Investigation of *in-vitro* Cytotoxic and Antibacterial Activity of Methanol Extract of *Crotalaria verrucosa* Leaves

A project submitted
by
Tasnova Nowrin
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This work is dedicated to my parents and sisters for their love and support.

Certification Statement

This is to certify that this project titled “Investigation of *in-vitro* Cytotoxic and Antibacterial Activity of Methanol Extract Of *Crotalaria verrucosa* Leaves” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University and this project is the result of the author’s original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the project paper itself.

Signed,

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Countersigned by the supervisor,

_____

Acknowledgement

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Abstract

Crotalaria verrucosa(*C. verrucosa)* has been traditionally used as a medicinal plant in Bangladesh for treating inflammation, skin diseases as well as heart diseases etc. Extracts of the various parts of this plant also possess therapeutic effects such as antipyretic, anti-diabetic, CNS depressant, antifertility and many more. Leaf extract of this plant contains alkaloids, flavonoids, glycosides, tannins, phytosterols, steroids and resins. The objective of this study was to evaluate *In vitro* cytotoxic and antibacterial activity of methanol extract of this plant. In this study, cytotoxic activity study was performed by MTT assay at different concentrations (2.5 mg/mL, 0.25 mg/mL, 0.025 mg/mL, and 0.0025 mg/mL) of plant extract to determine its cytotoxic effect using HeLa cell line. The cytotoxicity test revealed that the highest cell growth inhibition (93.2%) was shown at the highest concentration of 2.5mg/mL of this methanol extract whereas the lowest cell growth inhibition was shown at the two lowest concentrations of 0.0025 mg/mL and 0.025 mg/mL extract of this plant. The IC_{50} value of *C. verrucosa* methanol extract was 0.83 mg/mL. This test finding showed that the cell growth inhibition was concentration dependent. With increasing concentration of plant extract, cytotoxic activity also increases gradually. The antibacterial activity of this plant extract was determined by disc diffusion method against 3 bacterial strains such as, *Bacillus cereus*, *Streptococcus pneumoniae* and *Shigella dycenteriae.*A standard antibacterial drug, Kanamycin was used. The inhibitory zones were recorded in millimeters. The highest concentration 350 µg/disc of this plant extract showed a significant result against *Bacillus cereus* and the zone of inhibition was 17 mm whether kanamycin showed an inhibiting zone of 31mm. The leaf extract contains alkaloids, flavonoid, glycosides and tannins which may exhibit the potential cytotoxic and antibacterial activity in this experiment.These findings indicate the possibility of using traditional plant extracts in the treatment of bacterial infections as well as in cancer treatment and the results of this study was encouraging, to be sure the need for pre-clinical and clinical studies to determine of the real effectiveness and potential toxic effects.

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Chapter 1: Introduction

1.1 Phytotherapy, Pharmacology and its relationship to the medicinal plants

The medicinal utilization of natural products that may derived from natural sources for example, plants, animals alternately micro-organisms made the history of drug discovery. The importance of natural products was very important throughout the evolution of mankind. At the very beginning of the history of mankind, humans were used to chew some herbs to mitigate pain or to improve healing they used to wrap leaves around the wound which leaded them towards phytotherapy (Ji, Li& Zhang, 2009).

The term phytotherapy refers to the study of the medicine in which extracts of natural plants are used as medicines. Phytotherapy is different from pharmacology. It is using the not only a single chemical compound but the whole part of a medicinal plant. Traditional use of medicinal plants from around the whole world and phytochemistry are used to synthesize phytotherapy. People use the extracts of the whole plants or the particular parts of the plants for treatment and prevention of disease (Rates, 2000).

Medicinal plants can be defined in many ways. According to the WHO, “A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis.” When a plant is designated as ‘medicinal’, it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation (Sofowora, Ogunbodede, & Onayade, 2013).

Drug discovery starting with medicinal plants includes a multifaceted methodology joining botanical, phytochemical, biological as well as molecular techniques. Medicinal plant drug discovery proceeds with provide new heads against different pharmacological focuses including cancer, HIV/AIDS, Alzheimer's, pain etc. some of the plant derived natural products have been presented of the united states market, including arteether, galantamine, nitisinone, furthermore tiotropium (Balunas & Kinghorn, 2005)

1.2 History of Medicinal Plants

From the ancient time, when there no medicine was discovered, people used to look for drugs in the nature to cure diseases. In that time, the people did not have proper information about the causes of diseases as well as they did not have the idea about choosing the right plant or how it could be used. They lived so close to nature which might lead them to use medicinal plants for illness. Approximately 5000 years old written confirmation for medicinal plants used for preparation of medications has been found on a Sumerian clay slab from Nagpur. It contains 12 recipes using more than 250 medicinal plants for the preparation of medications. Some of them also contain alkaloid for example poppy, henbane and mandrake (Kelly, 2009).

According to the ancient history, Dioscorides was known as “the father of pharmacognosy” who wrote “De Materia Medica” by studying medicinal plants while travelling with roman army as he was a military physician by profession. His classical work contains lots of information about plant origin medications. Total 944 drugs were describe on it in which 657 of them were from plant origins along with the description of appearance, locality, mode of collection, how to make the medicinal preparation and their therapeutic effect (Petrovska, 2012).

According to Indian ayurvedic such as Sushruta, Jivika, Charaka and Vagabhatta, plants are used for the remedial treatment of wound healing from the ancient time. In traditional Chinese medicine, medicinal plants are also used for practicing of healing of different diseases for thousand years.

Some of these plants have been screened scientifically for the evaluation of their wound-healing activity in different pharmacological models and patients, but the potential of most remains unexplored. In a few cases, active chemical constituents were identified (Bhattacharya, 2012).

1.3. Significance of medicinal plants as traditional medicines

Medicinal plants are used from the ancient time as a treatment of different diseases which help to build the history of drug discovery. In most cases, a part of the plant like roots, leaves, barks, flowers or fruits are used to make the medicinal preparations. These parts of plants contain lots of chemical constituents along with the desired component. In contrast, by isolating that desired

component with the addition of bulk and making a dosage form leads to drug discovery. Though lots of new technologies are used such as combinatorial chemistry and computer-based molecular modeling design to create synthetic molecule day by day but plant derived medicines are having a high rate of acceptance by the patients. Moreover the medicinal plants have long history of clinical use which makes these more reliable (Veeresham, 2012).

A recent statistics of published by World Health Organization (WHO) shows that approximately 80% people of the whole world used natural medication as their primary healthcare at some extent. For instance, there are approximately 600-700 plant based medications are available in Germany and 70% of the German physicians are more interested to prescribe those medications. Moreover, the history of past 20 years in United States shows that uses of natural medication increased drastically due to less tolerance of synthetic drug by the patient and cost of the drug.

Huge numbers of the plants might utilized as stimulants, poisons, hallucinogens but there are lots of medicinal plants that contains such chemical constituents which produce definite physiological action to the human body and give therapeutic effect and increased the value of that plant as a medicinal plants. Some bioactive constituents that make a plant valued as medicinal plant are -

- Alkaloids
- Flavonoids
- Tannins
- Saponins
- Glycosides
- Phenols

Medicinal plants play an important role in phytotherapy.Some of the plants are used for other purposes also have medicinal uses are given in the Table 1.1 below-

Table 1.1: Plants are used for other purposes also have medicinal uses. (Edeoga, Okwu, & Mbaebie, 2005)

Species	Family	Traditional use	References
Cleome rutidosperma	Caparaceae	Leaves are edible. Anthelminthic and carminative.	Burkill, 1984 Gill, 1992
Eupithecia coccinea	Astcraccae	Treatment of fever and convulsions in children.	Agoha, 1981
Euphorbiaheterophylla	Euphorbiaceae	Vegetable and latex used for insect bites. Treatment for cough, bronchial paroxysmal asthma, hay fever, catarrh.	Edeoga and Gomina, 2002 Gill, 1992
Sessea bransilensis	Solanaccae	Treatment of malaria, toothaches, liver ailment and rheumatism and treatment of stomach disorder and asthma in children.	Gill, 1992
Richardia bransilensis	Rubiaceae	Cure for eczema. Treatment of boils. Active cure against avine malaria.	Burkill, 1984
Scoparia dulcis	Scrophulaiaceae	Antiviral, inhibitory and anti-tumor activity. Remedy for cough, chest pains and sore throat, treatment of gonorrhea.	Hayashi *et al*, 1993 Gill, 1992
Siphanta acuta	Malvaceae	Feed for livestock. Stops bleeding.	Egunjohi, 1969

1.4 Prevalence of cancer

Cancer is the second reason of mortality over cardiovascular disease. Statistics shows that, approximately 576,691 deaths were accounted from cancer which is almost 23% of total deaths in the United States in 2011. By evaluating the information of recent 5 years, the rate of death in cancer increased around men (1.8%) than women (1.4%). The rate of mortality also increased in children due to cancer (Siegel, Miller, & Jemal, 2015). In Bangladesh, new cases and mortality rate of cancer is increasing side by side along proportionally with the increasing population. There are approximately 13 to 15 lakhs of cancer patient along with 2 lakhs newly diagnosed cancer patient every annual year (Noronha *et al.*, 2012).

