

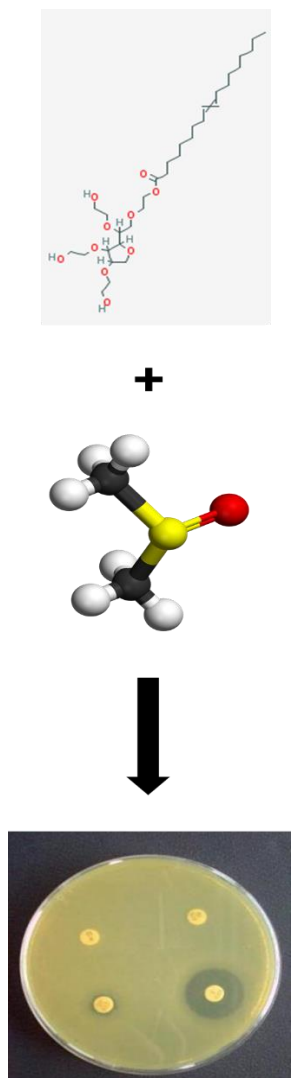
Evaluation of the Interference of Solvents Used in the Evaluation of Antimicrobial Activity of Liposoluble Natural Compounds

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Graphical Abstract



Abstract.

Because it is an activity already consolidated throughout the ages, the use of compounds from plants has been well studied and tested for definition or proof of its antibacterial activity. Despite the difficulties encountered as the solubility of essential oils, there are compounds that help in experiments, they are called solvents and emulsifiers and the most used in phytotherapeutic tests are: ethyl acetate, acetone, ethyl alcohol, methyl alcohol, neutral detergent (phosphates free), dimethylsulfoxide (DMSO), triton X-100 and polysorbate 80 (tween 80). In view of this fact, the present work seeks to identify concentrations of polysorbate 80 (Tween 80) and dimethylsulfoxide (DMSO) capable of performing an antibacterial activity, due to its wide use in the scientific environment, against the following bacterial strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25922 and *Enterococcus faecalis* ATCC 29212. The evaluation of the activity of the compounds was performed by the diffusion disc method. This method is the one recommended by the Clinical and Laboratory Standards Institute and is based

on the diffusion through the agar of a reagent impregnated in a disc of filter paper and the diffusion of the same leads to the formation of a halo of inhibition of growth of the microorganisms whose diameter is inversely proportional to the minimum inhibitory concentration. This method is qualitative, that is, it allows to classify the bacterial sample as susceptible, intermediate or resistant to antimicrobial. The tests were carried out with different concentrations of the reagents to determine the antimicrobial effect of the studied solvents. The experiments were run in triplicate at all concentrations using the compounds in combination (DMSO + Tween). The incubation was done in a greenhouse at 35 ± 2 ° C, for a period of 24 hours. The tests were performed and the results expressed in mm by the arithmetic mean of the diameter of the inhibition halos, formed around the discs. As results, no values were determined that determined antimicrobial activity, and it is not possible to determine MIC when the formed halo is equal to or less than 6 mm or when there is no formation thereof. In view of the results, it can be observed that the compounds may not present activities against the microorganisms tested or, due to their physico-chemical characteristics, suffer some interference, such as the diffusion difficulty in the agar, its insolubility in water and chemical complexity.

Introduction

The use of medicinal plants for the treatment, cure and prevention of diseases is one of the oldest forms of medicinal practice of mankind. As early as the early 1990s, the World Health Organization (WHO) reported that 65-80% of the population in developing countries depended on medicinal plants as the only form of access to basic health care [1].

In the scientific field, extracts and essential oils from plants are used as natural sources of new compounds to combat bacterial infections [2]. However, the estimation of the antibacterial activities of many plant-derived compounds is hampered due to their low solubility in water. Solubilizers, such as surfactants and solvents, 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 [3]. Among the most used solvents and emulsifiers in phytomedicine tests are: ethyl acetate, acetone, ethyl alcohol, methyl alcohol, phosphates free, dimethylsulfoxide (DMSO), triton X-100 and tween 80 [4].

Dimethyl sulfoxide (DMSO) is the organic solvent most commonly used in biochemical and cellular assays during drug discovery programs [7]; is an aprotic solvent of universal use with the ability to permeate biological membranes, and therefore is commonly used to obtain the appropriate biological availability of hydrophobic toxic substances [8]. Mi et al [9] also emphasizes that the popularity of DMSO in both the pharmaceutical and antimicrobial industries is due to several factors, including: (i) low toxicity, (ii) organic and inorganic dissolution capacity (iii) the ability to remain in a liquid state over a wide temperature range (e.g., 19Â ° C to 189Â ° C), (iv) ability to improve cell membrane permeability, and (v) miscibility in water and a wide range of organic solvents.

However, surfactants may interact with organisms and drugs affecting the in vitro activity of antimicrobial agents. According to Gomez-Lopez et al. [10] the surfactant could modify the solubility of the antifungal, developed in a medium and aid in the precipitation of the agent, leading to the increase of MIC. According to Hammer et al. [11], when using an emulsifying agent, it is necessary to take into account the possible interactions between this agent and the components of the essential oil, besides the possible antimicrobial activities that can be presented by the same. For them, these effects may vary according to the ratio of essential oil and emulsifier, which makes it essential to use this association appropriately.

