ANALYSIS OF BIOACTIVE PHYTOCHEMICALS AND STUDY OF ANTIMICROBIALACTIVITY FROM METHANOLIC EXTRACT OF ROOTS OF *STREPTOPUS STREPTOPOIDES*

A MINI RESEARCH REPORT

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CERTIFICATE OF APPROVAL

This mini research report entitled **"Analysis of bioactive phytochemicals and study of antimicrobial activity from methanolic extract of roots of *Streptopus streptopoides*"** by Anup Subedee, Department of Chemistry, Amrit Campus (ASCOL), IoST, T.U., is hereby submitted has been accepted by Institute of Science and Technology research committee.

RECOMMENDATION

The mini research report entitled "**Analysis of bioactive phytochemicals and study of antimicrobial activity from methanolic extract of roots of *Streptopus streptopoides*"** by Anup Subedee, Amrit Campus, T.U. is hereby completed under my mentorship. This is a novel work and has been performed genuinely and good ethics of research. This research is useful in the study of traditional medicinal plant and their application. This work is performed under the support of Mini Research Grant-2080 provided by Dean's office, IoST, T.U., Nepal. I recommend to proceed this document for further execution.

………………………………

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DECLARATION

This project work entitled "**Analysis of bioactive phytochemicals and study of antimicrobial activity from methanolic extract of roots of *Streptopus streptopoides*"** is hereby submitted to the Dean office, IoST, T.U., Nepal, as mini research project granted by IoST Dean office. This research/Project work is performed by me under the mentorship of Dr. Deval Prasad Bhattarai. I claim this work is original. This research work has not been submitted earlier elsewhere in full or part to any institute.

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It is my sincere pleasure to carry out this mini research work entitled, "Analysis of bioactive phytochemicals and study of antimicrobial activity from methanolic extract of roots of *Streptopus streptopoides"* under the mentorship of Assistant Prof. Dr. Deval Prasad Bhattarai. I express my earnest appreciation to him for his continuous backing in this research work as well as for motivation and support.

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ABSTRACT

Traditional practice of using medicinal plants have been the foundation of modern medicine. *Streptopus streptopoides* is a plant of traditional medicinal value. This report focuses in the phytochemical analysis of root parts of *Streptopus streptopoides* collected from Gorkha and study of biological activity of the extract. Methanolic extract of root was used for the analysis. Herein, three hundred grams of dried powder of the root of the plant was processed to extraction using methanol. The extract of *S. streptopoides* root solution was subjected to primary phytochemical screening. Methanol extract method revealed the presence of various phytochemicals. Total flavonoid content (TFC) in methanol has been reported to be 1.92±0.36 mg QE/g. It indicated the prescence of very low content of flavonoid in the plant sample. The total phenolic content (TPC) in methanol extract has been quantified to be 50.37 ± 0.44 mg GAE/g. Nauplii started dying at the concentration of 50 ppm. 50% (or more) of the tested nauplli died at the highest concentration of 100 ppm. Hence, LC50 of methanol extract is at 100 ppm (100 µg/mL).

Keywords: *Streptopus streptopoides,* phytochemical screening, alkaloids, Flavonoid, nauplii

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Unit-1

Introduction

1.1 Background of the study

Nepal is rich in flora and fauna, located in the lap of the stunning Himalayas of South East Asia, and has a diverse biodiversity and geographical distribution. In Nepal, there are approximately 1600 medicinal plant species out of 6000 reported species of plants (Tiwari et al., 2019). People from various areas of life use these plants in a number of ways, both directly and indirectly, in traditional medicine as well as pharmaceutical applications in modern medicine. Phytochemicals with biological activity have shown to be very useful as medicines and pharmacological agents (Sánchez-Ramos et al., 2021). As time passes, data show that many diseases and ailments are becoming more common as a result of poor dietary habits. Lately, diets have been heavy in fats and proteins. Plants offer a lot of therapeutic properties in the health care of human with wide range of applications. Taking a lot of natural plant foods provide lot of fiber, vitamins, natural protein, and other nutrients (Miliauskas et al., 2004).

Chemicals obtained from plants are called phytochemicals. These are bioactive and naturally synthesized by plants that act as anti-stress and anti-pathogenic agents. Phytochemicals are rich in primary metabolites and secondary metabolites. Phytochemicals are divided into several categories based on their metabolic origin. Metabolites are the compounds which are produced during the process of metabolism. Primary metabolites are chemical compounds like amino acids, nucleosides, nucleotides, carbohydrates, lipids, alcohol and organic acids. These are used by plants for normal growth and development as well as reproduction of plants. These compounds basically work for energy production, building blocks and cellular functions. On the other hand, secondary metabolites are involved in important ecological functions like defense mechanism, interaction with organism and protection against environmental stress rather than involving in life sustaining process. Plants frequently use these substances to protect themselves from bacteria, insects, and herbivores. Basically, chemical compounds of different groups like alkaloids, terpenoids, phenolics, glycosides and antibiotics are regarded as secondary

metabolites. To encourage the practice of herbal uses and to explore their efficacy, it is necessary to examine medicinal plants with a folklore reputation in more depth (Chew et al., 2011).

Phytochemical screening is the process of identifying the presence or absence of phytochemicals or metabolites of different parts of plant via their chemical extracts. In addition, it provides the basic know-how about the therapeutic significance of plant extract (Pant et al., 2017). The qualitative and quantitative estimation of chemical constitutes of such traditionally important medicinal plants shows the presence of metabolites, reducing sugars, free radicals, alkaloids, flavonoids, saponins, coumarins, anthraquinones, tannins, glycosides and many others phenolic compounds (Altemimi et al., 2017). Such compounds are featured by pain-relieving, antimicrobial, anti-inflammatory, and anti-diabetic effects, among many others. Amidst the talk of different phytochemicals, the safety and quality concern of the medical plants and herbal products are in forefront in the arena of natural product chemistry on a global scale. From immemorial times, various uses and application of herbal medicine has also be mentioned different Hindu ancient books including the Vedas (Kaggwa et al., 2022).

Understanding the chemical components of plants provide important information that helps us determine the true worth of traditional medicinal therapies (Fabricant & Farnsworth, 2001). Phytochemical research that is grounded in ethnopharmacological data is typically regarded as a successful strategy in the formulation of medicinal plant-based novel medication. (Hassan and Zainab Kazmi, 2015)

Microorganisms degrade all types of food, causing waste and loss even in industrialized countries (Tropea, et al., 2022). Food waste accounts for around 40% of worldwide food losses each year by the action of bacteria, yeast, molds, etc. (Alegbeleye et al., 2022). Microbes are also to blame for food-borne illnesses. Combining chemical preservatives with phytochemicals can lead to the synergistic effect (Bintsis, 2017).

Plant extracts as Antimicrobial

Traditionally, crude extracts from medicinal plants were employed to cure against various diseases (Gonelimali et al., 2018). Ethnoscience has directed in the applications of various phytochemicals like flavonoids, tannins, and terpenoids, either on the basis of scientific proof or on the basis of rule of thumb as these plants possess antibacterial, antifungal or antiviral properties in addition to antioxidant properties.

