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Antimicrobial Activity, Cytotoxicity and Phytochemical Analysis of *Sanguinaria canadensis* Native to South Dakota

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Abstract

A methanolic extract of *Sanguinaria canadensis* rhizome contains a mixture of benzophenanthridine alkaloids with the major component sanguinarine. American Indians used this rhizome in treatment of rheumatism, asthma, bronchitis, lung ailments, fever and warts. Lethal diarrhea in neonatal and post weaned pigs due to enterotoxigenic *Escherichia coli* (ETEC) is a major problem among the swine producers of South Dakota region. The increasing resistance of ETEC towards synthetic antibiotics is a matter of concern. Our aims in this study were 1) to determine the minimum inhibitory concentration (MIC) of methanol extract of *S. canadensis* against ETEC, 2) to evaluate the cytotoxicity activity against porcine intestinal epithelial cell lines, and 3) to quantify the amount of sanguinarine in the rhizome of *S. canadensis* native to South Dakota. The MIC value was found to be 6.25 mg/mL against ETEC whereas the 100% inhibition of IPEC-J2 cells was found to be at 0.312 mg/mL. The total amount of alkaloids in this plant was found to be 1247 mg in 100 gm of fresh plant material whereas the amount of sangunarine was 559.64 mg in 100 gm of fresh plant material. The percentage of sangunarine in fresh plant material was 0.55% and in total alkaloid is 44.8%. Our results showed that *S. canadensis* has antibacterial effect against *E. coli* but it is toxic to IPECJ2 cells *in vitro.* It is important to carry out an *in vivo* animal study to confirm its further use as feed supplements.

Subject Areas

Microbiology

Keywords

Antibacterial Activity, Enterotoxigenic *E. coli*, Cytotoxicity, Alkaloids,

Sanguinarine

1. Introduction

Sanguinaria canadensis, (Papaveraceae) is a spring blooming perennial herb producing a red sap or latex in the rhizome commonly called bloodroot. Bloodroot is indigenous to North America and it is mainly wild harvested in the eastern parts of the USA in the fall season [1]. American Indians used the root for the treatment of arthritis, asthma, bronchitis, lung ailments, fevers and the brightly colored juice from roots was applied to remove warts. The juice was also used for face painting and part of a love potion [2]. Fresh bloodroot sap or the root infusion can be used for treating ulcers, ringworm and other skin affiliations [3]. The red sap contains up to 9% isoquinoline alkaloids on a dry weight basis. The major compound is sanguinarine representing about 50% of the total alkaloids. In addition to sanguinarine, other alkaloids: chelerythrine, sanguilutine, allocryptopine, protopine, berberine and coptisine are usually present [1]. These compounds have been also shown to have anticancer and antimicrobial activities [4] [5]. However, sanguinarine is thought to be the most bioactive of the alkaloids [6]. Sanguinarine has demonstrated antimicrobial [5] [7], anti-inflammatory [8] [9], anti-platelet (inhibit platelet aggregation) [10], antiproliferative [11] [12] [13], and anti-angiogenic [14] activities. Sanguinarine is used as a feed additive known as Sangrovit. It was observed that Sangrovit led to significant increase in body weight of broiler chickens [15] and pigs [16].

S. candensis has been exploited commercially for more than two decades, for its antiplaque properties in oral hygiene [17]. Although its efficacy has been challenged [18], it is still widely available for this use. *S. canadensis* preparations have also found utility as non-antibiotic feed supplements to promote weight gain in livestock, especially in Europe [19].

Enterotoxigenic *Escherichia coli* (ETEC) is the foremost group of bacteria causing diarrhea in pigs and causing high morbidity and mortality [20]. ETEC is the etiological agent for both neonatal and post weaning diarrhea. Extensive diarrhea in weaning pigs can lead to a significant economic loss in swine industry [21] [22]. Antibiotics have been used traditionally to prevent and treat enteric disease including diarrhea induced by weaning stress [22]. However the continuous exploitation of antibiotics has led to drug and antibiotic residues in animal products and possible chance of spread of antibiotic resistant bacteria through the food chain [23] [24]. Livestock producers in many countries are trying to manage production without antibiotic growth promoters [25]. *S. canadensis* has not been tested for antibacterial activity against ETEC.

The objectives of our study were to 1) determine the MIC value of *S. Canadensis* against ETEC; 2) quantify total alkaloid and the percentage of sanguinarine in *S. Canadensis*; and 3) evaluate the cytotoxicity against porcine intestinal epithelia

cell lines.

