Extraction of secondary metabolites, phytochemical screening and the analysis of antibacterial activity in *Stevia rebaudiana*

Inspiring Excellence

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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DECLARATION

I hereby solemnly declare that the research work titled "**Extraction of secondary metabolites, phytochemical screening and the analysis of antibacterial activity in *Stevia rebaudiana***" 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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Abstract

Stevia rebaudiana is a plant of the Asteraceae family and native to Paraguay. Its sweetness and calorie free property has increased its demand commercially all over the world as a sugar substitute in food beverages and medicines. Stevia has many pharmacological and therapeutic properties including antimicrobial, antifungal, antioxidant and anticarcinogenic activity. Due to this importance the leaf was analyzed for preliminary phytochemical studies to find the presence of phytoconstituents such as alkaloids, flavonoids, steroidal compounds, sponins, tannins, phenols, and cardiac glycosides. In this study, the crude plant extract from three different extractions (ethanol, methanol, and water) were collected and tested for the presence of phytochemicals. Comparative analysis of the antimicrobial activity of the extracts was investigated against four bacterial strains (Bacillus *subtilis, Staphylococcus aureus, Streptococus peneumoniae* and *Shigella flexnari*). This was done using agar diffusion method and as controls kanamycin, tetracycline and chloramphenicol were used. The diameter of the clear zone of inhibition surrounding the disc was measured in millimeters. The results showed that methanolic and ethanolic extracts of *Stevia rebaudiana* contained the highest amounts of phytochemicals. Antibacterial activity results showed there is no inhibitory effect of stevia against *S.aureus* and *S.flexnari*. The highest amount inhibition was formed against *B.subtilis and S.pneumoniae.* It can be concluded from the results that methanolic, ethanolic and aqueous extracts of *Stevia rebaudiana* may be considered as an antibacterial agent against *S.pneumoniae* and *B.subtilis* and be used to source antibiotic substances.

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Chapter 1
Introduction

1.0 Background:

The therapeutic properties of various medicinal plants have been used for centuries to treat many human diseases. As estimated, between 60-90% of the population of developing countries use traditional herbal medicines considering them to be a normal part of primary healthcare (WHO,2002). The demand of herbal medicine has been increasing day by day by consumers as they perceive these forms of healing as safe and effective than the synthetic drugs. This trend of using alternative and complimentary healthcare has prompted scientists to investigate the various biological activities of medicinal plants (Wendakoon et al. 20111). These plant-derived products contain a great diversity of phytochemicals such as phenols, flavonoids, tannins and other phytoconstituents that possess numerous health related effects such as antibacterial, antidiabetic and ant carcinogenic properties.(Bidlack et al, 2000). *Stevia rebaudiana* (bert) Bertoni is one such plant that contains such properties.

1.1 History and use of *Stevia Rebaudiana:*

*Stevia rebaudiana (*Bert) Bertoni, commonly known as „the sweet leaf of Paraguay" is a herbaceous perennial plant of Paraguay. It is a small, semi-bushy, perennial shrub of the Asteraceae family originated in Paragua. (Katayama et al, 1976). The plant grows for about 30 cm in height (Fig 1). Natives of Paraguay centuries ago used the leaves of the herb to sweeten their bitter drinks. Stevia is native to the valley of Rio Monday in highlands of North-eastern Paraguay in South America where it grows in sandy soils near streams on the edges of marshland, acid infertile sand or muck soils. It is one of the 154 members of the genus Stevia, which produces sweet stevioside, diterpenoid glycoside in the eaves of this plat (Soejarto et al. 1982). It was first brought to attention of Europeans in 1887 when M.S Bertoni learned of its unique properties from the Paraguayan Indians and Mestizos (Lewis 1992). Dating back to the Guarani Indians of South America this plant has been used to sweeten tea for centuries. Its extracts are used today as a food additive by the Japanese and Brazilian and as a non-caloric sweetener but in the U.S it has been used as a supplement only. (Madan et al, 2009).

Fig: 1 *Stevia rebaudiana*

1.2 Scientific Classification:

- Domain: Eukaryota
- Kingdom: Plantae
- Order: Aterales
- Family: Asteraceae
- Tribe: Eupatorieae
- Genus: Stevia
- Species: *S. rebaudiana*
- Binomial name: *Stevia rebaudiana (*Bertoni) Bertoni

1.3 General characteristics of *Stevia rebaudiana:*

Commonly known as the Honey leaf and Candy leaf in Paraguay, this perennial semi-shrub grows up to 30 cm in height. Its leaves are sessile, 3-4 cm long, elongate-lanceolate or spatulate shape with blunt tipped lamina, serrate margin from the middle to the top and entire below. The upper surface of the leaf is slightly glandular pubescent. The step is weak pubescent at bottom and woody. The rhizome has slightly branching roots. Flowers of this plant are composite surrounded by an involucre of epicalyx. The capitulate are in loose, irregular sympodial cymes. Flowers are purple colored and the fruits of this plant are generally five-ribbed spindle-shaped achene (Medan *et al, 2009)*

1.4 Cultivation:

Commercial cultivation of *Stevia rebaudiana* was first reported in Paraguay in 1964. Seeds were sent to England in 1942 in an unsuccessful attempt to establish production. (Katayama et al. 1976; Lewis 1992). After a large effort aimed at establishing the crop in Japan, stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada since 1990 (Lee et al. 1979; Donalisio et al. 1982; Schock 1982; Goenadi 1983;) Current production of Stevia is centered in China and the major market is in Japan. (Madan et al, 2009).

Due to its semi-humid subtropical properties this plant that can be grown easily like any other vegetable crop. Agro-technologists of some countries are studying the various parameters like mean height, weight of leaves, growth per day, total biomass yield and stevioside content of the plant. It can be transplanted in February or March and the seed can be collected in late summer. Flowering under these conditions should occur between 54-104th day following transplanting, depending on the day length sensitivity of the cultivars used for seed production. (Madan et al, 2009). The concentration of stevioside in the leaves of Stevia increases when the plants are grown under long days (Mativer and Viana, 1979). The plant is harvested just prior to flowering when steviol glycoside content in the leaves is maximum. Seed viability and yield are affected by

growing conditions during pollination and can be affected by excessive rainfall during pollination.

To get a better leaf yield and rebaudiside-A concentration in leaves, a variety of plant breeding procedure has been used. Based on the cultivar descriptions from Japan, China and Korea, the sufficient genetic variability exists to make significant genetic gains in leaf yield of rebaudioside. Its seed is best stored at 0ºC, but even under low temperature conditions, germination declines by 50% over three years (Madan 2009). Glycoside studies revealed that synthesis is reduced at or just before flowering; delaying flowering with long days allows more time for glycoside accumulation. It follows that Stevia herbage production would be best in a long day environment where vegetative period is longer and the resultant steviol glycoside yield will thus be higher. Fertilizer requirements for Stevia grown as an annual crop are moderate (Das et al 2004).

1.5 Metabolic importance of Stevia:

The major compounds of Stevia as steviol glycosides are metabolized and eliminated through similar pathways in both humans and animals (Genus et al. 2007). Rebaudioside A in the digestive tract is first metabolized by microbes in the colon to stevioside which is further converted into glucose molecule and steviol. The released glucose molecule is used by the bacteria in the colon and is not absorbed into the blood stream. The metabolized components essentially leave the body and there is no accumulation. The metabolism of steviol glycosides to steviol means that the metabolic equivalency of the different steviol glycosides permits to apply the findings from studies with stevioside to the safety evaluation of rebaudioside A, and thus to the safety of Stevia (Koyama et al., 2003). A study on the human digestive tract demonstrates that steviol is not altered or changed at either high or low concentrations as observed through human faeces, indicating that steviol is in fact the final product of Stevia metabolism (Koyama et al., 2003). The study also showed that the majority of steviol glycosides are absorbed and glucuronidated (a bond intended to help them clear out of the blood) in the liver. The newly bonded glucuronide is released in the blood and filtered by the kidneys into the urine. Small amounts of glucuronidate that remain in the colon are excreted through fecal matter. Tests with stevioside compounds and the effect of gastric juices and digestive enzymes on them show their

failure to degrade or rearrange the compounds (Wingard et al., 1980). In vitro digestibility of steviosides by various digestive enzymes was examined and found that none of the enzymes digested the stevioside and intestinal microflora hydrolyzed it to both steviol and steviol-16, 17 alphaepoxide (Hutapea et al. 1997). Later, steviol 16, 17 alpha-epoxide was then completely converted back into steviol, which further excreted from the body in urine as steviol glucuronide (Chatsudthipong and Muanprasat, 2009).

