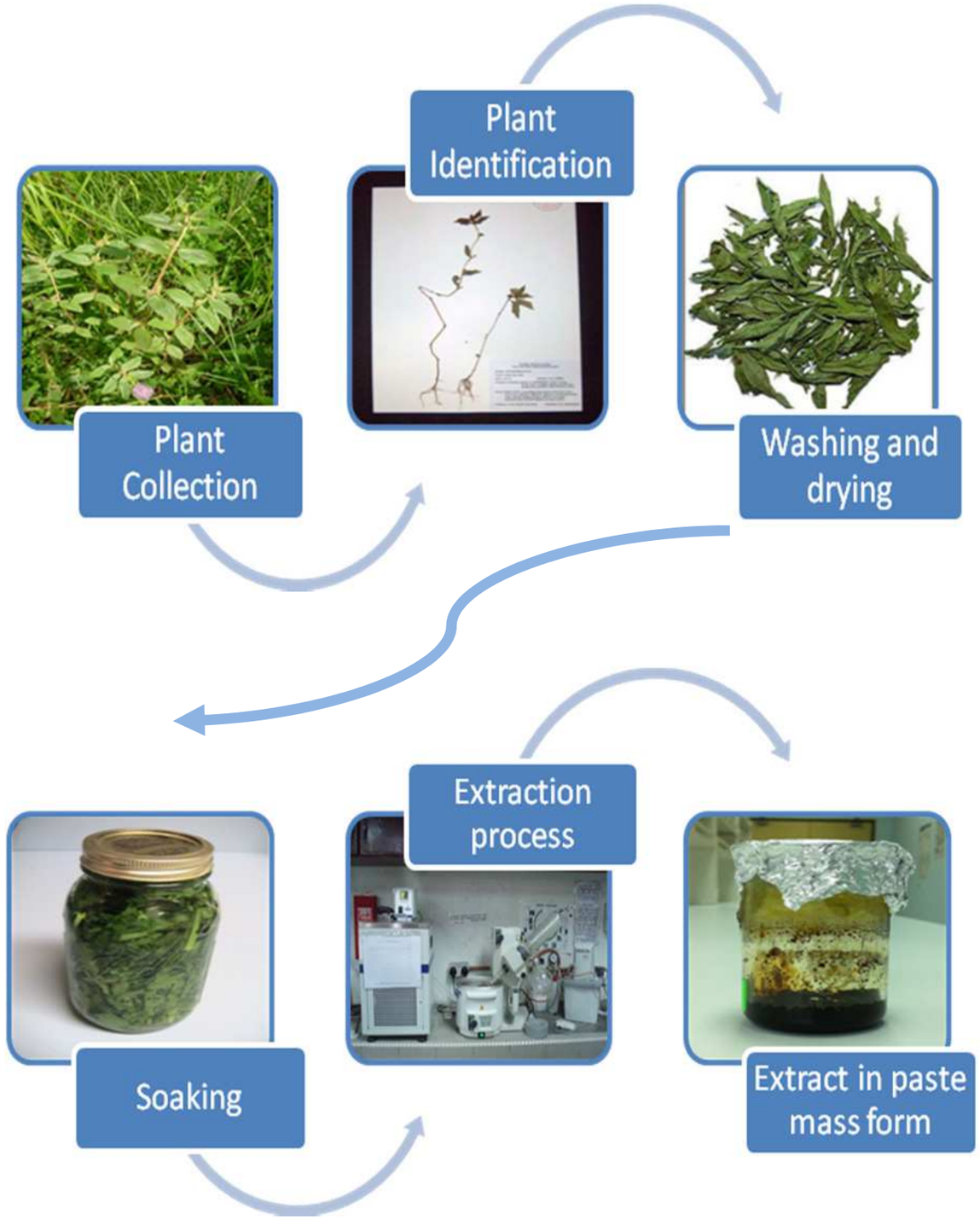

Fig. 3. An example of maceration method using *Euphorbia hirta* sample where organicsolvent extraction was performed by soaking 100g of powdered dried plant material in methanol (1.0 L) through occasional shaking and stirring for 7 days. The whole extract was then filtered and the solvent was evaporated to dryness *in vacuo* using a rotary evaporator at 40-50^{o}C to afford a paste mass.

