Advances in Biological Research 5 (5): 241-247, 2011
ISSN 1992-0067

Should Solubility and Zone of Inhibition Be the Only Criteria for Selection of Solvent in Antimicrobial Assay?

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Abstract: Effect of different solvents; dimethyl-sulfoxide (DMSO), dimethyl formamide (DMF), methanol, tween 80 and acetone on morphology, cytology and reproduction of *Aspergillus flavus, A. parasiticus, A. fumigatus* and *A. niger* was assayed for standardization of a suitable organic solvent for test/antimicrobial compound in antimicrobial assays. Comparison of antimicrobial sensitivity methods was also made. Inhibition of pigmentation, conidia germination and dichotomous branching in conidiophores was observed in all test fungi after treatment with DMSO and DMF. Increased mycelial width, conidia size, conidiophores size was found in presence of tween 80. It was observed that culture was more sensitive to solvents in dilution method as compared to diffusion method. Results showed that standardization of solvents could be achieved through screening their effects on various parameters such as morphology, cytology and reproduction of different organisms preferably prior to assay of antimicrobial properties of a compound.

Key words: *Aspergillus* spp. · Agar well diffusion · Broth dilution · Growth inhibition · Morphology · Reproduction

INTRODUCTION

In vitro antimicrobial sensitivity screening during search of novel antimicrobial agents from various sources such as plant extracts, essential oils and synthetic compounds is commonly done by diffusion (agar well diffusion and disk diffusion) and dilution (Broth dilution and agar dilution) methods [1]. As most of the test molecules are insoluble in water, diffusibility of the test compound is generally enhanced by use of suitable organic solvent. Dimethyl-sulfoxide (DMSO) and N, N-dimethyl-formamide (DMF) are commonly used as most organic molecules, carbohydrates, peptides, inorganic salts and gases are soluble in these solvents [2-4]. Apart from these acetone, methanol and tween 80 are also frequently used [5, 6] solvents. Further in most studies, the antimicrobial activity is assayed only by measuring the zone of inhibition produced [7, 8]. Since, most of the organic solvents used exert an inhibitory effect on the test organism [9-11]; antimicrobial activity of test compound is usually determined by subtracting the zone of inhibition produced by solvent from the zone of inhibition given by mixture of compound and solvent [12].

As the toxicity of solvent is organism/species specific [13, 14] and many reports suggest that commonly used solvents such as DMSO, acetone etc. are mutagenic [15] and induce membrane damage, cause formation of abnormal structures etc. in test organisms [16, 17]; large number of solvents against large number of organisms should be screened prior to *in vitro* antimicrobial testing for standardisation of solvent. Also, instead of single criteria i.e. zone of inhibition, other parameters should also be studied as some of the solvents may not show any direct effect on growth but may seriously affect the cytomorphology [18], reproduction, metabolic activity etc. of the test organism. Standard methods must be employed to generate adequate data to indicate the toxicity of respective solvent on the test organism.

Most antimicrobial assays are performed using *Aspergillus* as an indicator strain in antimicrobial assay since *Aspergillus* sp. is responsible for life threatening infections such as invasive aspergillosis etc. [19]. Hence in the present investigation; a comparative study of inhibitory effect of different solvents viz. DMSO, DMF, methanol, acetone and Tween 80 was assayed on the growth inhibition, cytomorphology and reproduction of

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Aspergillus spp by agar well diffusion and broth dilution methods with an aim to standardize the most suitable method and solvent to be used for antimicrobial assay.

MATERIALS AND METHODS

Organic Solvents: Analytical grade DMSO (Central drug house, Mumbai), DMF, methanol, acetone and tween 80 (Sara Fine Chemicals) were used for evaluation of effect on test fungi.

Test Fungi: Aspergillus flavus Navjot 4NSt, *A. niger* (procured from Department of Botany, BHU, Varanasi), *A. parasiticus* (MTCC 411) and *A. fumigatus* (MTCC 2550) (procured from IMTECH, Chandigarh) were used in the experiment. Fungal strains were maintained on Sabourd Dextrose Agar (SDA, Difco). 1×10^7 spores /ml spore suspension was prepared from 7 day old culture by washing the surface of cultures with sterile distilled water and used as an inoculum in agar well diffusion as well as broth dilution method.

