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Research Paper

Molecular and cytotoxicity investigations of *Phytolacca americana* (l.) root, leaf, and berry extracts

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Abstract

Background: Pokeweed anti-viral protein (PAP) and lectin are two of the toxic components of pokeweed, *Phytolacca americana*, suspected of affecting free grazing livestock and small herbivorous animals.
Objectives: This research aimed to investigate the antimicrobial activity of the pokeweed extracts against two bacterial strains, the gram negative *Escherichia coli* and the gram positive *Staphylococcus aureus*, to investigate the toxicity of the extracts to cells of tobacco, *Nicotiana tabacum*, callus, and to investigate the presence of selected toxic constituents present in pokeweed.
Methodology: Pokeweed plants were identified and brought to the laboratory and separated into roots and leaves. The berries were collected later in the growing season. Aqueous extracts were obtained by homogenizing the plant parts separately in sterile water followed by centrifugation. The supernatants were filter-sterilized and used for bacterial and tobacco callus growth inhibition assays. Total cytoplasmic proteins were also obtained by homogenizing the plant parts separately in protein extraction buffer and centrifuging. The supernatants were investigated for the presence of various toxins suspected of being present in pokeweed, using western blot analyses.
Results and Conclusions: Pokeweed constituents possess growth inhibitory effects to gram negative E. coli and to *N. tabacum* callus but not of the gram positive S. aureus, and that all three plant parts studied were rich in lectin and lectin-like constituents such as PL-A, PL-C, and PL-G. No PL-B was detected in any of the plant extracts.

Keywords: *Pokeweed; Cytotoxicity, Lectin; Antibacterial; Callus.*

1. Introduction

Pokeweed (*Phytolacca americana* L.) is a common perennial native plant found in the United States' northern, central and southern states. It is thought to be native to the African and the Latin American continents. Pokeweed belongs to the family Phytolaccaceae, which includes several small size trees and vines found in tropics. The genus Phytolacca comprises 35 species, most of them known for their secondary metabolites constituents (Owen 1988, Duke 2001, Ravikiran et al. 2011, USDA-NRCS 2015). In the United States, pokeweed grows naturally in or around fields and along undisturbed property fence lines. Being adapted to diverse growing environments, an individual pokeweed plant can grow up to four meters. The plant bears small flower clusters on green racemes that develop later into black-purple berries (Owen 1988, Ravikiran et al. 2011). At an early stage of growth, pokeweed leaves can be boiled with several changes of water and render them consumable, a dish know in the Southern United States as "Poke salet". Native Americans and other indigenous people have long used the juice from the berries as a dye and coloring agent (Duke 2001).
Pokeweed contains several chemical constituents that are toxic to livestock and other animals. Consumption of pokeweed leaves or roots was reported to have caused death. The symptoms of pokeweed poisoning include a burning feeling in the stomach and nausea followed by vomiting, and diarrhea (Owens 1988, Ravikiran et al. 2011, US-FDA Poisonous Plants Database 2015). When the plant toxins enter the bloodstream and central nervous system, the victim begins excessive salivation and sweating followed by blurred vision, weak pulse, and shallow breathing, paralysis and possibly death. Many of the chemical constituents of pokeweed are toxic and play major roles in the plant defense mechanisms against herbivores. The genus Phytolacca was determined to contain natural products such as the phytolaccasaponins B, E, and G, oleanolic acid, esculentoside E, the phytolaccasides A B D E & G (Suja et al. 1978, Owen 1988, Sparg et al. 2004, Chapagain et al. 2007, Ravikiran et al. 2011). Other chemical constituents of pokeweed include the poisonous alkaloids phytolaccine and phytolaccagenin, and two compounds similar to saponin in chemical properties (Suja et al. 1978, Sparg et al. 2004, Escalante et al. 2002). Also known to be present in pokeweed are the triterpenoid saponins jaligonic acid, phytolaccagenic (phytolaccacinic) acid, esculentic acid, and pokeberrigenic acid (Suja 1978, Sparg et al. 2004, Chapagain et al. 2007, Ravikiran et al. 2011).
In addition, pokeweed plants synthesize and store potentially harmful glycosides and a variety of glycosidase enzymes. The enzymes are sequestered in large quantities in the plant cell vacuoles and are released to the glycosides upon consumption of the plant, causing chemical reactions that liberate hydrogen cyanide (Opassin et al. 2003) as defense mechanism. The plant is also rich in proteins, starch, and tannins. Lectin and the pokeweed antiviral protein (PAP) are two toxic proteins present in pokeweed plant. Lectin belongs to a class of proteins with high carbohydrate-binding affinity. These proteins are known to cause red blood cells to clump together and to stimulate abnormal cell growth (Sussner

