Valorization of pomegranate peel wastes by Ferulic Acid Degrading Bacteria and Bio-vanillin production

A PROJECT SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated to Manonmaniam Sundaranar University

In partial fulfilment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

AJMAL RIZWANA A.S

(REG. NO. 21SPMB01)

Under the Guidance of

Ms. A. Maria Heartina Adlin Vaz M.Sc., SET

DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS),

THOOTHUKUDI – 628001

APRIL – 2023

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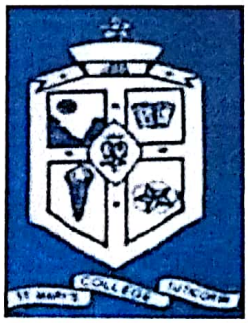
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CERTIFICATE

This is to certify this Project work entitled **"Valorization of pomegranate peel wastes by ferulic acid degrading Bacteria and Bio-vanillin production"** submitted to **ST. MARY'S COLLEGE (AUTONOMOUS), Thoothukudi** affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY, Tirunelveli** in partial fulfillment for the award of "Master of Science in Microbiology" is a bonafide research carried out by **Ms.AJMAL RIZWANA A.S** under the guidance and supervision of **Ms. A. Maria Heartina Adlin Vaz M.Sc., SET** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi for the academic year 2022- 2023.

SIGNATURE OF THE GUIDE

Dr. Joys Selva Mary Albert
Head
Department of Microbiology
St. Mary's College (Autonomous)
Thoothukudi

SIGNATURE OF THE HOD

SIGNATURE OF THE DIRECTOR
Director
Self Supporting Courses
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATURE OF THE PRINCIPAL
Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

18/4/2023
SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE : 05.04.2023

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DECLARATION

I hereby declare that the Project work entitled **"Valorization of pomegranate peel wastes by ferulic acid degrading Bacteria and Bio-vanillin production"** is a bonafide record of the work completed by me during the academic year 2023 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundharanar University. I also affirm that this is an original work done by me under the supervision of **Ms. A. Maria Heartina Adlin Vaz M.Sc., SET** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

A. Adlin Vaz

SIGNATURE OF THE GUIDE

Ajmal Rizwana. A.S.

SIGNATURE OF THE STUDENT

PLACE: THOOTHUKUDI

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CONTENTS

S.NO	TITLE	PAGE NO.
1.	INTRODUCTION	1-14
2.	AIM AND OBJECTIVE	15
3.	REVIEW OF LITERATURE	16-21
4.	MATERIALS AND METHOD	22-30
5.	RESULT AND DISCUSSION	31-46
6.	CONCLUSION	47
7.	SUMMARY	48
8.	BIBLIOGRAPHY	49-54
9.	ANNEXURE	55

Scanned with CamScanner

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
Table 1	physio-chemical properties of vanillin	4
Table 2	Colony morphology of the isolates	33
Table 3	Identification of the isolate	37

Scanned with CamScanner

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
Figure 1	Industrial applications of vanillin	2
Figure 2	Chemical structure and geometry of vanillin molecule	4
Figure 3	Overview of metabolic pathways leading to vanillin	6
Figure 4	Bar diagram representing the estimation of vanillin production	7
Figure 5	Vanilla plant at flowering stage and by-hand pollination	7
Figure 6	Chemical reaction sequence for producing vanillin from guaiacol	9
Figure 7	Image of banana tree where the soil sample 1 was collected	22
Figure 8	Soil sample 1	23
Figure 9	Image of banana tree where the soil sample 2 was collected	23
Figure 10	Soil sample 2	23
Figure 11	Pomegranate peel sustrate	24

Scanned with CamScanner

Figure 12	Pomegranate peel powder	24
Figure 13	Enrichment culture of Sample 1 and Sample 2	31
Figure 14	Nutrients agar plates containing the ferulic acid degrading bacteria	32
Figure 15	Pure culture of isolate 1	32
Figure 16	Pure culture of isolate 2	32
Figure 17	Pure culture of isolate 3	33
Figure 18	Primary screening of ferulic acid degrading bacteria by growing 3 isolates in M9 medium supplemented with Ferulic acid.	34
Figure 19	The organic layer after the acidification and extraction process	34
Figure 20	Paper chromatography done with the extract from primary screening	35
Figure 21	Gram's staining	38
Figure 22	Motility Test	38
Figure 23	Indole Test	39
Figure 24	Methyl Red Test	39
Figure 25	Voges- Proskauer Test	40
Figure 26	Citrate test	40

Scanned with CamScanner

Figure 27	Oxidase Test	41
Figure 28	Catalase Test	41
Figure 29	Starch Hydrolysis	42
Figure 30	Urease Test	42
Figure 31	Gelatin Hydrolysis	42
Figure 32	Bioconversion of pomegranate peel and pomegranate peel powder using isolate 2	43
Figure 33	Picture showing pellet and supernatant obtained from bioconversion medium after centrifugation	44
Figure 34	Supernatant collected from the bioconversion media after centrifugation	44
Figure 35	Acidified supernatant	45
Figure 36	Organic layer obtained after extraction using ethyl acetate.	45
Figure 37	Paper chromatography using bioconversion media	46

Scanned with CamScanner

Figure 27	Oxidase Test	41
Figure 28	Catalase Test	41
Figure 29	Starch Hydrolysis	42
Figure 30	Urease Test	42
Figure 31	Gelatin Hydrolysis	42
Figure 32	Bioconversion of pomegranate peel and pomegranate peel powder using isolate 2	43
Figure 33	Picture showing pellet and supernatant obtained from bioconversion medium after centrifugation	44
Figure 34	Supernatant collected from the bioconversion media after centrifugation	44
Figure 35	Acidified supernatant	45
Figure 36	Organic layer obtained after extraction using ethyl acetate.	45
Figure 37	Paper chromatography using bioconversion media	46

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LIST OF ABBREVIATIONS

S.NO	ABBREVIATION	EXPANSION
1	g	Gram
2	ml	Milli litre
3	N	Normality
4	R_f	Retardation Factor
5	M9	Modified M9 medium
6	HCL	Hydrochloric acid
7	CFU	Colony forming unit
8	NB	Nutrient Broth

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INTRODUCTION

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1. INTRODUCTION

1.1 FLAVOURS AND FRAGRANCES:

Flavours and fragrances, which are widely employed in the food and feed, cosmetic, and pharmaceutical industries, are in high demand on the global market. The manufacture of "natural" tastes, which are derived from botanical sources or tissue culture, makes up a very small portion of the flavouring compounds that are currently accessible and are created mostly by chemical synthesis. Consumer preference for "green," "eco-friendly," and "healthy" methods that are connected to "bio" or "natural" products has grown over the past few decades. Even though chemical procedures produce flavours with acceptable yields, they have a number of drawbacks, including a negative impact on the environment and poor product quality. Additionally, the labels "nature identical" (EC Flavour Directive 88/388/EEC) or "artificial" (US Code of Federal Regulations 21 CFR 101.22) placed on chemically generated substances reduce their commercial appeal.

Natural flavours are therefore preferred on the global market despite being much more expensive. However, due to low concentrations of the desired product in plants, which increases the extraction and purification procedures, as well as the harvest's dependence on seasonal, climatic, and political factors, the production of natural flavours by direct extraction from botanic sources can no longer satisfy the high market demand. There is a need for alternative natural sources for flavour synthesis, and biotechnology is by far the most alluring area of study. The benefits of using biotechnological methods often include gentle reaction conditions, high regio- and enantio selectivity that results in only one product isomer, the absence of toxic waste production, and less environmental issues.

