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Standardization of the Safety Level of the Use of DMSO in Viability Assays in Bacterial Cells

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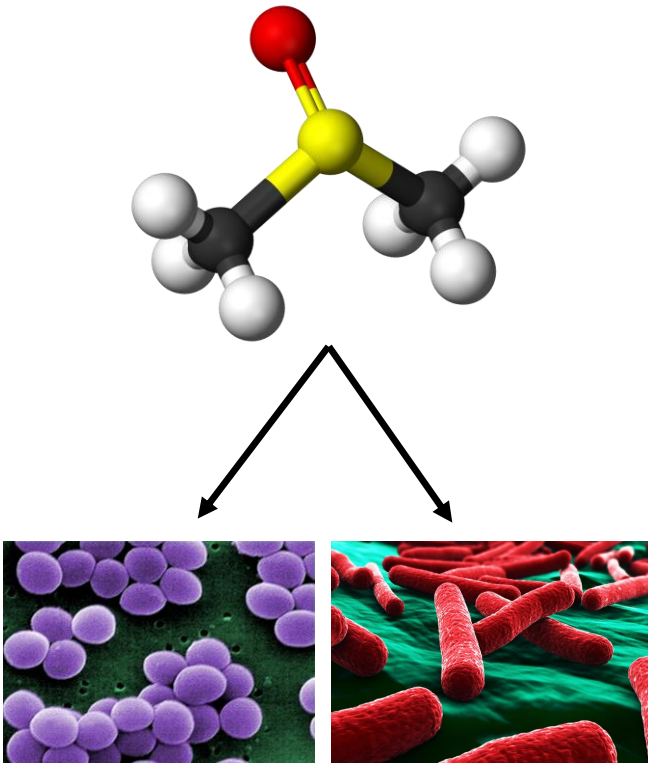
Graphical Abstract



Abstract.

The antibacterial potential of the most diverse medicinal plants has benefited humanity for centuries, and precisely because of this, the number of studies investigating the antimicrobial activity of essential oils and their components is increasing. However, the hydrophobic character of the essential oils has made the experiments difficult, requiring the use of organic solvents in the tests in order to avoid such complications. Among the most commonly used solvents are dimethylsulfoxide (DMSO/C_2H_6OS). To date, the literature has not yet determined a standardization of the usual concentration of DMSO suitable for bacterial experiments, so that its use does not check the efficacy of the tested phytoconstituent by interactions between the solvent and the exploited compound. In view of this reality, the present study intends to standardize the DMSO concentrations that do not interfere in the viability of the bacterial strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC

	25923, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25922 and *Enterococcus faecalis* ATCC 29212. In order to reach this objective, the disc diffusion method in Müller Hinton Agar of a reagent impregnated on a filter paper disc was used, using different concentrations of DMSO to determine the minimum inhibitory concentration (MIC) of the five microorganisms listed. The experiment was carried out in triplicate in order to safeguard the effectiveness of the method tested. The incubation was done in an oven at 35º C, for a period of 24 hours. For the test method the conclusions of the tests performed were expressed by the arithmetic mean of the diameter of the inhibition halos formed around the disks. The test demonstrates that for the concentrations and microorganisms tested, DMSO provides safety in its use.

Introduction

In the scientific field, extracts and essential oils from plants are used as natural sources of new compounds to combat bacterial infections. However, the estimation of the antibacterial activities of many plant-derived compounds is hampered by the low solubility of these compounds in water. Solubilizers, such as surfactants and solvents, among them dimethylsulfoxide (DMSO), have been used to solve this problem, but it may be difficult to distinguish the contribution in the antimicrobial activity of the solubilizer from the compounds under investigation [1].

DMSO was originally synthesized by Zaytsev in 1866, and has since been extensively investigated for possible industrial and biological utility, and a considerable amount of literature has developed on its properties and uses [2]. It is an organosulfur of formula C_2H_6OS of molecular weight 78 g/mol, boiling point 189 °C and freezing temperature 18.5 °C; an amphipathic molecule composed of a polar domain characterized by sulfinyl and two non-polar methyl groups, which makes it capable of solubilizing polar and non-polar substances and transposing hydrophobic barriers (Figure 1) [3].

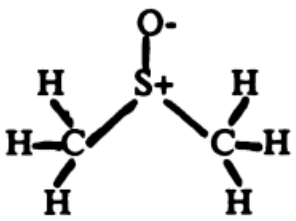


Figure 1: Polarized form of dimethylsulfoxide

These properties are important for pharmacological compounds that act as vehicles intracellularly as they allow non-water-soluble therapeutic and toxic agents to be generally soluble in DMSO [4].

An important biological action observed in DMSO is its ability to cross cell membranes; other pharmacological properties of DMSO include: immunomodulation; vasodilation; anti-platelet aggregation with protection against ischemic injury; diuretic, among others. The higher its concentration in water, the greater the penetrating capacity, the ideal range being between 70-90% of DMSO [5].

Thus, DMSO has been used as a commercial solvent since 1953, also possessing several other applications: therapeutic use, excipient for formulations in veterinary therapeutics, as a control group for testing natural products, for treating cells cultured in certain experiments and several in vitro studies. Despite the multiple applications of DMSO, its physiological and pharmacological characteristics as well as their effects are not fully understood, and further investigations into its pharmacological activities and which concentration of use is safe are required [6].

