Isolation and characterization of a bactericidal withanolide from *Physalis virginiana*

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ABSTRACT

Background: *Physalis virginiana* (Virginia Groundcherry) is a member of the family Solenaceae. Several species of the *Physalis* genus have been used traditionally by American Indians as medicinal treatments. **Materials and Methods:** This study investigated the antibacterial activity of chemicals extracted from *P. virginiana* through antibacterial disc and cytotoxicity assays. Isolation and purification of an antimicrobial compound was achieved through flash chromatography and preparative HPLC. Finally, identification of chemical structure was determined from ^{1}H and ^{13}C NMR and MS. **Results:** Disc assays showed that crude ethanol extracts were effective antibacterial agents against one gram-negative and seven gram-positive bacterial strains. Cytotoxicity assays indicated that it is less toxic than gentamicin controls. Isolation of the active component showed it to be a relatively polar compound. ^{1}H and ^{13}C NMR chemical shifts together with HRMS indicated a similar structure to withanolides previously identified from *Physalis angulata*. HRMS analysis showed a molecular mass of 472.2857 which corresponds to a molecular formula $C_{28}H_{40}O_6$. **Conclusion:** An antibacterial withanolide was isolated from *P. virginiana* using flash chromatography and HPLC separations. The chemical structure was determined by NMR and MS to be the withanolide physagulin V.

Key words: Antibacterial, cytotoxicity, physagulin V, *Physalis virginiana*, withanolide

INTRODUCTION

Physalis virginiana (Virginia Groundcherry) is a member of the family Solenaceae. American Indians traditionally used several species of *Physalis* as treatments for eye infections, open wound dressings, and as treatments for various gastrointestinal symptoms.[1] Although there is a paucity of data on the medicinal properties of *P. virginiana* extracts, compounds isolated from other species of *Physalis* have been shown to inhibit bacteria, fungi, parasites, and cancerous tumors.[2] Physalins, isolated from *Physalis angulata*, have demonstrated the ability to inhibit the growth of *Mycobacterium tuberculosis*.[3] Tuberculosis infections are of particular concern because of the growing resistance of the bacterium to current treatment methods.[4] Calystegins, isolated from *P. alkekengi* var. *francheti,* have the ability to inhibit the enzyme β-76 glucosidase.[5] Inhibition of this enzyme has widespread potential for treatment of diabetes, bacterial and viral infections, and as an insecticide.[6] A third class of compounds isolated from *Physalis* spp. are withanolides.[7,8] Withanolides are steroidal lactones which have been found in several species of Solanaceae.[9,10] Withanolides have been shown to have antiparastic effects against the protozoan *Trypanosoma cruzi*- the cause of Chaga's disease[11] and to have antibacterial properties.[12]

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The purpose of this study was to characterize the efficacy of *P. virginiana* extracts using antibacterial assays. The antibacterial activity of chemicals extracted with various polar solvents, as well as the effects of pH and heat stability were determined. The ultimate goal of this research was to identify specific compounds from *P. virginiana* that may be useful in development of new antibiotics. At each step in the isolation scheme, antimicrobial activity was confirmed by screening against eight susceptible bacteria.

Isolation and purification of an antimicrobial compound was accomplished with flash chromatography and preparative HPLC separations. Once purified, the active compounds were identified using NMR and mass spectrometry.

MATERIALS AND METHODS

Chemicals

Mueller-Hinton broth, sterile blank paper discs, and gentamicin Sensi-Discs (10 μg per disc) were obtained from Fisher Scientific. HPLC grade methanol and acetic acid were obtained from Sigma–Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

Bacteria

Twelve species of bacteria were used for these investigations (*Bacillus cereus, Corynebacterium xerosis, Micrococcus luteus* and *Micrococcus roseus, Staphylococcus aureus* and *Staphylococcus epidermidis, Streptomyces viridosporus, Mycobacterium smegmatis, Citrobacter freundii, Escherichia coli, Pseudomonas aeruginosa* and *Pseudomonas fluorescens*). The bacteria were chosen based upon their Gram stain (gram positive or negative) and their association with human infections.

