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Journal of Ethnopharmacology 106 (2006) 290–302
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Review

Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'

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Received 6 February 2006; received in revised form 1 April 2006; accepted 3 April 2006
Available online 18 April 2006

Abstract

Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. To secure this, a number of pivotal quality standards need to be set at the level of extract processing and primary evaluation in pharmacological screening models. This review provides a number of recommendations that will help to define a more sound 'proof-of-concept' for antibacterial, antifungal, antiviral and antiparasitic potential in natural products. An integrated panel of pathogens is proposed for antimicrobial profiling, using accessible standard in vitro experimental procedures, endpoint parameters and efficacy criteria. Primary requirements include: (1) use of reference strains or fully characterized clinical isolates, (2) in vitro models on the whole organism and if possible cell-based, (3) evaluation of selectivity by parallel cytotoxicity testing and/or integrated profiling against unrelated micro-organisms, (4) adequately broad dose range, enabling dose–response curves, (5) stringent endpoint criteria with IC_{50}-values generally below 100 μg/ml for extracts and below 25 μM for pure compounds, (6) proper preparation, storage and in-test processing of extracts, (7) inclusion of appropriate controls in each in vitro test replicate (blanks, infected and reference controls) and (8) follow-up of in vitro activity ('hit'-status) in matching animal models ('lead'-status).

Keywords: Natural products; Antibacterial; Antifungal; Antiviral; Antiparasitic; Screening

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Abbreviations: ATCC, American type culture collection; BSL, biosafety level; CFU, colony forming unit; CPE, cytopathic effect; EPTT, endpoint titration technique; EPS, extracellular polymeric substance; HMI, hirumi-9 medium; HSV, *Herpes simplex virus*; MBC, minimal bactericidal concentration; MH, Mueller-Hinton; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; NBT, nitro blue tetrazolium; NCCLS, national committee for clinical laboratory standards; PES, phenazine ethosulphate; SAB, Sabouraud; SI, selectivity index; $TCID_{50}$, 50% tissue culture infective dose; TSA, tryptic soy agar; TSB, tryptic soy broth; VRE, vancomycin-resistant *Enterococci*; WHO, World Health Organization

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doi:10.1016/j.jep.2006.04.003

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1. Introduction

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance (Okeke et al., 2005). Research on new antimicrobial substances must therefore be continued and all possible strategies should be explored. Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Clardy and Walsh, 2004). Only a minute portion of the available diversity among fungi, marine fauna and flora, bacteria and plants has yet been explored and ample opportunities lie theoretically ahead. Current research on natural molecules and products primarily focuses on plants since they can be sourced more easily and be selected on the basis of their ethno-medicinal use (Verpoorte et al., 2005). However, the chemical complexity of many natural products and the lack of assurance of a renewable supply have created a diminishing interest by the pharmaceutical industry, which in turn endorses the pivotal role of academia and public organisations in the protracted exploration and evaluation of natural products. Use of ethnopharmacological knowledge is one attractive way to reduce empiricism and enhance the probability of success in new drug-finding efforts (Patwardhan, 2005; Cordell and Colvard, 2005).

Any effort to identify pharmacological action entails the access to both robust bioassays and targeted collections of compounds and extracts for testing. Specific hurdles for ethnopharmacology include either the isolation and characterisation of bioactive molecules in the extract and the problem of "re-isolation" of known bioactive compounds or the standardization of plant extracts. In addition, fractionation of extracts frequently leads to a reduction or loss of biological activity by compound break-down or loss of additive or synergistic effects between analogue constituents.

Validation and selection of primary screening assays are pivotal to guarantee sound selection of extracts or molecules with relevant pharmacological action and worthy following-up. Primary bioassays are generally designed for rapid screening of large numbers of products or extracts. They are simple, easy to implement and produce results quickly and preferably at low cost. Compounds or extracts with a specific activity at a non-toxic dose, so-called "hits", then need further evaluation in secondary or specialized in vitro bioassays and in animal models to define "lead" status. Advanced assessment of kinetic and toxicological properties will ultimately define full 'proof-of-concept' and 'development-candidate' status (Verkman, 2004). A recent review of the literature (Rios and Recio, 2005) revealed that still too many articles on natural products claim so-called "exciting" antimicrobial activities, despite major flaws in used methodologies. Most frequent are the lack of sound criteria for activity, the omission of appropriate in-test controls, the inclusion of unrealistically high assay dosages and the nature of the bioassay itself (selection of target organism, endpoints, etc.).

In an effort to provide some guidance on how to improve quality of screening against infectious organisms, this article will focus on primary in vitro antiviral, antibacterial, antifungal and antiparasitic bioassays. Within the established need

to improve "good bioassay practices", recommendations for compound sourcing and processing, selection of appropriate organisms in bioassays, selection of reference controls, endpoint parameters and criteria for efficacy are proposed. Knowing that the perfect single approach does not exist, it is up to the scientist to define the set of criteria that will yield the greatest chances to attain a robust "proof-of-concept".

2. Selection of plants

Four standard approaches are available 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 (Fabricant and Farnsworth, 2001). The first, so-called phytochemical approach searches for classes of secondary metabolites containing various antimicrobial substances (e.g. alkaloids, isothiocyanates, e.a.). This approach is still very popular in developing countries because the tests are easy to perform; however, false-positive tests often render results difficult to interpret. In the second approach, all available plant parts are collected, irrespective of prior knowledge and experience. This methodology is expensive and laborious and depends heavily on the panel of test pathogens and the 'activity' criteria used. The third approach exploits the vast resource of published reports on antimicrobial activities. However, critical evaluation of sometimes contradictory test results is warranted and prior confirmation of the published results remains prerequisite. In the ethnomedical approach, oral or written information on the medicinal use of a plant forms the basis for selection and focused evaluation. Information from organized traditional medical systems (Ayurveda, Unani, Kampo and traditional Chinese medicine), herbalism, folklore and shamanism can be acquired from various sources, such as books, herbals, review articles (usually involving surveys of medicinal plants by geographic region or ethnic culture), notes placed on voucher herbarium specimens, field work and computer databases, such as NAPRALERT and USDA-Duke. A fifth, non-systematic approach is serendipity, where plant selection is based on ethnomedical use, but where the recovered bioactivity is new or unexpected. Among others, well known examples are the anticancer compounds vinblastine and taxol (Clardy and Walsh, 2004).

