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Abstract: Background: Due to the ever increasing burden of multidrug resistance (MDR) in human fungi, phyotherapeutic research has gained prominence nowadays. Traditional medicines derived from natural products constitute an important source of new drugs due to their low side effects and minimal drug resistance issues.

Objective: The aim of the present review is to examine the various in vitro assays to determine antifungal activities of natural products against fungal pathogens.

Results: The various antifungal susceptibility testing methods are described for natural compounds that are used for novel drug discovery. A number of biological assays ranging from the classical disk diffusion and broth dilution assay format to dye-based and fluorescent/luminescence reporter assays are in current use.

Conclusion: The importance of design and implementation of antifungal susceptibility testing methods is necessary to evaluate and exploit the diversity of natural products.

Keywords: Traditional medicine, antifungal susceptibility, MIC, agar dilution, disk diffusion, reporter assay.

1. INTRODUCTION

Fungal infections are increasing nowadays and causing life-threatening issues [1]. In spite of the significant advances being made in the improvement of antifungal drugs, only a limited number of antifungal drugs are currently available [2]. Moreover, recent emergence of multidrug resistance (MDR) to the currently available antibiotics has led scientists to revert to traditional medicines as an alternative form of health care [3]. Natural compounds have recently become of great interest due to their versatile applications. Natural compounds have been used for centuries as remedies for human diseases and offer a new source of biologically active secondary metabolites as antimicrobial agents. Medicinal plants are the richest bio-resources of drugs for traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemicals entitled for synthetic drugs [4]. India is one of the leading countries in Asia in terms of the wealth of traditional knowledge systems related to herbal medicine and employs a large number of plant species which includes Ayurveda, Siddha, Unani and Tibetan [5].

This article revisits the methods being employed earlier and recently in relation to investigations of the antifungal efficacy of medicinal plant extracts and their lead compounds [6]. Evaluation of the antimicrobial agent or formulation of plant origin begins with systematic biological evaluation of plant extracts to ensure efficacy and safety. Identification of active principles of extract, dosage formulations, efficacy and pharmacokinetic profile of the new drug are important points of the drug discovery process. Plants synthesize aromatic secondary metabolites, most of which are phenols or their oxygen substituted derivatives. Important subclasses in this group of compounds include phenols, phenolic acids, quinones, tannins, flavones, flavonols, flavonoids and coumarins. These compounds show evident antifungal effect and serves as plant defence mechanisms against pathogens [7-10].

2. METHODS FOR EVALUATION OF EFFICACY OF THE FORMULATIONS

2.1. Broth Microdilution

The dilution assays are reliable and widely accepted methods for determining an organism’s susceptibility
to inhibitors. Current dilution assays for testing antifungal drugs to yeasts have been standardized by Clinical and Laboratory Standards Institute (CLSI). CLSI method has greatly improved the reproducibility of antifungal susceptibility testing of Candida and Cryptococcus and serves as the "gold standard" for determining Minimal Inhibitory Concentration (MIC) [11, 12]. MIC is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation. Using broth micro dilution assay for naturally occurring pure compounds as well as extracts from plants, it has been noted that inhibitory activity of many such compounds against pathogenic fungus is widespread in nature. Many of these tested compounds include sesamol obtained from sesame oil, perillyl alcohol present in essential oil of lavender and cherries, citronellal from the essential oil of Cymbopogon plants that are active against Candida albicans [13-15]. Sharma et al. utilised the micro dilution assay to test the antifungal activity of extracts of Grewia asiatica L against C. albicans and A. fumigatus. Furthermore, hydroalcoholic extracts of G. asiatica however showed better inhibitory activity (31.5 μg/ml) in both organisms. In Ayurveda, ancient Indian medicinal system indicated the use of different plant parts of Grewia to cure inflammation, fever, burning sensation, wound healing, blood disorders, diabetes, ulcerative colitis and heavy menstrual flow [16]. In another study, Zhang et al. investigated the antifungal activity of a hydroalcoholic extract from Flos Rosae Chinensis combined with fluconazole against different fungi. They employed checkerboard assay, a common broth microdilution assay, which is suitable for dual antibiotic therapy [17, 18]. They showed the synergistic activity of Flos Rosae Chinensis in combination with Fluconazole (FCZ) against A. fumigatus, Microsporum and Trichophyton rubrum by calculating the fractional inhibitory concentration index. This method can also be used for a wide variety of microorganisms, it is not expensive and it presents reproducible results.