Disc susceptibility test

Sterile paper discs, 6 mm in diameter, were infused with 20 μl of the *P. virginiana* extract (40 mg dry wt. equivalent of plant material per disc).[13] The discs were allowed to completely air dry. Individual bacterial strains were streaked for to isolation using Mueller-Hinton (MH) agar. Isolated colonies were collected after appropriate growth times and used to inoculate MH broth. The inoculated broth was incubated for 24 h at 35–37 °C. *Mycobacterium smegmatis* required an extra 24 h of incubation prior to plating. Plates were inoculated with 100 μl of bacterial culture, spread evenly over the MH agar.[14] The extract-infused discs were then placed onto the agar. Each extract was screened with three replicates and a randomized disc placement. Each agar plate also contained one gentamicin-infused disc as the positive control and one solvent-infused disc as the negative control.[13] The inoculated plates were incubated for 24 h (*M. smegmatis* for 48 h) at 35–37 °C. After 24 h, the zone of inhibition (the diameter of no bacterial growth) was measured.[15]

Lethality bioassay

To examine cytotoxicity of the extracts, culture water was prepared by dissolving 15.0 g of aquarium salt in 1 l of water. Acidity was adjusted with sodium bicarbonate to ~pH 7.5. Cultures were maintained at 25 °C, and an aquarium pump was used to aerate the tank. *Artemia salina* eggs (brine shrimp) were added to the salt water. Two days after hatching, the brine shrimp nauplii were ready for the assay. Aliquots of freshly prepared salt water, containing three different concentrations of each extract were prepared. The salt water and extracts were added to yield a final volume of 5 ml per tube with extract concentrations made to 10, 100, and 1000 μg/ml.[16,17] Ten nauplii per replication were collected using a dissecting microscope and a plastic pipette and placed into each of the test tubes. Only *A. salina* that were active swimmers and appeared healthy were used in this assay. Twenty-four hours later the *A. salina* were observed again using the dissecting microscope, and the number of survivors was recorded.[18-20]

Solvent extractions

One gram of ground dried *P. virginiana* shoots and 10 ml of solvent were placed into a 100 ml bottle. The bottle was covered to protect it from sunlight, and the sample was shaken for 96 h at 100 rpm.[21] After 96 h, the sample was vacuum filtered using a VWR grade 415 qualitative filter paper. The solid plant material was then rinsed with another 10 ml of solvent.[14,22] Solvents were removed under vacuum at room temperature, and residual water was removed by lyophilization.[13] Dried extracts were brought to a final concentration of 2 g of plant material per ml in the extraction solvent.[13] Solvents tested were 100% methanol, 70% ethanol, 100% ethanol, and acetonitrile. An additional extraction was performed using water. The water sample was extracted under refrigeration to minimize bacterial growth. All extracts were screened for activity as described above.

pH testing

The initial pH the 70% ethanol extract was 5.9 ± 0.0. The pH of extracts was adjusted to 2, 4, 7, 9, and 11 using 1 M HCl or 1 M KOH.[22] Each sample was then infused into sterile paper discs (6 mm diameter). The disc susceptibility test was then performed from with these samples.

Heat stability

One milliliter of the 70% ethanol crude extract was placed into a sealed tube. This tube was placed into a water bath (90 °C).[23] Samples were heated for 0, 10 and 20 min.[22] Tubes were then removed from the water and brought to room temperature. Each heat treated extract was then screened using the disc susceptibility test.

Flash chromatography

Dried ground *P. virginiana* shoots (185 g) and 1850 ml of 70% ethanol were placed in a 4 l bottle. The bottle was covered to protect it from sunlight, and the sample was placed onto an orbital shaker for 96 h at 100 rpm.[21] After 96 h, the sample was vacuum filtered using a VWR grade 415 qualitative filter paper. The solid plant material was then rinsed with another 1850 ml of 70% ethanol.[14,22] The extract was brought to dryness under vacuum.[13]

The dried extract (33.67 g) was redissolved in 100% methanol. Once dissolved it was combined with 60 g of silica gel (Sorbent Technologies, porosity 60 Å, particle size 32–63 μm). The silica/extract combination was dried under nitrogen.

