Studies on antimicrobial activity, in vitro, of Physalis angulata L. (Solanaceae) fraction and physalin B bringing out the importance of assay determination

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Complex physalin metabolites present in the capsules of the fruit of Physalis angulata L. have been isolated and submitted to a series of assays of antimicrobial activity against Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 29213, S. aureus ATCC 25923, S. aureus ATCC 6538P, Neisseria gonorrhoeae ATCC 49226, Escherichia coli ATCC 8739; E. coli ATCC 25922, Candida albicans ATCC 10231 applying different methodologies such as: bioautography, dilution broth, dilution agar, and agar diffusion techniques. A mixture of physalins (pool) containing physalins B, D, F, G inhibit S. aureus ATCC 29213, S. aureus ATCC 25923, S. aureus ATCC 6538P, and N. gonorrhoeae ATCC 49226 at a concentration of 200 mg/µl, using agar dilution assays. The mixture was inactive against P. aeruginosa ATCC27853. E. coli ATCC 8739; E. coli ATCC 25922, C. albicans ATCC 10231 when applying bioautography assays. Physalin B (200 µg/ml) by the agar diffusion assay inhibited S. aureus ATCC 6538P by ± 85%; and may be considered responsible for the antimicrobial activity.

Key words: Physalis angulata L. - antimicrobial methods - physalins

Physalis is an important genus of the Solanaceae family. Most of the species are herbaceous annuals or perennials, native to tropical North and South America. Some species have edible fruits and the tea of their roots is known. Their anti-inflammatory and anti-spasmodic properties are also known.

Ray and Gupta (1994) and Tomassini et al. (2000) report some data on therapeutic applications and describe the pharmacological activity of the Physalis species as anti-parasitic, anti-viral, and anti-neoplastic. Trypanocidal activity was described by Freiburhaus et al. (1996) for P. angulata L. dichloromethane and ether extracts. Acetone, ethyl acetate, ethanolic extracts from leaves, stems, and roots of the specimen were assayed against Biomphalaria tenagophila yielding positive results for molluscicidal activity (Dos Santos 2003).

Antimicrobial activity was described by Ongulana and Ramstad (1975) for methanol/water extracts of P. angulata against Bacillus subtilis. Cáceres et al. (1995) reported the results of the antimicrobial tests with the ethanolic extract leaves of Neisseria gonorrhoeae in agreement with the reported popular use in Guatemala. Aqueous and ethanolic extracts of P. angulata inhibited the growth of Staphylococcus aureus and Escherichia coli (Sanches et al. 1997, Silva et al. 1999).

Pietro et al. (2000) described the tuberculostatic activity of P. angulata chloroform extracts from epigeal parts against Mycobacterium tuberculosis, M. avium, M. kansaii, M. malmoense, and M. intracellulare.

Januário et al. (2003) reported activity against M. tuberculosis for physalins B and D. Physalin F isolated from P. angulata ethanolic extract (Chiang et al. 1993) shows a potent antineoplastic activity. Soares et al. (2003) described the immunosuppressive activity of physalins B, F and G extracted from the stems of P. angulata L.

The continuous development of antibiotic resistance of pathogenic microorganisms and particularly of Streptococcus pneumoniae to penicillin (PRSP), Staphylococcus aureus to methicillin (MRSA), and Enterococcus to vancomycin (VRE) is a major health concern worldwide with economic, social and political implications.

The screening of plant materials and their isolated substances for new antimicrobial compounds represent an important potential source for new effective medicines. Several techniques are routinely available to test antimicrobial properties, among which, the most popular are the following:

Agar diffusion technique - Known as the Kirby-Bauer method, this assay was standardized by Bauer et al. in 1966. It is the test, which is most widely used in clinical practice and is recommended by Clinical and Laboratory Standards Institute (CLSI 2001).

The method measures microbial growth inhibition at the surface of an inoculate medium around a paper disk impregnated with the antimicrobial substance at a standard concentration. The result may show, or not, the pres-
ence of an inhibition zone around the paper disk, the diameter of this zone being a good indicator of the antibiotic activity.

**Bioautography** - This assay is a variation of the agar diffusion methodology. The sample to be analyzed is transferred from the chromatographic adsorbent to the inoculated agar. The spots containing the substances are visualized using microbial indicators (tetrazolium salts) as a growth detector of dehydrogenase activity (Kline & Golab 1965, Rahalison et al. 1994, Nostro 2000).

**Dilution tests** - These can be applied in solid (agar dilution) or liquid (micro and macrodilution broth method) media. The results obtained allow a quantitative estimate of antimicrobial activity. Several dilutions of the antimicrobial substance are incorporated to the liquid or solid media to determine the minimal inhibitory concentration (MIC). MIC is the smallest concentration of the antimicrobial agent, which is capable of inhibiting the growth of the microorganism in vitro. These data are important to determine the optimal dosage as well as the correct administration route of the antimicrobial substance in therapy.

