Chapter 19
Antifungal Activity of Medicinal Plant Extracts and Phytocompounds: A Review

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Abstract The epidemiological data suggest that the incidence and prevalence of serious mycoses continues to be a public health problem. The increased use of antifungal agents has resulted in the development of resistance to these drugs. The spread of multidrug-resistant strains of fungus and the reduced number of drugs available make it necessary to discover new classes of antifungals from natural products including medicinal plants. Historically, herbs and spices have enjoyed a rich tradition of use for their medicinal properties and provide unlimited opportunities for new drug leads because of the huge chemical diversity. Assays of bioactive compounds have been reported with good antifungal properties *in vitro* or *in vivo*. It is almost impossible to discuss the various characteristics of these plants such as mode of action and extraction of active compounds in a single review. Therefore, we have focussed here mainly on the antifungal plant extracts, their use against pathogeinc and drug resistant fungi. The various classes of compounds such as phenolics, terpenoids, saponins, and alkaloids, etc., are discussed in detail. The new emerging classes of antifungal proteins and peptides are also reviewed briefly. In this chapter, we also describe the technical aspects related to the methodology for screening and identification of antifungal compounds. The technical aspects regarding the use of reliable methodology of extraction, screening, bioautography, and identification of pure compounds from crude extracts and fractions are also discussed here.

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19.1 Introduction

Infectious diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide (World Health Organization 1998). Despite the significant progress in human medicine, infectious diseases caused by microorganisms such as fungi are still a major threat to public health. The impact is even more in developing countries due to the unavailability of medicine and the emergence of widespread drug resistance (Okeke et al. 2005).

Infections induced by pathogenic fungi are increasingly recognized as an emerging threat to public health (Wu 1994; Walsh et al. 1996). The increase in occurrence of fungal infections during recent years is due to a growth in the immunocompromised population, such as organ transplant recipients and cancer and HIV patients (Portillo et al. 2001). Certain commensal fungi, such as *Candida* species, cause infections when their human hosts become immunocompromised (Cannon et al. 1995). These problems are also associated with resistance to antibiotics and toxicity during prolonged treatment with several antifungal drugs (Giordani et al. 2001).

There are many synthetic and natural product-based drugs available for treating fungal infections, but they are not consistently effective (Lazar and Wilner 1990; Gearhart 1994; Goa and Barradell 1995). Furthermore, the development of resistance in fungi against most of the drugs has now been reported for several years (Cuenca-Estrella et al. 2000). The use of amphotericin B, known as the "gold standard", is limited because of its infusion-related problems and nephrotoxicity (Grasela et al. 1990; Fanos and Cataldi 2000). In addition, the low efficacy, and side-effects and resistance associated with the existing drugs, highlight the advent of safe, novel, and effective antifungal drugs. Plants produce a great deal of secondary metabolites, many of them with antifungal activity. Similarly, traditional medicine has made use of many different plant extracts for treatment of fungal infection and many of these have been tested for *in vitro* antifungal activity. Based on the knowledge that plants develop their own defense against fungal pathogens (Gurgel et al. 2005), they appear as an interesting source for antifungal compounds.

Medicinal plants have also been reported in traditional systems of medicine for the treatment of both human and animal mycoses, and are considered to be a valuable source for the discovery of new antifungal drugs (Mathias-Mundy and McCorkle 1995). Many books have reported and recorded the use of medicinal plants in the traditional system of medicine. Gerard's *Herball*, first published in 1597, has so far yielded 16 currently prescribed drugs (Cox 1998). In a study, Fabricant and Farnsworth (2001) reported that 94 species of plants are utilized for the production of 122 single-agent natural products that are being used as single-agent drugs around the world. Thus, even with this very incomplete database of global ethnomedical information, there is abundant opportunity for the discovery of new medicinal agents.

According to an estimate by the World Health Organization (WHO), about 80% of the world's population in developing countries depend essentially on plants for

their primary health care. However, only a few plants have been scientifically studied for the assessment of their quality, safety, and efficacy. In addition to these, recent evidence from the pharmaceutical companies shows that, for some complex diseases, natural products still represent an extremely valuable source for the production of new chemical entities, since they represent privileged structures selected by evolutionary mechanisms over a period of millions of years (Newman et al. 2003; Clardy and Walsh 2004; Koehn and Carter 2005).

19.2 Screening of the Crude Extracts and Antifungal Activity

Many studies have been carried out to screen medicinal plants for their antifungal activity. Various groups all over the world initiated antifungal programs for plants used in traditional medicine or as antiinfectious agents. The most extensive screening was sponsored by the National Cancer Institute (NCI, USA). However, the main focus of this study was not antifungal screening. In this program, around 30,000 species were screened from 1960 to 1981 (Douros and Suffness 1980, 1881; Cragg et al. 1997, 1994). The Central Drug Research Institute (CDRI), Lucknow, India conducted the second largest study and evaluated more than 2,000 plant extracts for several biological activities including antifungal, antibacterial, antidiabetic, antifertility, antihypercholesteremic, anti-inflammatory, antitumor, cardiovascular, central nervous-system depressant, cytotoxicity, diuretic, and others (Bhakuni et al. 1969, 1971; Dhar et al. 1968, 1973, 1974; Dhawan et al. 1977, 1980). A large number of known and novel bioactive compounds were isolated from the plants (Rastogi and Dhawan 1982). After these programs, various workers conducted research in different parts of the world, in both academic and pharmaceutical institutions. Some of the screening studies are summarized here.

Seventy-eight plants were selected from Indian traditional medicines on the basis of their use in the treatment of infectious diseases. Only 10% of ethanol extracts showed the activity at 1.6 mg/ml; however, 44% were active when tested at concentration of 6.25 mg/ml (Valsaraj et al. 1997).

Mehmood et al. (1999) demonstrated the activity of aqueous and alcoholic extracts of 37 Indian medicinal plants against the pathogenic yeast, *Candida albicans*, and dermatophytes, *Epidermophyton floccosum*, *Microsporum gypseum*, and *Trichophyton rubrum*. In this study, all the plants showed activity; however, 21 plants showed stronger activity against *C. albicans* and at least one dermatophyte. When compared, only 16 aqueous extracts showed the activity. Further, they demonstrated that MIC values of the five most active extracts ranged between 4 and 9 mg/ml.

In another extensive study, Ahmad and Beg (2001) screened ethanolic extracts of 45 Indian medicinal plants used in the traditional system of Indian medicine for their antimicrobial activity against certain drug resistant bacteria and a yeast, *C. albicans*, of clinical origin. Of these, anticandidal activity was detected in 24 plant extracts. Qualitative phytochemical tests, thin-layer chromatography, and

TLC bioautography of certain active extracts demonstrated the presence of common phytocompounds including phenols, tannins, and flavonoids as major active constituents. In addition to this, no correlation was observed between susceptibility of test strains with plant extracts and antibiotic resistance behavior of the microbial strains (*C. albicans*).

Similarly, Aqil and Ahmad (2003) demonstrated the activity of ethanolic extracts of 22 traditionally used Indian medicinal plants for their antifungal activity against five filamentous fungi (*Aspergillus niger, Alternaria alternata*, *Fusarium chlamydosporum*, *Rhizoctonia bataticola*, and *Trichoderma viride*) and a yeast, *C. albicans*, of clinical origin. Seventeen and nine plant extracts showed antifungal and anticandidal activity respectively.

Eleven traditionally used Argentinian medicinal plants were assayed for antifungal activity against yeasts, hialohyphomycetes, as well as dermatophytes, using the broth microdilution method. Of the tested methanolic extracts, *Eupatorium buniifolium*, *Terminalia triflora*, *T. mentagrophytes*, and *T. rubrum* were the most susceptible species, with MICs ranging from 100 to 250 μg/ml. *Lithrea molleoides* showed the broadest spectrum of action inhibiting all the tested dermatophytes at MIC of 250 μg/ml (Muschietti et al. 2005).

In another study, Turchetti et al. (2005) screened leaf extracts of *Camellia sinensis*, *Cupressus sempervirens*, and *Pistacia lentiscus* and the seed extract of *Glycine soja* against yeast and yeast like species implicated in human mycoses. Of these extracts, only *C. sinensis* exhibited broad activity towards *C. glabrata, Clavispora lusitatiae, Cryptococcus laurentii, Filobasidiella neoformans, Issatchenkia orientalis, Saccharomyces cerevisiae*, and *Prototheca wickerhamii* strains. MICs ranging from 300 to 4,800 μg/ml of extract (corresponding to 130–2,010 μg/ml total polyphenols) were observed.

In the same year Lamidi et al. screened 77 crude extracts from leaves and stem barks of 15 Gabonese plants used in traditional medicine for their cytotoxic, antileishmanial, and antifungal activities. The methanolic extract of *Polyalthia suaveolens* displayed a strong antifungal activity on all the tested strains ($IC_{50} < 1$ mg/ml). This extract was divided into six fractions; however, the antifungal activity observed for the crude extract could not be recovered in the corresponding fractions (Lamidi et al. 2005).