3. Extraction scheme

Irrespective of the adopted plant collection strategy, a critical step is the processing of the plant material that will be used in the panel of screens. Appropriate measures must be taken to guarantee that potential active constituents are not lost, altered or destroyed during the preparation of the extract. Being aware that individual constituents may require specific processing, for example at the later stage of bio-guided fractionation, a generic extraction scheme is best indicated for the preparation of crude extracts. Plant extracts are prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvents. For hydrophilic compounds, polar solvents such as methanol, ethanol or ethyl-acetate are used. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol 1:1 are used. In some instances, extraction in hexane is used to remove chlorophyll. Important to consider in the ethnomedical approach is the need to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug.

To detect active substances present in very small quantities in the extracts, a concentration step is usually required and is based on evaporation of the solvent in vacuo. It is advised to extract and evaporate at low temperature not to destroy any thermolabile constituent. Unfortunately, this concentration step often results in precipitation or co-precipitation thereby hampering proper performance and interpretation of the bioassay. Introducing pH differences may further enhance separation of acid, neutral and basic constituents. Of the many extraction schemes that have been published, the method proposed by Mitscher et al. (1972) and later adapted by Ieven et al. (1979) can be considered as a practical standard since it offers a logical, low-cost, feasible and highly performing starting approach (Fig. 1). In some instances and if logistics permit, a 'primary' fractionation of the total extract can be carried out prior to testing to separate polar from less-polar constituents by sequential use of solvents from high to low polarity (Vanden Berghe and Vlietinck, 1991). This permits better discrimination between fractions that exhibit aspecific activity or cytotoxicity and fractions that show selective antimicrobial activity. This 'primary' fractionation scheme may also contain dereplication steps to avoid re-isolation of known compounds; for example, acidic polysaccharides and tannins frequently produce a broad, non-selective activity against several micro-organisms, particularly viruses. It may therefore be appropriate to use acidic polysaccharide-free and/or tannin-free extracts, which can easily be obtained respectively by precipitation after adding 50% ethanol and/or chromatography on a polyamide 6 or 8 column, using water and methanol as eluents (Cordell et al., 1993).

4. Selection of the appropriate bioassay

Different screening approaches are available to identify the primary pharmacological activity in chemical and/or natural products. The screening option will largely depend on the specific nature of the disease being targeted and on the availability of practical and biologically validated laboratory models. As illustrated in Fig. 2, four levels of screening can be identified and the most rewarding strategy is to opt for models that remain as close as possible to the final target, i.e. the patient. In that respect, some compromises will have to be made for throughput, labour-intensiveness, costs and compound requirements. Whenever possible, activities discovered at one particular screening level should be confirmed using a model in the next higher evaluation level. For example, results obtained in a subcellular (enzymatic) screen should be confirmed against the whole organism. A good in vitro activity against the whole organism should then be linked to a confirmation test in an animal model.

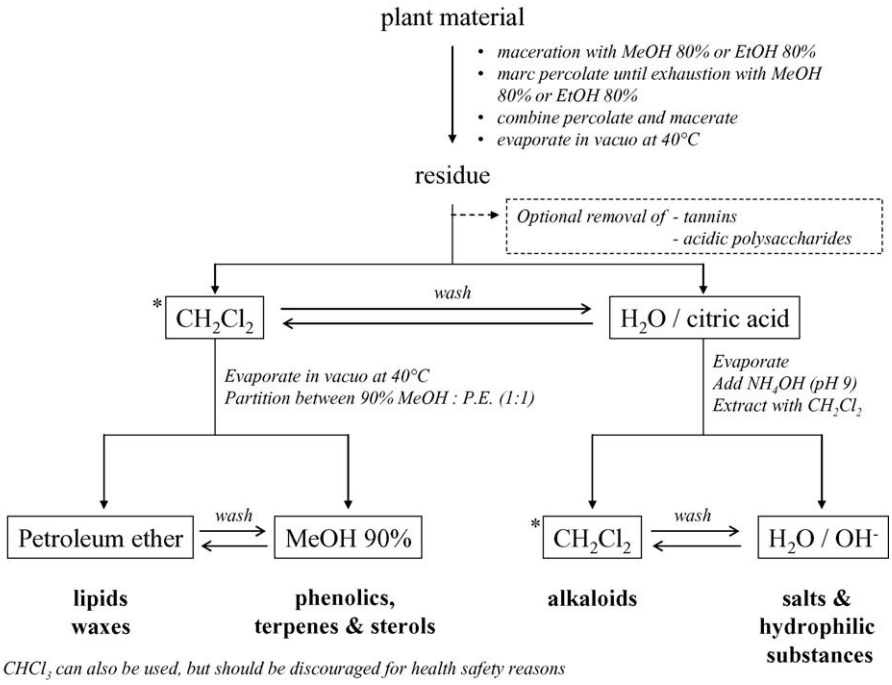

Fig. 1. Standard scheme for preparation of plant extracts for biological screening (Mitscher scheme adapted by Ieven et al., 1979).

For most infectious diseases, this can easily be achieved since validated in vitro and in vivo laboratory models using the whole organism are available.

4.1. Subcellular target approach

A theoretical first step in drug discovery is to identify and validate specific molecular targets. With the expanding knowledge of microbial biochemistry and genetics, it has now become possible to identify important microbial enzymes, receptors and processes at the molecular level that might be suitable targets for drug action. Compounds are identified that block the functioning of these targets and hence would inhibit microbial growth. For the listed microbial diseases, several targets have indeed been identified and new ones are still being discovered. Advanced genomic and proteomic research may enable a more rapid progression towards 3D-structural information and create possibilities for computer (in silico) drug design and/or screening (Kitchen et al., 2004). The initial "active" compounds ('hits') may result from high-throughput screening of large and diverse product libraries, or from a more rational approach if structural information on the target is available. On large-scale drug screening platforms, enzymatic screens are attractive because of ease of implementation and minimal compound requirements; however, their overall poor validation status has contributed to the failure of delivering initial expectations (Sams-Dodd, 2005). An opposite trend is even being noted where target validation is positioned after whole-organism screens (Hart, 2005). Since natural products and extracts are generally complex mixtures of quite diverse molecules, target-oriented screens are perceived