A 1000 ml flash column was prepared with 300 g of silica gel mixed with dichloromethane. One-third of the dried silica/extract mixture was added to the top of the flash chromatography column. The column was washed with 1500 ml each of 10%, 20%, 30%, 40%, and 100% methanol in dichloromethane. All washes were collected in 250 ml aliquot and labeled with the methanol percentage and the number of the fraction. Each 250 ml sample was screened against *B. cereus* for activity using the disc susceptibility test.

HPLC separation

Chromatography was performed using the Varian Prostar model 210 solvent delivery system and the Varian Prostar Model 330 detector. Absorption was measured at 254 nm. Separations were made with an Alltech C18, 250 mm × 22 mm column with a particle size of 10 µm, using degassed methanol and water as the solvents. The flow rate was 10 ml/min, and the column was equilibrated with 40% methanol. The gradient was: 40–60% in 5 min, 60–65% in 5 min, an isocratic elution at 65% for 10 min, followed by 65–73% in 28 min. Each fraction was dried under vacuum and subjected to the disc susceptibility test.

NMR

HPLC purified samples (2.0 mg) were analyzed in a Brucker 400 GRX NMR spectrometer. The sample was dissolved in methanol-d_4. Internal reference standard was tetramethylsilane (TMS). ^{1}H and ^{13}C NMR, DEPT, and 2D NMR (HMBC and HMBC) spectra were collected with standard pulse sequences.

Mass spectrometry

Samples were dissolved in a solution of methanol, and analyzed using a Kratos MS 50 HRMS EI+.

Statistical analysis

Means and standard deviations were calculated using SAS 9.1 (SAS Institute Inc., 100 SAS Campus Drive, Cary, NC). Analysis of variance, general linear model, correlation data, and Tukey's multiple comparisons were performed using JMP IN 5.1 (SAS Institute Inc., 100 SAS Campus Drive, Cary, NC).

RESULTS

Extract screening

The inhibition of bacterial growth, in comparison to that of gentamicin, is shown in Figure 1. The activity of root, shoot, and fruit extracts against 12 bacterial strains, both gram positive and gram negative, are shown. The extracts significantly inhibited growth in eight of the 12 bacterial strains, with four gram negative species (*Escherichia coli, Citrobacter freundii, Streptomyces viridosporus*, and *Pseudomonas aeruginosa*) showing complete resistance to the *Physalis* extracts.

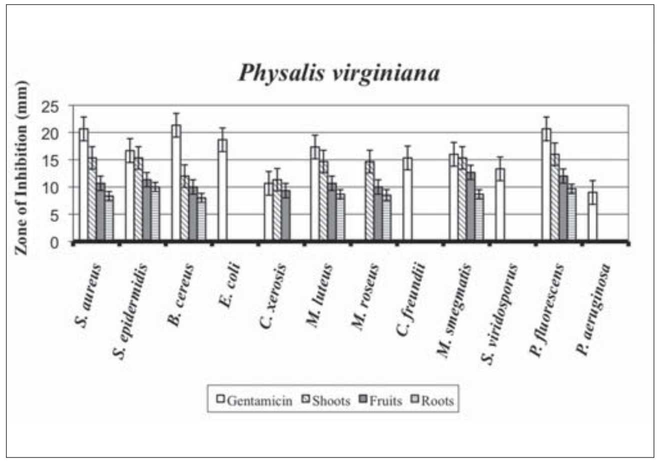

Figure 1: Zones of inhibition (mm) for the plant *Physalis virginiana* against the 12 bacteria. Positive control was gentamicin and negative control was ethanol. All ethanol controls had 0 mm zones of inhibition. Error bars indicate upper and lower 95% confidence intervals. Linear regression ANOVA results: (shoots) $R^2 = 0.9595$, Prob > $F \leq 0.0001$; (fruits) $R^2 = 0.9705$, Prob > F = <0.0001; (roots) $R^2 = 0.9839$, Prob > F = <.0001; (gentamicin) $R^2 = 0.9389$, Prob > F = <.0001

Figure 2 shows the mean efficacy, against eight bacterial strains, of *Physalis* extracts made using several solvents. The data clearly indicate that the polar antibacterial components found in this plant are most efficiently extracted by a 70% ethanol solution. The impact of changing the pH of the shoot extracts also had no significant effect on their efficacy. There was a trend for lower activities as the pH increased, but even at pH 11, the analysis showed no significant inhibition of activity. The antibacterial compounds in the shoot extracts were also shown to be heat stable, with no significant difference in extract efficacy with any of the treatments.