1.6 Therapeutic values of *Stevia rebaudiana:*

The ancient medicinal system of medicine has a long history regarding the use of *S. rebaudiana* (Megeji et al., 2005). Leaves of *S. rebaudiana* has been recommended as a treatment against various chronic and non-chronic diseases like diabetes, cardiovascular disease, cancer, renal disease, obesity, inflammatory bowel disease and dental caries. Stevia leaf extract has been used traditionally in the treatment of diabetes (Megeji et al., 2005; Soejarto et al., 1982). Their ingestion causes a slight suppression of plasma glucose levels and significantly increased glucose tolerance in normal adult humans (Curi et al., 1986). The effects of Stevia leaves and its extracted polyphenols and fiber on streptozotocin induced diabetic rats were studied besides its hypoglycemic effect and it also reduce the risk of oxidative stress (Shivanna et al., 2013). Overall, Stevia possess the ability to increase the insulin effect on cell membranes, increase insulin production, stabilize glucagon secretion and blood sugar levels, and improve glucose tolerance to ingested carbohydrates and lower post-prandial blood sugar levels in both animals and humans. Stevia acts at the cell membrane level much in the same way as a type of medication known as a calcium channel blocking agent. Labdane sclareol, compound present in leaf extract of Stevia has anti-tumorous and cytotoxic properties (Kaushik et al., 2010). Studies have demonstrated the inhibitory effects of Stevia leaf extracts and their polyphenolic constituents on tumor promotion and initiation. Stevioside, the Stevia leaf aglycones, steviol and isosteviol, and their metabolites have been reported to inhibit tumor promotion by blocking Epstein-Barr virus early antigen (EBV-EA) induction (Akihisa et al., 2004) as well as by reducing tumor formation.

1.7 Objective:

In this study, three different types of extracts from samples of *Stevia rebaudiana* (ethanol, methanol and water) were collected and investigated for the presence of phytoconstituents. Extracts were also used to test their antibacterial activity against 4 strains of bacteria *(Bacillus subtilis, Shigella felxnari, Staphylococcus aureus, Strreptococcus pneumoniae*). Although medicinal companies have produced a number of synthetic drugs against such microogranisms, 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 Stevia 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 *Stevia rebaudiana* and collection of extract.
- Phytochemical screening of collected *Stevia rebaudiana* plant extract.
- Comparative analysis of plant extracts antibacterial activity against four bacterial strains.

Chapter: 2

Materials and Method

2.1 Plant sample Collection:

A total of 500gm of powdered stevia leaves was collected from BRAC Nursery, niketon, Dhaka. The samples leaves were finely powdered without any impurities mixed in them. After collection they were stored at the laboratory at room temperature.

2.2 Preparation of Extracts:

A total of three types of extracts using three different solvents of ethanol, methanol and water were collected from the samples of *Stevia rebaudiana*.

2.2.1. Ethanoic Extracts:

For collection of ethanoic extracts 50g of stevia powder was packed in thimble and extracted in Soxhlet apparatus using 250ml of ethanol (Fig 1). 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 (Fig 3). 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.

Fig 1: Extract collection through Soxhlet Apparatus

2.2.2 Methanolic Extract:

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.

2.2.3 Aqueous extract:

Collection of aqueous extract was done in a different way than the other two. 40 gms of stevia powder was measured and mixed with 480 ml of distilled water (Fig2). This was left for 2 days in sterile environment. The liquid extract was then filtered through Whatman Filter paper no.40. The filtrate was kept in waterbath at 80-90 ºC till the extract was dried out. The dried extracts were then stored at 4 ºC in McCartney bottles.

Fig: 2 Aqueous Extract collection

Fig: 3 Dried Extracts in Petri dishes

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. Table 1 shows the different amount of extracts that were collected. After collection of extracts using spatula the McCartney bottles were tightly stoppered and stored in the refrigerator at 4 ºC. (Fig: 4)

Fig: 4 Storage of Extracts in McCartney Bottles

2.4 Preparation of Stock Solution for phytochemical assays:

For working the extracts were dissolved and a stock solution of 5µg/µl was made. This was done by mixing 0.5gm of crude extract with 100 ml of solvent. All three stock solution was made this way.

2.5 Biochemical Assays:

Preliminary screening of biochemical tests of all three 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 $FeCl_3$ 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 well diffusion method was used. A positive and negative 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 subtillis*
- *Streptocccus Pneumonae*
- *Staphylococcus aureus*
- *Shigella flexnari*

2.6.2 Preparation of extract solution for antibacterial activity test:

All three types of the dry extracts of *Stevia rebaudiana* that were collected were dissolved in 0.25% DMSO to make two different concentrations of extract solutions for the antibacterial activity test. 4gms of extract was dissolved in 50ml of 0.25% DMSO to prepare 80µg/µl. This was then diluted to prepare a solution that has a concentration of 60µg/µl. For each inoculation during the test the stock 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:

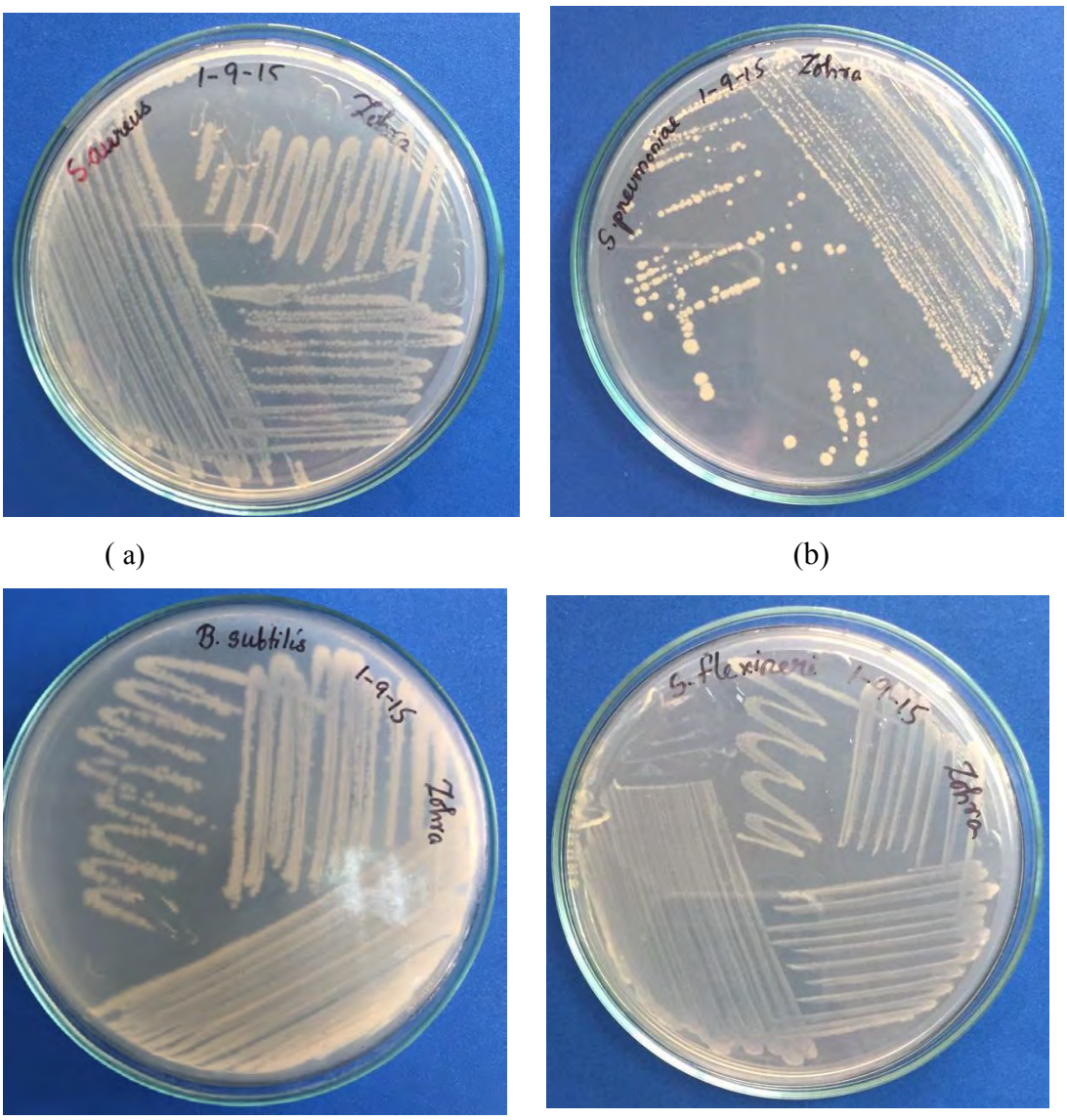
Ingredients	Amount
Beef,infusion form	30%
Caesin hydrolysate	1,75%
Starch	0.15%
Agar	1.7%
pH	Neutral

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 subcultured 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 5).



