Research Note

Inhibition of Growth, Enterotoxin Production, and Spore Formation of *Clostridium perfringens* by Extracts of Medicinal Plants

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ABSTRACT

The extracts of 14 plants used in the traditional medicine of Mexico were evaluated for their effects on the growth, spore formation, and enterotoxin production of *Clostridium perfringens* type A. The extracts of *Psidium guajava* L., *Haematoxylon brasiletto*, and *Euphobia prostrata* were the most effective inhibitors of growth, spore formation, and enterotoxin production. No enterotoxins were detected when extracts were added to the media at less than the MIC for growth.

*Clostridium perfringens* is a spore-forming bacterium associated with food poisoning, gas gangrene, infectious diarrhea, and several veterinary diseases (7, 8). The spore-forming ability of this microorganism enables it to survive critical conditions such as aerobic and food-processing environments (7). *C. perfringens* causes food poisoning upon ingestion, because a large number of vegetative cells of the microorganism can survive passage through the stomach. These cells can then sporulate and produce an enterotoxin in the small intestine (7).

The use of antimicrobial compounds from natural vegetation could be of considerable value in protecting stored food from contamination and in controlling plant and human diseases of microbial origin (1). In this study, the extracts of 14 plants commonly used to treat gastrointestinal diseases in the traditional medicine of Mexico were evaluated for their ability to inhibit the growth, spore formation, and enterotoxin production of *C. perfringens*.

MATERIALS AND METHODS

Plant extracts. A total of 14 plants (*Acacia farnesiana* L. [bark], *Citrus aurantium* L. [leaves, peel], *Euphobia prostrata* Aiton. [leaves], *Guazuma ulmifolia* [leaves], *Haematoxylon brasiletto* Karst. [bark], *Hypitis suaveolens* L. [stems], *Hypitis verticillata* Jacq. [stems], *Lippia dulcis* Trev. [leaves], *Psidium guajava* L. [leaves], *Rhizophora mangle* L. [roots], *Solanum hindsianum* [stems], *Solanum nigrum* L. [leaves], and *Tagetes erecta* L. [leaves], and *Taxodium mucronatum* Ten [bark]) were analyzed. The plants were purchased from retail markets in the metropolitan area of Monterrey, Nuevo León, Mexico. Twenty grams of washed plant material was immersed in 100 ml of 10 mM phosphate buffer (pH 7; aqueous extracts; distilled water was used for buffer preparation) or 96% ethanol (alcoholic extracts). The samples were then ground with a mortar and pestle to extract soluble material. Aqueous extracts were macerated at 4°C for 8 h, and alcoholic extracts were macerated at room temperature overnight. The macerated samples were then filtered with Whatman no. 5 filter paper and centrifuged at 10,000 × g for 20 min. Supernatants were concentrated with a rotary evaporator (Buchi R 3000, Switzerland) at 65°C and 30% rotation until a small volume (20 to 30 ml) was obtained. The concentrated extracts were dried, dissolved in 10 to 15 ml of phosphate buffer, filtered sterilized, and maintained at 4°C for no longer than 7 days. An aliquot was used to determine dry weight.

Cultures. The enterotoxin-positive strain FD-1041 of *C. perfringens* type A was used. This strain was originally provided by Stanley Harmon, of the Food and Drug Administration, Washington, D.C., and was maintained as a stock spore culture in cooked meat medium (Difco Laboratories, Detroit, Mich.) at −20°C. Active vegetative cultures were obtained by transferring two drops of the stock culture into test tubes containing 10 ml of fluid thioglycollate medium (Difco), heat activated at 75°C for 20 min. Supernatants were concentrated with a rotary evaporator (Buchi R 3000, Switzerland) at 65°C for 8 h, and incubated overnight for 16 to 18 h at 37°C (4).

A diffusion method was used for preliminary screening. Petri dishes (150 mm) were filled with 25 ml of nutrient agar (1.5% peptone, 1% yeast extract, and 1.5% agar). Aliquots (100 μl) of the bacterial culture (1 × 10⁷ CFU) were homogeneously inoculated onto the agar. Five holes (12 mm in diameter) were made in the seeded agar plate. Holes were then filled with 200 μl of each extract. Sterile phosphate buffer or 96% ethanol were used as controls for aqueous and alcoholic extracts, respectively. The dishes were then incubated for 24 h at 37°C under anaerobic conditions. Growth-inhibitory activity was evaluated by the absence of bacterial growth in the area surrounding the holes filled with the plant extracts. The relative inhibition of bacterial growth mediated by the various extracts was quantified by measuring the diameters of the resulting inhibition zones as shown in Table 1. The MIC for growth was determined for those extracts that...
TABLE 1. Relative antibacterial activities of aqueous and ethanolic extracts as determined by the diffusion method in a preliminary screening.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Alcoholic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia fernesiana L.</td>
<td>++</td>
<td>NI</td>
</tr>
<tr>
<td>Citrus aurantium L.</td>
<td>++ (leaves), NI (fruit)</td>
<td>++ (leaves), ++ (fruit)</td>
</tr>
<tr>
<td>Euphorbia postrata Aiton.</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Guazuma ulmifolia</td>
<td>+</td>
<td>NI</td>
</tr>
<tr>
<td>Haematoxylon brasiletto Karst.</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Hiptis suaveolens L.</td>
<td>NI</td>
<td>+</td>
</tr>
<tr>
<td>Hiptis verticillata Jacq.</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Lippis dulcis Trev.</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Psidium guajava L.</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Rhizophora mangle L.</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Solanum nigrum L.</td>
<td>+</td>
<td>NI</td>
</tr>
<tr>
<td>Solanum hindsianum</td>
<td>+</td>
<td>NI</td>
</tr>
<tr>
<td>Tagetes erecta L.</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Taxodium mucronatum Ten.</td>
<td>++</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Inhibition zone diameters were as follows: +, 0.1 to 0.5 cm; ++, 0.6 to 1 cm; ++++, 1.1 to 1.5 cm; NI, no inhibition detected.

