

Extracts of Edible and Medicinal Plants Damage Membranes of *Vibrio cholerae*

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Received 17 December 2009/Accepted 16 August 2010

The use of natural compounds from plants can provide an alternative approach against food-borne pathogens. The mechanisms of action of most plant extracts with antimicrobial activity have been poorly studied. In this work, changes in membrane integrity, membrane potential, internal pH (pH\textsubscript{in}), and ATP synthesis were measured in *Vibrio cholerae* cells after exposure to extracts of edible and medicinal plants. A preliminary screen of methanolic, ethanolic, and aqueous extracts of medicinal and edible plants was performed. Minimal bactericidal concentrations (MBCs) were measured for extracts showing high antimicrobial activity. Our results indicate that methanolic extracts of basil (*Ocimum basilicum* L.), nopal cactus (*Opuntia ficus-indica* var. Villanueva L.), sweet acacia (*Acacia farnesiana* L.), and white sagebrush (*Artemisia ludoviciana* Nutt.) are the most active against *V. cholerae*, with MBCs ranging from 0.5 to 3.0 mg/ml. Using four fluorogenic techniques, we studied the membrane integrity of *V. cholerae* cells after exposure to these four extracts. Extracts from these plants were able to disrupt the cell membranes of *V. cholerae* cells, causing increased membrane permeability, a clear decrease in cytoplasmic pH, cell membrane hyperpolarization, and a decrease in cellular ATP concentration in all strains tested. These four plant extracts could be studied as future alternatives to control *V. cholerae* contamination in foods and the diseases associated with this microorganism.

The search for natural antimicrobials to use in foods is encouraged by the high prevalence of food-borne diseases and the current popular preference of consuming only natural foods (31). Furthermore, the resistance of microorganisms to common and novel antibiotics is on the rise (38).

Some plant products have been historically used as natural antimicrobials to extend the shelf life of foods and as therapeutics used in folk medicine to treat diseases caused by pathogens (1). Currently, plant products are considered to be important alternative sources of new antimicrobial drugs against antibiotic-resistant microorganisms (38) and as preservatives of food (33). According to this trend, the use of natural compounds derived from plants for the prevention of pathogenic and spoilage microorganisms in foods has been extensively reported (31).

Because a large number of plant species still need to be analyzed for their antimicrobial activity against diverse bacteria, it is critical to develop simple systems for rapid antimicrobial screening. Toward this end, several methods have been described, including those based on the use of membrane-impermeable fluorescent probes (31). In these assays, probes may be found to passively diffuse through the cell wall of bacteria, acting as an indicator of a loss of membrane integrity, which frequently is taken as an indicator of cell viability (22).

Bacteria use two forms of metabolic energy: energy-rich phosphate bonds, such as ATP, and electrochemical energy provided by ion gradients (6). Measurements of these forms of energy, such as membrane potential, cytoplasmic pH, and ATP synthesis, can be used as indicators of loss of cell viability. Changes in membrane potential are an early indication of injury in bacteria, and the ability of a cell to maintain a stable membrane potential can be determined by probe uptake or exclusion (26). On the other hand, ATP plays a fundamental role in cellular energetics, metabolic regulation, and cellular signaling (4). An increase in cytosolic ATP concentration is a key event in the membrane depolarization of ATP-dependent K\textsuperscript{+} channels (12). A common method used to measure this compound employs bioluminescence to measure cellular ATP levels (45).

Membrane integrity is fundamental for the control of cytoplasmic pH in bacteria, which is essential for many physiological activities (28). The capacity of cells to maintain a pH gradient (higher pH inside than outside the cell) may also supply information about cellular viability (9). In principle, these bacterial vital signs (intracellular pH, ATP production, and membrane potential and integrity) could form the basis of a rapid search for novel antimicrobial agents.