Brine shrimp lethality

P. virginiana cytotoxicity values are shown in Figure 3. At all concentrations and of the shoot, root and fruit extracts showed no cytotoxicity in the brine shrimp assay. In all cases, survival rates were not significantly different from the controls. However, gentamicin was more toxic in this assay, at 1000 µg/ml the gentamicin was almost 100% lethal to the brine shrimp.

Withanolide purification

Thirty fractions were collected from the flash chromatography column. Of these, all but six showed no activity in disc susceptibility assay. Activity was demonstrated in the 10% and 30% methanol dichloromethane fractions. Further purifications were made using sample 10-5 (10% methanol, 1000–1250 ml elution fraction). The dried sample, weighing 139.8 mg, was dissolved in 100% HPLC grade methanol to a volume of 3.0 ml (46.6 mg/ml). A 20 µl sample was injected into the HPLC and fractionated.

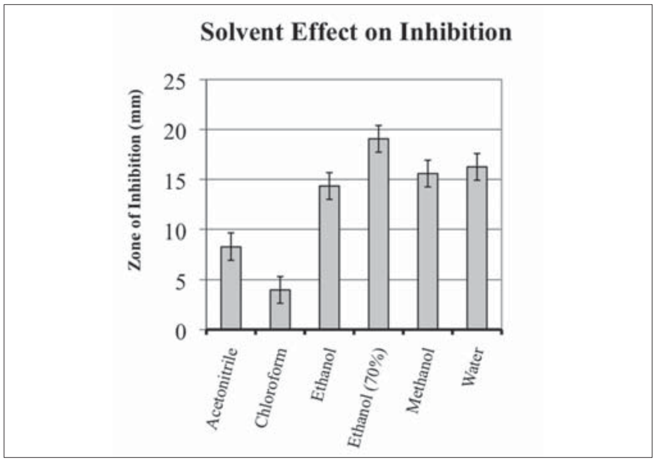

Figure 2: Zones of inhibition (mm) for *Physalis virginiana* shoots extracted in different solvents screened against eight bacteria. Positive control was gentamicin and negative controls were each of the solvents tested. All negative controls were zero. Error bars indicate upper and lower 95% confidence intervals. Linear regression ANOVA results: $R^2 = 0.8378$, Prob > $F \leq 0.0001$

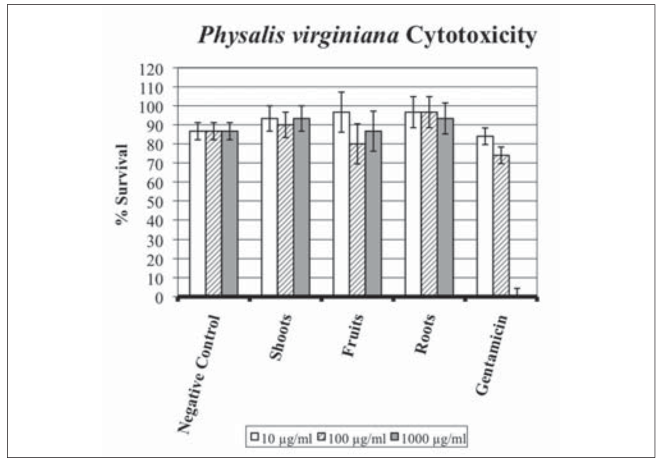

Figure 3: Cytotoxicity of *Physalis virginiana* screened against *Artemia salina* in concentrations of 10, 100, and 1000 µg/ml. Error bars indicate upper and lower 95% confidence intervals. Linear regression ANOVA results: (negative control) $R^2 = 0$, Prob > $F = 1.000$, r = 0.0000. (shoots) $R^2 = 0.1429$, Prob > $F = 0.6297$, $r = 0.1614$; (fruits) $R^2 = 0.5588$, Prob > $F = 0.0859$, $r = -0.1465$; (roots) $R^2 = 0.1$, Prob > $F = 0.7290$, r = –0.3152; (gentamicin) $R^2 = 0.9887$, Prob > $F = <.0001$, $r = -0.9934$

Samples were collected at one minute intervals. Each fraction was then tested using the disc susceptibility assay.