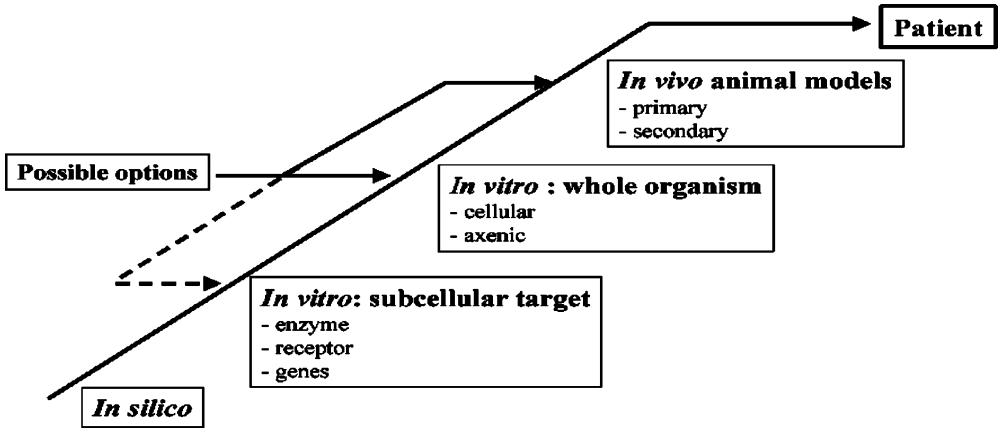

Fig. 2. General approaches in (anti-infective) drug screening.

inappropriate and will therefore not be considered here as a valid screening option.

4.2. Conventional "whole-cell" test systems

Conventional screening systems on the whole organism have considerable advantages over the target-oriented options (Table 1): they involve all targets and take bioavailability phenomena into account. All too frequently, compounds that are potent inhibitors of enzymes in vitro ultimately fail to confirm against the whole organism because, among other reasons, they do not pass the cell membrane.

In vitro models using the whole organism are the 'golden' standard and should be used whenever possible. Most micro-organisms can easily be cultivated either in cell-free medium or in cell cultures. Important to note is that any drug action on the micro-organism should be discriminated from aspecific cell toxicity, necessitating the inclusion of a parallel evaluation in host cell lines (cytotoxicity evaluation) and/or by inclusion of other microbial screens, covering a panel of bacteria, fungi, parasites and viruses. Most recommendations and examples given in this article refer to the standard 96-well microplate format.

Animal models in laboratory rodents are indispensable for confirmation of in vitro active drug candidates. Because of animal welfare considerations, high cost and labour-intensiveness, the number of animal tests is generally kept to an absolute minimum. In these models, a more profound pharmacological picture is obtained, as pharmacokinetic, metabolic and toxicological phenomena are taken into account. Since animal models are not within the scope of this article, referral for protocols is made to the book: Handbook of Animal Models of Infection (Zak and Sande, 1999).

Table 1
Advantages and disadvantages of the different drug screening approaches

In vitro screening approach	Advantages	Disadvantages
Whole organism vs. subcellular target	• Known and unknown targets are considered • More relevant biological test conditions • Indicated for empirical/random screening of 'unknown' chemicals and natural products	• No link to the mode-of-action • Existence of membrane barriers • Stringent endpoint for weakly active compounds • Difficult to run in high-throughput (HTS) mode
Integrated screening vs. single-model	• Rapid profiling for related and unrelated antimicrobial activities • 'Selectivity' criterion more important than 'potency' criterion	• More complex, labour-intensive, expensive • Need for integrated expertise • Higher compound requirements • More sophisticated data management

5. Compound handling and storage

The most frequently used solvents to make up test compound solutions include dimethyl sulfoxide (DMSO), methanol and ethanol. The latter two, however, have the disadvantage of rapid evaporation whereby the stated concentration of stock solutions cannot be maintained. Solutions in 100% DMSO have become the standard and are prepared at adequate strength ("master plates" at 20 mM or μg/ml for pure compounds and extracts, respectively). Added advantages of stock solutions in 100% DMSO are: (1) elimination of microbial contamination, thereby reducing the need for sterilisation by autoclaving or other strenuous methods, which affect the quality of the test sample and (2) good compatibility with test automation and integrated screening platforms, assuring for example good solubility during the serial dilution procedures. It is important to note that DMSO is potentially toxic for cells and many micro-organisms, and to avoid later interference in the biological test systems, the in-test concentration of DMSO should not exceed 1%. In practical terms, this entails the need for inclusion of an intermediate dilution step in water since minimum dispensing volumes for most standard pipettes are 5–10 μl. Because of the variability of individual compounds, there are no general storage conditions that guarantee sample integrity (Verkman, 2004). A practical recommendation for storage of compounds or extracts is either without solvent for long term storage or in 100% DMSO at −20 °C with minimal exposure to freeze–thaw cycles and humidity.

6. Integrated in vitro screening for selective activity

Too often, natural products are tested only against a single species or class of micro-organisms, mostly selected based on ethnomedical use data. When the product exhibits high activity, it is then identified as a potential 'hit'. However, this 'narrow focus' strategy overlooks the incidence of false-positives and suffers from the lack of discrimination from aspecific cell toxicity. This can easily be solved by inclusion of a parallel evaluation on host cell lines (cytotoxicity evaluation) and/or other microbial screens on bacteria, fungi, parasites and viruses (Maes et al., 2004). In antiviral screening, this selectivity is intrinsically included and reflected in the selectivity index (SI). Total extracts and derived fractions exhibiting strong non-selective action in the panel of in vitro screens can only be properly evaluated in animal models.

