In vitro Antibacterial Activity and Phytochemical Analysis of Ethanolic Leaf Extracts of *Cnidoscolus aconitifolius* and *Commelina diffusa*

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Abstract

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In recent years, the indiscriminate and arbitrary use of commercial antimicrobial drugs has resulted in multiple antibiotic-resistant pathogenic microorganisms. Natural products derived from plants can serve as important sources of bioactive compounds that can be used as alternatives in the treatment of various infectious diseases. *Cnidoscolous aconitifolius* (colloquially known as Chaya), is used in Belize, Guatemala, and Mexico as a leafy vegetable as well as a medicinal herb in the treatment of diabetes, skin disorders, and venereal diseases. *Commelina diffusa* (also known Dayflower), is used throughout the Americas for both culinary and medicinal purposes, most often as a diuretic and febrifuge. The present study investigated the *in vitro* antibacterial activity as well as phytochemical properties of ethanolic leaf extracts of *C. aconitifolius* and *C. diffusa*. Fifty (50) grams of dried leaves of *C. aconitifolius* and *C. diffusa* respectively, were pulverized in 100 mL of 95% ethanol and the solvent portion was separated and concentrated to 3 mL of each. Antibacterial activity was assessed using disc diffusion assay against Gram-positive *Streptococcus pyogenes* and Gram-negative *Escherichia coli* bacterial strains. Leaf extract of *C. aconitifolius* exhibited highest antibacterial activity (average zone of inhibition 10.16mm $\pm$ 4.34) against *S. pyogenes*, whereas the leaf extract of *C. diffusa* exhibited least antibacterial activity (average zone of inhibition 7.81mm $\pm$ 1.27) against *E. coli*. Thin layer chromatographic studies of *C. aconitifolius* and *C. diffusa* leaf extracts revealed several colored and UV-active fractions of phytochemical compounds at discrete R_f values. The results indicate that the active ingredients in these extracts could be plant secondary metabolites such as Terpenoids, Phenylpropanoids, and Alkaloids, which are known for their antioxidant, cytotoxic, and antimicrobial properties. Results obtained from the preliminary *in vitro* experiment support the ethnopharmacological uses of both plants. Further studies are needed to characterize the biochemical compounds of medicinal values.

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I declare that this is my own work, and that it does not contain material that has already been used to any substantial extent for a comparable purpose.

Name of Student: _____ Signature: _____
Date: _____

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This study was completed under the guidance and supervision of Dr. Thippi Thiagarajan, and Dr. Apolonio Aguilar. Without their help, this research would have not been possible, yet even a success. I must express sincere gratitude to both, for their ideas and knowledge that led to the culmination of this research and their suggestions that led to the finalization of this report. I am grateful to Dr. Thippi for his help in the microbiological aspect of the research and would also like to thank Dr. Aguilar for his time and patience in helping me to carry out all things chemistry related. I am also thankful to my family and friends, and to the lab technicians: Mr. Rolando Caballero, Mrs. Zeida Franco-Leon, and Ms. Azelea Gillett, who allowed me access to the lab and lab resources in order to complete the research. Their suggestions and support were vital to the success and completion of this research.

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1 Introduction

Natural products derived from plants have been integral to traditional healing systems throughout the world from time immemorial, constituting the 'backbone' for not only culture, but also history. Plants are used for a wide variety of purposes, including food preservation, pharmaceutical, alternative medicine, and natural therapies (Khan 2011). The use of bioactive natural products for medicinal purposes can be traced back hundreds, even thousands of years. However, their application as isolated and characterized compounds to modern drug discovery and development started only in the nineteenth century. Since then, it has been well documented that natural products play critical roles in modern drug development, especially so as antibacterial and antitumor agents (Veeresham 2012).

The hugely diverse plant kingdom, consisting of some 250,000 to 300,000 species, continues to evolve and adapt to a multiplicity of environmental conditions and to protect from pathogens and predators. On the contrary, whether by chance or design, the human species appears to have stabilized its genetic code. Many characterized human endogenous receptors, important in physiological function, are activated by plant-derived chemicals; for example the opioid, and the more recently discovered cannabinoid, receptors. It is not unreasonable to hypothesize that many more structure-activity relationships of physiological and pharmacological significance involving plant molecules have yet to be characterized (Gwynn and Hylands 2000).

Plants are important sources of phytochemicals – active compounds that can be derived directly or indirectly for drug use. Despite the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous, and natural products will continue to be extremely important as sources of medicinal agents (Veeresham 2012). In addition to the natural products which have found direct medicinal application as drug entities, many others can, and have served as chemical models or templates for the design, synthesis, and semi-synthesis of novel substances for treating various diseases (Obichi *et al.* 2015).

The medicinal use of plants has had a long history in the treatment of various diseases. To date, thirty-five thousand to seventy thousand plant species have been screened for their medicinal uses. Plants, especially those with ethno-pharmacological uses have been the primary sources of medicine for early drug discovery. Fabricant and Farnsworth (2001) reported that eighty percent of one hundred and twenty-two plant-derived drugs were related to their original ethno-pharmacological purposes. There is also growing evidence to suggest that old molecules are finding new applications through better understanding of molecular biology and clinical observations.

In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have been observed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. Plants that have antioxidant, antiviral, antibacterial, fungicidal, and cytotoxic agents have gained popularity in recent years as sources of new therapeutic agents as opposed to synthetic drugs. Plant-derived compounds have also had a long history of clinical use, better patient tolerance, and overall acceptance as opposed to synthetics. Diseases that are most challenging in today's society tend to be complex (involving various targeting mechanisms). Plant-derived drugs contain many phytochemically active components that are specific to such mechanisms, and hence may prove to be better treatment-wise as well as in regards to cost.

Cnidoscolus aconitifolius is a group of arborescent shrubs belonging to the Euphorbiaceae family. *C. aconitifolius* is an evergreen, drought deciduous plant that grows up to 3 to 6 m in height. The plant has alternate palmate lobed leaves, milky sap, and small flowers on dichotomously branched cymes. The leaves are large (32 cm long and 30 cm wide) and are found on chartaceous and succulent petioles. The crop originated as a domesticated leafy green vegetable in the Maya regions of Guatemela, Belize, and Southeast Mexico during the Precambrian period (Ross-Ibarra and Molina-Cruz 2002). It has been used as food, medicine, and an ornamental plant. *C. aconitifolius*, known as tree spinach (English) is colloquially referred to as Chaya.

Commelina diffusa belongs to the Commelinaceae family. *Commelina* is a genus of perennial herbs of neotropical origin that has been distributed in various places in India, China, Malaysia, Africa, Egypt, and the Americas. This genus of approximately 170 species are commonly called dayflowers due to their short-lived flowers (Gajurel and Shrestha 2010); they are less often known as widow's tears. The dayflowers are herbs that may be either perennial or annual. They are characterized by their zygomorphic flowers and by the involucral bracts called spathes that surround the flower stalks. These spathes are often filled with a mucilaginous liquid. Each spathe houses either one or two scorpioid cymes bearing several flowers. All members of the genus have alternate leaves (Aleman 2013).

The present study has been designed to determine the role of *C. aconitifolius* and *C. diffusa* leaf extracts in the *in vitro* antibacterial activity against human pathogens, namely, a Gram positive bacterium [*Streptococcus pyogenes* (*S. pyogenes*)] and a Gram negative bacterium [*Escherichia coli* (*E. coli*)]. Based on the literature surveyed, it is hypothesized that plant extracts of *C. aconitifolius*and *C. diffusa* will have potential *in vitro* antimicrobial properties.