There are some plant species whose antimicrobial activities have been widely researched. For e.g., Cinnamon, Garlic, Basil, Ginger, Sage, Mustard, etc. acts against the growth and development Gram +ve and Gram –ve bacteria (Liu et al., 2017). It has been reported that Nepal is rich with 118 ecosystem and is in forefront in the availability of bryophytes, pteridophytes and flowering plant accounting 6 %, 3 % and 2 %, respectively of the world flora.

1.2 Introduction to plant species

Streptopus streptopoides is a grassy perennial plant belonging to Liliace family. Literally, *streptos* means 'twisted' and *pous* means 'foot', in reference to twisted peduncle. This plant is primarily found in cool and moist hilly region and subalpine zones. In Nepal, this plant has been reported in moist, shaded environment such as deciduous and coniferous forests. It is found in mid-hilly region of Nepal like Gorkha, Chitwan and so on. This plant bears a height of 0.3m (1 ft.) to 1.5m (4 ft.). This plant is hermaphrodite (it contains both male and female parts). It blossoms from July to August. Its flower is small, white and bell-shaped with black and round seeds. It grows in damp soil. Light (sandy) and medium (loamy) soils are both suitable. It can grow in either full shadow (deep woods) or semi-shade (light woods). The leaves are alternating and lanceolate. The stem of the plant is zigzagged or slightly twisted; therefore, this plant is also called "twisted stalk".

Traditional and medicinal uses

The root of the plant is considered as to have disease resistance power. The local name of this plant is Khole Harchul and scientific name is *Streptopos streptoides*. This plant can be distinguished as root, stem, flower and fruit. Its root is white in color which can also be used as medicines. Its root is used for healing the fractured bones. Besides these, different parts of this plant is being used for the treatment of fever, inflammation and skin disease. Traditionally, its root is dried and grinded to powder and is mixed in the milk or water and drink so that the cracked bones can be healed faster. Basically, indigenous people and herbalists have used various parts of this related species for medicinal applications. Besides this, it can be used as an ornamental plant in shaded garden due to its attractive foliage.

Classification

Kingdom: Plantae

Family: Liliaceae

Subfamily: Calochortoideae

Genus: *Streptopus*

Species: S*treptopoides (Ledeb.) Frye & Rigg*

Local name: Khole Harchul

Figure 1. ***Streptopus streptopoides*** **plant**

1.3 Extraction Process

Extraction is the process of separating an active agent or a waste item from a solid or liquid mixture using a liquid solvent. It is a technique for separating one or more components from a liquid or solid mixture by utilizing a liquid immiscible solvent. It is a technique for separating organic compounds from mixtures. This method dissolves one or more compounds selectively in a suitable solvent. Compounds can be separated using the solvent extraction technique according to how soluble or insoluble they are in two different immiscible liquids. This technique is used to remove or separate the desired materials or chemical pollutants. When the term "extraction" relates to plant or animal tissues, it refers to the process of separating medicinally active components from inactive or inert components by using solvents in the extraction process (Wacowich-Sgarbi & Department, 2018). Basically, extraction process involves the following techniques:

A. Liquid-liquid extraction
B. Soli-liquid extraction
C. Super-critical fluid extraction
D. Soxhlet extraction
E. Ultrasonic extraction
F. Microwave-assisted extraction
G. Maceration
H. Percolation

Solvent extraction process needs to select the appropriate solvent to mix into the substance through which a component has to be extracted, percolation, separation and recovery. The solvent extraction process is facilitated by the solubility of the substance, working temperature, pH and solvent to sample ratio.

1.4 Separation of Compounds

Mixtures can be separated using filtration, separating funnels, sublimation, simple distillation, and chromatographic methods. They are all physical tools.

1.5 Antimicrobial activity

Antimicrobial is a substance which kills, obstruct the growth of microorganism like bacterium, virus, fungus and protozoans. Antimicrobial medications are categorized according to the germs they are most effective against (Neu & Gootz, 1996). For example, antibiotics treat bacteria, but antifungals treat fungi. The bulk of medications used in medicine today are synthetic. Drug-resistant bacteria have emerged, posing a challenge to clinical practice in the management of microbial illnesses (Ventola, 2015). Search for novel antimicrobial is getting more preference in the field of biomedical science due to increased challenges of multiple drug resistance. Plants are rich in bioactive compounds which can be a potential source for the preparation of new antimicrobial agent. Due to these worries, current research has been focused on screening naturally occurring compounds found in medicinally significant plants in an effort to create new and potent medications to address (Ojah, 2020).

1.6 Objectives

General objective of this work is was Phytochemical screening & examination of antimicrobial and cytotoxicity potency of *Streptopus streptopoides* root extract.

Specific objectives of this research were:

a. Extraction of methyl alcohol fraction of root
b. Phytochemical screening of the methanol extract.
c. Quantification of total phenolic content and total flavonoid content.
d. Antimicrobial study & cytotoxicity study of the prepared extract.

Unit-2

LITERATURE REVIEW

With the increased multi-resistance effect of bacteria and viruses, the uses of traditional medicinal plants are peered by biomaterial and natural products scientists for the formulation of medicinal plant-based medicine. Medicinal plants have their own pre-historic history and have been used by people of different region. Before, the onset of modern medicine, there were different healing system globally. Especially, in the south Asian region, Ayurveda was in forefront in the traditional system. The uses of medicinal plant in Ayurveda have very long and effective history. Charak and Shrushruta Sanhita are the two prominent books on Ayurveda which describes the uses of different medicinal plants for healing system. By this time also, so many literatures are available describing the medicinal uses of plants and their phytochemical study. Some of the notable study in the field of medicinal plants are briefly mentioned below.

Vaou et al. (2021) reported the study of *Antidesma madascarience Lam* and *Erythroxylum macrocarpum* as medicinal plants. In this work, aqueous and methanolic extract of plant leaves and twigs were studied for in vitro test against five Gram +ve and Gram –ve bacteria. Except for the methanol extract, neither plant's aqueous extracts demonstrated antifungal efficacy against *A. Niger*. Mahomoodally et al.,repored Study of plant phytochemicals reveals the presence of antimicrobial phytoconstituents such as tannins, phenols, alkaloids and flavonoids

Alqahtani et al. carried out a test for antimicrobial activities and phytochemical screening of seeds of Lepidium sativum which shows the presence of alkaloids, flavonoid, cholesterol, steroids, carbohydrates, phenols, and saponins. Herein, antimicrobial activity of crude extract against four bacteria was investigated.

Chew et al. (2011) studied the phytochemical screening and an vitro antimicrobial activities of *C. sappan* leaves collected form the forest area of Tamil Nadu In this work, extraction of crude contract was accomplished using Soxhlet apparatus. For this extraction, solvents such as petroleum ether, dichloromethane (DCM), ethyl acetate and methyl alcohol were used.