	Soxhlet extraction	Sonification	Maceration	Supercritical Fluid extraction (SFE)	Microwave assisted extraction (MAE)	Pressurized Liquid Extraction, (PLE)
Common solvents used	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Carbon dioxide or carbon dioxide with modifiers, such as methanol	Methanol, ethanol, or mixture of alcohol and water	Methanol
Temperature (°C)	Depending on solvent used	Can be heated	Room temperature	40–100	80–150	80–200
Pressure applied	Not applicable	Not applicable	Not applicable	250–450 atm	Depending on if it is closed or opened vessel extraction	10–20 bar
Time required	3–18 hr	1 hr	3-4 days	30–100 min	10–40 min	20–40 min
Volume of solvent required (ml)	150–200	50–100	Depending on the sample size	Not applicable	20–50	20–30
References	Zygmunt & Namiesnik, 2003; Huie, 2002	Zygmunt & Namiesnik, 2003; Huie, 2002	Phrompittayarat et al.,2007; Sasidharan et al.,2008a; Cunha et al., 2004; Woisky et al., 1998	Zygmunt & Namiesnik, 2003; Huie, 2002; Luque de Castro et al., 2000; Liu & Wai, 2001	Zygmunt & Namiesnik, 2003; Huie, 2002; Camel, 2000; Pan et al., 2001; Pan et al., 2002 Fang et al., 2000	Ong et al., 2000; Ong & Apandi, 2001; Lee et al., 2002; Ong, 2002; Ong & Len, 2003a; 2003b; Choi et al., 2003;

Table 1. A brief summary of the experimental conditions for various methods of extraction for plants material

As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. The choice of solvent depends on several factors including the characteristics of the constituents being extracted, cost and environmental issues. SFE has been used for many years for the extraction of volatile components on an industrial scale. An important advantage of applying SFE to the extraction of active compounds from medicinal plants is that degradation as a result of lengthy exposure to elevated temperatures and atmospheric oxygen are avoided. Using MAE, the microwave energy is used for solution heating and results in significant reduction of extraction time (usually in less than 30 min) compared with conventional liquid–solid extraction methods in which a relatively long extraction time (typically 3–48 h) is required. Another advantage of MAE is that it enables a significant reduction in the consumption of organic solvents, typically less than 40 mL, compared with the 100–500 mL required in Soxhlet extraction (Huie, 2002).

3. *In vitro* antifungal testing

3.1 Microbial strain

Potato dextrose agar (PDA) medium normally used by the scientist to maintained fungal isolates and consist of extract of boiled potatoes, 200 mL; dextrose, 20 g; agar, 20 g; deionized water, 800 mL at 28°C. Spore suspensions can be prepared and diluted in sterile potato dextrose broth (PDB) to a concentration of 10^7 spores per mL. The spore population needed can be counted using a haemocytometer. Subsequent dilutions can be made from the aforementioned suspension to adjust to the required concentration, which can be used in the antifungal test.

3.2 Screening for the antifungal effect

Screening for antifungal effect can be carried out by using the disc diffusion method. The plate containing 25 mL of PDA medium will be seeded with 1 mL of fungal conidial spore suspension containing 10^5 spores per mL from a 120-h-old culture. Three Whatman filter paper No. 1 discs of 6-mm diameter can be used to screen the antifungal activity. Each sterile disk will be impregnated with 20 mL of the extract corresponding with 100 mg/mL of crude extract, myconazole 30 μg/mL, as positive control, or vehicle as negative control. The plates will be refrigerated for 2 h to allow the compounds presents in the extract diffused and then will be incubated at 28°C for 5 days. Diameter of the inhibition zone will be measured, and the mean of the three replicates are taken (Bauer et al., 1966). The disc diffusion method is a qualitative test which could provide the information whether the crude extract possessed antifungal properties