2. Materials and Methods

2.1. Sample Collection and Processing

S. canadensis rhizome samples and voucher specimens were collected from the Sica Hollow State park of South Dakota in October 2010, as is the custom of the local Native Americans (personal communication with Dakota Tribal Elders). All samples were stored on ice and brought back laboratory for processing. These plant samples were then homogenized in a blender in 100% methanol and methanol plant extracts were mixed continuously on an orbital shaker for 24 hours, at 150 RPM in the dark as previously described [26] [27]. After complete extraction, the methanol extract was filtered using Whatman #1 filter paper. Methanol was removed from the sample using rotary evaporation under vacuum and the aqueous remainder of the extracts were frozen and lyophilized to dryness. The dried extracts were then stored at −20˚C until being used.

2.2. Bacterial Strains and Growth Conditions

Escherichia coli H157 K88-LT STB was obtained from the South Dakota Veterinary Diagnostic Laboratory, at SDSU. The assay medium for *E. coli* was tryptic soy broth (TSB) and tryptic soy agar (TSA) with 5% sheep blood. Bacterial strains were streaked for isolation using TSA with 5% sheep blood. Bacterial cultures for antimicrobial testing were prepared by inoculating 25 ml of TSB from fresh culture plates. Cultures were grown overnight in an incubator shaken at 200 rpm at 37˚C. For antibacterial activity assay the CFU was adjusted to 10^7 - 10^8 CFU/ml using spectrophotometer (absorbance 0.5 at 600 nm). Stock cultures were maintained in 50% glycerol and stored at −80˚C.

2.3. Bacterial Enumeration Assay for Minimum Inhibitory Concentration

The MIC values for *S. canadensis* extracts that were active against *E. coli* were measured by using broth dilution and bacterial enumeration as described by Al-Bakri and Afifi [28] with some modifications. The dried bloodroot extracts were solubilized with 10% ethyl alcohol (100 mg/mL). These solubilized extracts were place in sterile 96-well microtiter plates sequentially in columns using a 2-fold serial dilution (50 µl/well). Aliquots of bacterial suspension (50 µl) suspended in TSB growth medium (OD 0.5) were added to each of the wells. The final volume of each well was 100 µl. The final concentrations of plant extract in the wells of the serial dilutions were 50, 25, 12.50, 6.25, 3.125, 1.56, 0.78, and 0.39 mg/mL. Controls contained TSB growth medium alone, TSB growth medium inoculated with the bacterial suspension, or TSB growth medium inoculated with the bacterial suspension and 10% ethanol and TSB growth medium inoculated with the bacterial suspension and 50 µg/mL gentamicin.

The 96 well plates were then incubated at 37˚C for 18 hour. A viable cell count

was done by diluting (tenfold serial dilution) the original samples), then plating aliquots of these dilutions onto a blood agar plates and incubating them at 37˚C for 18 hours. The viable cells in the broth of different concentrations were determined by counting bacterial colonies.

2.4. Cytotoxicity Test

Dry extracted plant sample (10 mg) was diluted in 1 ml 10% ethanol to make a stock solution. From the stock solution different dilutions of plant extract were prepared using *IPEC-J2* media. Extracts were filtered with 0.2 mμ sterile filter and used immediately.

Porcine epithelial cell lines (*IPEC-J2*) were cultured in *IPEC-J2* media supplemented with Dulbecco's Modified Eagle Medium (DMEM), epidermal growth factor (1000X), ITS (Insulin, Transferrin, Selenium) (1000X), L-Glutamine 200 mM, antibiotics (100X) in 95% humidity, 5% CO_2 and 37˚C temperature.

2.5. Alamar Blue Assay

A microplate Alamar Blue Assay was performed to detect the toxic effect of plant extract on animal cells as described by the manufacturer's protocol (alamar Blue® assay, Invitrogen). Cytotoxicity against porcine epithelial cells was performed in the Immunology laboratory of Bio/Micro Department, SDSU. For this assay, normal porcine epithelial cells (IPEC-J2) harvested from cultured plates, were washed with phosphate buffer saline (PBS) and added trypsin. Cell suspensions were centrifuged for 5 min at 1200 rpm. Pellet was suspended in 5 ml IPECJ2 media. Dilutions were made and 7500 cells in 200 µl media was seeded in each well of a 96 well plate and incubated at 37˚C at 5% CO_2 for attachment. After 24 h cells were washed and 200 µL of different dilutions of the plant extracts 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078 mg/ml were added and further incubated another 48 h. Each concentration was tested for cytotoxicity in triplicate. At the end of each treatment 20 µL of Alamar Blue dye was directly added to the culture media making a final concentration of 10% Alamar Blue and incubated for another 4 to 5 hours. Finally fluorescence was measured using at an excitation of 540 nm and emission of 620 nm in an Eliza reader to determine the viability of living cells. Ethanol (70%) was used as control. Reading of the blank was taken and subtracted from the experimental reading to get the actual reading of the viable cells.