An integrated screening concept for anti-infective activity is described in detail and should be regarded as a practical operational example rather than a strict set of rigid instructions. Besides antibacterial, antifungal, antiviral and antiparasitic 'whole organism' assays, cytotoxicity on MRC-5 cells (human lung fibroblasts) is performed in parallel. Although many other cell types can be used for cytotoxicity evaluation, MRC-5 cells were selected because of their sensitivity and receptiveness for several viruses and parasites. Standardization across the different bioassays maximizes efficiency, minimizes cost and allows easy and reproducible data acquisition. It includes the use of:

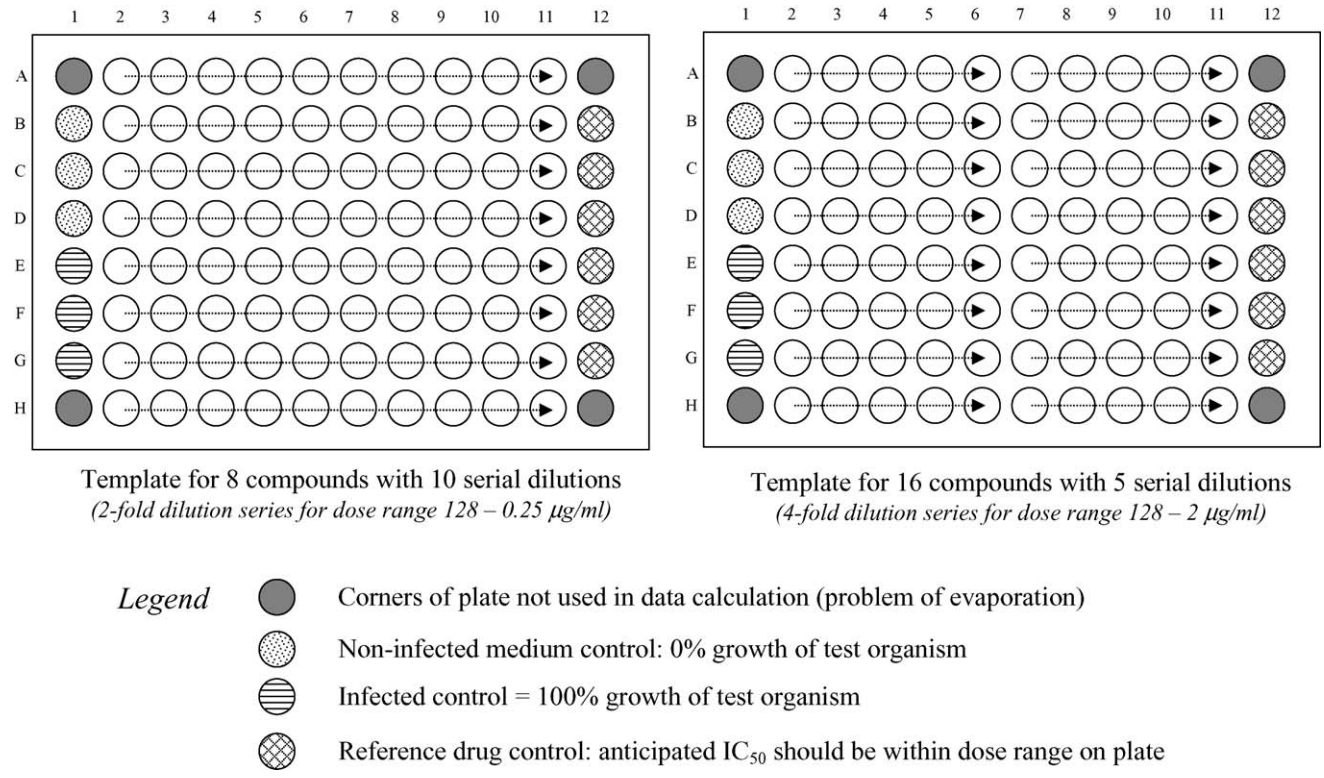

Fig. 3. Templates for primary integrated in vitro screening (common 96-well microplate format for all screens, separate plates are used for replicate testing).

1. Standard (20 mM or 20 mg/ml) stock solutions in 100% DMSO, stored at −20 °C in 96-well deep-well stacker plates. Since DMSO is highly hygroscopic, tight sealing is required.
2. Fixed concentrations in all screens (using 2- or 4-fold serial dilutions) with 100–150 μM or μg/ml as the highest in-test concentration. Higher concentrations generally result in a markedly increased incidence of false-positives.
3. Standard lay-out of 96-well microplates to facilitate plate production and to minimize human errors during the bioassay screen. The proposed plate layouts (Fig. 3), although not restrictive and to be modified depending on the specific objectives of the test, always includes the presence of negative, positive and reference controls.
4. Spectrophotometric reading of endpoints, whenever possible. It is advisable for compounds with known molecular weight to express activity/inhibition parameters in molar concentrations. For natural products or compound mixtures where the exact molecular weight is not known, concentrations are expressed in μg/ml.
5. Standard templates (spreadsheet), allowing rapid result processing and reporting. Endpoints include IC_{50}, IC_{90}, MIC and MBC-values.

7. Antibacterial and antifungal assays

7.1. Overview of standard assays

Antimicrobial activity of natural extracts and pure compounds can be detected by observing the growth response of various micro-organisms to samples that are placed in contact with them. 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. The general problems inherent to antimicrobial screening of plant extracts have already been discussed by several authors (Vanden Berghe and Vlietinck, 1991; Cole, 1994; Rios et al., 1988; Hadacek and Greger, 2000), so focus here will mainly be on the correct implementation and interpretation of the diverse laboratory models.

The antibacterial and antifungal test methods are classified into three main groups, i.e. diffusion, dilution and bioautographic methods. A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedance of the growth medium (Sawai et al., 2002). The latter method is not discussed here. It should be emphasized that many research groups have modified these methods for specific samples, such as essential oils and non-polar extracts and these small modifications make it almost impossible to directly compare results. It is therefore a 'must' to include at least one, but preferentially several reference compounds in each assay.

7.1.1. Agar-diffusion methods

In the diffusion technique, a 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 (inhibition diameter) is measured at the end of the incubation period. In order to enhance the detection limit, the inoculated system is kept at lower temperature for several hours before incubation to favour compound diffusion over microbial

growth, thereby increasing the inhibition diameter. Different types of reservoirs can be used, such as filter paper discs, stainless steel cylinders placed on the surface and holes punched in the medium. The hole-punch method is the only suitable diffusion technique for aqueous extracts, because interference by particulate matter is much less than with other types of reservoirs. To ensure that the sample does not leak under the agar layer, fixed agar is left on the bottom of the hole (Cole, 1994). The small sample requirements and the possibility to test up to six extracts per plate against a single micro-organism are specific advantages (Hadacek and Greger, 2000). The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar. In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Furthermore, agar-diffusion methods are difficult to run on high-capacity screening platforms.