Assays of Fungi Inhibitory Activity: Both agar well diffusion and broth dilution methods [20] were used to screen the antifungal activity of solvents. In agar well diffusion, 12 mm wells were bored in SD agar plates inoculated with 10 µl spore suspension (1×10^7 spore ml^{-1}) and were filled with 250 µl of 80, 50 and 10% concentration of respective solvent. In broth dilution method; 10, 50 (prepared by mixing the 1.25 ml and 6.25ml of 80% solvent with 8.75 ml and 3.75 ml of SD broth respectively) and 80% concentrations of solvent were used. Sterile distilled water was used as control. The plates and tubes were incubated for 7 days at 28 ± 1°C. Three replicates of the experiment were maintained. Zone of inhibition and mycelium weight of agar and broth cultures were measured respectively to observe the effect on growth.

Study of Effect of Solvent on Morphology of Vegetative and Asexual Reproductive Structure of Test Fungi: Microscopic observation of changes in mycelium width, conidia number, conidia size and vesicle size was done after 7 days of incubation with the test solvent using Olympus trinocular research microscope BX- 51 and image analysis software Olysia Bioreport 3.2.

Study of Effect of Solvents on Spore Numbers: To observe the effect of solvent on spore numbers, 10µl (1×10^7 spore's ml^{-1}) spore suspension of test fungi was inoculated onto plates containing SDA with and without organic solvent and incubated for 7 days. Spores were collected by flooding the plates with 3 ml distilled water and scraping the surface of the agar with a sterile glass rod. This procedure was repeated twice and both the fractions were mixed and serially diluted 4 times. The number of conidia was counted using a haemocytometer. Total number of conidia was calculated by following formula:

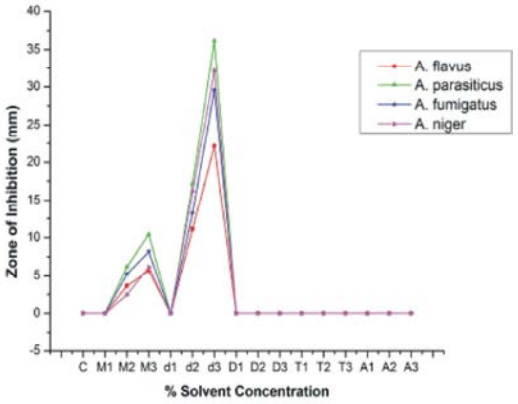
Number of spore /ml = N × 1/ 0.0025 × Dilution of Sample.
Where N = Average no. of conidia per small square.

Study of Effect of Solvent on Spore Germination: Effect of solvents on spore germination was also observed. Spores were collected from the 7 day old cultures (control and treatment) of test fungi by scraping the surface of culture plates with 10 ml sterile distilled water. Number of spores in suspension was calculated by the method discussed above and set to100 spores/ml by serial dilution. 1 ml of spore suspension was inoculated in 1 ml SD broth and the tubes were incubated for 8h at 28±1°C. Percent of spore germination was determined microscopically. A spore was considered germinated when the germ tube length was equal to or greater than half of the spore diameter [21]. Germinated spores were counted and recorded as % of total spore number.

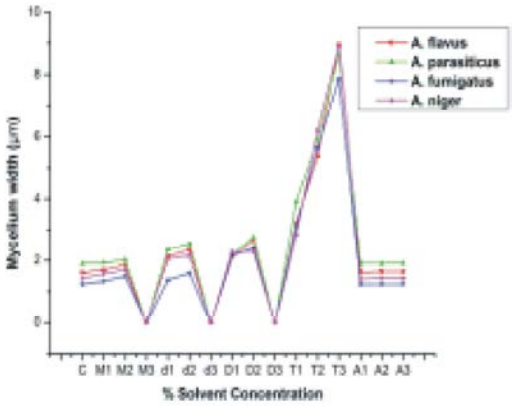
RESULTS AND DISCUSSION

Among the tested solvents, highest zone of inhibition was found with DMF followed by methanol. There was no zone of inhibition in case of DMSO, tween 80 and acetone. Among the test fungi, *A. parasiticus* was found most sensitive to both methanol as well as DMF as highest zone of inhibition was found, followed by *A. niger*, *A. fumigatus* and *A. flavus* (Fig. 1A). This observation confirms the species specific effect of a solvent. In broth dilution method (Fig. 1 B), highest inhibition of mycelial weight was observed with DMF followed by methanol and DMSO. No change observed in mycelial weight after treatment with acetone and tween 80.