1.4.1 Types of cancer

There are several types of cancer but most common types are bone cancer, bladder cancer**,** breast cancer**,** cervical cancer**,** colon cancer**,** kidney cancer**,** liver cancer**,** leukemia**,** stomach cancer**,** prostate cancer**,** thyroid cancer and many more (Crosta, 2015).

1.4.2 Cancer causing agents

According to WHO there are some common factors that triggers the cancer causing agent and those are given below:

Chemical carcinogens: Some chemical and environmental toxins are known as carcinogens which change the normal DNA and mutate them thus it helps in abnormal growth of cells. Particular substances accept been affiliated to specific types of cancer. Tobacco smoke is associated with abounding forms of cancer, and causes 90% of lung cancer. Tobacco also contains other carcinogens like nitrosamines and polycyclic aromatic hydrocarbons which is responsible for cancers such as lung, larynx, stomach, bladder, kidney, esophagus and pancreas etc.

Ionizing radiations: Melanoma and other malignancies can occur due to exposure of radiations and ultraviolet ray from the sun.

Viral and bacterial infections: Some cancers occur due to the infections of pathogens in the body. For example, Hepatitis B and C infections can cause liver cancers. Human Papilloma virus (HPV) can cause cervical cancer etc.

*Genetic factors:*Common examples are genetic breast cancer and ovarian cancer genes including BRCA1 and BRCA2. Li-Fraumeni syndrome includes defects in the p53 gene that leads to bone cancers, breast cancers, soft tissue sarcomas, brain cancers etc.

1.4.3 Medicinal plants for cancer treatment

The term cytotoxicity refers to a quality of a molecule or compound which basically responsible for the death of a cell. Cytotoxic agent is mainly used to treat cancer by inhibiting the proliferation of the cancer cell or by destroying them. Nowadays cancers become the biggest threat to increase the mortality rate in both developing and developed countries. Cancer is a kind of disease in which rapid cellular growth occurs in an organ of the body which may lead to a formation of a mass of tissue or tumor progression. In some cases, the abnormal growth of tumors may affect the neighboring cells or interfere with another organ. As a consequence patient may die. (Knudson, 2001)

By analyzing the history of last decades, it is proved that lots of improvement occurs in the research of molecular oncology. Though there are some facilities available to treat the cancer like chemotherapy, radiotherapy, surgery and immunotherapy but still the rate of cure is very low. New technologies like gene therapy and hormonal therapy are also making approach to replace other therapy. In spite of this new approaches, curable rate is still low because all of these therapies have undesired side effects, they are usually not accessible all the time and they are expensive.(Amit, Tamir, & Hochberg, 2013) In case of surgery, large amount of cortisol released which affect the immune system vulnerably and may cause cancer relapse. Furthermore, the use of chemotherapy is accompanied with severe side effects which lead to immunosuppression by inhibiting bone marrow stem cells proliferation. In the same way, Radiotherapy which is highly used is also responsible for lots of side effects. Some other side effects like bone necrosis, lung fibrosis, skin devascularization, ulceration, nausea, vomiting, and renal damage occurs with all the type of therapies.

On the other hand, there is a huge opportunity of using plant derived natural products against cancer over the therapies and their side effects. Medicinal plants are a good source of medication and gives better therapeutic effects besides it does not have that much side effects which all the therapies have. It also have high rate of acceptance by the patient (Fennell et al, 2004). From the ancient time, plants are used for the treatment of illness and lots of medicinal plants are used as a modern drug which may shows cytotoxicity activity (Rosangkima & Prasad, 2004). The antioxidant property of a plant is very useful to prevent and cure cancer other diseases by protecting the cells which is mainly triggered by free radical. Nowadays many plant derived compound like vinca alkaloid, flavonoid, phenols has been showed the cytotoxicity activity against many rodents and Human Cancer cell line (Rao & Yamada, 2013).

1.4.4 Principle of *in vitro* cytotoxic activity

In vitro cytotoxicity includes a cell accordance which will be looked after to particular medium along with nutrients and antibiotics. These permit the cells to stay healthy and uncontaminated as well as anchored of the surface. Cytotoxicity assays differ depending upon the type of experiment. Cell is cultured in a specific no per well or microtiter plate and extracts are added further to determine the viability of cells under a microscope. Apoptosis will occur to the cultured cells after adding the extracts even it can lead to secondary necrosis. Gradually the metabolisms of cells remain down which leads the cells to loss their membrane integrity and finally the cells release the cytoplasmic content into the medium. The number of viable cells in the medium determines the impact of the extract on the cell line. To be more specific, it shows whether the extracts had any cytotoxic activity or not (Nema & Khare, 2012).

1.5 Antibacterial agent

The term antibacterial refers to a natural or semi-synthetic or synthetic compound that kills or inhibits the growth of bacteria but causes no damage to the host. The word antibacterial was derived from the Greek words anti which means against, and bios means life which refers to all agents that act against microbial organisms.

Bacteria are an organism that has only one cell. They are different in nature. All the bacteria are not hazardous to human health. Some of the bacteria lives in human body and helps in metabolism.

Microorganisms like bacteria and viruses become susceptible easily with new drugs. But there is less possibility to invent new drugs to meet the increasing demand of antibacterial agent. Therefore, to prevent the antibacterial diseases, discovery of new drug is getting essential day by day. Medicinal plants are a good source of compound which can give lots of therapeutic effects as well as antibacterial effect (Prasad, Sudha, Khadri, & Riazunnisa, 2015).

1.5.1 Classification of antibacterial agents

Antibacterial agents can be classified into 3 groups according to their mechanism of action such as cell wall synthesis inhibitors, protein synthesis inhibitors and nucleic acid synthesis inhibitors.

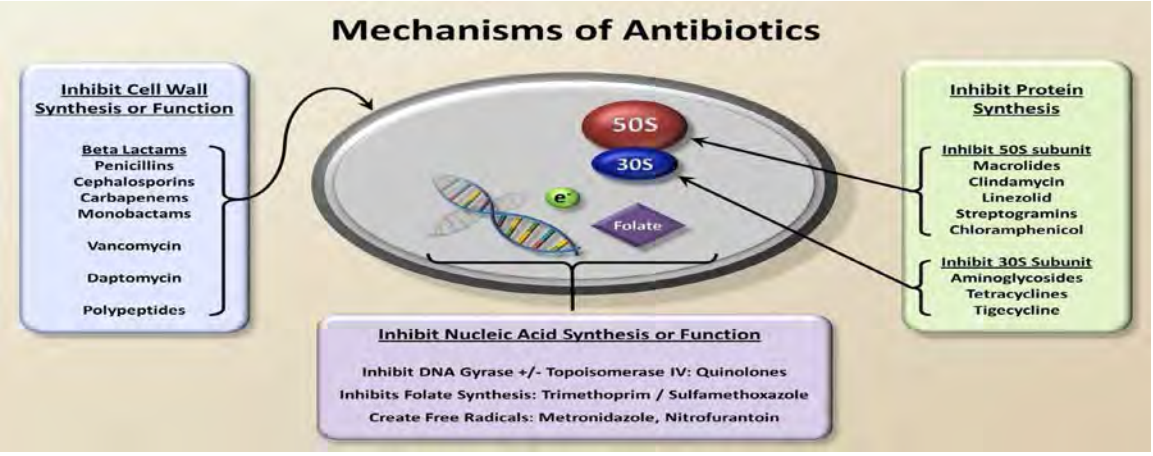

Figure 1.1: Classification of antibacterial agents according to their mechanism of actions

The Figure 1.1 is showing the classification of antibacterial agents shows that antibacterial agents exhibit bactericidal or bacteriostatic activity by inhibiting the bacteria's cell walls synthesis or inhibiting the 50s or 30s protein subunit synthesis. It can also stop the nucleic acid synthesis which may lead to bacterial cell death.

1.5.2 Principle of disc diffusion method

To determine the antibacterial activity, Disc Diffusion method is the most popular method. It is also known as Kirby-Bauer method. Though MICcannot be obtained from this method but the zone of inhibition can easily be obtained from this method. Muller Hinton Agar medium or Nutrient Agar medium is used to culture the microorganisms. A suspension of microorganism is made by swirling some freshly cultured microbes in the saline. Then it is streaked in a fresh agar medium and the disc containing the plant extract is placed on it carefully. Afterward it is incubated for 24 hrs to screen the microbial growth. Lastly, zone of inhibition is measured which is formed around the disc to determine the antibacterial effect of the plant extract.

1.6 Phytochemicals associated with anticancer activity

Medicinal plant contains so many constituents but some of them have the anticancer effect. Studies showed that one plant may contain several components which may act as anticancer agent like flavonoids, cardiac glycosides, phenols, alkaloids, taxanes, antimetabolites etc. but they have different mechanism of action to cancer cell (Felth, 2011).

1.6.1 Flavonoids

Flavonoids are polyphenolic phytochemicals which is very useful cure different disease. It is enlisted in non-essential dietary components found in food and beverages. Due to its medicinal uses researchers are more interested to study about those plants which contain flavonoids (Ahmad, Kaleem, Ahmed, & Shafiq, 2015).Classification of flavonoids are showing in Table 1.2.

There are lots of activity found antibacterial, anti-inflammatory and antioxidant as well as cytotoxicity activity by analyzing the flavonoid containing plant extract.