A total of 65 crude methanol extracts belonging to 56 plant species of 38 families from Tanzania were screened, using the broth microdilution method, for antifungal susceptibility testing of yeasts. Among the tested plant species, 45% (25 species) showed antifungal activity against one or more test fungi. The most susceptible yeasts were *C. neoformans*, followed by *C. krusei*, *C. tropicalis*, and *C. parapsilosis*. The least susceptible were *C. albicans* and *C. glabrata*. Strong antifungal activity was exhibited by extracts of *Clausena anisata*, *Sclerocariya birrea*, *Turraea holstii*, *Sterculia africana*, *Acacia robusta* sub sp. Usambarensis, *Cyphosterma hildebrandti, Elaeodendron buchannanii*, *A. nilotica*, *Jatropha multifida*, and *Pteridium aquilinum* (Hamza et al. 2006).

Braga et al. (2007) screened 20 plants used in Brazilian traditional medicine for their antifungal activity against *C. albicans* and *C. neoformans*. Among the 20

tested methanolic extracts, *Schinus terebintifolius*, *O. gratissimum*, *Cajanus cajan*, and *Piper aduncum* extracts were the most active against *C. albicans* (MIC of 1.25 mg/ml) whereas *Bixa orellana*, *O. gratissimum*, and *Syzygium cumini* exhibited the best activity against *C. neoformans* (MIC of 0.078 mg/ml).

Masoko et al. (2007) screened hexane, dichloromethane, and methanol leaf extracts of the 24 South African *Combretum* species against five fungal animal pathogens *C. albicans*, *C. neoformans*, *A. fumigatus*, *M. canis*, and *Sporothrix schenckii*. They reported MIC values in the range of 20–60 μg/ml; substantially better values have been reported in the literature for crude extracts. Methanol extracted the highest quantity from leaves, but the acetone extracts had the highest antifungal activity in practically all cases. The methanolic extracts of *C. moggii* and *C. petrophilum* were, however, most active against all the pathogens. All extracts of *C. nelsonii* were also very effective against all the pathogens. They also report time-dependent antifungal activity, and the MIC values determined after 48 h were usually 2 times higher than values determined after 24 h.

Screening of the antifungal activity of plants from the north-east of Mexico against some of the main etiological agents inducing pulmonary mycoses, *C. albicans*, *A. fumigatus*, *Histoplasma capsulatum*, and *Coccidioides immitis*, was conducted *in vitro*. Ten hydroalcoholic extracts from the 15 plants evaluated showed antifungal activity against at least one of these fungi. Furthermore, a differential extraction was conducted with solvents of different polarities, and 16 extracts showed activity ranging from 16 to 125 μg/ml against the different fungi (Alanís-Garza et al. 2007).

Extracts from 50 plant parts obtained from 39 different plants belonging to 22 families used to treat infectious diseases in Bunda district, Tanzania, were tested against *A. niger* and *C. albicans*. Only *Balanites aegyptiaca* stem bark exhibited a high antifungal activity against *C. albicans* (MIC 125 μg/ml and MFC 250 μg/ml) (Maregesi et al. 2008).

In another study conducted in Mexico, 14 plants were tested for their antifungal activity. Of these, *Fragaria virginiana*, *Epilobium angustifolium*, and *Potentilla simplex* demonstrated strong antifungal potential overall. *Fragaria virginiana* had some degree of activity against all of the fungal pathogens. *Alnus viridis, Betula alleghaniensis*, and *Solidago gigantea* also demonstrated a significant degree of activity against many of the yeast isolates (Webster et al. 2008).

In a study from Krisch et al. 2009, fruit juices and pomace (skin, seeds) extracts from blackcurrant (*Ribes nigrum*), gooseberry (*R. uva-crispa*), and their hybrid plant (jostaberry, *Ribes* × *nidigrolaria*) were evaluated against the 12 most frequently isolated human pathogenic *Candida* species. Growth of most of the *Candida* species was inhibited, with the exception of *C. albicans, C. krusei, C. lusitaniae and C. pulcherrima. R. nigrum.*

Uma et al. 2009 have also shown the anticandidal activity of *Asparagus racemosus*. In their study, the *in vitro* anticandidal activity of *A. racemosus* roots and tubers extract was investigated against *C. albicans, C. tropicalis, C. krusei, C. guillermondii, C. parapsilosis*, and *C. stellatoida,* which are isolated from vaginal thrush patients. The extract of *A. racemosus* showed a high degree of

activity against all the *C*. strains. The inhibitory effect of the extract against all the Candida tested was found comparable with that of standard antibiotics used.

Furthermore, a study from Tayel and El-Tras (2009) revealed the anticandidal activity of pomegranate. In this study, anticandidal activity of pomegranate peel extracts (PPE) and application of PPE aerosol as sanitizer agent against *C*. *albicans* contamination were investigated. PPE exhibited potent anticandidal activity against *C*. *albicans* strains compared with standard fungicides in both susceptibility techniques used. Methanol, ethanol and water extracts were the most effective for inhibiting *C*. *albicans* growth. PPE aerosol was an efficient method for complete sanitizing of semi-closed places against *C*. *albicans* growth.

19.3 Classes of Compounds and Their Antifungal Activity

Several papers and reviews have been published on the occurrence of antifungal compounds in relation to their role in plant resistance (Ingham 1973). However, literature and systematic reviews on the natural products as an alternative to antifungal drugs are still scanty. The distribution of antifungal compounds can be defined either on the basis of their taxonomic distribution or on the basis of their chemical classes. Table 19.1 shows the antifungal natural products belonging to all major classes of secondary metabolites such as phenolics, alkaloids, terpenoids, saponins, flavonoids, proteins, and peptides, etc. The importance of these groups of compounds against pathogenic/nonpathogenic fungi is described below.

19.3.1 Terpenoids

Terpenoids (or isoprenoids), a subclass of the prenyllipids (terpenes, prenylquinones, and sterols), represent the oldest group of small molecular products synthesized by plants and are probably the most widespread group of natural products. It has been reported in the literature that aglycones of terpenoids are more stable and active as compared to the glycosides. For instance four nonglycosidic iridiods were discovered in *Aliertial macrophylla* and two of the 1α and 1β hydroxydihdrocornin aglycones showed fungitoxicity against a range of *Clostridium* and *Aspergillus* species (Young et al. 1992). Some representative structures of this group are shown in Fig. 19.1 (bold numbers 1 to 8 in brackets below refer to these).

Six antifungal sesquiterpenes [5- and 7-hydroxycalamenene, drimenol (**1**), drimenal, viridiflorol, gymnomitrol, and chloroisopiagiochin D] were isolated by Scher et al. (2004) from a dichloromethame and methanol extract of the liverwort *Bazzania trilobata* (L.). These compounds showed antifungal activity against various fungi including *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phythophthora infestans*, *Pyricularia oryzae*, and *Septoria tritici*. Similarly polygodial, a sesquiterpene isolated from *Polygonum punctatum* (Polygonaceae), was

Table 19.1 Antifungal activity of plant extracts and their active principle

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
1.	*Ajania fruticulosa* (Asteraceae)	Fruits	Xantholides	Seven different xantholides	*Candida albicans, C. glabrata, A. fumigetus*	Lavault et al. (2005)
2.	*Alibertia macrophylla* (Rubiaceae)	Leaves	Nonglycosidic iridoid	l z- and Ijl-Hydroxydihydrocornin aglycones	*Cladosporium sphaeros- permum; C. cladosporioides; A. niger; Colletotrichum qloeosporioides*	Young et al. (1992), Luciano et al. (2004)
3.	*Aniba panurensis* (Lauraceae)	Whole plant	Alkaloid	6,8-didec-(1Z)-enyl-5,7-dimethyl-2,3-dihydro-1Hindolizinium	*C. albicans*	Klausmeyer et al. (2004)
4.	*Aquilegia vulgaris* (Ranunculaceae)	Leaves and stems	Flavonoid	4′-methoxy-5,7-dihydroxyflavone 6-C-glucoside	*A. niger*	Bylka et al. (2004)
5.	*Avena sativa* (Poaceae)	Root	Triterpenoid saponin	Avenacins	*Geumannomyces graminis var. tritici*	De Bertoldi et al. (2009)
6.	*Bauhinia manea* (Leguminosae)	Wood	Chalcone	Isoliquiritigenin; isoliquiritigenin 2′-methyl ether; echinatin	*Borryris cinerea; Saprolegnia asterophora and three other fungi*	Achenbach et al. (1988)
			Flavan Diphenylpropene	(2S)-7,4′ -Dihydroxyfiavan; (2S)-3,4′ -dih ydroxy-7-methoxyfiavan; (2S)-7, 4′-Dihydroxy-3′ -methoxyflavan; Obtustyrene		
7.	*Blumea balsamifera* (Asteraceae)	Leaves	Flavonoid	Luteolin	*A. niger*, *Trichophyton mentagrophytes* and *C. albicans*	Ragasa et al. (2005)
8.	*Camellia japonica* (Theaceae)	Leaf	Saponin	Camellidins I and II	*Pesraloria longisera*	Nagata et al. (1985), Kim et al. (2001)
9.	*Camptotheca acuminata* (Cornaceae)	Leaves	Flavonoid	Trifolin and hyperoside	*Alternaria alternata*, *Epicoccum nigrum*, *Pestalotia guepinii*, *Drechslera* spp. and *Fusarium avenaceum.*	Li et al. (2005)
10.	*Cassia tora* (Leguminoceae)	Seeds	Anthraquinone	Emodin	*Botrytis cinerea, Erysiphe graminis, Phytophora infestans, Puccinia recondita, Pyricularia grisea and Rhizoctonia solani*	Kim et al. (2004)
11.	*Celastrus hypoleucus* (Celastraceae)	Root	Triterpenoid	Pristimerin, celastrol	*Glomerelia cinguiata: R. solani*	Luo et al. (2005)
12.	*Centaurea sulphurea* (Asteraceae)		Sesquiterpene lactone	Costunolide, dehydrocostunolide	*C. sulphurea*	Barrero et al. (2000)
13.	*Chamaecyparis pisifera* (Cupressaceae)	Leaves and twigs	Diterpene	Pisiferic acid	*P. oryzae*	Kobayashi et al. (1987), Xiao et al. (2001)
14.		Fruits				