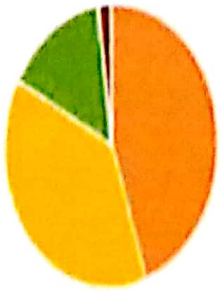
Additionally, as per recent US Food and European legislation, the term "natural" is no longer just applied to flavours derived from botanical components. The definition of "natural flavouring substance" as stated in article 3(2) c) of the European flavour regulation (EEC No 1334/2008) is: "Natural flavouring substance shall mean a flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal, or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes." New flavours are added to the GRAS list since the definition of "natural" in US food regulations is similar. (generally recognized as safe).

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Thus, in order to replace the conventional chemical procedures, the industrial attractiveness for the biotechnological production of natural flavours, also known as bio flavours, is constantly rising. As a result, numerous research projects have been conducted, mostly in the area of flavour creation from different natural precursors employing diverse microorganisms or single enzymes. Vanillin is by far the flavour that matters the most for biotechnological applications. Vanillin is one of the finest and most widely used flavours in the world, which isa crucial and well-liked spice and flavour enhancer. (Gokce S. Karatoprak 2022). The primary flavour and aroma component in vanilla is vanillin, which stands out the most. It is used in the most flavours and perfumes on the market today.

1.2 VANILLIN:

Vanilla is an orchid that belongs to the Orchidaceae family which has its origin in Mexico (Rao *et al.*,2000) Vanillin is the primary component of the vanilla beans extract which is responsible for the characteristic taste and smell of vanilla (Joanna C. Sadler *et al.*,2021)Natural vanilla is produced in roughly 2000 tonnes annually, however due to the expanding global market and growing popularity of natural flavours, the supply cannot keep up with demand. Just 1% of vanilla flavour is derived from a natural source the balance is made synthetically. However, due to the flavour's intricacy, only its primary component, vanillin, is made. (Luziatelli F *et al.*, 2019) One of the most popular flavours in the world, vanilla is most often used in the food and beverage industry to flavour things like chocolate, ice cream, and coffee. It is also frequently utilised in the perfume and cosmetics sectors. Vanillin, the primary ingredient in vanilla flavour, is also utilised in the pharmaceutical sector to create medications including L-DOPA, dopamine, and papaverine. It is also employed as a food preservative because of its antibacterial and antioxidant properties. (Sagar S. Arya *et al.*, 2021). The appliance of vanillin in the industries are illustrated in Figure1.



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Figure 1 – Industrial applications of vanillin, where ■ represents food and beverage industries (45%), ■ cosmetic and perfume industries (38%), ■ pharmaceutic industry (15%) and ■ other utilizations (2%) (Adapted from Eswaran 2017).

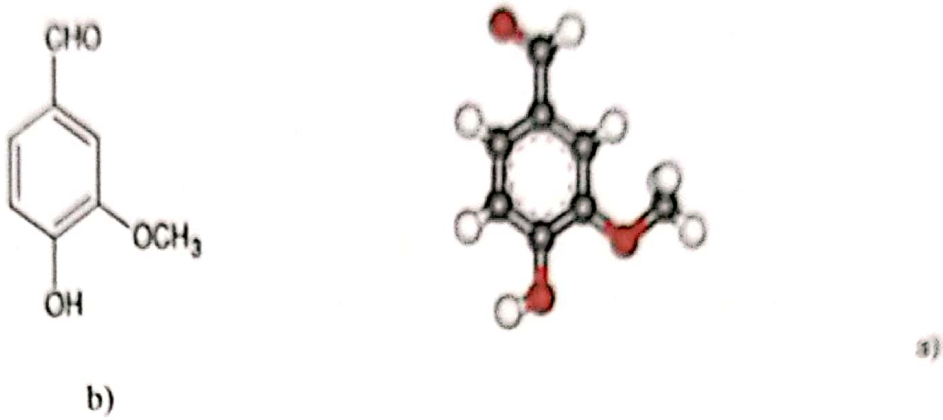
Vanillin is the primary and most often used taste in the world since it is quite flexible and most people like the aroma (Schrader *et al.*, 2004) However, less than 0.5% of the overall output of vanillin is derived from vanilla; the majority is created far more easily and inexpensively by chemical methods. In contrast to the price of the synthetic product, which is less than $15 per kilogram, vanillin derived from vanilla pods has a fluctuating and expensive price in the market, ranging between $1200 and $4000 per kg (Muheim *et al.*,1999). The restricted supply of vanilla pods that depend on climatically-related variations in harvest yields, economic and political decisions, as well as the labor-intensive cultivation, pollination, harvesting, and curing of vanilla pods, all contribute to the unpredictable and high cost of natural vanillin. The flavour industry is becoming more and more interested in producing vanillin from other natural sources because of the price difference between natural and synthetic vanillin and the rising consumer trend towards "green" and "eco - friendly" as well as "healthy" processes, which are associated with "bio" or "natural" products. (Priefert *et al.*, 2001). Since biotechnological procedures are regarded as natural under US and European law, they make excellent solutions to this issue. When a substance is "obtained by appropriate physical, enzymatic, or microbiological processes from material of vegetable, animal, or microbiological origin" and "corresponds to substances that are naturally present and have been identified in nature," it can be labelled as natural in the European Union. (European Commission, 2008). The term "natural flavour" in the United States "means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating, or enzymolysis, which contains the flavouring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf, or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional (U.S. Food and Drug Administration).

1.3 VANILLIN STRUCTURE AND PROPERTIES:

The physical form of isolated vanillin is a white, crystalline powder with a pleasant, sweet scent and a distinctive vanilla-like flavour. It is a simple phenolic molecule and is classified chemically as an aromatic aldehyde (3-methoxy-4hydroxybenzaldehyde). Aldehyde, ether, and phenol are some of its structurally functional groups, and its chemical formula is

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$C_8H_8O_3$ (Converti *et al.*, 2010). The chemical structure and geometry of vanillin are shown in Figure 2. Some relevant physio-chemical properties are summarized in Table 1.



a)

b)

Figure 2. Chemical structure (a) and geometry (b) of vanillin molecule.

Table 1. physio-chemical properties of vanillin

PROPERTY	VALUE
Solubility in water at 20° (g/L)	10
Molecular weight (g/mol)	152.15
Melting point (°C)	80/83
Boiling point (°C)	~ 285

Vanillin crystallizes from hot water at 81–82°C as colourless needles, according to Gildemeister *et al.*,(1899). It smells strongly and incredibly delicious, just like vanilla. Vanillin can be sublimated under controlled heating conditions without decomposing; however, when heated for an extended period of time at 105°C, vanillin decomposes and non-volatile compounds are produced. Because vanillin is somewhat insoluble in cold water, it can be recrystallized from water. Vanillin is easily soluble in alcohol, ether, chloroform, and hot water.

1.4 BIOSYNTHESIS OF VANILLIN IN *Vanilla planifolia:*

Vanillin is present in cured vanilla pods at a concentration of 1.0–2.0% w/w of dry weight where it is solely deposited in conjugated form, primarily as the D-glucoside. After pollination, the green vanilla beans are collected about six to eight months later, although they are still devoid of any traces of the vanilla flavour. (Walton *et al.*, 2003). Only by fermentation, also known as "curing," in which the glucoside of vanillin, glucovanillin, and related D

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glucosides are hydrolyzed by -D-glucosidase, free vanillin and related compounds (particularly 4-hydroxybenzaldehyde) are liberated. (Odoux *et al.*, 2003). In contrast to the process by which vanillin -D-glucoside is first created, the appearance of vanillin during curing is in theory straightforward. Although a number of biosynthetic pathways have been suggested, the full mechanism of vanillin synthesis is still unknown. Vanillin is generally acknowledged in the literature to be a byproduct of the shikimic acid pathway. This route involves the deamination of phenylalanine or tyrosine to a C6-C3 phenylpropanoid, which is then used as a precursor for the production of vanillin. Although the genesis of vanillin is generally accepted to be a phenylpropanoid C6-C3 molecule, there is still a great deal of debate around the chain shortening and other processes that lead from the hypothesised hydroxyl cinnamic acid precursor to vanillin –D glucoside.