Table 1.2: Classification of Flavonoids. (Shah, 2016)

Flavonoids	Structure	Compound
Flavone	Dihydroxy 5,7	Chrysin
	Trihydroxy 7,3',4'	Bulin
	Trihydroxy 5,7,4'	Apigenin
	Tetrahydroxy 5,7,3',4'	Luteolin
	Tetrahydroxy 5,7,3',4	Fisetin
Flavonol	Pentahydroxy 3, 5,7, 3', 4'	Quercetin
	Tetrahydroxy 3,5,7,4'	Kaempferol
Flavonone	Tetrahydroxy 5, 7, 3', 4'	Eriodictyol
	Dihydroxy 7, 4'	Liquiritigenin
Xanthone	1, 7 – Dihydroxy-3-methoxy	Gentisin
Isoflavone	Dihydroxy 7,4'	Formononrtin
	Trihydroxy 5, 7, 4'	Genistein
Biflavone	Hexahydroxy 2X (5,7,4'	Amentoflavone

Flavonoids not only act in a single stage of cancer development but also it works through the overall process by following several mechanisms. Model of carcinogenesis in normal cell to cancerous cell and potential consequences of flavonoids on cancer progression is given in the Figure 1.2.

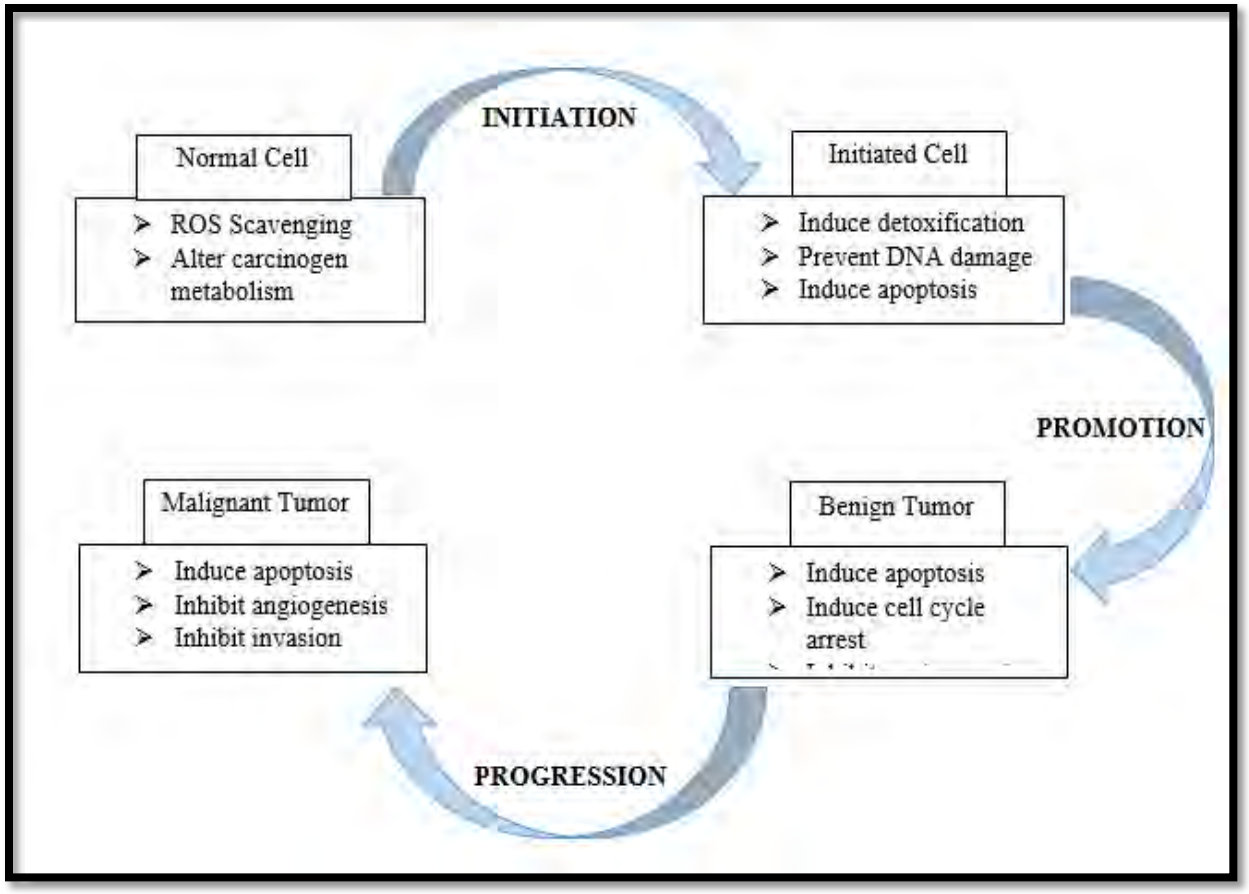

Figure 1.2: Model of carcinogenesis and potential consequences of flavonoids on cancer progression.

Flavonoids works in different stages for instance modulating mitogenic signaling, apoptotic signaling, cell-cycle regulation, angiogenesis, and metastatic effects in the cells. Thus flavonoids may inhibit the activity of DNA topoisomerase I/II, release of cytochrome C from mitochondria and subsequent activation of caspases-3, 8 and 9.Carcinogenesis is a complex multistep. It is initiated from a normal cell and by promotion and progression it leads to a cancer cell. Figure is showing that how flavonoids work on normal cell to cancerous cells to inhibit the cell growth or inducing apoptosis (Yadegarynia, 2012).

1.6.2 Alkaloids

Alkaloids are chemical compounds that containing a nitrogen atom in a ring structure. Many alkaloids which are found in medicinal plants play significant role in cancer research as they have anti proliferative and anti-metastatic effect.Alkaloids can be classified into many ways. Classification of plant derived alkaloids according to their ring structure has been shown in the Table 1.3. Some alkaloids like camptothecin, vinblastine, have effectively used for anticancer medications. Camptothecin basically act as a topoisomerase (I) inhibitor and vinblastine interacts with tubulin. berberine, evodiamine, matrine, piperine, sanguinarine, and tetrandrine are also isolated from plants and have cytotoxicity effect (Lu, Bao, Chen, Huang, & Wang, 2012). In addition, some of the alkaloid may target the mitochondria of a cancer cell because mitochondria are high proliferative and can resist cell death (Urra et al., 2013).

Alkaloids show cytotoxicity effect in different stage of cell proliferation. Vinca alkaloid such as vincristine and vinblastine binds in the surface or ends of the proteins of microtubules in a dimeric form. This binding of alkaloids is responsible for the disruption of the microtubule assembly which leads to the de-polymerizations of the microtubule. As a consequence, a mitotic arrest occurs at metaphase by dissolving the mitotic spindle thus it leads to cell death (Moudi, Go, Yien, & Nazre, 2013).

Table 1.3: Classification of alkaloids along with plants (Tuan& Hai, 2016)

Basic ring structure	Alkaloid	Botanical origin	Family
Pyrolodine	Hygrine	*Erythroxylon coca*	Erythroxylaceae
	Stachydrine	*Stachys tuberifera*	Labiatae
Pyrindine	Arecoline	*Areca catchu*	Palmaceae
	Ricinine	*Ricinus communis*	Euphorbiaceae
Piperidine	Connine	*Conium macultum*	Umbeliferae
	Lobeline	*Lobelia inflate*	Lobeliaceae
Tropane [Piperidine-Pyrrolidine (N-Methyl)]	Atropine	*Atropa belladone*	Solanaceae
	Cocaine	*Erythroxylon coca*	Erythroxylaceae
Quinoline	Quinine	*Cinchona officinalis*	Rubiaceae
	Quinidine	*Cusparin trifoliate*	
Isoquinoline	Papaverine	*Papaver somniferum*	Papaveraceae
	Berberine	*Hydrastis Canadensis*	Berberidaceae

1.7 Phytochemicals associated with antibacterial activity

Previous studies have shown that phenols, polyphenolic compounds, flavonoids, alkaloids, tannins, coumarins exhibit the antibacterial activity. Some plant derived compound with their antibacterial mechanism of actions is given in the Table 1.4 below-

Table 1.4: Some plant derived compounds with their antibacterial mechanism of actions. (Nandagopal, Sankar, Ramamurthy, Sathish, & Sridharan, 2011)

Class of compounds	**Possible mechanism of actions**	**Activity**
phenols, phenolic acids	Enzyme inhibition by oxidized compounds through reaction with sulfhydryl groups	Bacteriostatic
Quinones	Source of stable free radicals complex irreversibly with nucleophilic amino acids in proteins	Bacteriostatic
Flavanoids, flavones, flavanols	Complex with extracellular and soluble proteins and complex with bacterial cell walls, lipophilic flavonoids disrupt microbial membranes	Inhibits *Vibrio cholerae*, *Streptococcus mutans*
Tannins	Interaction with eukaryotic DNA	Antiviral
Coumarins, terpenoids	May involve membrane disruption by the lipophilic compounds	Antibacterial
Alkaloids	Intercalate with DNA	HIV infection
Lectin and polypeptides	Formation of ion channels in the microbial membrane or competitive inhibition to host.	Antibacterial

1.8 Bangladeshi medicinal plant

1.8.1 Overview of Bangladeshi medicinal plant

Within the Asian subcontinent around 2000 plants were enlisted having medicinal properties (Chopra, Handa & Kapur, 1958) and among them more than 500 medicinal plants currently grow in Bangladesh (Yusuf, Begum, Hoque, & Chowdhury, 2009). Some medicinal plants contain lots of chemical constituents along with the desired component which is responsible for the therapeutic effect. By isolating the desired component with addition of bulk material may leads to the drug discovery of that plant derived products. In some cases, clinical trial has been practiced by physicians and therapeutic effects were found in plants.