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
	Chisocheton paniculatus (Meliaceae)		Meliacin-type nortriterpenoid	1,2-Dihydroxy-6Cl-aceroxyazadirone and three similar meliacins	*Curvularia verruciformis; Dreschlera oryzae; Alternaria solani*	Bordoloi et al. (1993), Phongmaykin et al. (2008), Yang et al. (2009)
15.	*Combretum apiculatum* (Combretaceae)	Heartwood	Phenanthrene Dihydrostilbene	4,7-Dihydroxy-2,3,6-trimethoxyphenanthrene; 2,7-dihydroxy-3,4,6-trimethoxydihydro-phenanthrene; 4,4′-dihydroxy-3,5-dimethoxydihydrostilbene	*Penicillium expansum*	Malan and Swinny (1993), McGaw et al. (2001), Bisoli et al. (2008)
16.	*Commiphora rostrata* (Burseraceae)	Bark of stem	Alkanone	2-decanone; 2-undecanone; 2-dodecanone	*Aspergillus; Penicillium sp.*	Mcdowell et al. (1988), Hanus et al. (2005)
17.	*Croton lacciferus* (Euphorbiaceae)	Root	Benzoquinone	2,6- Dimethoxy benzoq uinone	*C. cladosporioides*	RatnayakeBandara and Wimalasiri (1988), Mohamed et al. (2009)
18.	*Datura metel* (Solanaceae)	Whole plant	Alkaloid	2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate	*C. albicans, C. tropicalis, A. fumigatus, A. flavus* and *A. niger*	Dabur et al. (2005)
19.	*Detarium microcarpum* (Leguminaceae)	Pulp	Diterpene	Clerodane diterpene	*C. cucumerinum*	Cavin et al. (2006)
20.	*Dioscorea balatas* (Dioscoreaceae)	Tuber	Oxygenated bibenzyl and Phenanthrene	3-Hydroxy-5-methoxybibenzyl 3,2′-dihydroxy-5-methoxybibenzyl (batatasin IV) (4{}); 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I) (41); 6,7-dihydroxy-2,4-dimethoxy- phenanthrene; 2,7-dihydroxy-4,6-dimethoxy-phenanthrene	Showed activity against 24 fungi	Takasugi et al. (1987)
21.	*Dolichos kilimandscharicus* (Lehuminodae)	Roots	Triterpenoid saponin	3-0-glucosides of hederagenin, bayogenin and medicagenic aeid	*C. cucumerinum*	Marston et al. (1988)
22.	*Dolichos lablab* (Fabaceae)	Fruits	Protien	Dolichin	*R. solani*	Lee et al. (2003)
23.	*Ecballium elaterium* (Cucurbitaceae)	Fruit	Cucurbitacin	Cucurbitacin I	*Boitylis cinerea*	Har-Nur and Meyer (1990)

24.	*Euodia lunu-ankenda* (Lauraceae)	Root bark	Phenylethanone	1-[2′,4′ -Dihydroxy-6′(3″-methyl-Z″ -butcnyloxy}-5′(3″-methyl- 2″-butenyl)Jphenylethanone and related compound	*C. cladosporioides*	Kumar et al. (1990), Kadavul and Dixit (2009)
25.	*Eupatorium riparium* (Asteraceae)	Roots	Chromene	Methylripariochromene A	*C. gloeosporioides*	RatnayakeBandara et al. (1992))
26.	*Glycosmis cyanocarpa* (Rutaceae)	Leaves	Sulfur-containing amide	Sinharine; methylsinharine	*C. cladosporioides*	Greger et al. (1992), Pacher et al. (2001)
27.	*Glycosmis mauritiana* (Rutaceae)	Leaves	Sulfur-containing amide	IIlukumbin; methylillukumbins A and B	*C. cladosporioides*	Greger et al. (1993), Pacher et al. (2001)
28.	*Haplophyllum sieversii* (Rutaceae)	Aerial part	Alkaloid	flindersine, anhydroevoxine and haplamine	*C. fragariae*, *C. gloeosporioides* and *C. acutatum*	Cantrell et al. (2005)
29.	*Helichrysum decumbens* (Asteraceae)	Leaf surface	Prenylated phenol	Phloroglucinol derivatives (25–27)	*C. herb arum*	Aiyegoro and Okoh (2009)
30.	*Helichrysum nitens* (Asteraceae)	Leaf surface	Methoxylated flavone and flavonol	Chrysin dimethyl ether; galangin trimethyl ether; baicalin trimethyl ether; five more highly rnethoxylated flavonoids	*C. cucumerinum*	Tornas-Barberan et al. (1988), Aiyegoro and Okoh (2009)
31.	*Hordeum vulgare* (Poaceae)	Leaves	Indole alkaloid	Gramine	*Erysiphe qraminis*	Wippich and Wink (1985)
		Inner epidermal cells	Phenylpropanoid	Unidentified	*Erysiphe graminis f.sp hordei*	Zarnowski et al. (2002)
32.	*Humulus lupulus* (Cannabaceae)	Resin	Flavanone	6-Isopentenylnaringenin	*T. rubrum*	Mizobuchi and Sate (1984), Natarajan et al. (2008)
			Chalcone	xanthohumol	*T. mentaqrophytes*	
33.	*Lupinus albus* (Legominosea)	Leaf surface	Isoflavone	Luteone; wighteone	*Helminthosporium carbonum*	Ingham et al. (1983), Bednarek et al. (2003), Erdemoglu et al. (2007)
		Roots	Isoflavone	Luteone; wighteone; licoisoflavones A and B; parvisoflavone B	*C. herbarum*	Tahara et al. (1984), Bednarek et al. (2003), Erdemoglu et al. (2007)
34.	*Lycopersicon esculentum* (Solanaceae)	Green fruits	Steroidal alkaloid	Tomatine	*Fusarium solani*	Defago and Kern (1988), Soylu et al. (2006), Vinaya et al. (2009)
35.	*Mammea longifolia* (Clusiaceae)	–	Coumarin	Surangin B	*R. solani and B. cinerea*	Deng and Nicholson (2005)

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
36.	*Mangifera indica* (Anacardiaceae)	Peel and pulp	Alkylated phenols	5-(12-*cis*-Heptadecenyl)-resorcinol; 5-pentadecenylresorcinol	*A. alternata*	Cojocaru et al. (1986), Kabuki et al. (2000), Bbosa et al. (2007)
37.	*Melia azedarach* (Meliaceae)	Seeds carnels	Hydroxy coumarins	Scopoletin	*Fusarium verticillioides*	Carpinella et al (2005)
38.	*Mollugo pentaphylla* (Molluginaceae)	Aerial parts	Triterpenoid	Mollugenol A	*C. cucumerinum*	Hamburger et al. (1989), Kim et al. (2008)
39.	*Musa* (Musaceae)	Unripe fruit peel	Amine	Dopamine (oxidation products)	*C. musae*	Muirhead and Deverall (1984), Mokbel and Hashinaga (2005)
40.	*Mutisia friesiana* (Asteraceae)	Aerial part	Coumarins	5-methylcoumarins, mutisicoumarones C and D.	*C. cucumerinum*	Viturro et al (2004)
41.	*Nicotiana tabacum* (Solanaceae)	Leaf surface	Diterpenoid	1.- and tJ-4.S,I3-Duvatriene-L3-diols	*Peronospora rabacina*	Reuveni et al. (1987), Akinpelu and Obuotor (2000)
42.	*Oryza officinalis* (Poaceae)	Leaves	Modified fatty acid	Jasmonic acid, Hydroxybenzoic acid	*P. oryzae*	Cho et al. (1998)
43.	*Persea americana* (Lauraceae)	Peel of unripe fruit	Long-chain alcohol	cis, cis l-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene	*C. gloeosporioides*	Prusky et al. (1983), Adikaram et al. (1992), Gomez-Flores et al. (2008)
		Peel of unripe fruit	Long-chain alcohol	1,2,4- Trihydroxyheptadec-l6-yne; 1,2,4-trihydroxyheptadec-16-ene; l-acetoxy-2,4-dih ydroxy-heptadec-Ie-yne	*C. cladosporioides*	
44.	*Picea sirchellsis* (Pinaceae)	Bark	Stilbene	Astringin; rhaponticin	*Paeolus schweinitzii; Sparassis crispa*	Woodward and Pearce (1988)
45.	*Pinus radiara* (pinaceae)	Needle surface	Long-chain fatty acid	Stearic acid: (!)-Hydroxydodecanoic acid; co-hydroxytetradecanoic ' acid; c;-hydroxyhexadecanoic acid	*Dothistroma pini*	Franich et al. (1983)
			Oxidized diterpene acid	7-Ketodehydroabietic acid ; 7-hydroxydehydroabietic acid; 15-hydroxypodocarpic acid		
46.	*Piper aduncwn* (Piperaceae)	Leaves	Chromene	Methyl 8-hydroxy-2,2-dimethyl-2H-chromene-6- carboxylate; 2,2-din:;etbyl-8-{3-metbyl-2-butenyl}-2H-chromene-6-carboxylic acid	*P. oxalicum*	Orjala et al. (1993), Guerrini et al. (2009)