et al. 2004). They are synthesized by the plant during its growth and development stage (Chrispeels & Raikhelb 1991) and accumulated in the plant's cellular vacuoles as a defense mechanism against herbivores. PAP is a ribosome inhibiting protein. It inactivates ribosomes by removing a single adenine from rRNA (Rajamohan et al. 1999). Some isoforms of PAP are known to depurinate viral RNA of HIV-1, therefore blocking the viral replication within cell. Aaron and Irvin (1980) showed that PAP has an effect on herpes simplex virus (HSV). These toxic proteins are of secondary metabolite nature for pokeweed plants and are synthesized for the sole purpose of defense against fungal and viral infection (Irvin 1975, Irvin et al. 1980, Ready et al. 1986, Irvin and Uckun 1992, Lodge 1993, Rajamohan et al. 1999).

Several studies have been initiated and conducted to test the antifungal properties of pokeweed extracts (Sindambiwe et al. 1998, Escalante et al. 2002, Sparg et al. 2004, Chapagain et al. 2007) and have concluded that the extracts of pokeweed and of other plant species have antifungal activities to a variety of fungal species. To date, very little is known on the effects of plant extracts on bacterial cells. Biswal et al. (2011) investigated the antibacterial effects of leaf extracts from Derris indica, a plant species belonging to the Fabaceae family. They found that the chemical constituents present in the leaf extracts are good antibacterial agents and are very effective against Staphylococcus aureus, Bacillus subtilis and Escherichia coli. In a separate study, Karadi et al. (2011) investigated the antimicrobial activities in the extracts of two plant species, *Musa paradisiaca* and *Cocos nucefera*, on three bacterial strains and three fungal species. Their results indicated that the extracts of both plant species showed antibacterial and antifungal activities against the organisms tested, and found that gram positive bacteria are more susceptible than gram negative bacteria. Upadhyay (2015) investigated the antimicrobial and antifungal activities of fruit latexes from ten laticiferous plants and found that the tested fruit latexes showed strong antifungal and antimicrobial activities.

Despite the number of reports of antifungal agents present in many plants species including pokeweed, only a few looked at their effects on bacterial cells. This research hypothesizes that extracts of pokeweed plants will show antibacterial activities and will suppress the growth other plant cells. The aims of the research are to investigate the inhibitory effects of pokeweed extracts to gram positive and gram negative bacteria and to tobacco callus, and to verify the presence of the lectins and lectin-like toxic proteins in pokeweed extracts.

2. Materials and methods

2.1. Water (aqueous) extraction for the bioassays

Pokeweed plants were collected from the surrounding areas of the City of Jacksonville and from the Jacksonville State University campus open field areas. The plants were brought to the laboratory and separated into roots and leaves. The berries were collected later in the growing season. The parts were extracted using glass-distilled water to test their effects on bacterial cells and on tobacco callus. The plant parts were homogenized separately at the ratio of 0.25 g of fresh sample/ml of glass distilled water using a Warring blender. The homogenates were filtered through two layers of cheesecloth and then centrifuged at 16,000 g for 10 minutes. The resulting supernatants were filter sterilized and kept in the freezer for use in the growth inhibition assays.

