Phytochemical screening and the analysis of antibacterial activity in *Chrysanthemum morifolium* Ramat

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DECLARATION

I hereby solemnly declare that the research work titled "Phytochemical screening and the analysis of antibacterial activity in *Chrysanthemum morifolium* Ramat" submitted by the undersigned has been carried out under the supervision of Ms. Jebunnesa Chowdhury, Assistant Professor, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original work. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Nazia Nowrid
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Abstract

Chrysanthemum morifolium Ramat is a plant of the family Asteraceae and native to Asia and northeastern Europe. It has many pharmacological and therapeutic properties including antimicrobial, antifungal, anti-inflammatory, immunomodulatory humoral and cellular, and mononuclear phagocytic activities; therefore widely and effectively being used in the treatment of many diseases as a herbal medicine. Essentially, medicinal plants are known to have the ability to synthesize a wide range of chemical compounds and secondary metabolites; also the active biological functions and medicinal values of such plants is attributed to the presence of bioactive compounds. In the present study, *Chrysanthemum* leaves were analyzed for preliminary phytochemical studies to find the presence of phytoconstituents such as alkaloids, flavonoids, steroidal compounds, saponins, tannins, phenols and cardiac glycosides. The crude plant extract from two different extractions (ethanol and methanol) were collected and tested. Comparative analysis of the antimicrobial activity of the extracts was investigated against five bacterial strains namely: *Bacillus cereus, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli and Shigella flexneri*. This test was done using agar disc diffusion method and as controls kanamycin, ampicillin, chloramphenicol, ciprofloxacin and cefotoxin were used. The diameter of the clear zone of inhibition surrounding the disc was measured in millimeters. The results showed that the ethanolic extracts of *Chrysanthemum morifolium* Ramat contained the highest amounts of phytochemicals, while the methanolic extract contained a substantial amount. Antibacterial activity results showed that the highest amount inhibition was formed against *B.cereus* and then in *S.aureus* and *S.flexneri*. It can be concluded from the results that methanolic and ethanolic extracts of *Chrysanthemum morifolium* Ramat may be considered as an antibacterial agent against *B.cereus, S.aureus, S.flexneri* and be used to source antibiotic substances.

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Chapter: 1

Introduction

1.0: Background:

For the treatment of human diseases, the therapeutic properties of many medicinal plants have been utilized since centuries. Between 60-90% of the population of developing countries use traditional herbal medicines considering them to be a normal part of primary health care (WHO, 2002). The demand of herbal medicines too has been on the rise as consumers perceive these forms of healing as safe and effective, compared to synthetic drugs. This trend of using alternative and complementary healthcare has prompted scientists to investigate the various biological activities of medicinal plants (Wendakoon et al. 2012). The significant and great diversity of phytochemicals is abundant in such plant derived products, namely - phenols, flavonoids, tannins and other phytoconstituents; each possessing numerous health related effects such as antibacterial, anti-inflammatory, immunomodulatory humoral and cellular etc. *Chrysanthemum morifolium* Ramat is one such plant that contains such properties.

1.1 History and use of *Chrysanthemum morifolium* Ramat*:*

The genus *Chrysanthemum,* golden flower in Greek, belongs to the Asteraceae family; it includes about 300 species (Kumar et al., 2005). The *Chrysanthemum* is distributed in two main centres, one in Mediterranean area, the other in China and Japan (Dowrick, 1952).

Chrysanthemum morifolium Ramat has significant importance as an instrinsic medicinal plant (Yeasmin et al., 2016). It is also used as a herb in both Japanese and Chinese traditional medicine. In Chinese medicine, *C. morifolium* Ramat is widely used as a dietary supplement or herbal tea (Chu et al., 2004; Lai et al., 2007) and has an antihepatotoxic and antigenotoxic effect (Lee et al., 2011); it exhibit an allelopathic activity (Beninger and Hall, 2005).

The hardy plant was first cultivated in China during the 15th century mainly as a flowering herb. It has been cultivated for more than 3000 years (Yeasmin et al., 2016). Practitioners of traditional Chinese, Japanese and Indian medicine have discovered new ways to use the flowers while modern scientists continue to discover why such beautiful flowers have potent effects on bacteria, viruses and fungi.

Chrysanthemum morifolium Ramat has various biological features, including anti-inflammation, antioxidant activity, cardiovascular protection, antitumorigenesis, anti-HIV, and anti-aging activities (Yen et al., 1996). *Chrysanthemum* tea can help detoxify blood, regulate blood pressure and calm the nerves. *Chrysanthemum morifolium* Ramat and its herbal infusions are used in the treatment of bacterial and viral infections, sinusitis, blood pressure, digestive, skin problems, influenza virus PR3, leptospira, HIV-1,

human colon cancer Colon205 cells, headache, dizziness, sore throat, hypertension, flu, cough etc. (Yeasmin et al, 2016).

Fig 1: *Chrysanthemum morifolium* Ramat leaf

1.2 Scientific Classification:

- ❑ Kingdom: Plantae
- ❑ Subkingdom: Tracheobionta
- ❑ Superdivision: Spermatophyta
- ❑ Division; Magnoliophyta
- ❑ Class: Magnoliopsida
- ❑ Subclass: Asteridae
- ❑ Order: Asterales
- ❑ Family: Asteraceae
- ❑ Genus: Chrysanthemum
- ❑ Species: *Chrysanthemum morifolium* Ramat

1.3 General characteristics of *Chrysanthemum morifolium* Ramat:

Chrysanthemum morifolium Ramat, family Asteraceae, is one of the oldest cultivated flowers. Despite many species of this genus, only one group, *Chrysanthemum morifolium* Ramat is grown on large scale (Anjum et al., 2007).

This majestic flowering plant is extremely popular all over the world. It generally starts to bloom during autumn season and in South Asian countries, its peak blooming period is month of December. The flowers have a great potential of export as cut flowers to many countries due to their beauty, and

outstanding varietal colours (Erler and Seigmund, 1986). Cut flowers have largest share in flower trade and chrysanthemum is one of the leading flowers with millions of stems sold in Europe and North America (Bhattacharjee and De, 2003).

International trade in flowers and plants is far more extensive than most people imagine, cut flowers account for largest share and *Chrysanthemum* is most popular florist flower. Sowing dates play an important role in the quality production and marketing of *Chrysanthemum* (Balaji et al., 2010)

1.4 Cultivation:

Chrysanthemum morifolium Ramat, occupying a prominent place in ornamental horticulture is one of the commercially exploited flower crops. In many countries, including the United States and Japan, it is considered as the number one crop (Verma et al., 2011). It is produced both as cut flower and as pot plant (Moura et al., 2014).

Though the *Chrysanthemum* is one of the important commercial flower crops, its yield and quality levels are low and hence there is a need to standardize the optimum dose of nutrients particularly the integrated nutrient management for improving the soil structure, physico-chemical properties and flower yield (Verma et al., 2011). Application of nitrogen (20 g/m^2) and phosphorus (20 g/m^2 P_2O_5) interacted positively resulting in maximum yield of *Chrysanthemum* Cv. Flirt (Beniwal et al., 2006). At present, these nutrients are supplied through chemical fertilizers.

Year-round production can be achieved in greenhouses by controlling climate conditions. In the winter months, especially in more northern latitudes, this means that high energy inputs are required because the greenhouses must be heated in order to maintain good plant quality and production levels (Van Der Ploeg and Heuvelink, 2006).

In *C. morifolium* Ramat, flowering time is predominantly determined by daylength. In cut *Chrysanthemum* cultivation, two different phases can be distinguished. First a period of long-day (LD; day-length more than 12 h) is maintained so that the plants grow vegetatively. Depending on the season, this period will last between 10 – 25 days (Carvalho, 2003). Then plants are grown under short-day (SD; daylength less than 12 h) conditions, leading to flower induction and development. The period between the start of the SD period and flowering under optimal conditions (reaction time) can vary between 6 – 11 weeks, although breeders apply a strong selection pressure on shorter reaction times, so that the cultivation period can be shortened (Spaargaren, 2002). During each of these phases, growth (e.g., the

production and partitioning of biomass) and development (e.g., leaf appearance rate, time to flowering, stem elongation, numbers and sizes of flowers) can be influenced by temperature (Wilkins et al., 1990; Carvalho et al., 2005).

For year-round cut-flower production, flowering time in the field is mainly regulated by bringing the photoperiod closer to (e.g. shading) or farther from (e.g. night break) the appropriate daylength for flowering. However, flowering time also largely depends on the temperature of the planting season, even when daylength is controlled. The optimum flowering temperature for *C. morifolium* is reported to be ~20 °C, and high temperatures delay flowering (Nakano et al., 2013).

1.5: Therapeutic values of *Chrysanthemum morifolium* Ramat*:*

Chrysanthemum morifolium Ramat is known to have antioxidant, anti-inflammatory, antimutagenic, antimicrobial, antifungal, antiangiogenic and nematocidal properties (Yeasmin et al., 2016). This plant is also known to produce flavonoids of medicinal value (Hu et al., 1994).

