Antimicrobial Activity, Cytotoxicity and Phytochemical Analyses of Rhus aromatica, Rhus glabra and Sanguinaria canadensis Native to South Dakota

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ANTIMICROBIAL ACTIVITY, CYTOTOXICITY AND PHYTOCHEMICAL ANALYSES OF *RHUS AROMATICA*, *RHUS GLABRA* AND *SANGUINARIA CANADENSIS* NATIVE TO SOUTH DAKOTA

BY

GITANJALI NANDAKAFLE

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

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusion of the major department.

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ABSTRACT

ANTIMICROBIAL ACTIVITY, CYTOTOXICITY AND PHYTOCHEMICAL ANALYSIS OF RHUS AROMATICA, RHUS GLABRA AND SANGUINARIA CANADENSIS NATIVE TO SOUTH DAKOTA

GITANJALI NANDAKAFLE

NOVEMBER, 2012

A total of twenty five South Dakota native medicinal plants were screened against enterotoxigenic Escherichia coli using a disk diffusion assay. Out of these 25 plants, three plants Rhus aromatica, Rhus glabra and Sanguinaria canadensis demonstrated the highest bacterial inhibition. The minimum inhibitory concentrations (MIC) of these plant extracts were determined using a bacterial enumeration assay. Alamar Blue in vitro cytotoxicity test showed that all extracts have some toxic effect to porcine intestinal epithelial cell lines. Phytochemical analysis of these plant extracts indicated the presence high concentration of flavonoids and condensed tannins in R. aromatica and that R. glabra contained higher amount of simple phenolic compounds. As R. aromatica showed antibacterial, anthelminthic and anticancer activity in our study, it was further studied for its essential oil components using gas chromatography and mass spectrometry. The major volatile compounds of its essential oils were identified as limonene, caryophyllene and cubebene consisting of 82% of the total essential oils. The total alkaloids content of S. canadensis was 1.24% of the fresh weight. HPLC analysis for sanguinarine quantification showed that it has 43% of sanguinarine out of total alkaloids.
**Key words:** Enterotoxigenic *Escherichia coli* (ETEC), Minimum Inhibitory Concentration (MIC), phytochemicals, flavonoids, essential oils, alkaloids.
CHAPTER-1

LITERATURE REVIEW

*Escherichia coli (E. coli)* and Antibiotic Resistance

*E. coli* is a gram negative, rod shaped, facultative anaerobe member of the family *Enterobacteriaceae* that lives in the lower intestine of warm blooded animals. Although most of *E. coli* strains are harmless, some types are pathogenic and can cause serious health problems. Infections due to pathogenic *E. coli* results in three clinical syndromes: (i) Urinary tract infections, (ii) Sepsis or meningitis, (iii) Enteric/diarrheal diseases (Nataro and Kaper, 1998). I will concentrate mostly on enterotoxigenic *E. coli* (ETEC), which was first detected as the main cause of diarrheal disease in piglets. The most common strategy of infection by virulent *E. coli* is; colonization of epithelial sites, circumvention of host defenses, proliferation and host damage. The virulent ETEC, contain at least one of the two defined groups of enterotoxins heat labile toxin (LT) or heat stable toxin (ST). The enzymatic activities of these enterotoxins cause diarrhea by stimulating excessive water and electrolyte loss from the intestine of the infected host (Nataro and Kaper, 1998). Virulent strains may express an LT only, ST only or both LT and ST. The LTs are oligomeric toxins that have similar structure and function as the cholera enterotoxin in *Vibrio cholera* (Sixma et al., 1993). LTI and LTII are two major serogroups of LT, LTI is found in *E. coli* strains that are pathogenic to both human and animal whereas LTII strains are pathogenic to mainly animals. The STs are small monomeric toxins with multiple cysteine residues, the disulfide bonds of these residues are the cause of its heat stability. The two major groups of STs are STa and STb, STa is
found in ETEC and several other gram negative bacteria, but STb is found only in ETEC (Nataro and Kaper, 1998). The second virulent factor of ETEC is fimbriae/adhesin mediated colonization in small intestine epithelial cells. There are five different adhesin types found in porcine ETEC; K88 (F4), K99 (F5), 987P (F6), F41 (F7) and F18 (Wilson and Francis, 1986). ETEC fimbriae explain the species specificity of the pathogen. For example ETEC strains with K99 expression are pathogenic to calves, lambs and pigs whereas strains expressing K88 are pathogenic to pigs only (Cassels and Wolf, 1995).

The two major clinical syndromes associated with ETEC are weanling’s diarrhea in pigs, and children and traveler’s diarrhea in humans. ETEC with the two virulent factors adhesin and enterotoxin are a major cause of death and illness in neonatal and recently weaned pigs (Francis, 2002). The fimbrial adhesin K88 and K18 generally occur in several antigenically distinct forms. Potentially pathogenic *E. coli* are usually present in the intestinal tract and feces of many normal swine. Poor sanitation, chilling environment and lowered immunity of the swine can increase the risk of infection. When proper management measures and vaccination programs fail, piglets need be treated with antibiotics (Hariharan et al., 2004). Antibiotics are also used as growth promoters and to control various infectious diseases by the swine producers. The ETEC isolates involved in diarrhea showed a high frequency of resistance to multiple antibiotics. The increasing resistance of *E. coli* is due in part to the use of various antibiotics in feed, water and injections (Amezcua et al., 2002). In a study by Boerlin et al (2005), a total of 318 *E. coli* isolates were obtained from diarrheic and healthy pigs and tested for susceptibility against 19 antimicrobials. The PCR test showed the presence of resistance genes for tetracycline, streptomycin sulfonamide and apramycin. In has been reported that *E.coli*
serotype O147: K89:K88a,c isolated from the fecal sample of preweaned pigs, showed resistance to apramycin, gentamicin, netilmicin, tobramycin, oxytetracycline and trimethoprim (Hunter et al., 1994).

Antibacterial drugs act on the bacteria cells, mainly in four different ways: (1) interferences with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis and (4) inhibition of metabolic pathways (Neu, 1992).

Bacteria may develop resistance to antibacterial drugs through a range of different mechanisms: (1) the organism may acquire genes encoding enzymes that destroy the antibacterial agent (2) bacteria may develop an efflux pump that expels the antibacterial agent from the cell before it can achieve its target site (3) it may alter the target site on the cell wall by acquiring several genes or (4) it may undergo mutations that limit contact of antibacterial agents to the intracellular target sites (Tenover, 2006).

To deal with the problem of antibiotic resistance, new bioactive components from traditional medicinal herbs have been a focus area for development of new therapeutic and clinical applications (Jung et al., 2011).

**Secondary Metabolites of Plant or Phytochemicals**

Secondary metabolites are organic compounds that have no direct involvement in growth and development of plants. Secondary metabolites play a vital role in the defense mechanism against herbivory and microbial pathogens. These products have also been shown to attract pollinators and seed dispersing animal and to serve as an agent for allelopathic interactions. These ecological functions affect plant reproductive fitness and help them to produce more descendants and pass the defensive traits on to the next
generations (Taiz and Zeiger, 1998). There are a myriad of natural compounds synthesized by plants but only several thousands of them have been identified. There are many more yet to be discovered in the search for new drugs, antibiotics, fungicides, insecticide and herbicides. Scientists are investing more time to identify the biological activities of these compounds (Croteau et al., 2000). Plant secondary metabolites largely fall into three major classes of compounds, alkaloids (12,000), phenolics (10,000) and terpenoids (25,000) (Bowsher et al., 2008).

**Alkaloids:** Alkaloids are large a group of over 12,000 nitrogen containing compounds with diverse structure and functions, which are known to occur in over 20% of plant species (Crozier, 2006). According to Bowsher et al. (2008) alkaloids are organic molecules with several ring structures containing one or more nitrogen atoms usually located within a carbon ring. The major alkaloid groups based on the structure of the carbon ring are protopine, piperidine, pyrrolidine, pyridine, quinoline, quinolizidine, quinazoline, imidazole, steroidal, tropane, pyrrolizidine, purine, isoquinoline and terpenoid indole alkaloids. Only a few alkaloids are derived from purines or pyrimidines, while majority of alkaloids are synthesized from amino acids. The role of alkaloids in plants is to defend from predators, especially mammals. Though many alkaloids are toxic when taken in large quantities, for example strychnine, atropine and coniine, when taken at lower doses many are useful medicines; morphine, codeine, aptropine, sanguinarine and ephedrine. Other alkaloids like cocaine, nicotine, and caffeine are used worldwide as stimulants or sedatives (Aniszewski, 2007). Alkaloids have a vast range of biological activities; antimicrobial, antiviral, anti-parasitic, anti-inflammatory, anticancer, anti-
malarial, analgesic and antineoplastic properties; they also display effects on the central nervous system (Aniszewski, 2007).

**Phenolic compounds:** A large part of the secondary products contain phenol groups and are classified as phenolic compounds. Plant phenolics have diverse chemical properties, some are soluble in organic solvents, some are water soluble and others are large insoluble polymers (Taiz and Zeiger, 1998). Phenolic compounds are the most widely studied phytochemicals and having been extensively used in various plant researches for their health benefits. The phenolics may be classified in to four major groups based on their basic structure; simple phenolics, flavonoids, tannins and lignins. Simple phenolics are divided into three main groups; phenylpropanoids (e.g. caffeic, ferulic, cinnamic and p-coumeric acids), coumarins (e.g. coumarin, esculetin and scopoletin) and benzoic acids (e.g. vanillin, salicylic acids, gallic acid) (Bowsher et al 2008).

The flavonoids are a highly diverse and large group of phenolics; more than 6000 have been identified so far. The basic structure of flavonoids consists of two six-carbon rings joined by a three carbon bridge that often forms a third ring. On the basis of the modifications of three rings, flavonoids are distributed into various groups; chalcones, flavanones, flavonols, flavons, isoflavons, flavan-3-ols and anthocyanins (Bowsher et al 2008).

Tannins are phenolic polymers capable of tanning leathers by reacting with protein. They occur in two forms; condensed tannins and hydrolysable tannins. Both tannins are structurally and biosynthetically different but they share the same biological activities. Condensed tannins are also known as proanthocyanidins and are flavonoid
polymers. Hydrolysable tannins are usually polymers of gallic acid (gallotannin) or ellagic acids (ellagitannin) together with D-glucose. Hydrolysable tannins are smaller in size and more readily hydrolyzed into carbohydrates and phenolic acids compare to condensed tannin (Bowsher et al 2008). The protein binding capacity of both the tannins may be the key for their antimicrobial action by inhibiting the bacterial adhesin, enzymes and cell envelope transporter proteins (Scalbert 1991, Cowan 1999).

Lignins are a group of very complex and heterogeneous polymers. They are composed of three different phenyl-propanoid subunits; p-hydroxy phenyl, guaiacyl and syringly. There are variations in composition of these subunits between plant taxa and one cell to another cell (Bowsher et al 2008).

Plant phenolics have a vast array of biological functions in the plant itself and in animals that consume them. For plants they produce flavors, scents, colors or visual signals to attract pollinators and repel herbivores. They also help in plant defense activity against bacterial and fungal pathogens. Some compounds are important for some specific function of plants like germination inhibition, ultraviolet protection, and signal transduction. (Bowsher et al., 2008). Polyphenols in our dietary fruits, vegetable and cereals are an important source of antioxidants in our body. Antioxidant properties of polyphenols help to reduce the damage of several diseases including cancer, cardiovascular diseases, osteoporosis, and degenerative disease linked with aging (Scalbert et al., 2005).
Ethnobotanical Survey

Infectious diseases are a major global cause of mortality and morbidity. Approximately 50% of deaths in tropical countries and 20% of deaths in the Americas occur due to pathogenic bacteria (Mahady et al., 2003). Despite the significant progress in microbiological research and control of microorganisms, periodic occurrences of epidemics due to drug resistance microorganisms and previously unknown microbes cause an enormous threat to public health. It is crucial to find alternatives for frequently used antibiotics. Plant products have been used as various remedies for thousands of years and it is often possible to treat drug resistance bacteria with natural antibiotics e.g. *Punica granatum, Eucalyptus maculate, Garcinia cola, Camellia sinensis, Mahonia aquifolium, Senna petersiana* contain compounds having antimicrobial properties (Sibanda and Okoh, 2007)

Ethnobotanical surveys play a vital role in the correct identification, utilization and application of the therapeutic potential of medicinal plants (Rojas et al., 1992, Silva et al., 1996, Soejarto et al., 2012). There are several reports of the inhibitory effect of traditionally used plant extracts on the growth of bacteria, fungi and viruses. Researchers worldwide are trying to authenticate the ethnobotanical uses of medicinal plants by scientifically testing their antimicrobial properties. Ethnobotanical surveys of indigenous plants used as folk medicine in Guatemala (Caceres et al., 1995), Argentina (Anesini and Perez, 1993), Ethiopia (Desta, 1993), Indonesia (Grosvenor et al., 1995), Malaysia (Mackeen et al., 1997), India (Valsaraj et al., 1997), Mexico (Navarro et al., 1996), Jordan and other middle eastern countries (Nimri et al., 1999), Turkey (Sokmen et al., 1999), Palestine (Essawi and Srour, 2000), Brazil (Holetz et al., 2002) and Lebanon.
(Barbour et al., 2004) have been reported to have antimicrobial properties against a broad range of microorganisms including *E. coli*.

The use of an ethnobotanical survey is an ideal approach to discover alternative medicine and validate the traditional use of various indigenous plants of a particular region. Here in my work I have made an attempt to validate the traditional use of some medicinal plants of South Dakota and the Northern Great Plains, which were traditionally used by Native Americans.

**Solvent Extraction of Plant material**

The extraction and isolation of secondary metabolites from plants is an area of interest for biochemical, chemotaxonomic, ecological, phytochemical, pharmacological, and plant tissue culture studies. Extraction and the separation of medicinally active components from the plant tissues are conducted using selective solvents through standard procedures (Handa et al., 2008). Plant tissues are extracted in various organic and aqueous solvents for several hours. The extraction with solvent causes a mass transfer of the active soluble compounds to the solvent which depends on the solubility of specific compounds in the solvent. A perfect solvent for extraction should easily dissolve the secondary metabolites, be easy to remove, be inert and nontoxic. Methanol, ethanol, chloroform and methylene chloride are usually used to carry out a preliminary extraction of plant parts. It has been reported that chloroform and methylene chloride may react with some alkaloids compounds like brucine, strychnine and ephedrine (Phillipson and Bisset, 1972). It is also been shown that the presence of a trace amount may cause decomposition, dehydration or isomerization of other compounds. Chloroform is regarded as nephro and hepato toxic; one must follow safety guidelines while using it.
Methylene chloride is less toxic but highly volatile. Methanol and 80% ethanol are more polar than chlorinated hydrocarbons and it is believed that alcohol solvents can penetrate plant membranes more efficiently and allow extraction of higher amounts of plant endocellular compounds. Alcohol has the ability to solubilize mainly polar metabolites collectively with medium and low polarity compounds extracted with less efficiency (Cannel, 1998). Aqueous-alcoholic solvents are found to have the optimum solubility characteristic for many initial extractions. Methanol and ethanol have been frequently used to extract plant materials for various purposes. It has been reported that aqueous methanol was found to be more effective in recovering maximum amounts of phenolic compounds from rice bran (Chatha et al., 2006) and Moringa oleifera leaves (Siddhuraju and Becker, 2003). Keeping these reviews in mind we chose methanol as the solvent for extraction in our study.