Based on this approach, Kaur et al. (2016b), performed preliminary phytochemical screening of different extracts applying a variety of color and precipitative chemical reagents. The extract's antimicrobial properties was tested against a fungal strain

Sharma et al. (2016) studied Phytochemical screening and antimicrobial activity of *Samanea saman* was studied. All of the organisms tested were suppressed by the plant extract. During phytochemical screening, tannins, flavonoids, saponins, steroids, and terpenoids were found. The research validates the use of herbs in traditional medicine

Ndhlala et al., reported Natural antioxidants, such as bioactive flavonoids, are becoming increasingly essential as a result of their indigenous origin and great efficacy in trapping/scavenging free radicals.

Trisha et al. (2022), reported one example of tea (black and green), which is widely drunk all over the world and contains a high concentration of polyphenolic chemicals Initial phytochemicals test ensured the availability of tannins, alkaloids, flavonoids, etc. in the extract of *Moringa oleifera*.

Aryal et al. (2019), studies, phenolic content is directly associated to antioxidative actions of fruits and vegetables, Petrovska, reported. The plant kingdom is an abundance of potential pharmaceuticals. Greenwell et al. reported Plant-based drugs are readily accessible, less priced, safe, and efficient, with little side effects. These plants have been claimed to provide health benefits, particularly as anticancer medications, and the likely mechanism of action of these extracts has been elucidated. Research has been done on the phytochemical screening of therapeutic plants and the impact of phytoconstituents on seed germination

Sánchez-Ramos et al., Phytochemical screening was critical in identifying the numerous phytoconstituents found in plant extracts. The aqueous extract's phytochemicals reduced development marginally.

Yusof et al. (2020), This study helped in determining the cytotoxic impact of phytoconstituents found in plant extracts on live cells.

Oloya et al. (2022b), reported antimycobacterial activity, toxicity, and phytochemical screening of some medicinally important plant extract used in local level in Uganda to treat tuberculosis were investigated. Coumarins, alkaloids, tannins, saponins, flavonoids, steroids, resins, and phenolic chemicals were found to be present there based on the phytochemical analysis.

Obakiro et al. (2022), reported except for *Albizia coriaria*, which appears to be quite harmful, the therapeutic plants that have been discovered have minimal toxicity and antibacterial activity.

Kunwar et al. (2013), reported the herb is highly valuable as medicine and is utilized in different ways by all social strata, either directly or indirectly, in the manufacture of traditional medicine and contemporary medicine.

Vincent et al., (2010), reported Pharmaceuticals and pharmacological effects have greatly benefited from phytochemicals with biological activity. Statistics show that as time goes on, a variety of diseases and disorders are increasing dramatically because of poor dietary habits. Modern diets tend to be heavy in fats and proteins. High therapeutic value exists in plants.

Iqbal et al. (2015), Since time immemorial, local plants with significant medicinal values have been utilized extensively to cure variety of illnesses. Among its many other healing qualities, they have analgesic, antibacterial, anti-inflammatory, and antidiabetic characteristics. Because of their native origin and powerful capacity to trap or scavenge free radicals, and bioactive flavonoids are of enormous significance and are now the focus of investigation. Tea (black and green), which is a popular beverage consumed worldwide and contains a significant amount of polyphenolic chemicals, is one such example.

Significant awareness regarding the medicinal plants has emerged in recent years. There is an abundance of possible drugs found in the kingdom of plants. Plant-based medications, also known as herbal medicines, have been used for centuries across various cultures for their therapeutic benefits. Such medications offer multitude of advantages, making them an attractive option for many individuals seeking alternative or complementary treatments. Importance of such medication is due to widespread availability, traditional knowledge, locally available materials, lower production cost, proven efficiency and holistic benefits.

Luitel et al. (2014), reported plants have been touted for their health advantages, particularly as anticancer medications, and their putative mechanisms of action.

Phytochemical screening, antimycobacterial activity and acute toxicity of crude extracts of some medicinal plants were studied by Oloya et al. (2022a) to treat against TB (Tuberculosis).

No extensive study has been found in the literature about *Streptopus streptopoides* of Nepal origin.

Unit-3

MATERIALS AND METHODS

3.1 Solvents

Methanol was used as the solvent

3.2 Chemical Required

Methanol (Fischer Scientific), Double distilled water (DDW), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, concentrated sulphuric acid (Merk, sp. Gr. 1.84 g/cc), Potassium hydroxide (Merk, Mol. Wt. 56.10), concentrated hydrochloric acid, aluminum chloride, phenol was obtained from the local supplier from Kathmandu, Nepal. All the reagent were used as-received without further purification. Mayer's reagent, Dragendroff's reagent, Fehling's (A and B) solution, etc. were prepared in laboratory under standard protocol.

3.3 Instrument and Equipment

During the study, the following instruments were used:

i. Digital balance
ii. Grinder
iii. Beakers
iv. Measuring cylinder
v. Sonicator
vi. FT-IR (PerkinElmerSpectrum IR,Version 10.6.2)
vii. UV lamp (UV 2510TS)
viii. Spectrophotometer (Labtronics LT-2802)
ix. Micropipettes
x. Conical flasks
xi. Test tubes
xii. Vial tubes
xiii. Water bath
xiv. Rota evaporator (IKA, RV 10 D S96)
xv. Precoated TLC

3.4 Plant material

Root of Khole harchul (*Streptopus streptopoides*) was obtained from the Gorkha.

3.5 Collection of Plant Part

The species was collected from Gorkha district in the Gandaki province of northern–central Nepal. *Streptopus streptopoides* root were collected from the Gorkha District with the GPS coordinates of 28° 28' 35.0220'' North and 84° 41' 23.1036''East. The sample were collected in the month of Kartik.