Some of the compounds in *Chrysanthemum* are flavonoids like luteolin, apigenin and acacetin, choline and vitamin B1. *Chrysanthemum morifolium* Ramat extract (CME) has the protective effect on cardiovascular diseases. Luteolin and apigenin are two major bioactive components in vivo when CME is orally administrated to experimental animal (Chen et al., 2007). Simultaneously, it is a good source of vitamins C and A, niacin, folic acid, pantothenic acid and also rich in calcium, magnesium, potassium, iron and phosphorus (Yeasmin et al., 2016)

The *Chrysanthemum* flower contains significant amounts of flavonoids and hydroxycinnamoylquinic acids that are considered to be the biologically active components (Beninger et al., 2004). Among them, acacetin 7-O-galactoside and apigenin 7-O-beta-D- (4”-caffeoyl) glucuronide were isolated as the anti-HIV compounds of this herb (Lee et al., 2003).

The flowering head of Chrysanthemum morifolium Ramat known in China as ‘‘Ju Huan”, is an important traditional Chinese medicine (TCM) used for scattering cold, cleaning heat and toxin and brightening eye, and used as an important component in many TCM formulas. In Chinese medicine, *C. morifolium* Ramat is widely used as a dietary supplement or herbal tea (Chu et al., 2004; Lai et al., 2007).

The tea of *Chrysanthemum* can help detoxify blood, regulate blood pressure and calm the nerves. For the treatment of bacterial and viral infections, sinusitis, blood pressure, digestive, skin problems, influenza

virus PR3, leptospira, HIV-1, human colon cancer Colon205 cells, headache, dizziness, sore throat, hypertension, flu, cough etc – *Chyrysanthemum morifolium* Ramat and its herbal infusions are effectively used. (Yeasmin et al, 2016)

1.6 Objective:

In this study, ethanolic and methanolic extracts from leaf samples of white flower producing *Chrysanthemum morifolium* Ramat plant were collected and investigated for the presence of phytoconstituents. Extracts were also used to test their antibacterial activity against 5 strains of bacteria (*Bacillus cereus, Staphylococcus aureus, Streptococcus pneumoniae, Shigella flexneri and Escherichia coli*). Although medicinal companies have produced a number of synthetic drugs against such microorganisms, their number of multi drug resistant bacteria (MDR) have been increasing since decades. Due to such increase of microorganisms and pathogens, medicinal plants such as Chrysanthemum have increasing significance in treating infections caused by such bacteria.

On the basis of this, the objectives of the present study are:

- Establishment of a suitable extraction process for *Chrysanthemum morifolium* Ramat and collection of the leaf extract using ethanol and methanol a solvent.
- Phytochemical screening of collected *Chrysanthemum morifolium* Ramat leaf extract.
- Comparative analysis of plant extracts antibacterial activity against five bacterial strains.

Chapter: 2

Materials and Method

2.1 Plant sample collection:

A total of 500gm of powdered *Chrysanthemum morifolium* Ramat leaves was collected from Krishibid Nursery, Agargaon, Dhaka. The samples leaves were finely powdered without any impurities mixed in them. After collection they were stored at the laboratory at room temperature.

Fig: 2

Fig: 3

Fig 2: *Chrysanthemum morifolium* Ramat saplings collected from Krishibid Nursery, Agargaon.

Fig 3: *Chrysanthemum morifolium* Ramat plants after blooming (grown in rooftop for research work)

2.2 Preparation of Extracts:

A total of two types of extracts using two different solvents of ethanol and methanol were collected from the samples of *Chrysanthemum morifolium* Ramat.

2.2.1. Ethanoic Extracts:

For collection of ethanoic extracts 50g of *Chrysanthemum* powder was packed in thimble and extracted in Soxhlet apparatus using 250ml of ethanol. The temperature was kept between 60-80ºC. The samples in the thimble of the soxhlet apparatus was kept boiling for approximately 4 hours till the solution becomes clear and the dark colored extract was collected at the bottom of the apparatus. This was then collected in petri dishes and left to dry for 24 hours. The dried extract having a sticky appearance was stored in 25 ml McCartney bottles at temperatures at 4 ºC in the refrigerator for further use. The whole process was repeated 3 times for the collection of a substantial amount of extracts for the study.

2.2.2 Methanolic Extracts:

The methanolic extracts were collected in the same way as the ethanolic extracts. 50g of powdered sample was weighed in the weighing machine and packed in the soxhlet apparatus with 250ml methanol. As the methanol boiled the extract was slowly collected in the flask below. The temperature here too was adjusted between 60-80 ºC. After leaving to dry overnight in petri dishes, the extracts were collected with spatula and stored in McCartney Bottles.

2.3: Storage of extracts:

The extracts were collected in 25ml McCartney bottles. In each bottles extracts were collected till the bottles are filled. They were dried till the extract had a very sticky appearance. After collection of extracts using spatula the McCartney bottles were tightly stoppered and stored in the refrigerator at 4 ºC.

2.4 Preparation of Stock working solution for phytochemical assays:

The ethanolic and methanolic extracts were dissolved in solvent and a stock working solution of 5µg/µl was made. This was done by mixing 0.5gm of crude extract with 100 ml of solvent (ethanol and methanol). All two stock solutions were made this way.

2.5 Biochemical Assays:

Preliminary screening of biochemical tests of all two extracts were done for testing various phytochemicals found in plants. The crude extracts were tested for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compound, flavonoids, saponins, tannins and cardiac glycosides. The following biochemical tests has been performed to confirm the presence or absence of the secondary metabolites in the plant extract.

2.5.2. Tests for alkaloids:

2.5.2.1 Hager's Test:

1 ml of extract was carefully mixed with 3 drops of freshly prepared Hager's reagent in a test tube. The formation of yellow precipitates showed a positive result and the presence of alkaloids in the extract.

2.5.2.2 Wagner's Test:

1 ml of extract was mixed together in a test tube with 3 drops of Wagner"s reagent prepared beforehand. The formation of brown precipitate showed the presence of alkaloids.

2.5.2.3 Dragendraff's Test:

2 ml of extract was taken in a test tube with 0.2ml dilute HCL and 1 ml of Dragendraff's reagent and left for a few mins. A positive result is indicated by the presence of an orange brown precipitate.

2.5.3 Test for steroidal compounds:

2.5.3.1 Salkowaski's test:

0.5g of extracts were dissolved in 2ml chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown color at the interface indicated the presence of a steroid ring.

2.5.4 Test for phenolic compounds:

Equal amounts of 1% ferric chloride solution and 1% potassium ferrocyanide was mixed. To 2ml extract, 3 drops of this freshly prepared mixture was added. The formation of a bluish-green color was taken as positive.

2.5.5 Test for Flavonoids:

2.5.5.1 Reaction with sodium hydroxide:

2 ml dilute NaOH solution was added to 3 ml of extract. The mixture was inspected for production of yellow color which is considered positive.

2.5.6 Tests for Saponins:

2.5.6.1 Froth Test:

0.5g of each type of extracts were dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 30 secs. It was then allowed to stand for 30 mins. A honeycomb froth above the surface that stays after 30 minutes is taken as a positive result.

2.5.7 Tests for Tannins:

2.5.7.1 Lead Acetate Test:

5 ml of each type of extract and a few drops of freshly prepared 1% lead acetate were dissolved together. Yellow precipitate shows a positive result.

2.5.8 Tests for Cardiac Glycoside:

2.5.8.1 Killer-Killani Test:

5ml of extract was treated with 2ml glacial acetic acid and 1 drop of FeCl3 and 1 ml concentrated H_2SO_4. A positive result shows a brown ring at the interface.

2.6 Antibacterial activity test:

To test the antibacterial property of the sample Agar Disc-Diffusion Assay was done. A positive control was used to compare the results.

2.6.1 Test Organisms:

The bacteria strains used in the experiment were collected from ICDDR, B and preserved in biotechnology laboratory, BRAC University.

List of Test Organisms:

- *Bacillus cereus*
- *Streptocccus pneumoniae*
- *Staphylococcus aureus*
- *Shigella flexneri*
- *Escherichia coli*

2.6.2 Preparation of working extract solution for antibacterial activity test:

Ethanolic and methanolic dry extracts of *Chrysanthemum morifolium* Ramat were dissolved in 0.25% DMSO to make two different concentrations of working extract solutions for the antibacterial activity test. 4gms of extract was dissolved in 50ml of 0.25% DMSO to get 80µg/µl. This was then diluted to prepare a solution that has a concentration of 60µg/µl. During the study, the working solution of extract was freshly prepared.

2.6.3. Media preparation:

For carrying out the antibacterial activity test, Muller Hinton Agar (MHA) was used. Nutrient Agar (NA) medium was used to prepare fresh cultures.