**Antimicrobial Susceptibility Testing Assays**

Antimicrobial susceptibility testing methods are divided into two types based on the principle applied in the technique:

I). Kirby Bauer disk Diffusion Susceptibility Test Protocol: In 1956 W. M. M. Kirby and his colleague A. W. Bauer at the University of Washington School of Medicine and the King County hospital proposed the single disk method for antimicrobial susceptibility testing (Winn et al., 2006). The Clinical and Laboratory Standards Institute (CLSI) formerly known as the National Committee for Clinical Laboratory Standards (NCCLS) has standardized the Kirby-Bauer disk diffusion assay with certain modifications (http://www.microbelibrary.org/component/resource/laboratory-test/3189-kirby-bauer-disk-diffusion-susceptibility-test-protocol). The purpose of this method is to determine
the susceptibility and resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds. The pathogenic organism is grown on Muller Hinton (MH) Agar plates in presence of various antimicrobial-impregnated 6 mm paper disks. The inhibition zone around the paper disks after incubation is the measure of the ability of the compounds to inhibit the growth of the pathogen.

This method is helpful in assessing various plant extracts for their antimicrobial activity. The theory behind this method is, when the filter papers, impregnated with the antimicrobials, are placed on MH agar they absorb water from the agar and the antimicrobial compounds begin to diffuse in to the surrounding agar. The size of the inhibition zone around the disk depends on the solubility, diffusion rate and the molecular size of the antimicrobial compounds and also thickness of the agar medium. The basic steps for the disk diffusion assay are: 1). Selection of pure colonies from freshly prepared culture. 2). Preparation and standardization of inoculum suspension: there are two methods for inoculum suspension direct colony suspension and log phase growth. For both methods the turbidity of the test suspension should be standardized to 0.5 OD at 600nm, which corresponds to $10^7-10^8$ CFU/ml. In direct colony suspension, colonies should not be older than 18-24 hours and suspended in MH broth or Tryptic soy broth. 3). Inoculation of agar plates: Before inoculating, the agar plates should be allowed to warm up to room temperature so that any excess moisture will be absorbed. The organism suspension needs to be vortexed to mix properly. Then 100 µl of suspension should be spread thoroughly and evenly on the plates by using a sterile glass spreader. 4) Applying antimicrobial disks: the 6 mm paper disk with antimicrobial agent should be applied on the inoculated plates within 15 min by pressing firmly for adequate contact with the agar
media. 5) Measurement of zone of inhibition; using mm scale the clear areas around the disk should be determined. Each plate should contain a positive and negative control to allow comparison of the results.

**Minimum Inhibitory Concentration:** According to NCCL “the minimum inhibitory concentration (MIC) of antimicrobial agent is the lowest concentration of antimicrobial agent that inhibits the bacterial isolate from multiplying and producing visible growth on the test system”. The broth microdilution method is widely used to perform MIC test.

**Broth Microdilution MIC protocol:** Broth microdilution MIC testing is performed in a 96 well plate following the protocol of NCCLS. Each plate should have one well for negative growth control (broth + inoculum) and positive growth control (inocula with 50µg/ml of gentamicin). The MIC is determined by taking 8 different concentrations of twofold serial dilutions of the antimicrobial agents (plant extracts) in the microtiter plates. Bacterial suspensions of innocula are prepared from 18-24 hour old cultures from agar plates. The broth cultures are incubated at 37°C until the negative controls achieve a turbidity of 0.1-0.5 OD at 600 nm. Each well is inoculated with 0.01-0.05ml of 10⁵-10⁶ CFU/ml of standardized bacterial suspension. The plate is covered with parafilm and incubated at 37°C for 18-24 hours (Reller et al., 2009). The optical density is determined just prior to incubation (T0) and again after 18 hour incubation (T18) at 600 nm. The OD of T0 is subtracted from the OD of replicates at T18. The adjusted OD of each negative control well is considered as 100% growth. The percent inhibition thus is determined using the formula; Percent inhibition= [1-(OD of test well/ OD of negative]
control well)]* 100. The MIC is reported as the lowest concentration of test substances at which showed 100% growth inhibition of the test organism (Sherlock Orla et al., 2010)

**Susceptibility Assay Using Bacterial Enumeration:** This assay is performed following the method by Al-Bakri and Afifi (2007). The 96-well plate cultures with different concentrations of plant extracts are prepared as described in broth microplate dilution method. Each well contained 100 µl of broth culture with a plant extract. The viable cells are determined by conducting tenfold serial dilution of these suspensions in 0.9% sterile saline. All dilutions are then plated on blood agar / tryptic soy agar plates and incubated at 37°C for 17 hours. The antimicrobial activity of the tested plant extracts are compared with the positive and negative results. Positive control is gentamicin (50 µg/ml) and negative control is only bacterial culture and solvent.

Percent viable counting reduction is calculated using the following formula.

100 - [(Experimental well viable count / Negative well viable count)* 100]

**Cytotoxicity Test Assay**

Mammal cell viability assays or cytotoxicity assays are useful in screening drugs, testing different chemicals and phytochemicals. These tests are based on three major parameters; i). Cellular metabolic activity which is measured by cellular ATP level or mitochondrial activity; ii). Cell membrane integrity disruption which is measured by the level of lactate dehydrogenase (LDH) in the extracellular medium; iii). Measurement of total cell protein or DNA which is proportional to number of live cells (http://www.noabbiodiscoveries.com/assays/invitro/cytotoxicity_studies.pdf).
**Alamar-Blue (AB) Cytotoxicity assay:** A cell proliferation assay needs to be simple, rapid, efficient, reliable, sensitive, nontoxic, and relatively inexpensive. Moreover it should not interfere with the test compound. Alamar blue has been in use since 1993 and shows all those characteristics to be an ideal dye for a cytotoxicity assay. It is a simple and rapid test, where the cell viability is assessed simply by adding 10% of the commercially available solution to mammalian or bacterial cell culture medium and measuring the fluorescence after incubation for certain time. **Working Principle of AB:** The active ingredient of AB reagent is resazurin which is a nontoxic, nonfluorescent, and cell permeable compound. Once it enters into the cell, it is reduced to a fluorescent red resorufin. Living cells continuously convert resazurin to resorufin and increase the color and fluorescence of the medium (O'Brien et al., 2000)

**Protocol:** The protocol is as per the AB cytotoxicity kit (Life Tecnologies, NY). A 96 well plate seeded with mammalian cells and treated with the testing materials to be prepared. Then 1/10 of the AB reagent is added directly to the cell in culture medium. If the number of cells is more than 5000 per 100µl then it needs to be incubated for 1-4 hours. If number of cell is small then the incubation time can be increased for more sensitivity of detection. The fluorescence of the colored solution of AB can be monitored by using a fluorescence excitation wavelength of 540-570 nm and emission at 580-620 nm. Determination of cytotoxicity of the test substances can be done by plotting fluorescence emission intensity values which correlate to an increase in reduction activity of the cells in the well plate.

**DNA-Based Proliferation Assay:** The DNA based cell proliferation assay is a colorimetric immunoassay for the quantification of viable cells. This assay is based on
the measurement of BrdU (5-bromo-2’-deoxyuridine) an analogue to thymidine, which can be incorporated into the newly synthesized DNA instead of thymidine. Cells are then fixed and DNA is denatured by using the FixDenat solution. After removing Fix-Denat solution, the anti BrdU-POD antibody is added, that binds to the BrdU incorporated DNA. The immune-complexes are detected by substrate (TMB, ready to use) reaction. The absorbance of the reaction product is taken to quantify the BrdU incorporated into DNA which is proportional to the cell proliferation.

REFERENCES


CHAPTER-2

SCREENING OF PLANT EXTRACTS NATIVE TO SOUTH DAKOTA FOR THEIR ANTIMICROBIAL ACTIVITY AGAINST ENTEROTOXIGENIC ESCHERICHIA COLI (E. COLI O157 K88 LT STB)

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ABSTRACT

*Escherichia coli* H157K88 LT STB are the foremost cause of lethal diarrhea in weaned pigs in South Dakota. The United State swine producers face huge losses due to bacterial infections. The increasing problem of antibiotic resistant *E. coli* requires finding an alternative source of antibiotics. Large numbers of phytochemicals have antimicrobial properties and have been used traditionally to treat different diseases. Based on the use of native plants by the Native Americans we chose 25 plants from South Dakota. Our study was aimed at investigating the antimicrobial effect of native South Dakota plants on enterotoxigenic *E. coli*. Some native plants have the potential to provide new classes of antibiotics with low cytotoxicity that may allow regional swine producers to protect young pigs from the lethal diarrhea. For our study fresh plant materials were extracted with methanol and then lyophilized. Screening of plant extracts for antimicrobial activity was done by using a disk diffusion assay. All experiments were performed in triplicate.
Out of twenty five plants three; *Sanguinaria canadensis*, *Rhus glabra* and *Rhus aromatica* showed antimicrobial activity with zone of inhibition of 13.66 mm, 9.66 mm and 8.66 mm respectively. The results suggest three plants native to South Dakota have potential antimicrobial activity against enterotoxigenic *E. coli*.

**Keywords:** Enterotoxigenic *E. coli*, antimicrobial activity, plant extract, disk diffusion assay
INTRODUCTION

Recently an increase in incidence of outbreaks of severe *E. coli* associated diarrhea has been observed worldwide (Fairbrother et al., 2005). Early weaning in pigs is profitable for the swine industry but is normally believed to be a traumatic incident that is often coupled with reduced feed intake and physical development. There is an increased prevalence of diarrhea and disease, overgrowth of pathogenic bacteria and mortality (Frydendahl, 2002, Fairbrother et al., 2005). Extensive diarrhea in weaning pigs could lead to a significant economic loss in swine industry (Zhang et al., 2007, Fang et al., 2009). Enterotoxigenic *Escherichia coli* (ETEC) are the foremost group of bacteria causing diarrhea in pig and is marked with high morbidity and mortality (Zajacova et al., 2012). ETEC is the etiological agent for both neonatal and post weaning diarrhea. ETEC that causes diarrhea in pigs possesses two types of virulence factors and both of these are essential to cause disease. Two major virulence factors in ETEC are specific fimbrial adhesins (F4 (K88), F5 (K99), F6 (987P), F18 and F41) and enterotoxins i.e. heat labile and/or heat stable (Zajacova et al., 2012). The fimbrial adhesins target specific receptors on porcine intestinal brush border epithelial cells, enabling the bacteria to colonize the cell surface and there excrete toxins whose action includes production of diarrhea in host animals (Dubreuil, 2008). ETEC possessing the K88 pilus (K88+ ETEC) are commonly associated with diarrheal disease in young piglets (Chandler and Mynott, 1998, Fairbrother et al., 2005).

Antibiotics have been used traditionally to prevent and treat enteric disease including diarrhea induced by weaning stress (Fang et al., 2009). 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 (Barton, 2000, Monroe and Polk, 2000). Livestock producers in many countries are trying to manage production without antibiotic growth promoters (Marcin et al., 2006). A study conducted in Ontario, Canada; found that porcine E. coli had resistance genes for tetracycline, streptomycin, sulfonamide and apramycin whereas a quinolone resistance gene was only detected in ETEC. It was also observed that antimicrobial resistance occurred more frequently in pathogenic than in other E. coli, so there may be a relationship between virulence factor and resistance gene (Boerlin et al., 2005). In Korea, research had been carried out to test the susceptibility of ETEC strains against 15 commonly used antibiotics, with 14 antibiotics out of 15 showing high MIC\textsubscript{90} values (Choi et al., 2002). A study reported that 73% of E. coli isolates were resistant to five or more antibiotics including tetracycline, gentamycin, streptomycin, sulfamethoxazole, tetracycline, kanamycin (Bischoff et al., 2002). Extensive use of antimicrobials most likely selects the survival of resistance bacterial species and these resistance genes can be transferred to other susceptible bacteria. This is not only reducing the numbers of available antimicrobials to the swine industry for bacterial infection but this resistance also causes a threat to human health (Pluske et al., 2002).

Novel natural products are needed to replace antibiotics. Plants produce a large variety of secondary compounds as natural protection against microbial and insect attacks. Plant extracts contain a vast source of different secondary products which have intrinsic bioactivities on animal physiology and metabolism (Marcin et al., 2006). A potential alternative to antimicrobial agents are traditional Chinese herbal medicines or their extracts which may impair microbial growth in early weaned piglets (Fang et al.,
Acanthopanax senticosus a tonic and sedative Chinese herb used as a dietary additive modified the cellular and humoral immune responses of weaned piglets by modulating the production of immunocytes, cytokines and antibodies, it also enhanced digestion and absorption of amino acids in weaned piglets (Kong et al., 2009). Chinese herbal medicine additives e.g. Astragalus membranaceus, Glycyrrhiza uralensis, Codonopsis pilosula, Poria cocos and Atractylodes macrocephola improve the gastrointestinal health and growth performance in weaned piglets and help to reduce the lower cecal and colonic Escherichia coli count (Ding et al., 2011). Fang et al (2009) investigated the efficacy of Acanthopanax centicosus extract as feed additive and showed that, it could regulate the microbiota composition and maintain a normal morphology of gut mucosa in weaning piglets, thereby decreasing diarrhea that resulted from the weaning stress. Coptidis rhizome, Lonicerae flos, Paeonia japonica methanol extracts (Korean Traditional herbal medicine) showed anti-diarrheal effects reducing diarrhea caused by E. coli in piglets (Jung et al., 2011). Essential oils from oregano, thyme clove and cinnamon are used as feed additive in livestock as antibiotics for improved growth (Lalles et al., 2009). Incorporation of purified plant extracts like cravacol, cinnamaldehyde and capsicum oleoresin in combination with formic acid promotes changes in the digestive function, microbial ecology and fermentation of weaned pigs (Manzanilla et al., 2004). Bromelain, a proteolytic extract obtained from pineapple stems effectively reduces ETEC induced diarrhea by temporarily inactivating the ETEC receptor in piglet small intestine (Chandler and Mynott, 1998). Aegle marmelos has been extensively used by the local people of India and it is well known for its anti-diarrheal properties. Brijesh et al (2009) validated its traditional use, they found that the hot
aqueous extract of *A. marmelos* significantly reduced the enteropathogenic *E. coli* adhesion and production of LT. *Punica gratum* pericarp is commonly used as local medicine for treatment of diarrhea in Thailand. The antibacterial activity of *P. gratum* was assessed against enterohemorrhagic *Escherichia coli* O157 and could be used to prevent toxin production by *E. coli* (Voravuthikunchai et al., 2005). Chen et al (2006) reported in his work that the ethyl acetate fraction of *Galla chinensis* blocked the binding unit of heat labile enterotoxin to the ganglioside on the surface of intestinal epithelial cells resulting in the suppression of LT-induced diarrhea. *Pentaclethra macrophylla* is a well-established herbal medicine used to manage diarrhea outbreaks in humans and animals in many states of Nigeria (Akah et al., 1999). Ethanolic extracts of three plants *Eupatorium cannabinum*, *Astilbe rivularis* and *Schimma wallichi* used in Sikkim, India as folk medicine were active against *E. coli* collected from feces of diarrheic calves and goats (Dubal et al., 2009). Throughout medical history, plant derived products have been shown to be valuable sources for the discovery and development of new drugs.

