Phytochemical screening and antimicrobial efficacy of *Ocimum sanctum* (Tulsi) and *Swertia chirayita* (Chirota) against *Escherichia coli and Salmonella* spp. isolated from poultry and their molecular study

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Roll No.: 0119/01

Registration No.: 00630

Session: 2019-2020

A thesis submitted in the partial fulfilment of the requirements for the degree of

Master of Science in Biochemistry

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JUNE 2020

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This is to certify, that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have made

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June 2020

DEDICATED TO MY
RESPECTED AND BELOVED
FAMILY AND TEACHERS

Acknowledgements

I would like to express my thanks and grateful feelings for everyone that have helped and supported me in various ways during my degree of **Master of Science in Biochemistry** under the Department of Physiology, Biochemistry and Pharmacology, Chattogram Veterinary and Animal Sciences University. Completion of a master degree is not an easy task for me, and I cannot image myself getting this far without everyone's help and support. First of all, I would like to thank my supervisor **Prof. Dr. S.K.M. Azizul Islam** Dept. of Physiology, Biochemistry and Pharmacology**,** Chattogram Veterinary and Animal Sciences University for his supervision and guidance in successful completion of this work. He is also funny and has a lot of stories. But when talking about research, he is a little bit of tough and really has a high standard and is not easy to be persuaded. Thanks to his serious attitude about research. Also, thanks to my co-supervisor **Prof. Dr Shahneaz Ali Khan** including other MS mate Lipa, Hoimonti, Srijan and other junior Liza, Benozir in the department.

I become a better researcher and learned the ability of critical thinking, problem solving, and trouble shooting and am more confident to face any difficult tasks that may confront me in the future.

Secondly, I would like to thank all members in the department. Thanks for their help on the method development, equipment operation, and data analysis. I feel so lucky to be working with this group of people who are fun, creative, and nice, which makes my master degree much easier and much less miserable.

Last but not least, I would like to thank my family and friends who supported me through all my way. There have been happy times and tough times and I am very grateful that they are always there so that I know I am not alone. With their support and help, I am stronger and braver to conquer any projects and solve any problems in the past, now, and future.

The author

June 2020

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Abbreviations

CVASU	Chittagong Veterinary and Animal Sciences University
et al	*Et al*ii/ *et al*iae/ *et al*ia
etc	Et cetera
FAO	Food and Agricultural Organization
HCl	Hydrochloric Acid
HNO3	Nitric Acid
mg	Mili Gram
MIC	Minimum Inhibitory Concentration
MBC	Minimum bactericidal concentration
PCR	Polymerase Chain Reaction

Abstract

The present study was conducted to evaluate the effect of ethanolic extract of Tulsi and Chirota against *Salmonella* spp., *Staphylococcus aureus* and *E. coli* and isolation of plasmid from antimicrobial resistant bacteria from June 2020 to September 2021 at Chittagong Veterinary Animal Science University (CVASU), Bangladesh. Tulsi and Chirota were collected from a local market in Chattogram. Then both Tulsi and Chirota were then immersed in Ethanol (70%) for 3-time: 7 days, 14 days, and 21 days. After 21 days, all three batches of Tulsi and Chirota were conducted phytochemical screening for proven the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, phenolic compound, glycosides, carbohydrates, reducing sugar, protein and amino acid, acidic compound, phytosterol, steroids and terpenes. And further investigate the efficacy of ethanolic extract of *Ocimum sanctum* (tulsi) along with some other commercial antimicrobial including ciprofloxacin, amoxicillin, enrofloxacin, colistin and sulfamethoxazole. Three different concentrations of 0.2 mg/µL, 0.3 mg/µL and 0.4 mg/µL ethanolic plant extracts were treated against *E. coli*, *Salmonella* spp. isolated from poultry and commercially isolated Staphylococcus aureus. *Ocimum sanctum* extracts showed zone of inhibition at 0.2 mg/µl 14 days extract against *E. coli* (7-9mm), S*almonella* spp. (17-19mm) and *Staphylococcus aureus* (14-15mm), which is similar to the intermediary zone sensitivity of ciprofloxacin, sulfamethoxazole and amoxicillin. Within commercial antibiotics, ciprofloxacin, amoxicillin, enrofloxacin and sulfamethoxazole were 100% sensitive, whereas colistin were found to be 100% resistant. After that, plasmid isolation was done to observe the plasmid in those resistant bacteria. It was found that plasmid was present in the resistance bacteria that inhibited the activity of Tulsi. In summary, Tulsi ethanolic extract showed antimicrobial efficacy against *E. coli*, *Salmonella* spp. and *Staphylococcus aureus* which can be used as a substitute of commercial antibiotics.

Keywords: *Salmonella* spp., *E. coli*, Phytochemical screening, Sensitivity, Plasmid isolation, PCR

Chapter 1: Introduction

Nature has provided a complete storehouse of remedies to cure the ailment of humanity. About 80% of the world's population depends wholly or partially on traditional medicine for its primary health care needs (Kunwar and Adhikari, 2005). According to World Health Organization (WHO), the practitioners of the conventional system of medicine treat about 90% of patients in Bangladesh, 85% in Burma and 80% in India (Siddiqui, 1993). The medicinal plants are rich in secondary metabolites (potential drugs) and essential oils of therapeutic importance. The vital advantages of medicinal plants' therapeutic uses in various ailments are their safety, economic, effectiveness, and easy availability (Atal and Kapoor, 1989; Siddiqui, 1993). Plants are the wealthiest resource of drugs of traditional systems of modem medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer, 1999). Traditional medicine system has excellent value. Many medicinal plants have been identified from indigenous pharmacopoeias, because of which plants are still making an imperative contribution to healthcare regardless of modern medicines, which has many advantages (Adebolu, 2005).

Tulsi has been recognized as a prime herb in Ayurvedic treatment for thousands of years. In the last few decades, scientists and researchers have carried out several studies to suggest the role of essential oils and eugenol in the therapeutic potentials of *Ocimum sanctum* L. (Rajeshwari, 1992; Sen, 1993). Eugenol is a phenolic compound and major constituent of essential oils extracted from different parts of the Tulsi plant (Gupta, 2002; Khanna, 2003). The main chemical ingredients in this plant are eugenol, carvacrol, methyl eugenol and caryophyllene. One of the qualities that make the Tulsi plant such a potent medicinal herb is its ability to reduce stress. Tulsi is abundant in essential oils and antioxidants, which are tremendously effective in reducing the effects of stress on the body. Phytochemical analysis of this medicinal herb can identify the nature if compounds present in the extract of *Ocimum sanctum*. This study evaluated the antimicrobial activity of extracts from the different parts *Ocimum sanctum* plant against three pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus.*

Swertia chirayita belongs to the Gentianaceae family and contains many compounds responsible for its therapeutic properties such as xanthones, flavonoids, terpenoids, and iridoids secoiridoid glycosides (Pant *et al*.2000). It is an annual and biennial herb with medicinal properties such as anthelmintic, hypoglycemic, hepatoprotective, antifungal, antibacterial, anticarcinogenic, antipyretic, and neuroprotective antioxidants (Joshi and Dhawan, 2005). It is a vital ingredient in many Ayurvedic health tonics, supplements, anti-diabetic and anti-cancer preparations, liver tonics, skin creams, soaps, and hair oils. This species was first introduced in the Edinburgh Pharmacopoeia in 1839 and is reported in British and American Pharmacopeias to be used as an infusion or a tincture. This plant is used locally in Nepal as an infusion prepared by grinding it and steeping it in water overnight. In addition, *Swertia chirayita* has only recently been brought into cultivation with limited success.

The medicinal values of plants lie in their phytochemicals, which produce concrete physiological actions on the human body. Phytochemicals are compounds present in plants used as food and medicine to protect against illness and maintain human health (Afolabi, 2007). Phytochemicals have an antioxidant or hormone-like effect that helps fight against many diseases, including cancer, heart disease, diabetes, and high blood pressure, and prevent cancer on the target tissues (Daniel, 2011). Medicinal plants help cure many diseases and have always promoted the search for different extracts from plants which could act as a potential source of new antimicrobial agent (Anmad, 1998 and Bushra Beegum, 2003). Spice plants (a plant that accumulates piquant aromatic substances in various organs) have been used traditionally as coloring agents, flavoring agents, preservatives, food additives, and antiparasitic, anthelmintic, analgesic expectorant sedative, antiseptic and anti-diabetic substances in many parts of the world (Lee, 2004). In addition, they possess biological activities such as that of antioxidants (Miura, 2002) and hypercholesteremic (Craig, 1999).

Infectious diseases are the leading cause of death worldwide. Therefore, antibiotic resistance has become a global concern, but the emergence of multidrug-resistant pathogens threatens the clinical efficacy of many existing antibiotics (Westh, 2004). The increasing prevalence of multidrug-resistant strains of bacteria and the recent appearance

of strains with reduced susceptibility to antibiotics raises the spectre of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Silver, 1993). Even though pharmacological industries have produced several new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). According to World Health Organization (Santos, 1995), medicinal plants would be the best source to obtain various drugs. About 80% of individuals from developed countries use traditional medicine and compounds derived from medicinal plants. Therefore, such plants should be investigated to understand better their properties, safety and efficiency (Ellof, 1998). Despite potent antibiotic and antifungal agents, resistant or multi-resistant strains are continuously appearing, impeding the need for an endless search and development of new drugs, which are safe, more dependable than costly drugs and which have no adverse side effects (Silver, 1993). The essential advantages of medicinal plants' therapeutic uses in various ailments are their safety, economic, effectiveness, and easy availability (Atal, 1989; Siddiqui, 1993). More than 35,000 plant species are used in various human cultures worldwide for medicinal purposes. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections (Jantan, 1998).

The medical world is on an immense requirement to discover antibiotics due to wide spread emergence of resistance among microbial pathogens against currently available antibiotics. However, traditional plants have been proved to be better source for antimicrobial drugs (Hammer *et al*., 1999). Historically medicinal plants have been placed at top among the source of novel drugs with anti- microbial activity. These traditional medicinal herbs have made considerable contributions to human health. In addition, plants are considered as one of the most important sources of secondary metabolites and essential oils (Singh *et al*., 2010). On one hand, the use of medicinal plants proved to be economical and effective and they are also easily available and safe to use (Govind and Madhuri., 2006). Extracted essential oils have also been shown to contain biologically active constituents that are insecticidal fungistatic. The disc diffusion method is the commonly used technique to

check the antimicrobial activity. An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, all protozoans (Gadiyar *et al*., 2017).

The growing importance of antimicrobial resistance as a problem for food safety has been recognized by various international organizations (WHO, 2011). This problem is multifaceted and intersectoral, and cooperation and the exchange of information between the sectors of agriculture, veterinary, food production and public health appear to be essential. The globalization of trade, which depends on the movement of goods, animals and food products, means that resistant bacteria can become widely distributed and transferred to consumers around the world. Although new microbiological hazards are detected in food, *Salmonella* spp. remain one of the most common foodborne pathogens worldwide. These bacteria are prevalent in the environment, and are found in both domestic and wild animals as pathogens or commensals. They can infect humans, mainly via the contaminated food: chicken, pork, dairy products, eggs, fruits, vegetables and others (Yang *et al*., 2010). Antimicrobial resistance in Enterobacteriaceae poses a critical public health threat, especially in the developing countries. Much of the problem has been shown to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species (Blake *et al*., 2003). Resistant bacteria are transferred from food animals to man via the food chain. After the ingestion of contaminated food, commensal and pathogenic bacteria in the gut can exchange mobile genetic elements mediating resistance. Recent epidemiological studies have revealed that human infections with resistant *E. coli* and *Salmonella* spp. are associated with prolonged illness, an increased risk of invasive disease and hospitalization, and excess mortality (Molbak, 2004). In this study we evaluate the antimicrobial effects of ethanolic leaf extracts of *Ocimum sanctum* against pathogenic bacteria (*Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis*) to determine their potentials as antibacterial agent and isolate the plasmid from the resistant bacteria.