2.1.1. Bacterial growth inhibition assay

Bacterial growth inhibition assays were performed in liquid LB media containing extracts of the roots, leaves, and berries separately. The experimental treatments consisted of a control liquid LB medium only, a liquid LB + root extract, a liquid LB + leaf extract, and liquid LB + root extract as shown in figure 1. The ratio of LB medium to extract was 1:1 for roots and berries. Because the 1:1 ratio for the leaf extracts was too dark green to calibrate the Spectronic 20^{+}, a 1:0.125 was used instead. Gram negative *Escherichia coli* and gram positive *Staphylococcus aureus* bacterial strains were inoculated into 10 ml liquid LB and cultured overnight. 1.3 ml aliquots of the overnight culture were added separately into 8 experimental flasks, supplemented with 0.3 ml of 20% glucose each. The flasks were incubated in a 37°C chambered-orbital shaker set at 130 rpm. Bacterial growth in each flask was assessed as the degree of turbidity measured using a spectronic 20^{+} at 600 nm (A_{660}) every 15 minutes for 3 hours. The assays were repeated three times and statistically analyzed using a one way classification (Steel and Torrie, 1980).

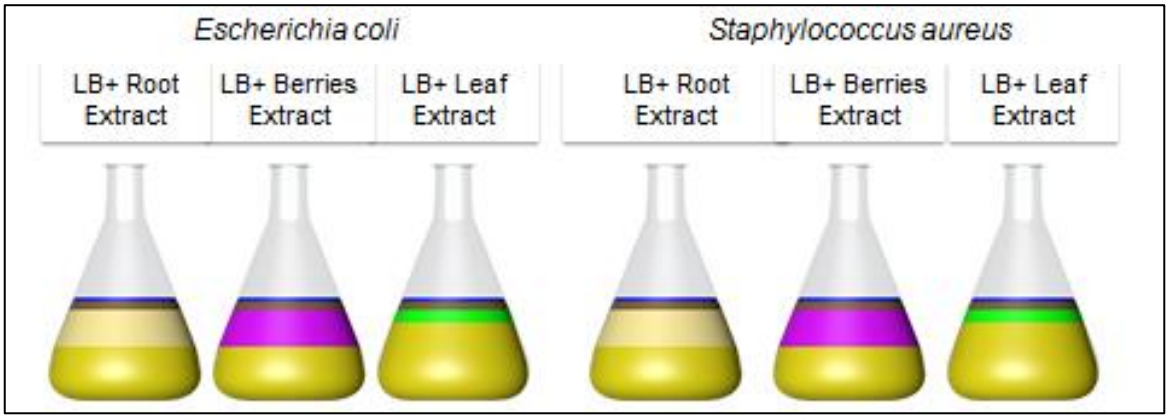


Fig. 1: Experimental Design of the Bacterial Growth Inhibition Assay. Layers in the Flask Represent from Bottom Up, Liquid LB, Plant Part Extract, and Glucose.

2.1.2. Callus growth inhibition assay

Callus growth inhibition assay was performed on Murashige and Skoog (MS) (1962) media containing the different extracts. The base medium consisted of MS macro and micro nutrients supplemented with Gamborg (1968) B-5 vitamins complex, 30g/L sucrose and 2mg/L 2, 4-D as growth regulator. This base medium was denoted 2MS. The experimental treatments consisted of a control 2MS base medium only, a 2MS + root extract, a 2MS + leaf extract, and a 2MS + berry extract. The ratios of the MS to extract were 1:1 for root and berry extracts, and 1:0.125 for leaf extract. The pH of each medium was adjusted to 5.8. TC agar was added at 7 g/L as gelling agent. The base media were autoclaved at 121°C (21 psi) for 15 minutes. After cooling down the media to touch, the filter-sterilized root, leaf, and berry extracts were added separately to the treatment flasks. Then the media were poured into tissue culture Petri dishes and allowed to solidify overnight. Four sizable pieces of *Nicotiana tabacum* callus were placed onto each experimental Petri dish and incubated for 3 weeks in a growth chamber programmed at 18 h light, 6 h dark, and 24˚C.

