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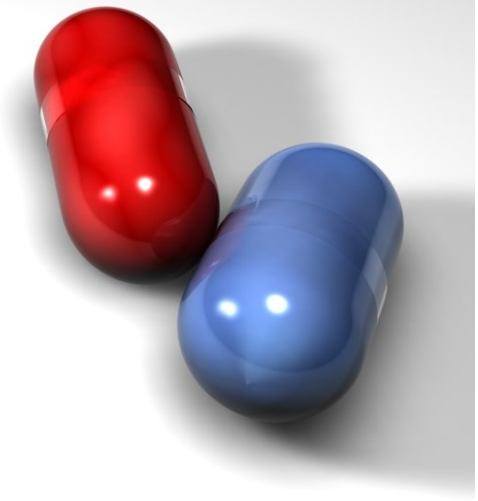

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Letter to the Editor

Improved mass screening of medicinal plants for antibacterial activity by leaf disc antibacterial assay

Sir,

The conventional method of testing antimicrobial activity of a plant includes the preparation of crude extract using organic solvent, separation, and purification of different compounds, and *in vitro* testing for antimicrobial activity by various methods. The whole process is an expensive, laborious and time-consuming, which often involves the use of large quantities of expensive chemicals (mainly solvents) and instruments. These problems demand an easy and efficient method of screening a large number of plants in a short time with minimum consumables and expenditure. Therefore, developing a simple, cost-effective, high throughput screening of plants for the antibacterial property is important for drug discovery.

Leaf samples in the form of discs were used in laboratory investigations such as transformation studies, to measure the physicochemical parameters of leaves, to monitor the biochemical process such as photosynthesis, to cultivate microbes and insects, to study the plant-microbe interaction and for gene expression studies. Similarly, leaf discs were also used (Case, 2001; Ndi 2007) to determine the antibacterial properties of plants. However, the method is in its basic form, and the data is not widely available in major scientific databases because of its informal publication. In addition to this, Ndi (2007) also highlighted some of the disadvantages of leaf disk diffusion assay. In this context, additional investigations are necessary to improve this assay for screening a large number of samples in a short time. Therefore, the objective of the present study was to improve the assay for good laboratory performance and for wider dissemination.

Three medicinal plants namely *Syzygium cumini, Punica granatum, Moringa oleifera* were selected for the present study. Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) was used as a test organism. The screening experiment was carried out in a 150 x 15 mm petri dish containing sterile Mueller-Hinton Agar (HiMedia Laboratories, Mumbai).

Fifty-nine plants species collected from the Yenepoya campus (12º48′42.56″ N 74º52′53.20″ E), Mangalore, Karnataka, India were successfully screened for their antimicrobial properties using this method. Initially, healthy whole leaves were washed with running tap water to remove any dirt and later surface sterilized by dipping in 70% ethanol for 1 min. Subsequently, the leaves were washed 5-6 times with autoclaved distilled water to remove the traces of ethanol, if any. Thus, sterilized leaves were air dried inside the biosafety cabinet for 30 min before use, and leaf discs (8 mm ⌀) was punched out from the leaf lamina using a sterile cork borer. To optimize the process and or to enhance the infiltration of phytochemicals into the surrounding media different types of pretreatments, alone or in combination were attempted. The pretreatment includes; a) Steaming: both the surface [i.e., astomatous (adaxial) and stomatous (abaxial)] of leaf disc (2.5 min on each side) on a sterile stainless steel mesh, b) Pricking: the leaf discs using sterile teasing needle, c) Solvation: flooding the leaf discs with 5 µL of 1% dimethyl sulfoxide (DMSO). Pretreated (a & b) leaf discs were placed on sterile Mueller-Hinton Agar media seeded [the inoculum of MRSA was prepared using a colony from the log phase of growth (18 hours) suspended in sterile normal saline] with MRSA. The addition of solvent (5 µL of 1% DMSO, pretreatment c) was carried out after placing the disc on the seeded media. The whole procedure was carried out inside the laminar air flow cabinet, under sterile conditions. Plates were incubated aerobically at 35-37°C for 24-48 hours in an inverted position. A separate study was also carried out for comparison purpose with the present method, where leaf discs were treated with three solvents namely methanol, ethyl acetate, and *n*-hexane to assess the extractability of phytochemical by the selected solvents (Table I). In this study, 5 µL of respective solvents were flooded on leaf discs, and the same volume of solvents was added to the well dug in the same agar media as a solvent control (to determine the solvent-induced toxicity on the test organisms).

The presences of antibacterials in the given plants were evaluated based on the zone of inhibition around the leaf disc and were expressed in millimeters. The experiments were carried out in triplicate. Differences among treatments were assessed with one-way analysis of variance (ANOVA) followed by LSD post hoc test.

