Investigation of In vitro Antioxidant and Cytotoxic Potential of Methanolic Extract of *Celosia argentea* Leaves

By

Nusrat Jahan Mitu

16146023

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Nusrat

Nusrat Jahan Mitu
16146023

Approval

The thesis titled "Investigation of In vitro Antioxidant and Cytotoxic Potential of Methanolic Extract of *Celosia argentea* Leaves" submitted by Nusrat Jahan Mitu (16146023) of Spring, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 8th October, 2020.

Examining Committee:

Supervisor:
(Member)

Md. Samiul Alam Rajib

Senior Lecturer, Department of Pharmacy

BRAC University

Program Coordinator:
(Member)

Dr. Hasina Yasmin

Associate Professor, Department of Pharmacy

BRAC University

Departmental Head:
(Chair)

Professor Dr. Eva Rahman Kabir

Chairperson, Department of Pharmacy

BRAC University

Ethics Statement

This study does not involve any animal or human trial.

Abstract

Celosia argentea, known as “morogful” in Bangladesh can be used as potential therapeutic agent and current medicines worldwide. The methanolic extract of leaves of *Celosia argentea* was chosen for investigation of antioxidant potential and *in vitro* cytotoxicity activity evaluation for the first time. The preliminary phytochemical screening revealed the presence of alkaloid, flavonoid, phenol, tannin, saponin in the crude methanolic extract of the plant. The leaf extract showed noteworthy antioxidant activities through total phenolic content, flavonoid content and antioxidant capacity. Total phenolic content was found to be 58.27 mg of gallic acid equivalent per gram at the concentration of 1200 μg/mL. Total flavonoid content was found to be 175.48 mg of quercetin equivalent per gram at the concentration of 1200 μg/mL. Finally, total antioxidant capacity was found to be 22.73 mg of ascorbic acid equivalent per gram at concentration of 1200 μg/mL. Therefore, overall antioxidant potential was very strong. MTT assay of methanol extract was prepared to assess the cytotoxic activity in HeLa cell line. Percentage of cell growth inhibition obtains 51.27% at highest concentration. To conclude, it can be inferred that extract showed strong antioxidant potential with high cytotoxic potential.

Keywords: Antioxidant, Cytotoxicity, *Celosia argentea,* MTT assay.

Dedication

Dedicated to my beloved parents for the unconditional love and support.

Acknowledgement

I would like to begin my gratitude to Allah for the help in the completion of this research and preparation of this paper.

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List of Acronyms

BHT	*tert*-butyl-1-hydroxytoluene
DMSO	Dimethyl sulphoxide
QE	Quercetin equivalent
TAC	Total Antioxidant Capacity
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
FCR	Folin Ciocalteu Reagent
WHO	World Health Organization
ASA	Ascorbic Acid
GAE	Gallic Acid Equivalent
LAF	Laminar Air Flow
ME	Methanolic Extract

Chapter 1

Introduction

1.1 General Information:

From the beginning of existence of human being in this world, illness is considered as a part in the occasions of each time. To get rid of this illness, distinctive solution of immunizations have been assumed for the diagnosis, treatment and prevention of the diseases. However, researchers are searching for such type of medications for some disease where no pharmaceutical products are available. Now-a-days most of the people take antibiotics or other medications for such type of disease where it is not necessary to take resulting harmful effect on long term use (Neill et al., 2013). For this reason, researchers are looking for such type of medications without having side effect. In this case medicinal plants can be a good alternative as they have some special constituents which shows more therapeutic effect with less or no side effects. From the ancient period of time plants are being used for different purposes with or without knowing their biological activity. Moreover, some chemical compounds are isolated from plants which also have biological or therapeutic activity. For example- garlic, ginger, aloe, pepper, turmeric are nutritious and also have therapeutic activity. Furthermore, some plants are used as active ingredients for the preparation of medications (Singh, 2015). The World Health Organization (WHO) has shown that about 80% of people are currently using natural medicines as their essential needs for health care. The use of medicinal plant treatment is increasing day by day as it has no or minimal side effect. Although the use of herbal medicines are expanding over time, the research in this field are not adequate. (Rafieian and Kopaei, 2012).

1.2 Relationship of Phytotherapy with medical plants:

Phytotherapy is the oldest medical practice in the world which can be defined as the way of treatment using medicinal plants. This technique is more traditional, but it is proven by modern science. The concept of phytotherapy was first discovered by french physician Henri Leclerc, who published multiple phytotherapy editions (Heinrich, 2013). So, phytotherapy concept is proven by modern science but it is more traditional concept. By realizing the benefits of medicinal plants, different countries use different medicinal plants in a different manner which are now included in pharmacopeias. For instance, phytotherapy products were introduced into health insurance coverage by South Korea and Japan. On the contrary, China, India, and Nepal evaluate herbal medicines as a wide range of healthcare services (Heinrich, 2013).

1.3 Objective and Rational of this study:

Approximately 80% of our country's population depends on drugs and medicinal plants to prevent disease (Sadi, 2012). This study's main purpose is to know about the constituents of *Celosia argentea* and their use for medical purposes. This research would demonstrate how the plant parts and their constituents are used effectively to prevent different diseases. Thus, pharmaceutical industry and medical science will be more beneficial by using the plants for medicine preparation.

1.4. Traditional drug research in Bangladesh:

Ayurvedic, homeopathic, unani are very popular and well known treatment methods in rural regions in Bangladesh. Such traditional medicines use different parts of plants with medicinal properties. For their primary health care and disease remedy, rural people are mostly reliant on medicinal plants Due to its fertile soil, climate and occasional variety, Bangladesh has about 5000 plant species (kadir, 2004). Approximately, 85% of the population living in rural areas in which nearly 80% rely on therapeutic plants. It was confirmed that many individuals use such medication frequently as herbal medicine is sometimes perceived to be relatively low cost (Halim,et al. 2007). Its success derives most of the time from the efficacy of the procedure with few or no side effects and is comparatively healthy (Elliot and Brimacombe, 2012). Because of unavailability of drugs and commodities, lack of trained providers, imposition of unofficial fees, a rural-urban imbalance in health provider's distribution, unfavorable opening hours and weak referral mechanisms; poor people have a little access to health-related services. Thus, these are contributing to low utilization of public facilities in Bangladesh. (Nawaz et al, 2009).

1.5 Name and taxonomic hierarchy of the plant:

Scientific Name: *Celosia argentea*

Local Name: Morogful

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Caryophyllales

Family: Amaranthaceae

Genus: *Celosia*

Species: *Celosia argentea*

1.6 The plant family: Amaranthaceae:

Amaranthaceae, amaranth family (order Caryophyllales) consisting of 175 genera and more than 2,500 species, where most of them are herbs and subshrubs that are distributed throughout the world. Some of them for example- beets and quinoa are essential food crops whereas others are produced as garden ornamentals. Members of this family mostly grow well in saline soil annually. Leaves are pulpy and arranged opposite along with the stems. Due to the presence of betalain pigments, the color of some stems, roots, leaves and flowers of many species is red. The small flowers can be bisexual or unisexual and the fruit may be a capsule, utricle, nutlet, drupe, or berry. Most of the species are C_4 plants in this family which means it is the largest collection of photosynthetic plants (The Editors of Encyclopedia Britannica, Amaranthaceae, 2017).

1.7 The plant Genus: *Celosia:*

Celosia, is a genus of about 45 species of herbaceous or flowering plants in the amaranth family (Amaranthaceae). It is widely distributed in tropical America and Africa. A variety of species including the cockscomb are grown as garden ornamental known as wool flowers which has large flower spikes. In West Africa Silver cockscomb is produced for its beneficial leaves and considered as an essential food crop.

1.8 General description of *Celosia argentea:*

Celosia argentea is an annual herb that grows up to 2 meter. It grows in the middle of summer that is from May to September. It reproduces by seeds and the seeds are very small in size. The color of the flower may vary from yellowish to reddish. Leaves are linear and the color is darker green. The plant needs full sunlight and well-drained soil to grow well. It is mostly found in the hill tracts of Chattogram and mostly known as morogful in Bangladesh. It is also found in India and China as well (Nariya et al., 2009).

Figure 1: Flower and Leaves of Celosia argentea (Nariya et al., 2009).