There are two main theories about how phenylpropanoid precursors are transformed into vanillin. (Havkin *et al.*,2008). According to one school of thinking, the aromatic ring on C6–C3 compounds (trans–cinnamic, p–coumaric acids) undergoes hydroxylation and methylation, giving rise to ferulic acid. This theory was first put forth by Zenk in 1965. The latter is subsequently subjected to -oxidation, which causes chain shortening to vanillin. The "ferulate pathway" is the name given to this plan. According to another theory, the initial metabolic process that results in the production of vanillin is the chain shortening of a phenylpropanoid. It is known as the "benzoate pathway" (Podstolski *et al.*, 2002).

Another possibility is that a shikimic acid route early intermediate directly generates the benzoate pool, avoiding the synthesis of phenylpropanoids and their subsequent breakdown into benzoate pathway intermediates. (Wildermuth *et al.*, 2001) Negishi *et al.* (2009) recently conducted tests using 14C-labeled substances in discs of green vanilla pods. According to preliminary findings, mature vanilla beans synthesize vanillin from 4-coumaric acid using ferulic acid, which is then glucosylated to create glucovanillin. The work to isolate, purify, and identify the essential enzyme that shortens the side chain of phenylpropanoid compounds is still ongoing.

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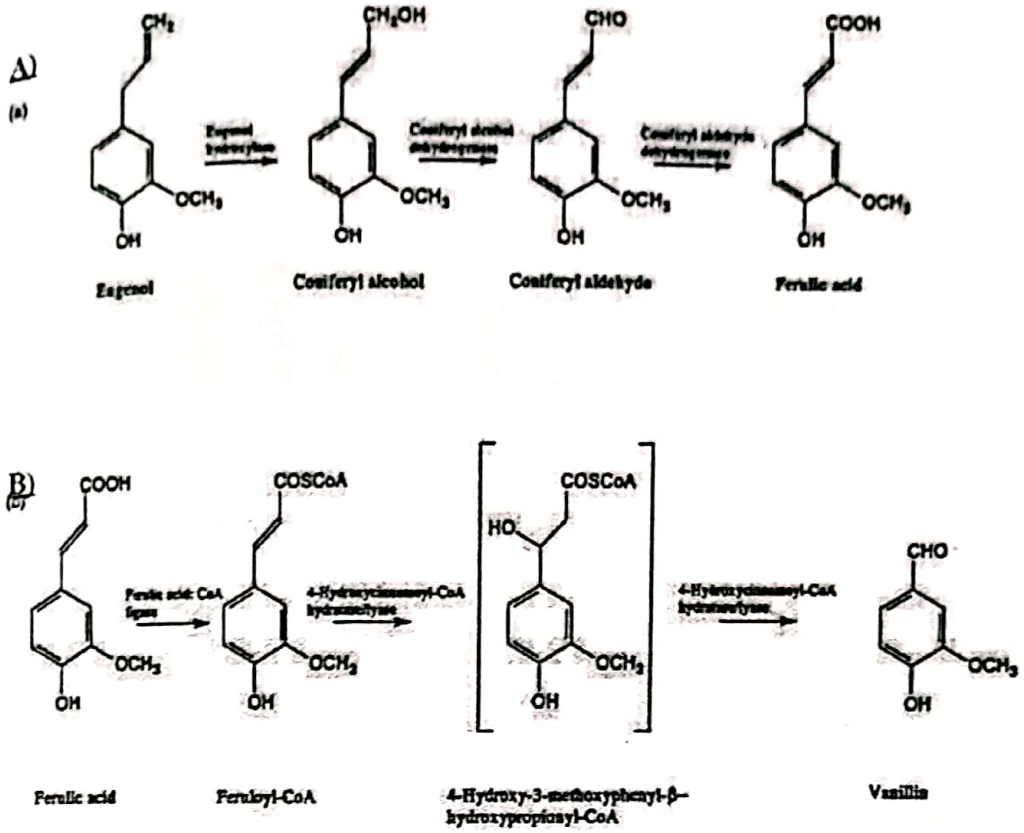


Figure 3. Overview of metabolic pathways leading to vanillin: (A) ferulate pathway and (B) benzoate pathway (Walton et al., 2003)

1.5 PRODUCTION OF NATURAL VANILLIN FROM *Vanilla planifolia:*

Natural vanillin is mostly derived from the bean or pod of the tropical orchid *Vanilla planifolia,* however it can also be found in smaller amounts in *V. tahitiensis* and *V. pompona*, which are also frequently referred to as "vanilla." For the purpose of making vanillin, *V. planifolia* is the one of them that is most frequently grown because of its flavourful properties (Anandaraj *et al.,* 2005). Native to Mexico, vanilla has been utilised since the time of the Aztecs. Spanish conquistadors later introduced it to Europe (Sinha *et al.,* 2008). The cultivation of vanilla involves vegetative growth. Vanilla is a perennial climbing orchid with sessile leaves and succulent green stems that produce aerial roots at the nodes. The global vanillin market size was estimated at USD 480.1 million in 2019 and is expected to expand at a compound annual growth rate (CAGR) of 7.1% from 2020 to 2025. Globally, rising demand from various end-use industries to enhance the flavour and aroma of the product is expected to be a key growth factor. It is one of the key ingredient used extensively in the food & beverage industry.

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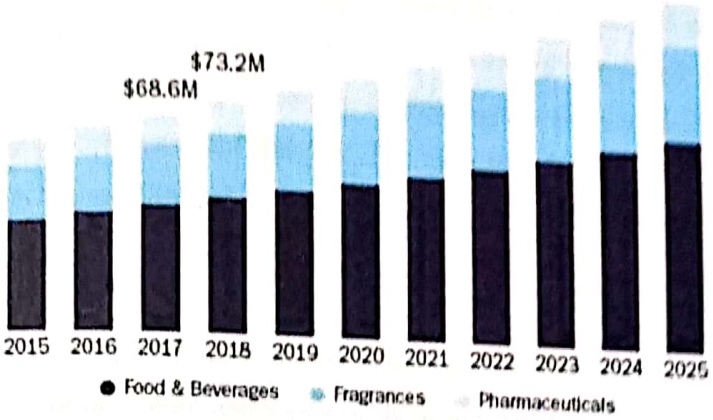


Figure 4. Bar diagram representing the estimation of vanillin production

Due to the blooms' tight structure, self-pollination is literally impossible (Figure 5) The vanilla flower is known to bloom for fewer than 24 hours, and pollination must occur between the hours of 8 and 11 AM in order for fruit development and fertilization to ensue. Hence, deliberate artificial pollination with a bamboo stick is required to obtain a high yield (Figure 5) The maturation of the beans takes between 10 and 12 months after conception. The timing to harvest varies by region and typically occurs six to eight months following fertilization. The first step in processing vanilla to make vanillin is curing freshly picked pods. The purpose of the curing procedure is to halt the organic vegetative activity and hasten the alterations that lead to the production of aromatic taste components. The curing process varies from one manufacturing area to the next, and this can significantly affect the quality and aromatic profile of traded pods. Despite the fact that there are various methods for curing vanilla, the two most common being the Mexican process (sun method) and the Madagascar process (Bourbon method), all of them share the same four fundamental stages: killing, sweating, drying, and packaging.