It has recently been found experimentally that DMSO has the ability to protect *Escherichia coli* from antimicrobial-mediated rapid death, interfering with the efficacy of the drugs tested, in this experiment DMSO inhibited death by ampicillin, kanamycin and two quinolones but had little effect on MIC. DMSO-mediated protection correlated with ROS reduction [7]. Another assay also reported a possible inhibition of microbial growth by interference of this solvent, thus, one can't rule out the possibility that DMSO interfered in the experiment, possibly causing a potentiating effect of the antimicrobial activity of tested compounds [8]. In view of such reports, caution is advised in using DMSO-dissolved antimicrobials for short-term death assays as well as accurate investigations to find out which DMSO concentration is most reliable for use in such experiments.

Materials and Methods

The microorganisms used for testing were Gram positive bacterial strains: *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212; and Gram negative strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Proteus mirabilis* ATCC 25922.

Disk diffusion tests with different concentrations of dimethylsulfoxide (DMSO) were carried out in order to identify concentrations of DMSO capable of carrying out an antibacterial activity in the five microorganisms. To test all concentrations, three petri dishes were used for each strain tested. The incubation was done in an oven at 35º C, for a period of 24 hours.

A suspension of the five microorganisms was performed, whose turbidity degree was 0.5 McFarland scale, corresponding to 1 x 10^8 CFU/mL, which was spread with the aid of a Drigalsky loop. After sowing on the plate, each disk was impregnated with different concentrations of the reactants and pressed against the plate in order to ensure complete contact with the agar surface, being applied individually and evenly distributed, so that the distance from the center of the edge of the board did not exceed 24 mm. The plates containing Müller-Hinton Agar were inverted and placed in an oven at 35 ºC for 24 hours after the application of the discs therein, and thereafter the halos were read.

The tests were performed and their results were expressed in mm by the arithmetic mean of the diameter of the inhibition halos formed around the discs during the disk diffusion test.

Results and Discussion

The disc diffusion method is performed to determine patterns of susceptibility to antibiotics according to the guidelines of the Institute of Clinical and Laboratory Standards (CLSI) [9]. It is an easy-to-perform and economical technique developed by the European Committee for Antimicrobial Susceptibility Testing (EUCAST), its standardized methods and clinical breakpoints have been adopted by clinical microbiology laboratories in Europe as well as in other parts of the globe [10].

From the evaluation of the results recorded in **Table 1**, it was verified that there was no inhibition halo formation at any of the concentrations tested, that is, there was no impediment to the growth of the investigated microorganisms. This demonstrates that the use of DMSO as a chemical solvent in experiments with the probed strains, in concentrations not exceeding 80%, according to the assay performed, is considered safe, not causing any inhibition of microbial growth.

Table 1: Verification of the antimicrobial activity of DMSO in different concentrations in the fusion disc method.

Microorganism	Substances / Halo (mm)							
	AMC	DMSO						
		80%	40%	20%	10%	5%	2,5%	1,75%	0,88%
S. aureus ATCC 25923	30	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
E. faecalis ATCC 29212	22	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
E. coli ATCC 25922	29	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
P. mirabilis ATCC 25933	22	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
P. aeruginosa ATCC 27853	33	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

AMC: amikacin disc (30 μg), Ø: absence of inhibition halo of bacterial growth.

The reading of the results was done by measuring the diameter of the halos, in mm, formed around the discs containing the reagents, when greater than 6 mm it becomes visible indicating susceptibility of the microorganism to the substance tested [11].

The experiment was carried out by Filipe et al. [12], where the antimicrobial activity of the leaves of *Cydonia oblonga* miller was tested against several microorganisms, including E. coli and S. aureus. DMSO at 20% concentration was used in the assay and, corroborating our finding, the experiment did not detect any interference with the growth of these bacteria at the concentration used.

Macieira [13] conducted a research on the antimicrobial activity of marine cyanobacteria, where eight bacterial species were selected: four Gram-positive (*Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium* and *Bacillus subtilis*) and four Gram-negative strains (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* enteric). In this experiment DMSO was used as a negative control and also proved to be a safe solvent, which offered no interference with the results obtained in the assay.

Soares and Benitez [14] in their research on the antimicrobial activity of botanical extracts and their interaction with antibiotics against two strains of *Listeria* spp isolated from food and the standard strain ATCC 7644. The authors demonstrated that DMSO only showed toxic action for to *L. monocytogenes* in concentrations above 12.5%.

Conclusions

The results obtained by the study evidenced the need to standardize an appropriate concentration of DMSO for use in these experiments, considering that there is a discrepancy in the findings of several authors on the antimicrobial effect of the different concentrations of this solvent used. The interpretation of the effects of DMSO on the microorganisms with which it interacts is of great importance in view of its expanded use as a solvent in the most diverse therapeutic and pharmacological studies.

From our experiments we can state that despite recent discoveries involving the use of DMSO with interactions and / or changes in the efficacy of certain drugs, for the strains tested in the trial, namely: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25922 and *Enterococcus faecalis* ATCC 29212, DMSO use was shown to be safe and innocuous at concentrations not greater than 80%.

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