Specific Objectives:

1) To determine the phytochemical constituent of *Ocimum sanctum*, *Swertia chirayita*, *Swietenia macrophylla*, *Phyllanthus emblica* and *Kaempferia galanga*;
2) To assess the antimicrobial susceptibility of Tulsi against *E. coli*, *Salmonella* spp. *and Staphylococcus aureus*;
3) Molecular characterization of resistant bacteria by plasmid isolation and PCR

Chapter 2: Review of Literature

The use of medicinal plants in traditional medicine has been described in literature dating back several 1000 years (Chang *et al*., 2016). Books on Ayurvedic medicine, written in the Vedic period (3500–1600 B.C.), describe practices, including medicinal plants, that formed the basis of all other medical sciences developed on the Indian subcontinent (Pattanayak *et al*., 2010). In modern complementary and alternative medical practice, plants are the primary source of therapeutics and each part of the plant, including the seeds, root, stem, leaves, and fruit, potentially contains bioactive components. (Jiang *et al*., 2015; Mandave *et al*., 2014; Sun *et al*., 2014). Bioactive compound means having an effect on a living organism. It is a type of chemical found in small amounts in plants and certain foods (such as fruits, vegetables, nuts, oils, and whole grains). Bioactive compounds are: flavonoids, carotenoids, carnitine, choline, coenzyme-q, creatine, dithiolthiones, phytosterols, polysaccharides, phytoestrogens, glucosinolates, polyphenols, anthocyanins, prebiotics, and taurine The main bioactive components in medicinal plants are combinations of secondary metabolites which are generally defined as small organic molecules produced by an organism that is not essential for their growth, development and reproduction (Singh *et al*., 2010; Wu *et al*., 2016). There are many advantages and benefits associated with medicinal plants, the main ones being their cost-effectiveness and global availability. Compared to other medicinal products, their safety and the lack of significant side effects are other clear advantages (Niu *et al*., 2011). However, plant metabolism is very variable. Before medicinal plant extracts or products are approved for primary health care, they must be standardized, subjected to stringent quality control and assessed to ensure their safety (Mantri *et al*., 2012; Olarte *et al*., 2013). The emergence and spread of antibiotic resistance and the evolution of new strains of disease-causing agents are of great concern to the global health community. Effective treatment of a disease entails the development of new pharmaceuticals or some potential source of novel drugs. Our community's common medicinal plants could be an excellent source of drugs to fight off these problems (Shisir *et al.,* 2019).

2.1 Tulsi

Ocimum tenuiflorum, also known as *Ocimum sanctum*, Holy basil, or tulasī, is an aromatic plant in the family Lamiaceae, native to the Indian Subcontinent and widespread as a cultivated plant throughout the Southeast Asian tropics (Warrier, 1995). It is an erect, much-branched subshrub, 30–60cm tall with hairy stems and simple opposite green or purple leaves that are strongly scented. Leaves have petioles and are ovate, up to 5cm long, usually slightly toothed. It has been one of the most valued and holistic herbs used over the years in traditional medicine in India, and almost every part of the plant has been found to possess therapeutic properties (Singh *et al*., 2010). Traditionally, Tulsi is used in different forms; aqueous extracts from the leaves (fresh or dried as a powder) are used in herbal tea or mixed with other herbs or honey to enhance the medicinal value. Traditional uses of Tulsi aqueous extracts include the treatment of different types of poisoning, stomachache, common colds, headaches, malaria, inflammation, and heart disease (Pattanayak *et al.*, 2010). Traditionally, *Ocimum sanctum* L. is taken in many forms, such as herbal tea, dried power or fresh leaf. Several recent investigations using these extracts have indicated anti-inflammatory, antioxidant and immune-modulatory and antistress properties (Singh *et al.*,1996). In addition, it has been reported to have radioprotective and anti-carcinogenic properties. Several medicinal properties have been attributed to *Ocimum sanctum* L. (Sethi *et al.*, 2003). *Ocimum sanctum* L. is a general vitalizer and increases physical endurance. Different parts of *Ocimum sanctum* L., like leaves, flowers, stems, roots, seeds etc., are known to possess therapeutic potentials. *Ocimum sanctum* L. has been well documented for its therapeutic possibilities and described as antiasthmatic and antiseptic drugs. Indian Materia Medica describes the use of aqueous, hydro-alcoholic, and methanolic extract of *Ocimum sanctum* leaves in various disorders, like bronchitis, rheumatism and pyrexia (Nadkarni *et al.*,1976).

Table 2.1: Phytochemicals present in *Ocimum sanctum* plant (Pattanayak *et al*., 2010)

Part of the plant	Phytochemicals
Leaf	Flavonoids, alkanoids, saponins, tannins, phenols, anthocynins, terpenoids, steroils,
Steam	Phenols, saponins, flavonoids, triterpenoids, tannins.
Seeds	Fatty acids, sitosterol.
Whole plant	Flavonoids, alkanoids, saponins, tannins, phenols, anthocynins, flavonoids, triterpenoids, tannins.

2.1.1 Pharmacological Properties of *Ocimum sanctum*

As it has been traditionally used, the pharmacological properties of the whole herb in natural form result from the synergistic interaction of many different active phytochemicals. *Ocimum sanctum* contains volatile oil, eugenol, ursolic acid, carvacrol, linalool, limatrol, methyl eugenol, sesquiterpine, caryophyllene, estragole. The sugars are composed of xylose and polysaccharides (Pattanayak *et al.,* 2010). Phytochemical investigation of *Ocimum sanctum* stem and leaves have shown constituents like saponins, flavonoids, triterpenoids and tannins (Pattanayak *et al.,* 2010). *Ocimum sanctum* L. contains Vitamin C, A and minerals like calcium, zinc and iron, chlorophyll and many other phytonutrients (Anbarasu and Vijayalakshmi, 2007).

2.1.1.1 Anti-microbial activity

Ocimum sanctum shows antimicrobial activity. Ethanolic, methanolic, and organic solvents extracts of *Ocimum sanctum* L. show broad inhibition zones against *Escherichia coli, Staphylococci sp., Shigella sp., Staphylococcus aureus* and *Enterobacteria* spp. (Rahman *et al.,* 2010). *Ocimum sanctum* also acts against *Pseudomonas aeruginous, Staphylococci sp., Salmonella typhi, Klebsiella pneumonia,* Proteus, *Candida albicans, Mycobacterium tuberculosis and Micrococcus pyogenes* (Mishra *et al.,* 2011). These results prove that *Ocimum sanctum* can act as an excellent antimicrobial agent against many microbes.

2.1.1.2 Anti-fungal activity

Ocimum sanctum shows an important property like antifungal activity. Aqueous, Hexane, Chloroform, n-butanol and other solvent extracts of *Ocimum sanctum* showed antifungal activity. *Ocimum sanctum* acts against bio-deterioration of food stuff during storage. Aqueous and acetone extract of *Ocimum sanctum* L. were also sensitive to many plant fungi such as *Alternaria tenuis, Helminthosporium* sp. and *Curvularia penniseli*. Essential oil of tulsi was tested on various types of fungal pathogen such as; *Alternaria solani*, *Candida guillermondii*, *Colletotricum capsici*, *Curvularia* sp. *Fusarium solani*, *Helminthosporium oryzae,* showed positive results. Essential oil and eugenol were found to check the growth of *Aspergillus flavus* (Marja *et al.,* 1999). Hence, *Ocimum sanctum* essential oil and eugenol can be used as plant-based safe preservatives against fungal spoilage of food stuff during storage (Khan *et al.,* 2010).

2.1.1.3 Antioxidant effect

The aqueous, hydroalcoholic and methanolic extracts of *Ocimum sanctum* show significant antioxidant activity, both in-vivo and in-vitro (Kelm *et al.,* 2000). Phytochemical investigations of *Ocimum sanctum* leaf extract show phenols (eugenol, cirsilineol, isothymucin, apigenin and vosamarinic acid) and flavonoids (orientin and vicenin). These pharmacophores (abstract description of molecular features) have been shown to possess potent antioxidant activity (cyclooxygenase inhibitory). Oral feeding of *Ocimum sanctum* provides significant liver and aortic tissue protection from hypercholesterolemia induced peroxidative damage (Geetha *et al.,* 2004).

2.1.1.4 Antidiabetic effect

Oral administration of *Ocimum sanctum* extracts led to the marked blood sugar lowering in glucose-fed hyperglycemic and streptozotocin-induced diabetic rats (Chattopadhyay *et al.,* 1993). The constituents of *Ocimum sanctum* extracts have stimulatory effects on physiological pathways of insulin secretion, which may underlie reported antidiabetic action. Another study suggested that *Ocimum sanctum* decreased the serum concentration of both cortisol and glucose and also exhibited an antiperoxidative effect. Therefore,

Ocimum sanctum may potentially regulate corticosteroid-induced diabetic Mellitus (Gholap *et al.,* 2004).

2.1.1.5 Wound healing effect

Several studies showed the wound healing property of the *Ocimum sanctum*. Wound healing activity of cold aqueous extract of *Ocimum sanctum* leaves and its effect on tumour necrosis factor-Alfa (TNF-Alfa) was assessed using excision model of wound repair in Wistar albino rats. After applying the *Ocimum sanctum* extract, the rate of epithelization with an increase in wound contraction was observed (Shetty *et al.,* 2008). Ethanolic extract of *Ocimum sanctum* was investigated for normal wound healing and dexamethasone depressed healing using incision, excision and dead space wound models in albino rats. The extract of *Ocimum sanctum* significantly increased the wound breaking strength in the incision wound model. The extract treated wounds were found to epithelialize faster and the rate of wound contraction was significantly increased compared to control wounds. A significant increase in wet and dry granulation tissue weight, granulation tissue breaking strength and hydroxyproline content in the dead space wound model, was observed. The extract significantly decreases the anti healing activities of dexamethasone in all the wound models. The results indicated that the leaf extract promotes wound healing significantly and can overcome the wound-healing suppressing action of dexamethasone (Shetty *et al.,* 2008).

2.1.1.6 Hypolipidemic activity

Ocimum sanctum L. leaves decrease serum lipid profile in normal and diabetic animals. The essential oil extracted from *Ocimum sanctum* L. leaves has lipid-lowering against hypercholesterolemia. The aqueous extract decreased lipid peroxidation (LPO) formation and increased antioxidant enzymes in plasma and rat liver, lung, kidney and brain (Hussain *et al.,* 2001).

2.1.1.7 Anti-carcinogenic property

Banerjee *et al*, reported anticancer activity of *Ocimum sanctum* against many carcinogenic agents (Banerjee *et al.,* 1996). Juice of fresh leaves of *Ocimum sanctum* has anticancer properties in cancer subjects. Alcoholic extracts of *Ocimum sanctum* act on the activities

of an enzymes cytochrome P-450. Cytochrome b_5 and aryl hydrocarbon hydroxylase in liver and glutathione-S-transferase (GST) and a reduced glutathione level in liver and lung. These enzymes and cofactors play an essential role in detoxifying carcinogens and mutagens. *Ocimum sanctum* leaves, when fed to experimental rats for ten weeks, significantly reduced the squamous cell carcinoma and hematoma incidences (Aruna *et al.,* 1992). These results show that the anticancer activity of *Ocimum sanctum*.

2.1.1.8 Antigenotoxic effect

Ocimum sanctum leaf extract protects against heavy metals like Chromium, Mercury induced genetic damage (Babu *et al.,* 2006). *Ocimum sanctum* extract-treated human lymphocyte culture could reduce experimentally induced mitotic index, sister chromatid exchange and replication index in a dose development manner (Siddique *et al.,* 2007).
In-vitro assay in *Allium cepa* (onion) root tips cells has been carried out to detect the modifying effect of *Ocimum sanctum* aqueous leaf extract against Chromium (Cr), and Mercury (Hg) induced genotoxicity (the ability of harmful substances to damage genetic information in cells). It was observed that the roots post-treated with the leaf extract showed highly significant ($p < 0.001$) recovery in mitotic index (MI) and chromosomal aberrations (CA) when compared to pre-treated (Cr, Hg) samples and the lower doses of the leaf extract were found to be more effective than higher doses (Siva *et al.,* 2016).

Table 2.2: Pharmacological properties of *Ocimum sanctum*

Part of the plant	Activity
Leaves	Anti-stress, antichronic, anti-hypolipidemic, antioxidant, anthelmintic, anti-malarial activity (against plasmodium vivex), antifungal (against ring worm and also skin diseases), anti-fertility activity, anti-cancer (carcinogenic), antiviral activity (Mishra *et al.,* 2011; Khan *et al.,* 2010; Geetha *et al.,* 2004).
Root	Root act as a diaphoretic in malarial fever, anti-larvicidal (against to mosquitoes), antifungal (*aspergillus niger*) (Khan *et al.,* 2010).
Flower	Antispasmodic agent (as smooth muscle relaxant).
Stem	Genitourinary system disorders.

Seeds	Reduced blood and urinary uric acid level in albino rabbits.
Whole Plant	Control diabetes mellitus, anti-dot for dog bite, scorpion bite and insects bite (Godhwani *et al.,* 1998; Gholap *et al.,* 2004; Hannan *et al.,* 2006; Komal *et al.,* 2012).

2.2 *Swertia chirayita* (Chirota)

Swertia, a genus in the family Gentianaceae include a large group of annual and perennial herbs, representing approximately 135 species. Swertia species are common ingredients in several herbal remedies. In India, 40 species of Swertia are recorded (Clarke 1885; Kirtikar and Basu, 1984), of which *Swertia chirayita* is considered the most important for its medicinal properties.