7.1.2. Dilution methods

In the dilution methods, test compounds are mixed with a suitable medium that has previously been inoculated with the test organism. It can be carried out in liquid as well as in solid media and growth of the micro-organism can be measured in a number of ways. In the agar-dilution method, the Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration able to inhibit any visible microbial growth. In liquid- or broth-dilution methods, turbidity and redox-indicators are most frequently used. 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, emphasizing the need for a negative control or sterility control, i.e. extract dissolved in blank medium without micro-organisms. The liquid-dilution method also allows determination whether a compound or extract has a cidal or static action at a particular concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth (static) or no-growth (cidal) after incubation. At present, the redox indicators MTT and resazurin are frequently used to quantify bacterial (Eloff, 1998; Gabrielson et al., 2002) and fungal growth (Jahn et al., 1995; Pelloux-Prayer et al., 1998). Resazurin has the advantage not to precipitate upon reduction, allowing direct reading. Easy and reproducible measurements can be obtained with a microplate-reader, but visual reading may also be used in cases where spectrophotometry is not available. Another assay exploits the principle that only living cells convert fluorescein-diacetate to fluorescein, producing a yellowish-green fluorescescence under UV light (Chand et al., 1994). However, it requires a more significant investment in equipment and validation is not easy: Sabouraud liquid medium can quench up to 95% of fluorescence, and sodium phosphate buffers hydrolyse fluorescein-diacetate. Fluorescent constituents present in crude plant extracts may also interfere (Clarke et al., 2001). In general, dilution methods are appropriate for assaying polar and non-polar extracts or compounds for determination of MIC and MBC/MFC-values. Using redox indicators or turbidimetric endpoints, dose–response effects allow calculation of IC_{50}- and IC_{90}-values, which are the concentrations required to produce 50 and 90% growth inhibition.

7.1.3. Bio-autographic methods

Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (a) direct bio-autography, where the micro-organism grows directly on the thin-layer-chromatographic (TLC) plate, (b) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (c) agar-overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison et al., 1991). Despite high sensitivity, its applicability is limited to micro-organisms that easily grow on TLC plates. Other problems are the need for complete removal of residual low volatile solvents, such as *n*-BuOH, trifluoroacetic acid and ammonia and the transfer of the active compounds from the stationary phase into the agar layer by diffusion. Because bio-autography allows localizing antimicrobial activities of an extract on the chromatogram, it supports a quick search for new antimicrobial agents through bioassay-guided isolation. However, this technique is not directly applicable in current high capacity screening designs.

7.2. Specific recommendations on antibacterial and antifungal screening

7.2.1. Panel of test organisms

The choice of test organisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferably used and should represent common pathogenic species of different classes. Various combinations are possible, but the panel should at least consist of a Gram-positive and a Gram-negative bacterium (Table 2). It has been well-established that Gram-positive bacteria are much more sensitive to drug action than Gram-negative bacteria, which is reflected by a higher number of random 'hits' during a screening campaign. Extracts with prominent activity against Gram-positive cocci should preferentially also be tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE), as they represent the greatest current medical need. ATCC strains are well-characterized and very popular for that purpose, but clinical field isolates may also be used if fully characterized by antibiogram. Another challenging new area in the microbiological world is biofilms (Mah and O'Toole, 2001). Although many bacteria grow in a free-living, 'planktonic' state, it is quite common for them to adhere to surfaces by producing extracellular polymeric substances (EPS), e.g. biofilms. Due to their higher resistance against antimicrobial agents, an interesting option in antibacterial research is to include a bacterial biofilm model (e.g. *Staphylococcus aureus* ATCC6538). A small set of reference fungi is used for primary screening and includes *Trichophyton mentagrophytes* and *Epidermophyton floccosum* as representatives of the dermatophytes. As opportunistic

Table 2
Proposed panel of test organisms for primary antibacterial, antifungal, antiviral and antiparasitic in vitro screening

Screen	Species	BSL[a]	Culture	CO_2[b]	Reference drug
Bacteriology (Gram-positive, Gram-negative, Acid-fast)					
- Gram-positive cocci	*Staphylococcus aureus*	2	Axenic in Mueller-Hinton	No	A broad selection of antibiotics is available (e.g. penicillin, ampicillin, norfloxacin, doxycyclin, kanamycin)
- Spore-forming Gram-positive rods	*Bacillus cereus* or *Bacillus subtilis*				
- *Enterobacteriaceae* – non-encapsulated	*Escherichia coli*				
- *Enterobacteriaceae* – encapsulated	*Klebsiella pneumoniae*				
- Non-*Enterobacteriaceae*	*Pseudomonas aeruginosa*				
- Acid-fast bacteria	*Mycobacterium* spp.	2 or 3	Axenic or cellular	No	e.g. Rifampicin
Mycology (yeasts and fungi)					
- Yeasts	*Candida albicans*	2[c]	Axenic in Sabouraud	No	Several broad-spectrum antifungals are available (e.g. amphotericin B, miconazole, ketoconazole, flucytosine)
- Dermatophytes	*Trichophyton mentagrophytes*, *Epidermophyton floccosum*				
- Opportunistic filamentous fungi	*Aspergillus niger*, *Fusarium solani*				
Virology					
- Single-stranded RNA, naked	*Coxsackievirus*	2	Cellular on VERO or other receptive cell	Yes	List of available reference drugs is limited. Acyclovir is used for Herpes virus.
- Double-stranded DNA, enveloped	*Herpes simplex virus*				
- Double-stranded DNA, naked	*Adenovirus*				
Parasitology					
- Malaria	*Plasmodium falciparum*	3*	Cellular (human RBC)	[d]	e.g. Chloroquine, artemether
- Leishmaniasis	*Leishmania donovani*	3*	Cellular (macrophages)	Yes	e.g. Amphotericin B, sodium stibogluconate
- Sleeping sickness	*Trypanosoma brucei*	2	Axenic HMI-medium	Yes	e.g. Suramin, melarsoprol, pentamidine
- Chagas disease	*Trypanosoma cruzi*	3	Cellular (MRC-5)	Yes	Nifurtimox, benznidazole

[a] BSL: biosafety level according to the European directive 2000/54/EC. Micro-organisms classified in BSL 3* may present a limited risk of infection for workers because they are not normally infectious by the airborne route.
[b] 5% CO_2 incubator.
[c] *Fusarium solani* is an opportunistic pathogen.
[d] Micro-aerophilic atmosphere (4% CO_2, 3% O_2, and 93% N_2).

filamentous fungi, *Aspergillus niger* and *Fusarium solani* are listed.