Composition of Nutrient agar:

Ingredients	Amount
Peptone	0.5%
Beef extract/ Yeast extract	0.3%
Agar	1.5%
NaCl	0.5%
Distilled Water	20 ml
Final pH	7.4±0.2

Composition of Muller Hinton Agar:

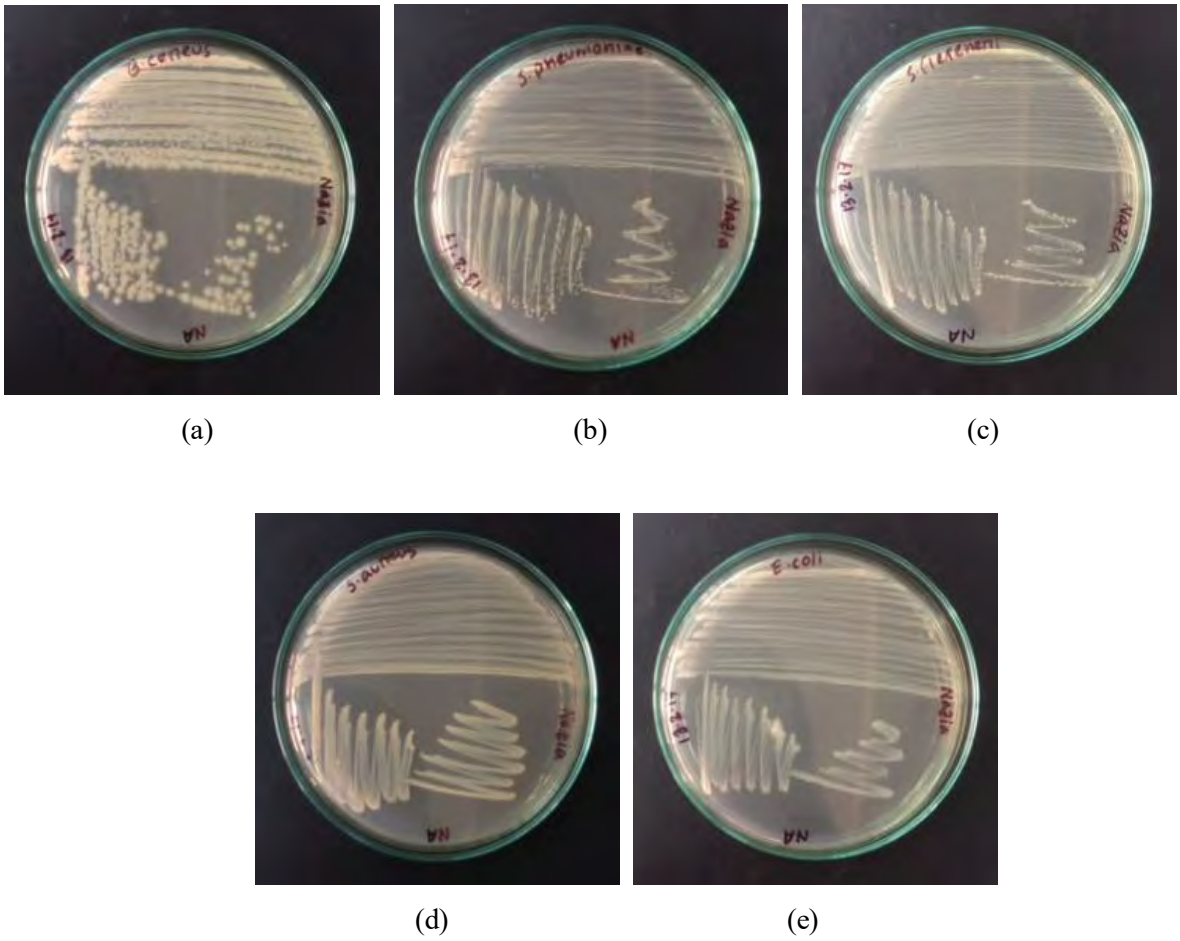
Ingredients	Amount
Beef, infusion form	30%
Casein Hydrolysate	1.75%
Starch	0.15%
Agar	1.7%
pH	Neutral

2.6.4. Preparation of fresh Nutrient Agar:

To prepare the required volume of this medium, calculated amount of each of the constituents were taken in a conical flask and distilled water was added to it make the desired volume. The media was then dissolved in a Bunsen burner until the solution in the flask turned crystal clear. It was then autoclaved for 1.5 hours to sterilize and remove all impurities. The media was poured in plates as soon as it was taken out of the autoclave to avoid it from solidifying. Fresh sterilized autoclaved plates that could hold 20 ml of media were labelled before. After pouring the media in the plates they were left to solidify inside the laminar air flow and then stored in the fridge for further use.

2.6.5 Preparation of Stock Culture:

In aseptic conditions organisms were sub cultured in freshly made NA plates from pure cultures using a sterile loop. The pure cultures were taken from the fridge of the departmental stock and left in the incubator for 30 minutes to thaw. Under a laminar air flow a metal loop was burned and used to streak the organisms in the freshly made nutrient agar plates. The inoculated plates were labelled and then incubated at 37ºC for 24 hours for optimal growth. These fresh cultures were then used for the antibacterial tests (Fig 4).



(a) (b) (c)

(d) (e)

Fig 4: Fresh culture used as stock in nutrient agar media (a) *Bacillus cereus* (b) *Streptococcus pneumoniae* (c) *Shigella flexneri* (d) *Staphylococcus aureus* (e) *Escherichia coli*.

2.6.6 Preparation of test plates:

For performing the biochemical tests Muller Hinton Agar (MHA) was used. Measured amount of powdered media was taken in a conical flask and dissolved on Bunsen burner and then autoclaved for 1.5 hours to sterilize it. 20 ml of media was poured in labelled sterile plates and left to solidify. They were stored in the fridge for further use.

2.6.7 Inoculation of test organism:

The test organisms were transferred using a loop in test tubes containing 5 ml of 0.9% saline to make cell suspension. They were then vortexed in a vortex machine for the organisms to mix properly in the saline solution. The concentration of the cells in each test tube was optimized using 0.5 McFarland solution that had an OD of 0.1 in 600nm wavelength when measured in a spectrophotometer. This was done so that there are equal number of cells in every cell plate. Using a cotton swab the bacteria from the cell suspension was immediately inoculated in the freshly prepared MHA media. The lawn was done multiple times in each plate by rotating them 90º each time to make sure there was uniform distribution of organisms in the media.

2.6.6 Placing extracts and controls in the plate:

The inoculated plates were labelled and four quadrants were made. They were each marked as positive control, crude, 80µg/ µl and 60 µg/ µl respectively. Then three sterilized petri dishes were taken and 1000 µl/ 1 ml of stock working solution, working extract solution in concentrations of 80µg/ µl and 60 µg/ µl were given petri dishes using a micropipette for each of ethanolic and methanolic extracts respectively. Then autoclaved filter paper discs were put in these petri dishes and allowed to soak for a good 5 minutes. After that, the discs were placed in the inoculated plates using a bent forcep according to the labelling of the quadrants. A positive control was used to compare the results. For positive control five different antibiotics were used against the organisms. Kanamycin was used against *Bacillus cereus,* Chloramphenicol against *Streptococcus pneumoniae*, Ampicillin for *Staphylococcus aureus,* Ciprofloxacin for *Escherichia coli* and lastly, Cefotoxin for *Shigella flexneri*. The plates were then incubated at 37 ºC for 24 hours.

2.6.7 Measuring Zones:

Following 24 hours of incubation of the test plates the clear zones were measured using a ruler. This was done by measuring the entire diameter of the clear zone and the results were recorded.

2.6.8 Measuring the activity index:

The inhibitory effects of the methanolic and ethanolic extracts were calculated and compared by measuring the activity index. This was done by using the following formula:

$$\text{Activity index} = \frac{\text{Zone of inhibition of extract}}{\text{Zone of inhibition of antibiotic}}$$

Chapter: 3

Results and Discussion

3.1 Results:

3.1.1. Amount of extracts collected:

The extraction was done using 2 different solvents; methanol and ethanol. Different amount of extracts were collected during the experiment. Table 1 shows the amount of extracts collected in grams after drying them in petri dishes.

Table 1: Amount of extracts collected from each experiment

Extraction	**Ethanol**	**Methanol**
1	25.61 gm	22.19 gm
2	28.34 gm	27.46 gm
3	20.04 gm	19.36 gm
Average	**73.99 gm**	**69.01 gm**

Comment: Therefore, from the above mentioned table, it can be concluded saying that the highest amount of extracts were collected when ethanol was used as solvent.

3.1.2 Biochemical Assays:

The biochemical assays were done to check the secondary metabolites present in the sample of *Chrysanthemum morifolium* Ramat that was collected. After performing several tests the secondary metabolites and its phytoconstituents present in it were found. A total of seven phytochemical tests were performed to see the presence of alkaloids, steroids, flavonoids, phenols, saponins, tannins and cardiac glycosides.

3.1.2.1 Test results for alkaloids: For testing alkaloids 3 different types of tests were carried out.

(a) Hager's Test: This test involving the addition of 1ml of extract to 3 drops of Hager's reagent shows yellow precipitation in presence of alkaloids. Fig 5 shows the test results for alkaloids in *Chrysanthemum morifolium* Ramat. The mild yellow precipitate in the ethanolic extract showed a moderately positive result and no precipitate in methanolic extract showed a negative result.