Pharmacological data reported in literature suggest that *Swertia chirayita* treats several ailments. However, there is a lack of adequate information on the safety evaluation of the plant. The pharmacological usefulness of *Swertia chirayita* requires the need for conservation-friendly approaches in its utilization. Providing high-quality genetically uniform clones for sustainable use and saving the genetic diversity of this species in nature is essential. In this regard, plant biotechnological applications such as micropropagation, synthetic seed production, and hairy root technology can play a significant role in a holistic conservation strategy. In addition to micropropagation, storage of these valuable genetic resources is equally essential for germplasm preservation. However, more advanced research is warranted to determine the activities of bioactive compounds *in vitro* and *in vivo*, establish their underlying mechanisms of action and commence the process of clinical research.

2.2.2 Medicinal Uses

S. chirayita a traditional Ayurvedic herb, is used by different indigenous population groups in multiple ways for several medicinal purposes (Table 2.3). Local people widely use the whole plant to treat hepatitis, inflammation, and digestive diseases (Bhatt *et al*., 2006). The wide range of medicinal uses includes the treatment of chronic fever, malaria, anaemia, bronchial asthma, hepatotoxic disorders, liver disorders, hepatitis, gastritis, constipation, dyspepsia, skin diseases, worms, epilepsy, ulcers, scanty urine, hypertension, melancholia,

and certain types of mental disorders, secretion of bile, blood purification, and diabetes (Karan *et al*., 1999; Banerjee *et al*., 2000; Rai *et al*., 2000; Saha *et al*., 2004; Chen *et al*., 2011). Recently, *S. chirayita* extracts showed anti-hepatitis B virus (anti-HBV) activities (Zhou *et al*.,2015). Traditionally, decoctions (method of extraction by boiling herbal or plant material) of these species are used for anthelmintic, hepatoprotective, hypoglycemic, antimalarial, antifungal, antibacterial, cardio stimulant, antifatigue, anti-inflammatory, antiaging and antidiarrheal, as protectant of the heart and also help lower blood pressure and blood sugar (Schimmer and Mauthner, 1996). Herbal formulations such as Ayush-64, Diabecon, Mensturyl syrup, and Melicon V ointment (Edwin and Chungath, 1988; Mitra *et al*., 1996) contain *S. chirayita* extract in different concentrations forits antipyretic, hypoglycaemic, antifungal, and antibacterial properties.

Table 2.3: Ethnobotanical uses of *Swertia chirayita* in traditional medicine.

Plant part used	Traditional uses	References
Whole plant	Used in several traditional and indigenous systems of medicines, such as Ayurveda, Unani, and Siddha	Mukherji, (1953); Kirtikar and Basu, 1984; Joshi and Dhawan, (2005)
Whole plant	Used in British and American pharmacopeias as tinctures and infusions	Joshi and Dhawan, (2005)
Root	Serves as a drug and an effective tonic for general weakness, fever, cough, joint pain, asthma, and the common cold	Kirtikar and Basu, 1984; Joshi and Dhawan, (2005)
Whole plant	The leaves and chopped stems are soaked overnight in water for headaches and blood pressure. A paste is prepared and filtered with 1 glass of water. The preparation is consumed once a day for 2–3 days	de *et al*. (2014); Malla *et al*. (2015)

Whole plant	For Tremor fever, whole *S. chirayita* plants are cut into small pieces and boiled in 1/2 L of water until the volume is reduced to less than half glass. The filtered water is stored in a glass bottle, and half a spoon is given to children once a day for two days. For an adult, the posology is one spoon once in a day for two days and varies to three times a day until cured	de *et al*. (2014)
Whole plant	Boiled in water and one cup of decoction is taken orally to cure malaria	Shah *et al*. (2014)
Whole plant	Paste of the plant is applied to treat skin diseases such as eczema and pimples	Joshi and Dhawan, (2005); Malla *et al*. (2015)
Whole plant	Liver disorders; stomach disorders like dyspepsia and diarrhoea, intestinal worms	Mukherji, (1953); Joshi and Dhawan, (2005)
Whole plant	Hiccups and vomiting, ulcers, gastrointestinal infections, and kidney diseases	Kirtikar and Basu, (1984)
Whole plant	Used in combination with other drugs in cases of scorpion bite	Nandkarni, (1976)
Whole plant	Used in excessive vaginal discharge	Jadhav and Bhutani, (2005)

Furthermore, the curative value of this herb has also been recorded in ancient Ayurveda medicine systems and other conventional medical systems. The widespread uses of *S. chirayita* in traditional drugs have resulted in considerable chemical analysis of the plant and active principles that attribute its medicinal properties. *S. chirayita* is also used in British and American pharmacopeias as tinctures and infusions (Joshi and Dhawan, 2005).

The whole plant is used in traditional remedies, but the root is the most bioactive part (Kirtikar and Basu, 1984).

2.2.3 Phytochemistry of *Swertia chirayita*

The widespread uses of *S. chirayita* as a traditional drug and its commercialization in modern medical systems have led to a rise in scientific exploration of its phytochemistry to identify the active phytochemicals. This has resulted in a considerable body of literature exploring the chemical constituents of this plant (Mandal and Chatterjee, 1987; Chakravarty *et al*.,1991, 1994; Mandal *et al*., 1992; Chatterjee and Pakrashi,1995; Pant *et al*., 2000). The wide-range biological activities of *S. chirayita* are attributed to the presence of a diverse group of pharmacologically bioactive compounds belonging to different classes such as xanthones and their derivatives, lignans, alkaloids, flavonoids, terpenoids, iridoids, secoiridoids, and other compounds such as chiratin, ophelic acid, palmitic acid, oleic acid, and stearic acid (Pant *et al*., 2000; Patil *et al*., 2013).The first isolated dimeric xanthone was chiratan in present indifferent parts of *S. chirayita*. Amarogentin is reported to be anti-diabetic (Phoboo *et al*., 2013), anticancerous (Saha *et al*., 2006; Pal *et al*., 2012), and antileishmanial (Ray *et al*., 1996; Medda *et al*.,1999), whereas Swertiamarin ($C_{16}H_{22}O_{10}$) has been tested for its anti-hepatitis (Wang *et al*., 2001), anticancer (Kavimani and Manisenthlkumar, 2000), anti-arthritic activities (Saravanan *et al*., 2014). It has been shown to exhibit anti-diabetic (Vaidya *et al*., 2013) properties. Mangiferin is also reported to have anti-diabetic, anti-atherosclerotic (Pardo-Andreu *et al*., 2008), anticancer, anti-HIV (Guha *et al*., 1996), antiparkinson (Kavitha *et al*., 2013), and chemopreventive (Yoshimi *et al*., 2001) activities. Swerchirinis known to be antimalarial, hypoglycemic (Bajpai *et al*.,1991; Saxena *et al*., 1996), hepatoprotective, pro-heamatopoietic (Ya *et al*., 1999), with blood glucose lowering activity (Sekar *et al*., 1987; Saxena *et al*., 1991) and weak chemo preventive pharmacological effects (Hirakawa *et al*., 2005). Swerchirin at different concentrations (1, 10, and 100μM) significantly enhanced glucose-stimulated insulin release from isolated islets (Saxena *et al*., 1993). Sweroside is reported to be antibacterial (Siler *et al*., 2010), hepatoprotective (Liu *et al*., 1994; Luo *et al*.,2009), preventative in treatment for hyperpigmentation (Jeong *et al*., 2015), and is also suggested as a promising osteoporosis therapeutic natural product (Sun *et al*., 2013). Amaroswerin is known for its

gastroprotective effects of the bitter principles (Niiho *et al*., 2006). Table 2.4 provides a summary focusing on the biological activity of the phytochemicals present in *S. chirayita*.

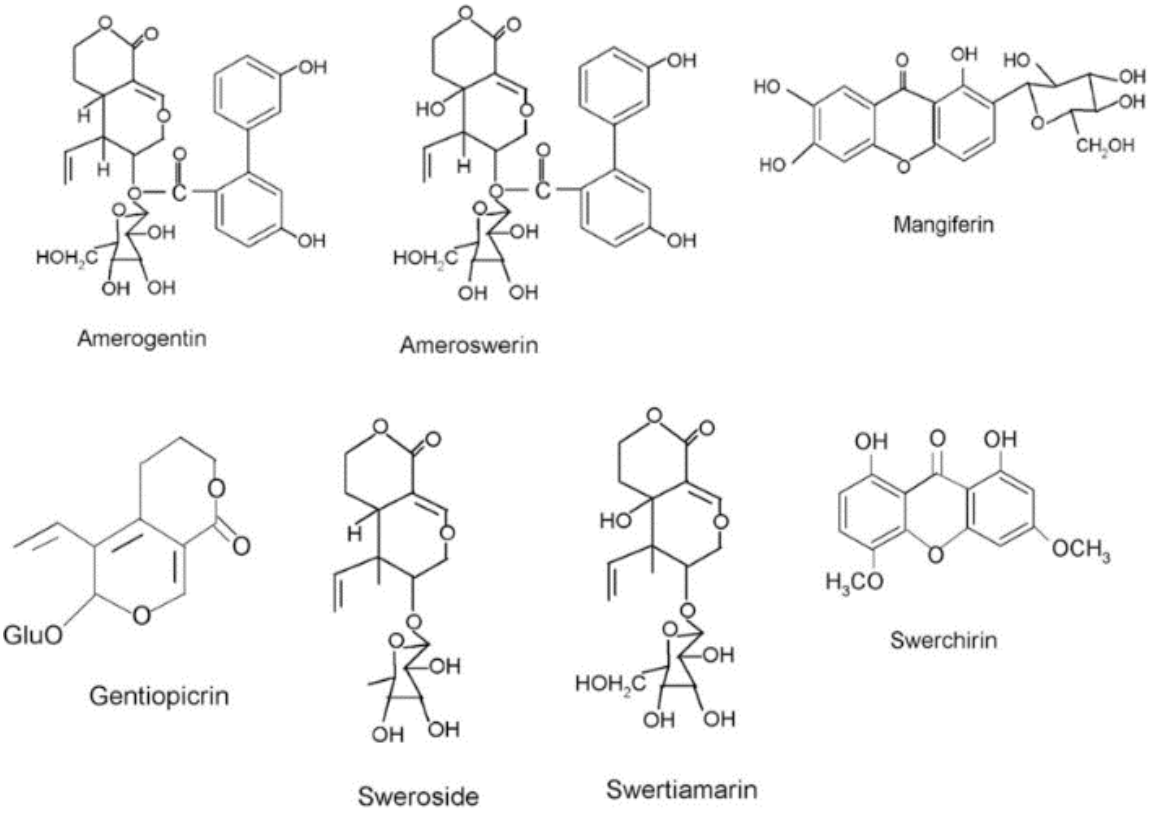

Figure 2.1 Chemical structures of phytoconstituents found in *Swertia chirayita*

Table 2.4 Important bioactive compounds isolated from *Swertia chirayita*.

Phytochemical	Biological activity	References
Amarogentin	Antileishmanial	Ray *et al*. (1996); Medda *et al*. (1999)
	Topoisomerase inhibitor	Ray *et al*. (1996)
	Anticancer	Saha *et al*. (2006); Pal *et al*. (2012)
	Anti-diabetic	Phoboo *et al*. (2013)

	Gastroprotective	Niiho *et al*. (2006)
Swertiamarin	Antiviral	Zheng and Lu, (1990)
	Immunomodulatory, antitumor, anti-HIV	Guha *et al*. (1996)
	Antioxidant	Sanchez *et al*. (2000)
	Chemopreventive	Yoshimi *et al*. (2001)
	Antiinflammatory	Kumar *et al*. (2003)
	Hypoglycemic	Muruganandan *et al*. (2005)
	Anti-diabetic, Antiatherosclerotic	Pardo-Andreu *et al*. (2008)
	Antiparkinson	Kavitha *et al*. (2013)
Swerchirin	Hypoglycemic	Bajpai *et al*. (1991); Saxena *et al*. (1996)
	Hepatoprotective, pro-heamatopoietic	Ya *et al*. (1999)
	Blood glucose loweringactivity	Sekar *et al*. (1987); Saxena *et al*. (1993)
	Chemopreventive	Hirakawa *et al*. (2005)
Sweroside	Antibacterial	Siler *et al*. (2010)
	Hepatoprotective	Liu *et al*. (1994);
	Hyperpigmentation	Jeong *et al*. (2015)
	Osteoporosis	Sun *et al*. (2013)
Amaroswerin	Gastroprotective	Niiho *et al*. (2006)
Gentianine	Antipsychotic	Bhattacharya *et al*. (1974)
	Antimalarial	Natarajan *et al*. (1974)
Oleanolic acid	Antimicrobial	Jesus *et al*. (2015)
	Antitumor	Soica *et al*. (2014)
	Antiinflamatory, antioxidant	Liu, (1995)
Ursolic acid	Antimicrobial	Jesus *et al*. (2015)
	Antitumor	Bonaccorsi *et al*. (2008)
Swertanone	Antiinflammatory	Kumar *et al*. (2003) Tabassum *et al*. (2012)

Syringaresinol	Hepatoprotective	Chakravarty *et al*. (1994)
Bellidifolin	Hypoglycemic	Basnet *et al*. (1995)

2.2.4 Pharmacological Activity of *Swertia chirayita*

The varied ethnobotanical uses of *S. chirayita* have led to various pharmacological investigations. Previous research demonstrates that the *S. chirayita* extracts exhibit a wide range of biological activities, such as antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, and others like antidiabetic and antioxidant activities (Verma *et al*., 2008; Alam *et al*., 2009; Arya *et al*., 2011; Chen *et al*., 2011; Laxmi *et al*.,2011). Concurrently, a diverse range of *in vitro* and *in vivo* test systems has been used to evaluate the pharmacological properties of *S. chirayita*. Evidence-based laboratory investigations indicate that aqueous, alcoholic and methanolic extracts of *S. chirayita* possess several promising pharmacological properties. The whole plant of *S. chirayita* has been reported to be used to treat antibacterial and antifungal activity (Alam *et al*.,2009; Laxmi *et al*., 2011; Rehman *et al*., 2011). The whole plant of *S. chirayita* has been reported for its anti-inflammatory and hypoglycemic activity (Banerjee *et al*., 2000; Kar *et al*., 2003; Alam *et al*., 2011; Das *et al*., 2012; Verma *et al*., 2013). Chen *et al*. (2011) investigated the 70% ethanolic extract of *S. chirayita* for antioxidant activities by using antioxidant tests, including reducing power and beta-carotene assay. The results showed that 70% of ethanolic extracts exhibited high DPPH (2,2-diphenyl-1-picrylhydrazy) scavenging activity (IC50=267.80μg/mL). Table 2.5 presents a summary focusing on the pharmacological evaluations using *in vitro* systems.