At present in Bangladesh, for the traditional medicine preparations over 250 medicinal plants are being used. Around 64 of medicinal plants along with their therapeutic effects are studied and reported over past two decades. Medicinal uses of certain medicinal plants in Bangladesh is given in the Table 1.5 below-

Table 1.5: Medicinal uses of certain medicinal plants in Bangladesh in traditional medicine preparation

Name of plant	**Medicinal uses**
Abroma augusta	Curing urogenital and female-related diseases
Allium sativum	Reducing blood cholesterol
Andrographis paniculaya	Curing fever and hepatic diseases
Bacopa monniera	Increasing the longevity of life ad as a brain tonic
Catharanthus roseus	Treatment of diabetes and cancer
Centella asiatica	Treatment of diarrhea and dysentery
Coccinea indica	Management of diabetes
Rauvolfia serpentine	Treatment of high blood pressure, insanity and insomnia
Terminalia arjuna	Treatment of heart disease

1.8.2 Selection of *C. verrucosa L*. for the present study

After going through literature reviews it was found that the cytotoxicity study and antibacterial activity study against *Bacillus cereus, Streptococcus pneumoniae* and *Shigella dysenteriae* of *C. verrucosa* was not performed previously. Thus to investigate the cytotoxic potential and antibacterial activity of *C. verrucosa* the initiative of present study was taken.

1.8.3 Introduction to *C. verrucosa*

C. verrucosa belongs from the Fabaceae family which is also known as Leguminosae. Leguminosae is the largest economically important family. It is also known as the pea, legume or bean family.

Out of 630 legume genera *C.*is the largest genera and has approximately 700specise. The other largest genera are *astagalus, acacia, Indigofera* and *Mimosa*. These five genera constitute about one-fourth of legume species.

Figure 1.3: *C. verrucosa* plant

1.8.4 Plant description

C. verrucosa is a flowering plant of legume family which is Native to Chittagong, Khulna, Mymensingh, Rajshahi and Sylhet. It is known as “Rattlepod” or “Blueflower”. In 1753, Linnaeus, C. von.recorded it in species Plantarum. “Bansan” or “Jhanjhania” are other names of *C. verrucosa* in Bengali. In Bangladesh it is also known as “Jhanja”. It starts giving fruits when its height is around 1-1.5m.

1.8.5 Traditional uses of *C. verrucosa*

C. verrucosa has been used in Bangladesh, India, Srilanka, China and many more countries. The leaves obtained from this plant have been used both internally and externally in the treatment of scabies and impetigo. From revewing the literature it was found that they are also used as an expectorant and as anti-emetic. They also used in diminishing salivation (Kumari *et al.*, 2010). It is also used traditionally in the treatment of dyspepsia, biliousness, in reducing fever, treating impurities of blood, in relieving heart complaints and in the treatment of throat and oral diseases. Amongst the Chakma and Marma tribes in Bangladesh, they used to treat skin allergies by using leaves of *C. verrucosa* (Yusuf, Begum, Hoque & Chowdhury, 2009).

By analyzing the phytochemical screening of this plant it has been found that *C. verrucosa* contains flavonoids, steroids, glycosides, tannins, alkaloids and necic lactone and it has been shown in the Table below-

Table 1.6: Chemical constituents of *C. verrucosa*

Chemical class	**Phytocompounds**	**Plant parts**	**References**
Flavonoids	Isovitexin	Seeds	Indian Journal of Pharmacy (1967 & 1972); Phytochem (1976)
Flavonoids	Vitexin-4'-O- glucoside	Seeds	Rastogi & Mehrotra (1993, p. 2)
Flavonoids	Vitexin (Apigenin-8C-glucoside)	Seeds	Rastogi & Mehrotra (1993, p. 2)
Phytosterols	β-sitosterol	Seeds and stems	Indian Journal of Pharmacy (1967 & 1972); Phytochem (1976)
Pyrollizidine alkaloids	Crotalaburnine (also known as Anacrotine)	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976), Roeder & Wiedenfield (2013)
Pyrollizidine alkaloids	Crotaverrine acetate (O-acetylcrotaverrine)	Seeds	Indian Journal of Pharmacy (1967 & 1972), Phytochem (1976).
Necic lactone	2-methyl-3-(2-oxo-[5H]-5-hydroxymethyl-5-methylfuran-3-yl)-propanoic acid	Leaves	Suri, O.P., Suri, K.A., and Dhar, K.L. (1989)

Plant taxonomy of C. verrucosa is given in the Table 1.7 below-

Table 1.7: Plant taxonomy

Rank	**Scientific name (common name)**
Kingdom	*Plantae*
Subkingdom	*Trachcobionta*
Superdivision	*Speratophyta*
Division	*Magnoliophyta*
Class	*Magnoliophyta*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Leguminosae*
Genus	*Crotataria L.* (Rattle box)
Species	*C.verrucosa L.* (Blue rattlesnake)

1.9 Rationale of the project

Many bioactivity properties such as anti-diabetic, *in-vitro* anticoagulant activity, thrombolytic and antibacterial, anti-fertility, antipyretic, CNS depressant potential, wound-healing and hepatoprotective activity study of *C. verrucosa* was performed before. However, it was found that the cytotoxicity and antibacterial activity study against *Bacillus cereus, Streptococcus pneumoniae* and *Shigella dysenteriae* of *C. verrucosa* was not performed previously. By

analyzing the phytochemical screening of this plant it has been found that *C. verrucosa* contains flavonoids, steroids, glycosides, tannins and alkaloids. Most importantly, these compounds can give the cytotoxic and antibacterial effect so thus, this study will focus mainly on determining the cytotoxic and antibacterial activity of methanol extract of *C. verrucosa.*

1.10 Aim of the project

The aim of this study is to investigate and evaluate the bioactivity study of the leaves extract of the local medicinal plant *C. verrucosa.*

1.11 Objectives of the project

After studying the literature review pertaining to the previous findings of *C. verrucosa,* the objectives of the project were made as follows with regards to using methanol leaf extract of *C. verrucosa*-

1. To determine the cytotoxicity effect by using human cell line from leaves extract of *C. verrucosa*.
2. To evaluate the antibacterial activity study from the leaf extract of this plant.

Chapter 2: Literature Review

2.1 Previously studied pharmacological properties of *C. verrucosa*

Literature reviews *C.verrucosa* have been done and the results showed that different pharmacological activities including antipyretic, thrombolytic, anti-diabetic, CNS depressant, antimicrobial, anti-fertility and wound healing activity have been carried out previously.

2.1.1 Antibacterial activity

The antibacterial activity was analyzed for *n*-butanol extracts of *C. verrucosa*(100µg/mL). It was done by measuring the diameter (mm) of the zone of inhibition utilizing the agar well dissemination technique against the bacterial strains specifically, *Klebsiella pneumonia* (G -ve), *Bacillus subtilis* (G +ve), *Proteus vulgaris* (G -ve), *Escherichia coli* (G -ve), and *Pseudomonas aeruginosa* (G +ve). In this study, gentamycin was utilized as the standard medication. It was seen that the zone of restraint in *C. verrucosa* extract for *K. pneumonia* and *B. subtilis* was 15mm each, 14mm for *P. vulgaris*, 13mm for *E. coli*, and 12mm for *P. aeruginosa*. Then again, for gentamycin, the zone of restraint for the above strains was 16mm, 20mm, 15mm, 18mm, and 20mm individually. Therefore, the *n*-butanol extract of *C. verrucosa* has an "expansive range" of antibacterial activity against a board of bacteria in charge of the vast majority of the normal maladies (Khadri, 2015).

2.1.2 Wound healing activity

Three injury models like incision, excision and dead space wounds were utilized as a part of this study. Two measurements of the concentrate with and without dexamethasone indicated noteworthy increments in mean hydroxyproline, dry weight of granulation tissue and complete protein content yet it was higher with dosage 800 mg/kg in contrast with the control. The group treated with dexamethasone demonstrated a noteworthy ($P<0.001$) decrease in the strength of breaking when contrasted with control group of wound model with incision type. Co-administration of *C. verrucosa* with dexamethasone fundamentally ($P<0.001$) expanded the breaking strength compared with the group that was treated with only dexamethasone. In

excision wound model, the rate of the injury withdrawal was fundamentally (P<0.01) expanded by two measurements of test extract on all the days aside from the lower dosage which displayed just on 12^{th}, 16^{th} days of medication treatment and it additionally switched the dexamethasone smothered injury constriction. It fundamentally (P <0.001) reduced the time required for epithelialization and turned around the epithelialization deferring impact of dexamethasone (P<0.001). *C. verrucosa* was found to have wound healing property. This was apparent by abatement in the time of epithelialization, increment in the rate of wound withdrawal, strength of skin breaking and dry weight content of granulation tissue. Thus *C. verrucosa* could be a decent agent for wound healing.