2.2. Agar Dilution

In this method a stock solution of the extract or natural compound is prepared in appropriate solvent and then incorporated in molten agar cooled to 50°C sometimes in a water bath. Various authors used different nutrient media depends upon the organisms to be tested. Inoculum preparation also differs between authors and others have used overnight culture dilutions of 1:100 [19] or 1:10 [20] in broth. However, European committee for antimicrobial Susceptibility (EUCAST) recommends an inoculum density of about 1-2 x 10^5 cfu/ml. The MIC is defined as the lowest concentration of the extract inhibiting the visible growth of microorganism on the agar plate. Agar dilution method has been used for the evaluation of Indian medicinal plants such as Azadirachta indica, Acacia nilotica, Curcuma longa and Withania somnifera against Aspergillus flavus, Ziziphus mauritiana [21]. The rhizome extract of Curcuma longa showed significant activity against Fusarium verticillioides. Many scientists used this method to evaluate the antifungal potential of Indian traditional plants against various fungal pathogens. Argyreia involucrata an Indian medicinal plant (Argyreia involucrata) belongs to the family Convolvulaceae which is commonly called as "morning glory and is active against human fungal pathogen C. albicans [22]. In another study, anti dermatophytic properties of Indian medicinal plants Cassia occidentalis, Lawsonia inermis, Cassia tora, Caesalpinia bonducella and Xanthium strumarium against various dermatophytic fungi has been documented [23]. Ethyl acetate leaf extract of C. bonducella was found to be most active among the all, which inhibited all test fungi with time and dose dependent activity. This plant extract retarded the growth of all the organisms at 10000 μg/ml up to 30 days.

2.3. Disk Diffusion

Disk method comprises the placing of filter paper disks containing test compounds on agar plate surfaces previously inoculated with the test organism. The test molecules or plant extracts then diffuse into the agar and inhibit growth of the test microorganisms. After incubation, mean diameters of growth inhibition zones are recorded. The diameter of the zone is propotional to the drug susceptibility of the pathogen [24]. The zone diameters of each drug are interpreted using the criteria published by the CLSI or those included in the US Food and Drug Administration (FDA) approved product inserts for the disks [25]. Advantage of this method includes simplicity as it does not require any special equipment and the results can be easily interpreted by clinicians. Many of the scientists used the disk diffusion method to check the antifungal activities of various plant formulations. More & Kharat 2016 reported the antifungal activities of root or stem extracts of Argeomone mexicana L. (Papaveraceae), commonly known as prickly poppy plant against some fungal pathogens [26]. It was observed that both cold aqueous and methanolic extracts of A. mexicana stem and leaves inhibited the growth of A. flavus, Mucor indicus, Aspergillus niger and Penicillium notatum. Mushrooms have been used in traditional medicines in many parts of the world. Mushrooms are one of the major sources of natural products exhibiting bioactivities including antimicrobial and antiviral activities [27]. The determination of antifungal activity of mushrooms extracts against C. albicans was investigated by disc method. Extracts of the shiitake mushroom showed efficacy towards C. albicans (inhibition zone diameter 14.5 for ethanol extracts and 19.0 for chloroform extracts). Many authors have reported promising
Many authors have reported promising antifungal activity of various traditional Chinese medicines. Zhang et al. used the disk diffusion methods to evaluate synergistic effect of Chinese herbal plant Flos Rosae Chinensis against C. albicans. Flos Rosae Chinensis has been used in the Chinese Pharmacopoeia for the treatment of menstrual disorders. On the agar plate the FCZ and Flos Rosae Chinensis combination yielded significantly clearer and larger zones than the zones of either drug alone on the plain agar plate indicating their synergistic activity [18]. In another study, Yan et al. evaluated the antifungal activities of genitopicrin, pseudolaric acid B using disk diffusion method against non-albicans Candida spp. It was found that pseudolaric acid B had the most potent antifungal effect and showed similar antifungal activity to all Candida spp. More interestingly, a synergistic effect of pseudolaric acid B in combination with FCZ was observed with C. albicans [28].

2.4. Poison Food Technique

Poisoned food method is mostly used to evaluate the antifungal effect against moulds. In this method, the antifungal agent is incorporated into the molten agar at a desired final concentration and mixed well. Then, the medium is poured into Petri dishes. After overnight preincubation, that can be done by a mycelia disc ranging from 2 to 5 mm, it is deposited in the centre of the plate. After further incubation under suitable conditions for the fungal strain tested, the diameters of fungal growth in control and sample plates are measured, and the antifungal effect is estimated by the following formula:

\[
\text{Antifungal Activity (\%) = ((Dc - Ds) / Dc) \times 100}
\]