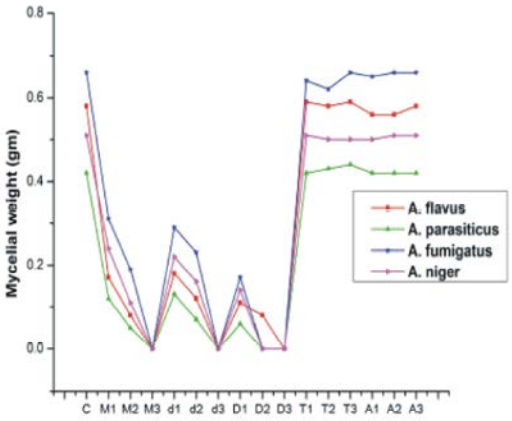
Results also show (fig. 1 A and B) that the toxic effect of DMF and methanol was concentration dependent as the zone of inhibition increased with increasing concentration. In broth dilution method, similar results were observed with DMF, methanol and DMSO and the order observed was 80%>50%>10%. This concentration dependent activity suggests that the solvent molecules act as extra cellular signals. It could be assumed that as the concentration of the solvent



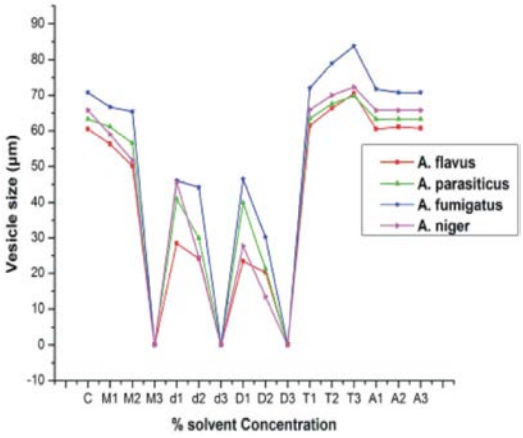
A. Inhibitory effect of various concentrations of orgainc solvents agains test fungi in agar will diffusion method.



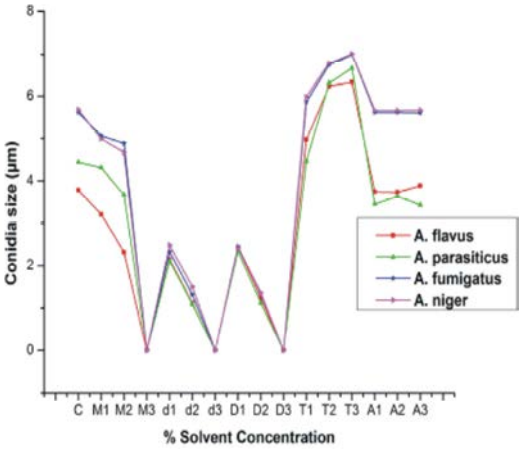
C. Effect of various concentrations of organic solvents on mycelialwidth of test fungi.



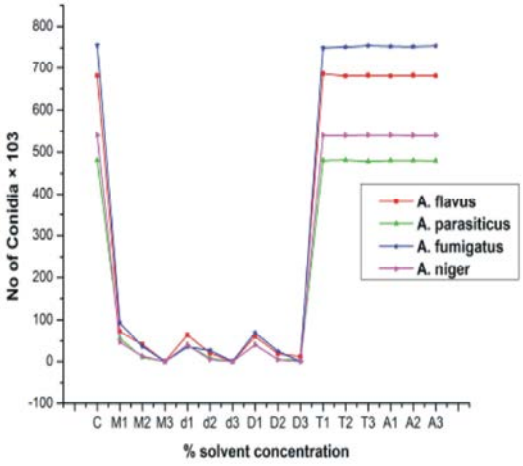
B. Inhibitory effect of various concentrations of orgainc solvents agains test fungi in borth diffusion method.