2 Literature Review

Table 1: Classification of Chaya (*Cnidoscolus aconitifolius*), Dayflower (*Commelina diffusa*), Gram-positive *Streptococcus pyogenes*, and Gram-negative *Escherichia coli*, *Gammaproteobacteria

	Cnidoscolus aconitifolius (Maya Tree Spinach, Chaya)	*Commelina diffusa* (Climbing Dayflower, Birdbill Dayflower)	*Streptococcus pyogenes* (Gram-positive)	*Escherichia coli* (Gram-negative)
Kingdom	Plantae	Plantae	Eubacteria	Eubacteria
Phylum	Tracheophyta	Tracheophyta	Firmicutes	Proteobacteria
Class	Magnoliopsida	Liliopsida	Bacilli	GPB*
Order	Malpighiales	Commelinales	Lactobacillales	Enterobacteriales
Family	Euphorbiaceae	Commelinaceae	Streptococcaceae	Enterobacteriaceae
Genus	*Cnidoscolus*	*Commelina*	*Streptococcus*	*Escherichia*
Species	*C. aconitifolius*	*C. diffusa*	*S. pyogenes*	*E. coli*

2.1 *Cnidoscolus aconitifolius*

C. aconitifolius is cultivated in domestic gardens rather than in agricultural fields, and as such can be used throughout the year (Donkoh and Atuahene 1990) (Figure 1). Regardless of the widespread use of this plant, scientific literature is yet to completely investigate the conventional uses and nutritional values of *Cnidoscolus* spp. However, there are some reports that describe the nutritive values of *C. aconitifolius* (Kuti and Konoru 2004). *C. aconitifolius* is most valued as a food source; however, it was, and continues to be an important medicinal plant (Ross-Ibarra and Molina-Cruz 2002. In fact, much of its recent spread into new areas may likely be attributed to its medicinal value.

Many claims have also been made for its medicinal efficacy as a treatment for various ailments, ranging from its ability to darken gray hair and strengthen fingernails, to its use in curing alcoholism, insomnia, gout, scorpion stings, and brain and vision impairment (Jensen 2009; Atuahene *et al.* 1999). Studies have shown that it has certain antibacterial properties, as well as a contraceptive effect (Dong *et al.* 2010). It has been observed in use as a diuretic,

circulation and lactation stimulant, and has also been recommended for diabetes, obesity, acne, kidney stones, and eye problems (Jimoh *et al.* 2009).

Adeniran *et al*. (2012), assessed the antibacterial activity of *Cnidoscolus aconitifolius* by the measurement of inhibition zones according to the parameters suggested by Kiehlbauch *et al*. (2000), where inhibition zones < 9 mm, were considered inactive; 9 to 12 mm, less active; 13 to 18 mm, active, and $>$ 18 mm, very active. The ethanol leaf extract was less active against *Escherichia coli* (12 mm), while the ethanol extracts of plant stems and roots were very active against *E. coli* with zones of inhibition of 23 mm and 24 mm respectively (Adeniran *et al*. 2012). The extract concentration of $20\,\mu$l/disc for the ethanolic extract was compared with control discs containing the standard antibiotic Tetracycline at $10\,\mu$g/disc; Gentamycine at $10\,\mu$g/disc; Contrimoxazole at $25\,\mu$g/disc; Chloramphenicol at $30\,\mu$g/disc.

Figure 1: *Cnidoscolus aconitifolius*

Awoyinka *et al*. (2007) noted that the fresh leaf water and ethanolic extracts of *C. aconitifolius* showed inhibitions on *Staphylococcus aureus*. The ethanolic extract had a zone of inhibition of 3.0 ± 0.1 mm against *S. aureus* whereas the fresh water leaf extract had a zone of inhibition of 2.0 ± 0.5 mm. The inhibition zones were compared with the control disc containing standard antibiotic Chloramphenicol at a concentration of $30\,\mu$g/disc. The Chloramphenicol zone of inhibition was recorded as 11.5 ± 0.1 mm. Iwuji *et al*. (2016) studied the antibacterial activities of the extracts of *C. aconitifolius* and standard drug control (Chloramphenicol) at different concentrations (0.25, 0.5, and 1 g/10 ml) against eight pathogenic bacteria, including *E. coli* and a *Streptococcus* sp. The aqueous extract (concentration: 1 g/10 ml) of the plant showed antimicrobial activity against *S. aureus* (21 mm) and *E. coli* (17 mm). Hydromethanolic leaf extracts also showed antimicrobial activity against *E. coli*, with zones of inhibition averaging 17 mm.

The healing and anti-inflammatory activities popularly attributed to *Cnidoscolus* spp. are strongly associated with its tannin content (Araujo-Gomes *et al*. 2014). *Cnidoscolus* spp. also contain saponins, which have immense significance as an antihypercholesterol, hypotensive and cardiac depressant agent. Iwuji *et al*. (2016) noted that the hydro-methanol leaf extract of *C. aconitifolius* gave a higher yield of chemical constituents (alkaloids) when compared with aqueous extracts. The hydro-methanol leaf extracts of *C. aconitifolius* were found to be more active on most of the pre-clinically isolated bacteria when compared with aqueous extract. The various studies conducted on the antimicroial properties of *C. aconitifolius* justified the use of the leaf, as well as root and shoot extracts in the traditional medicine to treat various infectious diseases (Awoyinka *et al*. 2007; Adeniran *et al*. 2012; Iwuji *et al*. 2016).

2.2 *Commelina diffusa*

Figure 2: *Commelina diffusa* Malaysia (Isaac *et al.* 2012).

In principle, *Commelina* species are used to cure various chronic and acute diseases (Ekeke and Agogbua 2018). Whole plant extracts were used traditionally in order to heal various chronic diseases such as atherosclerosis and diabetes (Isaac *et al.* 2012; Ujowundu *et al*. 2008). The leaves of *Commelina* spp. (Figure 2) are harvested from the wild for local consumption; in many areas they are viewed as a famine food and only eaten in times of scarcity. The plant also has local medicinal uses in Bangladesh and Malaysia, and is the source of a dye. The fresh plants are also sold by Chinese herbalists in

Commelina diffusa, also known as the climbing dayflower or spreading dayflower is used to reduce swelling and inflammation. It is commonly used in urinary tract infections, removing cough with sticky phlegm, and in diarrhea, hemorrhoids, eye irritation, conjunctivitis and other

eye problems like ophthalmia. The juice of the stem is also used for laryngitis, sore throats, acute tonsillitis, pharyngitis, otitis media and nose bleeding. Internally, the plant is used for abscess, boils, fever and malaria and for the treatment of insect, snake, and bug bites (David 1998). However, antioxidant and antimicrobial properties of plant metabolites are yet to be documented in detail.

Results from Khan *et al's*. (2011b) 'Evaluation of phytochemical and antimicrobial properties of *Commelina diffusa* Burm. f.' showed that the methanolic fraction of *C. diffusa* extract ($250\,\mu$l/disc) produced the highest zone of inhibition against the test bacteria among the tested solvents. Zones of inhibition for *S. aureus* and *E. coli* were 14 ± 1.5 mm and 11 ± 1.2 mm respectively. The standard drug Ciprofloxacin was used as a positive control, and was observed to inhibit the growth of the entire test bacteria with significant zones of inhibition (27 to 31 mm).