The HPLC separation of sample 10-5 yielded three peaks showing a range of bacterial inhibition. The first peak, retention time = 20.6 min, eluted at about 66.25% methanol; the second peak, retention time = 28.4 min, eluted at about 68.25% methanol; and the final peak, retention time = 43.4 min, eluted at about 72% methanol. Peak 2 demonstrated the strongest effect in the disc susceptibility assay and was chosen for further purification.

NMR and mass spectrometry

In comparison of isolated active compounds with published data,[11] main spectroscopic characteristics (NMR and MS) of withanolides were identified. A few of the observed chemical shifts were clearly not found in a compound of the same group. ^{1}H [Figure 4] and ^{13}C NMR showed no chemical shifts for acetate at C15 and no double bond at C24–C25 which accounts for the difference in structure from physagulin I identified from *P. angulata*.[11] HRMS analysis showed a molecular mass of 472.2857 [Figure 5] which corresponds to a molecular formula $C_{28}H_{40}O_6$ [Figure 6]. Proton and carbon NMR chemical shifts together with HRMS indicated a similar structure to withanolides previously identified from *P. angulata*.

DISCUSSION

The disc susceptibility test is a quick screening method which employs agar diffusion to identify the antimicrobial potential of a given plant extract.[24] After inoculation and incubation, active compounds displayed a zone of inhibition in which there was no bacterial growth.[13,25] Although this method provides a good method for quickly evaluating the inhibition of antibacterial hydrophilic compounds, it does not allow evaluation of hydrophobic compounds due to their limited diffusion in agar.

Initial studies, utilizing the disc assay, identified *P. virginiana* extracts as one of several plant species offering a potential source of new antibiotic compounds.[26] To our knowledge, this is the first demonstration of antibacterial activity from this species of *Physalis*. Extractions from the roots, shoots and fruits of this species consistently identified the shoots as the tissues containing the highest concentrations of this activity. Extractions of the tissues with several different polar solvents showed that 70% ethanol provided the highest activity levels in response to their diffusion in agar.[24,25]

The pH of this extract was 5.9 ± 0.0 and modification of the pH had a little effect on the biological activity. Screening of the *P. virginiana* extracts also demonstrated that the antimicrobial activity of the extracts was heat stable. These data suggested that the active compounds were unlikely to be proteins, but most probably hydrophilic secondary plant products.[23]

P. virginiana inhibited eight of the 12 bacteria screened. The whole plant (shoots, fruits, and roots) demonstrated activity, with the shoots being the most active and the roots the least active. Seven of the eight bacteria inhibited were gram positive bacteria, which may indicate that the antibacterial