The maximum zone of inhibition obtained was 15.8 ± 1.0, 17.7 ± 0.6 and 10.3 ± 0.6 for *S. cumini, P. granatum,* and *M. oleifera* respectively for the same pretreatment i.

e., steaming, pricking and flooding the leaf disc with 1% DMSO (Figure 1). Analyses revealed significant variation in the zone of inhibition for both *S. cumini* (p=0.032) and *P. granatum* (p=0.013) when compared to the control, i.e., leaf disc without any pretreatments. Similarly, the highest zone of inhibition obtained for *M. oleifera* was also in the same pretreatment. However, it approximated but did not reach statistical significance (p=0.118).

The diffusion of phytochemicals was visible in the form of color change around the discs (pale yellow or pale brown). Multiple inhibition zones or different degree of diffusion of phytochemicals was observed around a few discs (Figure 1; arrow heads); probably it was due to the migration of phytochemical with the difference in their polarity. Some of the inhibition zones were clear, distinct and well demarcated. However, the zones were translucent or diffusive around a few discs.

The physicochemical pretreatment of leaf disc significantly influences the release and diffusion of phytochemicals responsible for antibiosis when compared to the leaf disc without any treatments. Steam loosens and softens the plant matrix, thereby releasing the phytochemicals from plant tissues (Ameer et al., 2013). Puncturing the leaf disc surface enhances the release (volume) of phytochemicals effectively from both the surfaces, in addition to the edges. DMSO is a pharmaceutically accepted 'solvent excipient' (McKim and Strub, 2008) which act as a diluent and or vehicle for the easy diffusion of bioactive phytochemicals in the agar media. The zone of inhibition observed for (leaf disc +) various organic solvents in the present study was comparatively lower than the pretreated leaf discs (i.e., steaming, pricking and flooding with 1 % DMSO). In addition, inhibition zones were noticed for solvent controls (methanol and ethyl acetate). This shows the solvent-induced toxicity on the test organism (Rekha et al., 2006). However, the present method is devoid of such harsh solvents instead DMSO was used, for the reason that it is a widely used solvent in the screening experiments because of its least toxicity (Basch and Gadebusch, 1968).

Microbial contamination is common in *in vitro* culture techniques due to phylloplane flora. This issue was not addressed in the previous works (Case, 2001; Ndi, 2007), which is an important criterion for the contamination-free screening of leaf samples. Sodium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide, etc. (Mihaljević et al., 2013) are normally used for surface sterilization of plant explants before introduction into the sterile media. Similarly, in the present study, the leaves were surface sterilized using 70% ethanol for 1 min. The duration of incubation can be extended depending on the size and thickness of leaf disc, place of sample collection, etc. Rapid evaporation of ethanol and repeated washing of leaf samples will remove the traces of ethanol, thereby preventing the ethanol-induced toxicity on test organisms. Detergents and other toxic chemicals were not used as sterilizing

Table I

Zone of inhibition observed for different pretreatment in medicinal plants

Pretreatments		Medicinal plants		
		Syzygium cumini	*Punica granatum*	*Moringa oleifera*
		Zone of inhibition[a] (in mm)		
Leaf disc without any pretreatments (control)		13.7 ± 0.6[b]	15.3 ± 1.5[b]	7.8 ± 0.3[b]
Leaf disc + 1% DMSO		14.0 ± 1.0[b]	14.0 ± 0.0[b]	7.0 ± 1.0[b]
Leaf disc + 5 min steaming (2.5 min each side)		14.7 ± 0.6[b]	15.8 ± 2.0[b]	9.7 ± 0.6[b]
Leaf disc + 5 min steaming (2.5 min each side) + 1% DMSO		15.2 ± 0.8[c]	17.0 ± 2.0[b]	9.3 ± 1.2[b]
Leaf disc + 5 min steaming (2.5 min each side) + Pricking + 1% DMSO		15.8 ± 1.0[c]	17.7 ± 0.6[b]	10.3 ± 0.6[c]
Agar well + Methanol (solvent control)		11.3 ± 2.1[b]	6.3 ± 0.6[b]	6.0 ± 1.0[b]
Agar well + Ethyl acetate (solvent control)		12.3 ± 0.6[b]	0.0 ± 0.0[b]	5.7 ± 0.6[b]
Agar well + *n*-Hexane (solvent control)		0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Leaf disc + 3 solvents (Polar, mid polar, non-polar)	Lead disc + Methanol	14.0 ± 1.0[b]	15.7 ± 0.6[b]	9.7 ± 1.2[b]
	Lead disc + Ethyl acetate	10.7 ± 3.1[b]	15.3 ± 0.6[b]	9.7 ± 1.1[b]
	Lead disc + *n*-Hexane	15.0 ± 1.0[b]	14.0 ± 1.0[b]	9.7 ± 0.6[b]
Leaf disc + Pricking + 3 solvents (Polar, mid polar, non-polar)	Lead disc + Methanol	15.0 ± 0.0[c]	15.0 ± 1.7[b]	9.3 ± 1.2[b]
	Lead disc + Ethyl acetate	14.3 ± 0.6[b]	15.0 ± 0.0[b]	9.6 ± 0.6[b]
	Lead disc + *n*-Hexane	15.3 ± 0.6[c]	15.3 ± 0.6[b]	7.3 ± 6.4[b]