2.1.3 Anti-fertility activity

Number of examinations has been done on anti-fertility plants to approve their viability *C. verrucosa* is one of them. Upon writing survey, it was found that the plant has flavonoids (β-sitosterol) and other alkaloids, which are known as non-steriodal phyto-estrogens and created anti-fertility in animals. Assessment of the concentrates for antifertility movement by receiving the screening method reported in the writing. The trial models (Screening method) utilized as a part of the study of estrogenic activity and anti-implantation and abortifaicent activity.

The study indicated that 95% ethanolic extract, 70% ethanolic extract and aqueous extract of the aerial parts of *C. verrucosa* showed significant early abortifacient and anti-implantation activity in female wistar albino rats. At doses of 500mg/kg b.w. as 250mg/kg b.w. the extracts of *C. verrucosa* had significant estrogenic activity. Estrogenic activity was indicated by uterotropic response, vaginal cornification and increased uterine weight. In the treated group with *C. verrucosa* few biochemical changes like cholesterol, concentration of glucose and alkaline phosphate seemed to be high in comparison with the controlled group (Singh, 2011).

2.1.4 Hepatoprotective activity

Wistar albino rats treated with paracetamol demonstrated a noteworthy hepatic damage as saw from hoisted levels of hepato-specific enzymes and also serious alteration in various liver parameters. Total bilirubin, SGPT and SGOT in serum were expanded in paracetamol inebriated control animals. Treatment with the ethanolic extract of *C. verrucosa* causes noteworthy protection homogenate and ethanolic extract of *C. verrucosa*indicated protection against both paracetamols actuated liver damage against paracetamol increment in serum enzyme levels and bilirubin in a measurement responsive way. Also, LP, CAT, GSH, SOD and glycogen contents were assessed from liver. On the premise of results acquired, it can be reasoned that the ethanolic extract of *C. verrucosa* aerial parts appears to deliver hepatoprotective activity in wistar albino rats. No lethal indication or mortality was seen in 48 hrs of study in mice. These outcomes appear to support the traditional use of this plant in securing liver against hepatotoxicity.

2.1.5 Antipyretic activity

The study incites pyrexia in Wistar rats through subcutaneous injection of brewer's yeast (20% w/v in distilled water at 10mL/kg b.w.). After 19hrs, the underlying rectal temperature was recorded and the *C. verrucosa* extract of various fixations (100, 250, 500 mg/kg b.w.) were orally introduced to various rats and the results were practically identical to administration of standard medication, Paracetamol 150mg/kg. The diminishment in temperature was recorded at 1hr, 2hrs and 3hrs after the treatment. It was recorded that the specimen measurement of 500mg/kg b.w. created a significant decrease in temperature (from 40.33^{o}C to 37.48^{o}C) similar to that of Paracetamol 150mg/kg (from 40.42^{o}C to 37.51^{o}C). Also it was adequately presumed that the 500mg/kg b.w. has strong antipyretic property more prominent than 250mg/kg (temperature reduced from 40.67^{o}C to 38.55°C) while 100mg/kg b.w. does not reduce hyperthermia fundamentally (Nawrin et al., 2015).

2.1.6 Thrombolytic activity

Thrombolytic activities was studied progressively presumed that the leaf extract of *C. verrucosa* was unable to create a coagulation lysis action in contrast with the standard medication, Streptokinase. The rate of thrombolytic action of *C. verrucosa* leaf extract against RBC clump denaturation was seen to be 26.81% in contrast with that of Streptokinase (80.65%). In this study, 100 µL of *C. verrucosa* extract was taken for each elevated tube containing thrombus to which the 500 µL of blood taken from solid volunteers was included and weighted. Similar steps were conducted for the standard medication, Streptokinase. The tubes were then incubated at 37^{o}C for 90min after which the supernatant was expelled from the tubes, which was then reweighed to watch clot disturbance and the rate of thrombolytic activity was then figured (Nawrin et al., 2015).

2.1.7 Anti-diabetic activity

The anti-diabetic action of *C. verrucosa* has been examined by artificially creating type-II diabetes on wistar rats through intraperitoneal infusion of alloxan monohydrate. After 72 hrs, the blood glucose level was recorded to be above 140mg/dL, in this manner, it confirms the diabetic condition. From that point, 3 distinct measurements of *C. verrucosa* were orally given to the wistar rats: 100mg/kg, 250mg/kg, 500mg/kg b.w., the aftereffect of which were contrasted with the standard medication, glibenclamide 2.5mg/kg b.w. amid a period interim of 0, 7, 14 and 21 days utilizing glucose kits which were commercially available. The blood glucose level of the wistar rats incited with *C. verrucosa* concentrate of 500mg/kg b.w. result was seen to be 284.54mg/dL and 190.33mg/dL separately, while that of glibenclamide demonstrated a noteworthy abatement in blood glucose level from 289.41mg/dL to 177.12mg/dL. Hence it was concluded by the author that the ethanolic extract of *C. verrucosa* show anti-diabetic activity but not as much as that of glibenclamide (2.5mg/kg) (Nawrin et al., 2015).

2.1.8 CNS depressant

The CNS depressant action of *C. verrucosa* was seen on Swiss albino mice by means of "Hole cross test" and "Open field test". For both tests, diazepam 1mg/kg b.w. was chosen as the reference standard which was orally introduced to the mice 20min preceding the begining of the trial. The lessening in "Hole cross test" reflected in CNS depressant action of the example on the swiss albino mice, while for the "Open field test", the decrease in the number of squares crossed by the mice in the open field in 20min reflected in CNS depressant activity (Nawrin et al., 2015).

2.1.9 Anti-inflammatory activity

Carrageenan prompted rat paw edema and xylene impelled mice ear edema tests were executed as *in vivo* evaluation of anti-inflammatory activity. In addition, these techniques were upheld by the i*n vitro* heat prompted protein denaturation and haemolysis tests. In all trials *C. verrucosa*extract (CVE) indicates moderate to critical efficacy. CVE 600 mg/kg suppressed edema in contrast with both steroidal and nonsteroidal anti-inflammatory drugs. Additionally, *in vitro* study recommended that the leaf extracts at measurements of 300, 400 and 500 µg/mL have moderate to high restraint limit for auto antigen generation which applies extract's potential against Carrageenan and xylene impelled inflammatory method are settled and generally utilized for the appraisal of any medications or tests for its anti-inflammatory activities. *In vitro* trials were performed to support the result of *in vivo* tests. The result of the study showed that the leaf extract of *C. verrucosa* have impressive anti-inflammatory activity as equipped for hindering the auto antigen production to huge degree in comparison with the standard. The leaves have been accounted for to contain tannins and phenolic content which can be the attributor for these actions. The present investigation of anti-inflammatory activity reasons that the utilization of the plant leaf of *C. verrucosa* in illnesses of inflammation has investigative premise. This study likewise finishes up the safety of this plant for use and pronounces it to be non-lethal in acute use at the dosages executed (Nawrin et al., 2015).

Chapter 3: Methodology

3.1 Cytotoxic activity study by MTT assay

3.1.1 Solutions preparation

1% penicillin-streptomycin solution

Penicillin streptomycin solution usually known as pen-strep is used in MTT assay to control the bacterial contamination and maintain the sterile condition throughout the process. The solution contains a mixture of 10000 unit of penicillin per mL and 10 mg of streptomycin per mL

10 % fetal bovine serum

50 mL of Fetal Bovine Serum was added to the 500 mL of DMEM to prepare 10 % FBS.

Trypsin

0.25% trypsin was used in the medium.

2% DMSO solution

2% DMSO solution was prepared by adding 60 µL in 2940 mLof distilled water for control.

3.1.2 Used consumables

24-well plate, 15-mL tubes, tips, gloves, culture flask, cell culture media, 1% penicillin-streptomycin, gentamycin, serological pipette, trypsin etc.

3.1.3 Used instruments

Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA)**,** CO_2 incubator (Nuaire, USA)**,** trinocular microscope with Camera (Olympus, Japan)**,** hemocytometer.

3.1.4 Celltiter 96 assay kit

Celltiter 96® assay kit is an accumulation of qualified reagents that give a fast furthermore advantageous system with determination of the amount of proliferation and cytotoxicity (Ifere et al., 2010). It is a view of change of a cell division with tetrazolium salt under a formazan product that is undoubtedly distinguished utilizing a 96-well plate. A mixer of dye solution was added in 96 well plates to culture cells to perform this assay.

3.1.5 HeLa cell line

HeLa cell line was collected from Centre for Advance Research and Science (CARS). This cell line was cultured and maintained in DMEM (Dulbecco's Modified Eagles Medium) by using cell culture flask. It was stored in liquid nitrogen.

3.1.6 Preparation of the different concentrations of plant extract

The assay was be performed by using 4 concentration 2.5 mg/mL, 0.25 mg/mL, 0.025 mg/mL and 0.0025 mg/mL of the *C. Verrucosa* leaves extract. A concentration of 2.5 mg/mLwas made by adding 25 mg of leaves extract in 1 mL DMSO and it was the stock solution and 0.25 mg/mL concentration was made by diluting 2.5mg/mL solution 10 times by DMSO. 10 µL of sample 1 is added to the 90 µL of DMSO to make 0.25mg/mL concentration. In this way 0.025 mg/mL and 0.0025 mg/mL concentrations were made by serial dilution with. Then the samples were filtered through 0.45 µm syringe filter prior to examination.

3.1.7 Cell culture

3.1.7.1 Preparation of assay plates

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagles Medium) in addition with 1% penicillin-streptomycin, 0.2% gentamycin, 10% fetal bovine serum.