2.3 *Streptococcus pyogenes*

Group A Streptococcus (GAS) syn. *Streptococcus pyogenes* is one pathogenic bacteria that is most often associated with a wide spectrum of infections and disease states. GAS pharyngitis ("strep throat") and GAS pyoderma account for more than 600 million of infection cases annually. The vast majority of GAS infections are of short duration and are relatively passive in nature; however, invasive disease can be severe and life-threatening. Respiratory tract infections, including, acute otitis media, pharyngitis, pneumonia, acute bronchitis, and acute sinusitis are widespread and are considered major health concerns. In developing countries, they account for a significant amount of the overall morbidity and mortality in pediatric settings (Camara *et al*. 2013).

GAS is a gram-positive coccoid-shaped bacterium that grows in chains. GAS produces small white to grey colonies with a clear zone of β-hemolysis on blood agar. The GAS cell is a complex structure. In strains that divide rapidly, such as those in young cultures, and epidemic strains, the cell surfaces are covered with a hyaluronic acid capsule, giving the colonies a mucilaginous or water drop appearance. Microscopic hair-like fimbriae also protrude from the cell surface and into the hyaluronic capsular layer; this promotes adherence of the bacterium to epithelial skin cells and

extracellular matrix proteins (Ibrahim *et al.* 2013).

Though uniformly GAS are susceptible to penicillin and other antimicrobial agents, GAS infections continue to present inherent clinical and public health challenges. All *S. pyogenes* isolates are susceptible to penicillin, the drug of choice for the treatment of group A streptococcal infections. Penicillin failures may, however, occur due to causes which include inadequate dosage and poor patient compliance. Erythromycin is usually recommended as the alternative treatment for group A streptococcal pharyngotonsillitis only in patients allergic to penicillin or in cases of penicillin failure. Recently, the increase in the incidence of antibiotic-resistant clinical isolates of *S. pyogenes* underscores the need for continuous surveillance of antimicrobial resistance patterns, with natural alternatives being considered for supplementation (Baldassarri *et al.* 2006).

2.4 *Escherichia coli*

E. coli is a common commensal of the intestinal tract of humans and animals, however, it can also be found in water, soil, and vegetation. *E. coli* is the most frequently isolated bacteria from clinical samples, and is the pathogen most involved in urinary tract infections (UTIs); it is also one of the common agents responsible for ear infections, sepsis and wound infection. In developing countries *E. coli* is the agent most commonly responsible for food and waterborne diarrhea and causes high mortality in children under 5 years old. *E. coli* is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium. *E. coli* stains Gram-negative because its cell wall is composed of a thin peptidoglycan layer and an outer membrane. During the staining process, *E. coli* picks up the color of the counterstain safranin and stains pink. The outer membrane surrounding the cell wall provides a barrier to certain antibiotics such that *E. coli* is not damaged by penicillin (Alem *et al*. 2015).

Antimicrobial resistance in *E. coli* has been reported worldwide and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. A rise in bacterial resistance to antibiotics complicates treatment of infections. In general, up to 95% of cases with severe symptoms are treated without bacteriological investigation. Occurrence and

susceptibility profiles of *E. coli* show substantial geographic variations as well as significant differences in various populations and environments. The emergence of resistance is a global phenomenon, however the rates of antibiotic resistance remain different between developed and developing countries. This emergence complicates the management of infections and impacts the use of widely prescribed antibiotics in clinical practice. Periodic monitoring of antibiotic resistance in different bacterial isolates has become essential given the constant evolution of the bacterial ecology and the emergence of antibiotic resistance (Kibret and Abera 2011).

3 Materials and Methods

This research was conducted at the University of Belize's CHEMLAB and BIOLAB at the Central Campus in Belmopan City under the Faculty of Science and Technology. The research was carried out from February to May 2018.

3.1 Plant Materials

Fresh leaf samples of Maya tree spinach (*C. aconitifolius*) and Dayflower (*C. diffusa*) were collected from the Cayo district, Belize. Fifty (50) grams of each respective samples were washed with distilled water and air dried under controlled conditions for preparation of the dry leaf extracts.

3.2 Preparation of Plant Extracts

Fifty (50) grams of dried leaves of *C. aconitifolius* and *C. diffus*a respectively, were pulverized in 100 mL of 95% ethanol and the solvent portion was separated and concentrated to 3 mL of each. At the end of each respective extraction, the extracts were filtered using Whatman filter paper by vacuum filtration. Extracts were further concentrated in a water bath and stored until needed (Figure 3).

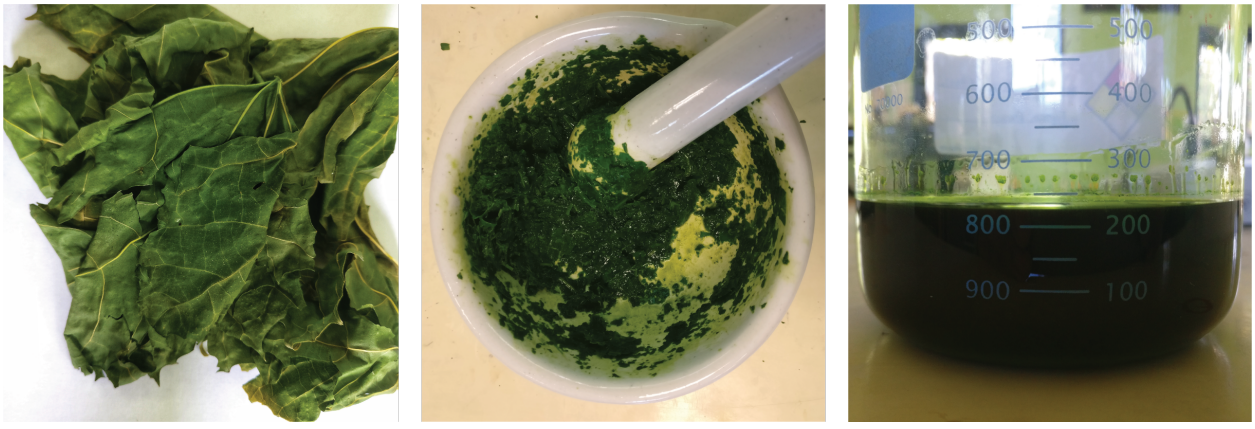

Figure 3: Dried Chaya, and Preparation of*C. aconitifolius* leaf extract with Mortar and Pestle

3.3 Bacteria and Growth Medium

For antimicrobial sensitivity assay two bacterial species, one Gram-positive - *Streptococcus pyogenes* and one Gram-negative - *Escherichia coli* were used as test organisms. Test organisms (bacterial isolates) were obtained and sub-cultured on Nutrient Agar and MacConkey Agar respectively, and incubated for 24 hours. Test organisms were then inoculated in 50 mL of Nutrient Broth and placed on an Orbit ™ Multipurpose Digital Vortexer for 24 hours; the stock cultures were subsequently maintained at 4^{o}C. Aseptic conditions were ensured by operating under the laminar air floor chamber, and the needed apparatuses were autoclaved for 20 minutes at 15 PSI.

3.4 Antibacterial Sensitivity Assay by Disc Diffusion Method

A standardized inoculum (100 microliters of stock solution) was introduced onto the surface of sterile agar plates, and a sterile glass spreader was used for the even distribution of the inoculum. Sterile filter paper discs (with a diameter of 5.75 mm) were impregnated with known amounts of extract using micropipettes, and were carefully placed separately on to the seeded agar plates. The plates were then incubated at 30^{o} C for 24 hours, after which the diameter of the clear zones around the discs were measured with a Vernier caliper and compared against zones of inhibition produced by the controls. Discs on which the solvent (used to dissolve the samples) were adsorbed and dried and used as negative controls.