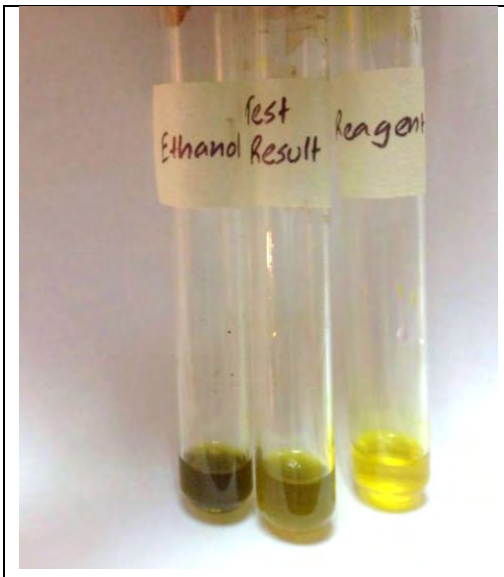
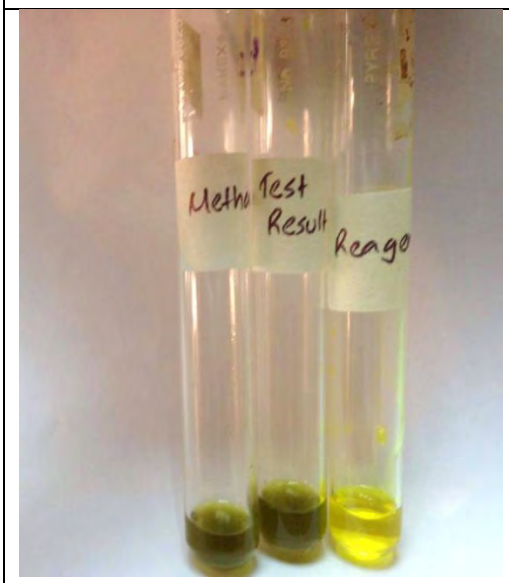
	(a) Hager's test for ethanolic extract showing moderately positive result.
	(b) Hager's test for methanolic extract showing negative result.

Fig 5: Results for Hager's test for alkaloids (a) Ethanolic extract (b) Methanolic extract.

* All tests were performed in triplicate

(b) Wagner's Test: This test involved adding 1 ml of extract with 3 drops of freshly prepared Wagner's reagent. The formation of brown precipitate is supposed to show a positive result (Fig 6). For the ethanolic extract there was a moderately positive result as the light brown precipitate can be seen, however methanolic extract does not have any precipitate and thus this result shows there is no alkaloid present in the methanolic extract.

	(a) Wagner's test for ethanolic extract showing moderately positive result.
	(b) Wagner's test for methanolic extract showing negative result.

Fig 6: Results for Wagner's test for alkaloids. (a) Ethanolic extract (b) Methanolic extract.

* All tests were performed in triplicate

(c) Dragendraff's test: This test was done by taking 2 ml of extract in 0.2ml HCL and 1 ml of Dragendraff's reagent and an orange precipitate shows a positive result. According to the results for the ethanolic and methanolic extracts, precipitate was seen giving a moderately positive result (Fig 7)

	(a) Dragendraff's test for ethanolic extract showing moderately positive result.
	(b) Dragendraff's test for methanolic extract showing moderately positive result.

Fig 7: Results for Dragendraff's test for alkaloids. (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

3.1.2.2 Test results for steroidal compounds: Tests for the presence of steroidal compounds were done using the salkowaski test where 0.5g of extract was dissolved in 2ml chloroform and a concentrated sulphuric acid layer gave a reddish brown color at interface gave a positive layer. The tests were done in this case for ethanol and methanol with a positive and negative result respectively (Fig 8).

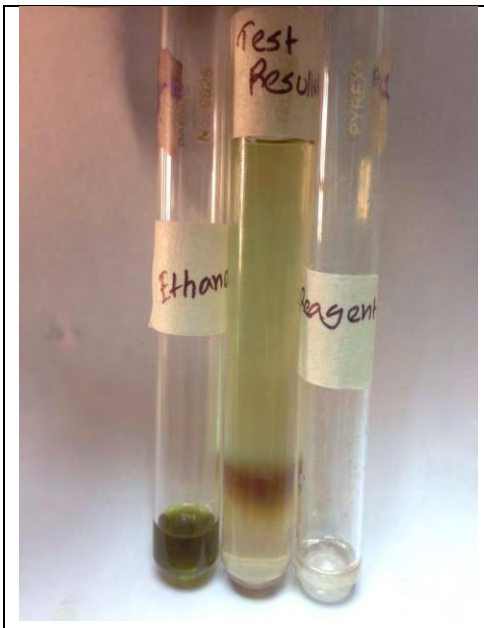
	(a) Salkowaski's test for ethanolic extract showing positive result.
	(b) Salkowaski's test for methanolic extract showing negative result as no reddish brown ring was formed at the interface.

Fig 8: Test results for steroidal compounds (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

3.1.2.3 Test results for phenolic compounds: Test for phenols showed positive results for ethanolic and methanolic extracts as bluish green color was formed. (Fig 9).

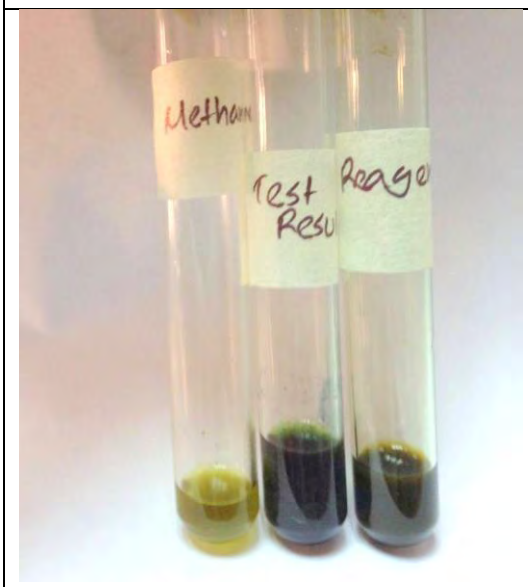
	(a) Ethanolic extract showing positive result.
	(b) Methanolic extract showing positive result.

Fig 9: Test results for phenols: (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

3.1.2.4 Test results for flavonoids: This test was done to find out the presence of flavonoids in *Chrysanthemum* by reacting it with 2ml NaOH which was inspected for the yellow color production for positive result.

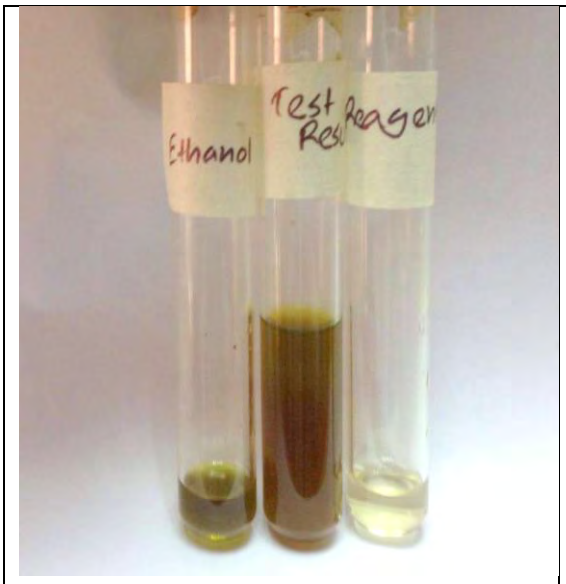
	(a) Ethanolic extract showing moderately positive result as some yellow could be seen.
	(b) Methanolic extract showing moderately positive result as some yellow could be seen.

Fig 10: Test results flavonoids (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

According to the results there was a moderately positive result for ethanolic and methanolic extracts as upon addition of NaOH, a slight yellow color indicated some presence of flavonoids in the sample (Fig 10).

3.1.2.5 Test results for Saponins: For testing the presence of saponin froth test was performed that required taking 0.5gm of extract dissolved in 10 ml solvent and shaking it for 30 sec vigorously. After 30 mins a honeycomb froth above the surface is taken as a positive result. In this experiment a moderately

positive result was found as traces were seen in ethanolic and methanolic extracts as the froth above the surface was slight (Fig 11)

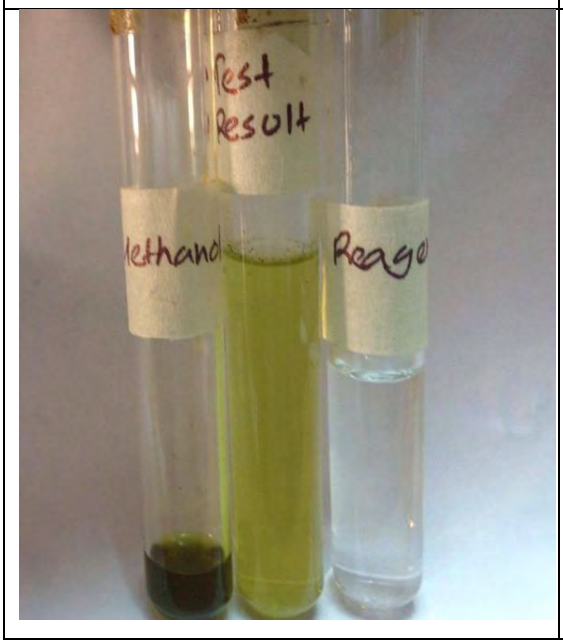
	(a) Froth test for ethanolic extract showing traces of saponins.
	(b) Froth test for Methanolic extract showing traces of saponins.

Fig 11: Test results saponins (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

3.1.2.6 Test results for tannins: For testing the presence of tannin lead acetate test was performed by adding 1% lead acetate to 5ml of extract. A yellow precipitate shows a positive result. For ethanolic and methanolic extract a slight yellow precipitation showed a moderately positive result (Fig 12).