1.9 Traditional use of *Celosia argentea:*

The whole plant is used as an antidote for snakebites (Yusuf et al., 2004). The leaves are used in the treatment of cancer in Chattogram hill tract. The roots are used for the treatment of colic, gonorrhea and eczema. It is used in the treatment of diarrhea, bloodshot eyes, blurring of vision, cataracts and hypertension and it has mydriatic action so not used by people with glaucoma as it dilates the pupils. In India for treating diabetes mellitus, the seeds are widely used (Mukulet al., 2007).

1.10 Other reported Species of *Celosia argentea*:

- *Celosia cristata*
- *Celosia nitida*
- *Celosia trigyna*
- *Celosia virgate* (Hossain et al., 2016)

Chapter 2

Methods

2.1 Collection and identification of plant:

For the study, *Celosia argentea* plant was chosen as no prior experiment was conducted for any of its properties except chemical constituents. The plant *Celosia argentea* was collected in October 2019 from Chattogram, Bangladesh. After collection, the plant was given to the National Herbarium of Bangladesh (NHB), Mirpur, Dhaka for the identification.

2.2 Extraction Procedure

The extraction process can be divided into 2 steps:

- Plant material preparation and drying
- Extraction

2.2.1 Plant material preparation and drying:

At first the leaves were separated from the stem and then for removing dust particles, the leaves were washed with clean water. After washing the leaves were placed for sun drying for almost three weeks. After proper drying the leaves were blended to make it powder form by using grinding machine. Finally, the powder was stored in an air tight container for further use and kept it in a dry and moisture free place.

2.2.2 Extraction of the plant material:

450 gm of the plant powder was taken in a clean beaker and soaked in one liter of methanol for 14 days. The beaker was sealed with foil paper and random stirring and shaking was done in a daily basis. After that, the mixture was filtered by a clean cloth and then by a fresh cotton plug. The filtrate was then placed in a Rotary evaporator for getting a concentrated methanolic extract. The rotary evaporator was rotated at 90 rpm at 50ºC until the concentrated methanolic extract

was produced. Afterwards, the extract was taken into petri dishes which was kept under Laminar Air Flow (LAF) for further drying. It was done for getting a semisolid, dry extract and also for evaporating the solvent from the extract. Moreover, in this process Laminar Air Flow was used to prevent the microbial growth to the plant extract. After keeping the plant extract under Laminar Air Flow for 15 days, a dry and semisolid extract was achieved which was covered with Aluminum foil. Finally, the covered petri dish was kept in refrigerator for further use.

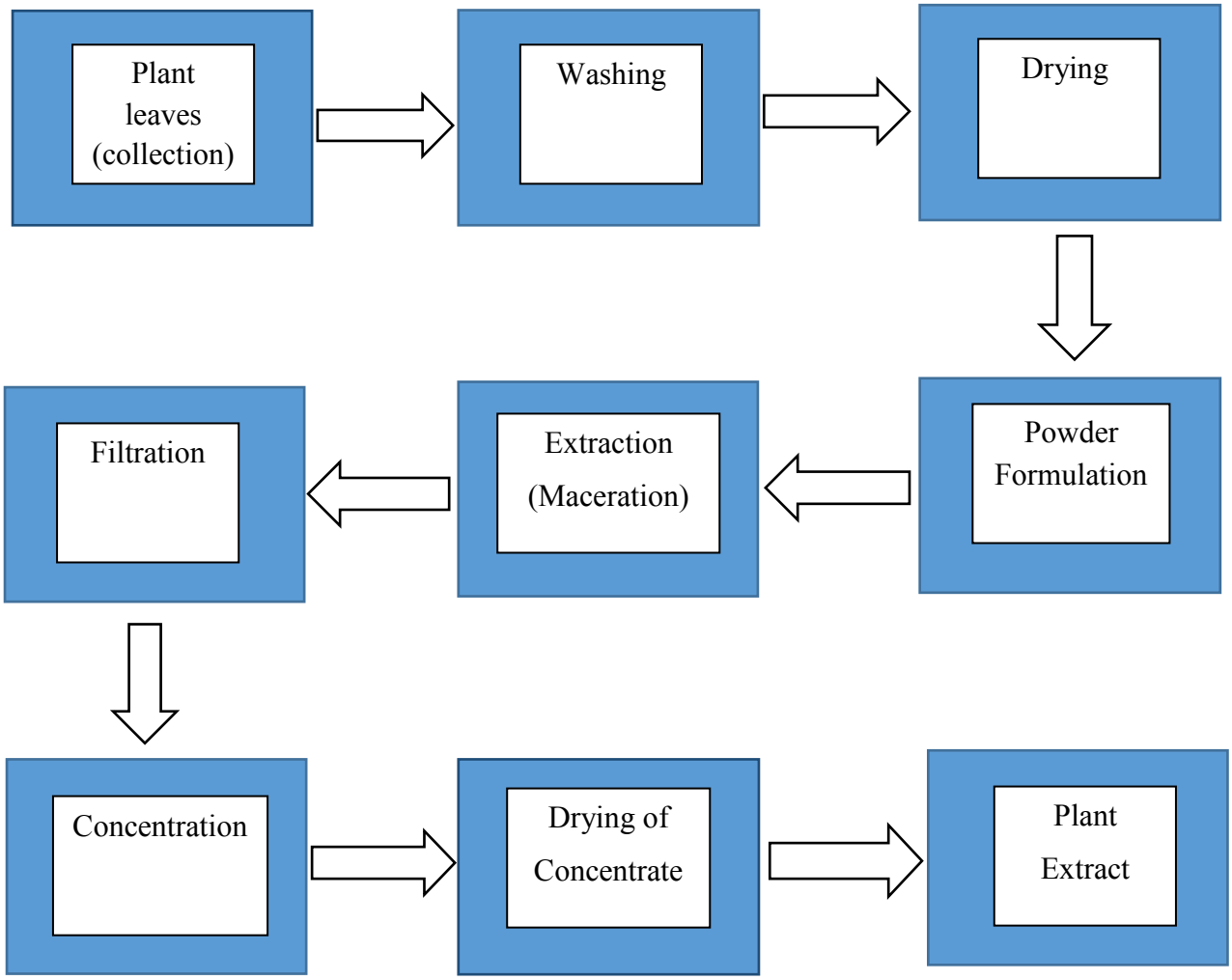

Figure 2: Step by step process for extraction of medicinal plant.

2.3 Phytochemical screening

Phytochemical screening was done on the crude extract of *Celosia argentea* in order to identify its qualitative chemical constituents such as alkaloids, carbohydrates, tannin, resin, flavonoids etc.

The following qualitative tests were performed for identification:

2.3.1 Detection of alkaloid

Three tests were performed for the qualitative determination of the alkaloids. 0.5 gm of methanolic extract of *Celosia argentea* was dissolved in 5 ml of 1% Hydrochloric acid, boiled in a water bath followed by filtration. Using the filtrate obtained the following tests were performed.

2.3.1.1 Hager's test

A few drops of Hager's reagent (1% picric acid solution) were added to 2 ml of the filtrate and the presence of alkaloids was confirmed by the formation of yellowish precipitate (Waldi, 1965).

2.3.1.2. Mayer's test

10 ml of Mayer's reagent was prepared by dissolving 0.1358 gm of Mercuric (II) chloride and 0.5 gm of Potassium iodide in 10 ml of distilled water.

After that, a few drops of Mayer's reagent were added to 2 ml of the filtrate along the sides of the test tube. The formation of a white or creamy precipitate indicated the presence of alkaloids (Trease & Evans, 1997).

2.3.1.3 Wagner's test

10 ml Wagner's reagent was prepared by dissolving 0.2 gm of Iodine crystal and 0.6 gm of Potassium iodide in 10 ml distilled water. To 2 ml of the filtrate, a few drops of Wagner's reagent were added. Formation of a brownish-black precipitate confirmed the presence of alkaloids in the sample (Wagner,1993).

2.3.2 Detection of carbohydrates

According to, carbohydrate can be qualitatively detected by weighing out 0.5 gm of methanolic extract of *Celosia argentea* and dissolving it in 5 ml of distilled water and then filtering the mixture. To the filtrate obtained, the following three tests were performed (Ramkrishnan et al., 1994):

2.3.2.1 Molisch's test

2 ml of filtrate obtained was treated with 2 drops of Molisch's reagent (alcoholic solution of α-napthol) and also 2 ml of concentrated Sulfuric acid was added along the sides of the test tube and the test tube was allowed to stand for a while. The presence of carbohydrate was confirmed by the formation of violate ring (Ramkrishnan et al., 1994).