47.	*P. regnellii* (*Piperaceae*)	Leaves	Neolignan	eupamatenoid-3, eupamatenoid-5	*Tricophyton rubrum, Tricophyton mentagrophytes ; Microsporum canis, M. gypsium*	Koroishi et al. (2008)
48.	*Polygala nicaeensis* (Polygalaceae)	Roots	Xanthone	1,7-Dihydroxy-4-methoxyxanthone; 1,7-dihydroxy-3,5.6-trimetboxyxanthone	*C. cucumerinum*	Marston et al. (1993)
49.	*Polygonum punctatum* (Polygonaceae)	–	Sesquiterpene	polygodial	*Zygosaccharomyces bailli*	Fujita and Kubo (2005)
50.	*Populus deltoides* (Saliaceae)	Leaf glands	Flavanone	Pinocembrin	*Melampsora medusae*	Shain and Miller (1982), Hoof et al. (2008)
51.	*Prunus yedoensis* (Rosaceae)	Leaves	Phenol Coumarin	Benzylalcobol	*C. herbarum*	Ito and Kumazawa (1992)
52.	*Psidium acutangulum* (Myrtaceae)	Twigs and leaves	Dihydrochalcone	3′ -F ormyl-2′.4′.6′ -trihydroxy-dihydrochalcone	*R. solani; Helminthosporium ceres*	Miles et al. (1991), Nowakowska (2007)
53.	*Rapanea melanophloeos* (Myrsinaceae)	Leaves	Trirerpenoid saponin	Sakurasosaponin	*C. cucumerinum*	Ohtani et al. (1993)
54.	*Rosmarinus officinalis* (Lamiacae)	Leaves and callus cultures	Enol ester of hydroxy-cinnamic acid	2-(3,4-Dihydroxyphenyl) ethenyl esters of caffeic acid (24)	*C. herb arum*	Banthorpe et al. (1989), Pintore et al. (2001), Oluwatuyi et al. (2004)
55.	*Rubia tinctorum* (Rubiaceae)	Root	Anthraquinone aglycone	Alizarin and emodin	*A. niger, Alternaria alternaria, P. verrucosum, Mucor mucebo, Doratomyces stemonitis and P. verrucosum*	Manojlovic et al. (2005)
56.	*S. woronowii* (Lamiaceae)	Aerial parts	Neo-clerodane diterpenoid	Jodrellin B	*Fusarium oxysporum f.sp. yeo persici*	Cole et al. (1991), Springob and Kutchan (2009)
57.	*Scutellaria violacea* (Lamiaceae)	Aerial parts	Neo-clerodane diterpenoid	Oerodin	*Fusarium oxysporum f.sp. yeo persici*	Cole et al. (1991), Springob and Kutchan (2009)
58.	*Sesamum angolense* (Pedaliaceae)	Root bark	Naphthoquinone	Naphthoxirenes	*C. cucumerinum*	Potterat et al. (1987)
59.	*Sorghum cultivars* (Poaceae)	Leaves and grains	Leuco-anthocyanidin	Flavan-4-ols	*Fusarium moniliforme; Curvularia lunara*	Jambunathan et al. (1986), Boddu et al. (2004), Kil et al. (2009)
60.	*Stemonoporus canaliculatus* (Dipterocarpaceae)	Bark	Stilbene trimer	Canaliculatol	*C. cIadosporioides*	Bokel et al. (1988), Springob and Kutchan (2009)
61.	*Theobroma cacao* (Sterculiaceae)	Flush shoot tissue	Proanthocyanidin	Polymeric procyanidin	*Crinipellis perniciosa*	Brownlee et al. (1990), Duke (2004)

(*continued*)

Table 19.1 (continued)

S. No.	Plant name (Family)	Part used	Chemical class	Compound (s)	Microorganism tested	References
62.	*Tithonia diversifolia* (Asteraceae)	–	Isocoumarins	Tithoniamarin	*Microbotryum violaceum, Chlorella fusca*	Yemele-Bouberte et al. (2006))
63.	*Triticum aestivum* (poaceae)	Leaves	A1kadienal	E-triticene; P-triticene, puroindoline	*C. cucumerinum*	Spendley et al. (1982), Liyana-Pathirana and Shahidi (2005), Dhatwalia et al. (2009)
64.	*Vernonanthura tweedieana (Asteraceae)*	Root	Sesquiterpene	6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one	*T. mentagrophytes*	Portillo et al. (2005)
65.	*Wedelia biflora* (Asteracea)	Leaf surface	Flavonol	7,3′-Di-O-methylquercetin	*R. solani*	Miles et al. (1993), Yoganandam et al. (2009)
66.	*Zingiber officinale* (Zingiberaceae)	Rhizomes	Diarylheptenone	Gingerenones A, B and C: isogingerenone B	*P. oryzae*	Endo et al. (1990), Samy (2005), Park et al. (2008)

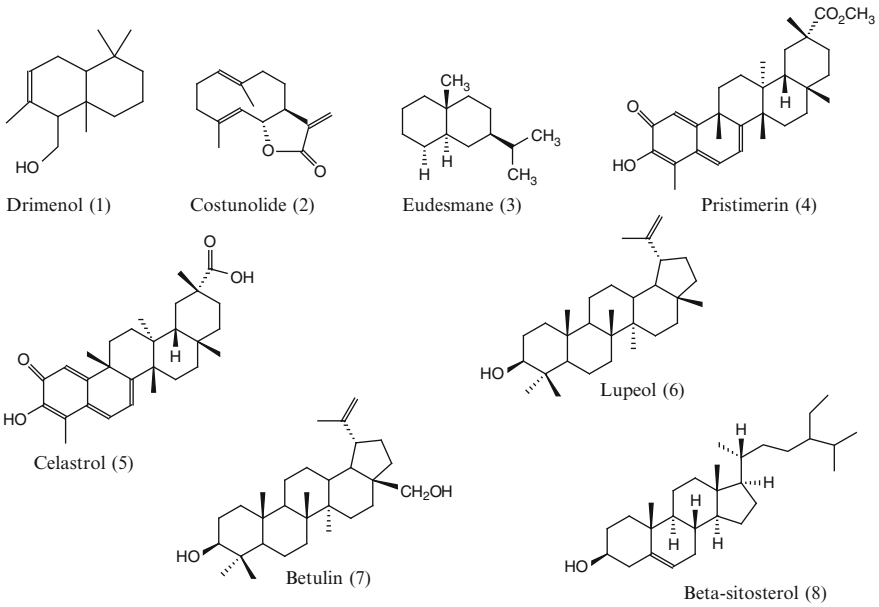


Fig. 19.1 Structures of antifungal terpenoids

found to exhibit a fungicidal activity against food spoilage yeast, *Zygosaccharomyces bailii* (Fujita and Kubo 2005).

The activity of the sesquiterpene lactones isolated from the *Centaurea* plants against the fungus *Cunninghamella echinulata* was evaluated. Costunolide (**2**) and dehydrocostunolide showed noticeable IC_{50} values, while more polar lactones were inactive. These results suggest that a relatively low polarity is one of the molecular requirements for the antifungal activity of sesquiterpene lactones. From other *Centaurea* species, *Centaurea thessala* and *C. attica*, two new eudesmanolides, 4-*epi*-sonchucarpolide and their 8-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxy) derivative, and one new eudesmane (**3**) derivative named atticin were isolated (Skaltsa et al. 2000). Fractionation of *Detarium microcarpum* (Leguminoseae) extract led to the isolation of four new clerodane diterpenes which showed antifungal activity against *C. cucumerinum* (Cavin et al. 2006).

Many diterpenoids show antifungal activity; a few of them are phytoalexins, while others are preformed. The preformed diterpenoids have been reported to be involved in the resistance of several conifers against phytopathogenic fungi (Franich et al. 1983). Two new diterpenes, fusicoserpenol A and dolabeserpenoic acid A, were isolated from leaves of *Hypoestes serpens* (Acanthaceae). Both of the compounds showed good antifungal activity using TLC bioautography at 10 μg concentration against *C. cucumerinum* (Rasoamiaranjanahary et al. 2003).

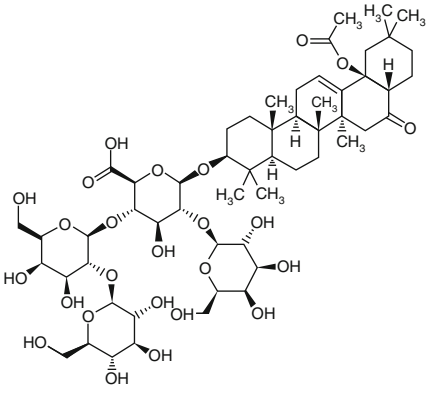
Diterpenoids 16α-hydroxy-cleroda-3,13-(14)-*Z*-diene-15,16-olide and 16-oxo-cleroda-3,13-(14)-*E*-diene-15-oic acid, isolated from the hexane extract of the seeds of *P. longifolia* (Annonaceae), also demonstrated significant antifungal

activity (Marthanda et al. 2005). Adou et al. (2005) isolated five new diterpenoids through bioactivity-guided fractionation, namely humirianthone, 1-hydroxy-humirianthone, 15*R*-humirianthol, patagonol, and patagonal. All five diterpenes showed activity against phytopathogenic fungi.