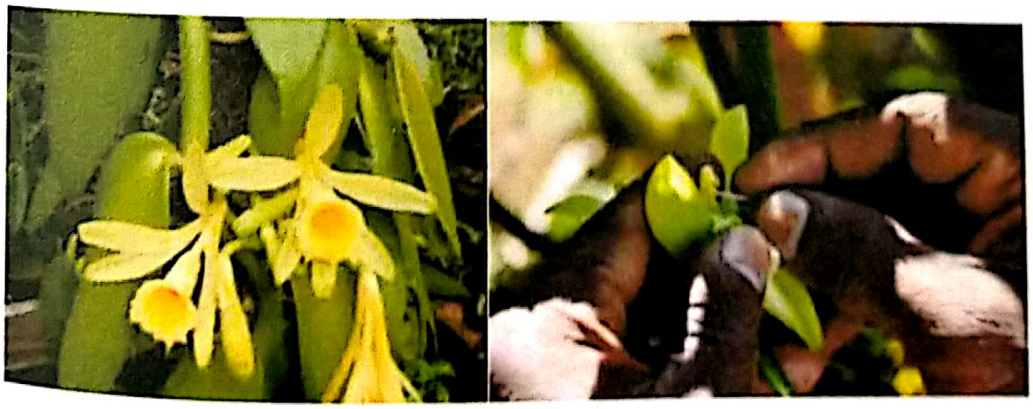


Figure 5. Vanilla plant at flowering stage and by-hand pollination

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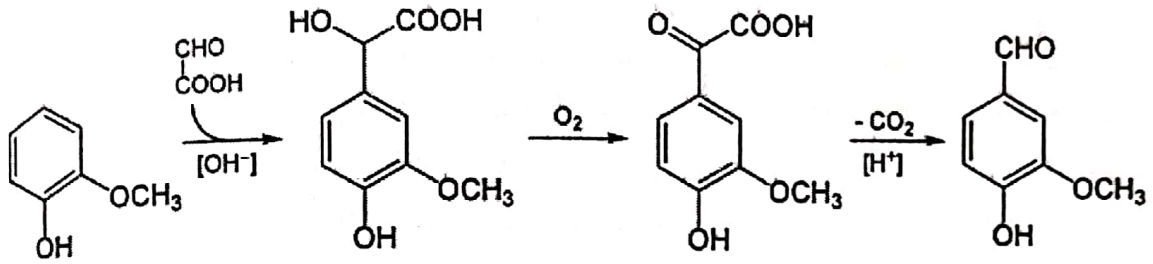
The killing procedure prevents vegetative growth after harvest and encourages the enzymatic processes that result in aroma and flavour. The action is referred to as "killing" because it alters the structure of plant cells in a variety of ways, including by blistering in hot water, withering in the sun or oven, and freezing. The most important step is the subsequent sweating since this is when vanillin and many other similar chemicals are liberated from their glucosides. In order to enhance enzymatic reactions and quick initial drying and prevent damaging fermentation, the temperature is thus elevated. The cured beans require additional drying after the sweating stage, which can take anywhere between 7 and 10 days, to lower their moisture level and prevent microbial deterioration. Pods are dried at room temperature for a third of their initial weight before being removed from the drying process. The pods are kept for one to several months in locked boxes after the final packaging procedure. During this time, a number of chemical and biological events, including oxidative degradation, esterification, and etherification, take place to provide the desired aroma and flavour. (Ramachandra Rao *et al.*,2000; Converti *et al.*, 2010; Exley, 2010). Agroclimatic conditions, political and economic considerations, as well as variations in crop yields, all contribute to the difficulty and expense of producing natural vanillin from botanical sources. The remainder of the international market's need for vanillin is satisfied by chemically synthesised vanillin made from lignin or fossil hydrocarbons like guaiacol because natural vanillin production only meets less than 0.5% of it. (Zamzuri *et al.*,2013).

1.6 PRODUCTION OF VANILLIN BY CHEMICAL SYNTHESIS:

In 1876, Tiemann created the first chemical procedure to produce vanillin. This method was commercially employed up until the 1920s and was based on the raw ingredient eugenol, which can be found in clove oil. Later, the residue of the wood pulp industry known as spent sulphite liquor, which is rich in lignin, was used to create vanillin. The lignin process entails adding oxidants to an aqueous solution of lignin while maintaining high temperatures and pressures, a very alkaline pH, and these conditions. Depending on whether or not catalysts are used, these oxidants may include air, oxygen, nitrobenzene, or metallic oxides. Vanillin is produced as a by-product of the oxidation and degradation of lignin along with other substances. Due to the need for intensive purification techniques due to the existence of these pollutants with chemical structures similar to vanillin, the lignin method is no longer widely used today, which also raises environmental concerns. Furthermore, due to the substantial chemical alterations necessary to produce the end product, vanillin chemically synthesised from lignin is labelled as synthetic vanillin by food legislation even though the precursor input comes from a natural source. (Rabenhorst *et al.*,2000). Today, guaiacol, a petrochemical raw

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ingredient, is used to make the majority of synthetic vanillin. There are numerous ways to create vanillin from guaiacol. The most competitive and important of these methods at the moment involve the use of guaiacol and glyoxylic acid. (Kumar *et al.*, 2012). This two-step procedure begins with a condensation reaction between guaiacol and glyoxylic acid in an alkaline medium. The resultant vanillyl mandelic acid is oxidatively decarboxylated to get vanillin. The sequence of reactions is shown in Figure 5.



gluaicol glyoxylic acid vanilliylmandelic acid vanillin

Figure 6. Chemical reaction sequence for producing vanillin from guaiacol (Kirk-Othmer Encyclopedia, 2005).

This approach yields vanillin from guaiacol that is largely free of by-products, simplifying the role of the later process of product purification. Although this cleaner method has lessened the environmental impact compared to lignin-derived production, guaiacol is a petrochemical product and, like vanillin synthesised from lignin, considerable chemical modifications yield a product that is considered synthetic. (Xu *et al.*, 2007).

1.7 BIOTECHNOLOGICAL PRODUCTION OF VANILLIN:

In addition to being labelled as natural, products made through biotechnological techniques have other benefits. These benefits include the high specificity for the substrate or the product, in the event that only one of the isomers is of interest, and the softer production conditions, which are less detrimental to the environment, when compared to a synthetic approach. Biotechnological production has the advantage over natural scent in that it is not reliant on agricultural factors, such as crop or climate, having an impact on price. Another benefit is the potential for a process to be scaled up and applied to the industry as a manageable procedure. (Krings *et al.*,1998; Kaur *et al.*,2013). Using fungi, bacteria, genetically altered microbes, and plant cells, biotechnological vanillin can be produced from natural substrates.

Ferulic acid, eugenol, and isoeugenol, carbohydrates, particularly glucose, lignin, aromatic amino acids, and phenolic stilbenes are the substrates that have been researched the most

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(Gallage *et al.*, 2015). Since ferulic acid is plentiful in nature, found in plant cell walls, and shares a molecular structure with the desired molecule, it has received the most attention of all the substrates mentioned above for the manufacture of vanillin (Gallage *et al.*, 2015; Priefert *et al.*, 2001). Vanillin can be produced from this substrate by a number of microorganism strains, the best investigated of which have been found to reach amounts exceeding 1 g.In a two-phase process involving these two microorganisms, L-1 is composed of *Amycolatopsis* ATCC 39116 (re-classification of *Streptomyces setonii* ATCC 39116) (Daguliset *al.*,2014; Muheimet *al.*, 1999), *Amycolatopsis sp.*V1 (Daugulis *et al.*, 2014), *Aspergillus niger* (Zheng *et al.*, 2007).