7.2.2. Growth medium

Mueller-Hinton (MH) agar or broth and tryptic soy agar or broth (TSA or TSB) are general growth media for bacteria, while Sabouraud (SAB) agar or broth is used for fungi. Growth of fastidious micro-organisms, such as *Streptococcus pneumoniae* and *Legionella pneumophila*, may require more complex media, enrichment of the incubation atmosphere with 5% CO_2 and/or extension of the incubation time. Slight differences in the composition of the growth medium can greatly affect the antibacterial activity of a compound. For example, addition of sheep blood to Mueller-Hinton medium increases the MIC of flavomycin from 0.12 to 256 mg/l (Butaye et al., 2000). Consequently, a definite choice of growth medium is essential to compare different antibacterial compounds or extracts. Mueller-Hinton medium allows good growth of most non-fastidious bacteria and is generally low in antagonists. It also meets the requirements of the NCCLS standard and is recommended as reference medium for agar- and broth-dilution tests (Anon., 2000, 2003).

7.2.3. Inoculum

The level of infection, i.e. inoculum concentration can have a profound influence on the antibacterial and antifungal potency

of a sample, endorsing the need for standardization of inoculates (Anon., 2003). In dilution methods, an inoculum of about 10^5 CFU/ml is adequate for most bacterial species while for yeasts and fungi between 10^3 and 10^4 CFU/ml is sufficient (Hadacek and Greger, 2000). A too low inoculum size (e.g. 10^2 CFU/ml) will create many false-positives, while a too high inoculum size (e.g. 10^7 CFU/ml) will hamper endpoint reading and increase the chances for false-negatives. Bacterial or yeast inoculates can be prepared from overnight cultures or from existing biofreeze stocks. It is recommended to collect from cultures during the logarithmic growth phase and always to take four or five colonies of a pure culture on agar to avoid selecting an atypical variant (Anon., 2003).

8. Antiviral assays

8.1. Overview of antiviral assays

Evaluation of antiviral activity in vitro includes antiviral efficacy and cell toxicity. Because different viruses grow in different cell systems, it is virtually impossible to design a single antiviral test that could be applied for all viruses. In addition, specific methodologies for assessment of antiviral agents may vary greatly from one laboratory to another, making direct comparisons of test results difficult. Various cell-based assays have been successfully applied for the antiviral or virucidal evaluation of single substances or mixtures of compounds, e.g. plant extracts (Table 3) (Vlietinck and Vanden Berghe, 1991). Antiviral agents interfere with one or more dynamic processes during virus biosynthesis and are consequently candidates for clinical use, whereas virucidal substances inactivate virus infectivity extracellularly and are rather candidates as biocides exhibiting a broad spectrum of germicidal activities. The methods commonly used for evaluation of in vitro antiviral activity are based on the different abilities of viruses to replicate in cultured cells. Some cause cytopathic effects (CPE) or form plaques. Others are capable of producing specialized functions or cell transformation. Virus replication can also be monitored by detection of viral products, i.e. viral DNA, RNA or polypeptides.

Table 3
In vitro antiviral screening options[a]

Test	Comment	Application
Plaque inhibition assay	Only for viruses which form plaques in suitable cell systems Titration of a limited number of viruses in the presence of a non-toxic dose of the test substance	A-S
Plaque reduction assay	Only for viruses which form plaques in suitable cell systems Titration of residual virus infectivity after extracellular action of test substance(s). Cytotoxicity should be eliminated, e.g. by dilution, filtration before the titration	V-S; V-M
Inhibition of virus-induced cytopathic effect (CPE)	For viruses that induce CPE, but not readily form plaques in cell cultures Determination of virus-induced CPE in monolayers, infected with a limited dose of virus and treated with a non-toxic dose of the test substance	A-S; A-M
Virus yield reduction assay	Determination of the virus yield in tissue cultures, infected with a given amount of virus and treated with a non-toxic dose of the test substance Virus titration is carried out after virus multiplication by the plaque test (PT) or the 50% tissue culture dose end point test (TCD_{50})	A-S; A-M
Endpoint titration technique (EPTT)	Determination of virus titer reduction in the presence of 2-fold dilutions of test compound	A-S; A-M

[a] Determination of the viral infectivity in cultured cells during virus multiplication in the presence of a single compound (A-S) or a mixture of compounds, e.g. plant extracts (A-M) or after extracellular incubation with a single compound (V-S) or a mixture of compounds (V-M).

8.2. Specific recommendations on antiviral assays

8.2.1. Panel of viruses

There is a great variety of viruses that can be selected for antiviral screening. The panel presented here has not been selected based on medical or clinical relevance but rather for practical feasibility in standard BSL-II laboratory infrastructure, easy culture on one cell type (VERO, MRC-5, e.a.) with formation of extensive CPE (Table 2). Their ability to multiply in the same cell culture allows an objective comparison of antiviral activities while toxicity testing can be minimized. Moreover, virus multiplication causes extensive CPE within about 1 week of infection, facilitating endpoint reading. The proposed virus battery includes HSV and adenovirus as representatives of DNA viruses and coxsackievirus as prototype of the RNA virus group. More specifically, coxsackievirus represents the *Picornaviridae*, which includes rhinoviruses and the enteroviruses. Titers up to 10^5 $TCID_{50}$/ml or higher are obtained, which markedly increases the sensitivity of the test system. The $TCID_{50}$ or 50% Tissue Culture Infective Dose is defined as that dilution of virus required to infect 50% of inoculated cell cultures. Finally, for most of these viruses suitable animal models are available, enabling in vivo confirmation.