3.1.7.2 Thawing of cells

HeLa cells were preserved in liquid nitrogen in cryovials. The cryovial was taken and rapidly defrosted by swirling the vial delicately using a waterbath at 37°C until there was a small piece of ice left in the vial. Afterward the thawed cells were transferred into a centrifuge tube which contained the DMEM medium drop wise under a laminar airflow hood. Thereafter, cells were suspended in the medium gently and transferred to the culture vessels.

3.1.7.3 Cell passage

To get a fresh cell suspension cell passaging was done by transferring the cells into a new medium. The used cultured media was washed by FBS and followed by addition of 800 µL of trypsin for detaching the cells from the top of the culture vessels. Then the cells were incubated and checked for the detachment under a microscope. After watching 90% of cells detached, 5 mL DMEM media was added to the vessels and blended using a pipette. Finally 1 mL of this solution was taken and mixed with 4 mL of DMEM in a new vessel and kept in an incubator for further use.

3.1.7.4 Harvesting of cells

The cell was harvested using trypsin in log phase growth. Then cell was counted and seeded into 96 well plates.

3.1.7.5 Counting of cells

Cell counting had been carried out by using a Hemocytometer which is given in Figure3.1. The Hemocytometer was prepared by cleaning and polishing the mirror like surface deliberately with ethanol and lens paper. For adding the cell suspension, the coverslip was put in the counting surface. A Pasteur pipet was used to introduce the fresh cell suspension into the hemocytometer. Enough suspension was presented so that the surface had been simply overflowed. Afterward the counting chamber was set in the microscope stage then the counting grid was focused. In a standard hemocytometers with Neubauer rulings 1 entire grid can be observed at 40X

magnification. The cells of the 4 large squares were counted. Either upper and left sides touching cells or lower or right sides touching cells were counted.

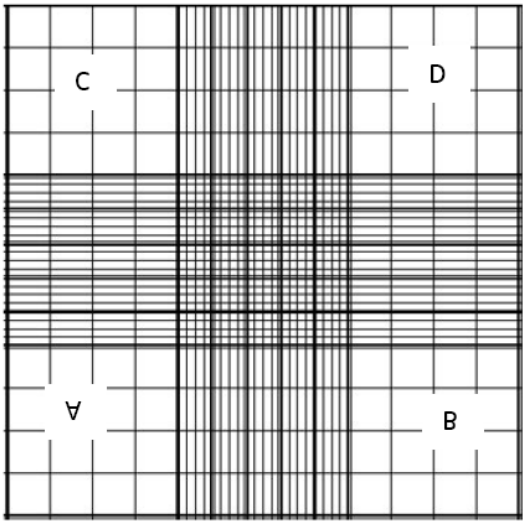

Figure3.1: Hemocytometer

3.1.8. Procedure

Cytotoxic effect was performed in The Centre for Advanced Research in Sciences using their services. The MTT colourimetric assay was performed by using celltiter 96 non-radioactive cell proliferation assay kit (Promega,USA). Cells were seeded onto 96 well plates and incubated at 37 °C and 5% of CO_2 atmosphere. After 24 hrs of incubation, 10 µL of sample was added into each well. Then it was again incubated for 2 days. After 2 days of incubation, cytotoxicity was examined using celltiter 96 non-radioactive cell proliferation assay kit. Then the absorbance was measured at 570 nm using a 96-well plate reader. Same procedure had been followed for all the concentrations, negative control and positive control. Negative control was contained medium with 2% DMSO solution and blank was contained only medium. Duplicate wells were used for each sample. Cytotoxic activity was calculated by using a formula which is given below-

$$\%\ \text{of cytotoxic activity} = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of negative control}} \times 100\ \%$$

3.2 Antibacterial activity study by disc diffusion method

To determine the antibacterial activity ofmethanol extract of C.*verrucosa* leaves was used against 3 bacterial strains which is given in the Table 3.1 below-

Table 3.1: List of bacteria used in this study

Type of Bacteria	**Name of Bacterial Strains**
Gram (+)ve Bacteria	*Bacillus cereus*
	Streptococcus pneumoniae
Gram (-)ve Bacteria	*Shigella dysentreiae*

3.2.1 Preparation of plant extract sample

The methanol extract of C.*verrucosa* leaves was weighed up to 25 mg and added with 700 µL of methanol to prepare the stock solution. Then it was serial diluted to get 350 µg/disc, 250 µg/disc, 150 µg/disc respectively.

3.2.2. Preparation of nutrient agar medium

Nutrient agar medium was prepared by adding 4.25gm of nutrient agar into 100 mL water. Then it was thoroughly mixed until the agar was completely dissolved. After that the mixture was autoclaved for 20 min at 121°C. Afterward, it was cooled up to 45°C -50°C and poured into 4 sterile petri dishes equally in a horizontal surface so that it could have uniform depth and 100 mm petri dish got 25 mL of agar solution approximately. Then it was allowed to cool and solidify at room temperature.

3.2.3. Pre-culturing the bacterial strains

Strains were collected from long term preserved medium which were preserved in a ultra-low temperature (ULT) freezer at -80°C temperature and the long term STGG medium containing skim milk, trypsine, glucose and glycerine to store the strains. The bacterial strain was taken from there by a loop and streaked to a freshly prepared nutrient agar medium and incubated 24 hrs to revive those bacteria. After 24 hrs of incubation, new bacterial colony was formed and ready to use. Figure 3.2 is showing the subculture of 3 bacterial strains. To maintain the quality of freshly revived strain, petri dishes were sealed by using para film which is also helpful to maintain the moisture and avoid drying of bacterial colonies. Then the strains were preserved in a refrigerator for further use.

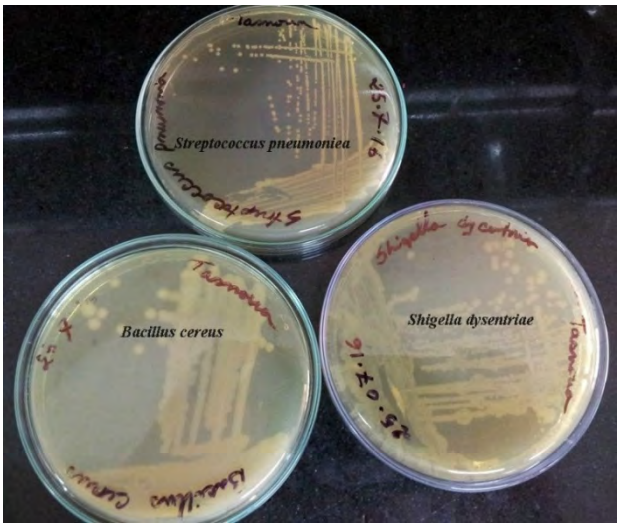

Figure 3.2: After 24 hrs incubation of bacterial subculture.

3.2.4 Preparation of bacterial suspension in 0.9% sterilized saline

0.9% NaCl solution was prepared in falcon tube and autoclaved for 20 min at 121°C. Then the saline was allowed to cool at room temperature. Every strain was collected from the media and dipped into the saline and mixed thoroughly to prepare the bacterial suspension.

3.2.5 Preparation of disc

The disc was made of Whitman paper with 6 mm diameter and autoclaved in a test tube. Then solutions of plant extract were added to get 350 µg, 250 µg and 150 µg concentrations per disc, respectively. Afterward, it was allowed to soak all the plant extract for 10-15 min.

3.2.6. Procedure

To observe the antibacterial activity of *C. verrucosa* leaves extract in 3 different microorganisms, a fresh medium was taken. The suspension of bacteria in sterile saline was taken and a cotton swab was dipped into it. The swab was gently squeezed against the tube to get rid of excess fluid. At first, the swab was used to streak the bacterial suspension to the nutrient agar plate in one direction and then it was streaking in right direction and after that it was streaked diagonally. Afterward, the swab was used to streak the strains at the end of the diameter of the nutrient plate. Then the agar plate was allowed to get dry for 5 min. 5 minutes later, the disc containing plant extract as well as kanamycin was placed individually on the surface of the plate by using forceps which is showing in the figure 3.3 below. Lastly, the petri dishes were incubated 24 hrs at 37°C to get the lawn growth of bacteria. All of this work was done under the biosafety cabinet.

Figure 3.3: After streaking the bacterial suspension followed by placing the disc containing methanol extract of *C. verrucosa.*

Chapter 4: Results

4.1 *In vitro* cytotoxic activity of methanol extract of *C. verrucosa* by MTT assay

The cytotoxic activity of *C. verrucosa* leaves extract was performed by MTT assay on HeLa cell line. Different concentration (0.0025 mg/mL, 0.025 mg/mL, 0.25 mg/mL, 2.5 mg/mL) of leaves extract were used to analyze the cytotoxic effect. 2% DMSO in DMEM medium was used as control. Absorbance was observed for each concentration and the results are given in the Table 4.1 and the Figure 4.1below-

Table 4.1: Cytotoxic activity of methanol extract of *C. verrucosa*leaves.