3.3 Determination of the minimal inhibitory concentration

The minimal inhibitory concentration (MIC) can be determined as the lowest concentration at which no growth occurs and is determined as follows: PDB medium will be prepared and sterilized in universal bottles, each containing 10 mL medium. Different amounts of the tested extract will be added to the broth medium to give the following concentration: 0.3125 to 100 mg/mL. To each flask 0.5 mL of Tween-80 will be added as emulsifying agent. The flasks will be inoculated with 0.5 mL fungal conidial spore suspension containing 10^5 spores per mL from a 120-h-old culture and will be incubated at 28°C for 5 days. The MIC value is determined as the lowest concentration of plant extract in the broth medium that inhibited visible growth of the test fungal strains. Each assay should be carried out in triplicate. The MIC test will be quantified the antifungal activity of plant extract.

3.4 Determination of minimum fungicidal concentration

The hyphal growth inhibition test can be used to determine the antifungal activity of the plant extract against fungal strains as previously described Picman et al. (1990). Briefly, dilutions of the test solutions dissolved in vehicle will be added to sterile melted PDA at 45°C to give final concentrations of 100, 10, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 mg/mL of plants extracts. The resultant solution will be thoroughly mixed and approximately 15 mL will be poured onto the petri plate. Plugs of 1 mm of fungal mycelium cut from the edge of actively growing colonies will be inoculated in the center of the agar plate and then incubated in a humid chamber at 25°C. Control cultures will be received an equivalent amount of vehicle. Three replicates will be used for each concentration. Radial growth is measured when the control colonies almost

reached 1.5 cm. Results will be expressed as the percentage of hyphal growth inhibited (Gamliel et al., 1989). Concentration response curves will be prepared in which the percentage of hyphal growth inhibition is plotted against concentration mg/mL. The concentration required to give 50% inhibition of hyphal growth IC_{50} will be calculated from the regression equation. Miconazole can be used as a positive control.

4. *In situ* antifungal activity

Electron microscopy (EM) is one of the many methods available for visual inspection of fungal strains. The effects of potential antifungal extracts from natural sources can also be evaluated by using the EM methods. Hence in this section the microscopical techniques such as Scanning (SEM) and Transmission (TEM) electron microscopy on the *in situ* antifungal activity by plant extract will be discussed.

4.1 Scanning electron microscopy

After treatment with plant extract, scanning electron microscopy SEM observation will be carried out on fungal strains. First of all, the plate containing 25 mL PDA medium will be seeded with 1 mL of the fungal conidial spore suspension containing 10^5 spores per mL from a 120-h-old culture. The extract 1mL, at the concentration of IC_{50} (obtained from the hyphal growth inhibition test), is then dropped onto the inoculated agar and will be further incubated for another 7 days at 28°C. A vehicle-treated culture can be used as a control. Five to ten mm segments will be cut from cultures growing on potato dextrose plates at various time intervals 1, 2, 3, 4, 5, 6, and 7 days for SEM examination (Sasidharan et al., 2008b). The specimen then placed on double-stick adhesive tabs on a planchette and the planchette placed in a petri plate. In a fume hood, a vial cap containing 2% osmium tetroxide in water will be placed in an unoccupied quadrant of the plate. After being covered, the plate will be sealed with parafilm, and vapor fixation of the sample proceeded for 1 h. Once the sample is vapor fixed, the planchette will be plunged into slushy nitrogen -210°C and then transferred on to the "peltier-cooled" stage of the freeze dryer, and freeze drying of the specimen will be proceeded for 10 h. Finally, the freeze dried specimen will be sputter coated with 5–10 nm gold before viewing in the SEM. The SEM is advantageous over several other microscopy methods as it is three-dimensional and almost the whole of the specimen is sharply focused. Furthermore, besides having a combination of higher magnification, larger depth of focus and greater resolution, the preparation of samples is also relatively easier, compared to the TEM method (Sasidharan et al., 2010). From the SEM micrograph (Fig. 4) we can observe the changes caused by the plant extract on fungal surface.