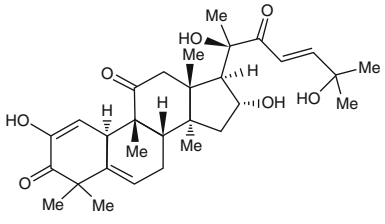
Two triterpenoid, pristimerin (**4**) and celastrol (**5**), isolated from the roots of *Celastrus hypoleucus* (Celastraceae), exhibited an inhibitory effect against diverse pathogenic fungi such as *R. solani* and *Glomerelia cinguiata* (Luo et al. 2005). Similarly Mollugenol A, a triterpenoid isolated from the aerial part of *Mollugo pentaphylla*, showed antifungal activity against *C. cucumerium* (Hamburger et al. 1989). In another study, four triterpenes, namely lupeol (**6**), betulin (**7**), β-sitosterol (**8**), 20(29)-lupene-3β-isoferulate, and two naphthoquinones, shinanolone and octahydroeuclein, isolated from the ethanolic extract of *E. natalensis* root bark, were investigated for their antifungal activity against *A. flavus*, *A. niger*, *C. cladosporioides*, and *Phytophthora* sp. Only β-sitosterol, 20(29)-lupene-3β-isoferulate, and shinanolone showed significant inhibition of *A. niger* at 10 μg/ml. Of these tested compounds, only octahydroeuclein was found to be significantly effective against *Phytophthora* sp. at 10 μg/ml. β-Sitosterol and octahydroeuclein inhibited the growth of *C. cladosporioides* significantly at the same concentration (Lall et al. 2006).

19.3.2 Saponins

An important source of constitutive antifungals is the saponins. Figure 19.2 shows two of these (bold numbers in brackets 9 and 10 below). Saponins are natural detergents that are effective antimicrobial, cholesterol-lowering anticancer compounds. These compounds chemically related to the triterpenoid group such as



Camillidin (9)



Cucurbitacin I (10)

Fig. 19.2 Structures of antifungal saponins

triterpene saponins, together with steroidal saponins, were also isolated as antifungal constituents.

A novel triterpene saponin, CAY-I, from the *Capsicum frutescens* L. (Solanaceae) plant commercially known as cayenne pepper, was investigated to determine its *in vitro* antifungal activity. CAY-1 showed activity against 16 different fungal strains, including *Candida* spp. and *A. fumigatus*, and showed highest activity against *C. neoformans*. Importantly the mechanism for this activity appears by disrupting the membrane integrity of fungal cells. The triterpenoid saponins camellidin I (**9**) and II from the leaves of *C. japonica* display antifungal activity against *Pestalocia longisate* (Nagata et al. 1985). In another study, glucoside of triterpenoid saponins 3-O-glucosides of hederegenins was isolated from Dolicus, and showed good activity against *Clostridium cucumerinum* (Tahara et al. 1984).

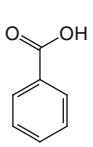
Gonzalez et al. (2004) isolated one novel saponin along with three others from the leaves of *Solanum hispidum*. All isolated compounds showed antifungal activity against both *T. mentagrophytes* and *T. rubrum*. However 6α-*O*-[β-*D*-xylopyranosyl-(1→3)-β-*D*quinovopyranosyl]-(25,*S*)-5α-spirostan-3β-ol, was found most active with IC_{50} values of 25 μg/ml. Similarly five saponins were isolated using bioactivity guided fractionation from *S. chrysotrichum*. All the isolated compounds showed antimycotic activity against *T. mentagrophytes*, *T. rubrum*, *A. niger*, and *C. albicans*.

A very important triterpenoidal saponin is cucurbitacin I (**10**). Although the whole family of cucurbitacins (cucurbitacin A, B, Q, I, and E, etc.) has very high activity, they have at the same time also shown potential toxicity. Cucurbitacin has been isolated from *Ecbellium elaterium* and found to be a very good antifungal against *B. cinera* (Har-Nun and Meyer 1990). Similarly Sauton et al. (2004) have isolated and demonstrated antifungal activity of steroid saponins from *Dioscorea cayenensis* against the human pathogenic yeasts *C. albicans*, *C. glabrata* and *C. tropicalis*. The saponins have been isolated from *A. racemosus*, *Astragalus verrucosus* Moris, *A. auriculiformis* and from *Hedera taurica* (Araliaceae), and have shown very good antifungal activity against *C. albicans*, *C. krusei*, and *C. tropicalis* (Pistelli et al. 2002; Mel'nichenko et al. 2003; Mandal et al. 2005; Rukayadi et al. 2008; Uma et al. 2009) (Table 19.1).

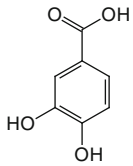
19.3.3 Phenolic Compounds

In recent years, a large number of studies have been published on antimicrobial activity of the phenolics compounds of natural origin. In many cases, these substances serve as plant defense mechanisms against microorganisms and insects, etc. Some plants give their odors like terpenoids, however others (quinones and tannins) are responsible for plant pigment. A large number of aromatic plants show antibacterial and often antifungal activity. These compounds include simple and alkylated phenols, phenolic acid, phenyl propanoids, coumarins, quinines, anthraquinones, and xanthones, etc. (Fig. 19.3: bold numbers in brackets 11 to 14 below refer).

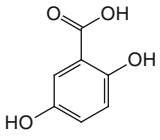
Fig. 19.3 Structures of antifungal phenolics



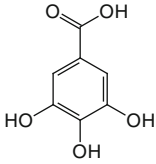
Benzoic acid (11)



Protocatechuic acid (12)



Gentisic acid (13)



Gallic acid (14)

Benzoic acid (**11**), protocatechuic acid (**12**) and gentisic acid (**13**), along with some other phenolic acids, have been reported as constitutive antifungal compounds (Tahara et al. 1984). However, gallic acid (**14**) is inhibitory to both pathogenic and saprophytic fungi. Phenylpropanoid or hydroxycinnamic acid include p-coumaric, ferulic, ferulic synaptic, and chlorogenic, etc. All these phenolics have been reported as strong antifungals against a number of plant and human pathogenic fungi (Kuc et al. 1956). Some new phenolic acid derivatives (crassinervic acid, aduncumene, hostmaniane, and gaudichaudanic acid) were isolated from four *Piper* species, *P. crassinervium*, *P. aduncum*, *P. hostmannianum*, and *P. gaudichaudianum* respectively, as major secondary metabolites. These compounds were reported to be fungitoxic against *C. cladosporioides* and *C. sphaerospermum* (Lago et al. 2004).

De-Leo et al. (2004)) isolated three new phenolic compounds together with a few known compounds from the leaves of *Baseonema acuminatum* (Asclepiadaceae). The compounds showed antifungal activity against two clinically isolated *C. albicans* strains in the range of 25–100 μg/ml. In the same year Lee et al. isolated four phenolic amides, dihydro-N-caffeoyltyramine, *trans*-N-feruloyloctopamine, *trans*-N-caffeoyltyramine, and *cis*-N-caffeoyltyramine, from an ethyl acetate extract of the root bark of *Lycium chinense* Miller. All had an antifungal effect against *C. albicans* with the potency of 5–10 μg/ml and showed no toxicity (hemolytic activity) against human erythrocyte cells (Lee et al. 2004). In another study two compounds were isolated from the essential oils of *Pulicaria odora*, a Moroccan medicinal plant, and identified as 2-isopropyl-4-methylphenol and isobutyric acid 2-isopropyl-4-methylphenylester. The study showed that the crude essential oil and the 2-isopropyl-4-methylphenol exhibited a very significant antibacterial and antifungal activity, while the isobutyric acid 2-isopropyl-4-methylphenylester was inactive for all tested strains (Ezoubeiri et al. 2005).

A bioassay-directed purification from a sunflower by column chromatography followed by HPLC allowed the isolation of a new compound, 3-acetyl-4-acetoxyacetophenone, and known compounds, demethoxyencecalin and 3-acetyl-4-hydroxyacetophenone. The new compound, 3-acetyl-4-acetoxyacetophenone, showed antifungal activity similar to the coumarin ayapin, previously described as a potent *Sclerotinia* inhibitor. A screening of seven sunflower genotypes in a field experiment showed a correlation between these compounds and resistance to *Sclerotinia* (Prats et al. 2007). Naldoni et al (2009) isolated benzophenones 7-epiclusianone and guttiferone-A from the pericarp and seeds of fruits of *Garcinia brasiliensis*. The pericarp hexane extract (PHE), seed ethanol extract (SEE), and both the compounds showed varying levels of activity against *C. albicans* (Table 19.1).

19.3.3.1 Flavones, Flavonoids, and Flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol, and flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan 1999). In the flavonoid group, reports on antifungal compounds from medicinal plants mainly concern those isolated from species of the fabaceae and moraceae families. However, reports on the antifungal activity of flavonoids of several medicinal plants belonging to other botanical families have also been found in the literature (Abad et al. 2007). Figure 19.4 shows a number of these (bold numbers in brackets 15 and 16 below), together with some of the coumarins from the next section (bold numbers in brackets 17 to 24).