	(a) Ethanolic extract showing traces of yellow.
	(b) Methanolic extract showing traces of yellow.

Fig 12: Test results tannins (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

3.1.2.7 Test results for cardiac glycosides:

The presence of cardiac glycosides was tested using the killer-killani test. This required 5 m of extract treated with 2 ml glacial acetic acid and 1 drop of $FeCl_3$ and 1 ml concentrated H_2SO_4. A positive result is supposed to show a brown ring at the interface. According to the results in this experiment a positive result was found for all two types of extract (Fig 13).

	(a) Ethanolic extract showing strong positive result as a brown ring could be seen at the interface.
	(b) Methanolic extract showing strong positive result as a brown ring could be seen at the interface.

Fig 13: Test results cardiac glycosides (a) Ethanolic extract (b) Methanolic extract

* All tests were performed in triplicate

The results of the phytochemical screening have been summarized in Table 2.

Table 2: Results of the phytochemical screening

Tests	**Reagents**	**E.E**	**M.E**
Alkaloids	Hager's	++	-
	Wagner's	++	-
	Dragendraff's	++	++
Steroids	Chloroform and concen. H_2SO_4	+++	-
Phenols	Ferric Chloride and Potassium ferrocyanide	++	++
Flavonoids	Sodium hydroxide	++	++
Saponin	Froth test	Trace	Trace
Tannin	Lead acetate	Trace	Trace
Cardiac glycoside	Killer-Kilani Test	+++	+++

E.E = Ethanolic extract, **M.E** = Methanolic extract

+++ = Highly positive

++ = Moderately positive

- = Negative

3.1.3 Antibacterial activity test results:

Antibacterial activity test for all two types of extract (ethanolic and methanolic) were performed using agar disc diffusion method. The zone of inhibitions were measured in millimeters (mms) and compared with the zone of inhibitions of antibiotics which were used as positive control. Antibacterial activity of the two different extracts with positive control has been shown in Table 5 and the comparison of zone of inhibition of extracts and antibiotics has been graphically presented in Fig 16.

3.1.3.1 Antibacterial activity of methanol extract:

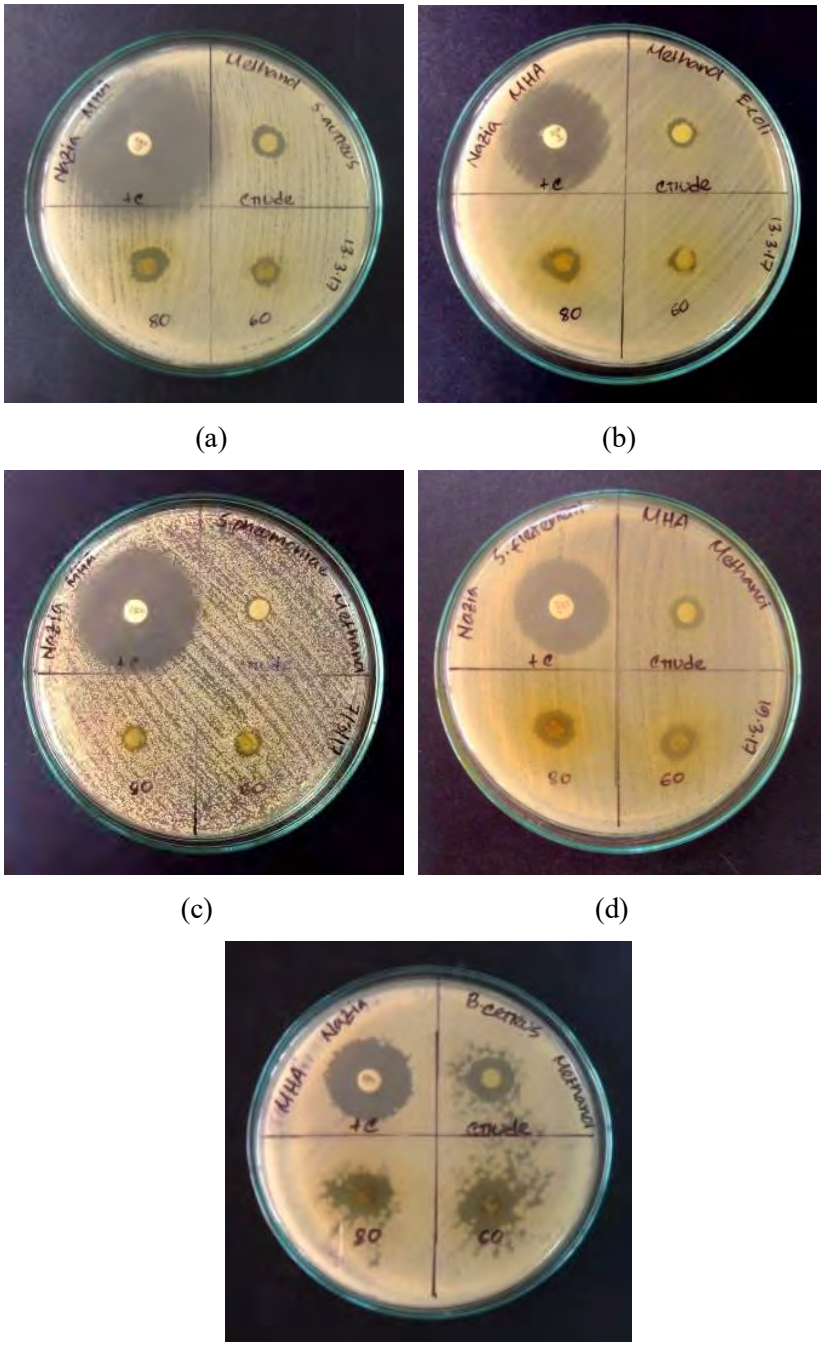
Among the five bacterial strains used *Bacillus cereus* showed 16mm clear zone against 80µg/µl and 15 mm in 60µg/µl plant extract, whereas the positive control showed a 25 mm clear zone. *Staphylococcus aureus* showed 13 mm clear zone against 80µg/µl and 10 mm in 60µg/µl plant extract, whereas the positive control showed showed a 42 mm clear zone. *Streptococcus pneumoniae* showed 9.25 mm zone against 80µg/µl plant extract extract and 8 mm in 60µg/µl plant extract, whereas the positive control

showed a 32 mm clear zone. *Shigella flexneri* showed 11mm clear zone against 80µg/µl and 11 mm in 60µg/µl plant extract, whereas the positive control showed showed a 25 mm clear zone. Finally, *Escherichia coli* showed 10mm clear zone against 80µg/µl and 9 mm in 60µg/µl plant extract, whereas the positive control showed showed a 30 mm clear zone A summary of antibacterial activity of methanol extract is included in Table 3 and the zones of inhibition has been included in Fig 14.

Table 3: Antibacterial activity of methanol extract

	Diameter(mm)	
Organisms	**80µg/µl**	**60µg/µl**
Bacillus cereus	16 mm	15 mm
Staphylococcus aureus	13 mm	10 mm
Streptococcus pneumoniae	9.25 mm	8 mm
Shigella flexnari	11 mm	11 mm
Escherichia coli	10 mm	9 mm

*All measurements are means of individual data obtained from triplicate tests



(a) (b)

(c) (d)

(e)

Fig 14: Antibacterial activity of methanolic extracts of (a) *S.aureus* (b) *E.coli* (c) *S.pneumoniae* (d) *S.flexneri* (e) *B.cereus*