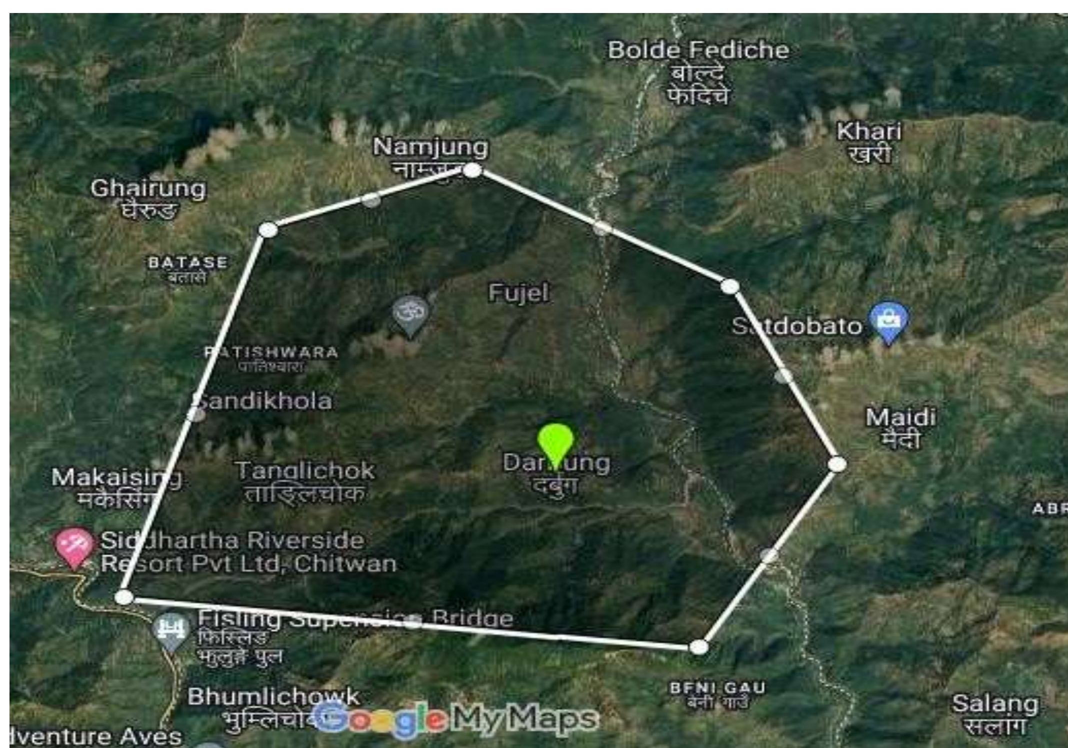

Figure 3.1: Map of sample collection area

3.6 Drying and Grinding

The collected samples of plant root were cleaned using tap water and then by DDW in laboratory. Then the samples were air dried for 10 days. The dried root was grinded to make fine powder using Herbal disintegrator machine (FW 177).

3.7 Preparation of methanol extract

The dried powder of the root was blended with methanol. The solution was then left for 14 days for percolation. Later on, the solution was filtered using Whatmann 42

filter paper. In this way, methanol extract was prepared and was employed for various tests.

3.8 Phytochemical tests

Table 1: Various tests for phytochemical constituents present in root extract of *Streptopus streptopoides*

S.N.	Analysis for		Procedure
1.	**Alkaloid**	**Mayer's Test**	Two drops of Mayer's reagent was put added into two milliliters of the extract solution (test solution).
		Dragendorff's test	3 drops of Dragondorff reagent was added into 2 mL of extract solution.
		Wagner Test	Two drops of Wagner reagents were put into the two milliliter of extract solution.
2.	**Flavonoid**	**Shinodha Test**	Magnesium turning and conc. Hydrochloric acid was put into three milliliter of root extract solution.
		Shibata's Test	Zinc dusts were added into the three milliliter of extract followed by shaking well. Then 1 mL of HCl solution was added.
3.	**Carbohydrate**	**Molish's Test**	Two milliliters of alcoholic naphthol solution were put into two milliliter of root extract. After vigorous stirring, few drops of conc. Sulphuric acids were added.
		Benedict's Test	One milliliter of Benedicts reagent was added into one milliliter of extract solution. Then the content was heated to boil for 2 minutes in water bath.
4.	**Fats and Oil**	**Spot Test**	About 5 milligram of root extract was pressed between the two folds of filter paper.

		Saponification Test	Few drops of alcoholic KOH solution (0.5 N) were added into about two milliliter of extract solution. One drop of phenolphthalein solution was put and the content was boiled for two hours in water bath.
5.	**Glycoside**	**Borntrager's Test**	Three milliliters of trichloromethane were put into two milliliter of root extract and vigorously shaken for three minutes. The trichloromethane layer was removed by adding 10 % of ammonia solution.
6.	**Tannin and Polyphenol**	**Ferric chloride Test**	Three drops of neutral iron (III) chloride solution were added into three milliliter o root extract.
		Lead acetate Test	Three milliliter of 10 % $Pb(CH_3COO)_2$ solution was put into one milliliter of root extract solution.
7.	**Protein**	**Biuret Test**	1 drop of 2 % copper sulphate, 1 mL of 95 % ethanol and KOH pellets were added into two milliliter of extract.
8.	**Saponin**	**Foam Test**	Five milliliter of root extract was mixed with ten milliliters of distilled water followed by vigorous shaking.
		Froath Test	Half-gram of root extract was put into two milliliter of water and shaken well.
9.	**Quinone**	**Quinone Test**	One milliliter of root extract was added into one milliliter of concentrated sulphuric acid.
10.	**Terpenoid**	**Terpenoid Test**	Three milliliter of root extract was mixed with two milliliter of plant extract and concentrated sulphuric acid was added.

3.9 Total Phenolic Content Study

Phenolic compounds constitute predominant class of chemicals present in plant and are extensively found throughout the plant realm (Saxena et al., 2013). These compounds belong to the class of chemicals containing –OH group bonded to aromatic chain, directly. Functioning as secondary metabolites, they play a crucial role as defensive agents. Phenolics possess various properties advantageous to human health, with their antioxidant capabilities being particularly noteworthy in safeguarding against diseases associated with free radical activity (Koche et al., 2016).

Folin-Ciocalteu colorometric analysis is based on the redox reaction and is employed for the estimation of total phenolic content present in the plant extract using methodology provided by Du et al., 2018 (Du et al., 2018). In this test, gallic acid was taken as reference (standard).

Preparation of standard gallic acid solution

Gallic acid solution of 1000 µg/mL concentration was prepared by dissolving 50 mg of gallic acid in 50 milliliters of 30 % DMSO. From the stock solution, other solutions of required concentration 250, 200, 100, 50, 25 µg mL^{-1} were prepared by the process of serial dilution.

Construction of Calibration Curve

In a beaker, 1 mL of each concentration of prepared gallic acid solution was taken. One milliliter of dimethyl sulphoxide (DMSO) was used as a blank. In each beaker, 1 mL Folin ciocalteu reagent (FCR) was added and left to stand for five minutes. Subsequently, 10 milliliters of 7 % sodium carbonate solution were put into each mixture content followed by shaking well. Then 13 mL of DW was poured into the beaker followed by incubation at 23 °C temperature for 90 minutes. After incubation is over, absorbance of each concentration was measured by spectrophotometer at a wavelength of 750 nm. Data were taken in triplicate. Then calibration curve was plotted at varied concentration taking the average absorbance value.

Preparation of sample solution

An extract solution of 5,000 µg/mL was made ready by dissolving calculated amount of sample in a calculated volume of 100 % DMSO. The concentration of extract was made 1000 µg/mL by dissolving 1 mL stock solution in 4 mL of 30 % DMSO and their triplicate absorbance was measured by adopting the same procedure as mentioned for gallic acid solution.

Calculation of Total Phenolic Content (TPC)

TPC of the root extract is quantified according to the following relation

Total phenolic content (C) = $\frac{c \times V}{m}$ . . . (1)

Here,

C is TPC (mg g^{-1}) in gallic acid equivalent, c is gallic acid concentration which is obtained from calibration curve, V is volume of extract solution and m is mass (in mg) of root extract.

The absorbance was obtained as triplicate data and the mean absorbance for each concentration was used for the calculation of the linear correlation coefficient (R^2) value.

The equation is $y = mx + c$ . . . (2)

Here, Y is absorbance of extract solution, m is slope in the graph, x is the concentration of root extract and c is intercept of the plot.

The concentration of extract was evaluated from graph of absorbance versus concentration of extract. Based on these data, total phenolic content was evaluated.