3.5 Thin Layer Chromatographic Analysis

3.5.1 Sample Application

Concentrated plant extracts were spotted to the origins of TLC plates. The extract solutions were drawn with capillary tubes and were spotted (three spots per extract per plate) separately on TLC plate at about 1 cm from base. Thereafter the plates were left to dry allowing evaporation of the solvent.

3.5.2 Development of Plates

For the development of the plates, three solvent systems were used; 10% Methanol/ Dichloromethane, 20% Ethyl acetate/ Cyclohexane, and 40% Ethyl acetate/ Cyclohexane. After the application of the extracts on the plate, the plates were kept in a beaker, and covered with watch glass (solvent saturated). The mobile phase was then allowed to move through the adsorbent phase up to 3/4th of the plate. At the end of the chromatographic development, the plates were removed, and the separated spots were visualized under daylight, low frequency Ultra-violet light, and subsequently stained with *p*-Anisaldehyde stain.

3.5.3 Preparation of *p*-Anisaldehyde Stain and Visualization of TLC Plates

To 462.5 mL of absolute ethanol, 17.5 mL of concentrated sulfuric acid and 5 mL of glacial acetic acid was added. 12.5 mL of *p*-anisaldehyde was then added, and the solution was stirred vigorously to ensure homogeneity. TLC plates were dipped in the stain solution, and heated to 105^{o}C using a heat gun, until maximum visualization of spots were observed.

3.5.4 Calculation of retardation factor (R_f)

The distance traveled by the solvent front and each respective separated spots were measured. Measurements were taken from the origin of the solvent front, and from the origin to the middle of each spot. R_f values were calculated by using the equation; $R_f = X_{sample}/X_{solvent}$; Where, X_{sample} = Distance traveled by substance and $X_{solvent}$ = Distance traveled by solvent front.

3.6 Statistical Analysis

The resulting data was statistically evaluated by a Student-t-test using SPSS Statistics (SPSS Inc; Version 17.0 2010), where the data was compared with the control results and a $p \leq 0.05$ was considered as significant.

4 Results and Discussion

4.1 Antibacterial Sensitivity Assay by Disc Diffusion Method

In this study, the antimicrobial activity of the ethanolic extracts of *C. aconitifolius* and *C. diffusa* was assessed to determine the zones of inhibition (in mm) against one gram positive (*S. pyogenes*) and one gram negative (*E. coli*) bacterium (Figure 4, 5, and 6). The ethanolic extract of *C. diffusa* had variable *in vitro* potential of antimicrobial activities against the two bacterial strains tested. Greater zones of inhibition were observed for the *C. aconitifolius* extract on both bacterial strains. *C. aconitifolius* had an average zone of inhibition of 10.02mm $\pm$ 1.21 on *E. coli*, with a high range of 11.45 mm, and a low range of 8.45 mm. *C. aconitifolius* had an average zone of inhibition of 10.16mm $\pm$ 4.34 on *S. pyogenes*, with a high range of 13.25 mm, and a low range of 6.60 mm. On the contrary, the extracts of *C. diffusa* had lower zones of inhibition. *C. diffusa* had an average zone of inhibition of 7.81mm $\pm$ 1.27 on *E. coli*, with a high range of 9.60 mm, and low range of 7.40 mm. *C. diffusa* had an average zone of inhibition of 9.98mm $\pm$ 1.83 on *S. pyogenes*, with a high range of 12.50 mm, and low range of 7.20 mm (Table 2).

Table 2: *In vitro* antibacterial activity of *C. aconitifolius*, and *C. diffusa* extracts. Effective Zones of Inhibition* (in mm diameter. Mean values followed by different superscripts in a column are significantly different (P$\leq$0.05)

Test Organisms	***C. aconitifolius* Extract**	***C. diffusa* Extract**	**Mean**	**Control (Negative)**
Escherichia coli	10.02 ± 1.21^{b}	7.81 ± 1.27	8.92	7.95 ± 0.55
Streptococcus pyogenes	10.16 ± 4.34^{a}	9.98 ± 1.83	10.07	8.71 ± 0.97

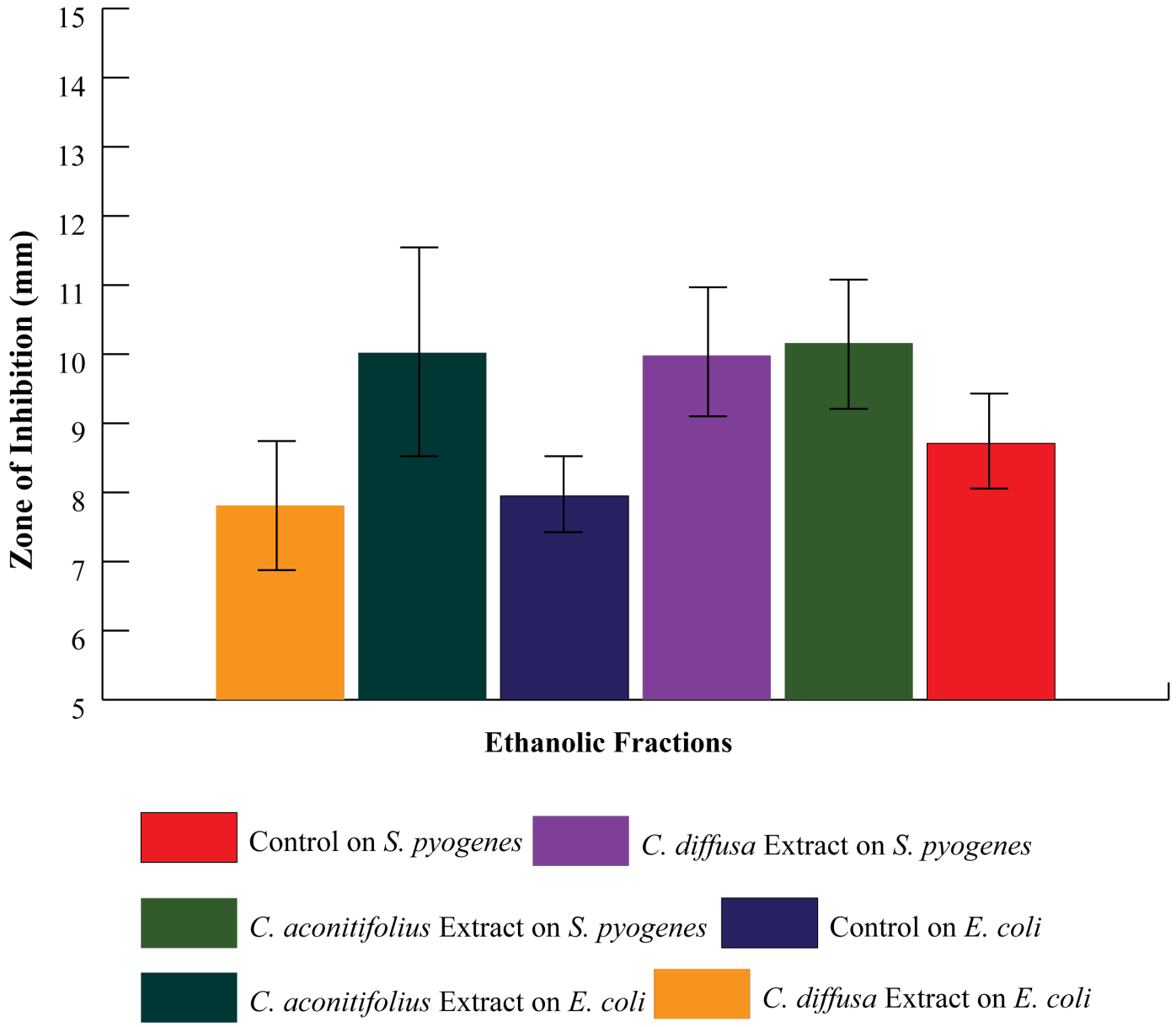

Figure 4: Effect of Ethanolic Fractions of *C. aconitifolius* and *C. diffusa* on *E. coli* and *S pyogenes*