Table 2.5 Evaluation of the biological activities of *Swertia chirayita*

Bioactivity evaluated	**Plant part(s) tested**	**Extracting solvent**	**Test Organism/Models**	**Control**	**References**
Antibacterial	Whole plant	EtOH	*Escherichia coli* ATCC 26922 *Pseudomonas aeruginosa* ATCC 25619 *Proteus vulgaris* ATCC 6380	Ciprofloxacin	Rehman *et al*. (2011)
Antibacterial	Whole plant	MeOH	*Bacillus subtilis* MTCC 736 *Bacillus polymyxa Staphylococcus aureus* MTCC 3160 *Escherichia coli* MTCC 723 *Salmonella typhi* MTCC 3216	Gentamycin	Laxmi *et al*. (2011)
Antibacterial	Stem	MeOH	*Bacillus subtilis* ATCC 6633 *Enterococcus faecalis* (ATCC 14506) *Staphylococcus aureus* (ATCC 6538)	Ceftriaxone, Ceftriaxone sodium, Cefuroxine, Ciprofloxacin, Gentamycine, Levofloxacin, Metronidazole ,	Khalid *et al*. (2011)

			Salmonella typhi (ATCC14028)	Tranexamicac id	
Antibacterial	Whole plant	DCM; EtOH	*Staphylococcus aureus*	Kanamycin 30µg/disc	Alam *et al*. (2009)
Antileishmanial	Aerial part	95% EtOH	*Leishmania donovani* UR6	-	Ray *et al*. (1996)
Antileishmanial	Whole plant	MeOH	*Leishmania donovani* AG83	-	Medda *et al*. (1999)
Antihelmintic	Whole plant	Water; MeOH	*Haemonchus contortus*	Levamisole 0.55mg/ml	Iqbal *et al*. (2006)
Anti-hepatitis B virus	Whole plant	50% EtOH	HepG 2.2.15 cells line	Tenofovir	Zhou *et al*. (2015)
Hypoglycemic	Whole plant	95% EtOH	N/A	Mice treated with vehicle	Kar *et al*. (2003)
Egg hatchability and larvicidal	Whole plant	HEX; EA; MeOH	*Aedes aegypti Culex quinquefasciatus*	Tween-80	Balaraju *et al*. (2009b)
Antidiabetic	Whole plant	95% EtOH; HEX	STZ-NAD (streptozotocin-nicotinamide) induced diabetic albino mice	Metformin (100µg/kg)	Grover *et al*. (2002)
Antiviral	Leaves/Stem	Water	Herpes simplex virus type-1	Acyclovir (1 mg/mL)	Verma *et al*. (2008)

Swertia plants bioactivities involve Anticarcinogenic, Hepatoprotective, Antioxidant, Hypoglycemic, Anthelmintic, Antibacterial, Antifungal, Antidiabetic, gut and airway modulatory metabolizing isozymes inhibitory, neuroprotective, HIV-I reverse transcriptases inhibitory, anticholinergic, and central nervous system (CNS)-depressant activities. The excellent performance of Swertia species arouses interest in studying the determination of their active compounds alone by enhancing the activity of those

compounds. The bioactivities of extracts and compounds derived from the genus S.*chirayita* were listed in Tables 2.6

Table 2.6 Biological Activities of *S. chirata* Extracts

Fraction	**Activity**	**Description**	**Reference**
Aqueous Infusion	Anticarcinogenic	Activate four detoxification enzymes: GST, SOD and CAT	Saha *et al*. (2004)
N-Hexane Purified Extract (Alcohol) 70% Ethanol Extract	Hepatoprotective	Restore the elevated levels of serum GPT, GOT, ALP and bilirubin in peripheral blood serum, alone with reduction of SOD, GSH and GPx induced by paracetamol.	Nagalekshmi *et al*. (2011)
N-Hexane Chloroform Fraction	Hypoglycemic Effect	The optimum effect was found when given orally at 250 mg/kg	Bajpai *et al*. (1991)
	Gut And Airways Modulatory Effects	Gut excitatory and inhibitory effects were mediated through cholinergic and Ca^{2+} antagonist mechanisms, respectively, as well as bronchodilatation, via Ca^{2+} channel blockade	Khan *et al*. (2012)
Methanolic Extract	Antibacterial Activity	*Bacillus flexus, Bacillus subtilis, Bacillus megaterium, Lactobacillus rhamnnosus, Pseudomonas oleovorans, Klebsiella pneumoniae, Salmonella enterica, Clostridium perfringens,*	Roy *et al*. (2015)

		Staphylococcus aureus, Escherichia coli	
Dichloromethane (CH2Cl2) Extract	Antimicrobial Activity	Zones of inhibition against *Staphylococcus aureus, Bacillus cereus, Bacillus subtili*s and *Escherichia coli* were 19 mm, 17 mm, 13mm and 15–17 mm, respectively	Alam *et al*. (2009)

According to the literature cited above, it is apparent that plant has been one of the essential sources of medicines since the beginning of human civilization. Plants are antioxidants, antidiabetic, hepatoprotective, antitumor, antifungal, antibacterial, antiviral etc. A structured and systematic study is authentic to assess the appropriate efficacy of medicinal tulsi and Chirota plants.

2.3 Plasmid

Plasmids are extra-chromosomal, self-replicating, double-stranded, circular DNA molecules, usually found in bacterial cells. However, plasmids are not necessary for the survival of bacteria under normal conditions. *In vitro* techniques such as code transformation can modify naturally occurring plasmids. Plasmids are vectors used as vehicles to carry genetic information to a second cell. Plasmid genomes generally include a 'backbone' of core genetic loci, which are somewhat conserved amongst broadly related plasmids of the same family (Phan *et al*., 2009), and associated with vital plasmid-specific functions as replication and mobility.

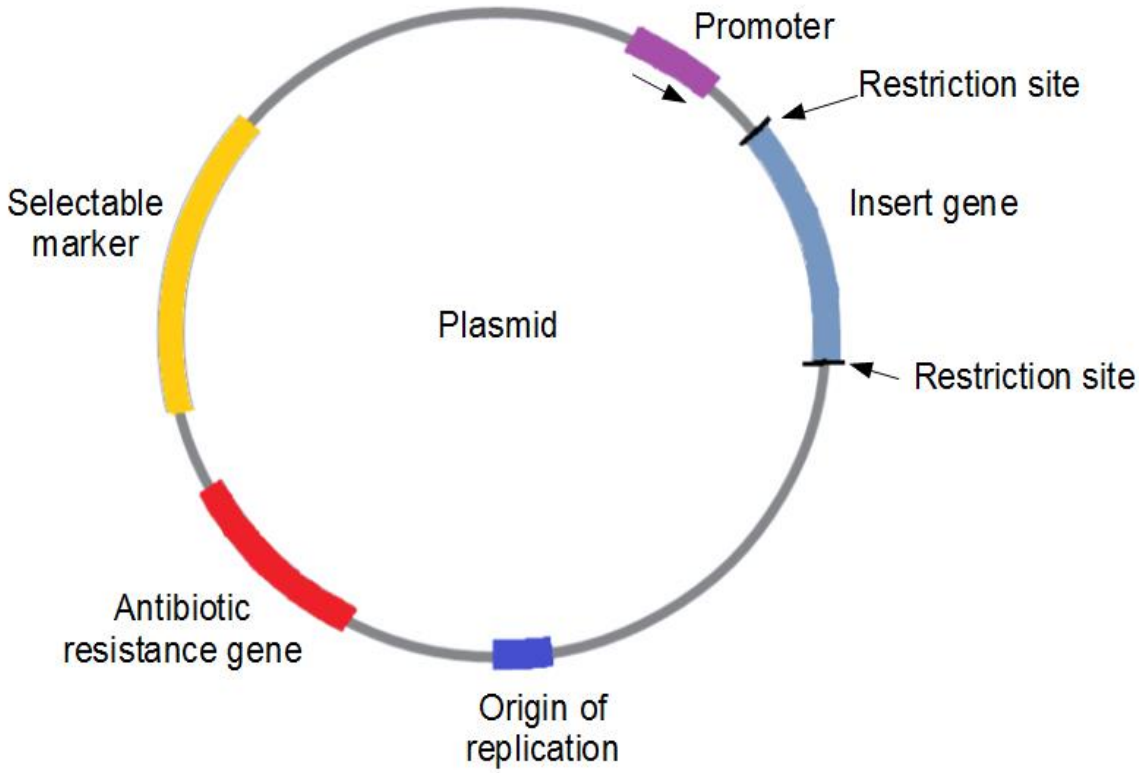

Figure 2.2 Plasmid structure

Accessory genes may also be present and often confer clinically relevant traits such as virulence and antibiotic resistance (Thomas and Summers, 2008). Plasmids can act as efficient vectors of horizontal gene transfer (HGT). Notably, during conjugation, a plasmid promotes its transfer (and that of a co-resident plasmid) from one bacterial cell to another (Norman et al., 2009). Therefore, accessory genes are frequently spread under being located on transmissible plasmids; moreover, they are commonly associated with more minor mobile elements such as transposons, facilitating intracellular mobilization amongst plasmids or to the chromosome (Stokes and Gillings, 2011). Due to their ability to transmit genes encoding adaptive traits across bacterial populations, plasmids can enable bacteria to evolve rapidly under environmental pressure (Heuer and Smalla, 2012). A striking example of adaptive bacterial evolution is that of antibiotic resistance, which is driven, in part, by the dissemination of resistance plasmids (plasmids conferring antibiotic resistance), and now threatens modern medicine (Carattoli, 2013; World Health Organization, 2014).

Classifying plasmids according to a typing scheme provides useful insights into the epidemiology of plasmid-mediated antibiotic resistance: for example, studying the composition of plasmid types can indicate whether an antibiotic resistance epidemic is driven by diverse plasmids or one dominant plasmid type (Valverde et al., 2009). In addition, hypotheses about resistance transmission during outbreaks can be refined according to the relatedness of resistance plasmids harboured by clinical strains (Pecora et al., 2015). The principal plasmid classification schemes are replicon based on backbone loci encoding plasmid replication and mobility functions (Carattoli et al., 2005; Garcillán-Barcia et al., 2009). While these single-locus typing schemes have been widely and successfully applied, they provide limited resolution (Fricke et al., 2009), restricting epidemiological inference: in an outbreak context, if two patients are infected by unrelated strains harboring resistance plasmids of the same type, this raises the possibility of plasmid transmission, but plasmid transmission cannot be conclusively ruled-in using single-locus plasmid typing alone; further higher-resolution investigation would be required (Foxman et al., 2005). If resistance plasmids are unrelated, plasmid transmission can be ruled-out, though a transmission link via resistance gene transposition is possible.

Plasmid typing may provide a stepping-stone to higher resolution analyses; identifying shared ('core') genes amongst related plasmids can inform the development of plasmid multi-locus sequence typing (pMLST) schemes (García-Fernández et al., 2011) or allow phylogenetic relationships to be reconstructed based on core gene single nucleotide polymorphisms (SNPs) (de Been et al., 2014). Unfortunately, determining high-resolution plasmid relationships is challenging: the tendency of plasmids to gain, lose and rearrange genetic content means sets of plasmids – even if of the same type – will tend to share few phylogenetically concordant core genes (Fondi et al., 2010; Tazzyman and Bonhoeffer, 2014), impeding subtyping and phylogenetic analysis (Maiden, 2006). Even backbone genes may not be well conserved across all plasmids of the same type (Lanza et al., 2014), and sometimes show mosaic phylogenetic origins (Sen et al., 2013).