2.3.2.2 Phenol test

Few drops of ferric chloride solution were added to 0.2 g of extract. A dirty green or green precipitate indicated the presence of phenolic compound (Ramkrishnan et al., 1994).

2.3.2.3 Lead acetate test

50 mg of extract was dissolved in distilled water and 3 mL of 10% lead acetate solution was added. The presence of phenolic compounds confirmed by white precipitate (Ghani, 2003).

2.3.2.4 Dilute iodine solution test

A few drops of dilute iodine solution were added to 3 mL of extract. The presence of phenolic compound was indicated by formation of transient red color (Ghani, 2003).

2.3.3 Detection of glycoside

2.3.3.1 Concentrated H2SO4 test

1 ml of concentrated hydrochloric acid was added in 1 ml of extract. Reddish color precipitate indicated the presence of glycosides (Tiwari & Bimlesh, 2011).

2.3.3.2 Legal's test

Alkaline sodium nitroprusside and pyridine were added in 2 ml of extract. Cherry red color solution indicated the presence of glycoside (Waldi, 1965).

2.3.4 Detection of phytosterols

2.3.4.1 Libermann Burchard's test

1 ml of chloroform was added to a small amount of extract which was filtered afterwards. 2 ml of acetic anhydride was added in the filtrate which was boiled and cooled. Lastly, 1 ml of concentrated Sulfuric acid was added to the solution. The presence of phytosterols was indicated by the Formation of brown ring at the junction (Soni and Sosa, 2013).

2.3.5 Detection of steroids

2.3.5.1 Salkowski test

To 1 ml of extract, 2 ml of chloroform and 1 ml of Sulfuric acid were added. The appearance of red color indicated the presence of steroids (Ghani, 2003).

2.3.6 Detection of tannin

2.3.6.1 Lead acetate test

A few drops of 1% Lead acetate solution were added to 1 ml of the crude extract and the formation of yellow colored precipitate indicated the presence of tannins (Tiwari and Bimlesh, 2011).

2.3.6.2 Potassium dichromate test

10% Potassium dichromate solution was prepared by dissolving 1 gm of Potassium dichromate in 10 ml distilled water. After that, 5 ml aqueous solution of crude extract was dissolved in 1 ml of 5% Ferric chloride solution. The formation of yellow precipitation indicated the presence of tannin (Ghani, 2003).

2.3.7 Detection of resin

According to, the presence of resin can be identified by adding 5-10 drops of acetic anhydride to 2 ml of the extract and heating the solution gently. This was then followed by addition of 0.5 ml of Sulfuric acid to the solution. The presence of resin was confirmed by the formation of a bright purple color (Soni and Sosa, 2013).

2.3.8 Detection of saponins

2.3.8.1 Froth test

The extract was diluted with distilled water and the volume was made up to 20 mL. The contents of the cylinder were shaken in a graduated cylinder for about 10-15 min. The formation of a foam layer of about 2 cm in height indicated the presence of saponins (Kokate, 1999).

2.4 Evaluation of antioxidant potential

There are different forms of antioxidant studies and three of them were chosen for the antioxidant analysis of *Celosia argentea*.

The three studies are:

- Total Phenolic Content (TPC)
- Total Flavonoid Content (TFC)
- Total Antioxidant Capacity (TAC)

2.4.1 Determination of Total Phenolic Content (TPC)

Phenolic compounds are the compounds that can be regarded as an integral component of protecting body cells from damage caused by hydrogen peroxide and the harm caused by unsaturated fats and lipid peroxides, absorbing and neutralizing free radicals (Sroka & Cisowski, 2003).

The phenolic compounds associate their antioxidant properties with redox properties that enable them to act as reduction agents, as donors of hydrogen and as single-oxygen quenchers (Proestos et al*.,* 2006). Folin-Ciocalteu method was the most popular method and by the enhanced version of this method. The total phenolic content of the methanolic extract of *Celosia argentea* was evaluated (Wolfe et al., 2003).

Reagents and chemicals

- Folin-Ciocalteu Reagent (FCR)
- Gallic acid monohydrate
- Sodium carbonate
- Methanol

2.4.1.1 Reagent and sample preparation

At first, by taking 2 mL of FCR (10 percent FCR) in a 20 mL volumetric flask, 20 mL FCR solution was prepared. 7.5 gm of sodium carbonate was weighed and dissolve in 100 ml volumetric flask and diluted with distilled water to 100 ml mark by this 100 mL of 7.5% Sodium carbonate was produced.

2.4.1.2 Sample and standard preparation

Firstly, the stock solution was prepared by measuring 12 mg of the extract dissolved in 10 mL of methanol. The sample concentrations were produced to extract 4 serially diluted concentrations by serial dilution of the sample stock solution: 1200 μg/mL to 200 μg/mL. Thus, standard was Gallic acid and it was prepared in the same way as the stock solution was prepared by dilution.

2.4.1.3 Preparation of the blank solution

In the experiment, same volume of FCR solution and Sodium carbonate was used as blank solution. 10 ml blank solution was prepared by adding 5 ml FCR, 4 ml Sodium carbonate and 1 mL of methanol.

2.4.1.4 Procedure

In test tubes, 1 mL of each of the fraction of sample and standard was taken and 5 mL of FCR solution was included into it. After that, 4 mL of Sodium carbonate solution was included. For 15s each of the mixtures had been vortexed and allowed to stand in a water bath for 30 minutes at 40°C.

Finally, by using a spectrophotometer the absorbance of standard and sample solutions was determined against blank at 765 nm.

The total phenolic content, C, for each of the fractions was expressed as Gallic acid equivalent (Gallic acid equivalent)/gm of the extract using the following equation:

$C = c \times V/m$

where, C = Total content of phenolic compounds

c = Concentration of Gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (gm)

2.5.1 Determination of Total Flavonoid Content (TFC)

The method of determining the total flavonoid contents of the extracts of *Celosia argentea* leaves explained by Kumaran and Karunakaran (2007).

Reagents and chemicals

- Quercetin (standard)
- Methanol
- Potassium acetate
- Aluminum chloride

2.5.1.1 Reagent preparation

100 mL of 10% Aluminium chloride solution was produced by weighing 10 gm of Aluminium chloride in a 100 mL volumetric flask. Then, the solution was diluted with distilled water to 100 mL mark.

100 mL of 1M Potassium acetate solution was produced by weighing 9.815 gm of Potassium acetate in a 100 mL volumetric flask. The solution was distilled with water to make 100 ml.

2.5.1.2 Sample and standard preparation

Firstly, the stock solution was prepared by measuring 12 mg of the extract dissolved in 10 mL of methanol. The sample concentrations were produced to extract 4 serially diluted concentrations by serial dilution of the sample stock solution: 1200 μg/mL to 200 μg/mL.

The standard was Quercetin and it was prepared in the same way as the stock solution was prepared by dilution, ranging from 1200 μg/mL to 200 μg/mL.

2.5.1.3 Preparation of the blank

The blank solution comprised 200 μL of 10% aluminium chloride solution, 200 μL of 1M Potassium acetate solution, 5.6 mL of distilled water and 4 mL of methanol. 10 mL of the blank solution was in total.

2.5.1.4 Procedure

In test tubes,1 mL of each of the fraction of 200 μL aluminium chloride, 200 μL potassium acetate solution was taken and 3 ml of methanol was added. Finally, to make 10 ml volume in total, 5.6 ml of water was added to the solution. After doing this, the test tubes were stand in the room temperature for 30 minutes.

Finally, by using a spectrophotometer the absorbance of standard and sample solutions was determined against blank at 465 nm.

The total flavonoid content, C, for each of the fractions was expressed as Quercetin equivalent (QE) using the following equati

$C = c \times V/m$

Where, C = Total content of flavonoid compounds

c = Concentration of Quercetin obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (gm)

2.6.1 Determination of total Antioxidant Capacity (TAC)

The TAC of leaves extract of *Celosia argentea* was determined by using a method described by Prieto et al. (1999).

Reagents and chemicals

- L-Ascorbic acid (Standard)
- Methanol
- Ammonium molybdate
- Concentrated Sulfuric acid
- Sodium triphosphate

2.6.1.1 Reagent preparation

To prepare 100 mL of 0.6M Sulfuric acid, 3.28 mL of 98% concentrated Sulfuric acid was weighed and taken in a 100 mL volumetric flask. The solution was diluted to a volume of 100 mL.

To prepare 100 mL of ammonium molybdate solution, 4.494 gm of Potassium acetate was weighed and taken in a 100 mL volumetric flask and diluting it with distilled water to 100 ml mark.