Two plates per treatments were made. The callus health and growth were assessed by measuring their sizes and by visual assessment.

2.2. Extraction and analyses of pokeweed proteins

Pokeweed total cytoplasmic proteins were extracted in TSB buffer (0.001M Tris-HCl pH 7.4, 0.15M sucrose, 0.001M $MgCl_2$, 0.05M KCl, 0.2% SDS, and 0.0001M PMSF). The plants parts were separately homogenized at 0.22 g of fresh weight/ ml TSB buffer. The homogenates were filtered through two layers of cheesecloth and centrifuged at 10,000g for 15 minutes. The supernatants were further centrifuged at 16,000g for 15 minutes. The resulting supernatants were retained as total extractable cytoplasmic proteins. The protein yields were determined according to Bradford (1976) using BSA as the standard protein.

2.2.1. SDS-PAGE analyses

The profiles of the extracted proteins were established by SDS-PAGE according to Laemmli (1970). 8 µg total proteins of each extract were mixed with protein loading buffer and electrophoresed in duplicate 12% acrylamide gels at 120 V for 2 hours. One gel was stained with Coomassie blue, de-stained and photographed and the second gel was processed for western blot analyses to investigate lectins and lectin-like phytolaccatoxins.

2.2.2. Investigating lectins and lectin-like proteins by Western blot analyses

Proteins from the second gel were electro-transferred onto a nitrocellulose membrane at 30 Volts, 40 Amps, overnight in refrigerator chamber. After 3 washes with the transfer buffer, the membrane was incubated in 5% nonfat dry milk, 0.02% Tween 20, 0.02% sodium azide in phosphate buffered saline (PBS) for 2 hours to block the unbound surfaces. The membrane was incubated in 1:100 rabbit anti-*Ulex europaeus* lectin primary antibody in PBS for two hours with shaking at 60 rpm at 4˚C. Following a 3 X 10 minutes wash with PBS, the membrane was incubated in 1:1,000 goat anti-rabbit (GAR) secondary antibody conjugated with alkaline phosphatase for 2 hours. The membrane was developed by incubating the enzyme-bound secondary antibody with a mixture of its substrate BCIP and NBT in PBS with light agitation. The experiment was repeated using an anti-*Ricinus communis* as the primary antibody and yielded similar results.

3. Results

3.1. Bacterial growth inhibition assays

The results of the time course growth inhibition assays were expressed as the turbidity of the culture media at 600 nm were averaged and presented in figures 2 & 3.