(a) (b)

(c) (d)

Fig: 5 Fresh culture used as stock in nutrient agar media. (a) Staphylococcus aureus (b) Streptococcus pneumonia (c) Bacillus subtilis (d) Shigella flexnari

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 (fig 6). 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.

Fig: 6 Lawn in MHA plate

2.6.6 Placing extracts and controls in the plate:

The inoculated plates were labelled and a cork borer was used to make a well in the media. 100 µl of stock extract that was previously prepared in concentrations of 80µg/ µl and 60 µg/ µl using freshly prepared 0.25% DMSO, were given in each well. A positive control and a negative control was used to compare the results. For positive control three different antibiotics were used against the organisms. Kanamycin was used against *Bacillus subtilis and Staphylococcus aureus,* Chloramphenicol against *Streptococcus pneumoniae* and tetracycline for *Shigella flexnari.* As

negative control 0.25% freshly prepared DMSO was given in one of the wells. The plates were then labelled accordingly and 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, ehtanolic and aqueous extracts were calculated and compared by measuring the activity index. This was done by using the following formula:

Activity Index (AI) = Zone of inhibition of extract/ 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 3 different solvents; methanol, ethanol and water. 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	Aqueous
1	29.62 gm	20.19 gm	22.46 gm
2	28.68 gm	27.46 gm	20.04 gm
3	30.52 gm	29.3 gm	19.54 gm

3.1.2 Biochemical Assays:

The biochemical assays were done to check the secondary metabolites present in the sample of *stevia rebaudiana* that was collected. After performing several tests the secondary metabolites and its pytoconstitiuents 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 8 shows the test results for alkaloids in *Stevia rebaudiana*. The yellow precipitate in the ethanolic and methanolic extract showed a positive result, however the aqueous extract had a significant negative result as there is no yellow precipitate.

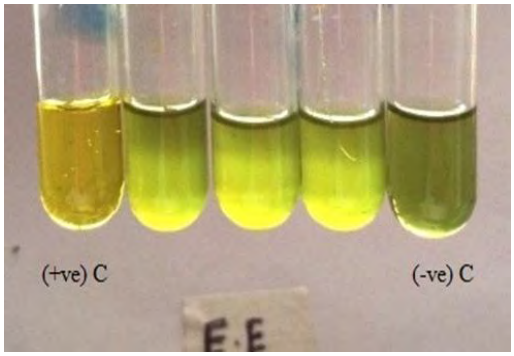
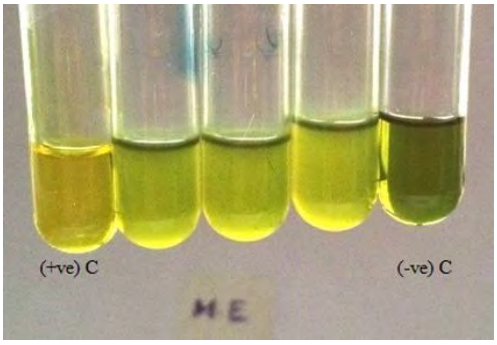
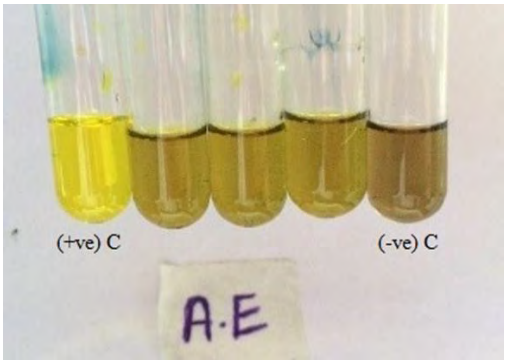
	(a) Hager's test for ethanolic extract showing strong positive result
	(b) Hager's test for methanolic extract showing moderately positive result
	(c) Hager's test for aqueous extract showing negative result

Fig: 8 Results for Hager‟s test for alkaloids (a) Ethanolic extract (b) methanolic extract (c) aqueous extract

b) **Wagner's Test:** This test involved adding 1 ml of extract with 3 dops of freshly prepared Wagner‟s reagent.

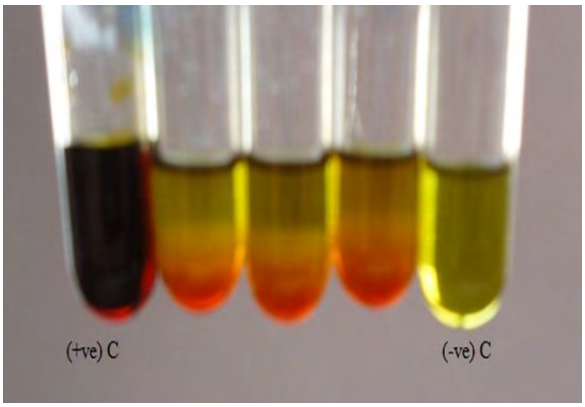
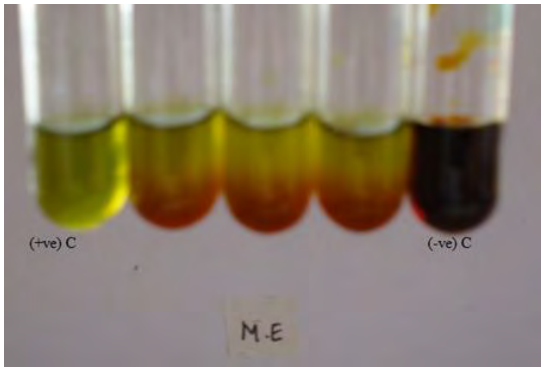
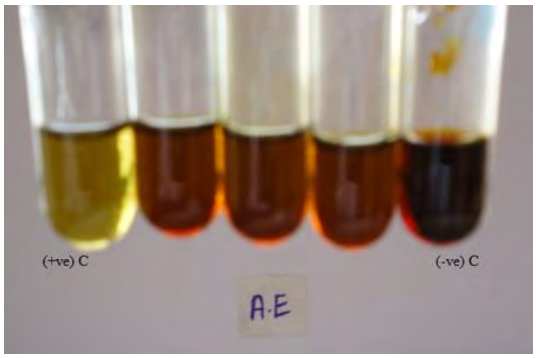
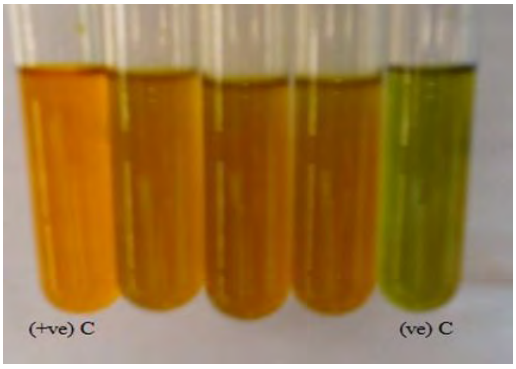
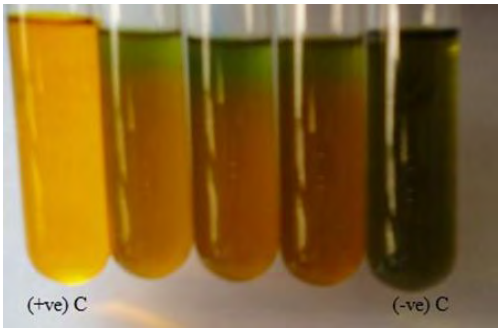
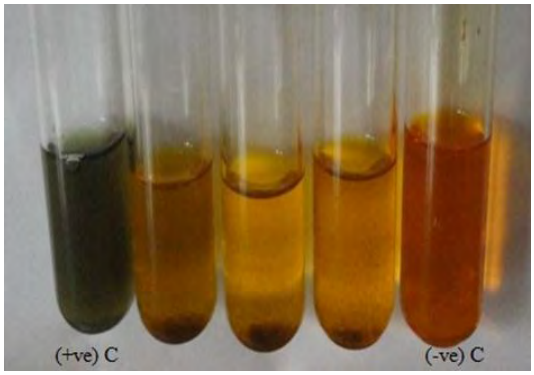
	(a) Wagner‟s Test for ethanolic extract showing strong positive result
	(b) Wagner‟s Test for methanloic extract showing strong positive
	(c) Wagner‟s Test for aqueous extract showing strong negative result