To prepare 100 mL of Sodium phosphate solution, 1.0645 gm of aluminium chloride was weighed and taken in a 100 mL volumetric flask and diluting it with distilled water to 100 mL mark.

2.6.1.2 Sample and standard preparation

Firstly, the stock solution was prepared by measuring 12 mg of the extract dissolved in 10 mL of methanol. The sample concentrations were produced to extract 4 serially diluted concentrations by serial dilution of the sample stock solution: 1200 µg/mL to 200 µg/mL. Thus, the standard was Ascorbic acid and it was prepared in the same way as the stock solution was prepared by dilution, ranging from 1200 µg/mL to 200 µg/mL.

2.6.1.3 Preparation of the blank

For blank solution, 3 mL of reagent solution and 300 µL of methanol was used.

2.6.1.4 Procedure

In test tubes 300 µL of each of the fraction of the samples were taken and then 3 mL of all the reagents were added. Both the sample and the standards were stand at 95°C in water bath for 90 min.

Finally, by using UV spectrophotometer the absorbance of the samples and the standards were measured at 695 nm. The total antioxidant capacity, A, for each of the fractions were expressed as Ascorbic acid equivalent (AAE) using the following equation:

$A = c \times V / m$

Where, A = Total content of antioxidant capacity

c = Concentration of Ascorbic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = Weight of the sample (gm)

2.5 Antimicrobial study by disc diffusion method

Various methods such as dilution method, automated antimicrobial susceptibility testing systems, disk diffusion method etc. can be used for Antimicrobial study. Among all of these method, the disc diffusion method is rapid, accurate and mostly inexpensive. Antimicrobial resistance monitoring may be used for various purposes, such as drug discovery, epidemiology and therapeutic outcome prediction (Balouiri et al., 2016).

2.5.1 Apparatus

- 6 mm diameter whatman paper discs
- Autoclave
- Petri dishes
- Sterile-cotton
- Micropipette
- Laminar Air Flow (LAF) hood
- Incubator
- Refrigerator
- Inoculating loop
- Sterile-forceps

2.5.2 Reagents

- Nutrient agar (mueller hinton agar)
- Antibiotics (Kanamycin 30, Amoxicillin 10)
- Ethanol
- Distilled water

2.5.3 Test organisms

Both gram positive and gram-negative organisms were taken for the test and they are listed in the table below:

Gram positive bacteria	**Gram negative bacteria**
Staphylococcus aureus	*Escherichia coli*
Bacillus subtilis	*Salmonella typhi*

Table 1: List of bacteria used in the study

2.5.4 Preparation of medium

Medium was prepared in 100 ml of distilled water by taking 3.8 mg Mueller Hinton agar in it. The mixture was then mixed thoroughly mixed until the agar had dissolved fully in water. After that the mixture was autoclaved for 20 min at 121°C. Then, the mixture was cooled up to (45-50) °C and poured into 4 sterile Petri dishes equally. Uniform depth of each plate was ensured after pouring into the dishes. The dishes were finally allowed to cool and solidify at room temperature.

2.5.5 Culturing of bacterial strains

The Nutrient Broth (NB) medium was prepared by weighing 0.25 gm NB that dissolved in 10 ml distilled water. The bacteria were then placed to the NB medium from the collected bacterial strains, and incubated for 24 hours to culture those bacteria

2.5.6 Preparation of disc

Disc was prepared from the whatman paper having 6mm diameter. Then solutions of plant extract were added to the disc to soak all the plant extracts and the process took for 10-15 min.

2.5.6 Procedure

A cotton swab had been dipped into the bacterial solutions. The excess fluid was pushed gently squeezed into the tube. After that, the swab was used to streak the bacterial suspension in one direction into the nutrient agar layer, then streaked diagonally. The agar plates were allowed to get dry for 5-7 min. Then, the discs containing plant extract were placed individually on the surface of the site using forceps. The standard was Kanamycin 30, Amoxicillin 10 and they were transferred on the plate surface. Finally, the Petri dishes were incubated for 24 hours at 37°C to get the lawn growth of bacteria.

2.6 Cytotoxicity study by MTT Assay

2.6.1 Used Consumables

96 well plate, 15 mL tubes, Tips, Gloves, PTFE Syringe Filter, Culture flask, Cell culture media, 1% Penicillin-Streptomycin, Gentamycin, Serological pipette, Trypsin etc.

2.6.2 Used Instruments

- Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA),
- CO2 incubator (Nuaire, USA),
- Trinocular Microscope with Camera (Olympus, Japan),
- Hemocytometer.

2.6.3 Celltiter 96 Assay Kit

The Celltiter 96 assay kit is an inventory of qualified reagents that offer a rapid, more preferable system with multiplication and cytotoxicity determination (Ifere et al., 2010). It is a perception of the shift in a cell division with tetrazolium salt under a formazan product that is incontestably differentiated using a 96 well plate.

2.6.4 10 % Fetal Bovine Serum

To prepare 10% FBS, 50 ml of Fetal Bovine Serum was included to 500 ml of DMEM. Fetal Bovin Serum is high in albumin, providing the cells expand faster with nutrient and optimal environment.

2.6.5 2% DMSO Solution

2% DMSO solution was prepared by adding 60 µl in 2940 mL of distilled water for negative control.

2.6.6 Trypsin

0.25 % trypsin was added to the medium.

2.6.7 Preparation of the Different Concentrations of Plant Extract

The assay was conducted by using 5 concentration 1200 µg/ml, 800 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml of the *Celosia argentea* leaves extract.

2.6.8 Cell Passage

Transfer of the cells into a new medium was performed to get a fresh cell suspension cell passage. Consequently, FBS washed the cultivated media and then applied 800 µl of trypsin to remove the cells from the top of the culture vessels. Instead, the cells were incubated and microscopically examined for detachment. After 90 % of cells were removed, 5 ml DMEM media were applied to the vessels and mixed with using a pipette. Eventually, 1 mL of this solution was withdrawn and mixed in a new vessel with 4 mL of DMEM and placed in a incubator.

2.6.9 Harvesting of Cells

The cells were harvested using trypsin in log phase growth.

2.6.10 Counting of Cells

Cell counting had been conducted using a hemocytometer. The hemocytometer was primed by purposely purifying and polishing the mirror with ethanol and lens paper. The coverslip was placed in the region of counting. The fresh cell suspension was inserted into the hemocytometer using Pasteur pipet. Enough suspension was added so the surface was actually overflowing. Moreover, the counting chamber was later placed in the microscope stage then centered on the counting grid. At 40X magnification can be observed in a regular

hemocytometer with rulings 1 whole line. The cells were counted on the 4 broad squares. Either upper or left sides touching cells or lower or right cells were counted.

2.6.11 Procedure

Cytotoxic effect was examined in Centre for Advanced Research in Sciences using their commercial services. In brief, Hela, a human cervical carcinoma cell line was maintained in DMEM (Dulbecco's Modified Eagles' medium) containing 1% penicillin streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine serum (FBS). Hela cells were seeded onto 96 well plate and incubated at 37 degree Celsius+ 5% CO_2. Next day, 25 microliter of sample (filtered) was added each well. Cytotoxicity was examined after 48h of incubation using cell counting kit-8 (CCK), a non-radioactive colorimetric cell proliferation and cytotoxic assay kit. Duplicate wells were used for each sample. Cytotoxic activity was calculated by using a formula which is given below- % of cytotoxic activity = (100 − Absorbance of test sample/ Absorbance of negative control) × 100

Chapter 3

Results

3.1 Determination of the percentage yield of the plant extract, *Celosia argentea*

Initial weight (beaker)	310 gm
Final weight (beaker + powder)	625 gm
Total weight of the powder plant	315 gm

Table 2: Total weight of the powder plant before maceration

Initial weight (beaker)	316 gm
Final weight (extract + beaker)	340.5 gm
Total weight of extract	24.5 gm

Table 3: Net weight of the plant extract after maceration

Interpretation:

In the maceration process and after drying of the methanolic extract of *Celosia argentea*, which was carried out during the experiment, a total weight of 24.5 gm of extract was obtained.