The present study deals with the antimicrobial activity of Physalin B and enriched physalin fractions (mixture of B, D, F, and G physalins) against pathogenic gram-positive microorganisms. Microorganisms were obtained from the Instituto Nacional de Controle de Qualidade e Saúde (INCQS) Fiocruz, Rio de Janeiro, Brazil.

**RESULTS AND DISCUSSION**

The bioautography screening method was the first method applied to detect the microorganism sensitivity to physalins. The bioautography involved a solvent that
completely dissolved the physalin pool and pure physalin B. Bioautography as described, with fully dissolved physalins was reproducible at all concentrations used.

After detection with MTT, *C. albicans* ATCC 10231, *E. coli* ATCC 8739, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 did not show sensitivity to the physalin pool while *N. gonorrhoeae* ATCC 49226, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538P strains showed positive results (Fig. 1).

The MIC for the physalin pool was based on the lowest concentration at which a positive effect was detected in bioautography assays.

Due to the insolubility of physalins in water or in aqueous 1.5% dimethylsulfoxide, it was not possible to use the microdilution broth technique.

Table I shows the MIC values for *N. gonorrhoeae* ATCC 49226, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538P for the physalin pool when applying the agar dilution assay. This technique is more versatile than microdilution broth due to the good stability of physalins in agar. The agar dilution assay suffers less from contamination and allows the determination of MIC for several microorganisms at the same time.

In agreement with the USP XXIII Pharmacopoeia (1995) and the FDA Code (1991), protocols recommended for the study of antibiotic potency against the strain of *S. aureus* ATCC 6538P were used for the potency determination of physalin applying the agar diffusion technique.

Table II shows the average values for inhibitory zones obtained after nine replications. Based on these results, it was possible to establish the linearity range presented in Fig 2. Table III shows the results of a comparative study between physalin pool and physalin B at three different concentrations using the agar diffusion technique.

**TABLE I**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (µg/ml)</th>
<th>50</th>
<th>90</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 6538P</td>
<td>100</td>
<td>200</td>
<td>3.1-1600</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>100</td>
<td>200</td>
<td>3.1-1600</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 29213</td>
<td>100</td>
<td>200</td>
<td>3.1-1600</td>
<td></td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em> ATCC 49226</td>
<td>100</td>
<td>200</td>
<td>3.1-1600</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Concentrations (µg)</th>
<th>Zone diameters (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>50</td>
<td>8.2</td>
</tr>
<tr>
<td>100</td>
<td>9.1</td>
</tr>
<tr>
<td>200</td>
<td>10.2</td>
</tr>
<tr>
<td>400</td>
<td>11.0</td>
</tr>
<tr>
<td>800</td>
<td>11.2</td>
</tr>
<tr>
<td>1600</td>
<td>11.0</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (µg)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physalin pool</td>
<td>100</td>
<td>9.0</td>
<td>7.0 – 9.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10.4</td>
<td>8.0 – 11.0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>10.7</td>
<td>9.4 – 11.0</td>
</tr>
<tr>
<td>Physalin B</td>
<td>100</td>
<td>7.4</td>
<td>6.0 – 8.0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>9.2</td>
<td>6.2 – 10.0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>10.0</td>
<td>8.6 – 11.4</td>
</tr>
</tbody>
</table>

between physalin pool and physalin B at three different concentrations using the agar diffusion technique. This result shows that, at the concentration of 200 µg/ml, pure physalin B exhibited ± 85% of the inhibitory zone observed with the total pool of physalins, at the same concentration.

In the bioautography assays, the physalin pool and physalin B did not inhibit *C. albicans*, *P. aeruginosa* or *E. coli* strains but did reveal efficacy against *N. gonorrhoeae* and *S. aureus*.

The physalin pool at 200 µg/ml yielded 100% inhibition for *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538P, and *N. gonorrhoeae* ATCC 49226, in agar dilution tests.

The above results point to the following conclusions: (a) the bioautography assay is a practical, reproducible test and easy to perform; (b) the agar dilution technique...
is more versatile than the broth dilution assay and does
not present the problems encountered with this latter
assay with the sample solution, contamination and MIC
determination; (c) the agar diffusion technique can be used
only for pure substances because when it is applied to
mixtures containing constituents, which exhibit different
diffusion factors, and, thus the results may be unreliable;
(d) the methods related here-in permit the choice of the
best tool for determination of the antimicrobial constitu-
ents from plant material.

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