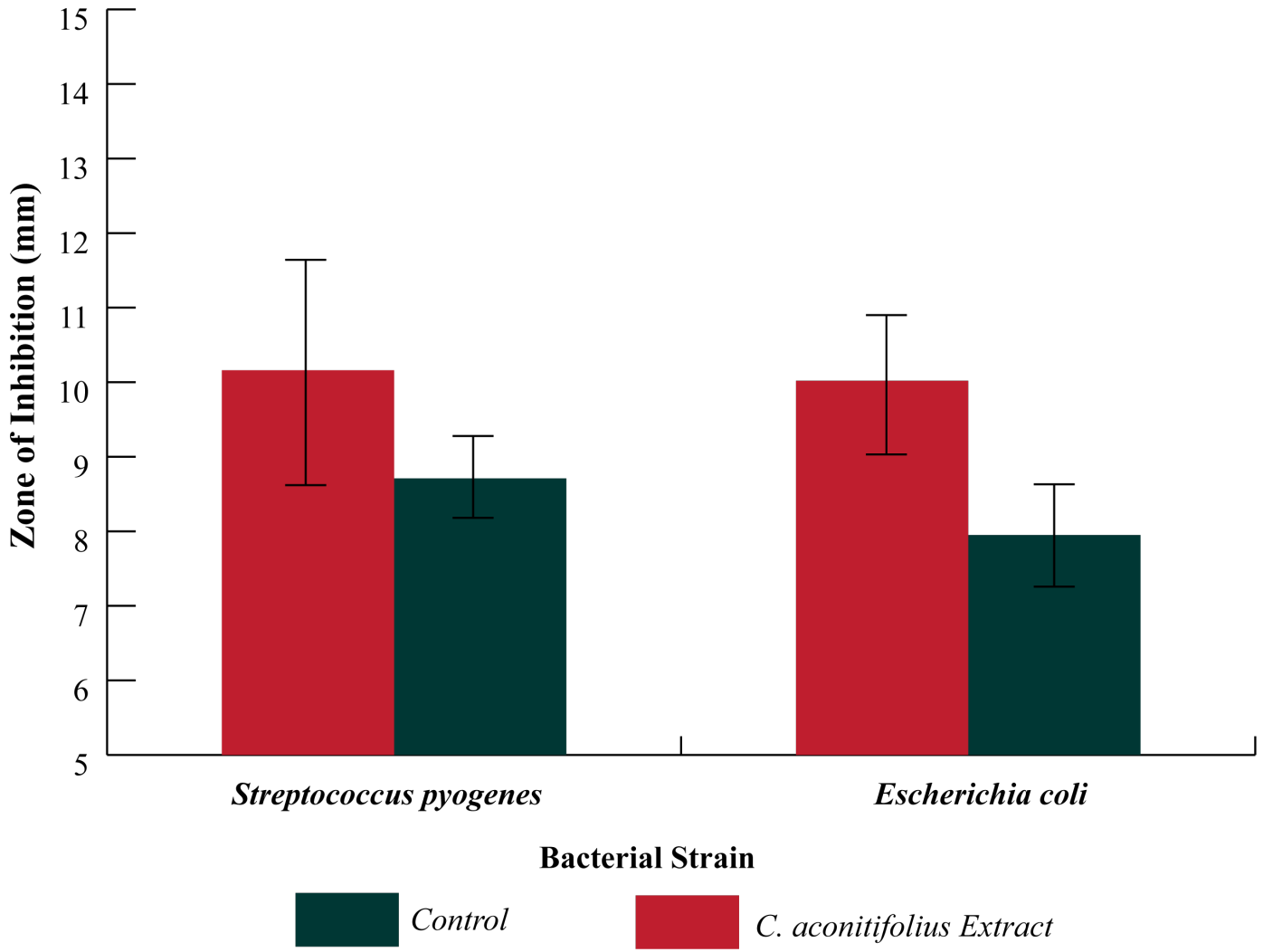

Figure 5: Effect of Ethanolic Fractions of *C. aconitifolius* on *E. coli* and *S pyogenes*