Native American medicinal plants are also traditionally used to prevent and treat variety of diseases. The traditional herbal remedies, used by indigenous people of Northern Great Plains were based upon holistic approach to treat the disease bodily and spiritually (Anthony.J, 2001). Native American Indian Tribes have a great understanding of how to use complex herbs in healing remedies. In fact it is believed that hundreds of our modern drugs have roots in Native American herbs. Several native plants from the Northern Great Plain were used to treat various bacterial diseases by Native Americans e.g. various species of *Echinacea*; *E. purpurea*, *E. angustifolia* and *E. pallida* indigenous to North America have been reported to treat upper respiratory infection (Melchart et al.,
1998), and otitis media in children (Mark et al. 2001). Daniel A. Moerman (2009), an ethnobotanist has documented 24,945 plants, used therapeutically by the Native Americans.

**OBJECTIVE**

The aim of our study was to screen indigenous plants Northern Great Plain for their antimicrobial activity against enterotoxigenic *Escherichia coli*

**MATERIALS AND METHODS**

*Collection of plant materials*

Selection and collection of plants materials were mainly based on their traditional use by American Indians for treatment of wounds, diarrhea and other illness caused by bacterial infection (Moerman, 2009). Plant materials were collected from South Dakota, from June through August (2010). Plant species (Table-2.1) were collected from different locations (Sica Hollow State Park, Oak Lake Field Station). All plants samples were prepared in the field or taken to the laboratory within 2 – 4 hours; they were cleaned with tap water and stored at -80°C. Field collections were made following standard herbaria protocols. To ensure positive identification of the collections, photographs and voucher specimens were prepared. Voucher specimens were mounted and are stored in a herbarium cabinet in our laboratory to provide authentication of the plants. A total of twenty five plants were selected for the initial screening.
**Extraction of plant materials**

Fresh plant samples were homogenized in a blender in 100% methanol and methanol plant extracts were mixed continuously on an orbital shaker for 24-72 hours, at 150 RPM. After complete extraction, plant materials were filtered using Whatman # 1 filter paper. Methanol was removed from the sample using a rotary evaporator and the remaining extracts were frozen and lyophilized to dryness. The dried extracts were then stored at -20°C for further use.

**Bacterial strains and growth condition**

*Escherichia coli* O157 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 a shaker incubator at 200 rpm at 37 °C (Klančnik et al., 2010). For antibacterial activity assay the CFU was adjusted to $10^7$-$10^8$/ml using spectrophotometer (absorbance 0.5 at 600nm). Stock cultures were maintained in 70% glycerol and stored at -80°C.

**Disk diffusion assay**

*In vitro* antimicrobial activities were screened by disk diffusion assay following the protocols of National Committee for Clinical Laboratory Standard (NCCLS, 2003) and the modified Kirby- Bauer disk diffusion method (Bauer et al., 1966). TSA plates with 5% sheep blood for disk diffusion test were obtained from BD (NJ, USA). The
plates were uniformly inoculated with 100µl of broth culture of 0.5 OD. The sterile paper disks of 6mm diameter were loaded with 20µl of plant extracts (2mg plant material per disk). The disks were allowed to air dry and then were placed on the blood agar plates. Each petriplate also contained one disk of gentamicin (10µg/disc) as the positive control and one disc of 20µl 70% ethanol for negative control. Each extract was screened with three replicates on the blood agar plates. The inoculated Petri plates were incubated for 18-24hrs at 37°C. The zones of inhibitions were measured in mm after 18hrs.

RESULTS

We screened 25 plants based on their medicinal properties (Moerman, 2009). Out of 25 plants, three plants *Sanguinaria Canadensis*, *Rhus glabra* and *Rhus aromatic* showed potential antimicrobial activity against ETEC (Table-1). The zone of inhibition for *S. canadensis* was 13.66 mm whereas 9.66 mm for *R. glabra* and 8.66 mm for *R. aromatic*. The zone of inhibition for control disk was 14.66 mm.

DISCUSSION

This is the first time, 25 native plants were subjected to preliminary screening for antimicrobial activity against ETEC to find a plant based antibiotics to treat diarrhea in pigs. It was clear from the present results, that methanol extracts of *R. aromatic*, *R. glabra* and *S. canadensis* exhibited pronounced activity against ETEC. They were further investigated in the next part of our research for their minimum inhibitory concentration (MIC) value against ETEC and cytotoxic activity and phytochemical analysis.
DISK DIFFUSION ASSAY (DDA)

Table 2. 1 Plant extracts screened for antimicrobial activity

<table>
<thead>
<tr>
<th>Plants Name</th>
<th>Zone of Inhibition (mm)</th>
<th>Plants Name</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arctostaphylos uva ursi</td>
<td>0</td>
<td>Oenothera biennis</td>
<td>0</td>
</tr>
<tr>
<td>Artemesia ludoviciana</td>
<td>0</td>
<td>Onosmodium molle</td>
<td>0</td>
</tr>
<tr>
<td>Astragalus canadensis</td>
<td>0</td>
<td>Pedicularis racemosa</td>
<td>0</td>
</tr>
<tr>
<td>Caltha palustris</td>
<td>0</td>
<td>Pediomelum argophyllum</td>
<td>0</td>
</tr>
<tr>
<td>Centaurea stoebe</td>
<td>0</td>
<td>Physialis virginiana</td>
<td>0</td>
</tr>
<tr>
<td>Chrysothamnus nauseosus</td>
<td>0</td>
<td>Plantago aristata</td>
<td>0</td>
</tr>
<tr>
<td>Cleome serrulata</td>
<td>0</td>
<td>Rhus aromatica</td>
<td>8.66±0.57</td>
</tr>
<tr>
<td>Corallorhiza sp.</td>
<td>0</td>
<td>Rhus glabra</td>
<td>9.66±0.57</td>
</tr>
<tr>
<td>Cyanoglossum officinale</td>
<td>0</td>
<td>Sanguinaria Canadensis</td>
<td>13.66±0.57</td>
</tr>
<tr>
<td>Geranium viscosissimum</td>
<td>0</td>
<td>Symphoricarpus albus</td>
<td>0</td>
</tr>
<tr>
<td>Mellilotus officinalis</td>
<td>0</td>
<td>Tanacetum vulgare</td>
<td>0</td>
</tr>
<tr>
<td>Monarda fistulosa</td>
<td>0</td>
<td>Control</td>
<td>14.66±0.57</td>
</tr>
</tbody>
</table>

Note: All numbers are average of three replications
REFERENCES


CHAPTER 3

ANTIBACTERIAL ACTIVITY, CYTOTOXICITY AND PHYTOCHEMICAL ANALYSIS OF RHUS AROMATICA AND RHUS GLABRA NATIVE TO SOUTH DAKOTA

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ABSTRACT

*Rhus aromatica* and *Rhus glabra*, commonly known as fragrant sumac and smooth sumac respectively, are native to South Dakota (SD). These two Sumac plants were extensively used by American Indians in their traditional medicinal practices. Both *R. aromatica* and *R. glabra* have been reported to have antimicrobial activity. A lethal diarrhea by enterotoxigenic *E. coli* is one of the major problems for the swine producers of SD. To find an alternative source of antimicrobial agents from native plant materials could provide an alternative solution to control diarrhea. The objectives of our research were a) To determine the minimum inhibitory concentrations of *R. aromatica* and *R. glabra* plant extracts that effectively inhibit *E. coli* H157 K88 LT STB growth and to evaluate their cytotoxicity in porcine intestinal epithelial cell lines, b) To quantify the major chemical components; total phenolics, flavonoids and tannin content of these two plants and to investigate the composition of essential oils in in *R. aromatica*. Plant materials were
extracted with 100% methanol. A bacterial enumeration assay was followed to determine the MIC value and Almar Blue cytotoxicity assay was used to determine cytotoxicity effect. Quantification of total phenolics, flavonoids and tannins of both plants were conducted using a spectrophotometer. Gas chromatography / mass spectrometry was utilized to identify different constituents of essential oil of *R. aromatica*. In our results, the MIC value of *R. aromatica* and *R. glabra* were found to be 25 and 12.5 mg/ml respectively and the AB cytotoxicity test against IPEC-J2 cells showed 50% inhibition in cell growth at a concentration of 1.25 mg/ml for both plants. *R. aromatica* has higher flavonoid and tannin content but *R. glabra* has a higher amount of total phenolics. This suggests that the antimicrobial properties of *R. aromatica* is due to its high flavonoid and tannin quantity and that of *R. glabra* is due to the presence of simple phenolics like gallic acids. Essential oil of *R. aromatica* contained Limonene, β-Cubebene and Caryophyllene as three major constituents and comprised of 82% of the total essential oil. These three compounds have been reported to have antimicrobial activities. It could be concluded from this research that *R. aromatica* and *R. glabra* may contain potential antimicrobial agents for controlling diarrhea in pigs due to enterotoxigenic *E. coli*, but further *In vivo* study is necessary to evaluate the cytotoxicity effect on animals. Furthermore the essential oil analysis of *R. aromatica* revealed that it could be a potential source of limonene to be used in food industry, pharmaceutical industry and other manufacturing industries.

**Key Words:** antimicrobial activity, cytotoxicity, phytochemicals, minimum inhibitory concentration, *Rhus aromatica*, *Rhus glabra*, enterotoxigenic *Escherichia coli*, essential oils, limonene, cubebene, caryophyllene
INTRODUCTION

The genus *Rhus* commonly known as Sumac (Family-*Anacardiaceae*) is found worldwide and contains more than 250 individual species (Rayne and Mazza, 2007)

*Rhus aromatica* and *Rhus glabra* are two common species widely distributed in most of the USA and are native to South Dakota. *Rhus aromatica*, commonly known as fragrant sumac is distributed from west to eastern South Dakota, Central Nebraska, Kansas, Oklahoma and Texas (www.fs.fed.us/database/feis). It is a small (4-5 ft), aromatic, nonpoisonous deciduous shrub and grows in dry, rocky soils, in hedges and thickets (Harper, 1881). American Indians used it as food and medicine. Ripe red fruits can be eaten dry or raw but the most popular use is as berry tea or sumac-ade (Moerman, 2009). Different parts of this plant have been used for various ailments. Infusions of roots, bark and leaves were used to treat diarrhea, stomach ache, tooth ache, sore throat, skin disease and eczema. They were also used to reduce fever, diabetes and excessive vaginal discharge. The bark and leaves of all sumacs has been used as astringent and barks can be used for tanning leather because of its high tannin content (plants.usda.gov/plantguide/pdf/cs_rhars.pd,)

(www.pfaf.org/user/plant.aspx?LatinName=Rhus+aromatica)

Dr J.F. McClanahan of Boonville, MO, introduced *R. aromatica* in medicinal practice in the year 1878, as a highly useful remedy in the treatment of diabetes, enuresis, uterine hemorrhage and diarrhea (Harper, 1881). An aqueous extract of *R. aromatica* showed strong antiviral activity against type-1 and type-2 herpes simplex virus (Reichling et al., 2009) Pinchak et al (2008) reported that the tannins of *R. aromatica*
have antibiotic activity against mastitis pathogens *E. coli* and *S. aureus*. Antinematodal activity has also been demonstrated, being used in the mixture of commercially available bionematicide Sincocin (Chitwood, 2002)

Little information is available as to the chemical composition of *R. aromatica*. Quantitative analyses of alcohol extracts of *R. aromatica* reported the presence of volatile and fixed oils, several resins and waxes, butyric acid, tannins, glucose, gums, oxalate (Harper, 1881).

*Rhus glabra* is commonly known as smooth sumac, dwarf sumac, scarlet sumac or upland sumac. It is a deciduous shrub native to North America found in 48 states of USA and Southern part of Canada. It grows as a shrub or small tree (from 6-15 ft.) in thickets, waste ground, open fields and roadsides. Native Americans used this plant as food and medicine. Young shoots, roots and fruits were used raw and ripened fruits were cooked or made in to a lemonade-like drink (Jackson and Bergeron, 2011). An infusion of bark or roots was used for the treatment of colds, diarrhea, fevers, sore mouth and throats, rectal bleeding, inflammation of the bladder, painful urination, and dysentery. It was also applied externally to treat burns and skin eruptions (Erichsen-Brown, 1989). All parts of this plant have medicinal value. Powdered bark is used as antiseptic salve; infusion of leaves is used to treat asthma, diarrhea, and stomatitis. A poultice of leaves is used for skin rashes; the leaves are chewed for sore gums and rubbed on sore lips. An infusion of berries was used in the treatment of diabetes and constipated bowel complaints (Jackson and Bergeron, 2011).

McCutcheon et al. (1992) reported the antimicrobial activity of *R. glabra*; they studied the crude methanolic extracts of 100 medicinal plants in British Columbia
(Canada), *R. glabra* showed the widest zones of inhibition in disc assays and the broadest spectrum of inhibition (active against all of the following species of bacteria: *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacter phlei*, *Pseudomonas aeruginosa* H187, *Serratia marcescens*, *Staphylococcus aureus* and *Salmonella typhimurium* TA98. Similarly in another study the same methanolic extracts from *R. glabra* branches inhibited the following nine fungal strain tested *Aspergillus flavus*, *A. fumigatus*, *candida albican*, *Fusarium tricuictum*, *Microsporum cookerii*, *M. gypseum*, *Saccharomyces cerevisiae*, *Trichoderma viridae* and *Trichophyton mentagrophytes* (McCutcheon AR et al., 1994). Saxena et al. (1994) reported that the methanolic extracts were active against several bacterial and fungal species, furthermore, three compounds were isolated which were the active constituents against bacteria: methyl gallate (MIC of 13µg/ml), 4-methoxy-3,5-dihydroxybenzoic acid (MIC of 25 µg/ml) and gallic acid (MIC of 1000 µg/ml). Presence of gallic acid derivates in *R. glabra* have also been reported by Khadem and Marles (2010). Chemical analysis of *R. glabra* showed the presence of 19.4-31.1% of polyphenols and essential oils together and that of 9.8-15.7 % of gallotannin (Campbell, 2010).

Quantification of chemical components of *R. aromatica* and *R. glabra* has not been fully reported. Here in this study we measured the quantity of polyphenols, total flavonoids and hydrolysable tannin and condensed tannin in both sumac species. Essential oils, aromatic and volatile products of plant secondary metabolism are widely applied in folk medicine, food flavoring, preservation and fragrance industries. Antimicrobial properties of essential oil have been known for many centuries. In recent years a large number of essential oils and their chemical components have been identified.
for their antimicrobial activities and other health benefits. Although *Rhus aromatica*
showed antibacterial, anthelminthic and anticancer activity (previous studies in our lab)
but it has not been studied yet for its essential oils and their constituents.