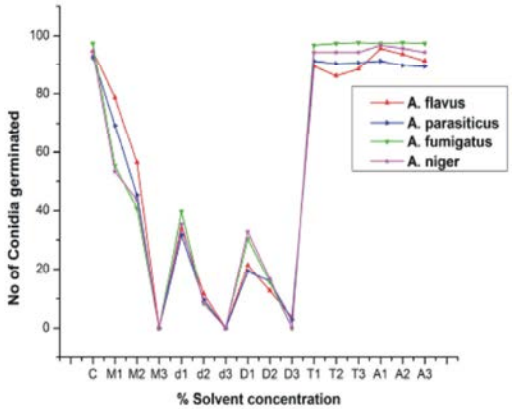
D. Effect of various concentrations of organic solvents on vesicle size of test fungi.



E. Effect of various concentrations of organic solvents on conidia size of test fungi.



F. Effect of various concentrations of organic solvents on conidia number of test fungi.



G. Effect of various concentrations of solvents on germination conidia of test fungi.

Fig. 1: Effect of test solvents on various parameters of test fungi.

increases the binding between ligands and receptor sites also increases which results in increased inhibitory effects. Toxicity of DMSO, DMF and methanol at different concentrations against various organisms such as dermatophytes, bacteria and fungi has been reported [10, 11]

In most studies, agar well diffusion method is used to determine the sensitivity [22, 23] whereas MIC is commonly determined by broth dilution method [24, 25].

When toxicity of all the solvents in both methods was compared, DMF and methanol was found more toxic in broth dilution method as compared to agar well diffusion method. Similarly DMSO was found nontoxic in agar well diffusion whereas it showed toxicity in broth dilution method (Fig. 1 A and B). The reason might be, in broth dilution method fungal spores are in direct contact with solvent which has more effect than agar well diffusion method. Results suggest that toxicity of the solvent should be first assayed using broth dilution method and then agar well diffusion method may be used for assaying the zone of inhibition as a function of inhibitory activity. Effect of all experimental solvents on differentiation, morphology, formation of reproductive structures and conidia pigmentation of test fungi are presented in fig 1 (C-G), fig. 2 (A-D) and fig. 3 (1-6). The seven day culture of control showed presence of green, yellow green, bluish green and black conidia in *A. flavus*, *A. parasiticus, A.fumigatus* and *A. niger* respectively, while temporary inhibition of pigmentation and formation of whitish or pale colored colonies were observed in cultures exposed to DMF and DMSO (Fig. 2 A, B, C and D). These white colonies revert to their original color when sub cultured on SDA. Delay in conidia formation was observed in all test fungi treated with methanol as after 5 days of noculation colored colonies were developed whereas in control conidiation was seen after 3 days. In case of Tween 80 and acetone there was no effect on pigmentation of conidia in all test fungi.

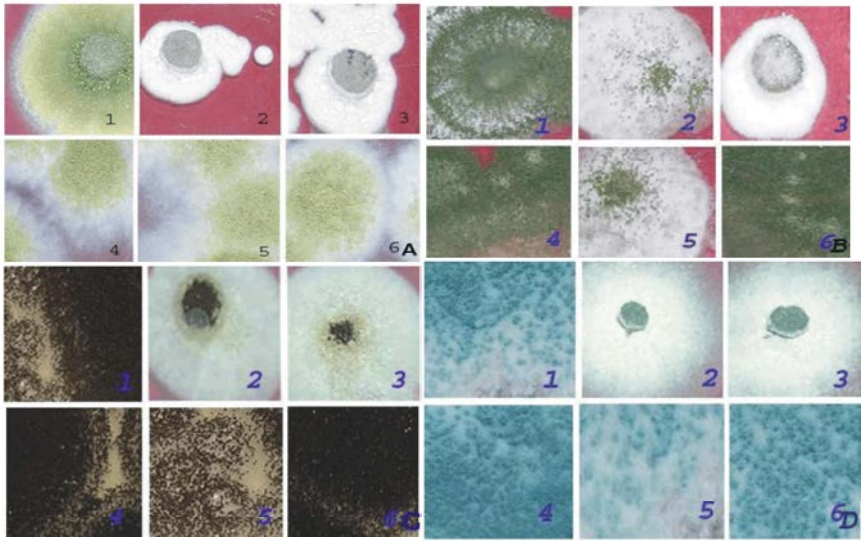


Fig. 2: Effect of solvents (1- control, 2- DMSO, 3-DMF, 4- tween 80, 5- methanol and 6- acetone) on pigmentation (colony colour) of test fungi (A) *A. flavus*, (B) *A. parasiticus*, (C) *A. niger*, (D) *A. fumigatus.*