Data are mean ± SD; Superscript 'a' means significant ($p<0.05$); Superscript 'b' means insignificant ($p<0.05$) when compared to control

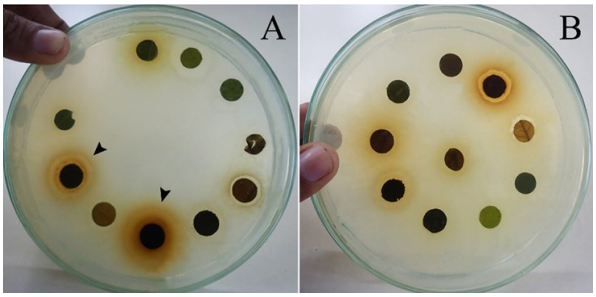

Figure 1: Petri plates (A) and (B) demonstrate screening of various plant species for their antibacterial activity by leaf disc antimicrobial assay. The diffusion of phytochemicals can be seen in the form of color change around the discs (pale yellow or pale brown). The zone of inhibition varied from disc to disc and can be seen around few discs indicating the positive results

agents, as avoiding the inhibitory effect on the test organisms and or to prevent false positive results due to the traces of surface sterilizing agents.

The theory behind the formation of the zone of inhibition may be compared to that of the standard Kirby-Bauer disc diffusion assay (Bauer et al., 1966), where the antibiotic diffuses away from the disk into the medium and inhibits the bacterial growth. The sensitivity/resistance of the test organisms is determined by the presence/absence a clear halo around the disk and is expressed as a zone of inhibition around the disc. Similarly, in the present work, simple diffusion of the phytochemicals are thought to be responsible for the anti-MRSA activity. Therefore, the improved method superficially resembles the standard Kirby-Bauer disc diffusion assay, however different in several aspects. Kirby-Bauer assay makes use of dried filter paper disks impregnated with specific concentrations of known purified antibacterials. However, the present method is different in the following features, a) It is a preliminary screening method only, to determine the antibacterial principles of plant species, b) this assay will reveal the antibacterial activity of unknown, heterogeneous compounds and the antibiosis may be due to single or synergistic activities of several compounds, c) the purified compound obtained in the conventional method by the several stages of lengthy, time-consuming procedure (solvent extraction, chromatographic separation, purification, etc) is not necessary in the present method, d) the phytochemical are tested in its native form without undergoing much changes in the chemical structure in the present study, otherwise introduced (modification/losses due to thermal degradation and oxidation) in conventional methods during the extraction process (De Castro and Garcia-Ayuso, 1998), e) the conventional methods make use solvents of particular polarity for the isolation of compounds, and there is a chance to miss the compounds of interest due to inappropriate solvent selection, which can overcome the present method.

Case (2001), was the first to demonstrate agar diffusion assay for testing the antimicrobial activities of plants using leaf disc. Later, Ndi (2007) used the same method (leaf disk diffusion assay) for preliminary screening of plants against different bacterial species. Ndi's (2007) experiments were based on the concept (secretion of phytochemicals through the cut edges of leaf disc, into the surrounding agar media, which may affect the growth of seeded microorganisms in the agar) of Ana et al., (2002). However, these assays were not widely used, probably due to the unavailability of the literature i.e., grey literature. Apart from these, the Green chemistry (Anastas and Warner, 1998) aims to reduce and or eliminate the use and generation of substances hazardous to human health and the environment. As a result, it plays a major role in sustainable development. Similarly, any progress in the area of biotechnology has its basis in the principles and policies of green chemistry. Therefore there is a need for promoting the application of the principles of green chemistry in different areas of scientific research including analytical science. Therefore, in the present investigation, the antibacterial leaf disc assay was improved for good laboratory performance. A large number of plants can be screened in a short time with minimum consumables, and the results can be obtained in 24-48 hours. This method offers one of the significant advantages over the conventional method in terms of organic solvent consumption. The other important feature is that it enables the selection and or elimination of plants in the early stages of screening, which can greatly save time, chemicals, labor, etc. It is a simple, inexpensive, efficient and eco-friendly screening method for plant antibacterials.

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