2.6. Determination of Total Alkaloid Contents

The total alkaloid contents were measured with Bromocresol Green solution (BCG) solution as described by [29]. For quantification, 50 mg of methanolic extracts were solubilized in 2N HCL and filtered through a coarse sintered glass filter. One ml of the solution was taken in a separatory funnel, washed with 10 mL chloroform three times, extracts were taken in separate tube and pH was adjusted to neutral with 0.1 N NaOH. BCG, (5 mL) and phosphate buffer (5 mL) were added and the

mixture was shaken for 1 - 2 min. The complex was extracted serially with 1, 2, 3 and 4 ml of chloroform and diluted to a total volume of 10 ml with chloroform. Absorbance of the sample was recorded at 417 nm. The concentration of alkaloid was quantified using atropine as standard.

2.7. Isolation and Quantification of Sanguinarine

The total sanguinarine was measured with HPLC as described by Sucheomelova *et al.* [30] with some modifications. One mg of dried plant extract was dissolved in one ml of phosphate buffer (0.01 M) solution and used for HPLC analysis. Phosphoric acid, heptane sulfonic acid, triethylamine, acetonitrile of HPLC grade and sanguinarine chloride (10 mg) were obtained from Fisher scientific (USA). An Agilent 1100/1200 HPLC with quaternary pump, UV-Vis multi wave length detector with autosampler control by ChemStation. The wavelength used for analysis was 280 nm and reference wavelength was 360 nm. An Eclipse XDB, 5 µm, 4.6 mm × 150 mm C18 column was used. The mobile phases were prepared from the stock solution containing heptanesulfonic acid (0.01 M) and triethylamine (0.1 M) in redistilled water, pH was adjusted to 2.5 with phosphoric acid (H_3PO_4). A-solvent contained 25% acetonitrile and B-solvent 60% acetonitrile (v/v). The elution profile was as followed to run the sample 0 - 1 min 20% B in A; 1 - 10 min 50% B in A; 10 - 20 min 100% B in A; 20 - 25 min isocratically 100% B flow; 25 - 30 min 20% B in A. The flow rate was 1.5 ml/min, the injection volume was 10 µl and detection was carried out at 280 nm wavelength. The peak of sanguinarine was identified on the base of retention time and UV/Vis spectrum.

A calibration curve based on five concentrations of sanguinarine chloride standards with a range of 0.01 - 0.05 mg/ml were obtained by plotting the peak areas of the standard alkaloid versus concentrations. All samples were run in triplicates. The statistical parameters were analyzed by linear regression.

3. Results

For data analysis dose-response relationships and inhibitory concentrations for antimicrobial activity and cytotoxicity test were determined from regression equation. Each experiment was conducted with three replicates and the results were calculated as mean ± SD. From the bacterial enumeration assay (**Figure 1**) the minimum inhibitory concentration of *S. canadensis* was found to be 6.25 mg/mL. The half maximal inhibitory concentration (IC50; concentration of *S. canadensis* where the 50% of the *E. coli* inhibited) was calculated to be 0.99 mg/mL.

Alamar blue cytotoxicity assay result showed that the extract of concentration of *S. canadensis* at 0.312 mg/mL or above had 100% inhibitory effect on the IPEC-J2 cell lines, whereas at concentration 0.156 mg/mL showed 50% inhibition of cell growth (**Figure 2**). The HPLC chromatogram showed the peak for major alkaloid sanguinarine (SA) including others CHE, chelerythrine; CHR, chelirubine; CHL, chelilutine; MA, macarpine; SR, sanguirubine; SL, sanguilutine (**Figure 3**) The total alkaloid quantification and percentage of sanguinarine