8.2.2. Endpoints

An intrinsic component of the antiviral testing is the determination of a selectivity index (SI) towards the supporting host cell. The SI refers to the ratio of the maximum drug concentration causing either 50% or 90% inhibition of growth of normal cells (CC_{50}, CC_{90}) and the minimum drug concentration

at which 50% or 90% of the virus is inhibited (IC_{50}, IC_{90}). Reports of antiviral activity of extracts/compounds even at very low concentrations but without SI-data are of limited value.

9. Antiparasitic assays

9.1. Overview of antiparasitic assays

Contrary to the antibacterial, antifungal and antiviral test systems that are based on common test conditions and endpoints, bioassays for parasites are more exclusive since they tend to be highly species-specific (Maes et al., 2004). There are indeed numerous parasites for which new drugs are needed but it would be beyond the scope of this review to attempt to give recommendations for all. Therefore, only protozoal diseases that have been defined by the World Health Organization (WHO) as important health risks will be covered, i.e. malaria, African sleeping sickness, leishmaniasis and Chagas disease (Remme et al., 2002; Pink et al., 2005). These tropical diseases may gain in importance as part of the ongoing globalization of society and as the discrepancy between developed and developing countries will become more controversial. The proposed in vitro laboratory procedures and test designs (Table 2) can be implemented in laboratory settings with limited technical resources. As already mentioned, the primary screening model should be as sensitive as possible to enable it to pick-up weakly active compounds, but at the same time also recognize and eliminate false-positives. To improve on the performance of the in vitro models, the following design options should be considered:

- Use of well-characterized drug-sensitive parasite strains. Clinical isolates should preferably be reserved for secondary profiling of established 'hits' at a later stage. Care for selection should be taken in disease entities that can be caused by different parasite species. For example, different *Leishmania* species are available, but visceral species are to be preferred in view of their higher sensitivity to available reference drugs, the availability of validated models and because they represent a greater medical need. For sleeping sickness, it makes little difference whether *Trypanosoma gambiense*, *Trypanosoma rhodesiense* or *Trypanosoma brucei* are used, although the latter is preferred because its non-pathogenicity for man. For malaria, *Plasmodium falciparum* is the only species for which an in vitro model is available.
- Use of sensitive endpoint reading techniques. Evaluation of parasite multiplication and total parasite burdens can be performed using different methods, which are specific for each parasite species. For example, simple microscopic reading of Giemsa-stained preparations for determination of the parasite burdens can be used for malaria, leishmaniasis and Chagas disease. The results are expressed as % reduction of parasite burden compared to control wells and the IC_{50} and IC_{90} (50% and 90% inhibitory concentration) values are calculated.

9.2. Description of antiparasitic bio-assays

9.2.1. In vitro model for Leishmania

As already mentioned, the visceral species *Leishmania donovani* or *Leishmania infantum* are best suited for drug screening. The use of promastigotes must be discouraged, because of lower level of validation. To prepare the inoculum for infection, amastigotes are harvested from the spleen of infected donor hamsters. Murine peritoneal macrophages are generally used as host cell and are obtained after intraperitoneal stimulation with 2% starch in water. The peritoneal cells are harvested about 24–48 h later and plated in 96-well microplates at about 10^4 cells/well. After adding 10^5 amastigotes per well and 5 days of incubation, parasite burdens are microscopically assessed after Giemsa staining. The recent establishment of axenic amastigotes might allow the implementation of colorimetric or fluorometric methods without the need for host cells (Debrabant et al., 2004).

9.2.2. In vitro model for African trypanosomes

Because of the non-pathogenicity for man, a drug sensitive *Trypanosoma brucei* strain is preferred for primary screening purposes. If appropriate laboratory containment can be guaranteed, strains of *Trypanosoma gambiense* or *Trypanosoma rhodesiense* can be used as well. The bloodstream (trypomastigote) forms are axenically grown in Hirumi-9 (HMI) medium (Hirumi and Hirumi, 1989) at 37 °C under an atmosphere of 5% CO_2. Assays are performed in 96-well tissue culture plates, each well containing about 10^4 parasites. After 4 days incubation, parasite growth is assessed by adding Alamar Blue™ or resazurin for fluorimetric reading (excitation 530 nm; emission 590 nm) after 4 h at 37 °C (Raz et al., 1997).

9.2.3. In vitro model for Chagas disease

According to existing bio-safety guidelines, *Trypanosoma cruzi* is a biohazard class-3 pathogen and all laboratory work should be carried out under BSL-3 containment. Particularly strict safety precautions must be adopted (working in a LAF safety cabinet, wearing gloves, a safety mask and a laboratory safety gown). Extreme care has to be taken with needles to prevent puncture accidents. The Tulahuen strain of *Trypanosoma cruzi* is nifurtimox-sensitive and can be maintained on MRC-5 cells. Assays are performed in 96-well tissue culture plates, each well containing the compound dilutions together with 3×10^3 MRC-5 cells and 3×10^4 parasites. Recently, a strain transfected with β-galactosidase (Lac Z) gene has been developed enabling colorimetric reading after addition of chlorophenolred β-D-galacto-pyranoside as substrate (Buckner et al., 1996).

9.2.4. In vitro model for malaria

Of the four species that infect humans, only *Plasmodium falciparum* can be cultured in vitro. Several strains can be used and it is advised to include drug-sensitive as well as drug-resistant strains in the primary screening panel. Drug-sensitive strains are for example GHA, FCR3, NF54 and D6 that are susceptible to chloroquine, quinine and pyrimethamine. Drug resistant strains include W-2 and K1, known to be resistant to chloroquine, quinine and pyrimethamine, but susceptible to

mefloquine. The strains are maintained in continuous log phase growth in RPMI-1640 medium supplemented with 10% human serum and 4% human erythrocytes (Trager and Jensen, 1976). The human serum can also be replaced by lipid-rich bovine serum albumin (AlbuMAXII). All cultures are conducted at 37 °C under micro-aerophilic (4% CO_2, 3% O_2 and 93% N_2) atmosphere. The screening assay is an adaptation of the procedure described by Desjardins et al. (1979) and Makler et al. (1993) as the parasite lactate dehydrogenase assay. Assays are performed in 96-well tissue culture plates, each well containing the compound dilutions together with the parasite inoculum (1% parasitaemia, 2% haematocrit). After 72 h of incubation at 37 °C, plates are stored at −20 °C until further processing. After thawing, 20 μl of haemolysed parasite suspension from each well is transferred into another plate together with 100 μl Malstat™ reagent and 10 μl of a 1/1 mixture of phenazine ethosulphate (PES, 2 mg/ml) and NBT (Nitro Blue Tetrazolium Grade III, 0.1 mg/ml). The plates are kept in the dark for 2 h and change in colour is measured spectrophotometrically at 655 nm. Alternatives to the Malstat assay for quantification of parasite growth is [^{3}H]-hypoxanthine incorporation assay (addition of 0.2 μCi and reading with liquid scintillation counter after 24 h), the use of the DNA fluorochrome Picogreen® (Corbett et al., 2004) or simple microscopic reading of Giemsa-stained smears.