Whole-genome sequencing (WGS) data can now be obtained for many bacterial isolates within short timescales at relatively low cost (Metzker, 2010). While sequencing reads from a bacterial isolate represent plasmid (s) and the chromosome, Whole genome

sequencing (WGS) based studies have often focused on the host strain chromosome as the unit of interest (Croucher and Didelot, 2015). For strain-level clinical surveillance to elucidate antibiotic resistance transmission routes, dissemination should primarily involve the clonal transmission of particular antibiotic-resistant strains. However, recent analyses indicate that plasmids may transmit between strains frequently, even over short timescales (Conlan et al., 2014; Sheppard et al., 2016). Therefore, the chain of transmission no longer corresponds to strain transmission; resistance plasmid dissemination across strains recruits different recipient strains into the outbreak, too, resulting in a 'plasmid outbreak'. Although difficulties in determining high-resolution plasmid relationships may limit insight, these dynamics mean that plasmid analysis across various strains is essential, including short-term surveillance studies (Adler and Carmeli, 2011).

2.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a new, popular molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. The technique allows a small amount of the DNA molecule to be amplified many times, in an exponential manner. With more DNA available, analysis is made much easier. PCR is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing. The technique was developed in 1983 by Kary Mullis, PCR is now a common and important technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel prize in Chemistry along with Michael Smith for his work on PCR (Bartlett and Bartlett, 2003). The PCR is commonly carried out in a reaction volume of 10-200 ml in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use an effect which permits both heating and cooling of the block holding the PCR tubes simply by

reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube. PCR can be used for Diagnosis of many human diseases, broad variety of experiments and analyses (Wang *et al*., 2012). PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000-fold higher than that of other methods (Rahman *et al*., 2013).

PCR also permits identification of non- cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non- pathogenic from pathogenic strains by virtue of specific genes. PCR is used to amplify a short, well-defined part of a DNA strand. This can be a single gene, or just a part of a gene. As opposed to living organisms, the PCR process can copy only short DNA fragments; usually up to 10 kb (kb stands for kilo base pairs). Certain methods can copy fragments up to 40 kb in size, which is still much less than the chromosomal DNA of a eukaryotic cell -for example, a human cell contains about three billion base pairs. (Rahman *et al*., 2013).

Chapter 3: Materials and Methods

The study was conducted for a period from October 2020 to July 2021 in the laboratory of Physiology, Biochemistry and Pharmacology Department at Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. *E. coli, Salmonella* and *Staphylococcus aureus* ATTC 29213 bacteria that were used in this experiment were collected from the Research Lab.

3.1 Experimental Design

The study was undertaken to evaluate the effects of ethanolic extract of Tulsi and Chirota at different concentration.

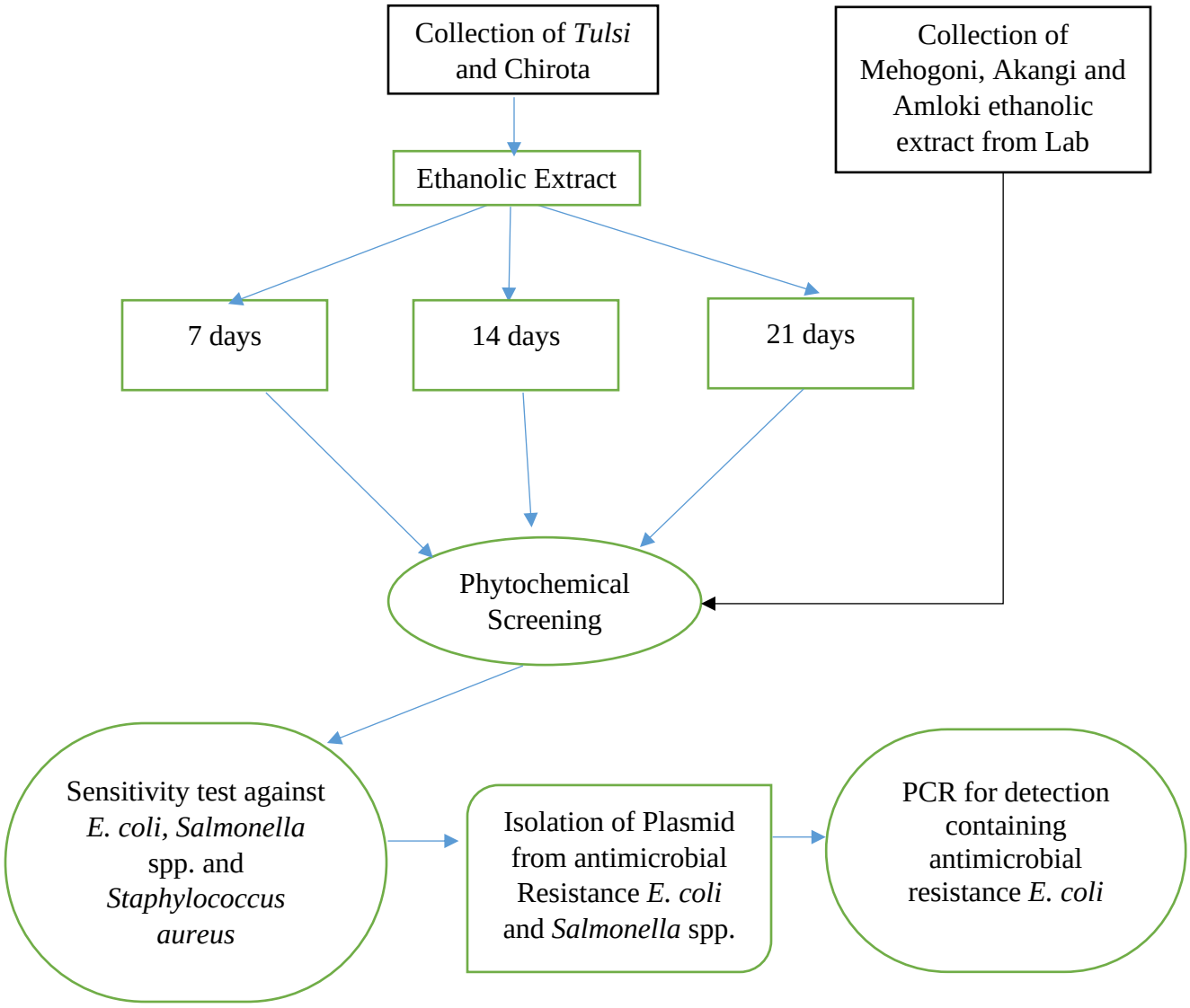

Figure 3.1 Flow chart of Experimental Design

3.2 Plants used in this study

Scientific Name	Local Name	Family	Plant part used
Ocimum Sanctum	Tulsi	Lamiaceae	Leaves
Swertia chirayita	Chirota	Gentianaceae	Whole plant

3.3 Collection of plant materials

Mature and disease free Tulsi were collected from DC hill Nursery in Chattogram district and Chirota were collected from CVASU.

3.4 Drying and grinding

Collected plant leaves were thoroughly cleaned by washing and discarding all the unwanted materials The aged leaves were discarded. The leaves were air dried at room temperature for ten days to remove moisture. The dried leaves were milled to fine powder with a blender, and then a sieve was used to obtain fine dust and preserved them into airtight plastic bag in the dark until used.

3.5 Preparation of ethanolic extracts

50 g of Tulsi and Chirota were taken into a bottle with 500 ml 80% ethanol. Then the mixture was stirred for 10 minutes and left for three periods. Seven days, 14 days and 21 days. The mixture was shaken and kept in a cool dark place every day overnight. After 7 days one batches of Tulsi and Chirota were taken for filtration. The whole mixture of Tulsi and Chirota was filtered separately through Whatman's Filter Paper No.1. Then the filtrates were taken into a round bottom flask of rotator vacuum evaporator. After the evaporation, the extracts were preserved in a petri plate and stored in a refrigerator (4°C)

3.6 Collection of *E. coli, Salmonella* spp. and *Staphylococcus aureus*

E. coli and S*almonella* spp. were collected from commercial poultry which were brought for treatment at the Department of Physiology, Biochemistry and Pharmacology. 1gm of liver was mixed with 9 ml of peptone water. Then incubate it for 24 hours at 37°C. After that MacConkey and Eosin methylene blue (EMB) agar was prepared. By inoculating loop, streaking was done in both agars. Then Incubate at 37°C overnight to observe the final outcome. After 24 hours large pink colony in MacConkey agar was indicative of *E. Coli.*

For *Salmonella* spp. incubate bacterial peptone water at 42°C and grow in the Xylose Lysine Deoxycholate (XLD) agar. Black centered red colony indicates the presence of *Salmonella* spp. Commercial *Staphylococcus aureus* was used in this test. The reference number is "ATTC *S.aureus* 29213".

3.6.1 Preparation of Stock

For the preparation of stock, 2-3 colony of bacteria was taken in a 5 ml of Brain heart infusion (BHI) broth which was freshly prepared. Then incubate the broth at 37°C overnight in incubator for bacterial growth. After that, 700 µL was taken from overnight culture and 300 µL of 50% glycerol was mixed in Eppendorf tube. Finally for long time preservation stored the Eppendorf tube at -40°C.

3.7 Phytochemical activity test

Phytochemicals present in the leaf extracts were screened as per standard protocols for phytochemical constituents such as alkaloids, glycosides, tannins, saponins, phenols, flavonoids, phenolic compound, steroids and terpenes,.

Phytochemical examinations were carried out for all the extracts of Tulsi, Chirota, akangi, amloki and mehogoni as per the standard methods (Sofowora, 1996).

3.7.1 Detection of Alkaloids:

3.7.1.1 Mayer's Test: The mayer's reagent was prepared by dissolving 1.36 g of mercuric chloride in 60 ml of distilled water. It added a solution of 5 g of potassium iodide in 20 ml distilled water. Both the solutions were mixed and diluted to 100 ml with distilled water. A few drops of the above reagent were added to a little of the test residue. The formation of cream-colored precipitate showed the presence of alkaloids.

3.7.1.2 Wagner's test: 1.27 g of iodine and 2 g of potassium iodide were dissolved in 5 ml of water and the solution was diluted to 100 ml with water. When few drops of this reagent were added to the test residue, reddish brown precipitate forms indicating the presence of alkaloids.

3.7.2 Detection of Flavonoids

3.7.2.1 Alkaline Reagent Test: Extracts were treated with 4-5 drops of sodium hydroxide solution. The formation of intense yellow colour, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

3.7.3 Detection of Saponins

3.7.3.1 Foam test: About 2g of the plant extract was mixed with 10ml of distilled water and shaken vigorously for a stable, persistent froth. The appearance of froth indicates the presence of saponins.

3.7.4 Detection of Tannins

3.7.4.1 Lead acetate test: 2ml of plant extract was combined with 2ml of distilled water. 0.01g lead acetate was added to this combined solution and shaken well. The development of white turbidity and precipitate indicates the presence of tannins.

3.7.5 Detection of Phenolic Compound

3.7.5.1 Ferric chloride test: About 2ml plant extract was taken to water and warmed at 45-50^{0}C. Then 2 ml of 0.3% $FeCl_3$ were added. The formation of green or blue color indicates the presence of phenols.

3.7.6 Detection of Glycosides

3.7.6.1. Cardiac Glycosides (Killer-Killani test): 1 ml of glacial acetic acid containing traces of ferric chloride and one ml of concentrated sulphuric acid were added to the test residue and observed for the formation of reddish brown colour at the junction of two layers. The upper layer turned bluish green in the presence of glycosides.

3.7.6.2. Anthraquinone Glycosides (Borntrager's test): 1 ml benzene was added to the test residue and 0.5 ml of dilute ammonia solution, no pink to red colour was formed due to the absence of glycoside.

3.7.7 Detection of Carbohydrates

3.7.7.1. Molisch's test: The Molish's reagent was prepared by dissolving 10 g of α-naphthol in 100 ml of 95% alcohol. A few mg of the test residue was mixed with 2 drops of Molisch's reagent. To this solution, 1 ml of concentrated sulphuric acid was added from the inclined test-tube's side. No reddish violet ring at the junction of the two layers appeared in the presence of sugars.

3.7.8 Detection of Reducing sugar

3.7.8.1 Benedict's Test: 0.5 ml of aqueous extract of the plant material was taken in a test tube. 5 ml of Benedict's solution was added to the test tube, boiled for 5 minutes and allowed to cool spontaneously. No red colour precipitate of cuprous oxide was formed in the absence of a reducing sugar.

3.7.8.2 Fehling's solution Test: A little of the test residue was dissolved in water, and a few ml of Fehling's solution was added to it. This mixture was then warmed. If a red precipitate of cuprous oxide was obtained, reducing sugars were present.

3.7.9 Detection of Protein and Amino Acid

3.7.9.1 Xanthoproteic test: To the 2 ml of extract, 0.5 ml of concentrated nitric acid were added by the side of the test tube. The absence of yellow color showed the absence of proteins and amino acids.