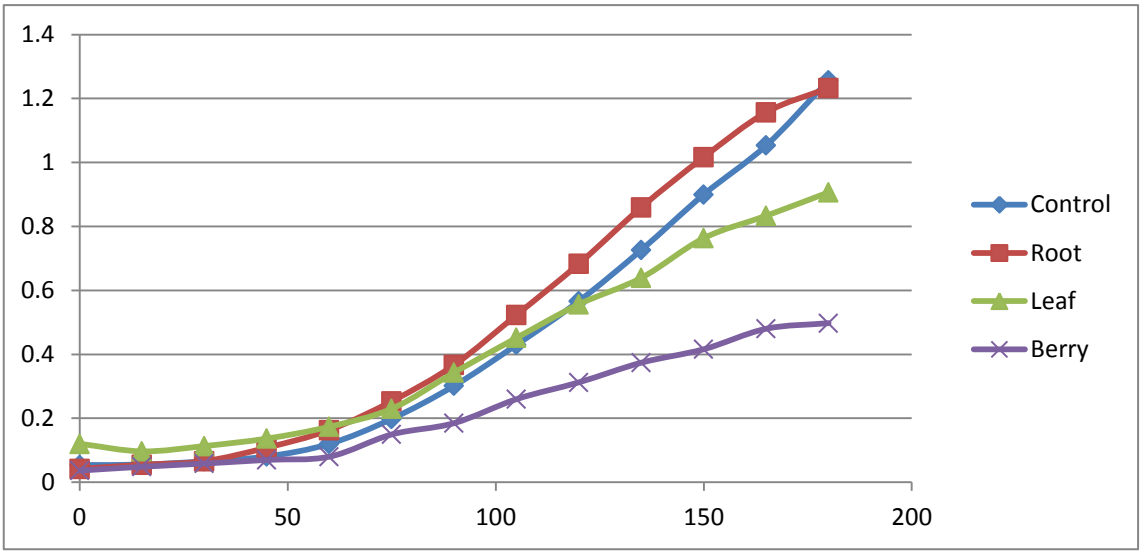


Fig. 2: Time Course Growth Curve of the Escherichia Coli Grown in Lb Media Supplemented with Pokeweed Root, Leaf, and Berry Extracts.

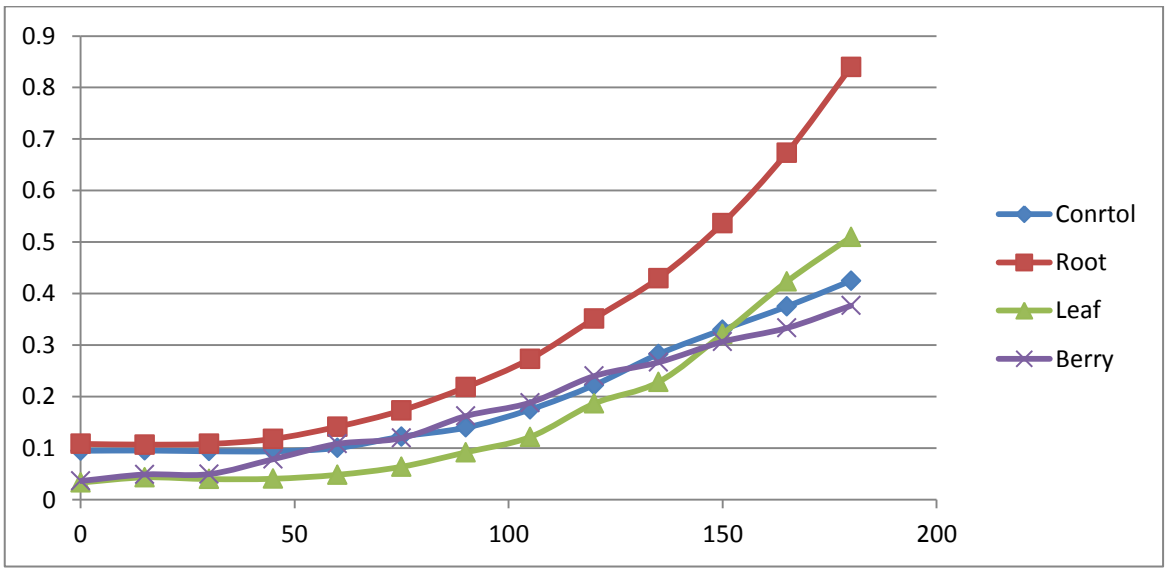


Fig. 3: Time Course Growth Curve of the Staphylococcus Aureus. Grown in LB Media Supplemented with Pokeweed Leaf, Root, and Berry Extracts.

The endpoints, A_{600} at time t = 180, were used for statistical analyses. The average A_{660} for *E. coli* growing in control LB media was 1.257 but decreased to 1.233 when cultured in the presence of root extract. The averages of the LB + leaf extract and LB + berry extract treatments were 0.907 and 0.498 respectively. Statistical analyses indicated that there is a significant difference between the control and the leaf treatment, and between the control and the berry treatments at 5% probability level. The averages A_{660} at t = 180 for *S. aureus* growing in control LB media was 0.425 and 0.840 for the LB + root extract treatment. The averages of the LB + leaf extract and of LB + berry extract treatments were 0.510 and 0.377 respectively (tables 1 & 2). No statistical differences were detected at 5% probability level.