**OBJECTIVES**

The aims of this study were **a**) To determine the minimum inhibition
concentration (MIC) of methanol extracts of *R. aromatica* and *R. glabra* against
enterotoxigenic *E. coli* (*E. coli* O157 K88 LT STB) **b**) To evaluate the cytotoxicity
activity against porcine intestinal epithelial cell lines **c**) To quantify total phenolics,
Flavonoids and Tannin content of *R. aromatica* and *R. glabra* and **d**) To identify different
volatile compounds in the essential oils of *R. aromatica*.

**MATERIALS AND METHODS**

*Collection of Plant Materials*

Collection of plant materials was done as described in chapter # 2 (Screening of plant
extracts, above)

*Extraction of plant materials*

Protocol for extraction was followed as per the protocol mentioned in chapter # 2.

*Bacterial strains and growth condition*

Bacterial strain and growth condition was maintained following the method as given in
chapter # 2.

*Determination of MIC value by Bacterial Enumeration Assay*
The MIC values for the sumac extracts that were active against *E. coli* were measured by using broth dilution and bacterial enumeration (by viable count method) by Al-Bakri and Afifi (2007) with a slight modification, 50 µl aliquots of bacterial suspension in TSB growth medium of 0.5 OD were added to the wells of sterile 96 well microtiter plates already containing 50 µl of two fold serially diluted plant extracts. The plant extracts used in this method were solubilized with 10% ethyl alcohol. The final volume of each well was 100 µl. Control wells were prepared by taking only broth, broth with culture (Negative control), Broth culture with 10% ethanol (Negative control) and broth culture with 50 µg/ml gentamicin (positive control). 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 (each plant extract of different concentrations of 0.39- 50 mg/ml treatment well), then plating aliquots of dilutions onto a blood agar plate and incubating them at 37°C for 18 hours. After incubation the colonies were counted and the original number of the viable cells was calculated by using the dilution factors.

**Cytotoxicity test**

A microplate Alamar Blue Assay was performed to detect the toxic effect of plant extract on animal cells. Alamar blue assay measures cellular metabolic activity based on the conversion of non-fluorescent dye resazurin, which is converted by mitochondrial and other enzymes to pink resorufin (O’Brien et al., 2000)

Cytotoxicity against porcine epithelial cells was performed in the Immunology laboratory of Bio/Micro Department, SDSU following the method of O’Brien et al. (2000) and Ajaiyeoba et al. (2006). Briefly, porcine epithelial cells were harvested in
culture plate in IPECJ2 media. Growth inhibition was determined using a 96 well microplate assay in which 7500 porcine epithelial cells in IPECJ2 media were plated per well and the plates were incubated at 37°C at 5% CO₂ for 24 hours. After 24 hours of incubation the 96 well plates were observed for the growth of epithelial cell layer. Once single layer had formed, plant extracts were dissolved in 10% ethanol to make solutions of different concentrations 10mg/ml- 0.078mg/ml by twofold serial dilution. Ten µl of plant extracts of each concentration were replaced into media. Each concentration was tested for cytotoxicity in triplicate. Again the plate was incubated for 24 hours at 37°C and 5% CO₂. The medium was then removed and the cells were washed with fresh media 2 times. Then wells were filled with 90µl media and 10µl of Alamar Blue. After a further four hours of incubation, fluorescent Almar Blue was detected using a microplate fluorometer with a fluorescence excitation wavelength 540 nm and fluorescence emission wave length of 600 nm. Ethyl alcohol (70%) was used as positive control.

**Determination of Total Phenolics:** Total phenolics were determined following the method of Singleton and Rossi (1965).

For this assay fresh plant materials were homogenized in a blender and one gram of each sample was transferred to a 15 ml falcon tube. Three plant samples were extracted with 15 ml mixture of acetone, water and acetic acid (70: 29: 0.5). All three samples were vortexed and allowed to stand for one hour at room temperature for complete solvent extraction. The supernatant was filtered through Whatman # 1 filter paper and rinsed with 10 ml of water. The filtrate was concentrated up to half of the total using a rotary evaporator under partial vacuum at 40°C. The concentrated samples were then diluted with Nanopure water and brought up to a final volume 15ml.
Measurement of total phenolics by Folin-Ciocalteu assay: one hundred microliter of plant sample (prepared previously), 100 µl Folin-Ciocalteu reagent and 2.3 ml water were mixed in a 10 ml tube. The mixture was then allowed to stand for 5-8 min at room temperature. Ten ml of 7% sodium carbonate solution was added followed by the addition of 20 ml Nanopure water. The solution was then mixed and allowed to stand for 2 hour. Absorption was measured spectrophotometrically at 750 nm. Standardization of Gallic Acid: The total phenolics content was standardized against gallic acid and expressed as milligrams of gallic acid equivalents. Standard gallic acid solutions were prepared in a concentration range of 10 mg/ ml to 50 mg/ml (Appendix-1). Reagent blanks using distilled water were also prepared. The data for the total phenolic content of three plant extracts were expressed as milligram of gallic acid equivalent per 100 gm dry mass.

**Determination of Total Flavonoids:**

The total flavonoids were determined by Aluminum chloride spectrophotometric assay following the method by Atanassova et al (2011). Samples for flavonoid extraction were prepared by extracting 0.5 gm of dried ground plant sample with 80% aqueous methanol on an ultrasonic bath for 20 min. An aliquot of extract (2 ml) was centrifuged for 5 min at 14000 rpm. Total flavonoids content was measured with al aluminum chloride assay. One ml aliquot of plant extract was mixed with 4ml of distilled deionized (dd) water in a 15 ml falcon tube. To the tube 0.3 ml of 5% NaNO₂ was added, then after 5 min 0.3 ml of 10 % AlCl₃ was added. After one minute, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with dd water. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm with an
UV-VIS spectrophotometer. A standard solution of catechin was prepared in a concentration range of 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml (Appendix-1). The total flavonoid content of the dry plants were expressed as milligrams of catechin equivalents per 100 grams of dry mass (mg in CE/100 gm dw). All samples were analyzed in duplicate.

**Determination of Total Hydrolysable Tannin (gallotannins and elagitannins)**

**Gallic tannin by Rhodanine Assay:** Gallo tannin was determined as per the method described by Inoue and Hagerman (1988).

**Reagents:** rhodanine (2-thio-4-ketothiazolidine) reagent was prepared 0.667% rhodanine by weight in methanol, gallic acid stock solution contained 0.1 mg/ml gallic acid in 0.2 N H$_2$SO$_4$.

**Extraction, hydrolysis and determination of gallotannin:** fresh leaves were ground in liquid nitrogen and lyophilized. One hundred mg of lyophilized plant leaves was extracted with 1 ml of 70% (v/v) acetone/water in a sonicater at 4°C. The extract was vacuum filtered through a coarse sintered glass filter into an ampule. The filter was washed with 5 ml of 2 N H$_2$SO$_4$ which was added into the sample and remaining solid was discarded. The samples were then frozen in dry ice –isopropanol. The ampules were vacuum sealed and the samples were heated in a block at 100°C for 26 hours. Once hydrolysis was completed the ampules were opened and all samples were transferred to graduated tubes. The samples were diluted to 50 ml with distilled water. One ml of the diluted sample was taken in a graduated tube and 1.5 ml of rhodanine reagent was added. After 5 min, 1 ml 0.5N aqueous KOH solutions was added. After 2.5 minutes the mixture
was diluted to 25 ml with distilled water. After 10 min the absorbance was read at 520
nm. A standard curve of gallic acid was made in a concentration range of 0.02, 0.04,
0.06, 0.08 and 0.1 mg/ml in 0.2 N H₂SO₄ (Appendix-1).

*Determining of Ellagitannin (hydrolysable tannin) (Pyridine assay)*: We
followed the method of Wilson and Hagerman (1990) in this assay.

**Reagents**: 2N H₂SO₄, Wash solution (acetone: water: conc HCl- 70: 30: 1- v/v/v), 1 %
NaNO₂ in water, stock solution of ellagic acid 0.5 mg/ml, pyridine, water bath (30° C),
glass cuvette, glass fiber filters.

**Sample preparation**: In a constricted Pyrex test tube, 50 mg of dry sample and 5 ml of 2N
H₂SO₄ were added. Tubes were vacuum sealed and then samples were placed at 100°C
heating block for hydrolysis for 10 hour. After hydrolysis the samples were cooled.
Tubes were opened and chilled in an ice bath. The samples were vacuum filtered and the
insoluble materials were washed several times with ice cold wash solvent. As ellagic acid
is insoluble in ice cold solvent, all other pigments were washed away. All the insoluble
material with the filter membrane was carefully placed in a test tube and 10 ml pyridine
was added. The mixture was vortexed to dissolve ellagic acid. The solution was filtered
with a glass filter and the insoluble material was removed. Into the pyridine solution 0.1
ml conc. HCl was added and the mixture vortexed. It was placed in a 30° C water bath for
5 min, then 0.1 ml NaNO₂ was added and vortexed. Absorbance was taken at 538 nm at
time 0 min. Samples were again put back in the water bath. After exactly 36 min
absorbance was again read. The difference between absorbance is a function of ellagic
acid concentration. Timing is critical in this assay as the color continues to change during
the reaction.
**Determination of soluble and insoluble condensed tannin:** Acid butanol method by Makkar et al (1999) was followed with some modification.

**Solvent and reagent preparation:** 1% acidic methanol (10 ml HCl in 990 ml of MeOH to make 1 L of acidified methanol), 0.2%(w/v) DMACA (p-dimethyleaminocinnamaldehyde) (200mg DMACA was dissolved in 100ml 3M HCl/50% methanol, v/v). To make 3M HCl, 25 ml conc. HCl was mixed with 75 ml of H2O. Acid butanol (Butanol: HCl) v/v: 95/5). Iron Reagent: 2% Ferric ammonium sulfate in 2N HCl (16.6 ml conc. HCl was brought up to 100 ml in distilled water) 0.5 gm FeNH4(SO4)2 x 12 H2O in 25 ml of 2N HCl (stored in a dark bottle).

Procyanidin B2 solution (standard): A stock solution was prepared by dissolving 1 mg of procyanidin with 1.25 ml of 80% methanol and stored in a screw capped bottle sealed with parafilm, in the refrigerator.

**Sample preparation:** Frozen plant materials (10 gram) were ground and extracted with acidified methanol. Extractions were shaken every five min for 30 min. After 30 min the materials were centrifuged for 20 min at 7500 rpm. After each centrifugation supernatant was filtered through a Whatman No. 1 filter paper with suction filtration. The pellet was extracted three more times as above. After the materials have been fully extracted, the supernatants were combined and brought to 50 ml with additional acidified methanol. This extract was used for soluble proanthocyanidin assay. The pellet was dried under nitrogen gas and then lyophilized. The freeze dried pellet was used for the condensed tannin assay.
Soluble Proanthocyanidin Assay: Fifty µl of the supernatant was mixed with 4.95 ml of DMACA. Samples, standard and the blank were read after 10 min at 630 nm in the spectrophotometer.

Insoluble condensed Tannins: the freeze dried pellets from above procedure were crushed in a mortar and pestle and then defatted by extracting with 10 ml of hexane three times (30 min each). Each time the sample was centrifuged for 20 min at 7500 rpm and the supernatant was discarded. The pellet was dried under nitrogen to remove hexane, ground with mortar and pestle and sieved through 60 micron mesh. In a 50 ml glass screw top tube a 100 mg sample and 30 ml acid butanol reagent were added. To this solution 0.5 ml of iron reagent was added and vortexed. The cap was then replaced tightly and the tubes were then placed in the boiling water bath. Samples were boiled for an hour and then cooled to 20° C. All of the deeply colored solution was pipetted into a 100 ml flask, being careful to avoid any insoluble material. The procedure of extraction was repeated two more times for each plant sample. The total solution was collected and made up to 100 ml. Absorbance was read at 550 nm. Standard curve of procyanidin B2 was prepared by taking different concentration of 2.5, 5, 10, 20 and 40µg/ml (Appendix-1).

Super critical CO₂ Extraction and GC-MS Analysis of R. aromatica Essential Oil Analysis

Plant materials were air dried and ground in a Wiley mill and sieved through 0.5 mm diameter particle size sieve. For super critical CO₂ extraction, 5gm of plant materials was packed into the extraction vessels of volume size 20 ml between two layers of glass
beads and filter paper of appropriate size was placed on the top of glass beads. The extraction vessel was mounted on the top of the extraction instrument with CO₂ inlet attached to the bottom and CO₂ outlet to the top (Fig 3.3). The temperature was set up to 37°C and pressure for CO₂ was 2000-10,000 PSI. The extraction type was both static and dynamic; in static type CO₂ was allowed to flow through the inlet into sample by keeping the outlet valve closed for 15 min, then the extracted plant materials were collected through the outlet for 15 min by continuous flow of CO₂, which is called dynamic extraction. After extraction the compounds present in the essential oil were determined using gas chromatography coupled with mass spectrometer (GC-MS).

**Instrument Details:** we used 7890A GC system and 5976C VL MSD with triple axis detector, DURAWAXFSOT (30m x 0.25 mm, film thickness 0.25 µm) column type with flow rate 1.2 ml/min. GC methods for oil separation were as followed; Injection temperature 250°C, injection volume 0.1 ml, oven temperature 50 °C on hold for 10 min and increased at 4 °C/min to 220 °C for 10 min and then increased at 1 °C/min to 240 °C. For Mass Spectrometer a 5975C VL MSD with triple axis detector system with ionization voltage 70 eV was used. NIST library was used to identify the probable oil spectra. The percentages of the compounds were obtained by calculating the percent area of the chromatograms.

**RESULTS**

Both *R aromatica* and *R. glabra* showed antimicrobial activity against Enterotoxigenic *E. coli*. The MIC value of *R. aromatica* and *R. glabra* were found to be
25 mg/ml and 12.5 respectively (Fig-3.1). The result of AB cytotoxicity test against IPECJ2 cells showed more than 50% inhibition in growth of cells at about 1.25 mg/ml of plant extract concentration (Fig- 3.2). The total polyphenol content of *R. aromatica* was less than *R. glabra* but the quantity of flavonoids was two times more compared to *R. glabra*, gallotannin was also significantly higher in *R.aromatica* (Table-3.1). The quantities of ellagitannin, soluble and insoluble condensed tannin were very small compared to the other three compounds (Table-3.1).

In *R. aromatica*, more than 50% of total polyphenols were in flavonoids and gallotannin. However, in *R. glabra* about 20% of total phenolics represented flavonoids and gallotannin. It appeared that *R.glabra* contained more simple phenolic compounds like gallic acid and dihydroxy benzoic acid etc. From this result it could be hypothesized that the antimicrobial properties of *R aromatica* are due in part to the presence of flavonoids, gallotannin and some contribution of condensed tannin. In the case of *R. glabra* the most biologically active compounds appear to be the simple phenolics.

A list of the compounds present in the essential oil of *R. aromatic* is provided in Table- 3.2 and the chromatograms shown in Fig- 3.4. The essential oil was extracted from the whole plant parts of *Rhus aromatica* by supercritical CO₂ extraction and a total of 11 compounds accounting for 99.99% of the total oil composition were identified by a combination of GC-FID and GC-MS analysis. The major compounds identified were Limonene (43.25%), β-Cubenene (27.91%), Caryophyllene (11.01%).
We selected *R. aromatica* for evaluation of essential oil as it contains many aromatic compounds which have not been previously evaluated. This class of compound has been shown to have antibacterial, anthelmintic and anticancer activity in our study.