Where Dc is the diameter of growth in control plate and Ds is the diameter of growth in the plate containing tested antifungal agent. Kumar & Tyagi studied the antifungal effects of the herb Bergenia stracheyi which belongs to genus Bergenia and family Saxifragaceae against six plant fungal species viz. A. niger, Alternaria alternata, Colletotrichum gloeosporioides, Fusarium oxysporum, Rhizoctonia solani and Ganoderma lucidum. Screening with poisoned food technique showed that different extracts exhibit different extent of antifungal activity against all test fungi [29]. Poisoned food technique was used to evaluate the antifungal activity of Cymbopogon citratus leaves against A. flavus and Mucor Sp. [30]. Similarly, Li et al. successfully employed the poisoned food technique to evaluate the antifungal effect of garlic oil, an extract of garlic against C. albicans [31]. They treated C. albicans with different concentrations (0.04, 0.09, and 0.17, 0.35 μg/ml and beyond) of garlic oil. No colonies were identified on the 0.35 μg/mL and beyond garlic oil-treated Petri dishes during the seven-day incubation. Hence, the MIC of garlic oil against C. albicans was determined to be 0.35μg/mL.

2.5. Spore Germination Assay

In addition to the above mentioned assays, antifungal activity of plant extracts can be evaluated by spore germination assay using the slide technique [32]. Plant extract of desired concentration and volume are added to the surface of dried slides as a film or in a cavity of a slide. Fixed volume and standard concentration of spore suspension of test fungi are spread over the film whereas in controlled treatment, distilled water is added in place of spore suspension. Slides are then placed on a glass rod in Petri dish under moistened conditions and incubated for 24h. After incubation, slides are fixed in lacto phenol cotton blue and observed microscopically for spore germination. Percentage spore germination is calculated according to the following formula.

\[
\% \text{ Spore Germination} = \frac{\text{Germinated spores (No.)}}{\text{Total Spores (No.)}} \times 100
\]

Spore germination assay has been used to study the antifungal effects of natural products against various plant, animal, and food borne pathogens. Several studies were reported on the effectiveness of spore germination assay as a tool for antifungal testing of natural products using combined with micro dilution method [33]. Similarly, gymnemic acids (GAs) purified from Gymnema sylvestre leaves, the Ayurvedic traditional medicinal plant inhibited yeast-to-hypha conversion in C. albicans. Moreover, GAs inhibited the formation of invasive hyphae from C. albicans-infected Caenorhabditis elegans worms and rescued them from killing by C. albicans. They also inhibited the conidial germination and hyphal growth of the filamentous fungal pathogen A. fumigatus. Thus, GAs could be useful for various antifungal applications due to their traditional use in herbal medicine [34].

2.6. Time Kill Assay

The time-kill is a labour intensive assay used to determine the relationship between the concentrations of the drug with its antifungal activity. This is a time consuming method; however, it is very useful for deriving real time exposure data. Time-kill assay allows for the presentation of a direct relationship in exposure of the pathogen to the antifungal and allows for the continuous monitoring of a cidal effect over time. The selected pathogen is exposed to the antifungal agent at selected time intervals and aliquots are then sampled and serially diluted. These dilutions are then plated out onto agar and incubated at the required incubation conditions for the pathogen. After incubation, the colony forming units (CFU) are counted. These results are interpreted from a logarithmic plot for the amount of re-
main ing viable cells against time. Time-kill test is the most appropriate method for determining the fungicidal effect of natural products. It is a strong tool for obtaining information about the dynamic interaction between the antimicrobial agent and the microbial strain. The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect [35, 36]. In addition, time-kill assay is also employed to study synergism or antagonism between drugs in combinations. Using this method synergism can be studied between two or more natural products or between natural product and conventional drugs. For example, it is reported that the synergistic properties of extracts of traditional plants (Terminalia catappa, Terminalia mantaly and Monodora tenuifolia) in Cameroon against C. albicans, Candida glabrata, Candida parapsilosis and Cryptococcus neoformans [37]. The results indicated that all extracts tested alone were fungistic. On the other hand, the combinations exhibited fungicidal activity cases against the tested yeasts. In another study, syner-
gistic activity of Glabridin (Gla) isolated from Glycy-
rrhiza glabra in combination with FCZ against FCZ-
resistant clinical isolates of C. albicans, Candida tropi-
calis and Cneformans were observed. Gla alone showed fungicidal effect at16 µg/ml and led to a de-
crease of 3.57–log10 CFU/ml at 24 h. No appreciable antifungal activity of FCZ alone at 8 µg/ml was ob-
berved, but the combination of FCZ (8 µg/ml) and Gla
(4, 8 or 16 µg/ml) yielded 3.14, 3.62 or 4.10–log10
CFU/ml reductions compared with Gla alone at. Be-
sides, the combination of Gla at 16 µg/ml and FCZ at 8
µg/ml almost resulted in a complete cell-killing at 24 h
suggesting its synergetic activity [38].