Table 1: Summary of the Average Turbidity of the Bacterial Growth Inhibition Assays Expressed As A_{600} at the End Points, Time T = 180 Minutes for Escherichia Coli. (*) Indicates Significant Difference from the Control.

Treatments	Average	Deviations from	Significance
The control	using $LSD_{0.05}$		
In Control LB	1.257	-	-
LB + root extract	1.233	0.024	not significant
LB + leaf extract	0.907	0.35*	significant
LB + berry extract	0.498	0.759*	significant

Table 2: Summary of the Average Turbidity of the Bacterial Growth Inhibition Assays Expressed as A_{600} at the End Points, Time T = 180 Minutes for Staphylococcus Aureus. (Ns) Indicates No Significant Difference from the Control.

Treatments	Average	Deviations from	Significance
The control	using $LSD_{0.05}$		
In Control LB	0.425	-	-
LB + root extract	0.840	-0.415	ns
LB + leaf extract	0.510	-0.085	ns
LB + berry extract	0.377	0.048	ns

3.2. Callus growth inhibition assays

The results of the callus growth inhibition assays were averaged and presented in Figure 4. The average size of the callus cultured on the control 2MS was 10 mm. On day 8, the callus increased to an average of 18 mm and to 25 mm on day 15. The average size of callus cultured on 2MS + root extract was 13 mm on day 0 and did not change by day 15. The callus masses have undergone extensive dehydration and browning. Callus cultured on 2MS + leaf extract and the ones cultured on 2MS + berry extract were extensively desiccated and discolored, and showed no change in sizes.

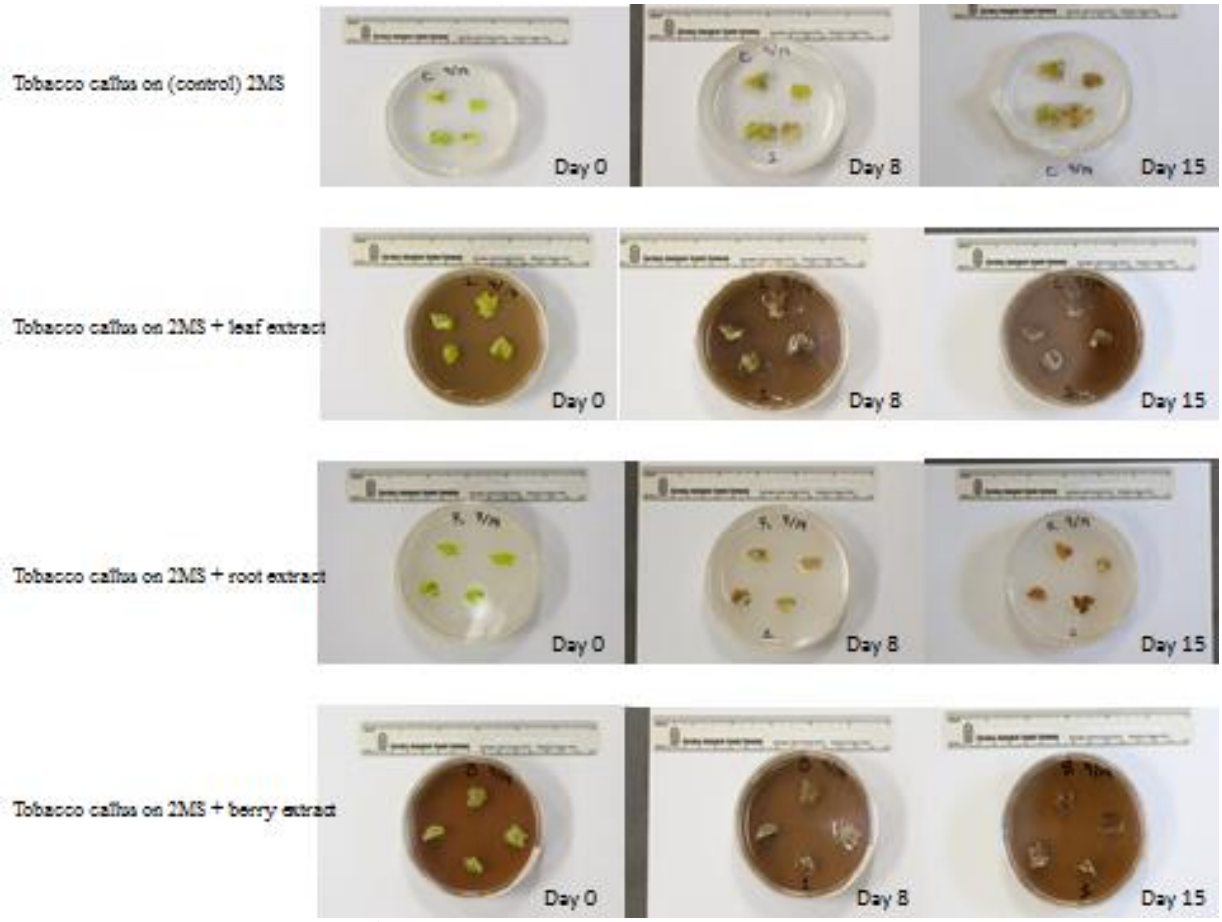
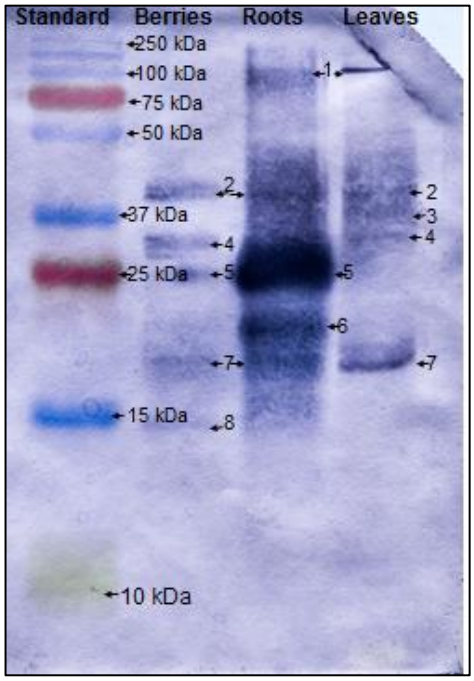