Calculation of percentage yield of the extract

Extract yield percentage (%) = $(W_1 \times 100)/W_2$

Where, W_1= Net weight of extract after maceration

W_2=Total weight of powder taken for maceration

Percentage yield in methanol = $(24.5 \times 100)/315$

=7.7%

Interpretation:

The total weight of the extract after maceration was found 24.5 gm while it was 315 gm before maceration; hence, the % yield of *Celosia argentea* was estimated at 7.7%

3.2 Phytochemical screening of *Celosia argentea*

Alkaloid	+
Carbohydrate	--
Flavonoid	+++
Phenol	+++
Phytosterol	-
Steroid	--
Tannin	+++
Glycoside	--
Resin	-
Saponin	+
Sterol	-

(+ means present in single test method, ++ means present in two experimental method, - means absent single test method, -- means absent in two experimental method)

Table 4: Phytochemical analysis of methanolic extract of Celosia argentea leaves

Interpretation:

Celosia argentea's phytochemical screening of methanolic leaf extract revealed the existence of alkaloid, flavonoid, phenol, tannin, saponin, while carbohydrate, phytosterol, steroid, glycoside, resin and sterol were absent.

3.3 Antibacterial activity of *Celosia argentea* leaves extract

The process of disc diffusion was used to conduct the antibacterial test on the leaf extract of *Celosia argentea*. The sample solution contained 250 µg/ml, 500 µg/ml, 750 µg/ml of disc, four separate bacterial cultures and the standard (ampicillin, kanamycin) was used as positive control. The experiment had found that the leaves extract of *celosia argentea* have no antibacterial effect on *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Salmonella typhi* by showing in the experiment their inhibition zone. The zone of inhibition of kanamycin against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Salmonella typhi* was 25 nm, 18 nm, 15 nm, 25 nm respectively and the zone of inhibition of ampicillin against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Salmonella typhi* was 26 nm, 21 nm, 26 nm, 30 nm respectively. For dilution of the plant extract, Methanol was used as solvent and methanol did not show any antibacterial activity.

Concentration of Sample (µg/ml)	**Bacterial strains and zone of inhibition (mm)**			
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*	*Salmonella typhi*
250	-	-	-	-
500	-	-	-	-
750	-	-	-	-
Kanamycin	25	18	15	25
Ampicillin	26	21	26	30

Table 5: Antibacterial activity of methanolic leaf extract of Celosia argentea.

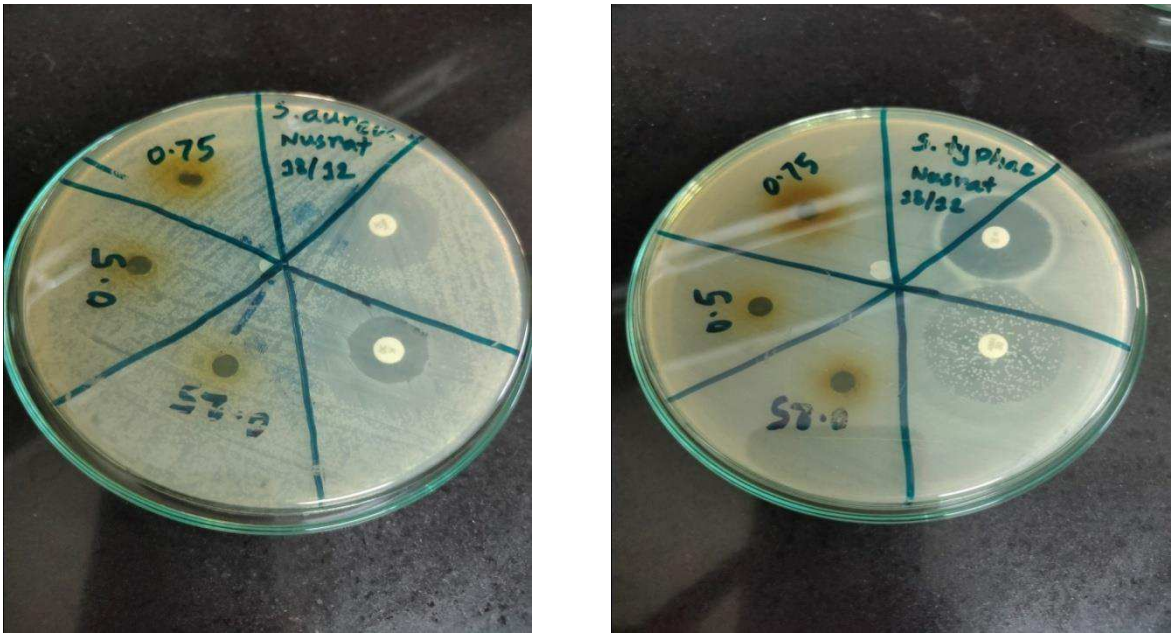

Figure 3: Zone of inhibition shown by standard (Kanamycin and ampicillin) on S.aureus, S.typhi respectively.

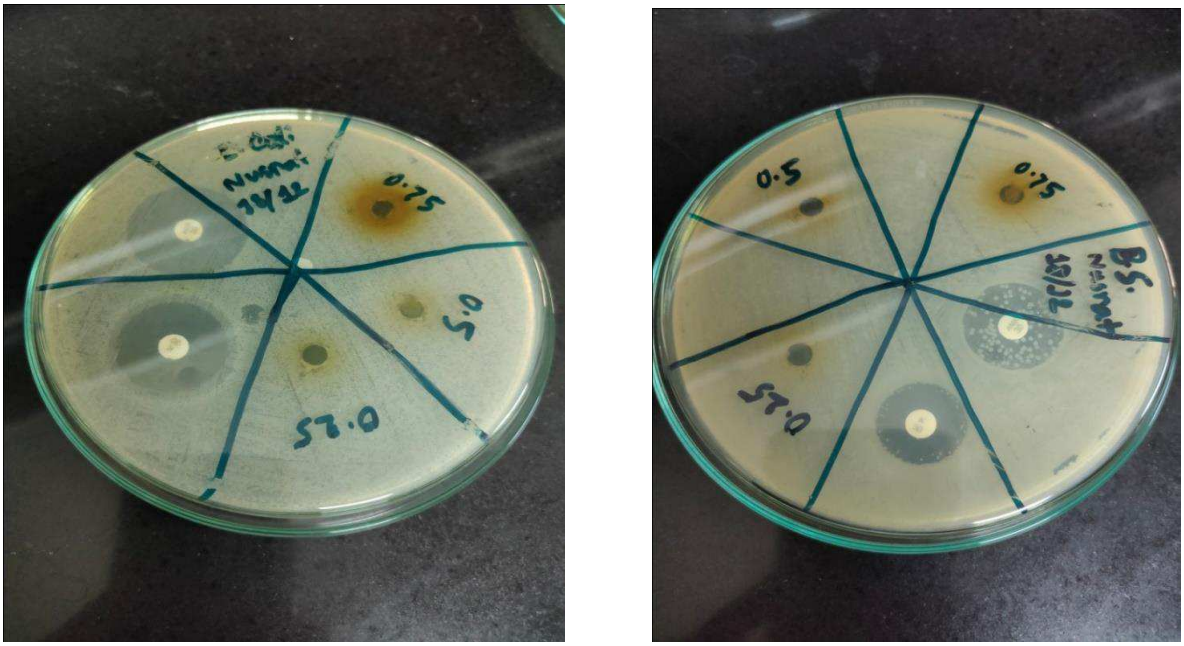

Figure 4: Zone of inhibition shown by standard (Kanamycin and ampicillin) on E.coli, B.subtilis respectively.

3.4 In vitro Cytotoxicity Test of *Celosia argentea* by MTT Assay on HeLa Cell Line

The cytotoxic effect of methanolic extract of *Celosia argentea* was done by MTT assay on HeLa cell line. Specific sample methanol extract concentrations (50 µg/ml,100 µg/ml,200 µg/ml,800 µg/ml and 1200 µg/ml) were used to examine the cytotoxic activity. 2 % DMSO had been used as a negative control in the DMEM medium. Absorbance for each one of the concentrations was recorded at 450nm including their average was measured. The Survival rate of cells and % of HeLa cell line growth inhibition in different concentrations as well as their LD50 values are given in the table 6. Images which are added in figure 4 are also recorded.