3.10 Total Flavonoid Content

Flavonoids are significant group of natural compounds basically categorized as plant secondary metabolites with polyphenolic structure. These compounds are widely prevalent in fruits, vegetables, along with green plants. Possessing diverse beneficial biochemical and antioxidant effects, flavonoids are implicated in combating various diseases like cancer, Alzheimer's disease (AD), and atherosclerosis (Karak, 2019). Their wide range of health-promoting benefits makes them essential for use in pharmaceuticals, cosmetics, nutraceuticals, and medical products. Flavonoids are vital due to some promising properties like anti-oxidative, anti-inflammatory, antimutagenic and anti-carcinogenic properties in addition to their ability to change the crucial cellular enzyme function (Panche et al., 2016). TPC of the root extract

was evaluated using $AlCl_3$ colorimetric test using quercetin as standard (Kalita et al., 2013).

Preparation of standard Quercetin solution

Quercetin solution of 1000 μg mL^{-1} concentration was prepared as a standard solution by dissolving ten milligram of quercetin in 10 mL of 100 % dimethylsulphoxide (DMSO) solution. Then quercetin solution of different concentration (100 μg/mL to 3.125 μg/mL) were made by diluting the stock solution by serial dilution method.

Construction of Calibration Curve

Each of the 400 μL concentration of prepared quercetin solution was taken separately in a test tube. 400 μL DMSO was taken as blank. In each test tube, 2.2 mL DW, 1.2 mL ethanol, 100 μL 0.3 M $AlCl_3$ and 100 μL of 10 % sodium potassium tartarate solutions were added. Whole mixture content was then mixed thoroughly and incubated for half an hour in the dark at 25 °C. After the incubation is over, absorbance of each solution and blank solution was observed by UV spectrophotometer at 415 nanometer wavelengths. To get triplicate data, the same procedure was carried out twice more. The average values taken for calculation.

Preparation of sample solution

An extract solution of 5,000 μg/mL was prepared by dissolving calculated amount of extract in calculated volume of 100 % DMSO. The concentration of extract was made 1000 μg/mL by dissolving 1 mL stock solution in 4 mL of 30% DMSO. Their absorbance in triplicate was taken adopting the same technique performed for standard quercetin.

Calculation for Total Flavonoid Content (TFC)

Total flavonoid content was quantified using the following relation.

$$\text{Total flavonoid content (C)} = \frac{c \times V}{m} \qquad \ldots (3)$$

Here, C is TFC (in mg/g) in quercetin equivalent, c is quercetin concentration which is obtained from calibration curve. Its unit is mg/mL. V is volume of root extract solution which is expressed in mL and m is mass of plant extract in milligram.

Absorbance of the study solution (extract solution or test solution) was measured in triplicate, goodness of fit (R^2) was evaluated from mean absorbance value of concentration. A mathematical linear equation of Y = mx+ c is mentioned below.

$$Y = mx + c \quad . . . (4)$$

Here, Y is absorbance of extract, m is slope of plot, x is concentration of extract solution and c is intercept of straight line.

The concentration of extract was quantified based on the above equation. TFC can be calculated using the relation.

3.11 Antibacterial Activity

The impact of crude plant extract at a certain dosage on the organism's species was explored in biological screening. In this study, antimicrobial study was studied taking Gram positive, Gram negative and antifungal strain. Antibacterial study of the plant extract was carried out via agar well diffusion technique which was used to test for bacterial growth inhibition and zone of inhibition (ZOI) was calculated (Dingle *et al.,* 1953).

Screening and Evaluation of Antibacterial Activity

Preparation of microbial culture media

Thirteen gram of Lysogeny Broth (LB, Sisco Research Laboratories Pvt. Ltd, India) was dissolved in one-liter DW to create the liquid broth (LB) medium. The content was autoclaved for 25 mins at 121 °C and 15 psi pressure. Then the content was cooled to 40–50 °C, the sterilized medium was transferred into 15 mL falcon tubes that had been previously sterilized (5 mL each). Each tube was co-cultured with bacterial seed using the prepared media, and the tubes were incubated for one day.

Muller-Hinton Agar media preparation

In order to create the Mueller-Hinton Agar (MHA) plates, thirty-nine gram of Muller-Hinton agar powder (Sisco research laboratories Pvt. Ltd, India) were dissolved in one-liter DW. This content was autoclaved for 25 minutes at 121 °C and 15 psi pressure. Then the content was cooled to 40–50 °C, the sterilized medium was transferred into 25 mL sterile petri dishes. Until it was utilized, the prepared media was kept in the refrigerator. In order to conduct antimicrobial testing, the media plates

were labelled well, and one hundred fifty microliter of bacterial suspension was applied onto the surface of plates using sterile cotton swab. Each well of 10 mm diameter and 3 mm depth was created onto the surface of the media plates. Each well was loaded with 100 microliter sample aliquot of 100 mg/mL concentration dissolved in DMSO and standard Kanamycin solution (5 mg L^{-1}, 10 µL loaded) in the corresponding wells. These media plates were incubated at 37 °C for a day. Then the antimicrobial study was carried out by quantifying the zone of inhibition.

3.12 Cytotoxicity test

The Brine shrimp lethality assay is incredibly easy to use, reasonably priced, and only needs a little amount of test material. It is crucial for the initial plant extract cytotoxicity test, which is predicated on the extract's capacity to destroy laboratory-cultured larvae (nauplii). For a full day, the nauplii are exposed to varying concentrations (from lowest to maximum) of plant extract. To determine the extract's effectiveness, the number of living nauplii is counted and expressed as a percentage of mortality.

Brine shrimp lethality assay (cytotoxicity)

Brine shrimp growth condition: Artificial Sea water was made using 30 g/L salt in water and 100 mg brine shrimp eggs were inoculated in the prepared artificial sea water. The shrimp eggs were hatched at 22-29 °C under continuous air pump supply. The nauplii hatched after 72 hours approximately were used for the cytotoxicity assay. 20 nauplii were used for each sample concentration in 96 well plates. The results tabulated here are obtained after 24 hours of assay.

Sample preparation and serial dilution:

10 mg of sample was carefully weighted into clean e-tube and was dissolved in one milliliter of dimethylsulphoxide (DMSO). Then the content was diluted with 9 mL of DW to prepare the solution of 1000 ppm concentration. Serial dilution was made for all the lower concentration needed for analysis. Lower concentrations of 10, 50, 100, 500, and 800 ppm were made from 1000 ppm, respectively. 96 well plate was used for cytotoxicity analysis with the volume capacity of 0.5 mL of each well. Each well was inoculated with 20 nauplii, after which each diluted sample (0.4 mL) was loaded in each well. Each sample was analyzed in triplicate for result accuracy. Experiment was

continued for complete 24 hours with close observation for 8 hours. All the nauplii died after 24 hours of analysis. A sample is considered cytotoxic if 50 % of the analyzed nauplii dies (mortality 50% or above).

Unit-4

Results and Discussion

4.1 Yield of Extract

From 600 g fresh plant root, 300 g of dried *Streptopus streptopoides* powder was produced which was percolated in 500 mL of methanol to prepare methanolic extract. Then the extract was used for further analysis.