Considering the reports presented, and taking into account also the fact that, to date, no standard amount of these agents has been defined, the present study aims to standardize the minimum inhibitory concentrations of Tween 80 and DMSO, considering its wide use in the scientific milieu, against the following bacterial strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25922 and *Enterococcus faecalis* ATCC 29212.

Materials and Methods

Diffusion tests were performed with different concentrations of polysorbate 80, (ween 80), and dimethylsulfoxide (DMSO) capable of performing an antibacterial activity of the five microorganisms. Therefore, to test all concentrations, three petri dishes were used for each strain tested. The incubation was done in an oven at 35° C, for a period of 24 hours.

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

The tests were performed and the results expressed in mm by the arithmetic mean of the diameter of the inhibition halos formed around the discs during the disk diffusion test. Then, five microorganisms were suspended, the turbidity degree of which was 0.5 McFarland scale, corresponding to 1 x 10⁸ CFU / mL, which was spread with the aid of a swab. After sowing each disc 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 disc to the edge not exceed 24 mm. Plates containing Müller-Hinton Agar were inverted and placed in an oven at 35 ° C for 24 hours after application of the disks therein.

Results and Discussion

The disc diffusion test is accepted by the FDA (Food and Drug Administration) and established by CLSI (Clinical and Laboratory Standards Institute). This method was idealized by Bauer et al. in 1966, and since then it has been one of the methods most used in clinical microbiology laboratories in

Brazil to test antimicrobial susceptibility. The principle of this method is based on the diffusion through agar of an antimicrobial agent impregnated on a filter paper disc, which leads to the formation of a bacterial growth inhibiting halo whose diameter is inversely proportional to the minimum inhibitory concentration. This method is qualitative, that is, it allows to classify the bacterial sample as susceptible, intermediate or resistant to antimicrobial [9-11].

The reading of the results was performed by measuring the diameter of the halos, in mm, formed around the disks containing the reagents that when greater than 6 mm becomes visible and indicates susceptibility of the microorganism to the substance tested [2].

Tables 1 and 2 were designed with the purpose of indicating the sizes of inhibition halos that were or were not formed by the microorganisms studied, whose values represent the arithmetic mean of the results in triplicate.

Table 1: Verification of the antimicrobial activity of the association of DMSO + TWEEN 80 against gram positive strains in different concentrations in the fusion disc method.

Bacterial strains	AMC	Substances / Halo (mm)						
		DMSO/Tween 80 (%)						
		40/32	20/16	10/8	5/4	2,5/2	1,25/1	0,625/0,5
<i>S. aureus</i> ATCC 25923	30	Ø	Ø	Ø	Ø	Ø	Ø	Ø
<i>E. faecalis</i> ATCC 29212	22	Ø	Ø	Ø	Ø	Ø	Ø	Ø

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

As noted in **Table 1**, it can be noted that there was no inhibition of growth of the tested microorganisms. This shows that a reaction may have occurred between the solvents, dimethylsulfoxide and polysorbate 80, not allowing them to exert their action on the bacterial strains tested. In addition, several other factors may have contributed to this result, such as the chemical characteristics of the solvents that may hinder the dispersion in the culture medium, as well as other interferences of the test adopted.

Other interferences were reported by Nascimento [8], who found that the disc diffusion method may have several interferences, such as oil volatility, diffusion difficulty in agar, its insolubility in water and chemical complexity.

Table 2 shows the results of the tests on the gram negative bacteria *E. coli*, *P. mirabilis* and *P. aeruginosa*, where it was also evident the absence of interference in the compounds for the bacterial strains.

Table 2: Verification of the antimicrobial activity of the association of DMSO + TWEEN 80 against gram negative strains in different concentrations in the fusion disc method.

Bacterial strains	AMC	Substances / Halo (mm)						
		DMSO/Tween 80 (%)						
		40/32	20/16	10/8	5/4	2,5/2	1,25/1	0,625/0,5
<i>E. coli</i>	29	Ø	Ø	Ø	Ø	Ø	Ø	Ø

ATCC 25922

<i>P. mirabilis</i>	22	Ø	Ø	Ø	Ø	Ø	Ø	Ø
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ATCC 25933

<i>P. aeruginosa</i>	33	Ø	Ø	Ø	Ø	Ø	Ø	Ø
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ATCC 27853

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

Gram negative microorganisms have a more complex morphology than gram positive bacteria, that is, they have an extra membrane (external membrane), which hinders the action of solvents, become an obstacle to their reach and action. In addition, gram positive microorganisms differ in the organization of their structures that are found externally to the plasma membrane where there is a structure, which is thick and has a large layer of peptidoglycan called the outer membrane [12, 13]. Another difficulty in the analysis of the results obtained in this experiment is that there is no standardization for the technique to be used in this type of assay for said reagents.

In a study developed by Estrela [14], it was observed that substances with different capacities of diffusion and dissociation using the techniques of liquid medium and diffusion in agar, and observed that the first one would be more effective precisely because of the difficulty that some substances have to diffuse on agar.

Conclusions

From the results found, it became evident the need to standardize the concentrations in use of the appropriate reagents, since several experiments are carried out using these microorganisms with variations in the concentrations of the same ones, without knowing that they can interfere in some way in the result of the experiment.

New studies should be carried out, since this is a pioneer in this line of research, seeking to identify the concentrations of solvents used that may cause interference or even inhibit the growth of the mentioned microorganisms or others, including using other methodologies with less interference.

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