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Working with Natural Products (Extracts): Certain Useful Suggestions to Avoid Trouble

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Bioactive natural products is an important area of research, and a large number of researchers are engaged in screening natural products (crude extracts of natural origin) for desired bioactivity(ies). This article discusses some of the issues usually encountered in this area of research, and how they can be tackled.

Introduction

The trend in the current world is to *go green*. With respect to medicine, nutraceuticals, and cosmetics, it means to search for novel bioactive preparations from natural sources. A considerable number of researchers are engaged in natural product research, wherein they screen a huge number of extracts of plant or marine or microbial origin for the desired bioactivity. Over past 8 years our lab has been involved in screening of plant extracts for antimicrobial activity against drug-resistant human pathogenic microbes and phytopathogens, with a special focus on their ability to inhibit quorum-sensing in bacteria, and to kill bacterial cells in biofilms. In this article, I describe the issues frequently encountered during this type of work, and how some of them can be tackled. Scientists already having sufficient experience in this field are likely to be aware with such troubles and would have handled such problems in their own innovative ways, but this article is mainly targeted to those who are relatively new to the field or are just preparing to enter this exciting area of research.

Selection of an Appropriate Extraction Method

The primary aim of any extraction protocol in general, is to achieve high extraction efficiency. But it should be realized that high extraction efficiency is not always associated with high efficacy. Extraction methods employing heat (e.g. Soxhlet) may be good with respect to extraction yield, but they carry the inevitable risk of degradation of thermolabile constituents. It is worth considering the use of newer methods such as Microwave Assisted Extraction (MAE), ultrasound based extraction, extraction under reduced pressure, etc. which do not require extended heating of the material to be extracted. In a comparative study (Kothari et al., 2012) of various methods for extraction of antioxidant and antibacterial compounds from plant seeds, we found Soxhlet method to be the best in terms of high extraction efficiency, and extraction of phenolic compounds. Microwave assisted extraction with intermittent cooling, room temperature extraction by shaking, and ulrasonication assisted extraction (UAE) proved good at extracting antibacterial compounds from plant seeds. Latter also proved effective at extracting antioxidant compounds. Extraction efficiency was found to have no notable correlation with any of the parameters assayed. Methanol proved most suitable solvent for extraction of flavonoids. Selection of an extraction method or solvent, which are not the most appropriate options, may cause a researcher to miss the identification of a particular bioactivity in the given natural preparation, even when it is there waiting to be detected.

Ineffective reconstitution may again reduce the possible benefits offered by high extraction yield. To make the reconstitution of the dried extract effective, shaking or mild heat may be applied to increase the reconstitution efficiency. Sometime shift from one method to another (or from one solvent to another) can solve the problem. For example, when *Tamarindus indica* seeds are extracted in polar solvents (e.g. methanol or ethanol), after drying it forms a smooth shiny film (rather than becoming dried powder), which makes it difficult to be reconstituted into any solvent for bioassay. In such cases, one needs to do trial and error with multiple solvents and/or extraction methods, so as to find out which will offer minimization of film formation.

Precipitation of the Test Preparation in the Assay Medium

Usually the test materials (e.g. plant parts) are extracted in water or different organic solvents, and dried. The dried extracts are then reconstituted in an appropriate solvent (most commonly water or DMSO) for the bioassay. But many of these extracts will get precipitated in the complex media (for example the Muller-Hinton broth widely employed in antimicrobial susceptibility assays) used for the bioassay. Precipitation problem is more likely to occur if the extraction is carried out in a non-polar solvent, and then the resulting extract is put into a water-based medium (invariably all biological media will

be water-based only) for assay. If this precipitation cannot be avoided, then it simply becomes impossible to work with such extracts. To solve such a problem, two different approaches can be tried. One is to employ lower concentrations of the test extract, as some of the extracts get precipitated in a given medium only at higher concentrations, but not at lower ones. If the desired biological activity is exerted by the extract at sufficiently low concentration without precipitation, then this approach can work. Another approach is to use a minimal medium with little or no organic ingredients in it. Mostly the precipitation problem is due to binding of the few of the constituents of the extract with some of the organic medium ingredients. However, this approach will not work if the test organism (or cells) are fastidious with respect to their nutritional demands (e.g. The fungus *Malassezia furfur* will require supplementation of the growth medium with oil). One must always bear in mind that precipitation is bound to reduce the available concentration of the test compound in the assay system.

While working with bacterial biofilms, usually the sugar-rich organic media are employed for biofilm formation (as with *Streptococcus mutans*). If the test preparation is getting precipitated in such a medium, then one can first form the biofilm using this complex medium (containing no amount of the test antimicrobial), and then once the biofilm has been formed, the medium can be decanted from the microtiter plate (in whose wells the biofilm has been allowed to form). Following this the test antimicrobial preparation dissolved either in water or a minimal (inorganic) medium can be added to the biofilm containing wells. This strategy of using two different media (one for biofilm formation, and another for dissolving the test antimicrobial) will allow testing of the potential antimicrobial agent without getting a chance to precipitate.

Precipitation may occur even during storage of the extract at low temperature. This is because the solvation power of the solvent in which the dried extract is reconstituted may get compromised at low temperature (during storage under refrigeration). This makes it important to bring the extracts at room temperature before using them for a bioassay. Though it seems trivial, students sometimes may not show enough patience to wait till it comes to the room temperature.