4.2 Phytochemical Screening Analysis

The phytochemical screening of *Streptopus streptopoides* root extract in Methanolic, Hexane, and Aqueous extracts was done qualitatively. Proteins, quinones, saponins, glycosides, and mucilage as well as alkaloids, carbohydrates, terpenoids, flavonoids, phenolic compounds, and tannin were tested. Analysis of phytochemicals reveals the availability of Alkaloids, Carbohydrates, Phenolic compound and Tannin, Quinones, and Saponins in Aqueous extract. As obtained results of phytochemical assay of the methanolic extract exhibited the availability of carbohydrates, alkaloids, flavonoids and phenolic compounds.

Fig 4.1: Digital photographs showing a Phytochemical test of root extract of *Streptopus streptopoides*

The presence of different phytochemicals from methanol extract are mentioned in the table 4.1.

Table 4.1: Results of phytochemical test of root extract of *Streptopus streptoids*

S.N.	Phytochemicals	Methanol fraction/extract
a.	Alkaloids	+++
b	Carbohydrates	++
c.	Flavonoids	+
d.	Phenol and Tannins	+
e.	Proteins	+
f.	Quinones	-
g.	Terpenoids	+
h.	Saponins	-
i.	Fats and Oils	-
j.	Glycosides	-

Absence (-), Presence (+), and more than one plus indicates strongly presence

Figure 4.2. Digital images for test of (a) alkaloid, (b) flavonoids

4.3 UV-Visible Absorption Spectroscopic studies

Spectroscopy is commonly used to determine the presence of unsaturation in organic compounds. It can provide valuable information about the electronic structure of a compound, including the presence of π-bonds and the conjugation of double bonds (Pratiwi & Dani Nandiyanto, 2022).

UV-Visible Absorption Spectrum of methanol extract of *Streptopus streptopoides* is shown in figure 4.2. In the given figure, a prominent peak was observed at 337 nm. Furthermore, a shoulder peak was observed at 406 nm. Meanwhile, a hump was observed for methanolic extract in the range of 290-470 nm.

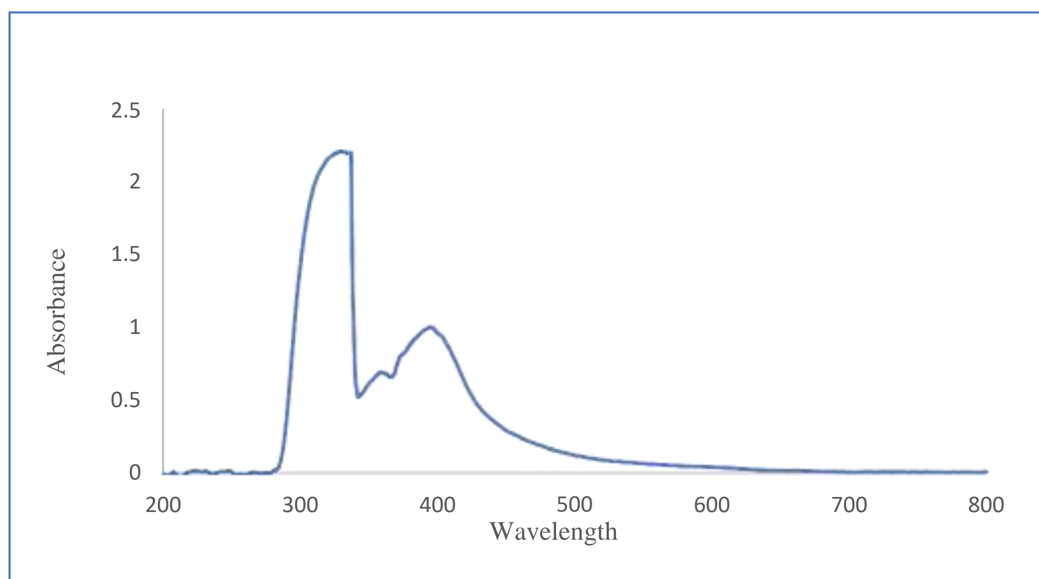

Figure 4.2: UV-visible absorption spectroscopic studies

4.4 Fourier Transform Infrared Spectroscopic Study

The components contained in the extract were identified and their distinctive peaks and functional groups were found using the FT-IR spectra based on the infrared radiation region's peak value. FTIR spectroscopy gives information about bonding condition, aliphatic and aromatic group and especially functional group of compound present in organic compounds. By analyzing the stretching frequency in the FTIR spectrum, the bond between carbon and hetero atoms can be identified. Overall, FTIR

spectroscopy is a powerful tool for characterizing organic molecules and gaining insights into their composition and structure (Dani Nandiyanto *et al.,* 2019)

The broad peak from 3100 cm^{-1} to 3400 cm^{-1} refers to the methanolic extract's hydrogen-bonded phenolic group. The carbon and hydrogen (C-H) bond is shown by the peak at 2940 cm^{-1}. Peaks near 2835 cm^{-1} correspond to a particular kind of carboxylic acid. Nitrosamine peaks at 1447 cm^{-1}. Absorbance band of 1112 cm^{-1} represents the primary alcohol's C-O stretching. The peak near 1021 cm^{-1} corresponds to an alkyl amine. All in all, the FTIR spectra reveal the availability of various phytochemicals in methanol extract of *Streptopus streptoides*.

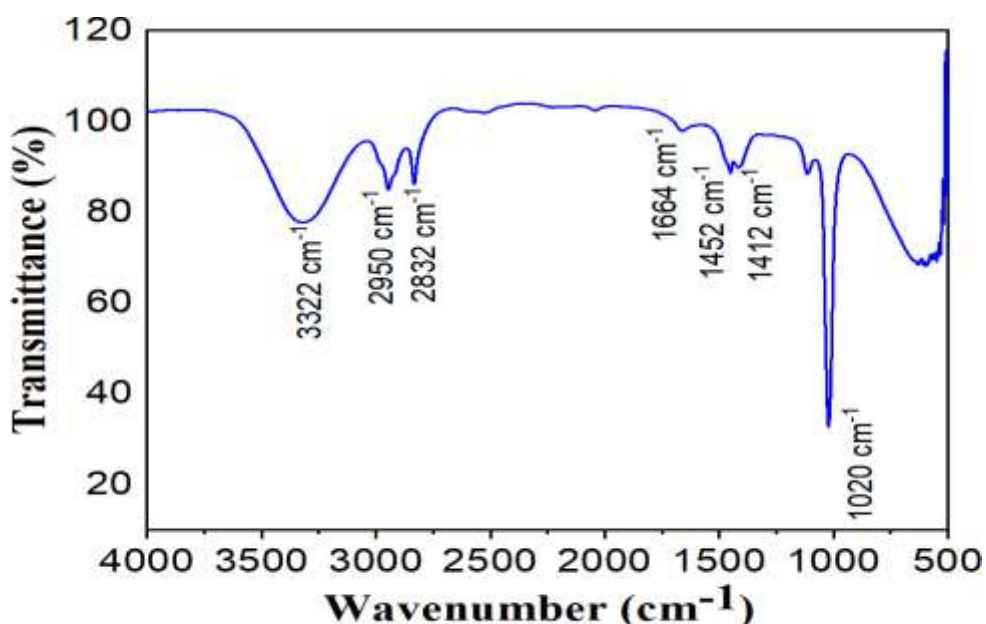

Figure 4.3: FT-IR spectrum of methanol extract

4.5 Antimicrobial Activities of Methanolic solution of root extract

One of the most important requisites of phytochemicals is their antimicrobial properties which includes antibacterial test, antifungal test. The antimicrobial activities of methanolic extract of the root part of *Streptopus streptoides* was studied taking Gram positive, Gram negative and fungal strain. The observed results are mentioned in table 4.2.