Fig.4: Experimental Design and Results of the Tobacco Callus Growth Inhibition Assay. Rows Represent Treatments on Days 0, 8, and 15.

3.3. Total protein analyses by SDS-PAGE and by western blot

The total cytoplasmic protein analyses performed in this research indicated that leaf extract had the most protein content of 0.80 µg/ml, followed by the root extract with 0.60 µg/ml and the berry with 0.35 µg/ml. The proteomic map by SDS-PAGE revealed several distinct bands of proteins whose sizes are shown in figure 5. There are 4 protein bands that are common to root and leaf extracts but not in the berry extract. Two additional protein bands were visible in the leaf extract but not in the root or the berry extract. The frequencies of occurrence of these protein bands are

shown in table 3. The results of Western blot analyses are shown figure 6. The sizes and natures of the proteins were summarized and presented in table 4.



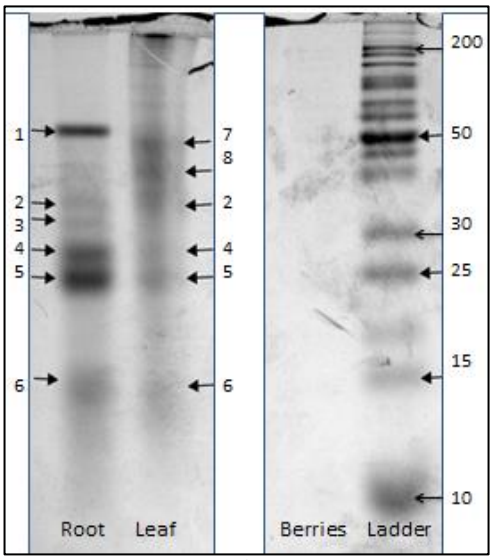


Fig. 5 & 6: Pokeweed Proteins Profiles, Resolved by SDS-PAGE (Left) and the Lectin-Like Proteins Detected by Western Blot Analyses (Right).