**DISCUSSION**

This study provides the first report of antimicrobial activity of *R. glabra* and *R. aromatica* against enterotoxigenic *E. coli*. Additionally we provide detailed phytochemical analyses of both these plants that have not been reported before. We have quantified polyphenols, flavonoids and tannins of both *R. aromatica* and *R. glabra* and we also identified the compounds of the essential oils in *R. aromatica*.

Previous studies have reported the antibacterial and antiviral activity of *R. aromatica* (Min et al., 2008, Reichling et al., 2009). The 30% ethanolic extract of *R. aromatica* containing gallic acid and tannin showed marked antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Chakraborty and Brantner, 2000). Methanolic extracts of *R. glabra* exhibited maximum inhibition capacity out of 100 tested plants (McCutcheon et al., 1992). The active constituents of *R. glabra* against the bacteria were shown to involve methyl gallate, 4-methoxy 3-5 dihydroxy benzoic acid and gallic acid (Saxena et al., 1994). As ETEC is resistant to commonly used antibiotics, both *R. aromatica* and *R. glabra* have the potential to treat ETEC related diarrhea.

An in vitro cytotoxicity study was conducted to understand the level of toxicity of *R. aromatica* and *R. glabra* extract; both plant extracts showed some extent of toxicity towards IPEC-J2 cells lines. Several studies have reported the toxic effect of antibiotics
when tested in vitro, still we use those antibiotics to treat various infectious diseases. Tetracycline, a widely used antimicrobial drug, showed cytotoxic effects in cultured human blood lymphocytes in vitro (Çelik and Eke, 2011). In another study, four antibiotics benzylpenicillin, cefuroxime, dicloxacillin and erythromycin showed cytotoxicity to three different cell lines in vitro (Lanbeck and Paulsen, 1995). However to explain the toxic effect for the use on these plant extracts, it is important to conduct in vivo studies. The condition of an animal body system is different than of cells grown in the culture plates. A short term (9 day) study of R. aromatica did not show any toxicity when fed to sheep (Acharya et al., 2012). Therefore our result indicated that R. glabra and R. aromatica extracts could be potential feed additives to reduce diarrhea in post weaned and neonatal pigs.

Plant polyphenols are a major group of phytochemicals having various functional properties in supporting human and animal health. Many vegetables and fruits in our daily diet contain polyphenols (Ignat et al., 2011). There are several plants traditionally used as medicines that have been found to have phenolic compounds as their major constituents. Five commonly used mint (Lamiaceae) plant methanol extracts were shown to have a correlation between the amount of phenolic compounds and antimicrobial activity. HPLC analysis for phenolics showed that carvacrol, apigenin, rosmarinic acid, rutin, catechin, caffeic acid are the major compounds present in those plants (Askun et al., 2009). Many medicinal herbs and spices with high phenolics show antimicrobial activity, it was observed that antimicrobial activity and phenolic content of extracts against each bacterium studied showed highly positive relationship (R² = 0.73-0.93) (Shan et al., 2007). Aegle marmelos a traditional medicinal plant widely used to treat
variety of diseases has a high phenolic content (Gheisari and Zolghadri, 2011). In a study, Thai vegetables were evaluated for their antioxidant and antimicrobial activity and total phenolics content. It was observed that four vegetables having high phenolic content were more effective against *Staphylococcus aureus*, *Salmonella typhumurium* and *E. coli* (Daduang et al., 2011). A mixture of black tea and green tea polyphenols; epigallocatechin, epigallocatechin-3-O-gallate, castalgin and prodelphinidin with 3,4,5 trihydroxy phenyl group show low MIC values against food borne pathogenic bacteria (Taguri et al., 2004).

Flavonoids, abundant in apples, reduce significantly *E. coli* O157 biofilm formation without affecting the commensal *E. coli* K-12 biofilm (Lee et al., 2011). Flavon-3-ols and their polymeric condensation products are the most common dietary flavonoids and are regarded as the functional ingredient of fruits, vegetables, grains, processed foods and herbal medicines. Flavon-3-ols are well known for their antioxidant, antimicrobial, anticanccer, antiviral and cardio protective agent (Aron and Kennedy, 2008).

Proanthocyanidin or condensed tannin is another group of phenolic compounds that is regarded as the active factor of several medicinal plants (De Bruyne et al., 1999). Proanthocyanidin extracted from *Pelargonium sedoides* was found to have antiadhesive activity against *Helicobacter pylori* in a dose dependent manner (Wittschier et al., 2007). Hydrolysable tannin extracted from *Rhizophora epiculata* barks showed significant antibacterial and anti-yeast activity against 23 bacterial strains and four yeasts respectively by altering the membrane permeability (Lim et al., 2006). *Rhus aromatica* contains condensed tannin and hydrolysable tannin and it showed moderate growth
inhibition of three mastitis pathogens a dose dependent manner (Min et al., 2008). From the above report it was evaluated that tannins obtained from different sources possessed different antimicrobial activity depending on their chemical composition. It does not depend on the concentrations of specific tannin. In our sample, *R. aromatica* contained 2.5 mg of condensed and 112.5 mg of hydrolysable tannin in 100 g plant material, and showed significant activity.

*R. glabra* phytochemical analyses indicated the presence of predominantly simple phenolic compounds in comparison to flavonoids and tannins. Saxena et al. (1994) reported the presence of three phenolic acid compounds in *R. glabra* having antimicrobial activity; 3, 4, 5- trihydroxy benzoic acid, 4- methoxy-3,5-dihydroxybenzoic acid, and gallic acid. This supports our result that simple phenolic compounds are the major constituents for antimicrobial activity of *R. glabra*.

*R. glabra* has also been shown to contain significant amounts of oils; 12% of the oil was sterols, 11% other free alcohols, 19% free acids, 13% triglycerides, 39% non-glyceride esters and 9% hydrocarbons (Buchanan and Otey, 1979). These may also play a role in the antibiotic activity of the extracts.

Table: 3.2 showed a list of 11 compounds present in the essential oils of *Rhus aromatica*. Limonene, β-Cubenene and Caryophyllene are the three major compounds according to the percentage of area in the chromatogram (Fig-3.4). Limonene (1-methyl-4-(1-methylene) cyclohexane) is a naturally occurring simple monocyclic monoterpenel, a major constituent of citrus fruit essential oils and also contributes to many other plant essential oils. Limonene is considered as an ecofriendly and low toxic
solvent and is listed in the code of Federal Regulation as generally recognized as safe (GRAS) for flavoring agent in food items and various cleaning applications (Sun, 2007). It is also used as a natural alternative medicine to treat various health problems; gall stone dissolution (Igimi et al., 1976), occasional heart burn and gastro esophageal reflux disorder (Wilkins, 2002), and to act as an anticancer agent (Giri et al., 1999, Crowell et al., 1992). It also shows antimicrobial, antifungal, antiviral, antiparasitic, antioxidant and anti-inflammatory properties (Paduch et al., 2007, Hamdan et al., 2010). The antimicrobial properties of essential oils are due to their ability to penetrate into the phospholipid bilayer membrane and their ability to interfere with the enzymatic reaction for energy uptake (Kalemba and Kunicka, 2003). Our finding of 43.25% limonene in R. aromatica may make it an ideal source of limonene for commercial uses.

The second major compound found in the essential oil of R. aromatica was β-Cubebene, a sesquiterpene. It is a constituent of essential oils in many plants that demonstrate antimicrobial properties (Porter and Wilkins, 1999, Solis et al., 2004). The volatile oil of Satureja cuneifolia characterized as containing cubebene as an important compound, showed broad spectrum antimicrobial activity against multidrug resistance pathogens (Skocibusic and Bezic, 2004). Essential oils from flowers, leaves and stems of Helichrysum armenium contained limonene, cubebene and caryophyllene as major components and showed antibacterial and antifungal activities (Oji and Shafaghat, 2012).

Caryophyllene, a natural bicyclic sesquiterpene is a constituent of various essential oils and found largely in many different spices and food plants such as Oregano, Cinnamon and black pepper (Orav et al., 2004, Jayaprakasha et al., 2003, Mockute et al.,
It is the third major compound of *R. aromatica* essential oil. Caryophyllene from *Alpinia purpurata* showed substantial antimicrobial activity against gram positive and gram negative bacteria (Santos et al., 2012). It has been also reported to have antioxidant (Jayaprakasha et al., 2003), anti-inflammatory (Gertsch et al., 2008) and antifungal (Yang et al., 1999) activities.

**CONCLUSION**

Our study reported that *R. aromatica* and *R. glabra* have potential antimicrobial activity against ETEC, though they showed some toxic effect to porcine intestinal epithelial cells *in vitro*. Further *in vivo* study is necessary to use these extracts as feed additives for pigs to control ETEC related diarrhea. The phytochemical analysis of these plant extracts showed that the antimicrobial activity of *R. glabra* may be due to the presence of simple phenolic compounds, whereas that of *R. aromatica* may be due to presence of flavonoids and condensed tannin and essential oils. The volatile oil analysis of *R. aromatica* revealed the presence of three major compounds; limonene, caryophyllene and β-cubebene. Previous reports and our study support that all three major compounds possess antibacterial, anthelmintic, anticancer activities. However, *in vivo* study and clinical trial would be required to justify the pharmaceutical use of *R. aromatica*. It could be suggested that *R. aromatica* is a good source of limonene for industrial use.
Table 3.1 Phytochemical Analysis of *R. aromatica* and *R. glabra*

<table>
<thead>
<tr>
<th>Plants Name</th>
<th>Total Polyphenols mg/100gm PM</th>
<th>Flavonoids mg/100gm</th>
<th>Hydrolysable Tannin mg/100gm</th>
<th>Condensed Tannin mg/100gm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhus aromatica</em></td>
<td>1484.11 ± 13.55</td>
<td>868 ± 9.93</td>
<td>112.31 ± 5.05</td>
<td>0.322</td>
</tr>
<tr>
<td><em>R. glabra</em></td>
<td>2072.91 ± 34.68</td>
<td>409.3 ± 5.33</td>
<td>26.28 ± 0.88</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.2 List of essential oils (GC-MS)

**Rhus aromatica** Essential Oil

<table>
<thead>
<tr>
<th>SN</th>
<th>RT (min)</th>
<th>% Area</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.343</td>
<td>43.25</td>
<td>D-Limonene</td>
</tr>
<tr>
<td>2</td>
<td>19.772</td>
<td>1.85</td>
<td>Nonynoic acid</td>
</tr>
<tr>
<td>3</td>
<td>22.82</td>
<td>2.3</td>
<td>Pyridine</td>
</tr>
<tr>
<td>4</td>
<td>24.384</td>
<td>11.01</td>
<td>Caryophyllene/ aromadendrene</td>
</tr>
<tr>
<td>5</td>
<td>26.415</td>
<td>27.91</td>
<td>β-Cubebene</td>
</tr>
<tr>
<td>6</td>
<td>27.811</td>
<td>1.94</td>
<td>Benzenepropan / Brocresine</td>
</tr>
<tr>
<td>7</td>
<td>36.612</td>
<td>2.42</td>
<td>Bicyclo [3.1.0] hexan-3-one</td>
</tr>
<tr>
<td>8</td>
<td>39.484</td>
<td>3.45</td>
<td>Benzofuran-2-one</td>
</tr>
<tr>
<td>9</td>
<td>49.921</td>
<td>1.79</td>
<td>3-Methoxyamphitamine</td>
</tr>
<tr>
<td>10</td>
<td>61.943</td>
<td>1.39</td>
<td>Cyclopropene</td>
</tr>
<tr>
<td>11</td>
<td>62.006</td>
<td>2.68</td>
<td>Octahydro-1,3-Cycloheptadiene</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>99.99</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 *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 Y-axis is the CFU/mL.

RA- *Rhus aromatica*, RG- *Rhus glabra*, NC- Negative Control
Figure 3.2: Inhibition of IPEC-J2 cell growth at different concentrations of plant extract, incubated at 37°C, 5% CO₂ for 24 hours. The X-axis is the plant extract concentration in mg/mL and the Y-axis is the % inhibition of IPEC-J2 cells.

RA - *Rhus aromatic*, RG - *Rhus glabra*, NC - Negative Control
Figure 3. 3 Supercritical fluid extractor (SFE-CO₂) instrument.
Figure 3.4: GC-MS Chromatogram of *Rhus aromatica* extract
Figure 3.5: Molecular structure of Limonene, Caryophyllene and Cubebene
REFERENCES


CHAPTER-4

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 being sanguinarine. American Indians used this rhizome in treatment of rheumatism, asthma, bronchitis, lung ailments, fever and warts. It has been studied extensively for its antibacterial, antiproliferative, anti-inflammatory, immune-modulatory activities. Lethal diarrhea in neonatal and post weaned pig, due to enterotoxigenic E. 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 I) to determine the minimum inhibitory concentration (MIC) of methanol extract of S. canadensis against ETEC II) to evaluate the cytotoxicity activity against porcine intestinal epithelial cell lines and III) to quantify the amount of sanguinarine in the rhizome of S. canadensis
native to South Dakota. The MIC value of *S. canadensis* was measured using a bacterial enumeration assay and the Alamar Blue cytotoxicity assay was followed to evaluate the cytotoxicity against IPEC-J2 cell lines. Sanguinarine was quantified with high performance liquid chromatography. 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 0.0312 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 sanguinarine was 543 mg in 100 gm of fresh plant material. The percentage of sanguinarine in fresh plant material was 0.54% and in total alkaloid is 43%. The result of our study explained that the alkaloid content of *S. canadensis* depends on both genetic and environmental factors.

**Key Words:** antibacterial activity, Enterotoxigenic E. coli, cytotoxicity, alkaloids, sanguinarine
INTRODUCTION

In the screening process of native plants (Chapter-1), Sanguinarine canadensis showed significant antimicrobial activity against enterotoxigenic E. coli (ETEC). Therefore, we chose this plant to determine its minimum inhibitory concentration against ETEC, cytotoxicity activity against porcine epithelia cells and phytochemical.

Sanguinaria canadensis, of the Papaveraceae family, is a spring blooming perennial herb producing a red sap or latex in the rhizome. Common names are bloodroot, red pucoon, Indian paint, tetterwort, Pauson and snakebite (Pengelly and Bennett, 2011). Bloodroot is indigenous to North America and it is mainly wild harvested in eastern part of the USA (van Wyk and Wink, 2004). 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 as love potion (Foster and Duke, 2000). Sanders (2002) discussed, the use of bloodroot for treating ulcers, ring worm and other skin afflictions. The red sap contains alkaloids that make this plant valuable. It 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 (van Wyk and Wink, 2004). Other alkaloids like chelerythrine, berberine and protopine have been also shown to have anticancer and antimicrobial activities (Malikova et al., 2006, Mahady et al., 2003). However, sanguinarine is thought to be the most bioactive of the alkaloids (Senchina et al., 2009). S. canadensis rhizomes are used by herbal practitioner for its reputed antimicrobial properties. Sanguinarine has
demonstrated antimicrobial (Mahady et al., 2003, Beuria et al., 2005), anti-inflammatory (Dvorak et al., 2006, Chaturvedi et al., 1997), anti-platelet (inhibit platelet aggregation) (Jeng et al., 2007), anti-inflammatory (Dvorak et al., 2006, Chaturvedi et al., 1997), and anti-angiogenic (Basini et al., 2007) 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 (Vieira et al., 2008) and pigs (Blank et al., 2010).