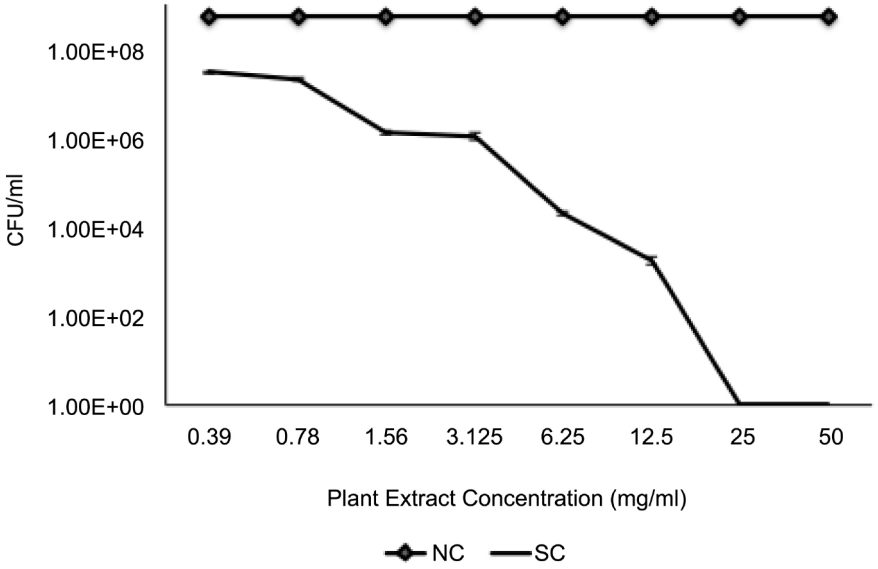

Figure 1. *Escherichia coli* H157 LT/STB showing increased colony forming unit (CFU/ml) with decreasing concentration of plant extract, incubated at 37˚C for 18 hours. The X-axis is the plant extract concentration in mg/mL and the Y-axis is the CFU/mL. SC-*Sanguinaria canadensis*, NC-Negative Control.

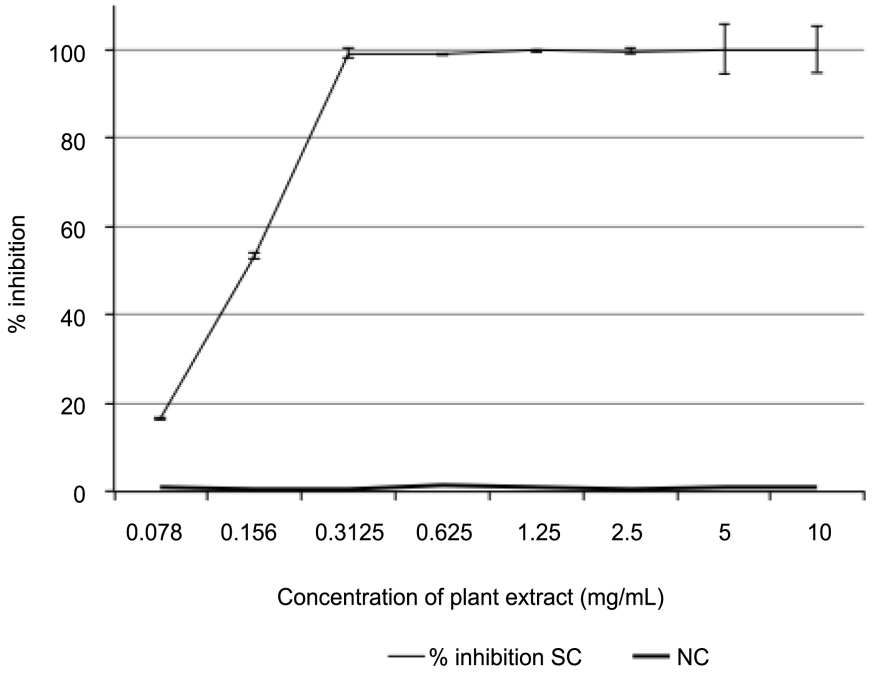

Figure 2. Inhibition of IPEC-J2 cell growth at different concentrations of *S. canadensis* extract, incubated at 37˚C, 5% CO_2 for 24 hours. The X-axis is the plant extract concentration in mg/mL and the Y-axis is % inhibition of porcine epithelial cells (IPEC-J2).

and other major alkaloids were calculated by using the linear regression equation of standard curve (Table 1). We found the amount of total alkaloid was to be 1.24% of the fresh weight (approximately 1247 mg/100g of fresh weight). The