3.7.10 Detection of Acidic Compound

3.7.10.1 Sodium bicarbonate test: To the alcoholic extract sodium bicarbonate solution was added and observed for the formation of effervescence.

3.7.11 Detection of Phytosterol

3.7.11.1 Liebermann-Burchard's test: 2 mg of the extract was dissolved in 2ml of acetic acid anhydride, heated to boiling, cooled and then 1ml of concentrated sulphuric acid was added along the side test tube. A brown ring formation at the junction confirmed the test for phytosterols.

3.7.12 Detection of Terpenoids

3.7.12.1 Salkowski test: Approximately 2mg of dry extracts was shaken with 1ml of chloroform, and a few drops of concentrated sulfuric acid were added along the side of a test tube. A red-brown color formed at the interface indicated the test as positive for triterpenoids.

3.8 Antimicrobial discs

Each of the discs was cut from Whatman's No.1 filter paper with an approximate diameter of 6 mm using a puncher. The prepared filter paper disc was sterilized by autoclaving at 121°C for15 minutes and impregnated with 30µl extract of the experimental plant. The plant extract was dissolved in 0.2% DMSO (Di-Methyl-Sulp-Oxide) to prepare three different concentrations such as 0.2 mg/µL, 0.3 mg/µL and 0.4 mg/µL. Total six types of doses were prepared including 14 days and 21 days batch of these 3 concentrations.

3.9 Culture and sensitivity test

A total of 28 cultures of *E. coli* (n=14) and *Salmonella* spp. (n=14) were used for sensitivity test against plant extracts and commercial antibiotic such as ciprofloxacin, amoxicillin, enrofloxacin, colistin and sulfamethoxazole. This commercial antibiotic is generally used in poultry sector. The colonies were dissolved in PBS (Phosphate Buffer Saline to obtain the optimum turbidity against the 0.5 McFarland standard concentrations. After equivalating the turbidity, the bacterial culture was ready for sensitivity test.

3.10 McFarland Standard preparation

McFarland Standards were used as the reference in order to adjust the turbidity of the liquid/ bacterial suspension in the vial or tube in the microbiology laboratory. It helps to maintain and/or ensure that the number of bacteria will be within a given range to standardize microbial testing. The McFarland standard can be prepared of the varying concentration ranging from the 0.5 to 4 concentration and depending upon the concentration, the cell count density varies. However, the most commonly used concentration for the antimicrobial susceptibility testing and the culture media performance testing is usually done by 0.5 McFarland standard in the microbiological laboratories (McFarland, 1907).

0.5 Mcfardland turbidity standards were prepared by mixing 1% of sulfuric acid and 1% of barium chloride to obtain a solution with specific optical densities. To prepare a 0.5 Mcfardland standard, 0.05 mL of 1%$BaCl_2$ and 9.95 mL of 1% H_2SO_4 solution and stirred to maintain a suspension. A 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension $1.5x10^8$ colony-forming unity (CFU/ml). In the presence of good lighting, visually compare the turbidity of test suspension with that of the McFarland standard by comparing the clarity of the lines on the a Wickerham card. If the test suspension is too light, inoculate with additional organisms or incubate tube until turbidity matches the standard. For dilution, use a sterile pipette and add sufficient broth or saline to obtain turbidity that matches the standard (McFarland, 1907).

3.11 Preparation and components of Agar

Mueller-Hinton Agar (MHA)

To prepare 1000 mL of Mueller-Hinton agar (MHA), 10 g of tryptone, 15g of nutrient agar, 5 g of sodium chloride, and 5 g of yeast extract were weighed and added to the conical flask, 1000mL of distilled water was added and mixed. To dissolve all ingredients completely, boil for 10 minutes, sterilize by autoclaving for 15 minutes, and then subculturing the test microorganisms.

Blood agar

To prepare 1000 mL of Blood agar, 10 g of Casein, 15g of nutrient agar, 5 g of sodium chloride, and 2 g of yeast extract were weighed and added into the conical flask.1000mL of distilled water was added and mixed. To dissolve all ingredients completely, boiling for 10 minutes, and was sterilized by autoclaving for 15 minutes. Then add 50 ml of cow blood when the temperature decreases at 45-50°C and mix well. Then the agar is used for subculturing of the test microorganisms.

3.12 Antimicrobial Activity test of plant extracts

Agar Disc Diffusion Assay

The antibacterial activity test of crude extracts and fractionated compounds of experimental plant against *E. coli* and *Salmonella* spp. were carried out by disc diffusion method. The Sterile Muller Hinton agar was prepared for each organism as follows.

Twenty millilitres of sterile Muller Hinton agar (MHA) (maintained at 45-50°C in a molten state), poured into sterilized Petri dishes. After solidifying Muller Hinton agar, 0.1ml of test organism was spread on MHA plate. Then the filter paper discs were placed on the surface of the agar plate at an equal distance from each other and 15 mm from the edge of the plate as described by Clutter buck *et al*. (2007). Each disc was pressed down to ensure complete contact with the agar surface and each container was inverted and placed in an incubator set at 37°c for 24 hours. The antimicrobials susceptibility was evaluated after 24 hours by measuring zone of inhibition using plastic ruler in mm.

3.13 Plasmid Isolation

Plasmid DNA was extracted using an alkaline lysis method (Birnboim and Doly, 1979). The sample was resuspended in 100 µL ice-cold resuspension buffer [50 mM glucose, 25 mM TrisCl (pH 8.0), 10 mM EDTA (pH 8.0)]. Bacterial cells were lysed with 200 µL lysis solution [0.2 N NaOH, 1% (wt/vol) sodium dodecyl sulfate (SDS)] for 4 min and neutralized with 150 µL of chilled 3 M potassium acetate, pH 4.8. The samples were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant containing the plasmid was mixed with an equal volume of isopropanol and incubated at −20°C for 15 min. Samples were centrifuged at 14,000 rpm for 30 min at 25°C. The supernatant was removed and 500 µL of 70% ethanol was added to the pellet and centrifuged at 14,000 rpm for 5 min at 25°C. The pellet was resuspended in 50 µL MilliQ water. Plasmids were visualized on a 1% agarose gel stained with GelRed run at 70 V for 60 min.

3.14 PCR

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (Saiki *et al*., 1985). *Taq* DNA Polymerase is an enzyme widely used in PCR (Powell *et al.,* 1987). The following guidelines are provided to ensure successful PCR. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations, or amplicons greater than 5 kb may require further optimization. The followings were the primer sequences: (5'-ACA ACT TGG TGA CGA TGT TGT A-3') (Forward primers) and lpxARKK2m (5'-CAATCATGDGCDATATGASAATAHGCCAT-3') (Reverse primer). The 20 µl reaction mixture constituted 10 µl New England Biolabs*Taq* 2X Master Mix (containing *Taq* DNA Polymerase, dNTPs, MgCl2, KCI and stabilizers), 0.5 µl each forward and reverse primer and 2µ of DNA extract template 6 µl Nuclease free water. The thermal cycling included 95℃ for 5 min followed by 94 ℃ for 1 min (denaturation), 52 ℃ for 1 min (annealing), 72 ℃ for 1 min (extension) for 35 cycle and with final extension 72 ℃ 5 min. PCR products on the 1% agarose gel were visualized through ethidium bromide staining.100 bp DNA was used (New England BioLabs) as standard molecular ladder.

Procedure

This process assembled all reaction components on ice and quickly transferred the reactions to a thermocycler preheated to the denaturation temperature (95°C).

Component	**25 µl reaction**	**Final Concentration**
10X Standard *Taq* Reaction Buffer	2.5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	0.5 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	0.2 µM (0.05–1 µM)
Template DNA	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	1.25 units/50 µl PCR
Nuclease-free water	up to 25 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin. Transfer PCR tubes from ice to a PCR machine with the block preheated to 95°C and begin thermocycling.

The PCR process consists of a series of twenty to thirty-five cycles. Each consists of three steps

1. The double-stranded DNA has to be heated to 94-96°C in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are now single- strand only. Time 1-2 minutes up to 5 minutes. Also, Taq-polymerase is activated by this step.
2. After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 50°C below their melting temperature (45-60°C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Time 1-2 minutes.
3. Finally, the DNA-Polymerase has to fill in the missing strands. It starts at the annealed primer and works its way along the DNA strand. This step is called extension. The extension temperature depends on the DNA-Polymerase. The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. As a rule- of-thumb, 1 minute per 1 kbp

   The PCR product was identified by its size using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that consists of injecting DNA into agarose gel and then applying an electric current to the gel. As a result, the smaller DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing it with a DNA ladder, which contains DNA fragments of knows size, also within the gel (Rahman *et al*., 2013).

Chapter-4: Results

4.1 Phytochemical

Table 4.1: Qualitative phytochemical screening ethanolic extract of *Ocimum sanctum* (Tulsi) leaf

Phytochemicals	**7 Days extract**	**14 Days extract**	**21 Days extract**
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	-	-	+
Phenolic compound	+	+	+
Glycosides	+	+	+
Carbohydrates	+	+	+
Reducing sugar	-	+	+
Protein and amino acid	+	+	+
Acidic compound	+	-	-
Phytosterol	+	+	+
Steroids and terpenes	+	+	+

The phytochemicals analysis in *Ocimum sanctum* (Tulsi) leave ethanolic extracts in three different batches were summarized in Table 4.1. Various bioactive molecules were found in Tulsi leaf extract from the phytochemical screening.

Table 4.2: Qualitative phytochemical screening ethanolic extract of *Swertia chirayita* (Chirota)

Phytochemicals	7 Days extract	14 Days extract	21 Days extract
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	-	+	+
Tannins	+	+	+
Phenolic compound	-	+	-
Glycosides	-	+	+
Carbohydrates	+	+	+
Reducing sugar	+	+	+
Protein and amino acid	-	+	+
Acidic Compound	-	-	-
Phytosterol	+	+	+
Steroids and terpenes	+	+	+

The phytochemicals analysis in *Swertia chirayita* (Chirota) leave ethanolic extracts in three different batches were summarized in Table 4.2. In 14 days extract more bioactive molecules were found other than two batches in Chirota extract from the phytochemical screening.

Table 4.3: Qualitative phytochemical screening of *Swietenia* macrophylla (Mehogoni), Kaempferia galanga (Akangi) and Phyllanthus emblica (Amloki).

Phytochemicals	**Mehogoni**	**Akangi**	**Amloki**
Alkaloids	+	-	+
Flavonoids	+	-	+
Saponins	+	-	+
Tannins	+	+	+
Phenolic compound	-	-	+
Glycosides	-	-	+
Carbohydrates	+	+	+
Reducing sugar	+	-	+
Protein and amino acid	-	-	+
Acidic compound	-	-	-
Phytosterol	+	+	+
Steroids and terpenes	+	-	+

The phytochemicals analysis of mehogoni, akangi and amloki leaves ethanolic extracts were summarized in Table 4.3. In these three plants only amloki has more phytochemical molecules other than two plants from the phytochemical screening

4.2 Antimicrobial Sensitivity

A total number of 28 bacterial isolates of *E. coli* (n=14) and *Salmonella* spp. (n=14) were used for sensitivity test against plant extracts and commercial antimicrobial such as ciprofloxacin, amoxicillin, enrofloxacin, colistin and Sulfamethoxazole.

In the tested samples, *E. coli* was 20% sensitive to 0.2 mg/µL concentration in broiler species and 100% resistant to 0.3 mg/µL and 0.4 mg/µL concentration in all species showed in Table 4.4.

Table 4.4: Susceptibility of *E. coli* to Tulsi (n=14)

Species (n)	**Tulsi sample Concentration**					
	0.2 mg/µL		0.3 mg/µL		0.4 mg/µL	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
Broiler (10)	20%	80%	0%	100%	0%	100%
Layer (2)	0%	100%	0%	100%	0%	100%
Sonali (1)	0%	100%	0%	100%	0%	100%
Duck (1)	0%	100%	0%	100%	0%	100%

The antimicrobial susceptibility of *Salmonella* spp. on the tested sample was 17% sensitive in broiler specices on each concentration and layer and sonali was 100% resistant showed in Table 4.5

Table 4.5: Susceptibility of *Salmonella* spp. to Tulsi(n=14)

Species (n)	**Tulsi sample Concentration**					
	0.2 mg/µL		0.3 mg/µL		0.4 mg/µL	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
Broiler (12)	17%	83%	17%	83%	17%	83%
Layer (1)	0%	100%	0%	100%	0%	100%
Sonali (1)	0%	100%	0%	100%	0%	100%

In summary, a total of 2 samples of *E. coli* out of 14 and 2 samples of *Salmonella* spp. out of 14 showed sensitive against *Ocimum sanctum* extract (Table 4.6).

Table 4. 6: Agar Disc diffusion Result of *E. coli* and *Salmonella* spp.