*S. candensis* has been exploited commercially for more than two decades, for its antiplaque properties in oral hygiene (Dzink and Socransky, 1985) and as non-antibiotic feed supplements to promote weight gain in livestock especially in Europe (Persons and Davis, 2005).

**OBJECTIVES**

The objectives of our study were to a) determine the MIC value of *S. Canadensis* against ETEC b) evaluate the cytotoxicity against porcine intestinal epithelia cell lines and c) quantify total alkaloid and the percentage of sanguinarine in *S. canadensis*.

**MATERIALS AND METHODS**

*Sample collection*

*Sanguinarine canadensis* samples were collected from the Sica Hollow State park of South Dakota in October 2010.

*Extraction of plant materials for bacterial enumeration and cytotoxicity assays*

Fresh plant samples were homogenized in a blender in 100% methanol and methanol plant extracts were mixed continuously on an orbital shaker for 24-72 hours, at
150 RPM. After complete extraction, the methanol extract was filtered using Whatman #1 filter paper. Methanol was then removed from the sample using rotary evaporation under vacuum and the remainder extracts were frozen and lyophilized to dryness. The dried extracts were then stored at -20°C for further use.

**Bacterial strains and growth condition**

*Escherichia coli* O157 K88 LT STB were obtained from the South Dakota Veterinary Diagnostic Laboratory, at SDSU. The condition of the ETEC strain was maintained as described in Chapter-1.

**Bacterial Enumeration assay for Minimum Inhibitory Concentration**

The MIC value of *S. canadensis* was determined by bacterial enumeration assay as per the protocol described in chapter – 2.

**Cytotoxicity test**

The cytotoxic effect of *S. canadensis* on porcine intestinal epithelial cell lines was evaluated using Alamar Blue cytotoxicity assay as per the protocol given in chapter – 2.

**Determination of Total Alkaloid Contents**

We followed a simple and rapid spectrophotometric method for the determination of total alkaloids (Shamsa et al., 2008). This method is based on the reaction of alkaloids with bromocresol green (BCG) which produces yellow colored products.

Bromocresol green (1X 10⁻⁴) was prepared by heating a mixture of 69.8 mg BCG, 3 ml 2N NaOH and 5 ml of distilled water. Once BCG was completely dissolved the solution
was diluted to 1000 ml with distilled water. Phosphate buffer solution of pH 4.7 was prepared by adjusting the pH of 2 M sodium phosphate (71.6 gm Na$_2$HPO$_4$ in 1 L of dd water) with 0.2 M citric acid (42.02 g of citric acid in 1 L dd water). Atropine standard solution was prepared by dissolving 1 mg of atropine in 10 ml of dd water.

Aliquots of atropine standard solution of different concentrations (0.04, 0.06, 0.08, 0.1 and 0.12 mg/ml) were transferred to separate 15 ml falcon tubes. To each tube 5 ml of phosphate buffer (pH 4.7) and 5 ml of BCG solution was added. The mixture was vortexed with chloroform, 1 ml of chloroform was added and vortexted then chloroform was removed by using a 1ml pipette and the process was repeated for four times. All chloroform was collected in a 15 ml tube and diluted to 10 ml with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against a blank prepared as above but without atropine (Appendix-2).

For alkaloid determinations, 50 µg plant extract was dissolved with 1 ml 2N HCl, then it was filtered and transferred into a 15 ml tube. The solution was washed 3 times with chloroform; the pH of the solution was then adjusted to neutral with 0.1 N NaOH. Absorbance of samples was taken after treating them in the same procedure as the standard.

**Isolation and Quantification of Sanguinarine Alkaloid**

A method by Suchomelova et al. (2007) was followed with slight modifications.

**Reagents and Chemicals:** Phosphoric acid, heptanesulfonic acid, triethyleamine and acetonitrile of HPLC grade were obtained from Fisher scientific (USA). Standard for
sanguinarine in the form of sanguinarine chloride (10 mg) was purchased from Fisher Scientific.

**Instrumentation:** 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 280nm and reference wavelength was 360nm. An Eclipse XDB, 5 µm, 4.6 mm X 150 mm C18 column was used.

**HPLC analysis:** five concentrations 0.01, 0.02, 0.03, 0.04 and 0.05 mg /ml were prepared by dissolving sanguinarine chloride (Fisher Scientific, USA ) in phosphate buffer (pH 2.5).Mobile phase was prepared from the stock solution containing heptanesulfonic acid (0.01 M) and triethyleamine (0.1 M) in redistilled water, pH was adjusted to 2.5 with phosphoric acid($\text{H}_3\text{PO}_4$). A-solvent contained 25% acetonitrile and B-solvent 60% acetonitrile (v/v). **Elution profile:** 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. 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 elution.

Ten grams of the *Sanguinaria canadensis* root was ground with 200 ml of 1% acidic methanol and extracted five times in seven days. The extract was partially evaporated by rotary evaporator under vacuum and then lyophilized to complete dryness. One mg of dried plant extract was dissolved in one ml of buffer solution and used for HPLC analysis. 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 (Appendix-2). All samples were run in triplicates. The statistical parameters were analyses by linear regression method. The HPLC results were used to identify and quantify sangunarine alkaloid in *Sangunaria canadensis* of South Dakota, Sica Hollow state park.

**RESULTS**

From the bacterial enumeration assay (Fig-4.1) the minimum inhibitory concentration of *S. canadensis* was found to be 6.25 mg/mL. Alamar blue cytotoxicity assay result showed that the extract of concentration of *S. canadensis* at 31.25 µg/ml or above had 100 % inhibitory effect on the IPEC-J2 cell lines, whereas at concentration 15.625µg/mL showed 50% inhibition of cell growth (Fig- 4.2).

From the phytochemical analysis of *S. Canadensis*, typically alkaloids, we found the amount of total alkaloid is 1.24% of the fresh weight that was 1247mg of total alkaloid in 100 gm of fresh plant materials (Table -4.1). The amount of sanguinarine in 100 gm fresh plant material was 543mg which is 0.54% of the total plant material and 43% of the total alkaloids (Table-4.2).

**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
(Schmeller et al., 1997)

A number of alkaloids have been isolated from S. canadensis including sanguinarine, chelerythrine, protopine, oxysanguinarine, alpha-allocryptopine, beta-allocryptopine, chelirubin, chelitutine sanguirubine and sanguilutine (Tin-Wa et al., 1970). It has been reported that sanguinarine has antibacterial activity against a range of gram positive and gram negative bacteria predominantly found in mouth (Godowski, 1989). Sanguinarine is effective in prevention of plaque formation and gingivitis by inhibiting bacterial adherence and is safe to use in toothpaste and mouth rinse (Godowski, 1989, Dzink and Socransky, 1985, Harper et al., 1990, Kopczyk et al., 1991).

Sanguinarine has been shown to strongly induced filamentation in gram positive and gram negative bacteria and prevent cell division by inhibiting cytokinesis (Beuria et al., 2005). A recent study demonstrated that MRSA (multidrug resistant Staphylococcus aureus) strain treated with sanguinarine lysed the cell and altered morphology (Obiang-Obounou et al., 2011). Sanguinarine and some structurally related alkaloids like chelerythrine, chelidonine, barberine inhibit extracellular lipase activity of Candida rogusa (Grippa et al., 1999). Methanol extracts of the rhizomes of S. canadensis has been shown to inhibit the growth of Heliobacter pylori in vitro (Mahady et al., 2003).

The above 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 31.25 µg/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 5mg/kg animal body weight is safe (Kosina et al., 2004). In another report, 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 (Becci et al., 1987). It is also reported that *S. canadensis* can be used as a feed additive in place of synthetic antibiotics for livestock (Clark, 2002). Clinical safety report showed that sanguinarine was not absorbed orally, did not induce mucosal irritation or sensitization in repeated doses and did not induce mutagenicity *in vivo* and *in vitro* (Godowski, 1989).

The major alkaloid sanguinarine was quantified by HPLC. Sanguinarine [13-methyl (1, 3) benzodioxolo (5, 6-c)-1, 3-dioxolo (4, 5-i) phenanthridinium] (Fig-5) 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 heliobacter pylori strain.
with an MIC range of 6.25 µg/ml- 50 µg/ml (Mahady et al., 2003). Godowski (1989) reported, in vitro study of 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. In one study (Godowski, 1989) the percentage distribution of benzophenethridine alkaloid in wild type S. canadensis was; Sanguinarine = 50%, Chelerythrine = 25%, Sanguilutine = 10%, Cheliluitine = 7%, chelirubine = 6%, Sanguirubine = 2%. The percentage yield of S. canadensis varies with the region of growth and time of harvest, as Newton et al (2002) reported that the % yield of S. canadensis was 27%, however the % yield (Methanol crude extract) was 11.5% of S. canadensis from SD (our sample). We quantified sanguinarine in S. canadensis root (collected in October, 2010, Sica Hollow State Park, SD). Previous researchers have suggested that bioactive compounds such as alkaloids are at their highest concentration in mid spring (Graf et al., 2007).

The amount of sanguinarine in 100 gm of fresh rhizome of S. canadensis contained 543.3 mg of sanguinarine which is about 43% of the total alkaloids. It is consisted about 0.54 % of the fresh weight. This result indicated that the variation in the percentage of sanguinarine may be due to the different region of growth, different cultivar 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 (1997) 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 (Salmore and Hunter, 2001). Graf et al (2007) reported in their study, in wild plants the alkaloids yield was higher compare to cultivated varieties and month to month 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 (Graf et al., 2007).

**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.
TABLES

Table 4.1 Total alkaloids in *S. canadensis*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Total Alkaloids mg/100gm fresh plant materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sanguinaria canadensis</em></td>
<td>1247±22.04</td>
</tr>
</tbody>
</table>

Table 4.2 Quantification of sanguinarine in *S. canadensis*

<table>
<thead>
<tr>
<th>Peak area of Sanguinarine in <em>S. canadensis</em></th>
<th>Concentration of Sanguinarine mg/100 gm of plant material</th>
<th>% distribution of sanguinarine</th>
</tr>
</thead>
<tbody>
<tr>
<td>730.7 ± 0.3</td>
<td>543.046</td>
<td>43 %</td>
</tr>
</tbody>
</table>
Figure 4.1 *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 4. 2 Inhibition of IPEC-J2 cell growth at different concentrations of *S. canadensis* extract, incubated at 37°C, 5% CO₂ 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).
Figure 4. 3 HPLC chromatograms of *S. canadensis* extract
Figure 4. 4 HPLC chromatogram for sanguinarine chloride standard
Figure 4.5 Molecular structure of Sanguinarine

Molecular structure of Sanguinarine
13-Methyl-[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-]phenanthridinium
en.wikipedia.org/wiki/File:Sanguinarine_structure.png
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CONCLUSION

In our research, we first screened 28 plants against nonpathogenic *E. coli* ATCC25922 and found that 5 plants; *Clematis ligusticifolia*, *Rhus aromatica*, *Monarda fistulosa*, *Centaurea stoebe* and *Onosmodium molle* were effective in inhibiting the growth (Appendix-3). However, these plants except *R. aromatica* did not show any inhibitory effect against enterotoxigenic *Escherichia coli* (ETEC). As our long term goal of this research project was to identify potential plant based medicine to control ETEC causing diarrhea in weaned pigs. We further screened 25 native plants of South Dakota for their antimicrobial properties and we identified two more plants *Rhus glabra* and *Sanguinaria canadensis* with potential antibiotic activity against ETEC.

All three plants, *R. aromatica*, *R. glabra* and *S. canadensis* were further investigated for their minimum concentration of extract to inhibit ETEC growth, cytotoxic effect against porcine intestinal epithelial cells and phytochemical composition. Our results indicated that the MIC values of extracts against ETEC were higher than concentrations of plant extracts needed to inhibit the porcine intestinal cell line. Further *in vivo* study is required to use these plant extract as an alternative to control ETEC related diarrhea.

Phytochemical analyses of *R. aromatica* and *R. glabra* showed the presence of phenolic compounds in high amounts. *R. aromatica* contains significant quantities of flavonoids and condensed tannins and volatile compounds, whereas *R. glabra* has higher amount of simple phenolics. It seemed from previous studies and our results that the antimicrobial properties of *R. aromatica* are due to the presence of flavonoids, condensed
tannins and volatile compounds. The GC-Ms analysis of *R. aromatica* essential oils showed that limonene, caryophyllene and cubebeene are the major compounds comprising 82% of the total oils. Out of these three, limonene was in the highest percentage (43%). Previous research reports widely support the antimicrobial activities of all three compounds. It could be assumed that the essential oils of *R. aromatica* also another reason for its antimicrobial activity. In *R. glabra*, the antimicrobial properties appeared to be due to simple phenolic compounds.

The total alkaloid content and % sanguinarine of *S. canadensis* was quantified. The result showed that, *S. canadensis* native to South Dakota has total alkaloids of 1.247%. The percent of sanguinarine in total alkaloid was 43. In our work, it showed significant inhibition of ETEC. There is evidence from *in vivo* studies that the use of *S. canadensis* in animals has not shown any toxic effect although it showed toxicity against porcine cell lines *in vitro*. As, sanguinarine has been reported to have antimicrobial, anticarcinogenic, anti-inflammatory activities and because sanguinarine is extensively used a feed additives in European countries, it may prove to be a useful tool to control diarrhea in pigs.
APPENDIX: 1

Figure 3.6 Standard (Catechin) for total phenols

\[ y = 0.0123x + 0.0174 \]
\[ R^2 = 0.9945 \]

Figure 3.7 Standard (Quercetin) for total flavonoids

\[ y = 0.0752x - 0.0919 \]
\[ R^2 = 0.9574 \]
Figure 3. 8 Standard (Catechin) for soluble condensed tannin

Figure 3. 9 Standard (Procyanidin B2) for insoluble condensed tannin
Figure 3. 10 Standard (Gallic acid) for gallotannin

Figure 3. 11 Standard (Ellagic acid) for ellagitannin
APPENDIX: 2

Figure 4. 6 Standard (Atropine) for Alkaloids

Figure 4. 7 Standard Sanguinarine
APPENDIX-3

IN VITRO ANTIMICROBIAL ACTIVITY OF NATIVE SOUTH DAKOTA PLANT EXTRACTS ON ESCHERICHIA COLI ATCC#25922

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Abstract

In vitro antimicrobial activity of 28 extracts of plants native to South Dakota were screened and evaluated against Escherichia coli. A disk diffusion assay was used for initial screening and antimicrobial activities of potential plant extracts were further assessed by a broth dilution method to determine their MIC values. Out of 28 plant extracts five plants; Clematis ligusticifolia, Monarda fistulosa, Rhus aromatica, Centaurea stoebe and Onosmodium molle showed significant antimicrobial activity. In the disk diffusion assay the percentage of inhibition for C. ligusticifolia was maximum. The minimum inhibitory concentration (MIC) for C. ligustifolia was 0.031gm /ml, for C. stoebe was 0.062gm /ml, O. molle and M. fistulosa it was 0.125gm /ml and for R. aromatica 0.25gm /ml. In vitro cytotoxicity tests indicated that C. ligustifolia and R. aromatica show some toxicity to animal cells (IPECJ2) whereas C. stoebe did not have impact on cell growth compared to controls. However O. molle and M. fistulosa increased the growth of IPECJ2 cell. Based on our findings it can be concluded that these five
plants have antimicrobial activity against *E. coli*. Further investigation to determine which of their secondary metabolites (the phytochemicals) are biologically active will be pursued.