4.2 Transmission electron microscopy (TEM)

Further confirmation of SEM finding can be obtained from TEM study. To study the antifungal activity through TEM method the hyphal specimens (1×3 mm^2, with approximately 1 mm thickness of underlying agar blocks) of test fungal strains will be excised from the margin of actively growing SDA culture treated with plant extract using a sterilized razor blade. The specimens are then fixed with modified Karnovsky's fixative (Karnivsky, 1965) consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer solution (pH 7.2) at 4°C overnight. Subsequently, the fixed specimens are washed with the solution three times for 10 min each. The specimens were then will be post-fixed in the solution with 1% (w/v) osmium tetroxide at 4°C for 2 h and then will be washed briefly with

distilled water twice each. The postfixed specimens will be *en bloc* stained with 0.5% (w/v) uranyl acetate at 4°C overnight and then will be dehydrated once in a graded ethanol series of 30, 50, 70, 80, and 95% and three times in 100% ethanol for 10 min each. The specimens will be further treated with propylene oxide twice for 30 min each as a transitional fluid and then will be embedded in Spurr's resin. Ultra-thin sections (approximately 50 nm in thickness) will be cut with a diamond/ glass knife using an ultra-microtome. The sections will be mounted on copper grids and will be stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) for 7 min each. Finally the sections will be observed with a transmission electron microscope. From the TEM micrograph we can observe the changes caused by the plant extract on fungal cytoplasm (Fig. 5).