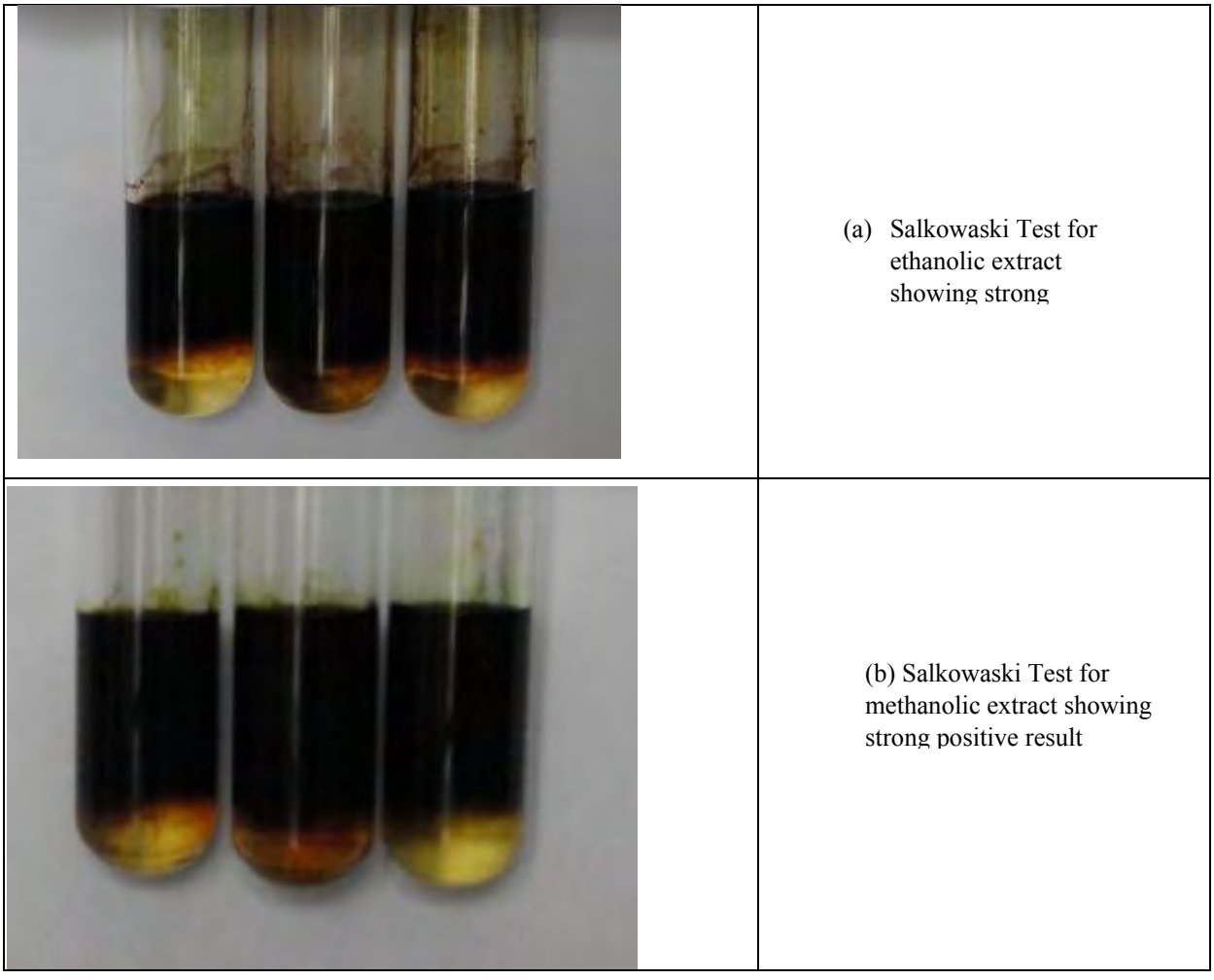
Fig: 9 Results for Wagner‟s test for alkaloids. (a) ehtanolic extract (b) methanolic extract (c) aqueous extract

The formation of brown precipitate is supposed to show a positive result (Fig 9). For the methanolic extract and ethanolic extract there was a strong positive result as the brown precipitate can be seen, Aqueous extract does not have any precipitate and thus this result shows there is no alkaloid present in the aqueous extract.

	(a) Dragendraff‟s‟s Test for ethanolic extract showing negative result
	(b) Dragendraff‟s‟s Test for methanolic extract showing moderately positive result
	(c) Dragendraff‟s Test for aqueous extract showing strong positive result

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 aqueous extract precipitate was seen giving a strong positive result. However for methanolic extract a slight precipitation gave a moderately positive result and a negative result for ethanolic extract (Fig 10).

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. Two tests were done in this case for ethanol and methanol with a strong positive result (fig 11).



(a) Salkowaski Test for ethanolic extract showing strong

(b) Salkowaski Test for methanolic extract showing strong positive result

Fig 11: Test results for steroidal compounds (a) ethanolic extract (b) methanolic extract

3.1.2.3 Test results for phenolic compounds

Test for phenols showed strong positive results for ethanolic and methanolic extracts but a negative result for the aqueous extract (Fig 12).

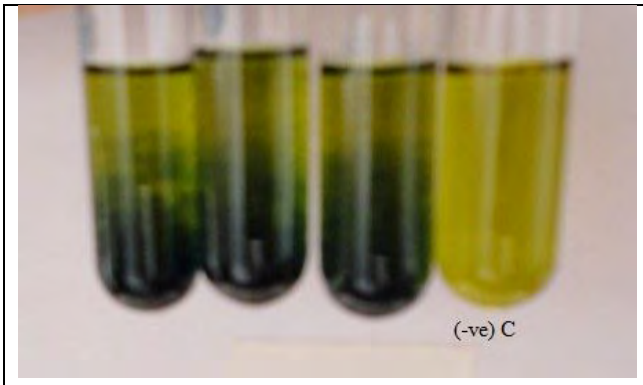
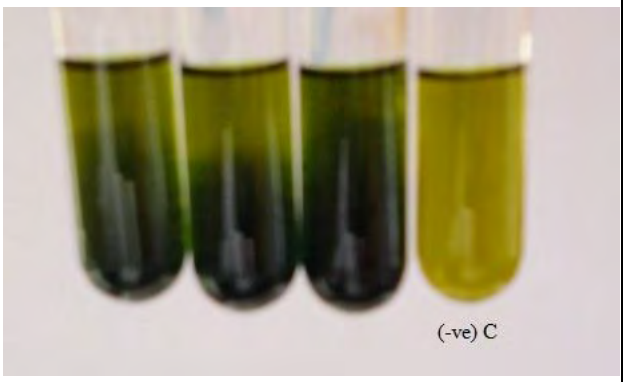
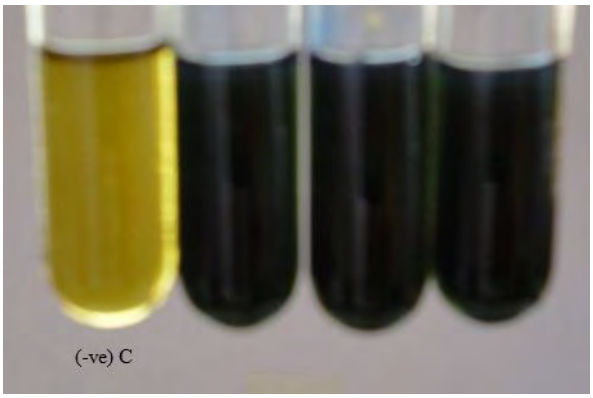
	(a) Ethanolic extract showing strong positive result for phenol
	(b) Methanolic extract showing strong positive result for phenol
	(c) Aqueous extract showing negative result for phenol

Fig: 12: Test results for phenols. (a) ethanolic extract (b) methanolic extract (c) aqueous extract

3.1.2.4 Test results for flavonoids:

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

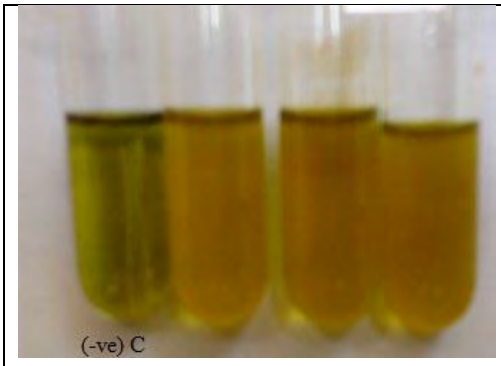
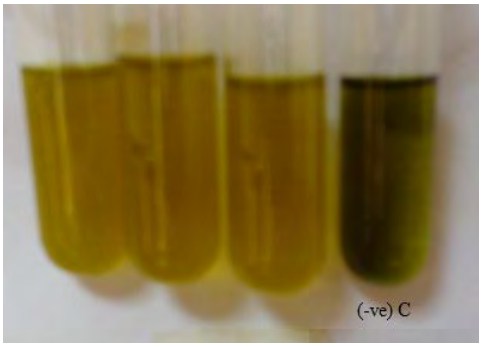
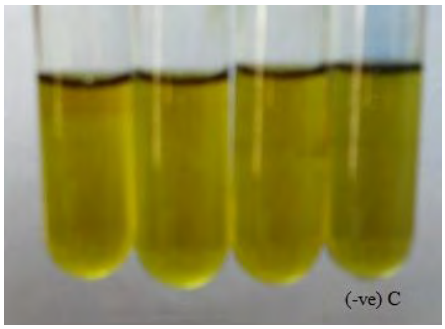
	(a) Ethanolic extract showing strong positive result for flavonoids
	(b) Methanolic extract showing strong positive result for flavonoids
	(c) Aqueous extract showing negative result for flavonoids

Fig 13: Test results flavonoids (a) ethanolic extract (b) methanolic extract (c) aqueous extract

According to the results there was a strong positive result for ethanolic and methanolic extract as they produced a yellow color upon addition of NaOH. For the aqueous extract a slight yellow color indicated traces of flavonoids in the sample (fig 13).