Key word: antimicrobial activity, *Escherichia coli*, methanol extract, MIC, cytotoxicity

**Introduction:**
Around the middle of 20th century, major advances in antibacterial drug development and other means of infection control helped human beings triumph over many infectious diseases. Development of penicillin in the early 1940s changed the situation radically with respect to bacterial infection (Tenover, 2006). However the attainment of complete eradication of infectious diseases is almost impossible. Bacteria respond to antibiotics by developing various modes of resistance as fast as we use antibiotics. Antimicrobial resistance in bacteria is one of the biggest global concerns at present (Siddiqi et al., 2011). Multidrug resistant isolates in several infectious agents are a cause for serious concerns. *Escherichia coli* isolates from environmental, animal and human sources have been identified as resistance to antibiotics (Heike & Reinhard, 2005). This in part is the consequence of improper use of antibiotics as medicine. Pathogenic organisms have acquired some degree of resistance toward antimicrobials through various mechanisms (Wose Kinge et al., 2010). Three types of antibacterial resistance strategies have been suggested; a drug efflux pump has been observed in several bacteria which prevent accumulation of antibiotics. A second mechanism is deactivation or destruction of the antibiotics by hydrolytic enzymes in the periplasmic space and a third strategy for antibiotic resistance is appears to be reprogramming of the target macromolecules to reduce the affinity of antibiotics for their RNA (Walsh, 2000; Tenover, 2006; Alviano &
Alviano, 2009). Resistance has led to need for development of alternate sources of antibiotics to control \textit{E. coli} infections.

Ancient herbal traditions are the source of origin for numerous contemporary medicines (Al-Bakri & Afifi, 2007). For the past few decades researchers have been trying to investigate novel antibiotics from plant products. Medicinal plants used in folk medicine may still provide guidance for developing new antibiotics. Higher plants produce large numbers of secondary metabolites with different biological activities. Medicinal plants have been used for centuries to treat various diseases all over the world (Vaghasiya & Chanda, 2007). So far, only a small percent of prescribed plant species on the earth have been studied for their therapeutic value. Plant phytochemicals could provide an alternative to synthetic antibiotics, as extracts having different target sites than current antibiotics may be effective against drug resistant pathogens. The drug industry is exploring phytochemicals extensively to develop new antibiotics which can overcome resistant pathogens without any toxic effect to their host (Oskay & Sari, 2007). Plants contain a large number of secondary compounds like alkaloids, phenolic compounds and flavonoids having antimicrobial properties.

There are several reports presented on the antibacterial activity of organic and aqueous plant extracts. \textit{Casearia sylvestris} is a popular medicinal plant of South America and being used against several diseases; diarrhea, gastric ulcer, inflammation, herpes and it was found that the ethanol extracts from leaves of this plant have potential antimicrobial activity (da Silva et al., 2008). In a recent study methanol extracts of \textit{Globularia alypum} have shown significant effects on the growth of \textit{E. coli} (Bogdadi et al., 2007). \textit{Berberis Asiatica}, a plant that is traditionally used in Nepal and India to treat wounds, contains
alkaloids that show strong activity against gram negative bacteria (Bhandari et al., 2000). Antimicrobial activity of native plants of Jordan; Gundelia tournefortii L. and Pimientella anisum L. have shown enhanced activity against antibiotic resistant strains of E. coli whereas, Origanum syriacum L, Eruca sativa Mill have synergistic effect when used with the antibiotic clarithromycin (Darwish & Aburjai, 2010). Leaf extracts of three different species of Aloe; Aloe barbadensis, A. chabaudii and A. arborescens, used in folklore veterinary medicine in Zimbabwe were evaluated for their antimicrobial activity and the study showed that E. coli are sensitive to all three extracts (Mbanga et al., 2010). Dubey et al (2009) screened medicinal plants of India for their antimicrobial activity against E. coli and found four plants Terminalia catappa, Syzygium cumini, Eucalyptus hybrida and Holarrhena antidysenterica have significant antimicrobial activities.

Native Americans have used herbs medicinally for thousands of years as a part of their holistic approach to good health (Moerman, 1998). Anthony (2001) in his book has described a number of herbal remedies used by Native Americans to treat illness and heal injuries. Borchardt et al (2008) found seven plant species that showed some effect against E. coli from Minnesota and Wisconsin. In South Dakota there are many varieties of plants having been used as folk medicine, only few of them have been studied scientifically for their antimicrobial properties. Our main objective in this study was to identify South Dakota medicinal plants having significant antimicrobial activity that could be exploited as a source of antimicrobial agents. We also evaluated their potential for treating bacterial infection without significant toxicity to mammalian cells. In this article we have identified five medicinal plants having antimicrobial activity against E. coli.
Material and Methods:
Selection of plants: Plants were selected based on their traditional use by Native Americans (Moerman, 2009).

Sample collection: Plant specimens for our study were collected from the Northern Great Plain from June through August 2010. Plant species were collected from different locations and were weighed and stored at -80°C if not used immediately. Identification of the plants species was made based upon the USDA plant database nomenclature. Voucher specimens were prepared and stored in the herbarium.

Extract Preparation: Plant samples were homogenized in a blender with methanol (being a highly polar solvent capable of separating a wide range of secondary metabolites) in a ratio of 25gm fresh weight/ 250ml methanol. Plant materials were extracted with methanol for about 24 hours in dark. The plant samples were then vacuum filtered using VWR grade 415 qualitative filter paper. Methanol was removed from the sample using a rotary evaporator under decreased pressure. The residue was solubilized in 5 ml of 70% ethanol with concentration representing 5 g fresh plant weight per ml of ethanol in the final plant extract.

Bacterial strains and growth conditions: *Echerichia coli* ATCC # 25922 were obtained from the South Dakota Veterinary Diagnostic Laboratory, at SDSU. The assay medium for *E. coli* was Trypton Soy Broth (TSB) or Agar (TSB, TSA,Oxoid Ltd Hamsphire, UK). Bacterial cultures for antimicrobial testing were prepared by inoculating 25ml of TSB from fresh culture plates. Cultures were grown overnight in a shaker incubator at 200rpm and 37°C (Klancnik et al 2010). For antibacterial activity assay the CFU is
adjusted to $10^5$-$10^6$/ml using a spectrophotometer (absorbance 0.5 at 600nm). Stock culture were maintained at 70% glycerol and stored at -80°C.

**Antimicrobial testing methods:**

**Disk Diffusion Assay (DDA):**

The disk diffusion assay was performed following the protocols of National Committee for Clinical Laboratory Standards (NCCLS, 2003) a modified Kirby-Bauer disk diffusion method (Bauer et al., 1966). One hundred µl of bacterial culture was evenly spread on a Mueller Hinton agar medium in a petri dish. Sterile paper disks, 6mm diameter were impregnated with 20ul (5g fresh plant material/ml) of extract. The disks were allowed to completely air dry. Then these disks were placed on the inoculated agar plates. Each extract was screened with three replicates. One disk of gentamicin (10µg per disk) was placed on each plate as positive control and one disk of ethanol dried as above, as negative control. The inoculated petri dishes were incubated for 18-24 hours at 37°C. After incubation the diameter of the inhibition zones were measured in mm.

**Broth Microdilution method for Minimum Inhibitory Concentration (MIC)**

The MIC value of the plant extracts that were active against *E. coli* in the disk diffusion assay was then measured. For the MIC value the method (OrdoñEz et al., 2003; Sherlock Orla et al., 2010) were followed with some modifications.

Using 96- well plate (Corning, NY), columns 1 and 2 were filled with 100 µl sterile water and column 3 with 100 µl of 70% ethanol. In the remaining wells, the first row was filled with 90 µl of sterile water. The remaining wells were filled with 50 µl of sterile water. Then, 10 µl of each extract dissolved in 70% ethanol was mixed with the 90 µl of water
in the first row. To obtain a twofold serial dilution of each extract ranging from 3.9 to 500 mg/ml of plant extract in fresh weight, 50 µl of the first row were aspirated with a micropipette and mixed with the second row. This process was repeated till the last row with remaining 50 µl was discarded resulting in all the wells containing a total of 50 µl of water and extract.

Then, 50 µl of TSB with bacteria (10^6 CFU/ml) were added to all the wells except those in the first column, which served as blanks. Fifty µl of TSB without bacteria was added to column one. Column 2 was negative control and column 3 was positive control. Finally the 96 well plate was incubated at 37°C for 18 hours. The bacterial growth was observed by taking absorbance at 600 nm. All assays were performed in triplicate, with absorbance of extract only and blanks subtracted to adjust for background absorbance.

The MIC value was termed as the minimum concentration of plant extract that inhibited growth of *E. coli* to a level < 0.05 at 600nm. Growth at this level cannot be observed through microscope (OrdoñEz et al., 2003).

The minimum bactericidal concentration was also determined by using the plate streaking method. In this method a loopful of the content of the well was streaked onto a sterile tryptic soy agar plate and allowed to incubate for an additional 24 hours, if there was bacterial growth within 24 hours it was concluded that extract acted as a bacteriostatic. If after 24 hour there no visual sign of growth the extract was determined to be bactericidal.

**Cytotoxicity test**
DNA – based proliferation assay: The DNA based proliferation assay was performed as per the instruction given by Roche Molecular Biochemicals. Briefly, undifferentiated
porcine intestinal epithelial cells that were derived from jejunum (cell line - IPECJ2) (7500) were cultured in 100 µl media in a 96 well flat bottom plate and incubated for 24 hours at 37°C. Ten µl of media was replaced with 10µ1 of plant extract with a final volume 100µl in each well. Cells were incubated for 18- 24 hours at 37°C. All cells in 96 well plate were labeled with BrdU labeling solution (10µl per well). The plate was incubated for 18 hours at 37°C, the media was aspirated, and 200µl of FixDenat solution was added to each well and incubated for 30 min at room temperature. FixDenat solution was removed and 100ul anti-BrdU-POD working solution was added and incubated for 90 min at room temp, followed by washing and the addition of substrate solution. Plates were incubated for 20 min and quenched with H₂SO₄. Absorbance was measured at 450 and 690 nm (for back ground correction) were measured.

Results

Disk Diffusion Assay

Twenty eight plants species were collected for our study (Table#1). The antimicrobial activity of these plant extracts were screened by disk diffusion assay. Out of twenty eight plants five plants showed significant antimicrobial effect on E. coli. Centaurea stoeba, Rhus aromatica, Monarda fistulosa and Onosmodium molle showed moderate inhibition to E. coli growth whereas Clematis ligusticifolia showed maximum inhibition (fig # 1). C. ligusticifolia was found to be highly effective against E. coli having zone of inhibition of 24.75 mm which is greater than gentamicin (fig # 1). R. aromatic and C. stoebe showed zone of inhibition that was more than 50% as large as that caused by gentamicin. M. fistulosa and O. molle showed inhibition distances less than 50% of that of gentamicin.
The disk diffusion assay provides an initial screening method showing that extracts from these 5 plant species have the potential for use as antibiotics. To further assess these plants we determine their MIC value by broth dilution method and evaluate whether the effect of these extracts were bactericidal or bacteriostatic.

**Broth Microdilution Method:**

The minimum inhibitory concentration (MIC) is the lowest concentration at which an antimicrobial substance will inhibit microbial growth under specified conditions. This concentration is bacteriostatic as it inhibits the growth but does not kill the bacteria completely. *C. ligusticifolia* showed the lowest MIC value, 0.031 g/ml whereas the highest MIC value was for *R. aromatica* 0.25 g/ml (table # 2)

The minimum bactericidal concentration (MBC) is defined as the lowest concentration at which the test compounds kill the bacteria. The MBC values for all five plants were found to be 0.5gm/ml (Table # 2).

**Cytotoxicity Test:**

To determine the toxic effect of these plant extract on animals we performed cytotoxicity test using DNA- Based proliferation assy. The result from this assay showed a wide range of effect of the plant extracts on the animal cell (IPEC-J2) (fig # 2). *Clematis ligusticifolia* and *Rhus aromatica* both showed cytotoxicity to the IPECJ2 cells. A total of eight different concentrations had been examined from 0.0039 gm/ml to 0.5gm fresh weight /ml. *C. ligusticifolia* did not show any change in its toxic effect even at the lowest concentration whereas *Rhus aromatica* decreased in cytotoxicity as the concentration of the extract decreased (fig # 3 and 4).
The extract of *C. stoebe* did not alter the growth of IPECJ2 cells, when compared to the control. The extracts of *M. fistulosa* and *O. molle* appeared to increase the growth of IECJ2 cells at their higher concentration. These extracts significantly increased incorporation of BrdU by greater than two fold compare to control (fig # 2). *R. aromatica* and *C. ligusticifolia* reduce the cell growth below 50% compare to control.

**Discussion:**

All those plants we had tested have been used traditionally by the American Indians. Many of these species were utilized alone or in combination with other plants to treat a wide range of ailments. Only five plants out of 28 were effective against *E. coli*. The result of MIC assay showed that some of these plant extracts may provide potential sources of new antibiotics. The BrdU assay indicated that only *C. ligusticifolia* was the most toxic to cultured IPEC-J2 cells and thus *R. aromatica* also showed some toxicity against IPECJ2 cells. Tetracyclines are broad spectrum antibiotics and widely used for various bacterial infections showed cytotoxic effects in human blood lymphocytes cells *in vitro* (Celik & Eke, 2011). Some erythromycin base and its chemical derivatives; used to treat variety of human infections, have been reported to show cytotoxicity in human liver cell lines when treated *in vitro* (Viluksela et al., 1996). Three antibiotics (ciprofloxacin, clyndamicin and metronidazole) tested on human gingival fibroblast cell cultures showed cytotoxicity effect in a dose dependent manner (Ferreira et al., 2010). Though these antibiotics showed toxicity *in vitro* test, still they are in use to treat bacterial infections. To confirm the safety use of *C. ligusticifolia* and *R. aromatica* further *in vivo* study is necessary.
The genus *Clematis* (Family – *Ranunculaceae*) includes 350 species which grow worldwide (Dong et al., 2010). Pharmacological studies have shown that extracts of many species of this genus demonstrate antimicrobial activity (Buzzini and Pieroni, 2003). Khan et al (2001) reported that *Clematis papuasica* leaves and stem bark have broad spectrum antimicrobial activity. Buzzini and Pieroni (2003) studied antimicrobial activity of *Clematis vitalba* and Kyung et al (2007) reported the antimicrobial activity of *Clematis apfifolia* DC. Bioactive components of different species were identified and isolated by researchers. Phytochemical researches carried out on *Clematis mandshurica* revealed the presence of triterpenoid saponins (Dong et al., 2010), lignans, alkaloids (Shi et al., 2006b), macrocyclic glycoside (Shi et al., 2007) and phenolic glycosides (Shi et al., 2006a). Wu et al (2010) studied the therapeutic action of *Clematis chinensis*. However the nature and chemical constituents of *Clematis ligusticifolia* has not been extensively studied. The Native American used an infusion of leaves on horses for wounds, was also used for treatment of skin disease, ulcers and colds (Sweet.M, 1998). *C. ligusticifolia* extract inhibited *E. coli* even more than gentamicin (positive control). It also inhibited cell growth in the IPEC J2 culture across a range of serial dilutions. Although this extract was highly effective against *E. coli*, additional research needs to be performed to determine how these extracts can be used as antibiotics and which of the compounds in the extracts are responsible for their activity.