Table 3: Summary of Pokeweed Proteins, in Kda, Visualized by SDS-PAGE, Stained with Coomassie Blue.

Protein sizes (kDa)	Plant parts where detected		
	Root extract	Leaf extract	Berry extract
51.6	X		
49.5		X	
41.5		X	trace
35.2	X	X	
33.2	X		
29.4	X	X	
27	X	X	
21.4	X	X	
15			trace

Table 4: Summary of Pokeweed Proteins, in kDa, Detected by Western Blot Analyses Using an Anti-Lectin Primary Antibody.

Proteins sizes (kDa)	Plant parts where detected		
	Root extract	Leaf extract	Berry extract
108.68		X	X
35.68	X	X	X
32.82			X
29.56	X		X
25.02	X	X	
21.76		X	
19.8	X	X	X
17.26	X		

4. Discussions

Despite the high number of researches that have investigated the effects of plants secondary metabolites on viral and fungal diseases (Sindambiwe et al.1998, Escalante et al. 2002, Sparg et al. 2004, Sussner et al. 2004, Chapagain et al. 2007), only a few made mention of their effects on bacterial cells. This research investigated the antibacterial activities of pokeweed extracts on a gram negative *Escherichia coli* and gram positive *Staphylococcus aureus*. The results indicated that pokeweed extracts had a negative effect on the log phase of the growth curve of *E. coli*, but not of *S. aureus*. The root, leaf and berry extracts were found to inhibit the growth of *E. coli*. The berry extract expressed the most growth inhibition based on cell densities at the end of the 180 minutes assay. The analyses of variance indicated the existence of significant differences between the effects of berry extract and the control, and between the berry extract and the root extract. Neither the leaf nor the berry or the root extracts showed significant effects on the growth curve of the gram positive *S. aureus*. This suggests that pokeweed extracts may not have inhibitory effects on the gram positive *Staphylococcus aureus* but more research is needed. In the absence of published data on the antimicrobial effects of pokeweed, this research concluded that there are some biologically active compounds in pokeweed that have negative effects on the life of *Escherichia coli*. This is in agreement with Biswal et al. 2011, Karadi et al. 2011, and Upadhyay 2015, who concluded that some plants species have constituents that have antibacterial effects. Furthermore, this research found that tobacco callus could not be cultured on MS media containing pokeweed extract. The callus tissues incubated on MS media containing root, leaf, and berry extracts experienced browning as the results of chemical oxidation by the pokeweed constituents.

Lectin is one of pokeweed toxic proteins known to cause cell damage and stimulate abnormal cell growth. Chrispeels and Raikhelb (1991) have demonstrated that many plant species contain classes of lectin at different concentrations. This research detected the presence of one of lectin isoform of MW 110kDa in pokeweed root and leaf extracts but not in the berry extract. Kino et al. 1995 found that pokeweed roots have three mitogenic lectins called PL-A, PL-B, and PL-C, with molecular masses of 22, 48, and 21 kDa, respectively. Sussner et al. (2004) identified the protease phytolaccain G (PL-G) with molecular mass of 25 kDa to have chemical properties similar to lectin. This research confirmed the presence of PL-G, PL-A, and PL-C in the root extract. No PL-B was detected in any of the three extracts (table 4) and no extract was PL-A detected beside in the root extract. Beside the roots, PL-G was also detected in the berry extracts but not in the leaf extract. On the other hand, PL-C was detected in the berry, the root, and the leaf extracts.

5. Conclusion

This investigation has concluded that some phytochemical constituents of pokeweed plants possess growth inhibitory effects to gram negative *Escherichia coli* and to *Nicotiana tabacum* callus,

and that pokeweed plants contains a wide variety of toxic components that are lectins or lectin-like in properties.

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