2.7. FACS/Fluorimetry

Since, last few years many authors investigated the antifungal activities of many drugs using Flow cytome-
try. The rapid detection of damaged cells by this ap-
proach depends on the use of appropriate dyes staining
[39, 40]. For example, propidium iodide (PI), a fluores-
cent and intercalating agent, is widely used as DNA
stain. Several other dyes such as syto9, fluorescein di-
acetate etc have been used to test antifungal activities
using this method [41, 42]. Several studies were re-
ported on the effectiveness of flow cytometer as a tool
for antifungal testing of essential oils against A. flavus,
using staining with PI for membrane damage evalua-
tion [43]. Flow cytometry also used to test cell size/
granularity which are indicative of cell death when ex-
posed to the natural products [44-46]. In addition to the
lysed cells, subpopulations such as dead, viable and
injured cells can be clearly discriminated. The injured
cells are described as stressed cells displaying cellular
components damage and subsequent impairment of
reproductive growth. Flow cytometry also used to
study the mode of actions of various natural products
such as DNA damage, reactive oxygen species genera-
tion, apoptosis, cell surface properties, mitochondrial
dysfunction etc. It has been reported that, curcumin a
polyphenolic compound has been used traditionally in
Asia for medicinal, culinary, and other purposes show
membrane disruptive function against C. albicans.
These authors studied potassium ion leakage from the
fungal cytosol and dissipation in membrane potential
was detected by bis-(1, 3-dibutylbarbituric acid) trim-
ethine oxonol [DiBAC4(3)] using flow cytometry [47].
Flow cytometric analysis showed that treatment with
berberine, a natural herb, leads to alterations in the in-
tegrity of the plasma and mitochondrial membranes
and DNA damage, which led to cell death, probably by
apoptosis [48]. Indeed, flow cytometric method allows
the detection of antimicrobial properties and estimates
the impact of the tested molecule on the viability and
cell damage of the tested microorganism in a rapid and
reproducible manner. Despite of wide spread use this
methodology for antimicrobial susceptibility testing
currently appears unlikely due to the inaccessibility of
this equipment in various laboratories.

2.8. ATP Bioluminescence

ATP bioluminescence assay is based on the capacity
to measure adenosine triphosphate (ATP) produced by
bacteria or fungi. As ATP is the chemical form of en-
ergy of all living cells, it is present in more or less a
constant amount in a cell. Therefore, its quantification
is used to estimate the microbial population in a sam-
ple. Mitochondrial ATP production is the major energy
source for intracellular metabolic pathways. If there is
a dysregulation in ATP generation, it affects the me-
tabolic activities. The ATP content assay has been used
to determine compound cytotoxicity in mammalian
cells and more recently on Thielavia subthermophilafungal growth [49-51]. This assay is a homogenous
assay with ‘one step’ reagent addition in which no cell
wash step is required. A luminescence signal is gener-
ated upon the release of cellular ATP that is detectable
following a10 minute incubation. A unique feature of
the ATP content assay is that the signal-to-basal ratio is
characteristically much higher in comparison to the
AlamarBlue assay, partly due to low innate lumines-
cence background generated by cells, reagents or
plates. Hence the intracellular ATP level decides
whether a cell dies by apoptotic pathway or necrotic
pathway. Sun et al. [52] used ATP content assay 1536-
well plate format to measure the sensitivities of thou-
sands of known drugs against Exserohilum rostratum.
They demonstrated that it has advantages over the con-
ventional antibiotic susceptibility testing which re-
quires lot of time and accuracy. Similarly, Haque et al.
[53] measured the ATP level in C. albicans cells for
the integrity of mitochondria and cytotoxic effect after
treatment with terpenoid such as Lupeol. It is suggested
that the elevated cytosolic ATP level is essential for apoptotic cell death process hence increased cytosolic ATP level promotes cell death [54]. Similarly, depletion of ATP in cells triggers necrotic cell death. So, decrease in ATP content after Lupeol treatment causing cell death may be by inducing necrosis. Tetraterpenoid does not alter the level of ATP which indicates that it may cause cell death by following alternate mechanisms rather than programmed cell death.

CONCLUSION

The present article attempts to highlight the most common methods used to study antifungal efficacy of natural compounds including traditional methods. So, it would be advantageous to further standardize methods of extraction and in vitro antifungal efficacy testing so that the search for new biologically active plant products could be more systematic and interpretation of results would be facilitated.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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