Sample Concentration		**Absorbance @ 450nm**	**Survival of cell (HeLa) %**	**% of Cell growth Inhibition**	**LD_{50} (µg/ml)**
Control	2% DMSO	3.845	100	0	1152
Sample (µg/ml)	50	3.796	95.51	4.49	
	100	3.687	93.64	6.06	
	200	3.785	93.93	6.37	
	800	2.808	67.27	32.73	
	1200	1.985	48.73	51.27	

Table 6: Cytotoxic effect analysis of methanolic extract of Celosia argentea

Interpretation:

The survival percentage of the HeLa cell was perceived through the trinocular microscope, it is perceptible that highest cell toxicity was noticed on HeLa cell line at 1200 µg/ml and

moderate cytotoxicity at 50 µg/ml concentration of sample extract. When the concentration of sample extract was higher (1200 µg/ml), highest cell death was detected (51.27% cell death where survival of HeLa cells were 48.73% after 48 hours of incubation). So, it can be concluded that 1200 µg/ml of sample concentration provided the satisfactory cytotoxic potential as highest percentage of cell death 51.27%) was observed compared to other concentrations. At the other three concentrations (800 µg/ml,200 µg/ml, 100 µg/ml, 50 µg/ml), extracts exhibited weak cytotoxic activity as the percentage of inhibition was 32.73%, 6.37%, 6.06% and 4.49% respectively.

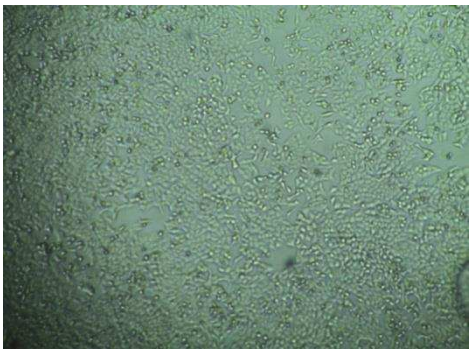

Figure 5(a): 2% DMSO Control

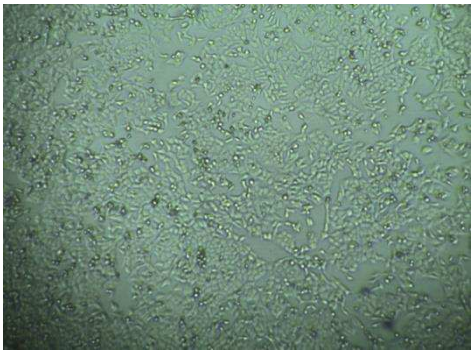

Figure 5(b): 50 µg/ml

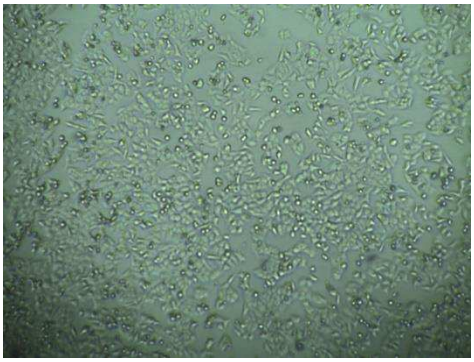

Figure 5(c): 100 µg/ml

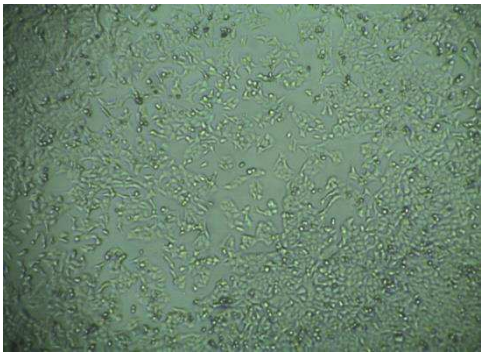

Figure 5(d): 200 µg/ml

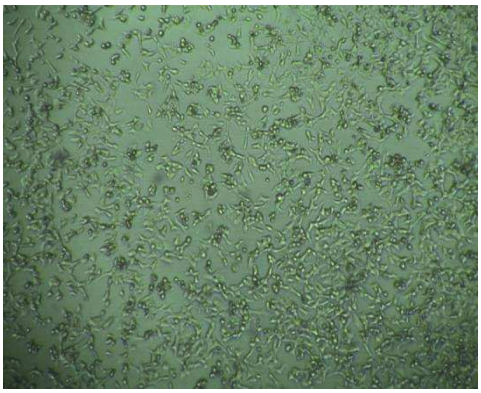

Figure 5(e): 800 µg/ml

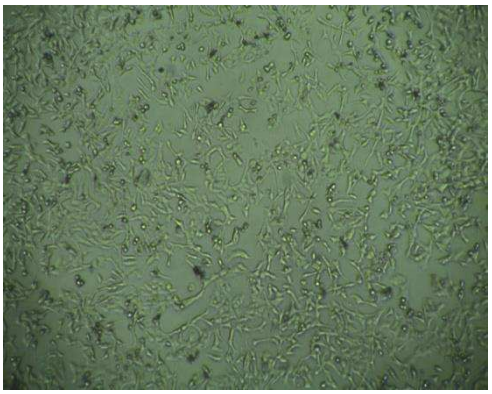

Figure 5(f): 1200 µg/ml

Figure 5: Cell viability of methanol extract of Celosia argentea at different extract concentrations of 50 µg/ml, 100 µg/ml, 200 µg/ml, 800 µg/ml, 1200 µg/ml and 2% DMSO as control respectively after incubating 48 hours in HeLa cell line.

Interpretation:

Figure 5 demonstrates the cell viability of HeLa cells in both plant extract concentrations and control (2 % DMSO). Here, Figure (a) reflects the control where DMSO was added by 2 % and no plant extract was added. In the figure (b), due to introduction of leaf extract of 50 µg/ml concentration, cell viability decreased; only 4.49 % cell growth inhibition was observed. Slightly less viability of the cell was observed, although the extract concentration of 100 µg/ml and 200 µg/ml was applied in figure (c) and (d). Due to the addition of 800 µg/ml concentration where the percentage of cell growth inhibition was 32.73, visible cell growth inhibition was seen in the next figure (e). Subsequently, the highest cytotoxicity of plant extract was found in the last picture as it destroyed the greatest number of cells ans was the highest percentage of cell growth inhibition; around 51.27%. This shows that plant extract at concentration of 1200 µg/ml displays tremendous cytotoxic potential with cell inhibition of 51.27%.

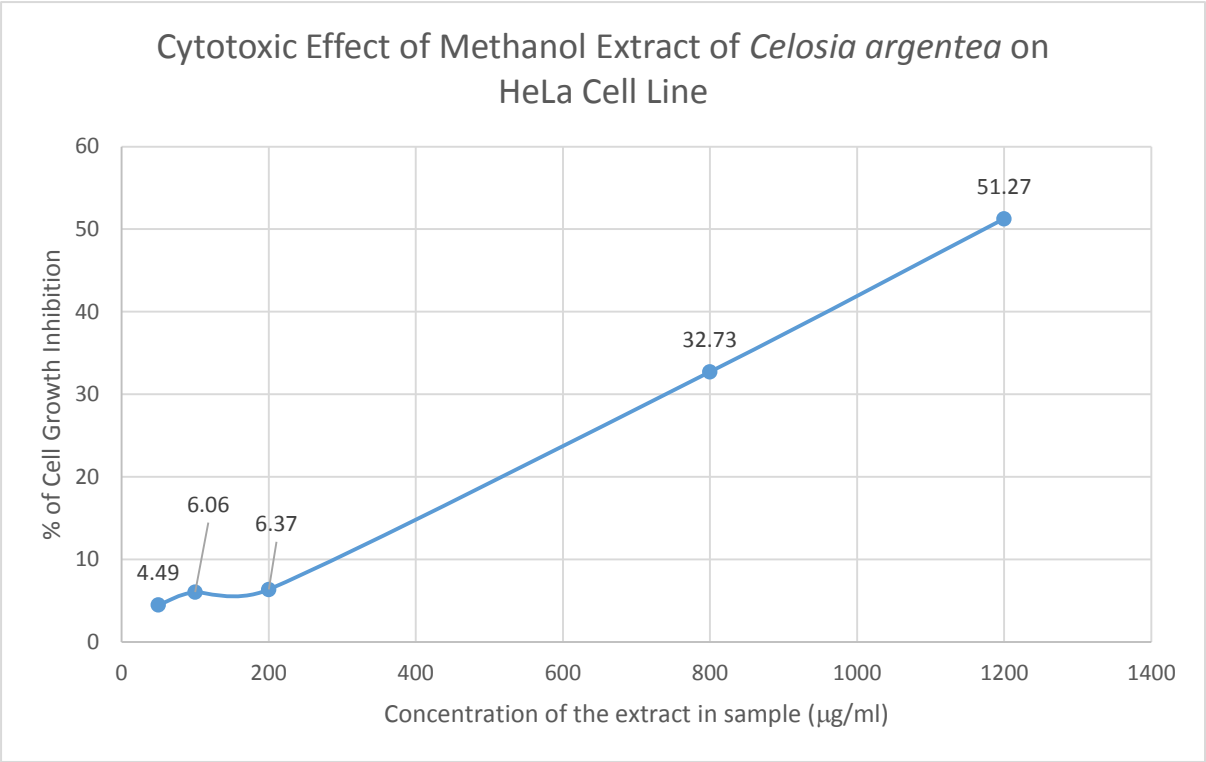

Figure 6: Cytotoxic effect of methanol crude extract on HeLa cell line

Interpretation:

Figure 6 refers with the graphical representation of cytotoxic effect of methanol extract of *Celosia argentea* on HeLa cell line. From the bar chart, it is found that at 50 µg/ml plant extract concentration the lowest percentage of cell inhibition was. In contrast, the maximum percentage of cell inhibition was demonstrated at plant extract concentration of 1200 µg/ml.