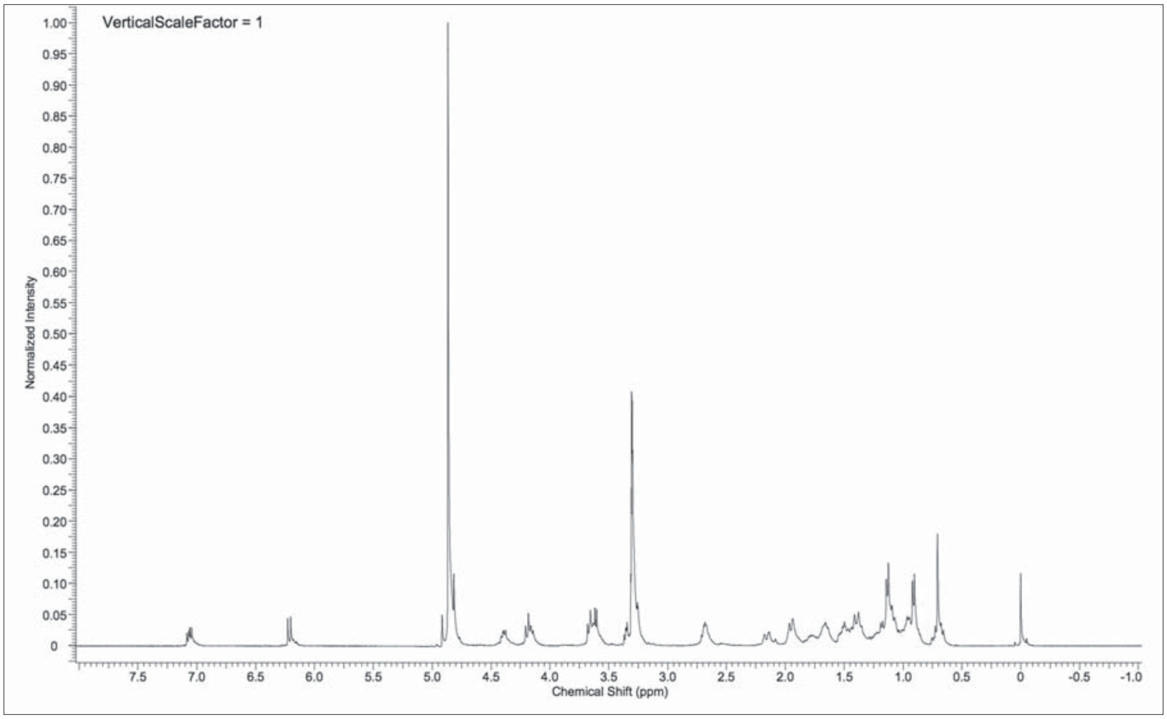

Figure 4: ^{1}H NMR spectra of physagulin V. The spectrum was obtained at 400 MHz in MeOD at 27 °C with an acquisition time of 3.9715 s.