Two new flavones from *Artemisia giraldi*, identified as 6,7,4′-trihydroxy-3′,5′-dimethoxyflavone and 5,5′-dihydroxy-8,2′,4′-trimethoxyflavone, together with 5,7,4′-trihydroxy-3′,5′-dimethoxy-flavone, have been reported to exhibit activity against *A. flavus* (Zheng et al. 1996). Galangin (**15**), a flavonol commonly found in propolis samples, has been shown to have inhibitory activity against *A. tamarii, A. flavus, C. sphaerospermum, Penicillium digitatum*, and *P. italicum* (Afolayan and Meyer 1997). A new prenylated flavanone recently isolated from the shrub *Eysenhardtia texana* has been identified as 5,7,4′-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone and shown to possess activity against the opportunistic pathogen *C. albicans* (Wachter et al. 1999).

Alcerito et al. (2002) analyzed the epicuticular wax of the leaves of *Arrabidaea brachypoda* for its flavonoid content and isolated four compounds: 3′,4′-dihydroxy-5,6,7-trimethoxyflavone, cirsiliol, cirsimaritin, and hispidulin. They are known flavonoids, and showed antifungal activity against *C. sphaerospermum*. Eighteen prenylated flavonoids, purified from five different medicinal plants, were evaluated for their antimicrobial activity by determination of MIC using the broth

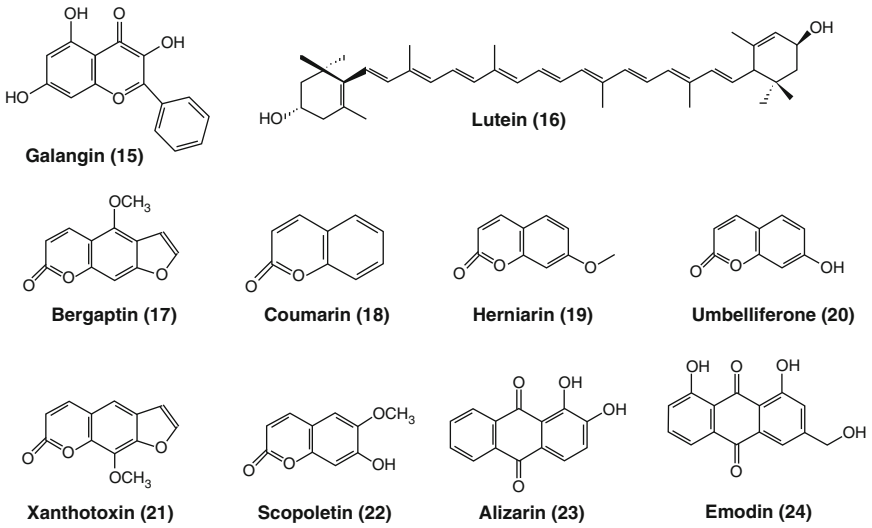


Fig. 19.4 Structures of antifungal flavonoids and coumarins

microdilution methods against four bacterial and two fungal strains (*C. albicans, Saccaromyces cerevisiae*). Of these isolated flavonoids, papyriflavonol A, kuraridin, sophoraflavanone D, and sophoraisoflavanone A exhibited a good antifungal activity with strong antibacterial activity. However, broussochalcone A was effective to *C. albicans* only (Sohn et al. 2004).

The leaves of *Blumea balsamifera* afforded icthyothereol acetate, cryptomeridiol, lutein (**16**), and beta-carotene. Antimicrobial tests indicated that the first compound has moderate activity against the fungi *A. niger, T. mentagrophytes,* and *C. albicans*, while two have low activity against *A. niger, T. mentagrophytes,* and *C. albicans* (Ragasa et al. 2005). The new kaempferol 3-O-β-d-glucopyranosyl (1→2)-O-β-d-glucopyranosyl (1→2)-O-[α-l-rhamnopyranosyl-(1→6)]-β-d-glucopyranoside (1) has been isolated from carnation (*Dianthus caryophyllus*) along with two known C- and O-flavonoid glycosides. The isolated compounds exhibited antifungal activity against different *F. oxysporum* f. sp. *dianthi* pathotypes (Galeotti et al. 2008). In addition to studies into simple phenolic derivatives and flavonoids, those into other antifungal phenolic compounds from natural sources also included coumarins and anthraquinones.

19.3.3.2 Coumarins

Various coumarins isolated from the traditionally used medicinal plants in Brazil showed that the isolated coumarins are active either alone or in combination against a number of fungi, *C. neoformans*, *M. gypseum*, *T. rubrum*, and *T. mentagrophytes* (Stein et al. 2006).

Ojala et al. (2000) tested six commercial coumarins, bergaptin (**17**), coumarin (**18**), herniarin (**19**), umbelliferone (**20**), xanthotoxin (**21**), and scopoletin (**22**), and found that they are active against *F. culmorum* (Fig. 19.4). Tithoniamarin is a new isocoumarin dimer isolated from *Tithonia diversifolia* (Hemsl) Gray (Asteraceae). Preliminary studies showed that tithoniamarin has antifungal and herbicidal activities against *Microbotryum violaceum* and *Chlorella fusca* (Yemele-Bouberte et al. 2006). Alizarin (**23**) and emodin (**24**), anthraquinone aglycone of *Rubia tinctorum*, has shown antifungal activity (Manojlovic et al. 2005).

19.3.4 Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. There are many reports on alkaloids showing activity against human pathogens, for example, the isoquinoline alkaloid jatrorrhizine, a range of peptide alkaloids, the quinolizidine alkaloid dietamnine and the pyrrolizidine alkaloids, etc., (Harborne and Baxter 1993). Berberine (**25**), a well-known alkaloid, has been reported for its strong antifungal activity against a number of organisms including human pathogenic fungi *C. albicans, T. mentagrophytes*, *M. canis*, *T. rubrum*, *E. floccosum*, and *M. gypseum* (Greathouse and Walkins 1938; Freile et al. 2006). Similarly, tomatine (**26**) from tomato and α-solanine from potato are examples of steroidal glycoalkaloids, and have very strong antifungal activity (Grayer and Harborne 1994). A few alkaloids are shown in Table 19.1 and Fig. 19.5 (bold numbers in brackets 25 to 28 refer).

A new alkaloid, puntarenine, which was isolated along with berberine from the organic extract of the whole plant *Xanthorhiza simplicissima*, was found to exhibit good activity against the dermatophytic fungus *T. mentagrophytes* and the yeast *S. cerevisiae* (Okunade et al. 1994). Dabur and co-workers isolated a novel alkaloid, 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate, from the plant *Datura metel* L. (Solanaceae). The compound was found to be active against all the species tested, namely *C. albicans*, *C. tropicalis*, *A. fumigatus*, *A. flavus,* and *A. niger* (Dabur et al. 2005). Klausmeyer et al. (2004) isolated a novel alkaloid, 6,8-didec-(1*Z*)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium, from organic extract of *Aniba panurensis* using bioactivity-guided fraction. In vitro bioassay demonstrated the activity of this alkaloid against a drug-resistant strain of *C. albicans*.

Slobodníková et al. (2004) tested the crude extract of *Mahonia aquifolium* along with its two main alkaloids, berberine and jatrorrhizine, for their *in vitro* antifungal activity against 20 strains of *Candida* sp. isolated from chronic vulvovaginal candidoses. Both the compounds show varying levels of minimum inhibitory concentrations, illustrating strong antifungal activity. Bioassay-guided fractionation of the extract showing growth-inhibition against *Colletotrichum fragariae*, *C. gloeosporioides*, and *C. acutatum* has led to the isolation of the bioactive alkaloids, flindersine (**27**), anhydroevoxine, and haplamine (**28**). Of these,

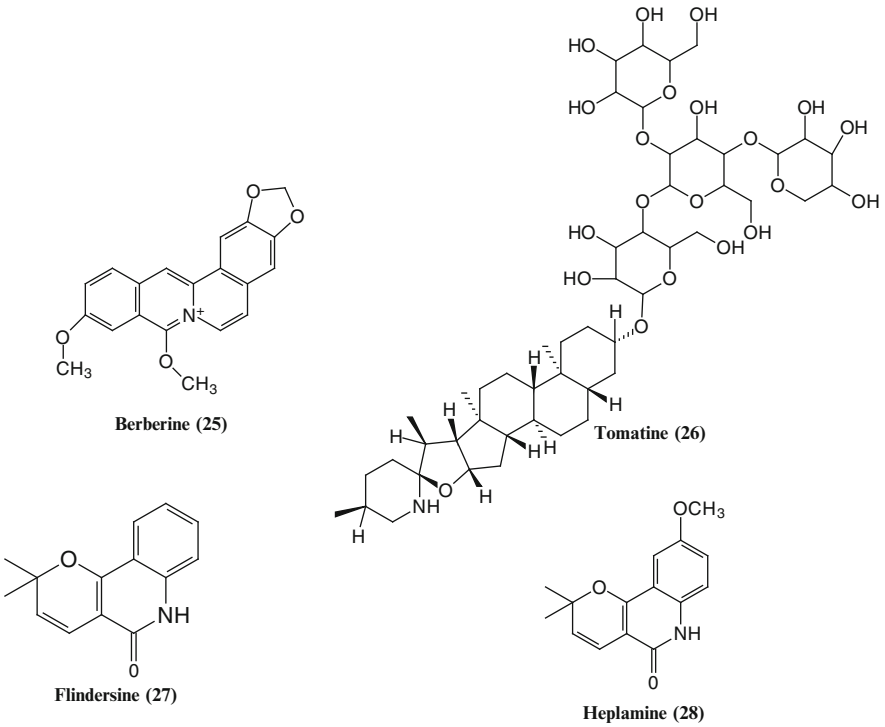


Fig. 19.5 Structures of antifungal alkaloids

flindersine and haplamine demonstrated the highest level of antifungal activity (Cantrell et al. 2005).