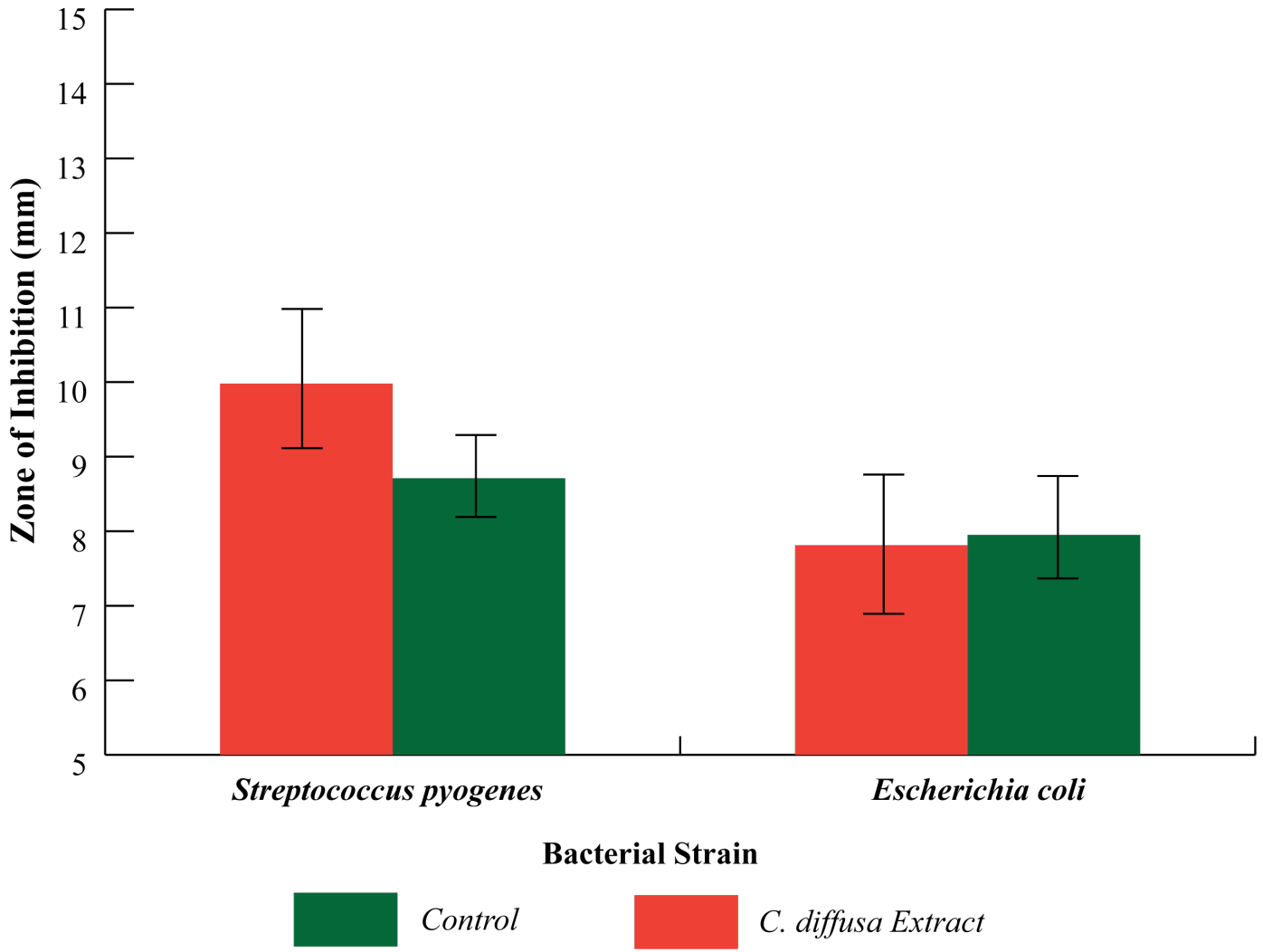

Figure 6: Effect of Ethanolic Fractions of *C. diffusa* on *E. coli* and *S pyogenes*

As was observed in Table 2m bacterial strains of *S. pyogenes* were more susceptible to both *C. aconitifolius* and *C. diffusa* ethanolic leaf extracts. The results of the antibacterial activity are presented in Figure 7.

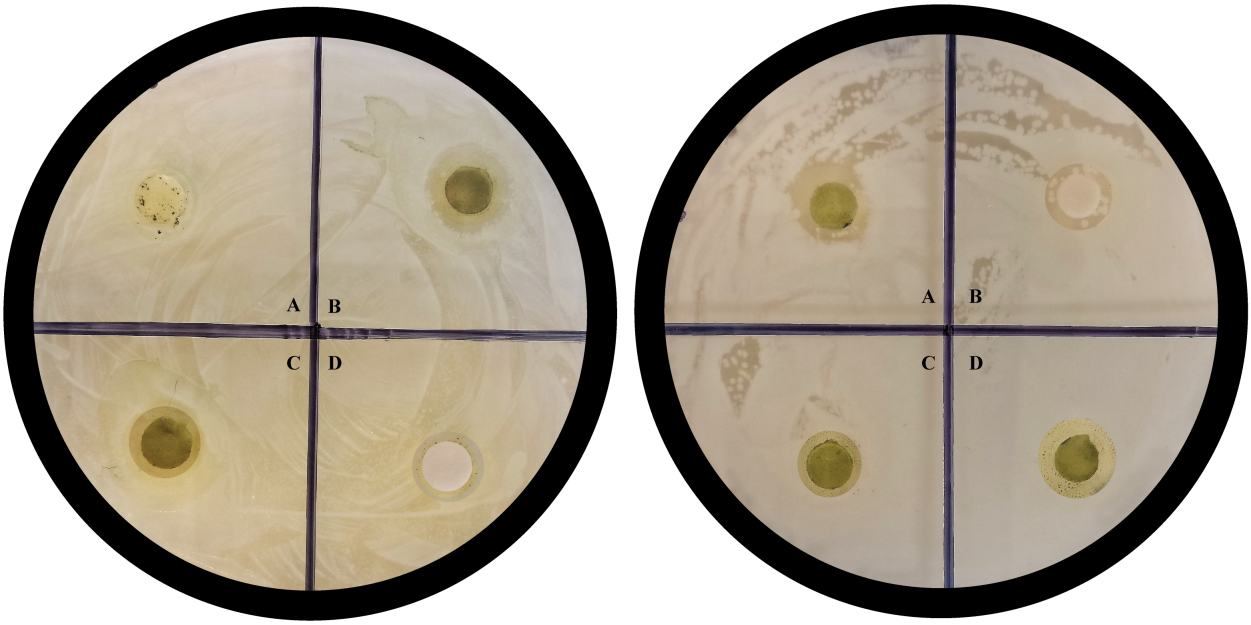

Figure 7: Antibacterial sensitivity assay; Left (*E. coli* - B = Disc impregnated with *C. aconitifolius* extract, C = Disc impregnated with *C. diffusa* extract, D = Negative Control (Disc soaked in Ethanol). Right (*S. pyogenes* - A,D = Disc impregnated with *C. aconitifolius* extract, C = Disc impregnated with *C. diffusa* extract, B = Negative Control (Disc soaked in Ethanol)

4.2 Thin Layer Chromatographic Analysis

Plants have been used since ages because of the antimicrobial and antioxidant properties due to various secondary metabolites that are synthesised in some or all its parts. Results of thin layer chromatographic analysis are summarized in Table 3 and 4. This investigation revealed the presence of various important phytochemicals in both plant extracts. These bioactive phytochemicals are the basis of therapeutic potential of medicinal plants and are useful in the treatment of several diseases. The medicinal significance of the plant could be attributed to the bioactive phytochemical compounds that generate characteristic physiological actions on humans.

Table 3: TLC profile of ethanolic extract of Chaya (*C. aconitifolius*) under daylight, UV and staining reagents

Spots	**Daylight**	**UV_{254}nm**	***p*-Anisaldehyde + H_2SO_4**	**R_f**
1	Brown green	Light purple	–	0
2	Light brown	–	–	0.12
3	–	Light purple	–	0.18
4	–	Light purple	–	0.28
5	Light green	–	–	0.30
6	Light green	–	–	0.36
7	–	–	Blue	0.46
8	Green	–	–	0.50
9	Light green	–	Purple	0.56
10	–	–	Pink	0.62
11	–	Brown	Violet	0.63
12	–	–	Dark green	0.70
13	Yellow green	–	Dark green	0.94
14	–	–	Light pink	0.94
15	–	–	Light purple	0.98

Table 4: TLC profile of ethanolic extract of Dayflower (*C. diffusa*) under daylight, UV and staining reagents

Spots	**Daylight**	**UV_{254}nm**	***p*-Anisaldehyde + H_2SO_4**	**R_f**
1	Green	Light brown	–	0
2	Light brown	–	–	0.12
3	Light green	–	–	0.30
4	Light green	–	–	0.36
5	–	–	Blue	0.46
6	Green	–	–	0.50
7	Light green	–	Purple	0.56
8	–	–	Green	0.62
9	–	Brown	Dark green	0.63
10	–	–	Dark green	0.84
11	Yellow green	–	Dark green	0.94
12	–	–	Green	0.94
13	–	–	Light purple	0.98

When the color of the spots from the TLC of the ethanolic extracts were observed under daylight, UV_{254} nm, and *p*-Anisaldehyde + H_2SO_4, fifteen spots appeared in different colors with

different R_f values ranging from 0 to 0.98 for the *C. aconitifolius* extract (sum of both eluent system). With the same treatment, and in identical experimental conditions ethanolic fractions of *C. diffusa* produced 13 spots of different colors and R_f values which ranged from 0 to 0.98. UV-active compounds were seen mostly in the 10% Methanol:Methylene Chloride TLC system (Figure 8).