3.5 Antioxidant activity study of *Celosia argentea*

3.5.1 Determination of total phenolic content (TPC)

Total phenolic content was calculated for the methanolic extract (ME) of *Celosia argentea* leaves by using Folin-Ciocalteu reagent. The value of the total phenolic content of the extract was calculated and distinct with standard solutions of Gallic acid, based on the absorbance values of the extract solution (Table 7) equivalents.

SI No.	Conc. of the Sample (µg/ml)	Absorbance	Regression Line	Regression Coefficient (R^2)
1	200	0.078	Y= 0.0001x + 0.0488	0.9895
2	400	0.095		
3	800	0.14		
4	1200	0.2		

Table 7: Standard curve preparation by using Gallic acid

Interpretation:

With the increasing concentration of *Celosia argentea* methanolic leaves extract from 200-1200 µg/ml, it is found that the total phenolic content also increased from 0.078 to 0.2 mg of Gallic acid per gram of dried extract. It is noted that, as the concentration of hence, it implies that the antioxidant activity of plant extract also increased with an increase in total phenolic content. Gallic acid calibration curve equation was found to be y = 0.0001x + 0.0488 which would be used to calculated the total phenolic content of *Celosia argentea* methanolic leaves extract. It also obtained a regression coefficient (R^2) of 0.9895 suggesting a strong relationship between the concentration and the absorbance.

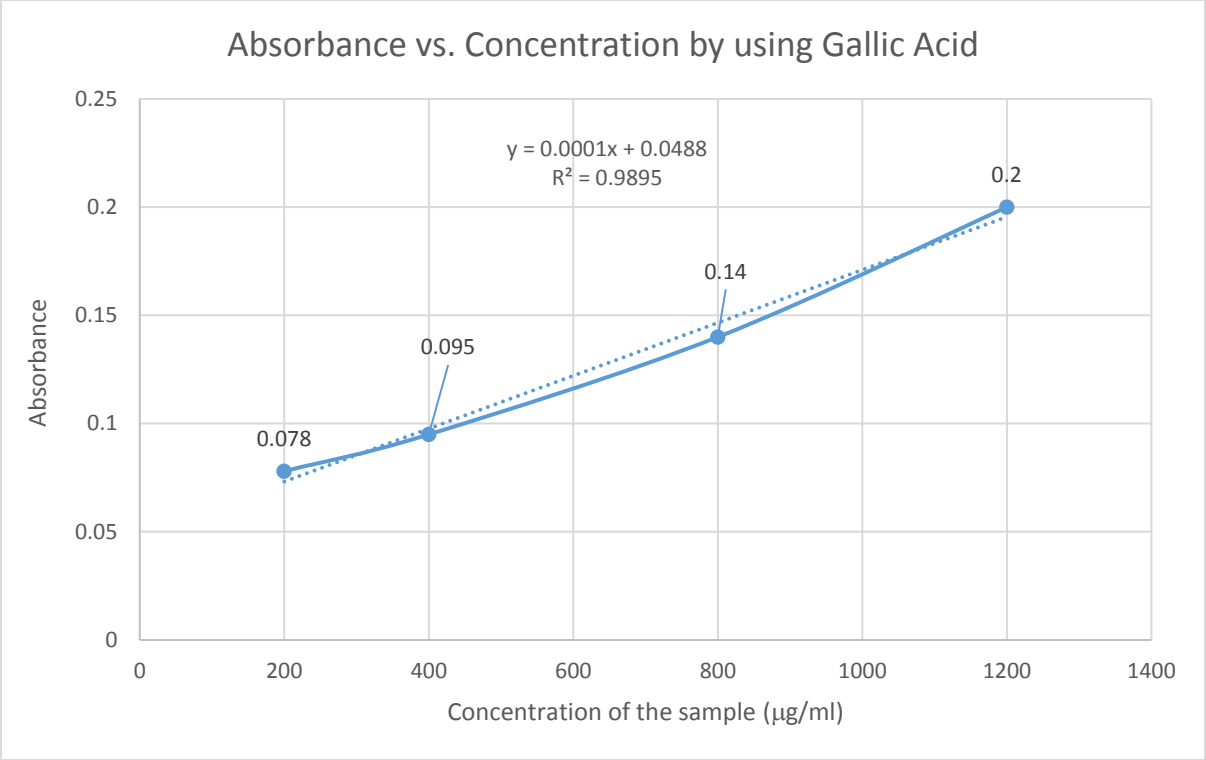

Figure 7: Standard curve of Gallic acid for total phenolic content determination

3.5.2 Determination of Total Flavonoid Content (TFC)

Total flavonoid content was calculated for the methanolic extract (ME) of *Celosia argentea* leaves by using Quercetin which was used as a standard. The value of the total flavonoid content of the extract was calculated and distinct with standard solutions of Quercetin, based on the absorbance values of the extract solution (Table 8) equivalents.

SI No.	Conc. of the sample (µg/ml)	Absorbance	Regression line	Regression coefficient (R^2)
1	200	0.22	Y = 0.0002x + 0.2169	0.8775
2	400	0.31		
3	800	0.34		
4	1200	0.39		

Table 8: Standard curve preparation by using Quercetin

Interpretation:

With the increasing concentration of *Celosia argentea* methanolic leaves extract from 200-1200 µg/ml, it is found that the total flavonoid content also increased from 0.22 to 0.39 of Quercetin per gram of dried extract. It is noted that, as the concentration of hence, it implies that the antioxidant activity of plant extract also increased with an increase in total flavonoid content. Quercetin calibration curve equation was found to be y = 0.0002x + 0.2169 which would be used to calculate the total flavonoid content of *Celosia argentea* methanolic leaves extract. It also obtained a regression coefficient (R^2) of 0.8775 suggesting a strong relationship between the concentration and the absorbance.