Scientific validation of the antimicrobial properties of plants has been extensively reported (10). However, little information is available about the mechanisms of action of antimicrobial compounds in bacteria. Several proposed mechanisms include membrane damage, changes in intracellular pH, membrane potential, and ATP synthesis (22, 42). In this work, we demonstrate that damage to membrane integrity, as well as changes in membrane potential, internal pH (pH\textsubscript{in}), and synthesis of ATP, occurs in *Vibrio cholerae* after exposure to particular extracts of plants.

**MATERIALS AND METHODS**

*Preparation of plant extracts.* Plant specimens (n = 27) were obtained from local supermarkets or collected in gardens of the Universidad Autónoma de Nuevo León. Voucher samples were deposited at the herbarium of the Botanical...
TABLE 1. Plants used in this work and growth inhibition of Vibrio cholerae strains by aqueous, ethanolic, and methanolic extracts

<table>
<thead>
<tr>
<th>Common name (part used)</th>
<th>Scientific name</th>
<th>Aqueous</th>
<th>Ethanol</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1837</td>
<td>569-B</td>
<td>1837</td>
</tr>
<tr>
<td>Basil (whole plant)</td>
<td>Ocimum basilicum (L.)</td>
<td>NI</td>
<td>NI</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Artichoke (whole plant)</td>
<td>Cynara scolymus (L.)</td>
<td>NI</td>
<td>NI</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Peanut (fruit)</td>
<td>Arachis hypogaea (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Pumpkin (fruit)</td>
<td>Cucurbita pepo (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Sweet potato (fruit)</td>
<td>Ipomoea batatas (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Naseberry (fruit)</td>
<td>Manilkara zapota (L.)</td>
<td>NI</td>
<td>NI</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Nopal cactus (cladode)</td>
<td>Opuntia ficus-indica (L.)</td>
<td>NI</td>
<td>NI</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Japanese plum (fruit)</td>
<td>Prunus salicina (Lindl.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Coconut (fruit)</td>
<td>cocos nucifera (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Summer rape (root)</td>
<td>Brassica napus (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Cranberry (fruit)</td>
<td>Vaccinium macrocarpon (Ait.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Peach (fruit)</td>
<td>Prunus persica (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Asparagus (fruit)</td>
<td>Asparagus officinalis (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>White sagebrush (whole plant)</td>
<td>Artemisia ludoviciana (Nutt.)</td>
<td>NI</td>
<td>NI</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Raspberry (fruit)</td>
<td>Rubus idaeus (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Spearmint (whole plant)</td>
<td>Mentha spicata (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Irish lace (fruit)</td>
<td>Tagetes filifolia (Lag.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Sweet acacia (bark)</td>
<td>Acacia farneciana (L.)</td>
<td>Wildld.</td>
<td>NI</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Kiwi fruit (bark)</td>
<td>Actinidia chinensis (Planch.)</td>
<td>NI</td>
<td>NI</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Mango (fruit)</td>
<td>Mangifera indica (L.)</td>
<td>0.9 ± 0.01</td>
<td>0.8 ± 0.01</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Cantaloupe (fruit)</td>
<td>Cucumis melo (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Honey mesquite (bark)</td>
<td>Prosopis glandulosa (Torr.)</td>
<td>NI</td>
<td>NI</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Pineapple (fruit)</td>
<td>Ananas comosus (L.) Mettr.</td>
<td>0.8 ± 0.01</td>
<td>0.7 ± 0.01</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Cattowba grape (fruit)</td>
<td>Viitis labrusca (L.)</td>
<td>0.7 ± 0.01</td>
<td>0.7 ± 0.01</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Golden sword (flower)</td>
<td>Yucca filifera (Chabaud.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Lemon grass (leaves)</td>
<td>Cymbopogon citratus (Stapf.)</td>
<td>NI</td>
<td>NI</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Poblano pepper (fruit)</td>
<td>Capsicum annuum (L.)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations. NI, no inhibition.
TABLE 2. MBCs of selected methanolic extracts against two V. cholerae strains

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>MBC (mg/ml) for V. cholerae strain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil</td>
<td>Ocimum basilicum</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Nopal cactus</td>
<td>Opuntia ficus-indica</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Sweet acacia</td>
<td>Opuntia ficus-indica</td>
<td>3 ± 0.05</td>
</tr>
<tr>
<td>White sagebrush</td>
<td>Artemisia ludoviciana</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations.