Table 4.2 Results of antimicrobial test of methanolic extract of *Streptopus streptoides*

Microbes	ATCC name	Type	Zone of inhibition	
			+ve control Kanamycin (c+) (cm)	Sample
E.coli	ATCC8739	Gram -ve	2.6	1.2
Staphylococcus aureus	ATCC6538P	Gram +ve	2.6	1.2
Candida albicans	ATCC2091	Fungi	2.4	1.5
Enterobacter aerogenes	ATCC 29007	Gram -ve	2.3	2
Klebseilla pneumoniae	ATCC 700603	Gram -ve	2	0
Salmonella Typhi	ATCC 19430	Gram -ve	2.5	1.2
Shigelle dysenteriae	ATCC 13313	Gram -ve	1.8	0
Bcillus subtilis	ATCC 6051	Gram +ve	2.6	1.2
Staphylococcus epidermidis	ATCC 1228	Gram +ve	2.4	2.2

In this test, the zone of inhibition (ZOI) has been tabulated in cm. Kanamycin antibiotic in the concentration of 5 mg/mL has been used as +ve control (c+). Dimethyl sulphoxide (DMSO) has used as –ve control. Negative control did not show activity.

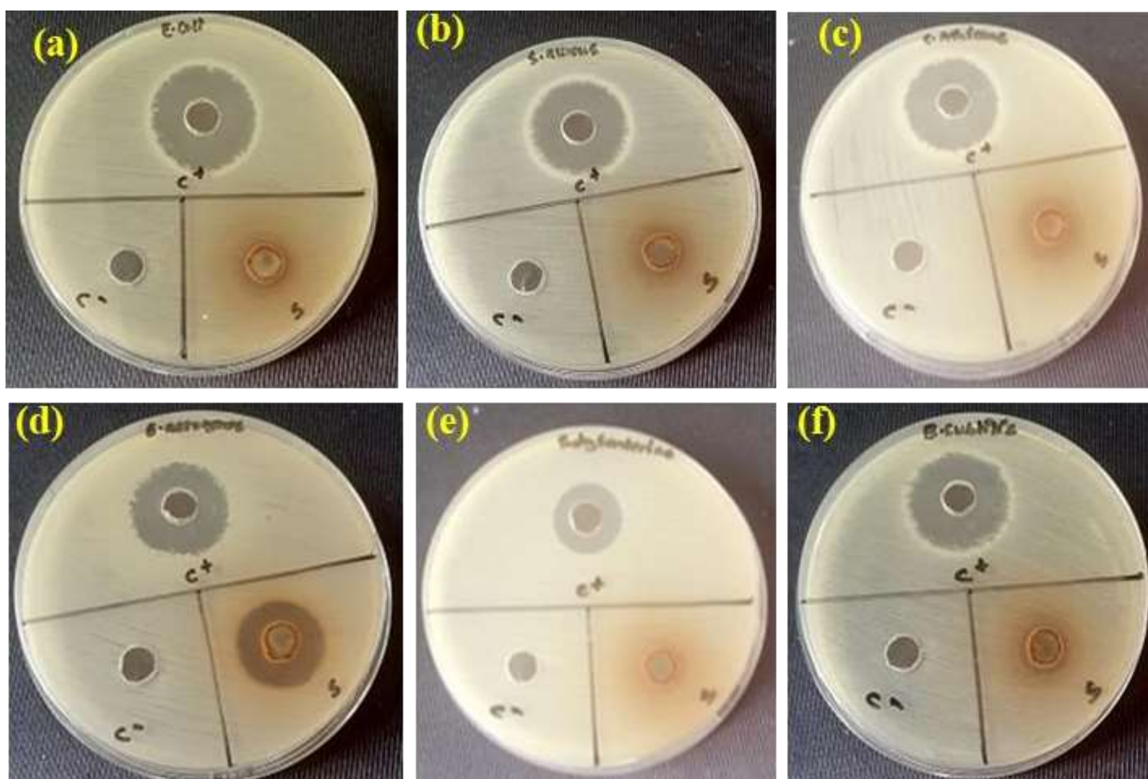

Figure 4.4: ZOI shown by different bacterial strain (a) *Escherechia coli*, (b) *Staphylococcus aureus*, (c) *Candida albicans*, (d) *Enterobacter aerogenes*, (e) *Shigella. dysenteriae* and (f) *Bacillus subtilis*

4.6 Cytotoxicity study of methanol extract

Cytotoxicity of the methanolic extract of *Streptopus streptoides* was studied. Result showed that Nauplii started dying at a concentration of 50 ppm. 50 % or more of the tested Nauplli dies at the highest concentration of 100 ppm. Therefore, LC50 of methanolic extract is at 100 ppm (100 μg/mL). The result is shown in table 4.3.

Table 4.3: Antimicrobial test of methanolic extract of *Streptopus streptoides*

Sample	Concentration (ppm)	Number of alive nauplii after 24 hours in each replication			Mortality rate %
		T1	T2	T3	
Methanol extract	0	20	20	20	0
	10	20	20	20	0
	50	15	17	14	23
	100	8	7	9	60
	500	3	2	2	88
	800	0	0	0	100
	1000	0	0	0	100

4.7 Total Phenolic Content (TPC) estimation

Construction of calibration curve

Total phenolic content in the root extract was determined by Folin Ciocalteu Reagent (FCR) colorimetric method. This reaction is based on the redox reaction. In this test, gallic acid was used as a standard to set the calibration curve. The absorbance versus different concentration (250 μg/mL to 25 μg/mL) of Gallic acid was plotted to get a calibration curve. The absorbance of the Gallic acid was measured in UV-vis spectrophotometer at 750 nm wavelength.

To plot the graph, absorbance was put in ordinate and concentration of gallic acid was put in abscissa. The calibration curve is shown in Figure 4.5.