Result	***E.Coli* (n)**	***Salmonella* spp. (n)**
Resistant	12	12
Sensitive	2	2
Total	14	14

Table 4. 7 : Zone of inhibition (mm) of *Ocimum Sanctum* ethanolic extracts against *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* at three different concentrations with 14 and 21 days extracts

Bacterial isolates		***E. coli* (n=2)**		***Salmonella* spp. (n=2)**		***Staphylococcus aureus* (n=2)**	
		Min-Max	**Mean±SD**	**Min-Max**	**Mean±SD**	**Min-Max**	**Mean±SD**
0.2 mg/µL	S_1	7-9	7.7±0.75	14-16	15±0.8	14-15	14.3±0.44
	S_2	8-9	8.4±0.49	17-19	18.1±0.87	16-17	16.6±0.44
0.3 mg/µL	S_1	0	0	5-7	6.1±0.87	11-13	12.2±0.75
	S_2	0	0	12-14	13.1±0.69	13-16	14.6±1.19
0.4 mg/µL	S_1	0	0	4.5-5	4.7±0.26	0	0
	S_2	0	0	8-9	8.5±0.49	11-14	12.5±1.23

S_1= 14 days, S_2= 21 days

Table 4.7 depicts that the highest zone of inhibition was found in the extracts of 14 days (S_1) and 21 days (S_2) having 0.2 mg/µL concentration against all three bacterial isolates. However, the concentration at 0.3 mg/µL and 0.4 mg/µL did not show any zone of inhibition against *E. coli* isolates. Other concentrations with different doses showed zone of inhibition with different diameters.

Table 4.8: The sensitivity and resistant pattern of different commercial antibiotics against *E. coli*, *Salmonella* spp. and *Staphylococcus aureus*

Antimicrobials	Zone of inhibition (in mm)																	
	Sample of *E. coli*					**Sensitivity**	Sample of *Salmonella* spp.					**Sensitivity**	Sample of *Staphylococcus aureus*					Sensitivity
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
Ciprofloxacin	27 **S**	28 **S**	31 **S**	31 **S**	30.5 **S**	100% **S**	23 **I**	24.5 **I**	25.5 **I**	27 **S**	26 **S**	40% **S** 60% **I**	22 **S**	22.5 **S**	23 **S**	24 **S**	25 **S**	100% **S**
Amoxicillin	17 **S**	18 **S**	19 **S**	19 **S**	18.5 **S**	100% **S**	20 **S**	20.5 **S**	20 **S**	21 **S**	21 **S**	100% **S**	15 **S**	15.5 **S**	15.5 **S**	16 **S**	15 **S**	100% **S**
Enrofloxacin	22 **I**	24 **S**	25 **S**	26 **S**	24.5 **S**	80% **S** 20% **I**	27 **S**	28 **S**	29.5 **S**	30 **S**	28.5 **S**	100% **S**	22 **I**	23.5 **S**	24.5 **S**	26 **S**	25.5 **S**	80% **S** 20% **I**
Colistin	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	100% **R**	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	100% **R**	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	100% **R**
Sulfamethoxazole	19 **S**	22 **S**	21.5 **S**	21 **S**	20.5 **S**	100% **S**	22 **S**	22.5 **S**	23.5 **S**	23 **S**	25 **S**	100% **S**	16 **S**	16.5 **S**	18.5 **S**	17 **S**	19 **S**	100% **S**

Note: S- Sensitive, I- Intermediary Sensitive, R- Resistant (CLSI, 2019)
Ciprofloxacin (S≥=26, I=22-25, R=≤21), Amoxicillin (S≥=15, I=12-14, R=≤11), Enrofloxacin (S≥=23, I=19-22, R=≤18), Sulfamethoxazole (S≥=16, I=11-15, R=≤10)

In table 4.5 reveals that amoxicillin and sulfamethoxazole showed 100% sensitive against *E. coli*, *Salmonella* spp. and *Staphylococcus aureus*. Colistin did not show sensitivity against all the isolates. Enrofloxacin showed 80% sensitive against *E. coli and Staphylococcus aureus*

4.3 Sensitivity Pattern

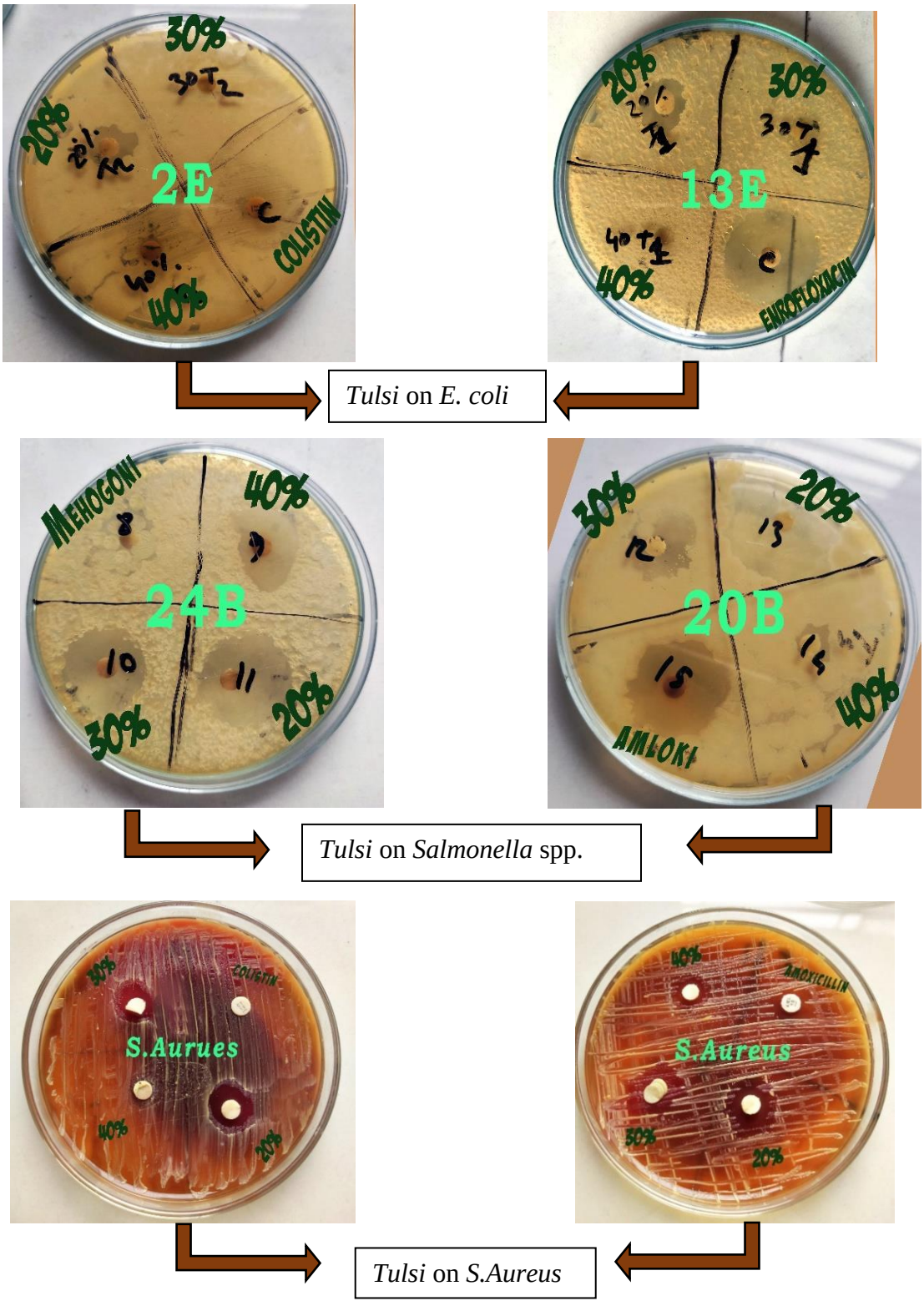

Figure 4.1: Sensitivity pattern of *E. coli*, *Salmonella* spp. and *S.Aureus* to Tulsi

4.4 Plasmid Identification

In order to determine whether *E. coli* and *Salmonella* spp. which showed resistant was plasmid encoded or not, extraction of plasmid was done on all bacterial isolates (14 samples from each species). Extraction plasmids were run on 1% agarose gel electrophoresis. These results showed that 28% *E. coli* and *Salmonella* spp. of the isolates contain plasmids, which molecular weight greater than 10 kbp in size as shown in the figure 4.2 and 4.3. These types of big plasmids indicates that it could contain lot of genes including antimicrobial-resistant ones (Huang *et al., 2012)*

Figure 4.2: *E. coli* Sample with ladder

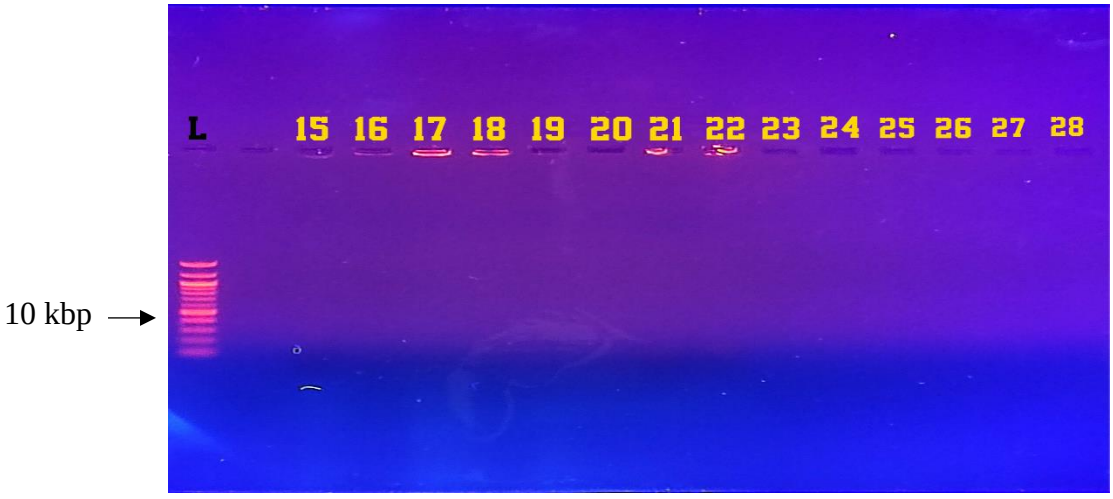

Figure 4.3: *Salmonella* spp. Sample with ladder

4.5 PCR for detection plasmid resistant bacteria

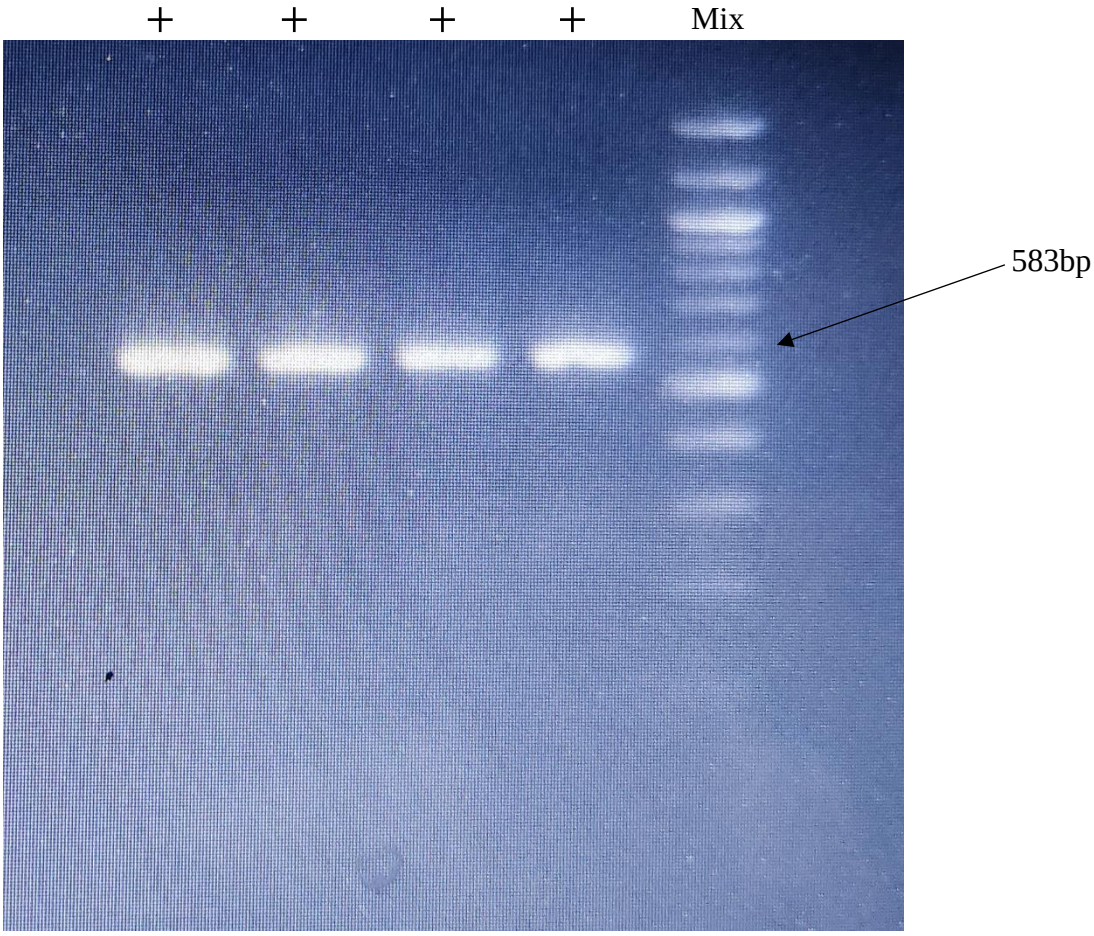

Figure 4.4: Agarose gel electrophoresis showing positive amplification of 583 bp fragments

Four resistant *E. coli* isolates were tested for PCR. Positive amplification of 583 bp fragments were detect all 4 given *E. coli* isolates.