Microorganisms are far better candidates for biotechnology applications due to their high growth rates and susceptibility to molecular genetics. These organisms can be chosen based on their propensity to develop on a purported precursor to vanillin as their only source of carbon and energy. Vanillin has been produced on a wide scale by microbes including bacteria, fungi, and yeast from a variety of substrates including lignin, eugenol, isoeugenol, and ferulic acid as well as vanillic acid, phenolic stilbenes, aromatic amino acids, and glucose.

1.8 FERULIC ACID - THE SUBSTRATE:

Plant cell walls contain a very large amount of ferulic acid, a hydroxycinnamic acid. It typically forms esters or ethers with lignin and hemicelluloses, as well as amides, and is linked to carbohydrates by covalent connections. By cross-linking with pentosane chains, arabinoxylans, hemicelluloses, and lignin during plant development, this molecule lends stiffness and hardens the cell wall, making these components less susceptible to hydrolytic enzymes. Despite being extracted primarily in its trans-isomeric form, the double bond that ferulic acid contains in its side chain is vulnerable to cis-trans isomerization. (Tilay *et al.*, 2008; Kumar *et al.*,2014). Ferulic acid can be found in a variety of foods, including fruits, vegetables, grains, leaves, and other agricultural products like beans, corn hulls, wheat, rice, and coffee seeds. Examples of foods that include ferulic acid include coffee, which contains 9.1–14.3 mg/0.1 kg, oranges, which contain 9.2-9.9 mg/0.1 kg, bananas, which contain 5.4 mg/0.1 kg, carrots, which contain 1.2–2.8 mg/0.1 kg, peanuts, which contain 8.7 mg/0.1 kg, and popcorn, which has 313 mg/0.1 kg (Zhao *et al.*,2008; Kumar *et al.*,2014). There are chemical extraction techniques, such as alkaline or acidic hydrolysis, to liberate ferulic acid from natural sources. (Tilay *et al.*, 2008). Ferulic acid in plant cell walls forms ester linkages that can be broken by alkaline techniques. Since hydroxycinnamic acids can also be degraded by acidic hydrolysis, this sort of hydrolysis results in a higher yield for the release of several phenolic acids, including ferulic acid. (Arranz *et al.*, 2010; Pan *et al.*, 2003; Acosta-Estrada *et al.*, 2014; Kim *et al.*, 2006).

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However, some other connections, for example, are not affected by alkaline treatment but can be broken by acidic hydrolysis, which is another sort of chemical linkage. (Xu *et al.*, 2005). The demand for healthy and natural products is rising today, thus the ferulic acid extraction methods mentioned above cannot be regarded as natural. The release of enzymes from natural sources can therefore replace the alternative. To break such polysaccharides, ferulic acid, which is found associated with hemicelluloses, namely arabinoxylans through ester bonds with arabinose residues, is required. Combinations of bacterial and fungal enzymes can be utilised for this type of extraction. For example, feruloyl esterase, acetyl xylan esterase, xylanase, and arabinoxylanase are examples of enzymes that must have ferulic acid esterase and carbohydrate hydrolase activity.(Uraji *et al.*, 2013; Dupoiron *et al.*, 2017) The yields obtained by enzymatic release are still lower than those obtained by alkaline extraction, according to the results published in the literature, and the process's disadvantage is that it is challenging to recover the enzymes for subsequent use (Uraji *et al.*, 2013; Dupoiron *et al.*, 2017; He *et al.*, 2015).

In addition to the biotechnological methods mentioned above for producing vanillin, ferulic acid and its derivatives have a wide range of uses that make them applicable to the pharmaceutical, cosmetic, and food industries. According to studies, ferulic acid exhibits antiinflammatory properties, has anti-diabetic, anti-aging, and anti-cancer properties due to its antioxidant activity brought on by the formation of a resonance phenoxy radical (Srinivasan *et al.*, 2007; Ou *et al.*, 2004; Acosta-Estrada *et al.*, 2014; Kumar *et al.*, 2014). Since ferulic acid can be used as an additive to preserve food, this feature is also significant in the food business. Ferulic acid can be used to improve the viscosity and create gels from polysaccharides such as pectin because of its ability to act as a cross-linking agent (Kumar *et al.*,2014; Ou *et al.*,2004).

Pomegranate (*Punica granatum*) is a fruit that originated in places like Iran, China, and Afghanistan but has gained popularity throughout the world. (Ismail *et al.*, 2012).Despite being rich in phenolic compounds, the pomegranate peel, which accounts for between 50 and 60 percent of the fruit's weight, is wasted in both the food industry and by consumers. However, considering that the world produces three million tonnes of pomegranates annually and that the fruit's peel and seeds make up about 54% of it, this amounts to 1.62 million tonnes of trash.

Because of the enormous amount of waste that is produced, it is essential to identify effective ways to valorize it by maximising the extraction of bioactive compounds from pomegranate wastes and then turning them into value-added goods. As a result, savings on resources like energy, water, and land used in production, harvesting, preservation, and distribution can be achieved while also benefiting the environment.It is crucial to find appropriate ways to

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maximise the nutritional and bioactive components of pomegranate waste and then convert this waste into value-added products to save energy, sustain resources, and protect the environment because of the sheer amount of waste that is produced for each edible percentage of pomegranate. Even while it might not be financially possible to extract the nutritional and useful components of pomegranates, looking into these uses is a critical first step towards finding a long-term solution for this excessive waste.

1.9 TOXICITY OF VANILLIN:

Aromatic aldehydes, like vanillin, are extremely reactive and can produce schiff bases, which can impede enzyme activity and cause cell death (Fleige *et al.*, 2013; Gallage *et al.*,2015; Sinha *et al.*, 2008). Additionally, studies have shown that phenolic chemicals, such as vanillin, can influence or prevent the creation of DNA, RNA, and proteins by forming covalent connections with them. Due to interactions with the cytoplasmic membrane made possible by the aromatic ring giving the molecule hydrophobicity, it can also have an impact on glucose uptake, pH homeostasis, and ion gradients. (Fleige *et al.*,2016). These appear to be the reasons why the producing microbes cannot collect vanillin in high concentrations. Low production yields are the fundamental issue with the industrial use of the biotechnological process because vanillin, due to its toxicity, is converted into less hazardous chemicals such as vanillic acid, vanillyl alcohol, and guaiacol instead of being accumulated. (Fleige *et al.*, 2013; Kaur *et al.*,2013).