10. Cytotoxicity assays

Cytotoxicity on host cells is a very important criterion for assessing the selectivity of the observed pharmacological activities and must always be included in parallel. Although many cell types can theoretically be used for that purpose, MRC-5 cells are given as an example. They are cultured in MEM medium, supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$ and 5% fetal calf serum. Assays are performed at 37 °C and 5% CO_2 in 96-well tissue culture plates with confluent monolayers. After 4–7 days incubation, cell proliferation and viability is assessed after addition of Alamar Blue™ or resazurin (McMillian et al., 2002). After 4 h at 37 °C, fluorescence is measured (550 nm excitation, 590 nm emission).

11. Test validation and reference compounds

It is evident that each test should contain at least one reference drug to ascertain test performance and proper interpretation of the screening results. Several reference drugs are available that are specific for the test organisms used (Table 2). Those presented here are commercially available through fine chemical suppliers and should be preferred. In addition, each test should contain a number of replicates, preferably as independent repeats. A practical solution is to combine negative, positive controls and reference controls on the same microplate, still allowing different dilution schemes and dosage ranges (Fig. 3).

12. Criteria for 'activity'

In anti-infective in vitro models, the activity of extracts or compounds is generally expressed by numeric values (IC_{50}, IC_{90}, MIC, etc.), but correct interpretation of these efficacy variables is not at all easy because a profound knowledge of the model and the used protocol is required. Nevertheless, stringent endpoint criteria for 'activity' can be set, still taking selectivity into account. Relevant and selective activity relates to IC_{50}-values below 100 μg/ml for extracts and below 25 μM for pure compounds. For some micro-organisms, even more stringent endpoints should be considered. It is well-known that different pathogens have specific sensitivities to the aspecific or toxic action of chemical molecules. For example, a same cell type becomes much more sensitive for in vitro cytotoxic effects when cultivated in serum-free conditions; Gram-negative bacteria are much less sensitive to 'drug action' than Gram-positive species and in random antiprotozoal screens, a consistent observation is that free-living organisms (for example, *Trichomonas* spp.) are much more robust than the blood protozoa *Plasmodium*, *Leishmania* or *Trypanosoma*. Among the latter, *Plasmodium* tends to be the most sensitive.

13. Rules of thumb for defining anti-infective potential in natural products

1. Identification and documentation of the plants (voucher specimen and number) and preparation and storage of extracts need to be reported. In a bioassay-guided fractionation, a scattered activity across the different fractions is mostly indicative for non-selectivity.
2. Test organisms are preferably ATCC strains as they are widely used and well-characterized. Defined clinical isolates may also be used, but only if the susceptibility to relevant reference compounds is reported.
3. In vitro models using the whole organism are the 'golden' standard and should be used whenever possible. If micro-organisms can be cultivated either in cell-free medium ('axenic') or in cell cultures, the latter one is preferred since it resembles more the in vivo situation.
4. Antimicrobial activity must be discriminated from non-specific toxicity by inclusion of a parallel cytotoxicity evaluation on mammalian cell lines or integration into a panel of unrelated microbial screens (bacteria, fungi, parasites, viruses).
5. Total extracts and derived primary fractions exhibiting strong non-selective action in the panel of in vitro screens can only be properly evaluated in animal models.
6. Extended dose ranges with at least three doses are needed for establishing representative dose–response curves. Descriptive values are IC_{50} and IC_{90}. MIC and MBC-values are also common endpoints in antibacterial and antifungal screening.
7. To correct for too many false-positives, stringent endpoint criteria must be adopted. For all anti-infective bioassays, IC_{50}-values should be below 100 μg/ml for mixtures and below 25 μM for pure compounds. Some micro-organisms even require more severe endpoint criteria.
8. A universal panel of test organisms does not exist and is largely determined by the specific drug-finding objectives and the adopted plant selection criteria. Taking into account

the sometimes broad traditional ethnomedical use, a minimum panel of test organisms is recommended (Table 2).

9. Inclusion of appropriate controls in each test replicate (blank-, infected- and reference controls) is necessary. For each test organism, one or more commercially available reference compounds can be considered (Table 2).
10. Differences in composition of the growth medium can greatly affect the potency of a compound. Mueller-Hinton is recommended for antibacterial and Sabouraud for antifungal testing in agar and/or broth dilution tests. For fastidious bacteria, supplementation with growth factors or other media are allowed.
11. The infective dose can have a profound impact on the test results. For most bacteria, 10^5 CFU/ml is adequate, while for yeasts and fungi about 10^3 and 10^4 CFU/ml is satisfactory. For viruses and parasites, optimal infective doses are species-specific.
12. For antibacterial and 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. For essential oils, addition of solvents or emulsifiers may be necessary, but their final concentrations should be limited and reported.
13. The phenomenon of 'additive' or 'synergistic' effects in mixtures or extracts frequently causes loss-of-activity during bio-guided fractionation efforts and hence precludes the identification or characterization of the relevant fraction that could be retained for further evaluation. In that case, further studies should focus on the defined crude extract.

Acknowledgements

Paul Cos is a postdoctoral researcher of the Fund for Scientific Research (FWO-Flanders, Belgium). The integrated screening concept for tropical parasitic diseases was developed with the support of UNICEF/UNDP/World Bank/WHO special programme for research and training in tropical diseases.

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