Chapter 5: Discussion

In our knowledge, this is the first comprehensive study that exposed phytochemical screening of plant extracts such as *Ocimum sanctum* (Tulsi), *Swertia chirayita* (Chirota), *Swietenia macrophylla* (Mehogoni), *Kaempferia galanga* (Akangi) and *Phyllanthus emblica* (Amloki) and to tested their antimicrobial activity against *E. coli, Salmonella* spp. and *Staphylococcus aureus* with different commercial antibiotics such as ciprofloxacin, amoxicillin, enrofloxacin, colistin, sulfamethoxazole and isolation of plasmid from the resistant bacteria and finally confirming the *E. coli* with PCR.

The study reveals that various secondary metabolites such as alkaloids carbohydrate, tannin, flavonoids, saponins, phytosterol, glycoside and terpenoid are presents in these leaf ethanolic extract. Leaves of *Ocimum sanctum* contain water-soluble phenolic compounds and various other constituents, such as eugenol, methyl eugenol and caryophylllene that may act as an immunostimulant. Saponins act as anti- hyperlipedemic, hypotensive and cardiodepessive properties (Bairwa *et al*., 2012). The phytochemical constituents such as alkaloids, steroids, flavanoids, tannins, phenols and several other aromatic compounds of plants serve as a defense mechanism against predation by many microorganisms, insects and other herbivore (Bonjar *et al*., 2004). Glycosides can act as cardiostimulants in case of cardiac failure (Sood *et al*., 2005). Tannins have antidiarrheal and haemostasis properties (Asquith *et al*., 1986). Flavanoids are responsible for antioxidant and immunostimulatory properties. Tulsi oil showed significant anti-inflammatory, analgesic, antipyretic and antimicrobial effects. It has also shown memory enhancing, antifertility, anticataract, antithyroid, antiulcer, antidiabetic, antiarthritic, antiamnesic, antihelmenthic, anticataract, hepatoprotective and no tropic activity (Rajesh *et al*., 2013). Alcoholic extract increased step down latency and acetyl cholinesterase inhibition and so used in the treatment of cognitive disorders. According to Cragg *et al*., 1999 and Khanna *et al*., 2003; alkaloids, glycosides, flavanoids and saponins are antibiotic principles of plants and these antibiotic principles are actually the defensive mechanisms of the plants against pathogens.

Leaves extract of *O. sanctum* affected both specific and non-specific immune responses and disease resistance against fungal and bacterial infection (Santra *et al*., 2017). It

stimulated both antibody response and neutrophil activity. The experimental studies have shown that ethanolic extract of *Ocimum sanctum* has antibacterial effect. The use of medicinal plants acts as a source of antimicrobial agent. *Ocimum sanctum* has been widely employed in traditional medicines. Hence phytochemicals from this plant can be used in variety of disorders troubling mankind. The herbs are cheap, available in large quantity around us and they pose no danger to the living organisms, the environment and the consumers and hence greatly helpful for living organisms (Raynor *et al*., 2011).

In recent decades, the intensification of animal production due to the increasing demand for products of animal origin has led to an increasing overall use of antimicrobials. In addition, the number of antimicrobials used also increases when specific diseases are being targeted or to prevent the spread of a particular disease, or in times of stress (Studdert *et al*., 1998).

While the prudent use of antibiotics is important to treat animal diseases, its overuse and misuse can contribute to antimicrobial resistance. The availability of antimicrobial drugs for therapeutic use in terrestrial animals is essential for animal health, welfare and productivity and contributes to food security, food safety and public health – and so in turn to the protection of livelihoods. The growing resistance to antimicrobial drugs could reverse these benefits; animals' resistance to antimicrobial drugs, makes treatments ineffective, increased severity of disease, reduces productivity and leads to economic losses (Parle and Bansal, 2006).

Considering the public health effects and prevention of the antimicrobial resistance in commercial poultry operation, potency and antimicrobial property screening of *Ocimum sanctum* were performed. Antimicrobial screening of *Ocimum sanctum* showed the highest zone of inhibition in 14 days (S_1) and 21 days (S_2) having 0.2 mg/µL concentration than other doses. According Vashney *et al.*, (2012), the highest zone of inhibition achieved against *E. coli* was 12 mm which is higher than the current study (9 mm). The highest zone of inhibition achieved by Vashney *et al.*, (2012) against *Salmonella* spp. was 30 mm which is higher than the current study (19). Here they also achieved a higher zone of inhibition in lower concentrations compared to the current study. In the current study, the extract of 14

days (S_1) of 0.2 mg/µL concentration gave a higher zone of inhibition than any other extract against *Salmonella* spp. According to Bishnu *et al.,* (2009) the highest zone of inhibition was found 6 mm at 0.25 mg/µL which is lower than the current study (19 mm at 0.2 mg/µL). Another study Praveen *et al.,* (2012) showed that the zone of inhibition of *S. aureus* found 22 mm which is higher than the current study (17 mm). The disc containing 0.3 mg/µL and 0.4 mg/µL failed to create any zone of inhibition against *E. coli* isolates. These variations might occur from the difference in the quality of *Ocimum sanctum* leaves or extraction efficacy.

The commercial antibiotics amoxicillin and sulfamethoxazole showed 100% sensitive against *E. coli, Salmonella* spp. and *Staphylococcus aureus*. Enrofloxacin showed 80% sensitive against *E. coli and Staphylococcus aureus.* But Colistin did not show sensitivity against all the isolates. The resistant might be the malpractice usage of commercial antibiotics in poultry. Many factors may have contributed to such level of resistance, including inappropriate use of antibiotics by health professionals, unskilled practitioners and over the counter availability of antibiotics etc. In Bangladesh, it is a common practice that antibiotics can be purchased without prescription, which leads to misuse of antibiotics by the public, thus contributing to the emergence and spread of antimicrobial resistance in community also (Islam, 2008). The current study has shown that some bacterial isolates showed 100% resistant which is similar to the Cardoso *et al.,* 2006 who found 100% resistant in *Salmonella* spp. and *E. coli.* Variation in the resistance pattern of antimicrobials might be caused by differences in bacterial isolates and types of antimicrobials uses and geographical location of the farms.

We investigated the resistance pattern of 14 isolates of *E. coli* and 14 isolates of *Salmonella* spp. isolated from poultry. This result suggests that the extent of resistance is to an antibiotic is associated with the extent of antibiotic use (Bebora *et al*.,1997; Mastour *et al*., 1999). *E. coli* isolated for the poultry farm was highly resistant to colistin even at 150µg/ml which could be the result of misuse and repeated use of colistin for the promotion of growth in the poultry farm (Allen *et al*., 1993). High resistance rate was also noted against ciprofloxacin and penicillin could be associated with the misuse of them as reported by Ginns *et al,* 1999. All of the isolates found multiple antibiotic resistance as reported

earlier Zhao *et al*. (2005); Guerra *et al.* (2003); Khan *et al*. (2002); Mulamattathil *et al*. (2000); Kariuki *et al*. (1999). In this study resistance of bacteria was found in *E. coli* and *Salmonella* spp. isolates. Plasmid DNA was extracted from both these isolates by using an alkaline lysis method. We analyzed randomly fourteen isolates of each for the presence of Plasmid DNA and among these four of them of each bacterial isolates which is 28% of total samples found to contain high molecular weight plasmid (greater than 10 kbp in size) with high resistant rate as reported by John *et al*. (2002). Our study concludes that multiple resistant *E. coli* isolates and plasmid containing multidrug resistant genes are present in the poultry farms and the poultry may act as a possible source of transfer of these highly resistant pathogens and their genes to human as reported previously Wooley *et al.* (2007), Mastour *et al*. (1999), we concur with the calls to ban the use of antibiotics for growth promotion and treatment in the poultry sectors.

For accurate diagnosis of some disease with more sensitivity and specificity PCR is a very common and widely accepted method now a days throughout the world and it is also gaining popularity in Bangladesh. Many advanced medical centers, modern diagnostic labs and medical institutions are using PCR as routine lab diagnostic and research modalities

All four resistant plasmid from *E. coli* isolates were tested for PCR. Positive amplification of 583 bp fragments were detect all 4 given *E. coli* isolates. That confirm that the isolates were *E. coli.*

Strength of the study

- Determine zone of inhibition on both gram positive and gram negative isolates.
- Extraction was done in 2 different plants of 3 batches such as 7 days, 14 days and 21 days.
- PCR was done to confirming the resistant bacterial isolates.

Limitations of the study

- Specific compound of the extract was done measured.
- Sample size was small.
- *In vivo* experiment was not done.
- Human pathogen was not introduced.

- Human clinical trial could not conduct to confirm its effectiveness.
- Budget was limited.

Chapter 6: Conclusion and Recommendation

The presence of various bioactive compounds in the *Ocimum sanctum* and *Swertia chirayita* extract justifies the uses for various ailments by living population. The obtain results from the phytochemicals confirmed the use of *Ocimum sanctum* and *Swertia chirayita* plant as traditional medicinal properties and also that some of the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused various pathogens. It is more beneficial to use tulsi as an herbal medicine as compare to chemically synthesized commercial drug. In this study tulsi plants extract showed a variable degree of sensitivity against *E. coli, Salmonella* spp. and *Staphylococcus aureus.* Whereas, different degrees of concentration and doses of the tulsi extract showed almost same zone of inhibition like certain commercial antimicrobials. That might be possible because *Ocimum sanctum* may contain compounds which are almost similar to the active compounds of commercially available antimicrobials.

Specific compound of the plant extract by Gas Chromatography-Mass spectrometry analysis should be conducted to measure the quantitative analysis of plant extracts. Large bacterial sample size should be taken for more accurate results. *In vivo* experiment should be done. Along with poultry bacterial isolates, antimicrobial susceptibility should be test from human pathogen against plant extract because human pathogenic microorganisms have developed antibiotic resistance. The existence of microbial strains with reduced susceptibility to antibiotics and increased number of antibiotic-resistant bacterial strains can be caused by indiscriminate use of broad-spectrum antibiotics.

It is suggested that future studies should include a broader range of antibiotics and a larger number of microbial spp. all over the country. Further study is warranted to determine the Whole Genome Sequence of resistant plasmid to know the gene responsible for the resistant.

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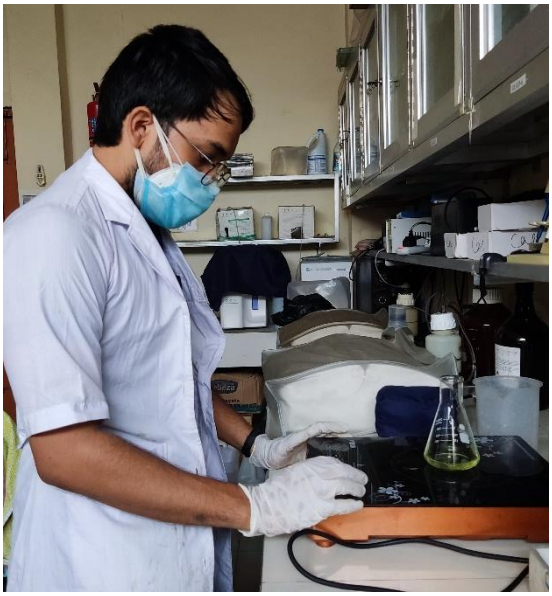
Appendices

Measuring Ethanol

Weighing

Rotary Evaporator



Heating Agar

McFarland Standard preparation

Applying sample in Bio-Safety cabinet

Blood Agar media

Measure Zone of inhibiton of sensivitiy bacteria

Brief Biography

Sajib Roy passed the Secondary School Certificate Examination in 2011 and then Higher Secondary Certificate Examination in 2013. He obtained his B.Sc. (Hon's) in Food Science and Technology from the Faculty of Food Science and Technology of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Now, He is a candidate for the degree of Master of Science in Biochemistry under the Department of Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University (CVASU). He has immense interest in clinical and molecular Biochemistry