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

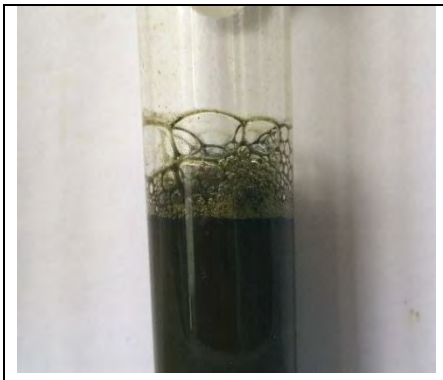
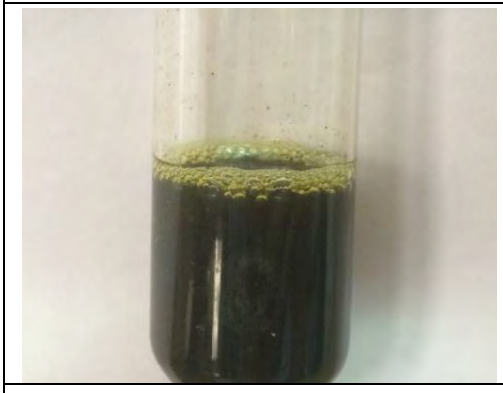
	(a) Froth test for ethanolic extract showing strong positive result for flavonoids
	(b) Froth test for methanolic extract showing traces of flavonoids
	(c) Froth test for aqueous extract showing moderately positive results for flavonoids

Fig 14: Test results flavonoids (a) ethanolic extract (b) methanolic extract (c) aqueous 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 strong positive result was seen for ethanolic extract as the froth was more and traces were seen in methaolic extract as the froth above the surface was slight. For aqueous extract a moderately positive result was seen (fig 14).

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 methanolic and aqueous extract a strong positive result was seen as there were more precipitate seen. However for ethanolic extract slight precipitation showed a moderately positive result (fig 15).

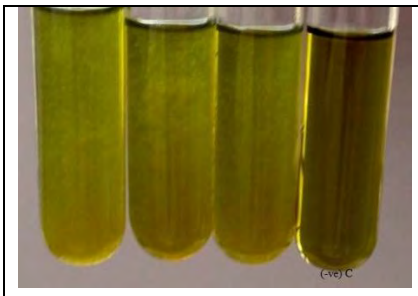
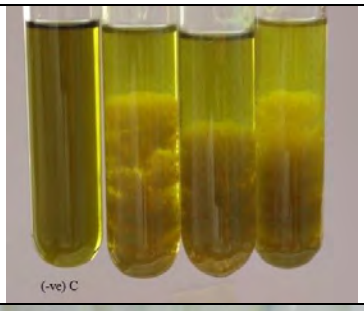
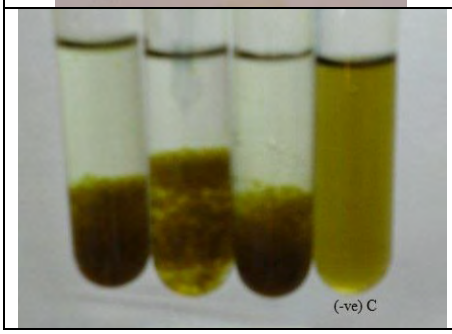
	(a) Lead acetate test for ethanolic extract showing moderately positive results for tannin
	(b) Lead acetate test for methanolic extract showing strong positive results for tannin
	(c) Lead acetate test for methanolic extract showing strong positive results for tannin

Fig 15: Test results tannins (a) ethanolic extract (b) methanolic extract (c) aqueous

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 strong positive result was found for all three types of extract (fig 16).

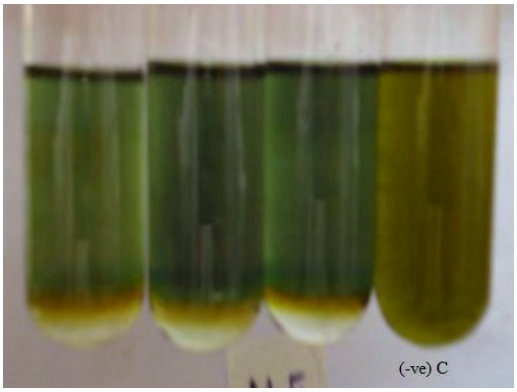
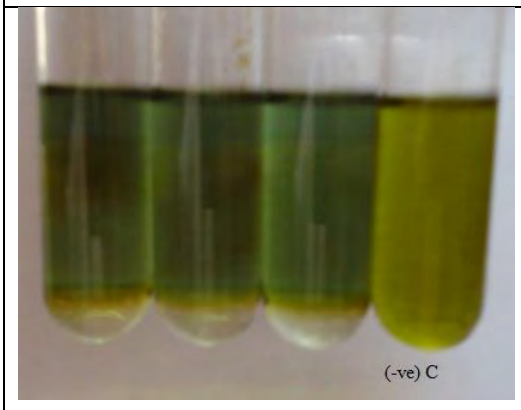
	(a) Lead acetate test for methanolic extract showing strong positive results for cardiac glycoside.
	(b) Lead acetate test for aqueous extract showing strong positive results for cardiac glycoside.
	(c) Lead acetate test for ethanolic extract showing strong positive results for cardiac glycoside.

Fig 16: Test results cardiac glycosides (a) ethanolic extract (b) methanolic extract (c) aqueous extract

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.**	**A.E**
Alkaloids	Hager‟s	+++	++	-
	Wagner‟s	+++	+++	-
	Dragendraff‟s	-	++	+++
Steroids	Chloroform and concen. H_2SO_4	+++	+++	
Phenols	Ferric Chloride and Potassium ferrocyanide	+++	+++	-
Flavonoids	Sodium hydroxide	+++	+++	trace
Sapoin	Froth Test	+++	Trace	++
Tannin	Lead Acetate	++	+++	+++
Cardiac Glycodise	Killer-Kilani Test	+++	+++	+++

A.E= Aqueous Extract; 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 three types of extract ethanolic, methanolic and aqueous were performed using agar 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 three 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. 17.

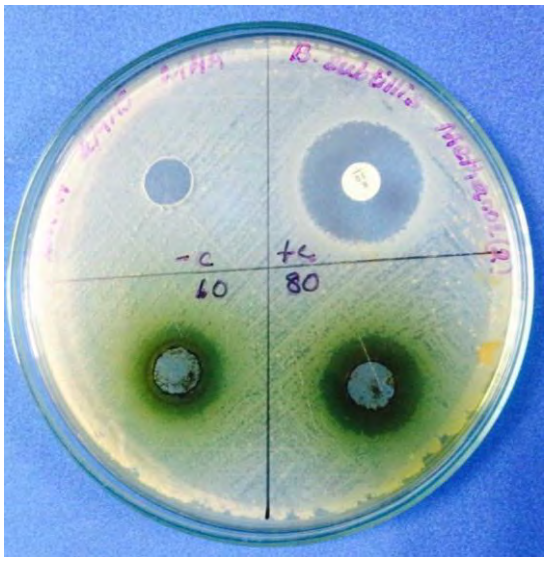
3.1.3.1 Antibacterial activity of methanol extract:

Among the four bacterial strains used *Bacillus subtilis* showed 17mm clear zone against 80µg/µl and 15 mm in 60µg/µl plant extract. *Staphylococcus aureus* and *Shigella felxnari* have no sensitivity. *Streptococcus pneumonia* showed 15 mm zone against 80µg/µl plant extract fig 17. A summary of antibacterial activity of methanol extract is included in table 3.