1.10 BIOCONVERSION OF FERULIC ACID:

One of the biotransformation that has received the greatest attention is the microbial conversion of ferulic acid to vanillin, which is also the most promising method for the production of bio-vanillin for commercial use. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a common plant substance that results from the metabolism of phenylpropanoid and, along with dihydroferulic acid, is a part of lignocelluloses. By cross-linking lignin and polysaccharides, ferulic acid gives cells their stiffness. (Ou *et al.*,2004). Ferulic acid (FA) can be obtained from agricultural by-products like corn hulls (31.0 g/kg), maize bran (30 g/kg), sugar beet (5-10 g/kg), rice endosperm cell wall (9 g/kg), wheat (6.6 g/kg), and barley grains (1.4 g/kg). FA is the main phenolic component found in the cell walls of monocotyledons. Enzymatic hydrolysis or treatment with a strong alkali can both release FA. For the purpose of producing vanillin with a "natural" label, the latter is the ideal option. (Hasyierah *et al.*, 2008; Mathew *et al.*,2006). Ferulic acid may also be the most advantageous candidate for the creation of bio-vanillin because it is the least poisonous of all the examined precursors. The

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biotransformation of ferulic acid to vanillin has been studied using a variety of microorganisms, such as *Rhodococcus ssp.*, *Actinomycetes spp.*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, *Rhodotorularubra*, *Debaryomyceshansenii*, *Halomonaselongata*, *Schizophyllum commune*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas sp.* EF3, JM109/pBB1 *Escherichia coli*, CGMCC0774 *Aspergillus niger* Lactic acid microbes, *Streptomyces sp.* V-1, *Amycolatopsis sp.* HR167, *Streptomyces sannanensis*, *Streptomyces halstedii* GE107678, *Streptomyces setonii* ATCC39116, and genetically modified microorganisms (Chen *et al.*, 2016). Additionally, more recent and preliminary investigations looked into the capacity of the bacteria *Staphylococcus aureus*, *Enterobacter* sp., *Lactobacillus sp.*, *and Staphylococcus aureus* to produce vanillin from ferulic acid (Sarangi *et al.*,2010), (Srwajgier *et al.*,2010). Vanillin was created in virtually all of the bacteria that have been the subject of studies to date in trace amounts as a transient metabolite that either quickly transformed into other products or was used as a carbon source by the microbe due to its toxic effects and product inhibition. Therefore, methods of metabolic engineering or process optimization, such as adding resin, were researched. Because they can produce vanillin using ferulic acid as their only carbon source, bacteria from the genus Pseudomonas sp. were discovered to be promising possibilities. FA, however, was swiftly transformed into vanillic acid or vanillyl alcohol by oxidation or reduction, respectively.

1.11 VALORIZATION OF AGRO-INDUSTRIAL WASTES FOR BIOVANILLIN PRODUCTION:

According to Bio energy and Food Security, "several kinds of biomass materials produced primarily in the food and fibre processing industries" are referred to as agro-industrial wastes (or by products). Since they are the most plentiful source of organic materials in the entire globe, they are significant, easily accessible, renewable natural resources. Examples of agricultural waste include straw, cereal bran, citrus peelings, cobs, stalks, and bagasse.A useful alternative to the high manufacturing costs of industrial fermentations could be the use of agroindustrial leftovers as substrates in biotechnological processes. (Bicas *et al.*, 2010).

The ability of E. coli JM109/pBB1 to manufacture vanillin from an alkaline hydrolyzate of maize cob was studied by Torres and colleagues in 2009. After 22 hours, the maximal vanillin concentration (239 ± 15 mg/L), vanillin yield on consumed ferulic acid (0.66 ± 0.03 mol/mol), and vanillin volumetric productivity (10.9 ± 0.7 mg L -1h-1) were found at an initial biomass content of 0.5 g (dry weight)/L. According to Shin *et al.* (2006), a filamentous fungus's enzyme pool might be used to produce vanillin from ferulic acid recovered from maize bran by

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an *Actinomycetes* strain of *Streptomyces setonii* (ATCC391161) (*Neosartorya spinosa* NRRL185). After 12 hours of cultivation, 98% of the ferulic acid was transformed into 0.43 mmol of vanillin with a 43% molar yield. When *Jatropha curcas* stem hydrolysate with 1.55 g/L of ferulic acid was created by steam explosion in 2009 by Vaithanomsat and Apiwatanapiwat, it was successfully employed as a substrate for the one-step vanillin production by *Aspergillus niger* and *Pycnoporus cinnabarinus*. Two fungi strains were utilised in a two-step procedure to generate vanillin using ferulic acid, which was produced from sugar beet pulp. The micromycete *A. niger* produced 920 mg/L of vanillic acid (molar yield: 88%) in the first stage after bioconverting ferulic acid to vanillic acid by propenoic chain breakdown, which was then decarboxylated to produce methoxyhydoquinone. It was claimed that the latter was the process's limiting pathway. In the second phase, a laccase-deficient strain of the basidiomycete *Pycnoporus cinnabarinus* converted vanillic acid to 237 mg/L of vanillin (molar yield 22%). Lesage-Meessen *et al,* (2002) found that *P. cinnabarinus* MUCL39533 could be fed with the autoclaved fraction of maize bran as a source of ferulic acid and with the culture filtrate from *A. niger* I-1472 as a source of extracellular enzymes. In these circumstances, 584 mg/L of vanillin was producedIt was also looked into whether rice bran could be a source of ferulic acid. *A. niger* CGMCC0774 and *P. cinnabarinus* CGMCC1115 were co-cultured to produce vanillin from waste rice bran oil residue, with vanillyl alcohol serving as a key intermediary. With the addition of 25 g of HZ802 resin and 5 g/L of glucose to the bioconversion medium, the yield of vanillin increased to up to 2.8 g/L. (Zhang *et al.*, 2007) The basidiomycete *Phanerochaete chrysosporium* was used by Barbosa *et al.*, (2008) to assess the potential for vanillin synthesis via solidstate fermentation on green coconut residue. Using the Plackett-Burman experimental design, the sun-dried green coconut husk was determined to provide the best solid support for the manufacture of vanillin.

This work focus on isolating the ferulic acid degrading bacteria from the soil of banana plantation and used pomegranate peels as a substrate for the production of bio-vanillin from ferulic acid by bioconversion process.

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AIM AND OBJECTIVES

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2. AIM AND OBJECTIVES

- To collect the soil sample from the banana plantation areas in Agastheeswaram, Kanyakumari and from local areas in Thoothukudi.
- To isolate ferulic acid degrading bacteria from soil of banana plantations using enrichment culture method.
- Identification of the isolate using morphological and biochemical methods.
- To perform primary screening technique for the determination of ferulic acid degrading bacteria.
- To perform bio conversion of ferulic acid to vanillin using the ferulic acid rich pomegranate peel waste using the isolate.
- To perform qualitative assessment of paper chromatography for determining the production of bio-vanillin.

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REVIEW OF LITERATURE

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Ramachandra Rao *et al.*, (2000) reviewed the history, botanical description, and chemistry of parts of the vanilla plant also described the production of vanillin by both chemical and biotechnological methods.

Priefert *et al.*, (2001) discussed the different biosynthesis routes and alternative biotechnology based approaches which involved in biotechnological vanillin production are based on bioconversion of lignin, phenolic stilbenes, isoeugenol, eugenol, ferulic acid, or aromatic amino acids, and on de novo biosynthesis, applying fungi, bacteria, plant cells, or genetically engineered microorganisms.

Podstolski *et al.*, (2002) demonstrated the usual 4-hydroxybenzaldehyde synthase activity from tissue cultures of vanilla orchid *vanilla plantifolia* in which it produce the flavor compound vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillin precursors such as 4hydroxybenzaldehyde.

Renu Agarwal *et al.*, (2003) demonstrated the biotransformation of ferulic acid to vanillin which was carried out using locally isolated bacterial cultures from manure, paddy straw and garbage samples which include *Pseudomonas sp.* was enriched with the ferulic acid thrice which produced very insignificant amount (1.13µg /100 mg) of vanillin.

Shiyi Ou *et al.*, (2004) reported that the ferulic acid have many physiological functions, including antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, and anti-cancer activities.