Bahçeevli et al. (2005) isolated A beta-carboline-, a tryptamine-, and two phenylethylamine-derived alkaloids along with three known aromatic compounds from the aerial parts and roots of *Cyathobasis fruticulosa*. The one new alkaloid, N-methyl-N-formyl-4-hydroxy-beta-phenylethylamine, showed marginal antifungal activity.

Rao et al. (2009) isolated a novel fibrecisine alkaloid [1,2-methylenedioxy-8-hydroxy-6a(*R*)-aporphine] with 21 known alkaloids including berberines, tetrahydroberberines, and aporphine derivatives. The bioassay result indicated that the berberines showed more potent activity than aporphine derivatives against the test *Candida* strains, while tetrahydroberberines showed very weak activity against *C. neoformans*. Similarly Meng et al. (2009) showed antifungal activity of the benzo[c]phenanthridine alkaloids from *Chelidonium majus* Linn against resistant clinical yeast isolates. Of the six compounds determined, 8-hydroxydihydro-sanguinarine and 8-hydroxydihydrochelerythrine demonstrated potent activity with the MIC ranges of 2–80 and 4–100 μg/ml, respectively. Dihydrosanguinarine, dihydrochelerythrine, sanguinarine, and chelerythrine had some degree of antifungal activity.

19.3.5 Proteins and Peptides

There are hundreds of antifungal peptides and proteins known, with more being discovered almost daily. They are produced by a multitude of organisms including leguminous flowering plants, nonleguminous flowering plants, gymnosperms, fungi, bacteria, insects, and mammals. In the last decade alone, many reports have been published on antifungal proteins and peptides.

Wong and Ng (2005a) isolated an antifungal peptide, vulgarinin, from the seeds of haricot beans (*Phaseolus vulgaris*) by using a simple protocol consisting of affinity chromatography on Affi-gel blue gel and gel filtration on Superdex 75. Vulgarinin manifested an antifungal activity toward fungal species such as *F. oxysporum, Mycosphaerella arachidicola, Physalospora piricola,* and *B. cinerea* (Wong and Ng 2005b). In the same year these authors reported lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*P. lunatus* L.), that has very good antifungal activity against *F. oxysporum, M. arachidicola*, and *B. cinerea*. Actinchinin, an antifungal protein, was isolated from the gold kiwi fruit. This protein exerts antifungal activity against *F. oxysporum* (Xia and Ng 2004).

Defensin has been isolated from the legume *Trigonella foenum-graecum*. The protein is characterized by the presence of eight cysteine residues, conserved in the various plant defensins and forming four disulphide bridges which stabilize the mature peptide. The protein exhibited antifungal activity against the broad host range fungus, *R. solani* and the peanut leaf spot fungus, *Phaeoisariopsis personata* (Olli and Kirti 2006). In addition to these, the work of Tzi Bun Ng (Ng 2004) has demonstrated the isolation of a number of antifungal peptides that have tremendous antifungal properties against a series of fungi.

19.4 Experimental Approaches

19.4.1 Selection of Plants

The choice of method for selecting plants for phytochemical and biological activity screening is often difficult. The large number of plants mentioned in traditional systems of medicine from all over the world requires a rationale for the discovery of interesting biologically active chemicals. Different approaches to chemical discovery can be distinguished; however, Fabricant and Farnsworth (2001) defined four standard approaches for selecting plants — (1) random selection followed by chemical screening, (2) random selection followed by antimicrobial assays, (3) follow-up of antimicrobial activity reports, and (4) follow-up of ethnomedical or traditional uses of plants against infectious diseases.

The first approach that defines searches for classes of secondary metabolites containing various antimicrobial substances (e.g., alkaloids, glycosides, isothiocyanates, etc.) is still very popular because the tests are easy to perform; however,

false-positive tests often render results difficult to interpret. The second approach for plant selection is quite labor-intensive, as it requires collection, identification, and analysis of all available plant parts, irrespective of prior knowledge and experience. However, third approach based on the follow up of current scientific literature available. The fourth approach includes plants used in traditional systems of medicine such as Ayurveda, Unani, Siddha, Kampo, and traditional Chinese medicine, Herbalism, folklore, and Shamanism and involves the use of databases, etc. A fifth, nonsystematic approach can be serendipity, where plant selection is based on ethnomedical use, but where the recovered bioactivity is new or unexpected, for example the anticancer compounds vinblastine and taxol (Clardy and Walsh 2004). Irrespective of the adopted plant collection strategy, when a plant has been identified, the first task should be the identification of bioactive compounds to the level of pure compound or to the level of major group of compounds.

19.4.2 Scheme for the Extraction and Identification of Active Principle

After selection of the plants, a major problem is the processing of the plant material that will be used in the panel of screening. Best strategies should be used to guarantee that the active principle is not destroyed, lost or altered. The first important measure should be the extraction period and efficiency. The extraction period can be shortened by grinding the plants finer and by shaking at a very rapid rate (Eloff and McGaw 2006). Extraction efficiency can be increased by sonicating the samples in a bath-type sonicator. Three to five extractions (10 min each, using a sonic bath) of very finely ground plant material with organic solvent of high polarity can extract up to 80% of the compounds on a dry-weight basis.

Choice of the solvent for extraction is also very important. Initial screening of the plants for biological activity starts with the use of aqueous or organic extracts followed by various fractionations. In one study Kotze and Eloff (2002) used 11–12 different extractants to extract and analyze the biological activity of *Combretum mycrophyllum*. Methanol, methylene dichloride, and tetrahydrofurane extract most components; however, diisopropyl ether, ethanol, ethyl ether, acetone, and ethyl acetate showed most biological activity with low quantity of other nonactive components. For hydrophilic compounds, polar solvents such as methanol, ethanol, or ethyl-acetate should be used; however, for extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol 1:1 should be used. In some instances, extraction in hexane is used to remove chlorophyll.

Various extraction and isolation strategies for pure compounds are reported in the literature, but successive fractionation using solvents of different polarity is commonly used. Figure 19.6 shows the simplified extraction and identification flow chart to be used in order to achieve/optimize bioactive phytochemical identification. After extracts/fractionation, a concentration step is usually required, which

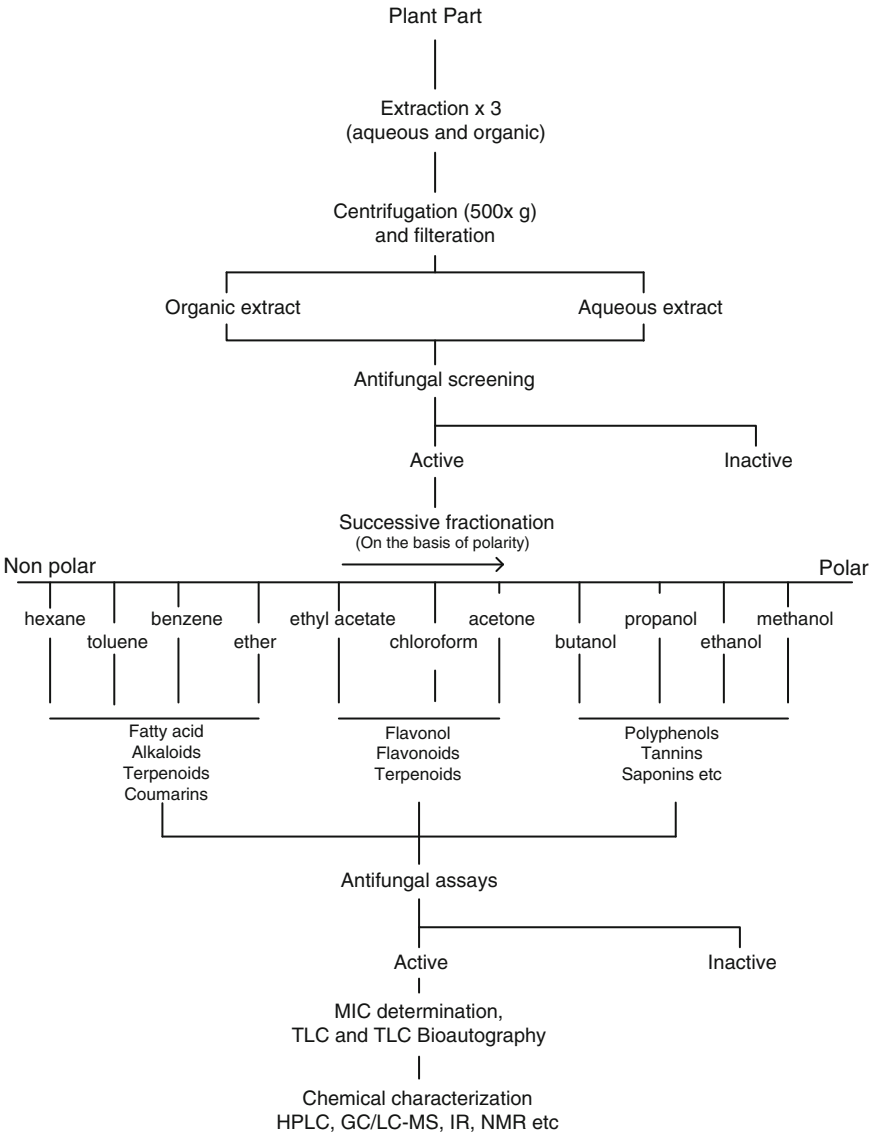


Fig. 19.6 Proposed scheme of extraction and identification of antifungal compounds from medicinal plants

should be based on evaporation of the solvent under vacuum at low temperature to avoid any loss of thermolabile constituent. In most cases, it is better if screening can be done prior to separating polar from less-polar constituents by the sequential use of solvents from high to low polarity. It saves labor and resources in the case of low or no activity.