Table 3: Antibacterial activity of methanol extract

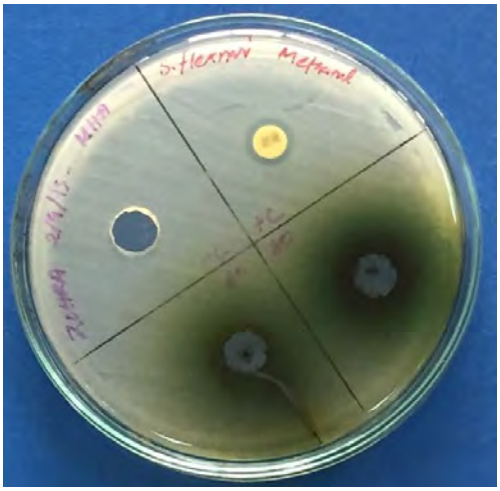
	Diameters (in mm)	
Organisms	**80µg/µl**	**60µg/µl**
Bacillus subtilis	17 mm	15 mm
Staphylococcus aureus	0 mm	0 mm
Streptococcus pneumoniae	15 mm	0 mm

Salmonella felxnari	0 mm	0 mm

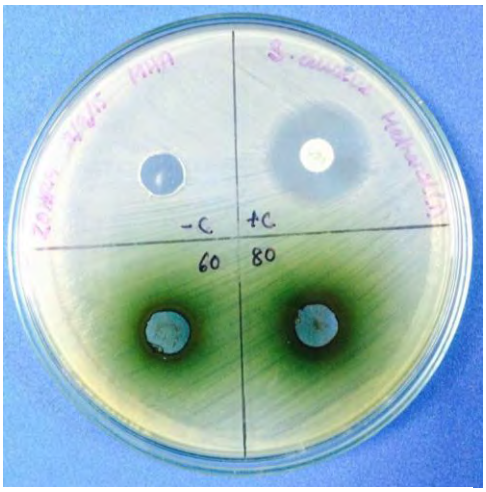


(a) Antibiotic activity test results of *B.subtilis*

(b) Antibiotic activity test results of *S.pneumoniae*



(c) Antibiotic activity test results of *S.flexnari*



(d) Antibiotic activity test results of *S. aureus*

Fig: 17 Antibacterial activity of methanol extract

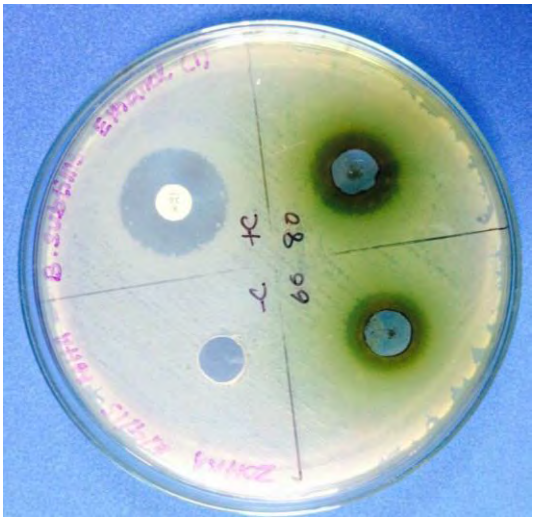
3.1.3.2 Antibacterial activity of ethanol extract:

Among the four bacterial strains used *Bacillus subtilis* showed 10 mm clear zone against 80µg/µl and 9 mm in 60µg/µl plant extract. *Staphylococcus aureus* and *Salmonella felxnari* have no sensitivity. *Streptococcus pneumonia* showed 20 mm zone against 80µg/µl and 9 mm in 60µg/µl plant extract. A summary of antibacterial activity of ethanol extract is included in table 4. Fig 18 shows the results of ethanolic extract in test plates.

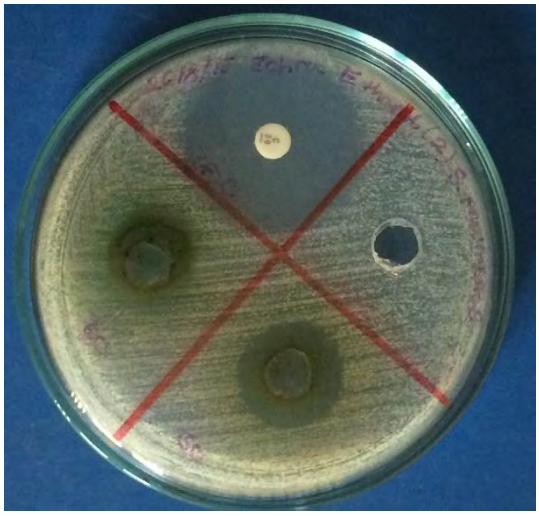
Table 4: Antibacterial activity of ethanolic extract

	Diameters (in mm)	
Organisms	**80µg/µl**	**60µg/µl**
Bacillus subtilis	10mm	9mm
Staphylococcus aureus	0mm	0mm
Streptococcus pneumoniae	20mm	9mm

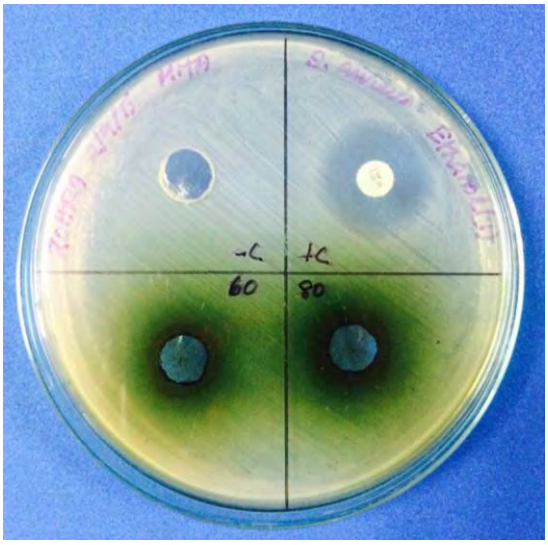
Shigella felxnari	0mm	0mm

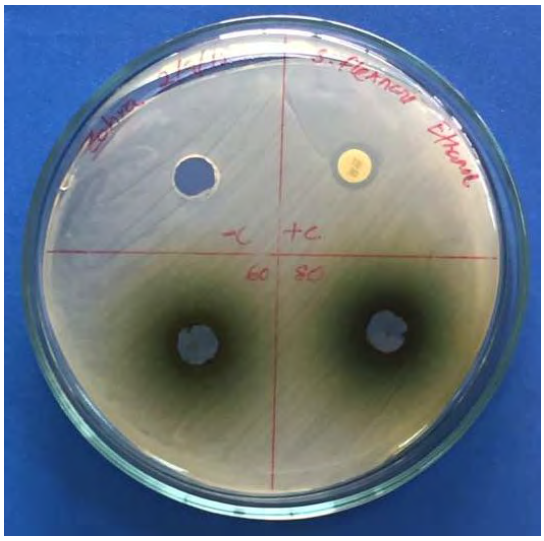


(a) Antibiotic activity test results of ethanol extract against *B.subtilis*



(b) Antibiotic activity test results of ethanol extract against *S,pneumoniae*





(c) Antibiotic activity test results of ethanol extract against *S,aureus*

(d) Antibiot
results o
against *S*

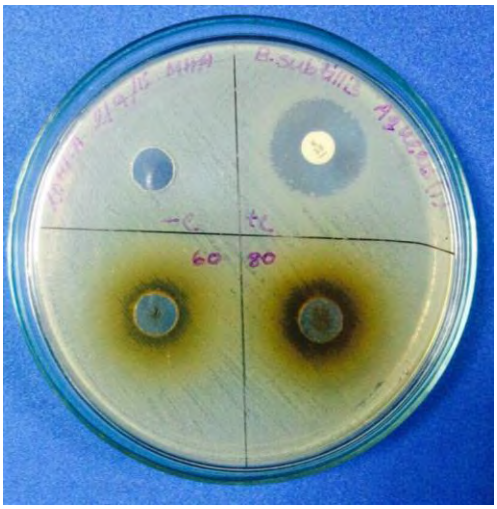
Fig: 18 Antibacterial activity of ethanol extract

3.1.3.2 Antibacterial activity of aqueous extract:

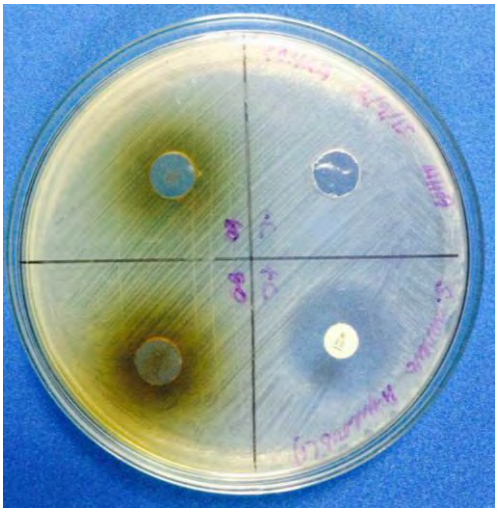
Among the four bacterial strains used *Bacillus subtilis* showed 15mm clear zone against 80µg/µl and 9 mm in 60µg/µl plant extract. *Staphylococcus aureus* and *Salmonella felxnari* have no sensitivity. *Streptococcus pneumonia* showed 25 mm zone against 80µg/µl and 18 mm in 60µg/µl plant extract. A summary of antibacterial activity of aqueous extract is included in table 5. Fig 18 shows the results of ethanolic extract in test plates.