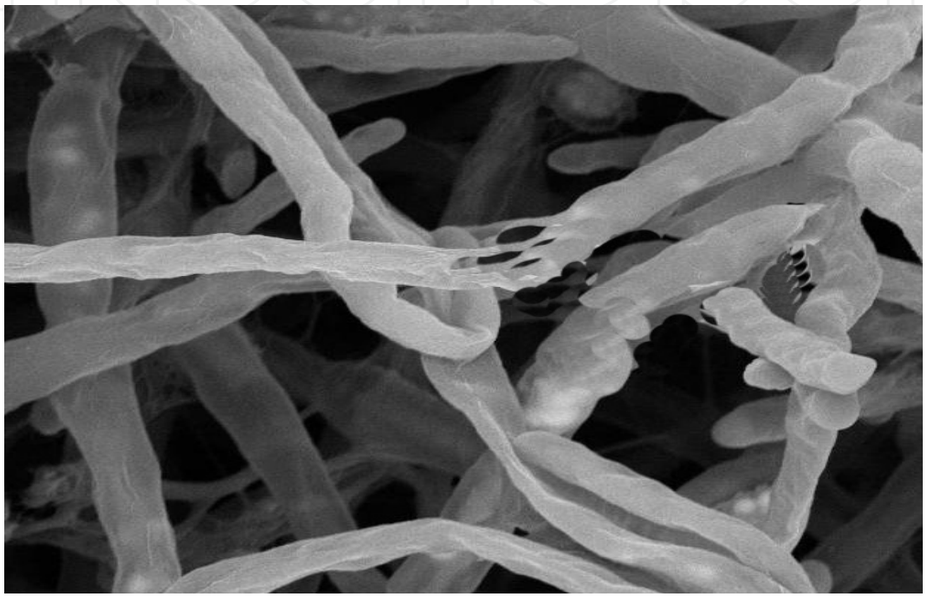

Fig. 4. SEM micrographs of *Aspergillus niger*

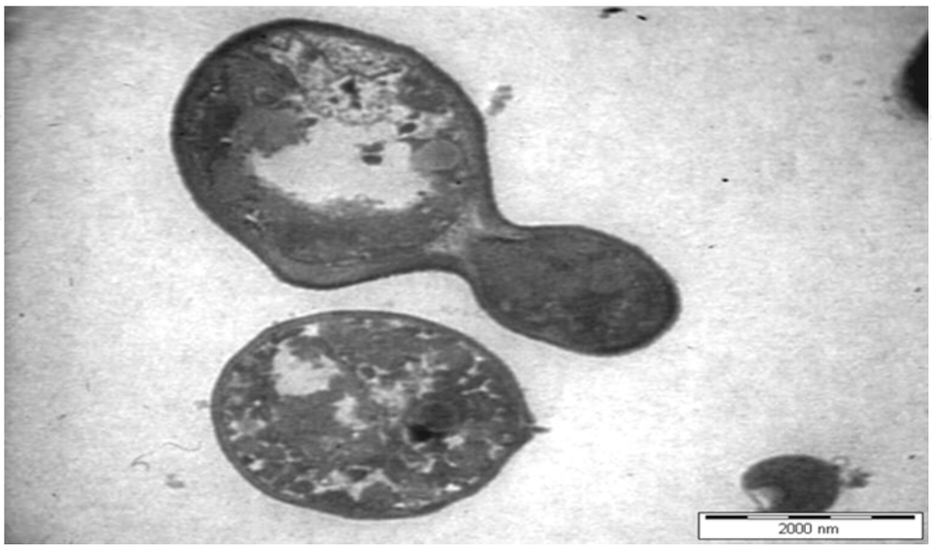

Fig. 5. TEM micrographs of *Candida albicans*

4.3 Confocal laser scanning microscopy (CLSM)

Further verification of SEM and TEM finding can be obtained from CLSM study. To study the antifungal activity through CLSM method the plant extract with MIC concentration will be prepared. 48 h fungal culture will be developed by culturing the fungal strains on SDA agar for 48 h. Controls without the plant extract or antimicrobials also will be included as control groups. The 48 h fungal culture will be gently transferred into a 12-well microtitre plate and rinsed with PBS for 15 s. The discs will be then immersed in 1 ml of the plant extract or antimicrobial agents and incubated at 37^{o}C in an aerobic incubator for 24 h. Subsequently, the extract or antimicrobial will be removed and the viability of the fungal cells will be assessed by Molecular Probes LIVE/DEAD BacLight Bacterial viability kit which comprise SYTO-9 and propidium iodide (PI) (Molecular Probes, Eugene, OR). After incubation with the dyes, the polymethylmethacrylate discs with biofilms will be placed on glass slides and live/dead ratio of cells will be quantified using the CSLM system (Thein et al., 2007). CLSM has become a precious tool for a wide range of studies in the biological and medical sciences for imaging thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometers.

5. Conclusion

The above mentions methods demonstrated the great potential in the development of antifungal testing to study the fungicidal properties of medicinal plants to develop fungicide. The main advantages of the presented methods are the following: easy; rapid; cheap and accurate. Our discussion demonstrates that the use electron microscopy is vital to reveal the cell injury caused by plants extract on fungal strains. The cell changes occurring in surface and cytoplasm of fungal cells following exposure to the plant extract could be visible using a combination of SEM and TEM studies.

6. Acknowledgment

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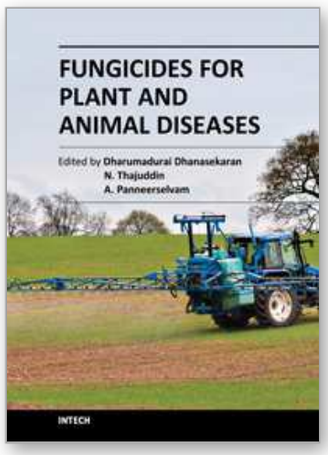

Fungicides for Plant and Animal Diseases

Edited by Dr. Dharumadurai Dhanasekaran

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A fungicide is a chemical pesticide compound that kills or inhibits the growth of fungi. In agriculture, fungicide is used to control fungi that threaten to destroy or compromise crops. Fungicides for Plant and Animal Diseases is a book that has been written to present the most significant advances in disciplines related to fungicides. This book comprises of 14 chapters considering the application of fungicides in the control and management of fungal diseases, which will be very helpful to the undergraduate and postgraduate students, researchers, teachers of microbiology, biotechnology, agriculture and horticulture.

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