Sample Conc. (mg/mL)	**Absorbance**	**% of survival of cells**	**% of inhibition of cells**	**Standard Deviation**	**IC_{50} value (mg/mL)**
Control 2 % DMSO	2.74	100	0	2.12	0.83
0.0025	2.74	100	0	2.12	
0.025	2.74	100	0	2.12	
0.25	1.79	65	35	6.36	
2.5	0.189	6.8	93.2	0.57	

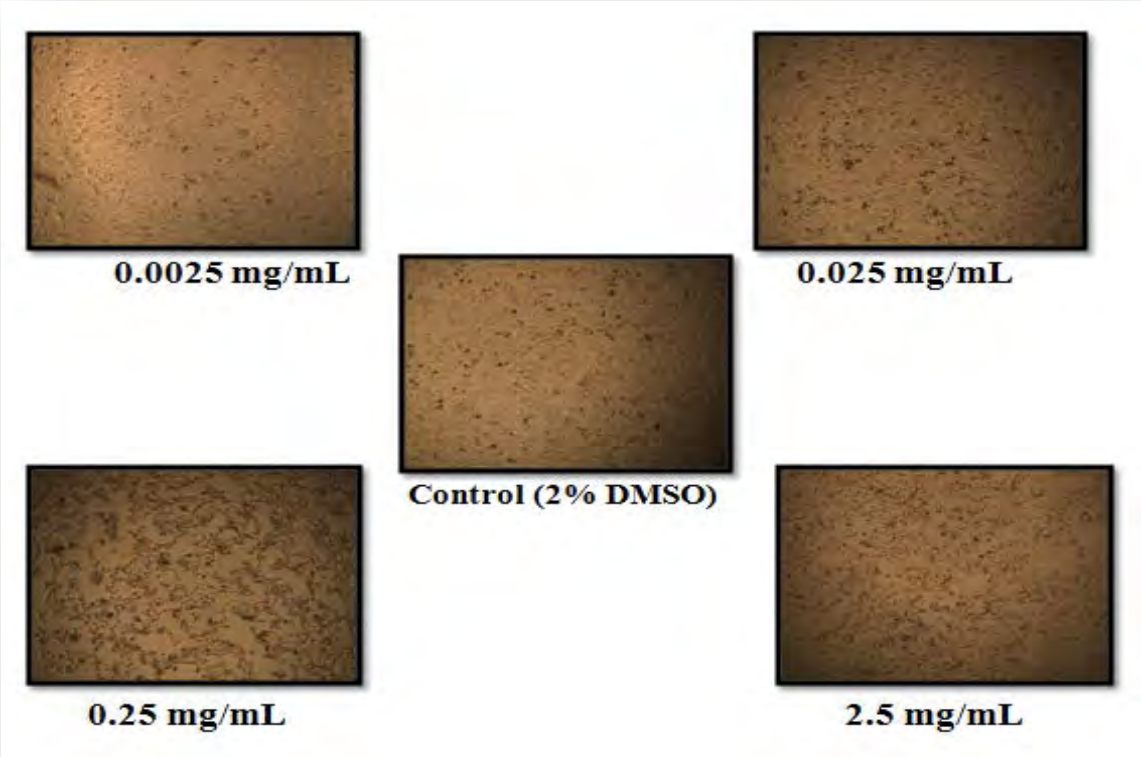

Figure 4.1: Cell viability of methanol extract of *C. verrucosa* at different concentrations and 2% DMSO as a positive control after incubating 48 hrs

HeLa cell was introduced by 0.0025 mg/mL, 0.025 mg/mL, 0.25 mg/mL, and 2.5 mg/mL concentration of *C. verrucosa* leaves extract in the medium and it was incubated for 48 hrs.

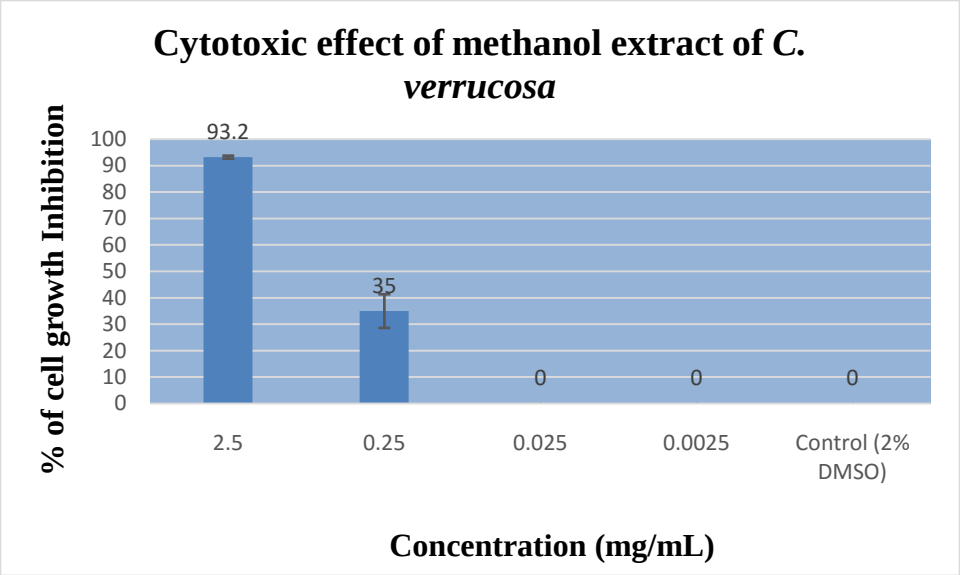

Figure 4.2: Graphical representation cytotoxic activity of *C. verrucosa* leave extract.

The Graphical representation in Figure 4.2 when the cells examinedafter incubation it was observed that survival of HeLa cell was 100% that means no cell death was occurred for 0.0025 mg/mL and 0.025 mg/mL concentrations. On the other hand, in 0.25 mg/mL concentration of *C. verrucosa* leaves extract survival of HeLa cell was 65% that means 35% of cell death occurred in this concentration. Moreover at the highest concentration survival of HeLa cell was 6.8% which indicates that 93.2% cell death occurred in this concentration after 48 hrs of incubation.

4.2. Antibacterial Activity

The antibacterial activity study of *C. verrucosa* leaves extract was done by disc diffusion method. The disc was containing 350 µg/mL, 250 µg/mL and 150 µg/mL respectively and these different concentrations were obtained by serial dilution. Kanamycin was used as positive control and methanol was used as a blank to observe that whether it has any antibacterial effect or not.

C. verrucosa leaves extract showed antibacterial activity against *Bacillus cereus*. The largest zone of inhibition which was 17 mm found at 350 µg/mL concentration against *B. cereus*. Lower concentration of *C. verrucosa* leaves extract also had some antibacterial activity against *B. cereus* but the zone of inhibition was too narrow. On the other hand, *C. verrucosa* did not show

any antibacterial activity against *Streptococcus pneumoniae* and *Shigella dycenteriae*. Kanamycin was used as positive control and it showed distinct antibacterial effect against all 3 strains. The zone of inhibition of kanamycin against *Bacillus cereus*, *Streptococcus Pneumoniae* and *Shigella dycenteriae* was 31mm, 20 mm and 21 mm, respectively. Methanol was used as negative control because it was used as solvent for dilution of plant extract and no antibacterial activity was observed for methanol. The Table 4.2 is showing the zone of inhibition against 3 strains at different concentration.

Table 4.2: Antibacterial activity ofmethanol extract of *C. verrucosa*leaves.

Bacterial strain	**Zone of Inhibition (mm)**		
Different conc. Per disc µg / disc	*Bacilus cereus* (mm)	*Shigella dysenteriae* (mm)	*Streptococcus pneumoniae* (mm)
350	17	-	-
250	6	-	-
150	-	-	-
30 (Kanamycin)	31	21	20
250 (methanol)	-	-	-

Figure 4.3: The effect of *C. verrucosa* against *Bacillus cereus.*

Figure 4.4: The effect of *C. verrucosa* against *Streptococcus pneumoniae.*

Figure 4.5: The effect of *C. verrucosa* against *Shigella dycenteriae.*

The antibacterial activity of *C. verrucosa* leaves extract has been shown in the Figure 4.3, 4.4 and 4.5 against *Bacillus cereus, Streptococcus pneumonia* and *Shigella dysenteriae*, respectively.

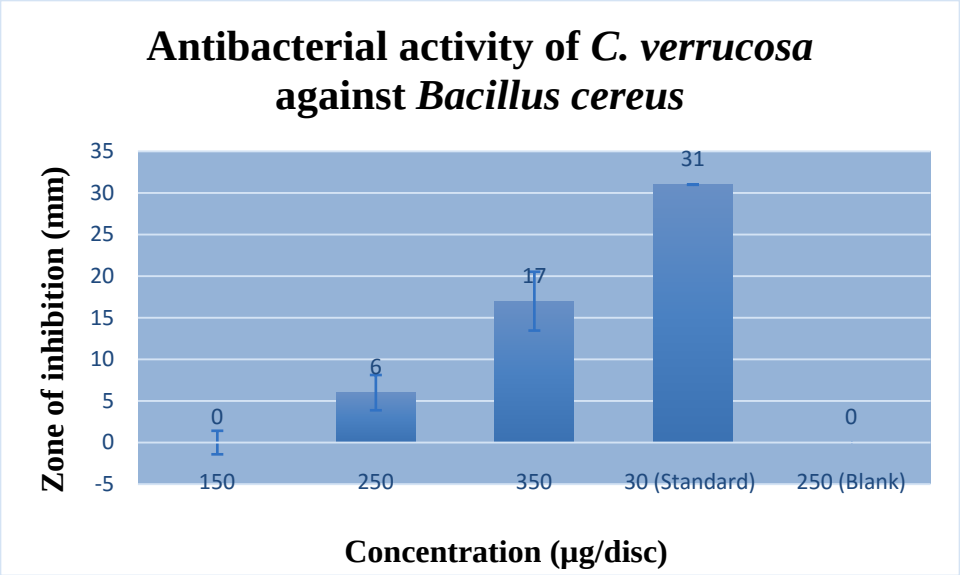

Figure 4.6: Graphical representation of antibacterial activity of *C. verrucosa* against *Bacillus cereus*.