Ryoghi mitsui *et al.*, (2004) examined the production of vanillin from ferulic acid by the newly isolated *Micrococcus sp.* TA1which is an alkalophilic bacterium which yields the amount of vanillin (35%) per amount of ferulic acid consumed under the conditions with continuous cultivation was higher than that (22%) with batch cultivation.

Slim Abdelkafi *et al.*, (2006) isolated *Halomonas elongata* strain from table-olive fermentation is the first halophilic bacterium to be shown to transform ferulic acid to vanillic acid under hypersaline conditions in which the optimal yield of vanillic acid (86%) was obtained after a 6h reaction using 5mM of ferulic acid and 4g of dry weight of cells are pre grown on ferulic acid and harvested at the end of the exponential phase.

Diana Di Gioia *et al.*, (2007) explored the possibility of obtaining vanillin from bioconversion of ferulic acid derived from enzymatic hydrolysis of wheat branch. The resting cells of an engineered *E. coli* strain, JM109 (pBB1), containing the genes responsible for conversion of

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ferulic acid into vanillin, were used to transform ferulic acid into vanillin. A vanillin molar yield of 50% was obtained

Xuemei Li *et al.*, (2008) reported a bacterium from Vanilla roots with the ability to utilize ferulic acid as the sole carbon source to produce vanillin. The strain was identified as *Enterobacter* sp. It was elucidated for the first time of a ferulic acid metabolism via decarboxylation to produce 4- vinyl guaiacol and a further conversion to vanillin in *Enterobacter* sp.

Prakash Kumar Sarangi *et al.*, (2009) studied isolation of ferulic Acid from Wheat Bran Using different *Streptomyces* isolates in which that the *Streptomyces* isolate S39 showed comparatively better ability than other two *Streptomyces* isolates was confirmed.

Pilanee Vaithanomsat *et al.*, (2009) conducted the feasibility Study on Vanillin Production from *Jatropha curcas* Stem Using Steam Explosion as a Pretreatment in which the obtained hydrolysate contained 1.55 g/L ferulic acid which after that was used as substrate for vanillin production by *Aspergillus niger* and *Pycnoporus cinnabarinus* . The maximum 0.65 g/L of vanillin were obtained with the conversion rate of 45.2% based on the initial ferulic acid.

Michael Dennis Sharp *et al.*, (2009) demonstrated the changed formulation of an ice cream mix, the vanilla flavor profile is clearly altered and by using several analytical technologies the investigation was done do determine the ability to aid in analyzing compounds in vanilla extracts and in model ice cream mixes.

Bicas *et al.*, (2010) reviewed the most important advances in biotechnological production of bioflavors and the use of some agro-industrial residues in such processes also discussed which expresses that this is a rising trend in biotechnology.

Converti *et al.*, (2010) reviewed the overview on the microbial production of vanillin describing the traditional ways of providing vanillin, specifically extraction and chemical synthesis and compared them with the new biotechnological options like bio transformations of caffeic acid, veratraldehyde and mainly ferulic acid and the most influencing factors for the bioproduction and recovery of vanillin.

Morahem Ashengroph *et al.*, (2011) conducted a study to screen strains of bacteria which are able to degrade ferulic acid, and to evaluate the possibility of forming vanillic acid via conversion of ferulic acid under resting cell conditions in which 1 g/l ferulic acid, vanillic acid reached a maximum value of 494mg/l (molar yield of 60%) after 45 h of reaction time using resting cells of strain SHL1 without further optimization study gives the first evidence for ferulic acid biotransformation by *B. licheniformis.*

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Ravendra Kumar *et al.*, (2012) summarizes the physicochemical properties, various synthetic procedures and the various pharmacological activities of vanillin derivatives which was studied to determine the various biological activities.

Balginder Kaur *et.al.*, (2013) emphasised the biotechnological approaches on the production of vanillin from eugenol, isoeugenol, lignin, ferulic acid, sugars, phenolic stilbenes, vanillic acid, aromatic amino acids, and waste residues by applying fungi, bacteria, and plant cells.

Baljinder Kaur *et al.*, (2013) screened a panel of LAB isolates for their ability to release phenolics from agro waste materials like rice bran and their biotransformation to industrially important compounds such as ferulicacid,4-ethylphenol, vanillicacid, vanillin, and vanillylalcohol.

Elena Dal Bello (2013) investigated the possibility of producing vanillin from ferulic acid released enzymatically from wheat bran by using resting cells of *Pseudomonas fluorescens* strain BF131p4 carrying an insertional inactivation of vdh gene and ech and fcs BF13 genes on a low copy number plasmid in which under optimized conditions, vanillin up to 8.46 mM (1.4 g/L) was achieved.

NazilaMotedayen *et al.*, (2013) examined the ability of 10 fungi strains for the degradation of ferulic acid and production of vanillic acid *Aspergillus niger* K8 was chosen as a more suitable fungus for conversion of ferulic acid to vanillic acid, due to its highest potential to produce high concentration of vanillic acid (116 mg/l) compared to other fungi. Bioconversion proves was further carried out with *Phanerochaete chrysosporium* ATCC 24725 for production of vanillin from vanillic acid produced by *A. niger* K8.

Zamzuri *et al.*, (2013) reviewed an overview of bio vanillin production from agro wastes and emphasizes the current application of vanillin as well as the utilization of bio vanillin as an alternative food flavor in which the natural vanillin and the bio-vanillin was compared.

Gallage *et al.*, (2015) outlined the current state of biotechnology-based vanillin synthesis with the use of ferulic acid, eugenol, and glucose as substrates and bacteria, fungi, and yeasts as microbial production hosts.

Shafika *et al.*, (2015) demonstrated the use of methanol and aqueous extracts of pomegranate peels (*Punica granatum*) were screened for phenolic compounds and many techniques were done including the detection of antioxidant activities.

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ShashankMishra *et al.*, (2015) studied the conversion of ferulic acid to vanillin and vanillic acid with the cell extract of *Paenibacillus lactis* SAMS-2001 in which production of vanillic acid (57.3 % molar yield) from ferulic acid within 18 h was observed.

Peng chen *et al.*, (2016) demonstrated that the *B. subtilis* B7-S has the potential for use in vanillin production through bioconversion of ferulic acid which showed the maximum conversion efficiency about 63.30% after 3h of bioconversion.

Kamaliah Abdul Samad *et al.*, (2016) analysed the factors of temperature, pH, agitation, water to-substrate ratio, volume of inoculum, fermentation time, and type of co-culture by employing a 24 fractional Factorial design (FFD) for ferulic acid production from banana stem waste.

Ana Rita da Silva Bernardino (2017) studied the process of bio production of vanillin from ferulic acid by the *Amycolatopsis sp.* ATCC 39116 in bioreactor in which a higher vanillin volumetric productivity of 0.46 g from 0.69 g. of ferulic acid was obtained.

Evernessah Eva Dominus *et al.*, (2018) demonstrate the potential of pineapple crown to produce bio sugar and bio-vanillin which were carried out using the solid state fermentation of by *Phanerochaete chrysosporium*.

Huszalina Binti Hussin (2018) investigated the potential of a one-step natural vanillin production from *C.citratus* leaves hydrolysate by *Phanerochaete chrysosporium*, the white-rot fungi of basidiomycetes in which using leaves within size of 125-249 µm appeared to be suitable for ferulic acid extraction, with 1.12g/L total recovery of ferulic acid by 55 minutes of boiling and under optimum conditions 0.20 g/L vanillin yield was obtained.