*All tests were performed in triplicate

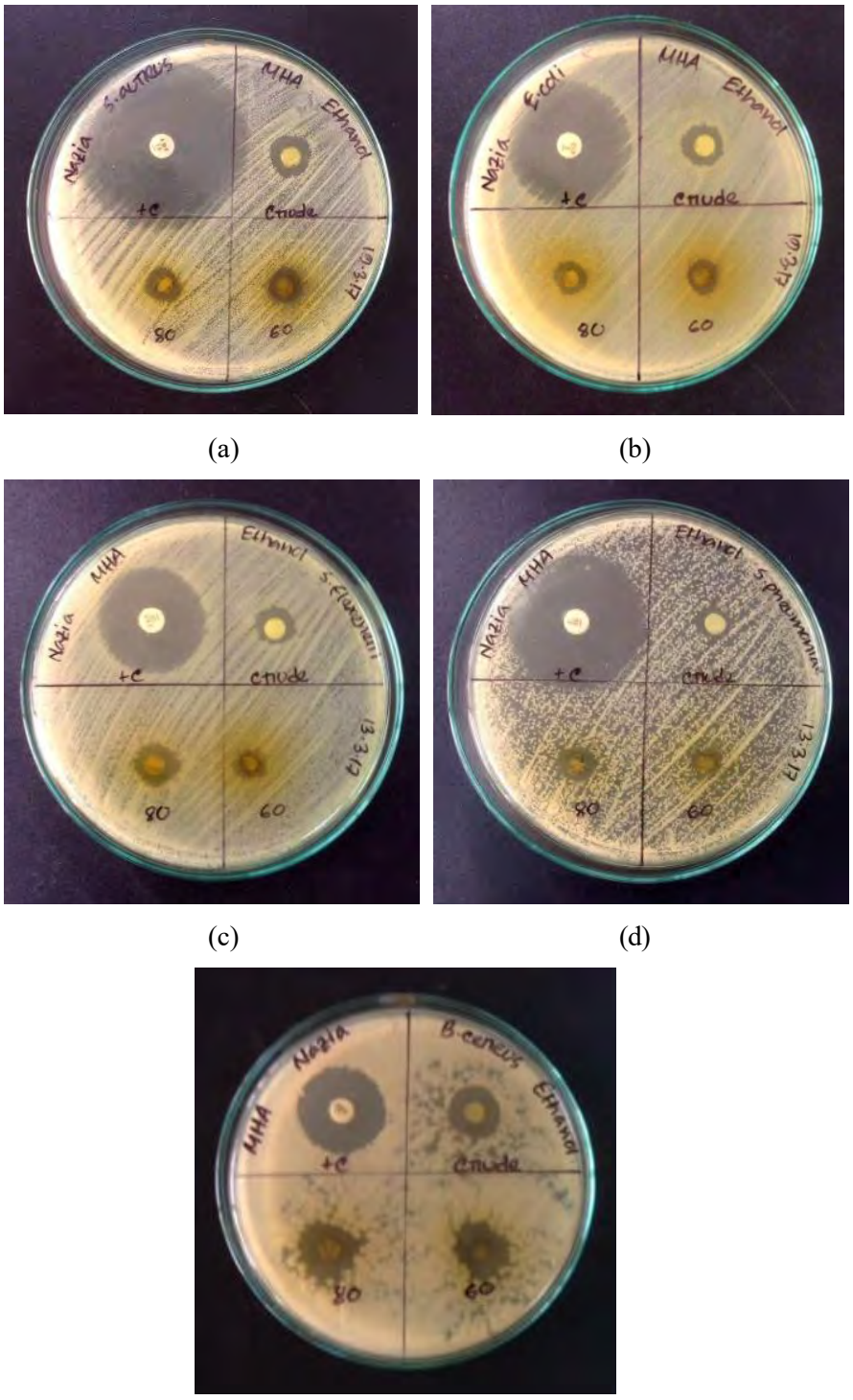
3.1.3.2 Antibacterial activity of ethanol extract:

Among the five bacterial strains used *Bacillus cereus* showed 16 mm clear zone against 80µg/µl and 15 mm in 60µg/µl plant extract, whereas the positive control showed a 25 mm clear zone. *Staphylococcus aureus* showed 12.5 mm clear zone against 80µg/µl and 10 mm in 60µg/µl plant extract, whereas the positive control showed a 42 mm clear zone. *Streptococcus pneumonia* showed 12 mm zone against 80µg/µl and 8.5 mm in 60µg/µl plant extract, whereas the positive control showed a 32 mm clear zone. *Shigella flexneri* showed 12 mm clear zone against 80µg/µl and 10 mm in 60µg/µl plant extract, whereas the positive control showed a 25 mm clear zone. Finally, *Escherichia coli* showed 11 mm clear zone against 80µg/µl and 10 mm in 60µg/µl plant extract, whereas the positive control showed a 30 mm clear zone. A summary of antibacterial activity of ethanol extract is included in Table 4. Fig 15 shows the results of ethanolic extract in five different test plates against five different bacteria used.

Table 4: Antibacterial activity of ethanol extract

	Diameter(mm)	
Organisms	**80µg/µl**	**60µg/µl**
Bacillus cereus	16 mm	15 mm
Staphylococcus aureus	12.5 mm	10 mm
Streptococcus pneumoniae	12 mm	8.5 mm
Shigella flexnari	12 mm	10 mm
Escherichia coli	11 mm	10 mm

*All measurements are means of individual data obtained from triplicate tests



(a) (b)

(c) (d)

(e)

Fig 15: Antibacterial activity of ethanolic extracts of (a) *S.aureus* (b) *E.coli* (c) *S.flexneri* (d) *S.pneumoniae* (e) *B.cereus*

*All tests were performed in triplicate

3.2 Antibacterial activity of the two different extracts with positive control:

The comparative results of the zones of inhibition produced by the different extracts and the controls has been shown in table 5. For positive control five different antibiotics were used against the organisms. Kanamycin was used against *Bacillus cereus*, Chloramphenicol against *Streptococcus pneumoniae*, Ampicillin for *Staphylococcus aureus*, Ciprofloxacin for *Escherichia coli* and lastly, Cefotoxin for *Shigella flexneri.*

Table 5: Antibacterial activity of the two different extracts with positive control (diameter shown in mm)

	Diameters (in mm)					
	Methanolic extract			Ethanolic extract		
Organisms	**80µg/µl**	**60µg/µl**	**(+ve)C**	**80µg/µl**	**60µg/µl**	**(+ve)C**
Bacillus cereus	16	15	25	16	15	25
Staphylococcus aureus	13	10	42	12.5	10	42
Streptococcus pneumoniae	9.25	8	32	12	8.5	32
Shigella flexnari	11	11	25	12	10	25
Escherichia coli	10	9	30	11	10	30

*All measurements are means of individual data obtained from triplicate tests

The bar graph below shows the comparison between the clear zones of the different types of extracts and the antibiotics used (Fig 16). The zone of inhibition (in mm) was placed in the X-axis and the extract types along with the organisms were placed in Y-axis. Different colored bars are used to indicate the concentrations of extracts and the antibiotics. Among the five different bacterial strains studied, *Bacillus cereus* showed best antibacterial activity against both ethanolic and methanolic extracts producing 16 mm and 15 mm zones of inhibition respectively. *Staphylococcus aureus* and *Shigella flexneri* showed 10-13 mm zones of inhibition whereas *Streptococcus pneumoniae* and *Escherichia coli* showed 8-12 mm zones of inhibition against both types of extracts.

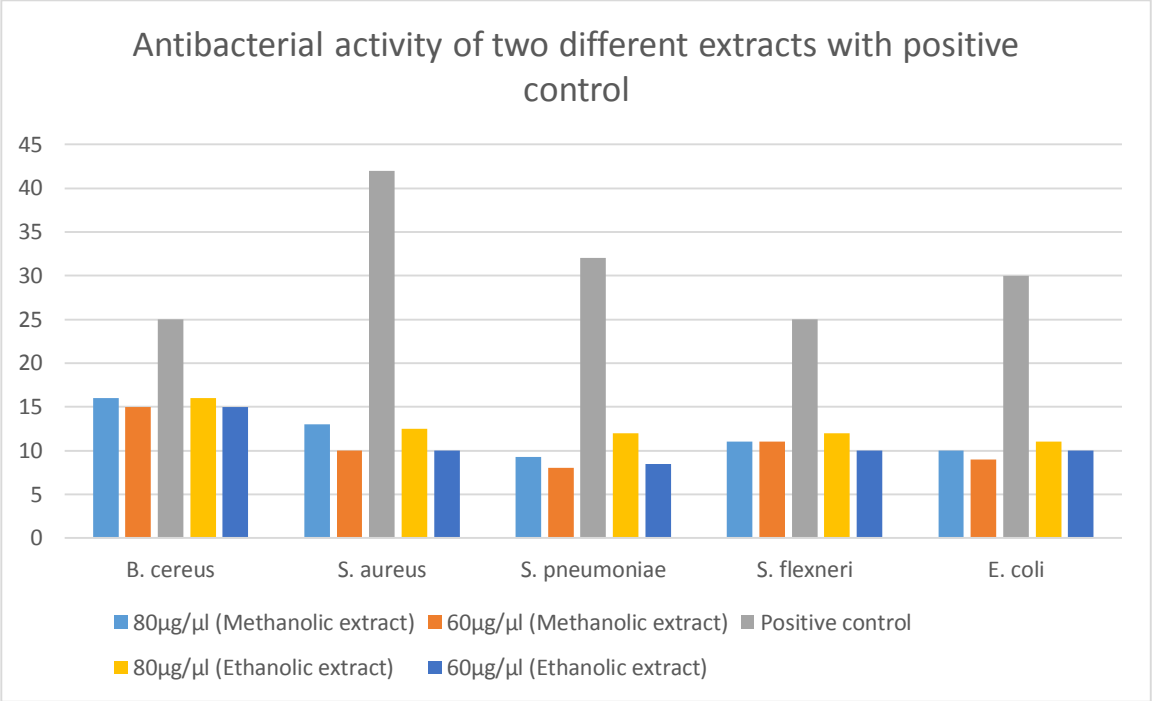


Fig 16: Comparison of zone of inhibition of extracts and antibiotics

*All measurements are means of individual data obtained from triplicate tests

Based on the zones of inhibitions, activity index of all the extracts were calculated and listed in Table 6. The following formula was used:

$$\text{Activity index} = \frac{\text{Zone of inhibition of extract}}{\text{Zone of inhibition of antibiotic}}$$

Among the five different bacterial strains, *Bacillus cereus* showed best activity index against both ethanolic and methanolic extracts. The values observed were 0.64 and 0.6 for 80µg/µl and 60µg/µl concentrations respectively. The second highest activity index has shown by *Shigella flexneri* and the observed value ranged from 0.4 to 0.48. Furthermore, the activity indexes of *Escherichia coli*, *Staphylococcus aureus* and *Streptococcis pneumoniae* ranged from 0.24 to 0.38 against both the types of extracts. Fig 17 shows a graphical representation of the activity index of *Chrysanthemum morifolium* Ramat.