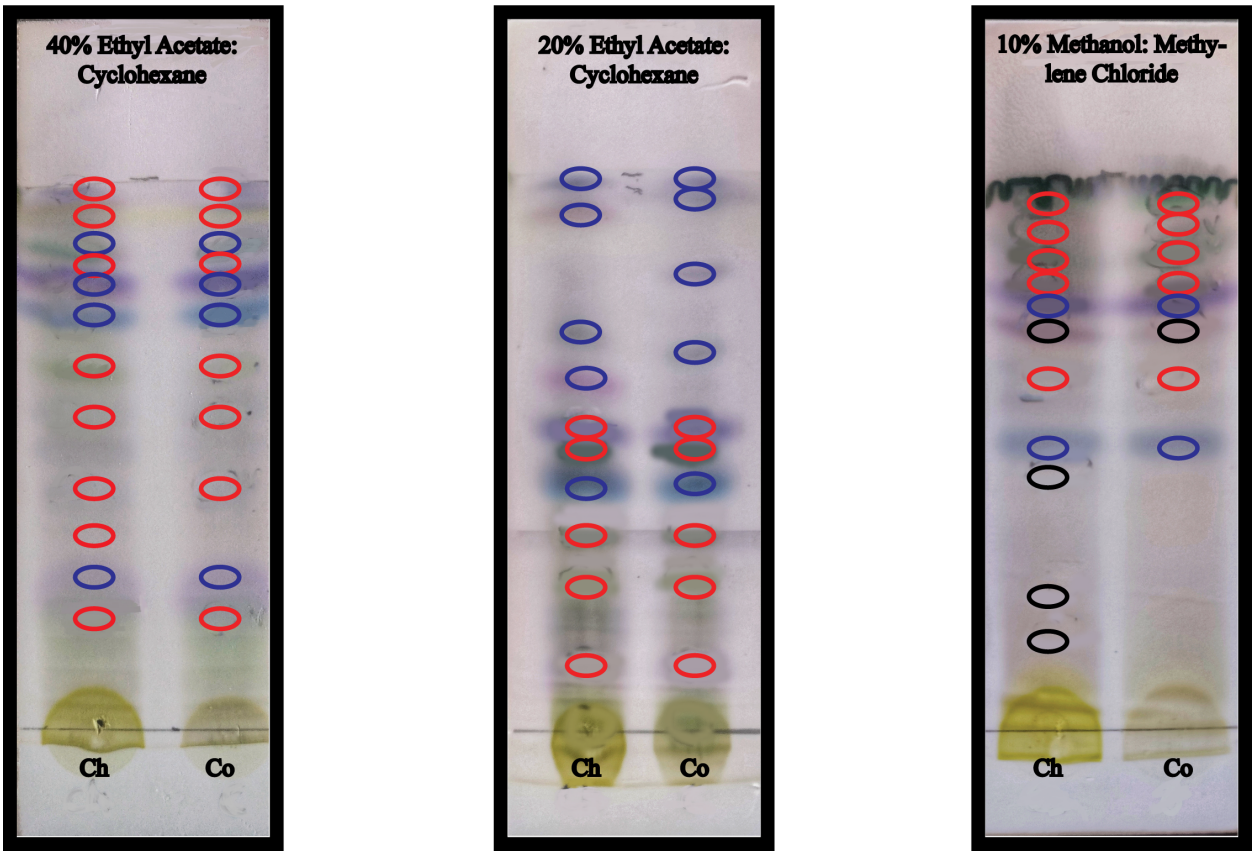

Figure 8: TLC profiling images of the two extracts with different solvent systems; Ch = Chaya (*C. aconitifolius*) extract, Co = Dayflower (*C. diffusa*) extract. Red circles indicate spots that were visible under daylight and after staining with *p*-Anisaldehyde, blue circles denotes spots that were visible only after the application of *p*-Anisaldehyde stain, black circles denote UV-active spots

p-Anisaldehyde is sensitive to most functional groups, especially those which are strongly and weakly nucleophilic. It tends to be insensitive to alkenes and alkynes. *p*-Anisaldehyde stains lichen constituents, phenols, terpenes, sugars, and steroids, which turn violet, blue, red or pink, and grey or green respectively when heated to 105°C. As depicted in Figure 8, several of these colored bands can be observed, indicating the presence of phytochemicals such as phenols and terpenes.

4.3 Statistical Analysis

Table 5: t-Test: Paired Two Sample for Means; E.c =*E. coli* (Control = 7.95), S.p = *S. pyogenes* (Control = 8.71), *p-value $\leq$ 0.05 is considered significant

	***C. aconitifolius* E.c**	***C. diffusa* E.c**	***C. aconitifolius* S.p**	***C. diffusa* S.p**
Mean	10.02	7.81	10.16	9.98
P(T$\leq$t) one-tail	**0.002***	**0.355**	**0.010***	**0.057**
t Critical one-tail	1.943	2.353	2.015	2.015
P(T$\leq$t) two-tail	0.004	0.711	0.020	0.114
t Critical two-tail	2.446	3.182	2.570	2.571

For the *C. aconitifolius* extract on *E. coli*; Null Hypothesis: There is no statistically significant difference between the means of the Zone of Inhibition of the *C. aconitifolius* extract and the Negative control, ethanol. Alternate Hypothesis: There is a statistically significant difference between the means of the Zone of Inhibition of the *C. aconitifolius* extract and the Negative control, ethanol. Given that p-value $0.002 < 0.05$, the null hypothesis is rejected, and we can conclude that there is a statistically significant difference between the means.

For the *C. diffusa* extract on *E. coli*; Null Hypothesis: There is no statistically significant difference between the means of the Zone of Inhibition of the *C. diffusa* extract and the Negative control, ethanol. Alternate Hypothesis: There is a statistically significant difference between the means of the Zone of Inhibition of the *C. diffusa* extract and the Negative control, ethanol. Given that p-value $0.355 > 0.05$, we fail to reject the null hypothesis, and conclude that there is no statistically significant difference between the means of the Zone of Inhibitions.

For the *C. aconitifolius* extract on *S. pyogenes*; Null Hypothesis: There is no statistically significant difference between the means of the Zone of Inhibition of the *C. aconitifolius* extract and the Negative control, ethanol. Alternate Hypothesis: There is a statistically significant difference between the means of the Zone of Inhibition of the *C. aconitifolius* extract and the Negative control, ethanol. Given that p-value $0.010 < 0.05$, the null hypothesis is rejected, and we can conclude that there is a statistical difference between the means.

For the *C. diffusa* extract on *S. pyogenes*; Null Hypothesis: There is no statistically significant

difference between the means of the Zone of Inhibition of the *C. diffusa* extract and the Negative control, ethanol. Alternate Hypothesis: There is a statistically significant difference between the means of the Zone of Inhibition of the *C. diffusa* extract and the Negative control, ethanol. Given that p-value $0.057 > 0.05$, we fail to reject the null hypothesis, and conclude that there is no statistically significant difference between the means of the Zone of Inhibitions. Comparison of the effectiveness of the extracts interspecifically and intraspecifically and the relative significance of the susceptible of each bacterial strain are provided in Table 6 below.

Table 6: t-Test: Two-Sample Assuming Unequal Variances; Intraspecific and Interspecific Variation between the Two Extracts. E.c =*E. coli*, S.p = *S. pyogenes*

	C. aconitifolius **E.c vs S.p**	***C. diffusa*** **E.c vs S.p**	***C. aconitifolius*** **vs** ***C. diffusa*** **E.c**	***C. aconitifolius*** **vs** ***C. diffusa*** **S.p**
P(T≤t) one-tail	**0.469**	**0.029***	**0.015***	**0.462**
t Critical one-tail	1.895	1.859	1.943	1.859
P(T≤t) two-tail	0.939	0.058	0.030	0.926
t Critical two-tail	2.364	2.306	2.447	2.306

In comparing the effects of the *C. aconitifolius* extract on *E. coli* and *S. pyogenes*, it was observed that there was no significant different between the means of the zone of inhibition cause by the extract on the two bacterial strains (the effective zone of inhibition on *E. coli* (10.02) and *S. pyogenes* (10.16)). This is so, despite the fact that larger zones of inhibition were generally observed on *S. pyogenes* than *E. coli*. However, given that p-value $0.469 > 0.05$, we can conclude that there is no statistically significant difference between the means of the Zone of Inhibitions.