Figure 4.6 is showing the graphical representation of antibacterial activity of *C. verrucosa* leaves extract and kanamycin against *Bacillus cereus*. The zone of inhibition of this plant extract was 17 mm and the zone of inhibition of kanamycin was 31 mm. other 2 strains the antibacterial activity leaves extract was absent in the media. Methanol also did not show any antibacterial activity. On the other hand, kanamycin showed antimicrobial activity against *Streptococcus pneumoniae* and the *Shigella dycenteriae*, zone of inhibition was 20 mm and 21 mm, respectively.

Chapter 5: Discussion

C. verrucosa is a well-known traditional herbal medicine. This plant is used in the south Asian countries like Bangladesh, Myanmar, Nepal, China, India etc. The leaves of this plant are widely used to treat biliousness, dyspepsia, fever, throat and mouth disease. It is also reported that the leaves give therapeutic effect in several heart diseases and it also used as CNS depressant and diuretic. Leaves are known as emetic and expectorant as well as have the ability to soothe skin allergies.

Cytotoxic activity study of *C. verrucosa* was never performed before. On the other hand, cytotoxic activity of other *Crotalaria* species was performed using different cancer cell line. A study was conducted with ethanol extract of *C. agatiflora* using XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) colorimetric assay on one noncancerous and four cancerous cell lines. Two pure compound, named as madurensine and doronenine was isolated from *C. agatiflora* and used as anticancer agent which showed significant number of cell death on cancerous U-937 cells (Le Roux, Hussein, & Lall, 2011). Another study showed that *C.retusa* has been shown cytotoxic activity against cancer cell line using MTT assay. The leaf extracts of *C. retusa* was contained saponins, phenols, tannins, alkaloids and sterols. Various parts such as leaf, stem, seed, pod and flower was used and all of them exhibit cytotoxic activity at dose dependent manner (Anim, Larbie, Appiah-Opong, & Aning, 2016).

Though cytotoxic study has been done with other species but there is no cytotoxic study of *C. verrucosa* was performed before. In current study, *in vitro* cytotoxicity activity was examined on cancer cell lines where different concentrations of leaves extracts were used to determine the cell viability. DMSO (2%) was used as positive control and viable cells was determined after incubating the medium for 48 hrs using micro plate reader. Obtained result showed that there was no cell death at low concentration but at high concentration significant number of death of cell has been found. It showed 35% and 93.2% cell death at0.25 mg/mL and 2.5 mg/mL concentrations of leaves extract, respectively. A dose response curve was drawn to determine the cytotoxicity effect of *C. verrucosa* leaves extract against concentration. This graph represented that the cell growth inhibition was concentration dependent. With increasing concentration of

plant extract, cytotoxic activity also increases gradually. From previous studies it is found that leaves extract of *C.verrucosa* contains alkaloids, flavonoids, glycosides, tannins, steroids and polyphenolic compound etc. these chemical compounds may cause the cytotoxicity. Literature search revealed that plant-derived compounds such as alkaloids, flavonoids, tannins and terpenoids exhibit the cytotoxic activity on cancer cell (Nwodo, Ibezim, Simoben, & Ntie-Kang, 2016). So the results ensured that the leaves extract of *C. verrucosa* have the potential to show cytotoxic activity against cancer cell line.

From the ancient time, a large number of medicinal plants serves as antibacterial agent and act against pathogen. Microorganisms like bacteria and viruses become susceptible easily with new drugs. But there is less possibility to invent new drugs to meet the increasing demand of antibacterial agent. Therefore, to prevent the diseases that occur due to bacterial infections, discovery of new drug is getting essential day by day. Medicinal plants are a good source of compound which can give lots of therapeutic effects as well as antibacterial effect. The main aim of this study to determine the bioactivity of *C. verrucosa* leaves extract. The antibacterial study of various parts of extracts *C. verrucosa* has been performed in *Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* previously. So in this current study antimicrobial study has been done by using other strains.

This study also focused on the determination of the antibacterial activity of *C. verrucosa* leaves extract using 3 different bacterial strains: *Bacillus cereus, Shigella dysenteriae, Streptococcus pneumoniae* by disc diffusion method. Kanamycin disc was used as positive control. Antibacterial activity study findings showed that the largest zone of inhibition was found in *Bacillus cereus* at 350 µg/disc concentration among 3 concentrations (350 µg/disc, 250 µg/disc and 150 µg/disc) and it was 17 mm. The other 2 strains did not show any zone of inhibition. A dose response curve was drawn to determine the antibacterial effect of *C. verrucosa* leaves extract against concentration. This graph represented that the cell growth inhibition was concentration dependent. On the other hand, kanamycin showed greater zone of inhibition against all 3 microorganisms. Previous study has shown that the methanol extract of *C. verrucosa* seeds showed 6 mm, 3mm and 1mm zone of inhibition against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* respectively (Prabhakar, Kamalakar, Vardhan, & Shailaja, 2015).

Another antibacterial activity study revealed that the *n*-butanolic extract of *C. verrucosa* showed inhibiting zone of 15 mm for *Bacillus subtilis* and *Klebsiella pneumoniae*, 13 mm *Escherichia coli*, 14 mm *Proteus vulgaris* and 12 mm *Pseudomonas aeruginosa* (Prasad, Sudha, Khadri, & Riazunnisa, 2015).

By searching literature, it was found that plant-derived compounds such as flavonoids, tannins, alkaloids as well as polyphenolic compounds are mainly responsible for antibacterial activity (Cowan, 1999). Phytochemical screening of this plant which was carried out by another research student in our lab revealed that *C. verrucosa* contains alkaloids, flavonoids, glycosides, tannins, streroids and polyphenolic compound etc. These chemical compounds may attribute to give the antibacterial effect. Thus, the leaves extract of *C. verrucosa* shows antimicrobial activity to specific microorganisms.

On a concluding note, this current study of *C. verrucosa* established that it has a strong cytotoxic effect as well as antibacterial effect.

Chapter Six: Conclusion

Medicinal plants have been played an important role in the history of drug discovery. Traditional medicinal plants were used from the ancient time to cure illness. Recently the researchers show much more interest to transform the medicinal plant or natural sources into drug cause lead compound from natural sources exhibit less side effects and reduce toxicity in the body. In his study, the aim was to investigate the *in vitro*cytoxic and antibacterial activity of methanol extract of *C. verrucosa* which is traditionally used to treat biliousness, dyspepsia, fever, skin disease as well as heart diseases.

The cytotoxicity activity study was done by using different concentration of methanol extract of *C. verrucosa* on HeLa cell line. The highest percentage of cell death (93.2%) occurs at 2.5 mg/mL concentration. The IC_{50} value is 0.83 mg/mL which indicated that, this plant extract exhibit strong cytotoxic activity in a concentration dependent manner.

The antibacterial activity study was performed by agar disc diffusion method using *Bacillus cereus*, *Streptococcus Pneumoniae* and *Shigelladycenteriae*. Kanamycin was used as positive control. The findings of this study showed that the methanol extract of *C. verrucosa* has concentration dependent antibacterial activity on *Bacillus cereus* and the largest zone of inhibition was 17 mm. On the other hand, kanamycin showed zone of inhibition of 31 mm, 20 mm and 21 mm against *Bacillus cereus*, *Streptococcus Pneumoniae* and *Shigella dycentiae* respectively.

On the concluding note, after performing the cytotoxicity assay of *C. verrucosa* leaves extract, it is established that the plant extract possesses cytotoxicity activity against cancer cell line thus may be used as anticancer agents. The findings of antibacterial activity study established that it has moderate antibacterial activity. From pervious study, it is found that *C. verrucosa* contains alkaloids, flavonoids, glycosides, tannins, streroidsand polyphenolic compound etc. These chemical compounds may attribute to give the cytotoxic as well as antibacterial effect. The presence of this compounds in the leaf extract of *C. verrucosa* which extrapolates to its promising cytotoxicity potential and antibacterial activity and it also justified the traditional uses of this plant.

The result of the present study established that methanol extract of *C. verrucosa* possesses moderate cytotoxic and antibacterial activity thus it broadening up a dimension full of different scopes for further studies:

- The study of the *in-vitro* cytotoxic and antibacterial activity of *C. verrucosa* encourages carrying out *in-vivo* study to demonstrate the pharmacological effect on laboratory animal-models.

- Isolation of those compound which are responsible for the screened activity in this project.

- Only Necic lactone was isolated from the leaves of *C. verrucosa* has been found. Thus, appropriate research initiatives may be carried out for the isolation of phytochemical constituents present in the leaves of *C. verrucosa*.

- Further bioactivity studies of this plant should carried out which are not explored yet. Such research initiatives into these activities might lead to the drug discovery, drug isolation and may serve this plant as a natural source for the development of novel drug compounds.

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