Table 6: Activity Index of the extracts collected from *Chrysanthemum morifolium* Ramat

	Methanolic extract		Ethanolic extract	
	80µg/µl	**60µg/µl**	**80µg/µl**	**60µg/µl**
Bacillus cereus	0.64	0.6	0.64	0.6
Staphylococcus aureus	0.31	0.24	0.30	0.24
Streptococcus pneumoniae	0.29	0.25	0.38	0.27
Shigella flexnari	0.44	0.44	0.48	0.4
Escherichia coli	0.33	0.3	0.37	0.33

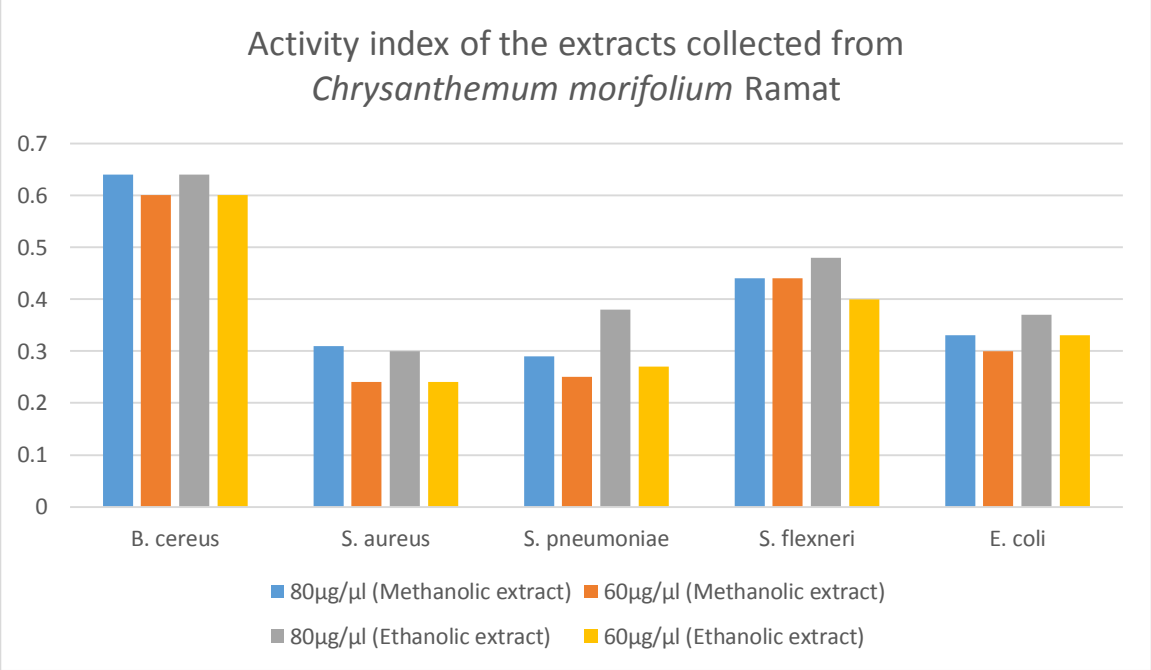


Fig 17: Graphical representation of the activity index of *C.morifolium* Ramat

3.3 Discussion:

The expanding bacterial resistance to antibiotics have become a growing concern worldwide (Gradam, 2000). Antibiotic resistant bacteria is considered a significant or major problem in the treatatment of patients (Lepape et al, 2009). Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistant strains (Alviano, 2009; Hemaiswarya et al, 2008). A large number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds. Medicinal plant extracts offer considerable potential for the development of new agents effective against infections currently difficult to treat (Wendakoon et al., 2012). *Chrysanthemum Morifolium* Ramat is one such plant that is well known for its application in treatment of many disease like detoxification of blood, regulation of blood pressure, calming the nerves, bacterial and viral infections, sinusitis, digestive and skin problems, influenza, headche, dizziness, sore throat, hypertension, flu and cough etc (Yeasmin et al., 2016)

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds. Many plant leaves have antimicrobial principles such as tannins, essential oils and other aromatic compounds. In addition, many biological activities and antibacterial effects have been reported for plant tannins and flavonoids. The current work deals with the collection of two different types of extract (methanol, ethanol) and the biochemical assay to find the presence of various phytochemicals including alkaloids, steroids, phenols, flavonoids, tannins, saponins, and cardiac glycosides in *Chrysanthemum morifolium* Ramat. The selected plant sample was collected and extraction was done using a Soxhlet apparatus. The study also reported the antimicrobial activity of *C.morifolium* Ramat against common pathogenic organisms such as: *B. cereus*, *S. pneumoniae, S.aureus, S.flexnari and E.coli*. The results obtained from the presence of phytoconstituents in all two extracts. Presence of alkaloids was confirmed through Hager's, Wagner's and Dragendraff's test. For the Hager's test and Wagner's test, ethanolic extract showed a positive result as a yellow precipitation was visibly seen. For the methanolic extract, a negative result was found in these two tests for alkaloids. The Dragendraff's test for alkaloids gave a moderately positive result for both the ethanolic extract and the methanolic extract. For steroidal compounds a positive result was seen for ethanolic extract. As a whole, the presence of phenols, flavonoids and cardiac glycosides were comparatively high. According to the sudy by Yeasmin et al., 2016 *Chrysanthemum morifolium* Ramat contains a high number of phenols, alkaloids, cardiac glycosides and flavonoids. In our study, we also found similar results for these phytoconstituents. Besides, traces of saponins were found in both the ethanolic and methanolic extract.

The results obtained also showed the antimicrobial properties of *C.morifolium* Ramat. Agar disc diffusion method was followed and the zones of inhibition were measured in millimeters. Kanamycin, Ampicillin, Ciprofloxacin, Cefotoxin and Chloramphenicol were used as positive controls. The largest clear zone was seen in *B.cereus*, producing 16 mm and 15 mm zones at concentrations of 80µg/µl and 60 µg/µl respectively, for both methanolic and ethanolic extracts. But Yeasmin et al, 2016 showed that methanolic extracts of *Chrysanthemum morifolium* Ramat has a smaller clear zone of 10.33±0.50. However, in case of ethanolic extracts, her study obtained a clear zone of 16.33±0.57 which is compliant to our data. Also, in *S.aureus*, our clear zone for 80µg/ µl of methanolic extract was found to be 13 mm, which is very close to the results obtained by Yeasmin et al, 2016 where the zone of inhibition was 11.50±0.88. Overall, the results of antibacterial activity showed that *C. morifolium* Ramat is most effective against *B.cereus, S.aureus* and *S.flexneri* in this experiment (Fig 16).

Chapter: 4

Conclusion

Chrysanthemum species are also known to contain a wide array of essential phytochemicals. These phytochemicals are the secondary metabolites present in smaller quantities in higher plants and they include alkaloids, steroids, flavonoids, tannins and many others (Peteros, 2010). It is therefore crucial to know the type of phytochemical constituent, thus knowing the type of biological activity which might be exhibited by the plant (Agbafor and Nwachukwu, 2011). Various studies have demonstrated the potential medicinal activities of *Chrysanthemum* plants (Ben Sassi et al., 2008). In addition there has been an immense interest in utilization of natural plant extracts as antimicrobial activity due to the increase in outbreak of food borne diseases and to minimize the health causing diseases over synthetic drugs (Gupta et al, 2013). The purpose of this study was to collect crude extracts and investigate the presence of phytoconstituents in *Chrysanthemum morifolium* Ramat that serves as an effective agent to treat many infectious diseases. The primary phytochemical analysis of ethanolic and methanolic extracts revealed the presence of alkaloids, flavonoids, steroids, phenols and cardiac glycosides. However, the extracts only showed traces of tannins and saponins. This shows that these phytochemicals could be responsible for the observed antimicrobial properties. Antibacterial tests show that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *B.cereus*, *S.aureus* and *S.flexneri*. The analysis of antioxidant, antidiabetic including the isolation, identification and purification of phytoconstituents and determining their respective antibacterial potencies to evaluate and formulate chemotherapeutic agents could be the future frontier for this investigation. From the present study it can drawn to conclusion that the traditional use of the plant *Chrysanthemum morifolium* Ramat for infectious diseases is promising against many bacteria and disease causing pathogens.

References

Beninger CW, Hall JC. Allelopathic activity of luteolin 7-0-β-glucuromide isolated from *Chrysanthemum morifolium* L. Biochemical Systematics and Ecology, 2005, 33: 103-111

Chu Q, Fu F. Guan Y, Ye J. Determination and differenciation of Flos *Chrysanthemum* based on characteristic electrochemical profiles by capillary electrophoresis with electrochemical detection, Journal of Agricultural and Food Chemistry, 2004, 52: 7828-7833

Dowrick GJ. The chromosomes of *chrysanthemum* I., The species, 1952, 6: 365-375.

Yeasmin et al. 2016. Phytochemical analysis and antioxidant activity of three flower colors *Chrysanthemum morifolium* Ramat. International Journal of Biosciences vol.9, no.2, p.69-77.