Antimicrobial effect of *M. fistulosa* (Family- *Lamiaceae*) has been studied by various researchers. Zhilyakova et al. (2009) studied the essential oils of *M. fistulosa* and showed that they have antibacterial activity against Gram negative bacteria. GC-MS analysis of Monarda oil revealed that Thymoquinone (simple monoterpenic quinine) is the
major constituent and that it shows antimicrobial activity against *Escherichia coli* (Inouye et al., 2006). Johnson et al (1998) reported the isolation of two bioactive monoterpenes: thymoquinone and thymol and also observed that the reduction of thymoquinone to thymohydroquinone reduced its activity. Our results showed that it is toxic to *E. coli* but not toxic to the animal cell line and therefore it may be used as a potential antibiotic to control *E. coli* infections.

The genus *Centaurea* (Family – *Asteraceae*) includes over 500 species that are found all over the world (Tekeli et al., 2011). Various species of *Centaurea* have been used traditionally to treat diseases such as hemmorhoids, abscesses and common colds. Twelve *Centaurea* species has been studied in Turkey for their antimicrobial activity out of these eight species (*C. balsamita*, *C. calolepis*, *C. cariensis* subsp. *maculiceps*, *C. cariensis* subsp. *microlepis*, *C. kotschyi* var. *kotschyi*, *C. solstitialis* subsp. *solsitialis*, *C. urvillei* subsp. *urvillei* and *C. virgata*) showed significant antimicrobial activity against different microorganisms (Tekeli et al., 2011). Guven et al (2005) reported that ethyl acetate extracts of *C. odyssei* and *C. kurdica* have significant antimicrobial effect. The chemical composition of *C. austro-anatolica* has been determined by GC-MS and the major components they found were caryophyllene oxide, spthulenol, n-tricosanol and geranyl isovalerate (Ugur et al., 2009). The main classes of components are oxygenated monterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated sesquiterpines and aromatic alcohols (Ugur et al., 2009). The key compound of essential oil in *C. sessilis* and *C. armena* was found to be β-eudesmol and was shown to have antimicrobial activity against both gram positive and gram negative bacteria (Yayli et al., 2005). Many species of *Centaurea* have been studied for their chemical constituents and it is found to be
effective against a broad spectrum microorganism, however there is little published research concerning the chemical constituents of *Centaurea stoebe* in particular. Further investigation to isolate the bioactive compounds of *C. stoebe* will be necessary.

Genus *Rhus* contains 250 species in the family of Anacardiacea found in temperate and tropical region worldwide. Various species have been used by the native people for medicine (Rayne & Mazza, 2007). Gundidza et al (2008) reported the presence of α-pinene (86.95%) in the essential oil of *Rhus lancea* having antimicrobial activity against *E. coli*. *Rhus coriaria* is used as a spice in Turkey and has been shown to have antimicrobial activity against *E. coli* 0157: H7 (food borne pathogen) (Nasar-Abbas & Halkman, 2004). Shabana et al (2008) reported that the major constituents of essential oil of *Rhus coraria* fruits are thymol, caryophyllene and embrene which are the key components with antimicrobial activity. It is also reported that *Rhus coraria* had significant antimicrobial activity against several gram positive and gram negative bacteria including *E. coli* (Fazeli et al., 2007). Mossa et al (1996) described the presence of free flavonoids such as persicogenin, velutin, (2S) 5,3’,4’-trihydroxy 7-methoxyflavonone and homoeriodictyol in *Rhus retinorrhoea*. Leaves, stems, barks, roots, fruits and the galls on *R. chinensis* have been shown to have therapeutic value for treating diarrhea, dysentery, rectal and intestinal cancer, diabetes mellitus, sepsis, oral disease and inflammation (Djakpo & Yao, 2010). Very little information is available about the phytochemical constituents and antimicrobial activity of *Rhus aromatica*, the fragrant sumac is native to the Northern Great Plains and used by the Native American for various medicinal purposes. Reichling et al (2009) have reported that the aqueous extract of *R. aromatica* has antiviral potency against herpes simplex virus type 1 and type 2. In our research we
demonstrated the antimicrobial activity of *Rhus aromatica* against *E. coli*. Further research is required to identify its active compounds.

*Onosmodium molle* (family- Boraginaceae) commonly known as false gromwell was used by the Native Americans as a dermatological aid, to treat lumbago and as veterinary medicine (Moerman, 2009). An infusion of *Onosmodium virginianum* root has been reported to help strengthening the renal apparatus (Cook, 1869). Inadequate literature is available for the confirmation of its use so far. In our study it showed antimicrobial property of *Onosmodium molle* against *E. coli* without having any cytotoxicity effect on porcine intestinal epithelial cells. Because of it selective toxicity effect against *E. coli*, it is more interesting to further investigate the bioactive compounds of *O. molle*.

**Conclusion**

This is the first report for *in vitro* antibacterial screening of Native South Dakota Plants against *E. coli*. The results suggest further investigation into the antimicrobial activity of the extracts of *Clematis ligusticifolia, Monarda fistulosa, Rhus aromatica, Centaurea stoebe* and *Onosmodium molle* against *E. coli* is warranted. It is also important to identify the secondary metabolites of these plants and their major active components having antimicrobial activity. Although monoterpane the bioactive components in *M. fistulosa*, there is a paucity of information concerning the influence growth environment and time of harvest on their antibiotic potential.

In this paper we showed that *M. fistulosa* and *O. molle* have inhibitory effect on *E. coli* but they are not toxic to the IPECJ2 cells. These two plants necessitate further study into their phytochemical inventory and their compounds that are active for treating infections.
C. ligusticifolia and R. aromatica have promising antibacterial activity against E. coli but they also showed toxicity to the IPECJ2 cells in vitro. The application of these two plants should be tested in vivo to understand the toxicity effect for further medicinal use of these plants.

The medicinal plants screened in this study may have metabolites which are effective for antibacterial activity needs to be further investigated.

Acknowledgement:

We thank South Dakota Agricultural Experiment Station and Biology/Microbiology Department of SDSU for the financial and technical support provided to complete this project.
Table 1: List of plants screened for antimicrobial activity (Moerman, 2009)

<table>
<thead>
<tr>
<th>S.N</th>
<th>Plants Name</th>
<th>Family</th>
<th>Common name</th>
<th>Medicinal use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Astragalus crassicarpus</em></td>
<td>Fabaceae</td>
<td>Milkvetch</td>
<td>Stimulant, hemostat, anticonvulsive, veterinary aid.</td>
</tr>
<tr>
<td>2</td>
<td><em>Arctostaphylus uva ursi</em></td>
<td>Ericaceae</td>
<td>Bearberry</td>
<td>Dermatological aid, oral aid, antidiarrheal, analgesic etc.</td>
</tr>
<tr>
<td>3</td>
<td><em>Artemisia ludoviciana</em></td>
<td>Asteraceae</td>
<td>Foothill sagewort</td>
<td>Gastrointestinal aid, antirheumatic, antidote</td>
</tr>
<tr>
<td>4</td>
<td><em>Balsamorhiza sagittata</em></td>
<td>Asteraceae</td>
<td>Arrowleaf Balsamroot</td>
<td>Dermatological aid, analgesic, gastrointestinal aid, pulmonary aid, burn dressing</td>
</tr>
<tr>
<td>5</td>
<td><em>Caltha palustris</em></td>
<td>Ranunculaceae</td>
<td>Yellow marshmerigold</td>
<td>Dermatological aid, cold remedy</td>
</tr>
<tr>
<td>6</td>
<td><em>Centaurea stoebe</em></td>
<td>Asteraceae</td>
<td>Spotted knapweed</td>
<td>Dermatological aid</td>
</tr>
<tr>
<td>7</td>
<td><em>Chrysothamnus nauseosus</em></td>
<td>Asteraceae</td>
<td>Rubber rabbitbrush</td>
<td>Dermatological aid and cold remedy, antidiarrheal</td>
</tr>
<tr>
<td>8</td>
<td><em>Clematis ligusticifolia Nutt</em></td>
<td>Ranunculaceae</td>
<td>Western white clematis</td>
<td>Analgesic, dermatological aid, cold remedy, veterinary aid</td>
</tr>
<tr>
<td>9</td>
<td><em>Cleome serrulata</em></td>
<td>Capparaceae</td>
<td>Rocky mountain beeplant</td>
<td>Gastrointestinal aid, dermatological aid, eye medicine</td>
</tr>
<tr>
<td>10</td>
<td><em>Conyza Canadensis</em></td>
<td>Asteraceae</td>
<td>Canadian horseweed</td>
<td>Antidiarrheal, orthopedic aid, antirheumatic, burn dressing, disinfectant</td>
</tr>
<tr>
<td>11</td>
<td><em>Corallorhiza maculata</em></td>
<td>Orchidaceae</td>
<td>Summer coralroot</td>
<td>Dermatological aid, cold remedy, pulmonary aid and blood medicine</td>
</tr>
<tr>
<td>12</td>
<td><em>Cyanoglossus officinale</em></td>
<td>Boraginaceae</td>
<td>Gypsyflower</td>
<td>Antihemorrhagic, cancer treatment, dermatological aid, kidney aid, tuberculosis remedy</td>
</tr>
<tr>
<td>13</td>
<td><em>Geranium viscosissimum</em></td>
<td>Geraniaceae</td>
<td>Sticky Geranium</td>
<td>Cold remedy, eye medicine, dermatological aid, Gynecological aid</td>
</tr>
<tr>
<td>14</td>
<td><em>Glycyrrhiza lepidota Pursh</em></td>
<td>Fabaceae</td>
<td>American Licorice</td>
<td>Antidiarrheal, gastrointestinal aid, ear medicine, pediatric aid, veterinary medicine,</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Family</td>
<td>Common Name</td>
<td>Uses</td>
</tr>
<tr>
<td>---</td>
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<td>------------------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>15</td>
<td><em>Melilotus officinalis</em></td>
<td>Fabaceae</td>
<td>Yellow sweetclover</td>
<td>Dermatological aid, cold remedy</td>
</tr>
<tr>
<td>16</td>
<td><em>Monarda fistulosa</em></td>
<td>Lamiceae</td>
<td>Wild bergamot bee balm</td>
<td>Dermatological aid, ough medicine, throat aid, toothache remedy, gastrointestinal aid, eye medicine for sore eye.</td>
</tr>
<tr>
<td>17</td>
<td><em>Oenothera biennis</em></td>
<td>Onagraceae</td>
<td>Evening primrose</td>
<td>Dermatological aid, hemorrhoid remedy</td>
</tr>
<tr>
<td>18</td>
<td><em>Onosmodium molle</em></td>
<td>Boraginaceae</td>
<td>Smooth onosmodium</td>
<td>Veterinary aid, dermatological aid, antirheumatic</td>
</tr>
<tr>
<td>19</td>
<td><em>Pediomelum argophyllum</em></td>
<td>Fabaceae</td>
<td>Silverleaf Scrupea</td>
<td>Used for fever, veterinary aid, used as laxative, dermatological aid</td>
</tr>
<tr>
<td>20</td>
<td><em>Perideridia gairdneri</em> (Hook and Arn) Mathias (root)</td>
<td>Apiaceae</td>
<td>Giardner’s Yampah</td>
<td>Antidiarrheal, cough medicine, dermatological aid, veterinary aid, diuretic and laxative</td>
</tr>
<tr>
<td>21</td>
<td><em>Physialis virginiana</em></td>
<td>Solanaceae</td>
<td>Virginia ground cherry</td>
<td>Used as stimulant</td>
</tr>
<tr>
<td>22</td>
<td><em>Plantago rugelii</em></td>
<td>Plantaginaceae</td>
<td>Blackseed plantain</td>
<td>Dermatological aid: poultice of fresh leaves applied to burn or any inflammation</td>
</tr>
<tr>
<td>23</td>
<td><em>Psoralea argophylla</em></td>
<td>Fabaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td><em>Quercus macrocarpa</em></td>
<td>Fagaceae</td>
<td>Bur oak</td>
<td>Antidiarrheal, dermatological aid, pulmonary aid, gastrointestinal aid</td>
</tr>
<tr>
<td>25</td>
<td><em>Rhus aromatic</em></td>
<td>Anacardiaceae</td>
<td>Fragrant Sumac</td>
<td>Dermatological aid, urinary aid, antidiarrheal, cold remedy, oral aid, pediatric aid</td>
</tr>
<tr>
<td>26</td>
<td><em>Senecio rapifolius</em></td>
<td>Asteraceae</td>
<td>Openwoods ragwort</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td><em>Symphocarpus albus</em></td>
<td>Caprifoliaceae</td>
<td>Common snowberry</td>
<td>Diuretic and venereal aid, dermatological aid</td>
</tr>
<tr>
<td>28</td>
<td><em>Tanacetum vulgare</em></td>
<td>Asteraceae</td>
<td>Common tansy</td>
<td>Anthelmintic, dermatological aid, antidiarrheal, cold remedy</td>
</tr>
</tbody>
</table>
Table 2: List of plants with their MIC and MBC (minimum inhibitory concentration)

<table>
<thead>
<tr>
<th>S.N</th>
<th>Plants Name</th>
<th>MIC g/ml plant extract in fresh wt</th>
<th>MBC g/ml Plant extract in fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Centaurea stoebe</em></td>
<td>0.062</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td><em>Clematis ligusticifolia</em></td>
<td>0.031</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td><em>Monarda fistulosa</em></td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td><em>Onosmodium molle</em></td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td><em>Rhus aromatic</em></td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>Gentamicin</td>
<td>0.025</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**Fig: 1** Zone of inhibitions for the plant extract against *E. coli*. Positive control was gentamicin and negative control was ethanol. All ethanol controls had 0 mm ZOI.

**Fig 2:** Incorporation of BrdU into DNA as a measure of cell growth, expressed as percent of incorporation into control cell DNA at the highest concentration of plant extracts, 0.5 g/ml.

**Fig 3:** IPEC-J2 cell growth at 8 different serially diluted concentrations of *R. aromatic* extracts.

**Fig 4:** IPEC-J2 cell growth at 8 different serially diluted concentrations of *C. ligusticifolia* extracts.
FIGURES

Fig-1

Fig-2
\[ y = -0.1515x + 1.8946 \]

\[ R^2 = 0.8834 \]

![Graph 1](R. aromatica)

![Graph 2](Dilution of Extract)

Fig-3

\[ y = 0.0017x + 0.1895 \]

\[ R^2 = 0.008 \]

Fig-4
References


Cook HWM. (1869). The Physio-Medical Dispensatory, Cincinnati, OH.