RESULTS

Preliminary screening and MBC determinations. A total of 81 extracts were prepared from the 27 plants evaluated for antimicrobial activity against V. cholerae. In the preliminary assay, 16 methanolic extracts showed good activity against both strains tested and were selected for further MBC analysis (Table 1). In addition, eight ethanolic extracts were effective against V. cholerae strains, while only three aqueous extracts displayed growth-inhibitory activities (Table 1).

MBC analysis indicated that methanolic extracts of basil (Ocimum basilicum L.), nopal cactus (Opuntia ficus-indica var. Villanueva L.), sweet acacia (Acacia farnesiana L.), and white sagebrush (Artemisia ludoviciana Nutt) exhibited the highest antimicrobial activities. The MBC of nopal cactus extracts was 3 mg/ml, and for basil the MBC ranged from 2 to 3 mg/ml, whereas sweet acacia and white sagebrush exhibited the lowest MBCs (0.5 to 1.0 mg/ml) against both bacterial strains (Table 2). The MBC of the other extracts was greater than 10 mg/ml, and these extracts were not examined further.

Effects of plant extracts on membrane integrity. The LIVE/DEAD BacLight system used to analyze membrane integrity after addition of methanolic extracts was effective at demonstrating the antimicrobial activities of the extracts. Epifluorescence microscopic observations revealed that the cells treated with extracts at 10× or 20× the MBCs were stained (100%) by propidium iodide, a characteristic of cells with membrane damage (data not shown). The MBCs of the four extracts against the two strains resulted in 1 to 14% stained cells, and 5× the MBC resulted in 8 to 41% stained cells. Non-membrane-damaged cells (stained green with Syto9 dye) appeared (<1%) in controls (with methanol in the absence of extracts).

Effects of plant extracts on pH. A clear change in cytoplasmic pH was observed after addition of methanolic extracts (Fig. 1). Exposure of V. cholerae strains to all extracts tested decreased the pH in significantly (P ≤ 0.05), from 7.2 to 3.9 ± 0.5 (standard deviation). Sweet acacia had the strongest effect on both V. cholerae strains; however, V. cholerae strain 1837 was more sensitive (P ≤ 0.05) to all the extracts tested except for sweet acacia.

Effects of selected extracts on membrane potential. DiBAC <sub>4</sub> (3), a fluorescent dye used for monitoring changes in the membrane potential of the V. cholerae strains, demonstrated that cells treated with methanolic extracts of basil, white sagebrush, and sweet acacia displayed cell membrane hyperpolarization, as evidenced by a decrease in fluorescence (negative values).

On the other hand, nopal cactus treatment caused rapid
depolarization, as evidenced by an increase in fluorescence (Fig. 2).

**Effects of selected extracts on total ATP concentration.** Our results indicated that all extracts provoked significant decreases \((P \leq 0.05)\) in cellular ATP concentrations in the two strains tested (Fig. 3). White sagebrush and sweet acacia had the strongest effects in both *V. cholerae* strains. Basil was less active, followed by nopal cactus.

**DISCUSSION**

The antimicrobial properties of plants have been recognized for a long time; however, in many cases, the mechanisms of action are poorly understood (15). In this work, the antimicrobial properties of aqueous, ethanolic, and methanolic extracts were evaluated using the well diffusion technique (31). From 81 extracts analyzed, 4 (basil, nopal cactus, sweet acacia, and white sagebrush) possessed the highest detectable anti-*Vibrio* activities. In all cases, the methanolic extracts were more active than the ethanolic or aqueous extracts. Methanolic extracts from plants consistently provide more antimicrobial activity than those extracted in ethanol or other more polar substances (10, 27). The higher antibacterial activity of methanol extracts is hypothesized to be due to the polarity of the solvent and to the capability to dissolve or diffuse into the medium used in the assays (10).