exhibited the strongest inhibitory activity. To make this determination, cells (1% inoculum) were grown in culture tubes containing 5 ml of brain heart infusion (BHI) broth in the presence of various concentrations of extracts (added in increments of 0.1 mg/ml). Cultures were then incubated at 37°C. Bacterial growth was quantitated by spectrophotometric analysis and is summarized in Table 2. The most potent growth-inhibitory extracts were further analyzed for their effect on spore formation. For this experiment, tubes (18 by 150 mm) with 10 ml of Duncan-Strong (DS) medium were inoculated (1% inoculum). Tubes were incubated in a water bath at 37°C, and sporulation was determined after 24 h of incubation. For the determination of sporulation, the cultures were heat shocked at 75°C for 15 min to kill the vegetative cells, and spores were enumerated by spore plate counting as described previously (5). Enterotoxin production was determined by enzyme-linked immunosorbent assay (6). All experiments were performed three times in duplicate. An analysis of variance was used to determine statistical significance.

RESULTS AND DISCUSSION

Of the plant extracts tested, we found that the aqueous and alcoholic extracts of *E. postrata*, *H. brasiletto*, *P. guajava*, and *R. mangle* were the most effective in inhibiting growth in nutrient agar (Table 1). Most of the other plant extracts examined inhibited bacterial growth either only in alcoholic extracts or only in aqueous extracts. No inhibitory activity was observed for extracts from *H. verticillata* and *L. dulcis* (Table 1). The MIC for growth inhibition was determined for extracts from *E. postrata*, *H. brasiletto*, *P. guajava*, and *R. mangle*, which exhibited the most extensive antimicrobial activity in the preliminary screening. The MICs differed for cells grown in BHI medium and those grown in DS medium and for aqueous and alcoholic extracts (Table 2). Alcoholic extracts from *E. postrata* and *P. guajava* exhibited the most potent growth inhibition (Table 2). The effects of *E. postrata*, *H. brasiletto*, and *P. guajava* extracts on sporulation were determined, and spore formation was found to be most potently inhibited by *E. prostrata* (*P > 0.05*) (Table 3). In most cases, the aqueous extracts were more effective than the alcoholic extracts in inhibiting the sporulation process. Sporulation and enterotoxin production are related processes (4). In no case was enterotoxin formation detected when 25, 50, or 75% of the MIC for growth was added to the media (data not shown). Thus, both processes were affected by the plant extracts.

To treat or prevent a disease, it is important not only to kill the pathogen that causes the disease but also to inhibit the production of that pathogen’s virulence factors. It has been shown that several antibiotics inhibit the growth and alpha-toxin production of *C. perfringens* (12). Natural products such as oleuropein, a phenolic compound extracted from olives, have been shown to inhibit growth and to inhibit the production of enterotoxin B and other exoproteins of *Staphylococcus aureus* (13). It has also been shown...
that garlic oil or onion oil can diminish the toxin production of \textit{C. botulinum} type A in meat slurry (3). Although the antimicrobial activity of some of the plants analyzed here has previously been established (10, 14), ours is the first study to report their effects on the bacterial sporulation and enterotoxin production of \textit{C. perfringens}.

It has been determined that quercetin, a compound found in \textit{P. guajava}, can inhibit peristalsis of the small intestine in vitro (9). Taken together with the inhibition of the growth and enterotoxin production of \textit{C. perfringens} by \textit{P. guajava} extracts demonstrated in the present study, these findings suggest that \textit{P. guajava} extracts may potentially serve as useful therapeutic agents against diarrhea caused by this bacterium (2). Furthermore, because of safety concerns and consumer preference for natural products, the use of natural compounds instead of synthetic forms is gaining prominence (11). Thus, the active plant extracts identified here could potentially be used as preservatives in food or as therapeutic agents for infectious diarrhea and other diseases caused by \textit{C. perfringens}. Current efforts directed at isolating and characterizing the primary active antibacterial compounds from these plant extracts are in progress.

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**REFERENCES**