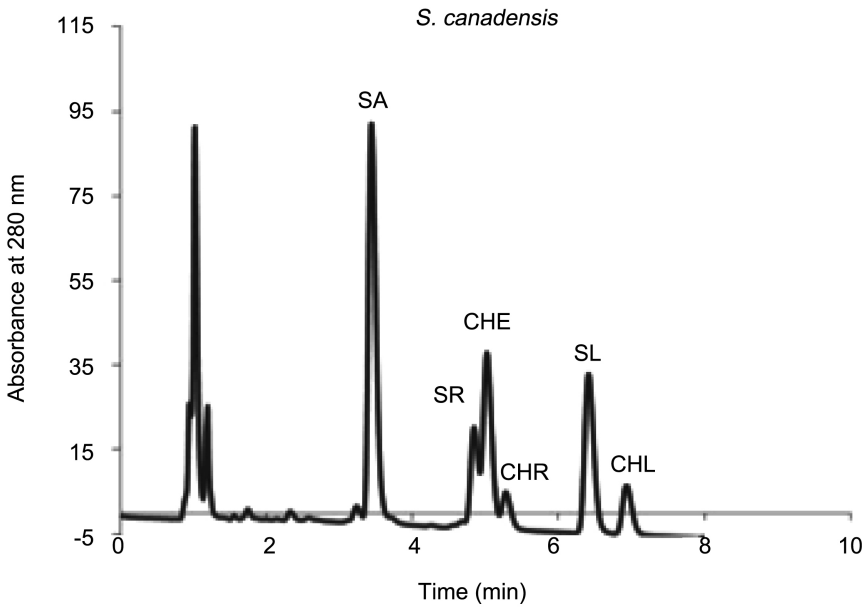

Figure 3. HPLC chromatograms of *S. canadensis* extract. SA, sanguinarine; CHE, chelerythrine; CHR, chelirubine; CHL, chelilutine; MA, macarpine; SR, sanguirubine; SL, sanguilutine; The X-axis represents the retention time in min and the Y-axis represent absorbance at 280 nm.

Table 1. Alkaloid content of *S. canadensis* root extract expressed as percentage of total alkaloid.

Name of Alkaloids	Peak Height	% of Total Alkaloids	mg/100g Fresh Plant Tissue
Sanguinarine (SA)	89.82	44.87	559.64
Sanguirubin (SR)	16.79	7.42	92.53
Chelerythrine (CHE)	37.27	17.92	223.53
Chelirubine (CHR)	2.359	0.018	0.23
Sanguilutine (SL)	30.31	14.35	179.01
Chelilutine (CHL)	4.305	1.016	12.68

amount of sanguinarine was 0.55% of the total plant material and 44.83% of the total alkaloids.

4. Discussion

This is the first report of testing *S. canadensis* extract for antimicrobial activity against enterotoxigenic *E. coli* and cytotoxic effect against IPEC-J2 cells lines. We also quantified the sanguinarine content of *S. canadensis* native to South Dakota.

The biological activities of *S. canadensis* have been reported previously in many research articles. Sanguinarine can be lethal to vertebrates, insects and also inhibit the growth of fungi, bacteria and virus. It inhibits choline esterase transferase, intercalates into DNA, inhibits DNA synthesis and reverse transcriptase, and affects membrane permeability [31].

A number of alkaloids have been isolated from *S. canadensis* including sanguinarine, chelerythrine, protopine, oxysanguinarine, alpha-allocryptopine, beta-allocryptopine, chelirubin, chelilutine sanguirubine and sanguilutine [32]. It has been reported that sanguinarine has antibacterial activity against a range of gram positive and gram negative bacteria predominantly found in mouth [33]. Sanguinarine is effective in prevention of plaque formation and gingivitis by inhibiting bacterial adherence and is safe to use in toothpaste and mouth rinse [17] [33] [34] [35].

Sanguinarine has been shown to strongly induced filamentation in Gram positive and Gram negative bacteria and prevent cell division by inhibiting cytokinesis [7]. A recent study demonstrated that MRSA (multidrug resistant *Staphylococcus aureus*) strain treated with sanguinarine showed lysed cell and altered morphology [36]. Sanguinarine and some structurally related alkaloids like chelerythrine, chelidonine, barberine inhibit extracellular lipase activity of *Candida rogusa* [37]. Methanol extracts of the rhizomes of *S. canadensis* has been shown to inhibit the growth of *Helicobacter pylori in vitro* [5]. Previously published reports have demonstrated the antimicrobial activity of *S. canadensis* in various aspects. However extracts of *S. canadensis* have not been tested against ETEC before. In our work we found that *S. canadensis* could be an effective alternative source of antibiotics against ETEC.