Of the four most active plants extracts, basil and nopal cactus have been commonly used as edible plants, while sweet acacia and white sagebrush have been used as medicinal plants. Basil has been used principally as a culinary herb, to add flavor to soups and sauces. The leaves are generally used fresh in salads, but these can also be dried to make a refreshing tea (25). Medicinal uses have also been reported, including antibacterial, digestive, and gastritic uses, for the treatment of colds and influenza disease, and for nausea and gastrointestinal threat (30).

Nopal cactus is a crop with multiple uses. It plays an ecological role in soil conservation, as well as in the production of edible fruits, vegetables, and other value-added products. Plant cladodes or cactus stems are used as fresh green vegetables (nopalitos and cactus) for human consumption. The fruit as well as the cactus stem are used to prepare value-added products, such as jam, squash, wine, pickles, body lotions, shampoos, and creams. Nopal cactus also has several medicinal and industrial uses (37).

Phytochemical analysis of these edible plants has identified several compounds that could be responsible for the antimicrobial activities observed in this study. Hussain et al. (19) reported that basil contains essential oils (principally linalool),

![FIG. 1. Effects of methanolic plant extracts on the intracellular pH of two *V. cholerae* strains. Ctr, control; MeOH, methanol control.](http://aem.asm.org/)

![FIG. 2. Effects of selected extracts on the membrane potentials of *V. cholerae* strains. Negative (hyperpolarization) and positive (depolarization) values produce a loss of cellular homeostasis. Ctr-MeOH, methanol control.](http://aem.asm.org/)
two components of the proton motive force, the pH\textsubscript{in} and the
membranes (22, 39, 43). In this work, several fluorometric tech-
iques has advantages, since plate counting, a very popular
method to give symptomatic relief of diarrhea. The tea made
with this plant has been used in traditional Mexican medicine
to alleviate intestinal pain (32). On the other hand, sweet
acacia has many traditional uses: the flowers are used as head-
ache remedies and for the treatment of indigestion; the fruit is
used to treat dysentery and skin inflammations (40). The
MBCs obtained with white sagebrush and sweet acacia were
relatively low, ranging from 0.3 to 0.9 mg/ml. Garcia et al. (14)
reported that ethanolic extracts of Acacia farnesiana and Aca-
cia ludoviciana had MBCs ranging from 4 to 7 mg/ml against
three strains of V. cholerae. These concentrations slightly differ,
but the discrepancy could depend on factors such as plant
source, time of collection, bacterial strain, and solvent used for
evaluation of the ATP hydrolysis reaction as a consequence of in-
creased permeabilization, which was detected as an increase in
nuclear propidium iodide staining.

The cFSE technique for measuring the pH\textsubscript{in} of bacteria is
based on intracellular conjugation of the succinimidyl group of
cFSE with the aliphatic amines of intracellular proteins and
subsequent elimination of free probe by a short incubation in
the presence of glucose (18). The method has been reported to be
effective for Gram-positive and Gram-negative bacteria; however, some interference can occur with Gram-negative
bacteria, because of the inability of cFDA-SE to cross the outer membrane of Gram-negative microorganisms. Preincu-
bation of bacteria with EDTA has been suggested to circum-
vent this problem (7). In this work, all the extracts induced
changes in the pH\textsubscript{in}, indicating that membrane damage had
occurred. Reduction in the internal pH in other organisms,
such as Staphylococcus aureus, has been observed to occur as
the result of treatment with oregano essential oil, thymol, or
carvacrol (22).