Table 5: Antibacterial activity of aqueous extract:

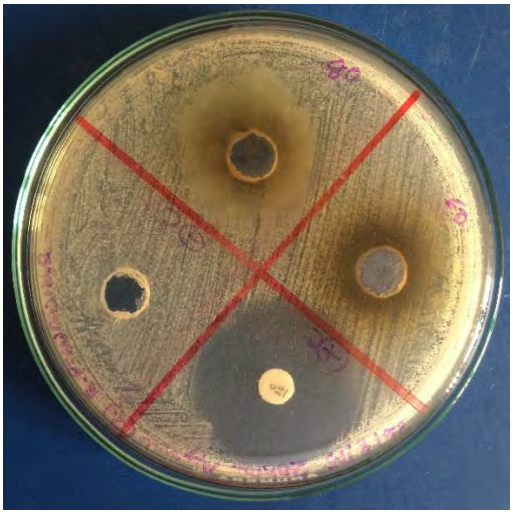
	Diameters (in mm)	
Organisms	**80µg/µl**	**60µg/µl**
Bacillus subtilis	15mm	8mm
Staphylococcus aureus	0mm	0mm
Streptococcus pneumoniae	25mm	18mm
Shigella felxnari	0mm	0mm



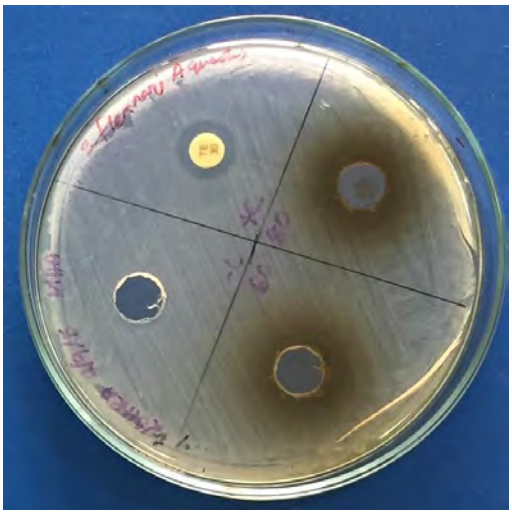
(a) Antibiotic activity test results of aqueous extract against *B.subtilis*



(b) Antibiotic activity test results of aqueous extract against *S,aureus*



(c) Antibiotic activity test results of aqueous extract against *S,pneumoniae*



(d) Antibiotic activity test results of aqueous extract against *S,flexnari*

Fig: 19 Antibacterial activity of aqueous extract

3.2 Antibacterial activity of the three different extracts with positive control

Table 5 showed the comparative results between the different extracts and the controls. For positive control against *B.subtilis* and *S. aureus* Kanamycin was used. For *S.pneumoniae* chloramphenicol was used and for *S. flexnari* tetracycline was used. According the results *S. aureus* and *S.flexnari* is completely resistant to all three types of extracts *Stevia rebaudiana.*

Table 5: Antibacterial activity of the three different extracts with positive control

	Diameters (in mm)								
	Methanolic extract			Ethanolic extract			Aqueous extract		
Organisms	**80µg/µl**	**60µg/µl**	**(+ve)C**	**80µg/µl**	**60µg/µl**	**(+ve)C**	**80µg/µl**	**60µg/µl**	**(+ve)C**
B.subtillis	17	15	20	10	9	20	15	8	20
S.aureus	0	0	22	0	0	20	0	0	22
S.pneumoniae	15	0	30	20	9	30	27	18	30
S. flexnari	0	0	10	0	0	10	0	0	10

3.3 Comparison of zone of inhibition and activity Index of the extracts collected from *Stevia rebaudiana*

The bar chart below shows the comparison between the clear zones between the different types of extracts and the antibiotics used fig 20. The zone of inhibition was measured in the X-axis and the extract types along with the organisms were kept in Y-axis. Different colored bars are used to indicate the concentrations of extracts and the antibiotics.

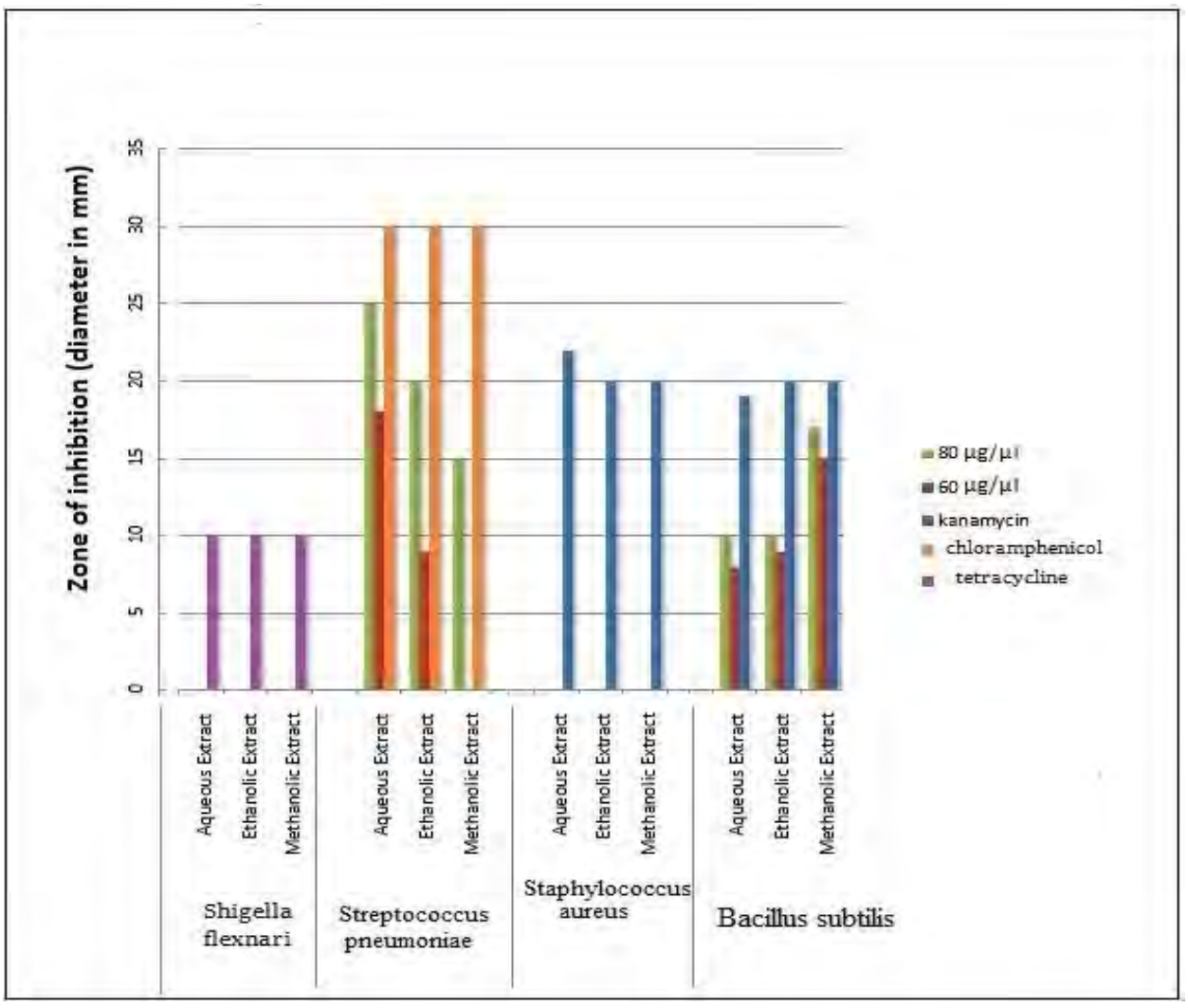


Fig 20: Comparison of zone of inhibition of extracts and antibiotics

Activity index of all the extracts were found out using the following formula:

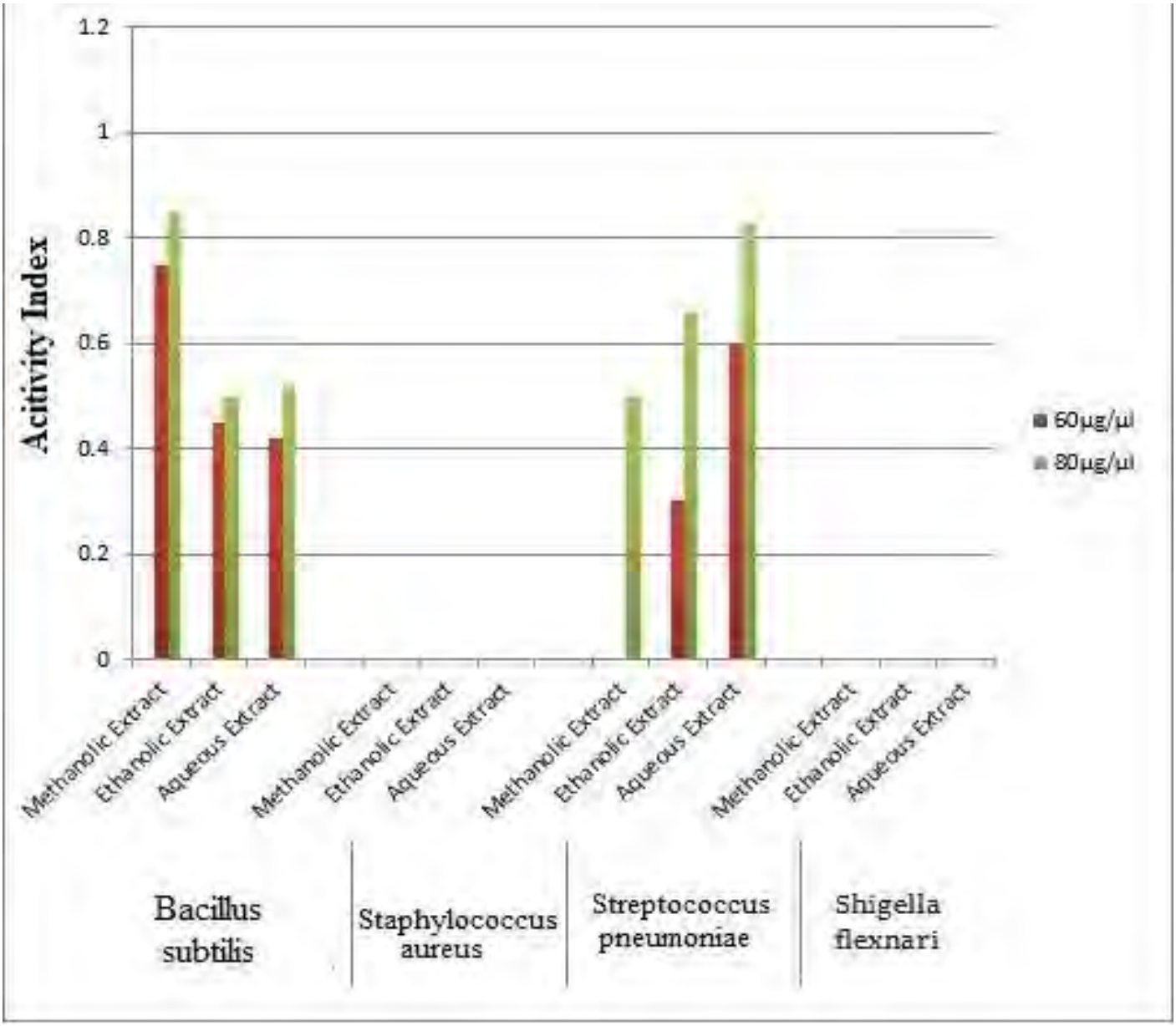
Activity index = zone of inhibition of extracts/ zone of inhibition of the antibiotics.

For *Staphylococcus aureus* and *Shigella flexnari* the activity index was zero. Fig 21 shows a graphical representation of the activity index of *Stevia rebaudiana.*

Table 6: Activity Index of the extracts collected from *Stevia rebaudiana*

	Methanol		Ethanol		Aqueous	
	80µg/µl	60µg/µl	80µg/µl	60µg/µl	80µg/µl	60µg/µl
B.subtillis	0.85	0.75	0.5	0.45	0.52	0.42
S.aureus	0	0	0	0	0	0
S.pneumoniae	0.5	0	0.66	0.3	0.83	
S. flexnari	0	0	0	0	0	0

I Fig: 21: Graphical representation of the activity index of *S.rebaudiana*



3.2 Discussion:

The expanding bacterial resistance to antibiotics have become a growing concern worldwide (Gradam, 2000). Intensive care physicians consider antibiotic resistant bacteria a significant or major problem in treat 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 (wendakoonn et al, 2011) Stevia is one such plant that is well known for its application in treatment of many disease like diabetes, candidacies, high blood pressure and weight loss. There has been few microbiological investigation of stevia plant extract against pathogenic species using an array of solvents. (Takaki, 1985). 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 three different types of extract (methanol, ethanol and water) and the biochemical assay to find the presence of various phytochemicals including alkaloids, steroids, phenols, flavonoids, tannins, saponins, and cardiac glycosides in *Stevia rebaudiana.*

The selected plant sample was collected and extraction was done using a Soxhlet apparatus. The highest amount of extract collected was from ethanol. The study also reported the antimicrobial activity of *S.rebaudiana* against common pathogenic organisms such as: *B. subtilis, S. pneumoniae, S.aureus, S.flexnari.*

The results obtained from the presence of phytoconstituents in all three extracts. Presence of alkaloids was confirmed through Hager‟s, Wagner‟s and Dragendraff‟s test. For the Hager‟s test ethanolic and methanolic extracts showed a positive result as the yellow precipitation was more visibly seen in these two. For the aqueous extract, a negative result was found in this test for alkaloids. The dragendraff‟s test for alkaloids however, gave a strong positive result for the aqueous extract and negative for the other two extracts. This result was similar to the study by Kujur et el, 2010. For steroidal compounds a strong positive result was seen for aqueous extract

as of the study by Kujur et el, 2010. In this experiment the tests for steroids couldn"t be done but there was a strong positive result for methanol and aqueous extracts. The presence of phenols, flavonoids and tannins were comparatively high except for the aqueous extract which was negative for phenols. According to the sudy by Singh et el, 2012 stevia contains a high number of penols, tannins and flavonoids. Traces of saponins were found in the methanolic extract compared to cardiac glycosides which were found to be in significantly higher amounts in *S. rebaudiana.*

The results obtained also showed the antimicrobial properties of *S. rebaudiana.* Agar diffusion method was followed and the zones of inhibition were measured in millimeters. Kanamycin, Tetracycline and Chloramphenicol were used as positive controls. The largest clear zone was seen in *S. pneumoniae.* For *B.subtilis* the clear zones were 17 mm at concentrations of 80µg/ µl for methanolic extract. A study by Kuntal et al, 2008 showed that methanolic extracts of stevia has a smaller clear zone. Another study by Tadhani et al, 2006 showed that methanolic extracts of stevia have smaller clear zones of inhibition agains B.subtilis. For S.pneumoniea a study by Sichani et al, 2012 showed bigger clear zones against Streptococcus species. In this experiment a clear zone of 15 mm was found for *S.peumoniae.* No zone of inhibition was found against S. aureus. A study by Tadhani et al, 2006 showed clear zones of more than 8 mms against *S. aureus*. Another study by Kuntal et al,2008 showed positive result for S. aureus. Another organism in this study that did not show any susceptibility to the extracts was *S.flexnari*. Aqueous extracts showed antibacterial activity against *S.aureus* and B.subtilis according to Tadhani et al, 2006. However in another study done by Jayaraman et al, 2008 these two organisms are resistant to aqueous extracts. For *B.subtilis* clear zones upto 10 mm was found in this experiment. For ethanolic extracts similar results were seen in the Kuntal et al, 2008. No clear zones were found with any of the in this experiment against *S. aureus* and *S. flexnari*. Overall, the results of antibacterial activity showed that *S. rebaudiana* is most effective against *S.pneumoniae* in this experiment (Fig 20).

Chapter 4

Conclusion

The extract of Stevia leaves has been subjected to various pharmacological, clinical and toxicological investigations and results revealed interesting therapeutic applications (Das et al, 2009). 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, 2009). The purpose of this study was to collect crude extracts and investigate the presence of phytoconstituents in *Stevia rebaudiana* that serves as an affect agent to treat many infectious diseases. The primary phytochemical analysis of ethanolic and methanolic extracts revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids, phenols and cardiac glycosides. Aqueous extracts had absence of phenols 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.subtilis and S.pneumoniae.*

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 *Stevia rebaudiana* for infectious diseases is promising against many bacteria and disease causing pathogens.

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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 EQUIPMENT™, United Kingdom