Dose Independent Response

It is relatively straightforward to interpret results of a bioassay where the response of the test organism is having a linear correlation with the concentration (dose) of the test substance (extract). But quite a few times, results are obtained where test organisms respond in a dose-independent fashion i.e. the higher doses may prove less effective than the lower doses. If a novice encounters such results, it may happen that he will doubt his experimental set-up, and get prompted to repeat the experiment with the hope for a linear response. Though it is not inappropriate to doubt the dose-independent type of results (they do need to be confirmed for their reproducibility, as any other results), one should be aware that while screening or evaluating the natural products such results may be obtained, and they should be interpreted properly. In case of antimicrobial susceptibility assays, such paradoxical results are described as *eagle effect* (Shah, 1982), first noticed by Eagle and Musselman in 1948.

Post Extract Effect

While evaluating the antimicrobial potential of the crude preparations, once the organism has been incubated in a medium containing such test preparation, plating usually is done on (antibiotic free) solid media to check whether the test preparation is microbicidal or microbistatic. Absence of growth after a particular length of incubation is taken as an indication of the cidal effect. However, incubation over an insufficient length of time may be misleading. It becomes necessary to differentiate the true microbicidal effect from the *post antimicrobial effect*. The latter refers to the persistent suppression of microbial growth after exposure to antimicrobial agents, and may last for several hours depending on the concentration of test agent and the susceptibility of the target organism. Identification of the agents exhibiting a post antibacterial effect (PAE)/post antifungal effect (PAFE) requires extended incubation following subculture in either time-kill or minimum lethal concentration (MLC) determinations in order to ensure the detection of slow-growing but not dead organisms (Pfaller et al., 2004). Such effect of commonly used antibiotics termed as post antibiotic effect (PAE; Ramadan et al., 1995) or post antibacterial/post antifungal effect (Pfaller et al., 2004) has been reported earlier in literature. With respect to natural extracts, it can be described as *post extract effect* (PEE; Ramanuj et al., 2012).

Antimicrobial Susceptibility Assays with Anaerobic Microbes, Molds, and Microbial Biofilms

To make valid comparison of results of different antimicrobial susceptibility assays, it is desirable that laboratories across the globe employ a single standardized methodology (e.g. a particular defined medium, inoculum density, incubation temperature and time, etc.). For the relatively non-fastidious (or less fastidious) pathogens like *Escherichia coli, Pseudomonas spp., Staphylococcus aureus,* etc. internationally accepted guidelines (e.g. those available from NCCLS) are available. However, while performing susceptibility assays with anaerobic bacteria, filamentous fungi, or microbial biofilms, such universally accepted guidelines either are not available, or the available methodologies are not applicable to all the test organisms. In case of fastidious organisms, a single medium can not be recommended which will

support growth of almost all test organisms (as the Muller-Hinton medium is able to support growth of a wide variety of common bacterial pathogens). Different organisms will have varying requirements (in terms of the type of surface provided, medium composition, length of incubation, etc.) for biofilm formation. With filamentous fungi, inoculum standardization also presents a challenge.

In case of slow growing organisms like fungi, relatively longer incubation (48 h or more) is required, this may give rise to the problem of evaporation of medium during incubation. To solve this, microtiter plates designed to minimize evaporation can be useful, or one may consider the option of using somewhat larger capacity wells. Alternatively fresh medium can be added at some time in between incubation (under sterile condition, of course!). Evaporation is usually maximum from the wells at the periphery of a plate, so either those wells can be kept unused, or parafilm can be used to seal the plates during incubation.

Necessity of Putting Appropriate Negative (Abiotic) Controls in Bioassays

While evaluating the natural extracts, they are reconstituted in some solvent (e.g. DMSO, ethanol, methanol, water, etc.). These solvents themselves may exert some effect on the test organism (or cells or animals; Houghton and Raman, 1998). To nullify this effect of the solvent *per se*, and to differentiate it from the effect of extract dissolved in it, it becomes crucial to include appropriate negative controls in the bioassay, where the test organism is under the influence of the solvent only, without any test substance dissolved in it. We (and other workers too) have found that the commonly used solvents can have a significant effect on microbial growth (Wadhwani et al., 2009). While screening the natural extracts for anti-quorum sensing property against bacteria, it should be kept in mind that these solvents may have some effect on bacterial quorum-sensing, without affecting bacterial growth. Our yet unpublished results show that such organic solvents can affect quorum-sensing regulated violacein production in *Chromobacterium violaceum*. Latter is a commonly used model of bacterial quorum-sensing.

Abiotic controls becomes of even more importance in case of coloured extracts (which is the case with most extracts), as while measuring optical density, one has to nullify the contribution of the absorbance due to extract itself.

Reporting the Effective Concentration as a Range Rather Than as a Single Value

Most of the times a given crude natural extract will exert the required level of bioactivity at a particular concentration, but at other times it may be a narrow concentration range (e.g. 15-20 µg/mL) within which the desired level of bioactivity is achieved. In such cases, one should not hesitate in reporting the effective concentration range, rather than keep trying to find a single concentration value. The latter may seem to be easier to explain and accept, but if the results within that narrow concentration range are reproducible, then there should be no hesitation in accepting them, neither on the part of the experimenter himself nor on the part of the reviewers.

I hope the opinions expressed in this article may provide useful working tips to new entrants in the field of bioactive natural products. It will be still better, if it can induce other experts in this area to share their laboratory experiences.

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