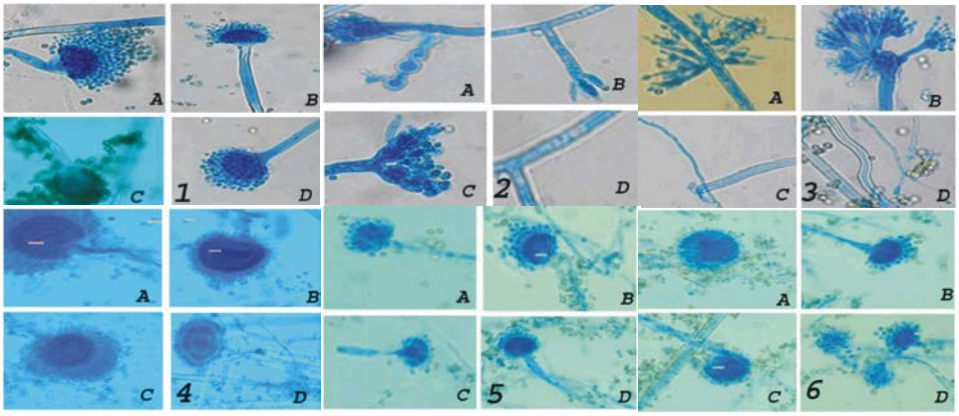


Fig. 3: Microscopic observations of effect of solvents on mycelial and reproductive structures of test fungi (400X) (1) (Control) (A. *A. flavus*, B. *A. parasiticus*, C. *A. niger*, D. *A. fumigatus*) (2) DMF (A. Multiple vesicle in *A. fumigatus,* B. Inhibition of vesicle formation in *A. flavus*, C. Dichotomous sporangiophore of *A. fumigatus*, D. Shrunken cytoplasm in *A. parasiticus*) (3) DMSO (A. Multiple sporangiophore and vesicle inhibition in *A. flavus*, B. Multibranched sporangiophore of *A. fumigatus*, C. Decreased mycelial width of *A. parasiticus*, D. Shrunken cytoplasm in *A. parasiticus*) (4) Tween 80 (Increased mycelial width, vesicle and sporangiophore size of A. *A. flavus*, B. *A. parasiticus*, C. *A. fumigatus*, D. *A. niger*) (5) Methanol (Decreased mycelial width and sporangiophore size of A. *A. flavus*, B. *A. parasiticus*, C. *A. fumigatus*, D. *A. niger*) (6) Acetone (No change from control, A. *A. flavus*, B. *A. parasiticus*, C. *A. fumigatus*, D. *A. niger*)

Abundant conidiophores showing characteristic stalked s tructure with globular head or vesicle-bearing bottle shaped phialides and chains of conidia were observed in control (Fig 3 (A)) whereas abnormal morphology in cultures was observed after treatment with all used solvents except acetone. Treatment with DMF, DMSO and methanol resulted in increased mycelial width as well as reduction in size, number and germination of fungal conidia and vesicle [(Fig. 1 (C-G)]. In case of Tween 80, mycelial width as well as conidia and vesicle size of all test fungi increased whereas number and germination of conidia was not changed [(Fig. 1 (C-G, 3 D)].No change was observed in acetone treated cultures [(Fig. 1 (C-G, 3 F)]. In addition, dichotomous branching, shrunken cytoplasm, inhibition of vesicle and phialides formation, multiple vesicle formation was also observed in all test fungi after repeatedly sub culturing on media with DMF and DMSO [(Fig. 3 (B-E)]. Toxicity and effect of DMF, DMSO, methanol and tween 80 on cytology, morphology, reproductive structures, germination of conidia/spores and sporulation of different fungi viz; *Aspergillus* spp. *Fusarium* spp. *Rhizopus* spp. were reported by various authors [26- 32].

It can be concluded that the organic solvent with good solvent property is not necessarily a perfect solvent for antimicrobial study. Toxicity of solvents must be studied by screening them against test organisms using more than one parameter prior to *in vitro* antimicrobial studies for standardisation of solvent.

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