In comparing the effects of the *C. diffusa* extract on *E. coli* and *S. pyogenes*, it was observed that there was a significant different between the means of the zone of inhibition cause by the extract on the two bacterial strains (the effective zone of inhibition on *E. coli* (7.81) and *S. pyogenes* (9.98)) Given that p-value $0.029 < 0.05$, we can conclude that there is statistically significant difference between the means of the Zone of Inhibition, and can also infer that the *E. coli* was less susceptible to the *C. diffusa* extract than *S. pyogenes*.

In comparing the effects of the *C. aconitifolius* and *C. diffusa* extract on *E. coli*, it was observed that there was a significant different between the means of the zone of inhibition cause by the two extracts (the effective zone of inhibition by *C. aconitifolius* (10.02) and *C. diffusa* (7.81)) Given that p-value $0.015 < 0.05$, we can conclude that there is statistically significant difference between the means of the Zone of Inhibition caused by the two extracts, and can also infer that the *C. aconitifolius* extract was more effective on *E. coli* than the *C. diffusa* extract.

In comparing the effects of the *C. aconitifolius* and *C. diffusa* extract on *S. pyogenes*, it was observed that there was no significant different between the means of the zone of inhibition cause by the two extracts (the effective zone of inhibition by *C. aconitifolius* (10.16) and *C. diffusa* (9.98)) Given that p-value $0.462 > 0.05$, we can conclude that there is no statistically significant difference between the means of the Zone of Inhibition caused by the two extracts.

4.4 Plants used in Folk Medicine

In folk medicines, different herbs and plants have been used for many thousands of years. However, it is also important to investigate such plants as potential alternatives to synthetics, since plant extracts are potential source of antibacterial compounds. This study showed *C. aconitifolius* and *C. diffusa* plant leaf extracts inhibited bacterial growth, but their effectiveness varied. Antibacterial activity has been attributed to the presence of different natural compounds. An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to rupture the lipid layer of the bacterial cell membrane and mitochondria, disturbing the cell structures and expose them for permeability. Heavy leakage from bacterial cells and excretion of vital molecules and ions will lead to death.

The presence of secondary metabolites in *C. aconitifolius* and *C. diffusa* is in line with earlier studies. According to the results of a phytochemical screening carried out by Adeniran *et al.* (2012), *C. aconitifolius* extracts showed the presence of tannins, saponins, cardiac glycosides, terpenoids and alkaloids. The presence of tannins support the use of the plant for its healing and anti-inflammatory properties. Results from the phytochemical screening carried out by Khan *et al.*

(2011), indicate that *Commelina* spp. showed positive inference in the test for alkaloids, ketonic compounds, and terpenoids. Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds of plant provide the protection against many microorganisms. The antibacterial activity of flavonoids may be due to their ability to make a complex with cell walls of bacteria, and with extra cellular and soluble proteins. Alkaloids are also known to have antimicrobial activity. The presence of saponins have also been attributed to many bactericidal, antiviral, cytotoxic, analgesic, and anti-inflammatory biological activities.

In the present study the ethanol extracts of leaves of *C. aconitifolius* and *C. diffusa* showed antibacterial activity against *E. coli* and *S. pyogenes*, which could be attributed to the presence of secondary plant metabolites. Generally speaking, gram-negative bacteria exhibit lower susceptibility to plant extracts, which is in accordance with the fact that they have a high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier. Recently, the increasing resistance rates of bacterial strains and control of the growth of pathogens are a big challenge. Developing more potent antibacterial agents using plant extracts is important in inhibiting the growth rate of pathogens.

Multi-drug resistant pathogens have been found to be sensitive to the crude plant extracts of *Commelina* spp. *Commelina* spp. were observed to contain bioflavonoids and phenolics, which are marker compounds for antimicrobial and antioxidant activities (Kuppusamy *et al*. 2014). The results of previous research revealed that extracts of *Commelina benghalensis*, and *Commelina nudiflora* parts may be useful as an alternative antimicrobial agent as natural medicine for the treatment of many infectious diseases. However, much like *C. diffusa*, further studies should be done on the fractionation and identification of their bioactive constituents (Khan *et al*. 2011a; Khan *et al*. 2011b; Kuppusamy *et al*. 2014).

The healing and anti-inflammatory activities popularly attributed to *Cnidoscolus* spp. are strongly associated with its tannin content (Araujo-Gomes *et al*. 2014). *Cnidoscolus* spp. also contain saponins, which have immense significance as an antihypercholesterol, hypotensive, and cardiac depressant agent. The various studies conducted on the antimicrobial properties of *C.*

aconitifolius justified the use of the leaf, as well as root and shoot extracts in traditional medicine to treat various infectious diseases (Awoyinka *et al.* 2007; Adeniran *et al.* 2012; Iwuji *et al.* 2016).

Thin layer chromatography is usually done for a better identification of the bioactive compounds. In the present study, the TLC profiling of all the plant extracts again revealed the presence of different plant metabolites. The different R_f values of the compounds provide an idea about the polarity of the plant constituents, which may also help in selecting a particular solvent system for further isolation of any compound from the plant extracts using chromatographic and spectroscopic techniques (Sonam *et al.* 2017). Compounds showing high R_f value in less polar solvent system have low polarity while those with a low R_f value have high polarity. Many species of Euphorbiaceae and Commelinaceae family have been commonly used in traditional and folk medicine. Hence, the present investigation on *C. aconitifolius* and *C. diffusa* plant extracts could be significant for the progress of new life preserving drugs. However, more advanced research is necessary to isolate and identify the bioactive compounds.

5 Conclusion and Recommendation

The results of the antibacterial sensitivity assay and phytochemical analysis indicate that *C. aconitifolius* and *C. diffusa* produce one (or several) secondary metabolites that confer antibacterial properties. Leaf extracts of *C. aconitifolius* exhibited higher antibacterial activity than *C. diffusa* on *E. coli*, however the antibacterial activity between *C. aconitifolius* and *C. diffusa* was not as statistically significant (p-value≤ 0.05). The results also indicate that *S. pyogenes* was more susceptible to the *C. diffusa* extract than *E. coli*. TLC profiling confirmed the presence of phenols, terpenoids, and other plant metabolites. Both plants showed antibacterial potential, supporting their ethnopharmacological use as an alternative to synthetics. However, advanced techniques of extraction, screening, identification, and isolation are needed to characterize the secondary plant metabolites, in order to see if they are new antimicrobial agents.

The study can be extended by using solvents of different polarities, different testing conditions, and different types of extraction procedures on various parts of the plants (including stems, roots, and flowers), and the respective extracts studied again on various pathogens, so as to find the best solvent for extraction, and the most active parts of the plant for optimal utilization of the plant crudely or for isolation of the active ingredients to serve as a template for synthetic drugs. The active components can be isolated, purified, and characterized to ascertain whether it is a new antimicrobial agent or not.

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