19.4.3 Solvent for Testing

The most frequently used solvents to make up test compound solutions for quantitative purposes include dimethyl sulfoxide (DMSO), methanol, and ethanol. However, methanol and ethanol have the disadvantage of rapid evaporation. Solution in 100% DMSO is a better option as it can avoid the contamination and has good compatibility with most of the compounds. But it is also important to note that DMSO is potentially toxic for cells and many microorganisms, so the in-test concentration of DMSO should not exceed 1%.

On the other hand, sometimes dried extracts are not freely soluble even if the same solvent is used. In these conditions, it is better not to dry the extract. The aliquots of the extract can be dried after activity evaluation, and active concentration can be achieved by back calculation (Eloff and McGaw 2006). It is always preferable to store dry extracts/compounds at −20°C. However, extracts of a few plants in methanol and acetone are reported to retain activity, so may be supposed to be stable when stored at room temperature in dissolved form (Eloff et al. 2001).

19.4.4 Antifungal Assays

Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method. There are several methods available for antifungal activity testing, which can be classified into three main groups, i.e., diffusion, dilution, and bioautographic methods.

19.4.4.1 Agar Well Diffusion Method

The agar well diffusion method is the most widely used technique for assaying plant extracts for their antimicrobial activity. In this technique, a well or reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (zone inhibition diameter) is measured at the end of the incubation period. Different types of reservoirs can be used, such as filter paper disks or holes punched in the medium. Of the various methods to conduct the assays described in the literature, however, the agar well diffusion method as described by Perez et al. (1990) can be easily used. We have adopted this method and used it since 1998 without any difficulty (Ahmad et al. 1998; Mehmood et al. 1999; Ahmad and Beg 2001; Aqil and Ahmad 2003; Aqil et al. 2005; Ahmad and Aqil 2007; Aqil and Ahmad 2007).

Small sample requirements and the possibility of testing six–eight extracts per plate against a single microorganism are specific advantages. In this assay 0.1 ml of diluted inoculum (10^5 CFU/ml) of test organism is spread on Saboroud Dextrose

agar plates. Wells of 8 mm diameter are punched into the agar medium and sealed with molten agar to prevent leaching of the compound. The wells are filled with 100 μl of plant extract of known concentration and solvent blank (≤1% DMSO) separately. The plates are incubated at 28 ± 2°C, for 72 h. The zone of inhibition of test organism growth around each well can be measured in mm. In order to enhance the detection limit, the inoculated plates can be kept at a lower temperature for several hours before incubation. In this way, compound diffusion can be enhanced over microbial growth, and better inhibition diameter is expected.

The agar well diffusion method also has some demerits. This method is not appropriate for the testing of nonpolar samples or samples that do not easily diffuse into agar, and it is not a preferred method for the testing of essential oils because of their volatile nature. The extra potency reported with this method is much less than for the dilution method, because the compound which is in contact with the test organism is much less than the loaded dose.

19.4.4.2 Broth Dilution Method

In this method, test compounds are mixed with a suitable medium and inoculated with the test organisms. Although it can be carried out in liquid as well as in solid media, the better way to perform it is in liquid media. The inhibition of fungal growth can be measured in a number of ways, for example by measuring optical density, by plating the known volume on the agar plates, or by the streak-plate method (Eloff and Mcgaw 2006; Aqil and Ahmad 2007). However, the most frequently used method measures turbidity and employs redox-indicators. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. However, test samples that are not fully soluble may interfere with turbidity readings. In these cases, individual plants or plant samples may give better results.

The major benefit of this assay is that it allows determining whether a phytocompound or extract has a fungicidal or fungistatic action at tested concentration. This can be determined by plating the samples from the test mixture and assessing growth or no growth. Kostiala and Kostiala (1984) compared the disk diffusion method with the broth dilution method using different antibiotics against *C. albicans*. They concluded that disk diffusion assay is equally good or better if short incubation (24 h) is used; however, the broth dilution method was found to be more sensitive when incubation was extended to 48 h.

Eloff (1998) developed microdilution and macrodilution techniques using 96-well microplates and sugar tubes respectively. This technique uses the redox indicator p-iodonitro tetrazolium violet (MTT) and resazurin to quantify fungal growth (Jahn et al. 1995; Pelloux-Prayer et al. 1998). Resazurin has the advantage of not precipitating upon reduction, allowing direct reading. Visual readings can also be used if a spectrophotometer is not available, as these compounds produce an intense red color.

19.4.4.3 TLC-Bioautography Technique

Bioautography can explain how many biologically active compounds are present in an extract. The best use of this technique is that it permits localizing of antimicrobial activities of an extract on the chromatogram; it supports a quick search for new antimicrobial agents through bioassay-guided isolation. This can be achieved using one of two approaches; (1) contact bioautography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, or (2) agar overlay bioautography, where a seeded agar medium is applied directly onto the TLC plate.

For direct bioautographic assay, we used the agar overlay assay approach as described by Slusarenko et al. (1989): 5–10 µl of each plant extract are spotted on E-MERCK chromatographic silica gel G f_{254}, 0.25 mm-thick plates of 3 × 8 cm. The chromatograms are developed using different solvent systems. One milliliter of spore suspension (10^5 spores/ml) is used for each 10 ml of media. Developed chromatograms are placed in autoclaved petri plates. Spores of test fungi in molten agar medium are poured over the chromatograms. The plates are incubated at 28°C for 72 h. A zone of inhibition of bacterial growth could be seen around the active chromatogram spot. The spot was also confirmed by flooding the plates with 0.02 mg/ml solution of *p*-iodonitro-tetzolium.

Although the technique is very sensitive, its applicability is limited to fungi that easily grow on TLC plates. The bioautography technique is more difficult with fungi, because they grow more slowly and contamination can be a problem. Another problem is the need for complete removal of residual low volatile solvents.

19.4.4.4 Media, Inoculum and Organisms

Sabouraud (SAB) agar or broth is a general growth media for fungi. Yeast inoculates can be prepared from overnight cultures or from existing biofreeze stocks. It is recommended that collection is made from cultures during the logarithmic growth phase, and that four or five colonies of a pure culture on agar shold always be taken, to avoid selecting an atypical variant (Anon 2003). In dilution methods, an inoculum of about 10^4 spores/ml is adequate for most yeasts and fungi (Hadacek and Greger 2000). However in our laboratory we have used 10^5 spores/ml for both the agar well diffusion (0.1 ml) and the broth dilution methods, and found good response in all the studies.

The choice of test organisms always depends on the specific purpose of the investigation. However in a primary screening, the use of drug-sensitive reference strains is preferable, which should represent common pathogenic species of different classes. A small set of reference fungi is used for primary screening and includes *T. mentagrophytes* and *E. floccosum* as representatives of the dermatophytes. As opportunistic filamentous fungi, *A. niger* and *F. solani* are listed (Cos et al. 2006).

19.5 Conclusion

There is a growing body of evidence indicating the benefits of medicinal plants for their use against pathogenic microorganisms. Plant-based remedies used in human and animal medicine are an essential part of the primary health care system in many countries. Extensive screening programs of plants used mainly in traditional medicine have resulted in the discovery of thousands of phytochemicals with inhibitory effects on different types of microorganisms *in vitro*. Studies from all over the world have indicated that several plant extracts and their phytocompounds have been identified in an effort to supplement the relatively sparse portfolio of antifungal drugs. There is a need to exploit these bioactive compounds in disease caused by pathogenic fungi.

Although hundreds of antifungal compounds have been identified, it is important to keep in mind that while *in vitro* activity does not necessarily verify the efficacy of a plant extract, it does provide a preliminary indication of the usefulness and potential toxicity of the plant. Problems such as narrow spectrum of activity, susceptibility to efflux pumps, protein binding, serum inactivation, and poor pharmaceutical properties are associated with most of these compounds. Therefore, new anti-infective agents must act on drug-resistant pathogens and may work on multiple targets.

For antifungal activities, follow-up of bioassay-guided fractionation can be performed with an assay of choice. Whenever possible, final reporting of active extracts or pure compounds should be done by applying the broth-dilution method. Stringent endpoint criteria with IC_{50}-values generally below 100 μg/ml for extracts and below 25 μM for pure compounds should be set. Those bioactive compounds which show promising *in vitro* activity should be subjected to *in vivo* studies to determine their efficacy, stability, and bioavailability.

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