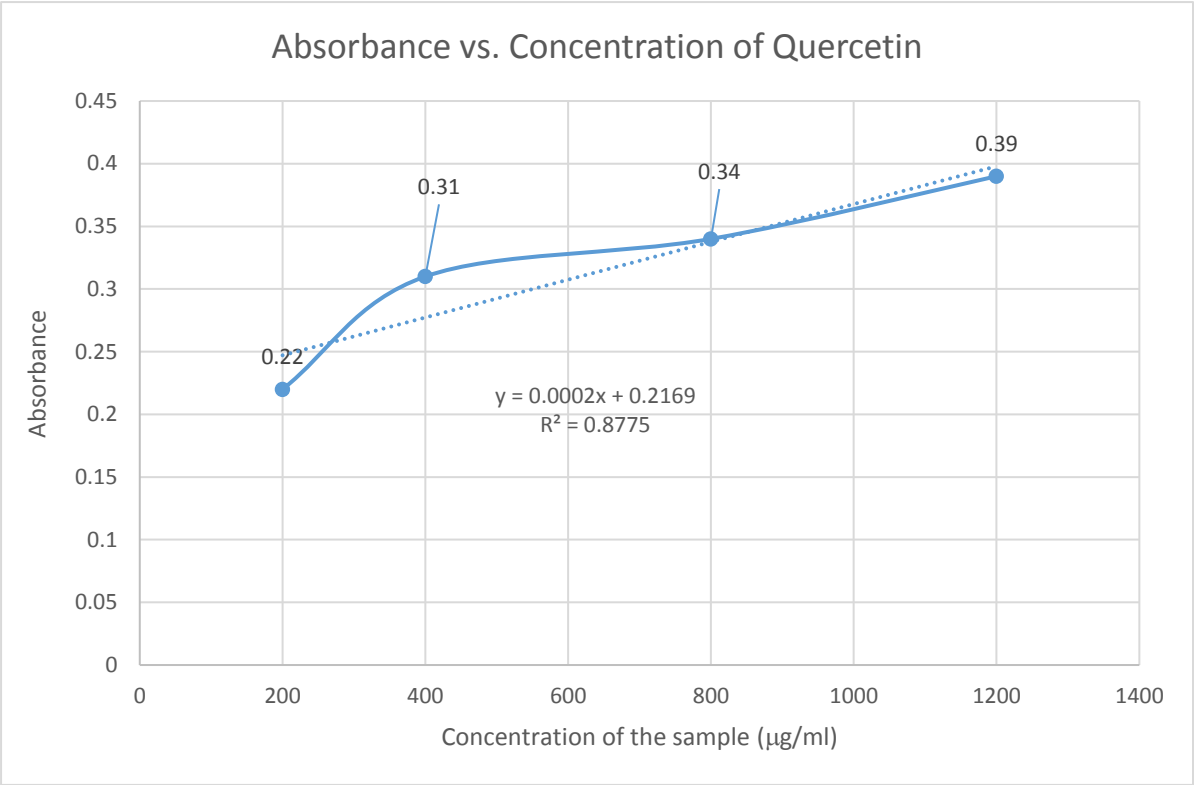

Figure 8: Calibration curve of standard Quercetin (at 415 nm) for determining TFC in ME of Celosia argentea leaves

3.5.3 Determination of Total Antioxidant capacity (TAC)

Total Antioxidant capacity was calculated for the methanolic extract (ME) of *Celosia argentea* leaves by using Ammonium molybdate which is a reagent. The value of the total Antioxidant capacity of the extract was calculated and distinct with standard solutions of Ascorbic acid, based on the absorbance values of the extract solution (Table 9) equivalents.

SI No.	Conc. of the Sample (µg/ml)	Absorbance	Regression Line	Regression coefficient (R^2)
1	200	0.160	Y= 0.0007x + 0.0126	0.9981
2	400	0.274		
3	800	0.582		
4	1200	0.843		

Table 9: Standard curve preparation by using Ascorbic acid

Interpretation:

With the increasing concentration of *Celosia argentea* methanolic leaves extract from 200-1200 µg/ml, it is found that the total Antioxidant capacity also increased from 0.22 to 0.39 of Ascorbic acid per gram of dried extract. It is noted that, as the concentration of hence, it implies that the antioxidant activity of plant extract also increased with an increase in total antioxidant capacity. Ascorbic acid calibration curve equation was found to be y = 0.000x + 0.012 which would be used to calculate the total antioxidant capacity of *Celosia argentea* methanolic leaves extract. It also obtained a regression coefficient (R^2) of 0.9981 suggesting a strong relationship between the concentration and the absorbance.

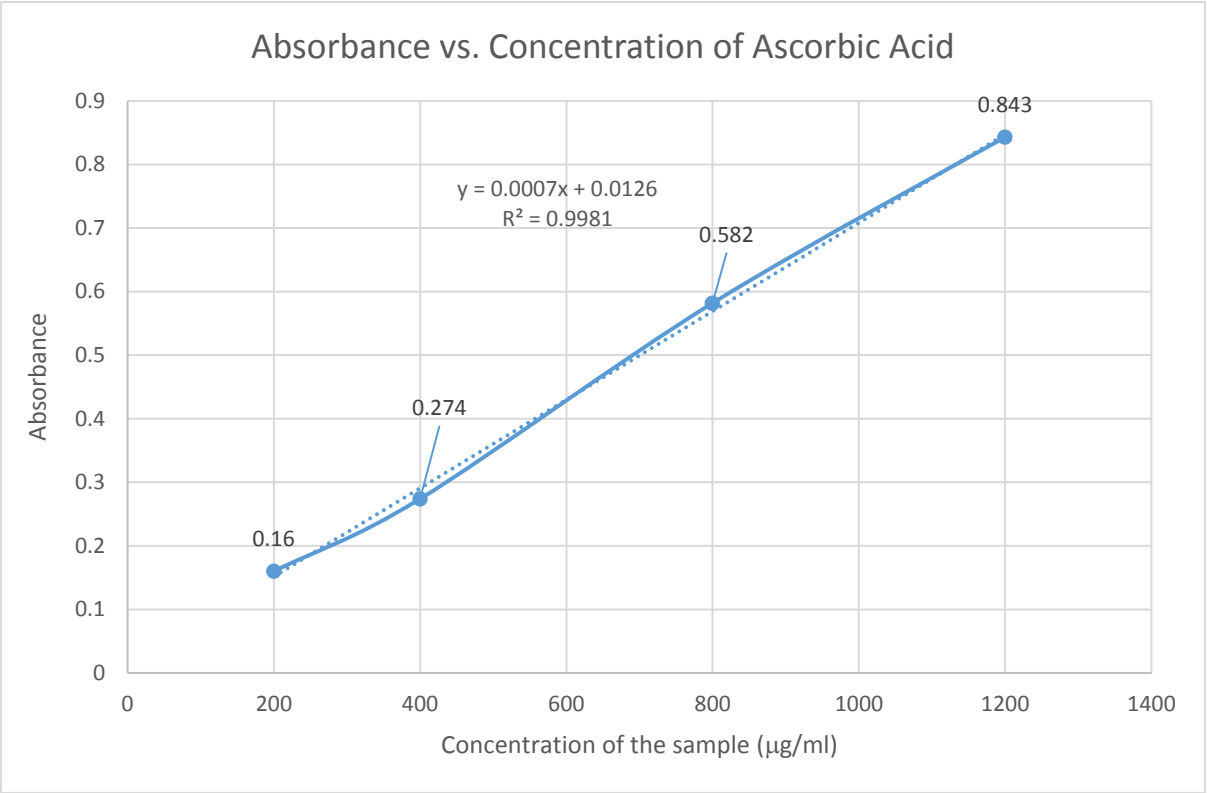

Figure 9: Calibration curve of Ascorbic acid (AA) at 695 nm for determining TAC in ME of Celosia argentea leaves

Chapter 4

Discussion

The antimicrobial test showed that the plant extract of *Celosia argentea* did not have any activity against both Gram positive and negative bacteria. As the plant did not show any antimicrobial activity so its antimicrobial potential cannot be evaluated by means of determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Additionally, cancer cell lines were selected for screening the cytotoxic activity before such as Michigan Cancer Foundation-7, breast cancer cells, prostate cancer cells, human pancreatic cancer cells, mouse breast cancer cells and mouse thymic lymphoma (Kaur et al., 2005; Sandhya et al., 2006). Among all of the solvents used for screening, methanol extract showed highest percentage in case of cytotoxicity potentiality. The cytotoxic effect analysis of plant extract showed that the survival rate of cell on HeLa cell line was decreased by increasing the concentration and the LD_{50} value was 1.152 mg/ml. Additionally, HeLa cell lines was used to investigate the cytotoxic potential of *Celosia argentea.* High cell toxicity was observed at 1200 μg/ml plant extract of methanol solvent in the cell lines. Chakma tribe people use *Celosia argentea* leaf extract 2-3 times a day to treat cancer (Rahman et al., 2006). This study also shows that methanol extract of *Celosia argentea* leaves decrease the survival rate of cancer cell with increasing concentration.

However, the study specifies that the leaves extract of *Celosia argentea* could be a significant contributor to the concept of drug development through the exhibit of a number of potential biological properties. The findings from the recent study of methanolic leaves extract of *Celosia argentea* gives a positive indication for using as a medicinal plant. To conclude, methanol extract of *Celosia argentea* signifies highest antioxidant activity and cytotoxic potential with no antimicrobial effect.

Chapter 5

Conclusion and Future Direction

The outcome of this study found that methanol extract of *Celosia argentea* leaves indicated both very high cytotoxic effect and antioxidant potential. As a result, High antioxidant potential and cytotoxic effect expand the scope for further research and experimentation.

- The findings of this study contributes to the development of further pharmacological and pharmacognostical action and assessment of those actions across various solvent systems.

- Several plant parts like root, bark, stem will be used to evaluate the cytotoxic and antioxidant potential using different solvents or the same methanol solvent.

- High levels of antioxidant activity and cytotoxic effect also provide a great potential for further identification and new compound research.

Chapter 6

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