Natural Wellbeing. (2017). *Chrysanthemum Morifolium - Natural Wellbeing*. [online] Available at: http://www.naturalwellbeing.com/learning-center/chrysanthemum_morifolium/

Yen GC, Chen HY. Relationship between antimutagenic activity and major components of various teas. Mutagenesis. 1996; 11(1): 37–41

Yeasmin et al. 2016. Evaluation of antibacterial activity of three flower colours *Chrysanthemum morifolium* Ramat against multi-drug resistant human pathogenic bacteria. International Journal of Biosciences vol.9, no.2, p.78-87.

Chen, T., Ping, L., Lu, X., Jiang, H. and Zeng, S. (2007). Absorption and Excretion of Luteolin and Apigenin in Rats after Oral Administration of *Chrysanthemum morifolium* Extract. *J. Agric. Food Chem.*, 55 (2), pp.273–277.

Mitra, M. and Pal, P. (2008). Performance of *Chrysanthemum X morifolium* Ramat. cv. 'Chandrama' grown at different levels of planting density and stem maintained per plant. *Natural Product Radiance*, 7(2), pp.146-149.

Verma, S., Angadi, S., Patil, V., Mokashi, A., Mathad, J. and Mummigatti, U. (2011). Growth, yield and quality of chrysanthemum (*Chrysanthemum morifolium* Ramat.) Cv. Raja as influenced by integrated nutrient management*. *Karnataka J. Agric. Sci.*, 24(5), pp.681-683.

Moura, F., Silva Vieira, M., Evangelista Junior, W., Silva, L., Oliveira, R., Rocha, A. and Costa, F. (2014). Cultivars, conduction, photoperiodic and quality chrysanthemum in Brazil. *Journal of Horticulture and Forestry*, 6(6), pp.53-57.

Anjum, M., Nawaz, A., Gul, S. and Naveed, F. (2007). Effect of various sucker sizes and planting times on flowering and vase-life of chrysanthemum. *Pak. J. Agri. Sci*, 44(3).

Van der Ploeg, A. and Heuvelink, E. (2006). The influence of temperature on growth and development of chrysanthemum cultivars: a review. *Journal of Horticultural Science & Biotechnology*, 81(2), pp.174-182.

Amin, N., Sajid, M. and Ahmad, H. (2014). Effect of sowing dates on enhancing the flowering time in chrysanthemum (Chrysanthemum morifolium). *International Journal of Biosciences*, 5(12), pp.152-159.

Higuchi Y, Sumitomo K, Oda A, Shimizu H, Hisamatsu T. 2012. Day light quality affects the night-break response in the short-day plant chrysanthemum, suggesting differential phytochrome-mediated regulation of flowering. Journal of Plant Physiology 169, 1789–1796 [PubMed]

Agrawal, P. K. and Bansal, M. C. 1989. Flavonoid glycosides, pp. 283–364, in P. K. Agrawal (ed.). Carbon-13 NMR of Flavonoids. Elsevier, New York

Hu, C.-Q., Chen, K., Shi, Q., Kilkuskie, R. E., Cheng, Y.-C., and Lee, K.-H. 1994. Anti-aids agents 10. Acacetin-7-O-β-D-galactopyranoside, an anti-HIV principle from *Chrysanthemum morifolium* and a structure–activity correlation with some related flavonoids. J. Nat. Prod. 57:42– 51.

Beninger, C. W., Abou-Zaid, M. M., Kistner, A. L., Hallett, R. H., Iqbal, M. J., Grodzinski, B., et al. (2004). A flavanone and two phenolic acids from Chrysanthemum morifolium with phytotoxic and insect growth regulating activity. Journal of Chemical Ecology, 30, 589–606

Lee, J. S., Kim, H. J., & Lee, Y. S. (2003). A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*. Planta Medica, 69, 859–861.

Kurata, R., Adachi, M., Yamakawa, O., & Yoshimoto, M. (2007). Growth suppression of human cancer cells by polyphenolics from sweet potato (Ipomoea batatas L.) leaves. Journal of Agricultural and Food Chemistry, 55, 185–190.

Lai, J. P., Lim, Y. H., Su, J., Shen, H. M., & Ong, C. N. (2007). Identification and characterization of major flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD–APCI/MS. Journal of Chromatography B, 848, 215–225.

Lograda, T., Ramdani, M., Chalard, P., Figueredo, G., Silini, H. and Kenoufi, M. (2013). Chemical composition, antibacterial activity and chromosome number of Algerian population of two chrysanthemum species. Journal of Applied Pharmaceutical Science, Vol. 3, pp.S6-S11.

Gupta, E., Purwar, S., Sundaram, S., & Rai, G. K. (2013). Nutritional and therapeutic values of *Stevia rebaudiana:* A Review. Journal of Medicinal Plants Research, 7(46), 3343-3353.

Kumar A, Singh SP, Bhakuni RS. Secondary metabolites of chrysanthemum genus and their biological activities, Current science, 2005, 89(9): 1489-1501

Wendakoon, C., Calderon, P., & Gagnon, D. (2012). Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. Journal of Medicinally Active Plants, 1(2), 60-68.

Who.int. (2002). *WHO | Traditional medicine*. [online] Available at: http://www.who.int/mediacentre/factsheets/2003/fs134/en/

Lee Hyun-Jung, Hwang Young-Il, Park Eunju, Choi Sun-Uk. Antihepatotoxic and antigenotoxic effects of herb tea composed of *Chrysanthemum morifolium* Ramat, Han'guk Sikp'um Yongyang Kwahak Hoechi, 2011, 40(1):78-83**.**

Erler R, Siegmund I. 1986. Year Book of the International Horticultural Statistics. 44 P. USA.

Bhattacharjee SK, De LC. 2003. Advanced Commercial Floriculture. Aavishkar Publishers, Distributors, Jaipur, India.

Balaji, Kulkarni S, Reddy BS. 2010. Effect of date of planting on yield and quality of chrysanthemum (*Chrysanthemum morifolium*) cv. Saraval. Karnataka Journal of Agriculture Science 23(2), 402-403.

Beniwal, B. S., Ahlawat, V. P., Sukhbir Singh and Dahiya, S. S., 2006, Influence of N and P application on flower yield and nutrient content of chrysanthemum (*Chrysanthemum morifolium* Ramat.) Cv. Flirt. Haryana J. Hort. Sci., 35(1&2): 790-80.

Nakano, Y., Higuchi, Y., Sumitomo, K. and Hisamatsu, T. (2013). Flowering retardation by high temperature in chrysanthemums: involvement of FLOWERING LOCUS T-like 3 gene repression. *Journal of Experimental Botany*, Vol. 64, No. 4, pp.909–920

Agbafor KN, Nwachukwu N. 2011. Phytochemical Analysis and Antioxidant property of leaf extract of Vitexdoniana and Mucunapruriens. Biochemistry Research International 2011, 1-4

Peteros NP. 2010. Antioxidant & cytotoxic activities & phytochemical screening of four Phillppine medicinal plants. Journal of medicinal plants research 4(5), 407-414.

Ben Sassi, A., Harzallah-Skhiri, F., Bourgougnon, N. and Aouni, M. (2008). Antimicrobial activities of four Tunisian Chrysanthemum species. *Indian J Med Res 127*, pp.183-192.

Carvalho, S. M. P. (2003). Effects of Growth Conditions on External Quality of Cut Chrysanthemum: Analysis and Simulation. Ph.D. Thesis. Wageningen University, The Netherlands. 171 pp

Spaargaren, J. J. (2002). Jaarrond chrysanten. Spaargaren, Aalsmeer, The Netherlands. 253 pp.

Wilkins, H. F., Healy, W. E. and Grueber, K. L. (1990). Temperature regime at various stages of production influences growth and flowering of *Dendranthema grandiflorum*. Journal of the American Society for Horticultural Science, 115, 732–736.

Carvalho, S. M. P., Abi-Tarabay, H. and Heuvelink, E. (2005). Temperature affects Chrysanthemum flower characteristics differently during three phases of the cultivation period. Journal of Horticultural Science & Biotechnology, 80, 209–216.

APPENDIX – I

(Reagents)

1. Hager's Reagent

Saturated solution of picric acid.

2. Wagner's Reagent

2 grams iodine and 6 grams of potassium iodide in 100ml of distilled water.

3. Dragendraff's Reagent

Bismuth Nitrate solution; 8 grams Bismuth Nitrate in 12ml 30% Nitric Acid. Dissolve 27.2 gram Potassium Iodide in 50ml distilled water and put into the Bismuth Nitrate solution. Dilute 100ml distilled water.

4. 1% Ferric Chloride

0.01 gram Ferric Chloride in 100ml distilled water.

5. 1% Lead Acetate

0.01 gram Lead Acetate in 100ml distilled water.

6. 1% Potassium Ferrocyanide

0.01 gram Potassium Ferrocyanide in 100ml distilled water.

APPENDIX – II (Instruments)

The important equipment used through the study are listed below:

Autoclave	SAARC
Freeze (-20ºC)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipett (20-200 µl)	Eppendorf, Germany
Oven, Model :MH6548SR	LG, China
pH meter, Model: E-2010C	Shanghai Ruosuaa Technology Company China
Refrigerator (4ºC) Model: 0636	Samsung
Safety Cabinet Class II Microbilogical	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water Bath	Korea
Weighing Balance	ADAM EQUIPMENTTM, United Kingdom