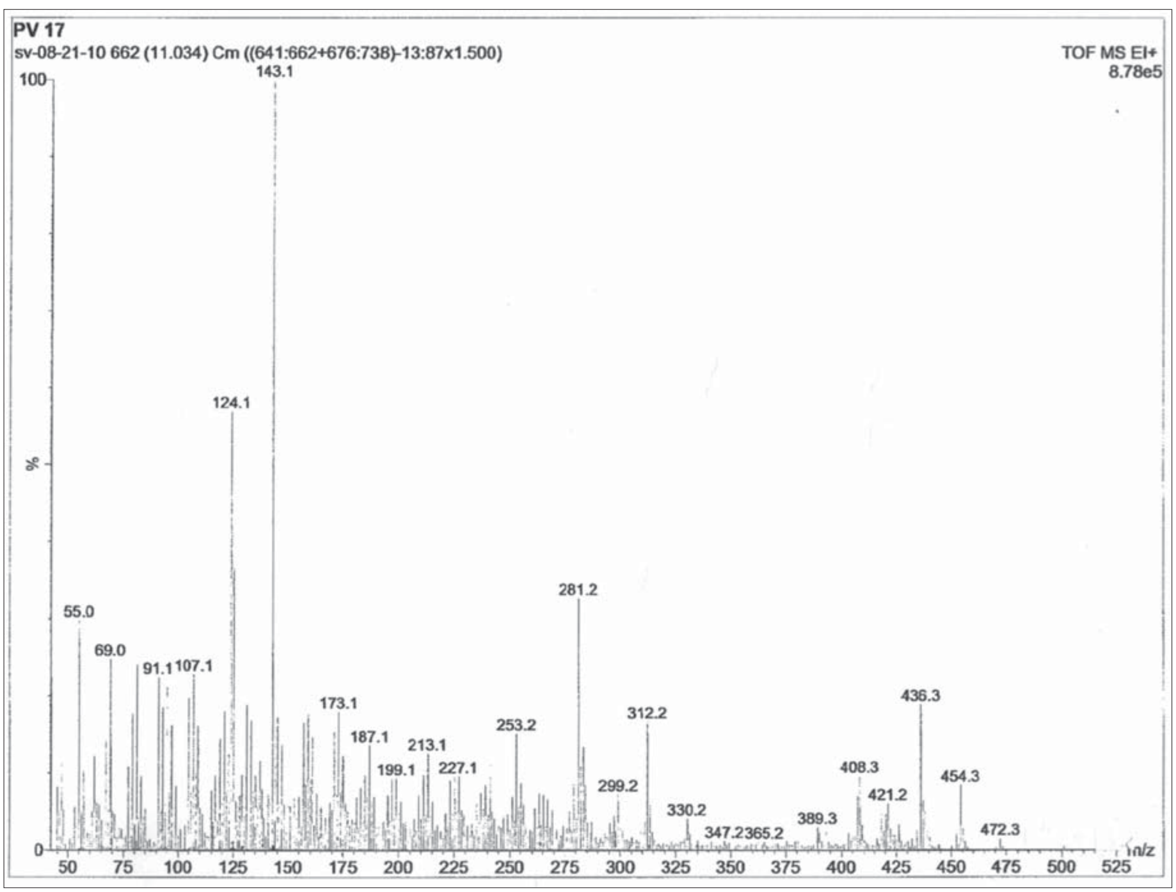

Figure 5: MS of physagulin V

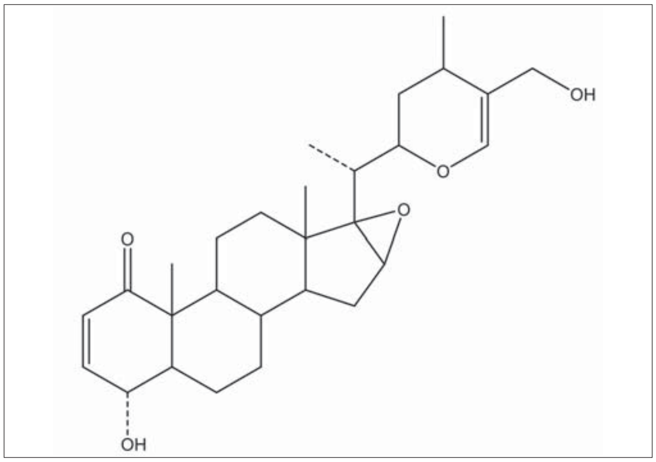

Figure 6: Chemical structure of compound **1** (physagulin V)

activity in *P. virginiana* is due to a small polar compound having low lipophilicity and not able to pass through the outer membranes present in gram negative bacteria.[27]

Crude extracts were further tested for cytotoxicity using the brine shrimp lethality test. Brine shrimp (*Artemia salina*) are small crustaceans which are sensitive to a broad range of cytotoxic compounds.[28] The amount of cytotoxicity of a given extract was determined by counting the number of surviving *A. salina* after 24 h of exposure to the given plant extract.[18-20] These results support the idea that the antibacterial components in *P. virginiana* should be tolerated by eukaryotes.

Identification of the most active antibacterial compound from *P. virginiana* was conducted using silica gel chromatography. The most active fraction was eluted in 10% methanol:90% dichloromethane. Although total activity was increased by adding water to the ethanol extractant, the elution of the most active component under these conditions suggests that there may be one or more polar factors that act synergistically with this compound to increase the overall activity of the crude extracts.

HPLC separation of the most active antibacterial silica gel elution fraction revealed three peaks representing one primary compound and two secondary components. Comparisons with other species of the *Physalis* genus and the physical data collected here suggest that the compound could be a withanolide. Withanolides were first discovered in the plant *Withania somnifera* (family Solenaceae),[29] and are predominantly found in plants of this family.[30,31] They are often located in the leaves of the plant, but in some species such as *P. peruviana*, they are quite prevalent in the roots.[29] As these compounds are often glycosylated,[32-35] the minor peaks may result from variation in glycosylation.

Withanolides are 28 carbon steroidal lactones[36,37] which are greatly oxidized.[7] Extraction of withanolides varies with the experimenter. Alcohol extraction with methanol or ethanol seems to be the method of choice, with others utilizing *n*-hexane and chloroform,[38] or a hot water extraction.[9]

Withanolides have been credited with the prevention of insect infestations and treatment of parasitic organisms.[9] These withanolides provide a chemical defense preventing the insects from eating the ripened fruit. Withanolides have also been proposed as a possible treatment for parasitic infections such as *T. cruzi* that causes Chaga's disease.[11] Furthermore, withanolides have also been shown to kill various infectious pathogens.[8,12,39]

CONCLUSIONS

Physalgulin V was shown to be an effective antibacterial *in vitro*. The crude extracts showed no apparent cytotoxicity in the brine shrimp assay. These results suggest that a further study of Physalgulin V and the other constituents in the *P. virginiana* extracts is warranted. Continued examination of this non-cytotoxic compound showing effective bacterial inhibition will continue. Exploration of the variation in glycosides and identification of other synergistic compounds, found in the crude extracts, are planned.

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