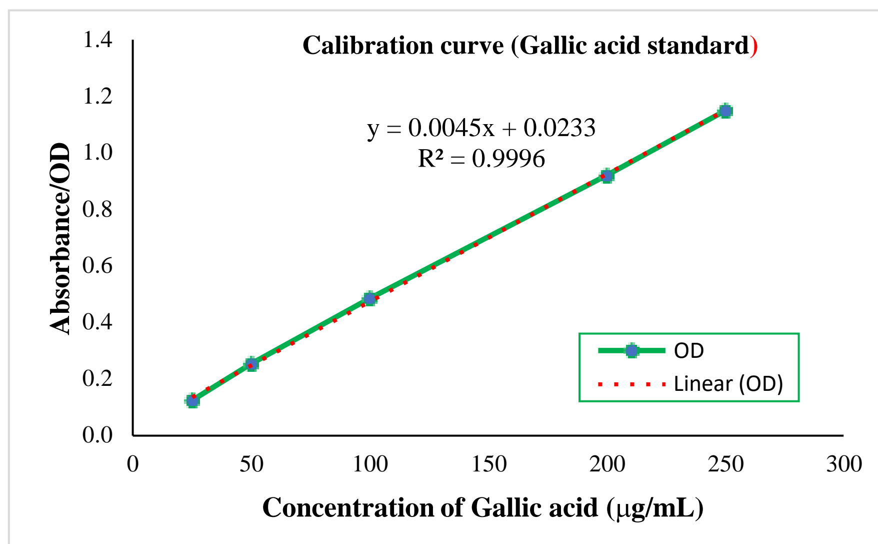

Figure 4.5: Calibration curve of Gallic acid

Calculation of Total Phenolic Content

The total phenolic content of the root extract (methanolic extract) was calculated using calibration curve. Absorbance was taken in triplicate. The data are tabulated in table 4.4.

Table 4.4: Total phenolic content in methanol extract

Extracts	OD of samples			OD of Control	TPC (mg GAE/g)			TPC Mean (mg GAE/g)	Std Dev
	I	II	III		I	II	III		
Methanol	0.324	0.323	0.321	0.072	50.82	49.93	50.37	50.37	0.44

Calculation shows the total phenolic content in methanol was found to be 50.37±0.44 mg GAE/g.

4.8 Total Flavonoid Content (TFC) estimation

Construction of calibration curve

The flavonoid content in total of the methanolic root extract was evaluated by $AlCl_3$ colorimetric test. Quercetin was used as standard to plot the calibration curve. The absorbance of the solution was carried out at 415 nm wavelength in UV-Visible spectrophotometer. The absorbance of different concentration from 100 to 3.125 µg mL^{-1} was measured. In this work, the calibration curve was plotted by putting

absorbance in ordinate and concentration (in µg mL^{-1}) of quercetin in abscissa. The pertinent calibration curve is shown in the figure 4.6.

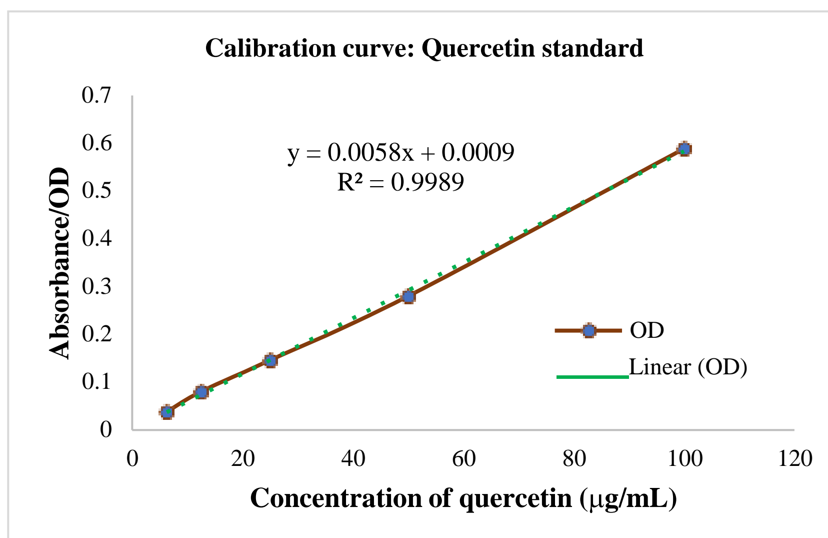

Figure 4.6: Calibration curve for standard quercetin

Calculation of total flavonoid content

The total flavonoid content in the methanolic extract of the root of *Streptopus streptoides* was evaluated using 1000 µg/mL solution of quercetin. In this experiment, the data were taken in triplicate. Total flavonoid content in methanol extract was calculated and is shown in Table 4.5.

Table 4.5 Total flavonoid content in methanol extract

OD of test solution			OD of Control	TFC (mg QE/g)			TFC Mean (mg QE/g)	Std Dev
I	II	III		I	II	III		
0.099	0.112	0.106	0.075	1.51	2.2	2.06	1.92	0.36

The calculated total flavonoid content in methanol extract was 1.92 ± 0.36 mg QE/g. It indicated the Prescence of very low content of flavonoid in the plant sample.

The phenolic and flavonoid compounds are regarded as secondary metabolites. They are the reactive oxygen species characterizing the therapeutic potential of the plant. In this plant phenolic content was found higher in comparison with flavonoid compound. The invitro and *in vivo* experimentation of the extract might provide more validity on the reactive oxygen species in correlation to the therapeutic spectrum of this plant.

Unit-5

Conclusions and Recommendation

5.1 Conclusions

Herein, methanolic extract of root of *Streptopus streptopoides* was separated by a series steps. The presence of phytochemicals was determined by general chemical tests including Mayer's test, Dragendorff's test, Wagner test, Shinodha test, Shibata's test, Molish's test, Benedict's test, Spot test, saponification test, ferric chloride test, lead acetate test, froath test, quinone test, and terpenoid test. Experimental evidences show the presence of carbohydrates, alkaloids, flavonoids and phenolic compounds in the phytochemical screening of *S. streptopoides* root extract.

The FTIR analysis illustrates the broad peak from 3100 cm^{-1} to 3400 cm^{-1} refers to the methanolic extract's hydrogen-bonded phenolic group. The carbon and hydrogen (C-H) bond is shown by the peak at 2940 cm^{-1}. Peaks near 2835 cm^{-1} correspond to a particular kind of carboxylic acid. Nitrosamine peaks at 1447 cm^{-1}. The absorbance band at 1112 cm^{-1} represents the primary alcohol's C-O stretching.

Total flavonoid content in methanol was found to be 1.92 ± 0.36 mg QE/g. It indicated the Prescence of very low content of flavonoid in the plant sample. The total phenolic content in methanol extract was found to be 50.37 ± 0.44 mg GAE/g. Nauplii started dying at the concentration of 50 ppm. 50% (or more) of the tested nauplii died at the highest concentration of 100 ppm. Hence, LC_{50} of methanol extract is at 100 ppm (100 µg/mL). The antibacterial activity test was performed using the ATCC technique, and the methanol extract of *S. streptopoides* root demonstrated antibacterial and anti-fungal activity effectively.

5.2 Recommendation

Different solvents have different capacity for the extraction of phytochemicals. In this context, it is imperative to study the plant extracts in other major solvents mentioned in this research work. Pure compounds can be extracted through column chromatography. The plant's high extraction capacity can produce a variety of active constituents for a variety of biological activities, which could be used to develop a powerful drug.

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