DiBAC\textsubscript{4} (3) is a fluorescent membrane potential dye that
has been used as an indicator of changes in membrane polar-
ization (41). The fluorescence emitted by the dye is enhanced
when the dye crosses the cell membrane as a result of mem-
brane depolarization (less negative charge inside the cell) (44).
Three of the methanolic extracts analyzed (basil, white sage-
brush, and sweet acacia) caused a hyperpolarization (more
negative charge inside the cell) of cellular membranes. Al-
though a depolarization effect was expected, hyperpolarization
has also been reported as an important type of membrane
damage (45). Recent studies of this phenomenon have con-
cluded that hyperpolarization occurs primarily due to a pH
change (from acidic to neutral) or by increasing movement of
ions, specifically K\textsuperscript{+}, which diffuses out of the cell membrane
through K\textsuperscript{+} channels and affects cellular homeostasis (5). Ex-
tracts of nopal cactus increased fluorescence in the treated
cells due to membrane depolarization (less negative inside the
cell) as a result of a decrease of the membrane potential (20).

When metabolic ATP was measured in the presence of
methanolic plant extracts, the results showed that all four ex-
tracts caused a decrease in the cytoplasmic ATP concentration
of V. cholerae. Although a direct effect of the extracts on
increased ATPase activity cannot be ruled out, it has been
demonstrated that natural products present in plant extracts
affect the permeability of Listeria monocytogenes membranes,
leading to a subsequent decrease in cytoplasmic ATP as a
consequence of envelope damage (8). Gonzalez et al. (16)
reported that diminished ATP concentrations could be caused
by release of cytoplasmic ATP and unabated hydrolysis by the
proton-pumping ATPase, which results in rapid depletion of
the intracellular ATP pool. Two mechanisms have been pro-
posed to explain the ATP hydrolysis: (i) a shift in the equilib-
rium of the ATP hydrolysis reaction as a consequence of in-
organic phosphate loss through the membrane due to impaired
permeability, and (ii) depletion of the intracellular ATP pool
and dissipation of proton motive force components (42). The
effect of antimicrobial compounds on the diminishing proton
motive force is strongly correlated with the leakage of specific
ions (2). Several authors have reported that natural preserva-
tives, including essential oils, phenols, and bacteriocins, can
promote the loss of cellular components, such as ions, ATP,
nucleic acids, amino acids, etc. (11, 22, 42).
The cell membrane is an active structure that acts as a barrier between the cytoplasm and the extracellular medium. It is essential for maintaining optimal internal conditions for metabolism and energy transduction. The plant extracts studied here were able to disrupt the cell membrane, resulting in increased permeabilization, changes in the pH_{in} and the membrane potential, and a decrease in the cytoplasmic ATP concentration, which together resulted in bactericidal activity. Secondary effects that may be involved and further decrease viability include the inhibition of several enzymes caused by leakage of essential ions, loss of turgor pressure, and alterations in macromolecular synthesis and other processes in the bacterial cell.

We conclude that the extracts of two edible plants (basil and nopal cactus) and two medicinal plants (white sagebrush and sweet acacia) cause damage to the membrane of *V. cholerae*, exerting profound physiological changes that lead to bacterial death. Additional experiments will be needed to provide information about other microbial sites or aspects affected by these extracts. Experiments are in progress to determine the specific compounds responsible for the antimicrobial activity of each extract. These four plant extracts could be studied as future alternatives to control *V. cholerae* contamination in foods and the diseases associated with this microorganism. However, for application in foods, appropriate methods for the nontoxic extraction of the active compounds would have to be developed. Also, the effect of active compounds on the beneficial and normal microbial flora in the human body would have to be determined, as well as the risks and benefits of potential applications in humans, including toxicity studies.

ACKNOWLEDGMENTS

This research was supported by the Consejo Nacional de Ciencia y Tecnología de México (CONACYT) and PAICYT-UANL. Eduardo Sánchez was supported by a scholarship from CONACYT.

We thank Ronald Labbe for his helpful discussions.

REFERENCES