Cytotoxicity assay against IPEC-J2 cell showed that *S. canadensis* is toxic at a concentration of 0.312 mg/mL, however there is evidence that sanguinarine can be used as a feed additive and that it is safe for animal use *in vivo*. In a ninety days feeding experiment, sanguinarine and chelerythrine were administered to pigs to see the effect on their health status, and it was reported that an average daily oral dose of sanguinarine up to 5 mg/kg animal body weight is safe [38]. A short term toxicity study of sanguinarine was conducted on rats as model animal; acute oral toxicity, acute intravenous toxicity, acute dermal toxicity, 14 day feeding study and 30 day toxicity study. The result of this study showed that the acute oral LD50 of sanguinarine in rats was 1658 mg/kg body weight, the acute intravenous LD50 was 29 mg/kg and acute dermal LD50 was found to be 200 mg/kg. However no toxic effects were observed in rats fed up to 150 mg/kg of sanguinarine in the diet for 14 days and by gavage feeding with up to 0.6 mg/Kg body weight for 30 days. These data suggest that sanguinarine is very poorly absorbed in the gastrointestinal tract [39]. It is also reported that *S. canadensis* can be used as a feed additive in place of synthetic antibiotics for livestock [3]. A clinical safety report showed that sanguinarine was not absorbed orally, did not induce mucosal irritation or sensitization in repeated doses and also there was no induction of mutagenicity *in vivo* and *in vitro* [33].

The major alkaloid sanguinarine was quantified by HPLC. Sanguinarine [13-methyl (1, 3) benzodioxolo (5, 6-*c*)-1, 3-dioxolo (4, 5-*i*) phenanthridinium] has been shown to have number of biological activities and higher therapeutic values than other alkaloids of *S. canadensis*. Sanguinarine was the most active among three major alkaloid found in *S. canadensis* inhibited the growth of all 15 *Heli-*

cobacter pylori strain with an MIC range of 6.25 μg /mL - 50 μg/mL [5]. Godowski [33] reported, sanguinarine has antimicrobial activity against a broad range of oral bacteria and its anti-plaque activity is due its ability to inhibit bacterial adherence. From electron microscopic studies he also observed that bacteria exposed to sanguinarine aggregate and show morphological irregularities. Based on previous literature it is obvious that sanguinarine is the major active constituents of *S. canadensis* having potential antimicrobial activity. We quantified sanguinarine percentage in total alkaloids of *S. canadensis* to be 44.82%. The percentage of sanguinarine in wild type *S. canadensis* was reported to be about 50% [33]. The percentage yield of *S. canadensis* varies with the region of growth and time of harvest, Newton *et al.* [40] reported that the % yield *S. canadensis* was 27%, however the % yield (Methanol crude extract) was 11.5% of *S. canadensis* from SD (our sample). Previous researchers have suggested that bioactive compounds such as alkaloids are at their highest concentration in mid spring [41]. In 100 gm of fresh rhizome of *S. canadensis* contained 559.64 mg of sanguinarine, which is about 44.82% of the total alkaloids. It is consisted about 0.55% of the fresh weight. This result indicated that the variation in the percentage of sanguinarine may be due to the different regions of growth, different cultivars or time of harvest. Alkaloid composition of *S. canadensis* has been found to be dependent on both environmental and genetic factors. There have been several studies indicating the impact of eco-physiological factors on the level of sanguinarine. Marino *et al.* [42] demonstrated that the vegetative growth of *S. canadensis* changes with elevated sunlight and nutrient availability, explaining the patchy distribution of *S. canadensis* in the wild. Other factors influencing alkaloid content were season of harvest, moisture content and sometimes elevation [43]. Graf *et al.* [41] have reported the wild plants alkaloid yields were higher compared to cultivated varieties and those months to months variability in the concentration of alkaloid was greater. Cultivated *S. canadensis* has larger rhizomes of more consistent size than does the wild type. Selective breeding and culturing should improve the dry mass production and increase the concentration of key alkaloids [41].

5. Conclusion

In conclusion, we found that *S. canadensis* showed antimicrobial activity against enterotoxigenic *E. coli*. It may be used as feed additives to control diarrhea in neonatal or weaned pigs irrespective of its toxicity against IPEC-J2 cells *in vitro*. There is evidence to support the use of *S. canadensis* as feed additives in place of synthetic antibiotics. It will be interesting to find out the quantity of sanguinarine at different periods of growth, different harvest times and other different growing conditions.

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