Misaki Maeda *et al.*, (2018) studied the production of vanillin and vanillic acid through aerobic oxidation of Japanese cedar (*Cryptomeria japonica*) at 120 °C for 72 h in aqueous alkali solutions with several $Bu4N^+$ and OH^- concentrations (1.25, 2.50, and 3.75 mol/L), where $Bu4N^+$ is an enhancer of the vanillin formation in which vanillin and vanillic acid were produced with the maximum yields of 21.0 and 1.7 wt % respectively, at the 1.25 mol/L $Bu4N^+$ and 3.75 mol/L OH^- concentrations using this treatment of the Japanese cedar with this alternative medium exhibited the moderately decreased vanillin yield of 14.6 wt%, which is, however, much higher than the vanillin yield obtained with a simple 1.25 mol/L NaOH solution.

Francesca Luziatelli *et al.*, (2019) reported that recombinant plasmid-free *E. coli* strains are promising candidates for the production of vanillin at industrial scale and that a reduction of the cost of the bioconversion process requires approaches that minimize the toxicity of both ferulic acid and vanillin.

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Giovanni Camera Rodaa *et al.*, (2019) compared two different membrane processes (pervaporation and dialysis) in view of their utilization in a membrane reactor, where vanillin is synthesized in a green and sustainable way in which ferulic acid from agricultural wastes is used as a precursor.

Ping-Hua Zhang *et al.*, (2019) explored the biodegradation of ferulic acid, by an endophytic fungus *Colletotrichum gloeosporioides* TMTM-13 associated with *Ostryarehderiana* Chun that has great potential for practical application in ferulic acid contaminated environments and the fungal biomass reached a maximum of almost 1.177 g under concentrations of 400–450 mg which indicated that ferulic acid ultimately degraded to vanillin, vanillic acid, aceto vanillone, and dihydro coniferyl alcohol.

Javier García-Hidalg *et al.*, (2020) has demonstrated the microbial degradation of lignin and its related aromatic compound using isolated *Pseudomonas* sp. strain 9.1 from historical waste deposits released from pulp and paper mills along the Baltic Sea coast. The strain accumulated vanillyl alcohol during growth on vanillin in which the deletion of PP 2426 in *P. putida* GN442 engineered to accumulate vanillin resulted in a decrease in by-product (vanillyl alcohol) yield from 17% to -1%.

Kamaliah Abdul Samad *et al.*, (2020) exploits the soil bacteria for production of ferulic acid (FA) to get high yield via co-culture fermentation of banana stem waste (BSW). The maximum FA yield of 394.76 mg/kg was achieved using co-culture of *Bacillus sp.* MB2, *Bacillus sp.* WB8A and B.
pumilus strain WB1A, which was 2.5-fold higher than FA produced by single culture

Pei Ling Tang *et al.*, (2020) conducted a study to evaluate the potential of pineapple peel (PP) and pineapple crown leaves (PCL) as the substrate for vanillic acid and vanillin production and about 202 ± 18 mg L−1 and 120 ± 11 mg L−1 of ferulic acid was produced from the Pineapple peel and pineapple crown leaves in which about 7 ± 2 mg L−1 of vanillic acid and 5 ± 1 mg L−1 of vanillin was successfully produced from Pineapple peel extract via *Aspergillus niger* fermentation.

Sagar S. Arya *et al.*, (2021) summarized the potential bioactivates of vanillin and its derivative with an aim to change the perspective from being a popular flavour to a new age therapeutics molecule.

Joanna C. Sadler *et al.*, (2021) reported a novel pathway in engineered *Escherichia coli* for the direct upcycling of Poly(ethyleneterepthalate) derived monomer terephthalic acid into the value added small molecule vanillin, a flavour compound ubiquitous in the food and cosmetic

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industries, and an important bulk chemical in which after optimisation, 79% conversion to vanillin was achieved, a 157-fold improvement over the initial conditions.

Huseyinkarakaya *et al.*, (2022) investigated the possibility of usage biphasic system in bioconversion of isoeugenol to vanillin by *Pseudomonas putida* (HUT 8100) and the amount of vanillin achieved was about 11.95 g L-1 with 6.2% molar yield within 15 days of bioconversion.

Rita Valerio *et al.*, (2022) discussed the recovery and fractionation of bio-vanillin, from a bioconversion broth, by pervaporation and by vacuum distillation, coupled with fractionated condensation.

Sujatha *et al.*, (2022) employed to create vanillin using *Bacillus subtilis* to ferment wheat bran with 0.05 percent ferulic acid as an persuader and antimicrobial potential of the bio-vanillin was determined.

Tahir Mehmood *et al.*, (2022) demonstrated vanillin production via solid-state fermentation (SSF) by *Enterobacter hormaechei* using different ferulic acid-rich fruit peels as substrates includes pomegranate, banana, and orange. After 24 hours *Punica granatum* (pomegranate) peels yielded maximum bio-vanillin (0.09 mg/g) which offers a cost-effective approach for the industrial production of bio-vanillin.

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MATERIALS AND METHODS

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4. METHODOLOGY

4.1 SAMPLE COLLECTION:

a) SOIL SAMPLE:

Two soil samples were collected at the region of banana tree plantation in Agastheeswaram, Kanyakumari and another from local areas in Thoothukudi in a sterile polythene bag.



Fig:7Image of banana tree where the soil sample 1 was collected



Fig :8 Soil sample 1

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Fig 9: Image of banana tree where the soil sample 2 was collected



Fig 10: Soil sample 2

b) SUBSTRATE:

Fruit peels of *Punica granatum* (pomegranate) was collected. The fruit peels were rinsed, cut into smaller pieces, and oven-dried at 60°C for 48 h. Then, divide it into two equal amounts and one half were grounded into a powder form by using an electrical grinder and another half remains as a peel that act as a source of ferulic acid.

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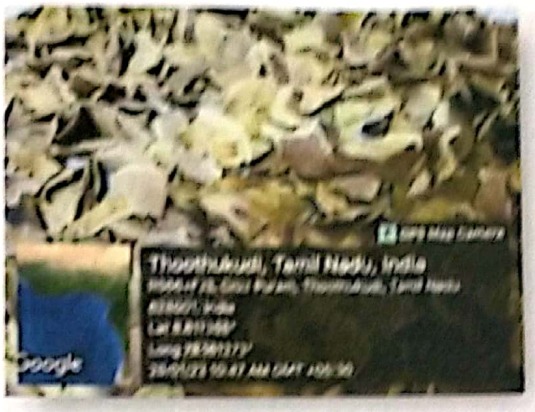


Fig 11: Pomegranate peel sustrate



Fig 12: Pomegranate peel powder

4.3 PREPARATION OF ENRICHMENT CULTURE:

For the isolation of ferulic acid degrading bacteria, enrichment culture was prepared using the soil sample which was collected in a sterile bag and it was brought to the laboratory. Then Ten g of each wet soil sample was suspended in 90 mL of sterile distilled water and this soil suspension was used as inoculum for enrichment cultures. Prepare sterile nutrient broth (NB) 200ml (100 ml in a flask) and then each soil suspension was added separately in 100ml of nutrient broth (NB) medium containing 0.3 g beef extract, 0.5 g peptone and 0.5% NaCl. Ferulic acid (1%) was added to the medium. Then the flasks were incubated at 30°C at 150rpm for 24h.

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4.3 ISOLATION OF FERULIC ACID DEGRADING BACTERIA FROM ENRICHMENT CULTURE:

Prepare sterile nutrient agar plates and hundred µl of enrichment culture was spread on prepared nutrient agar plates using sterile L-rod and again incubated at 30°C for ½ h. After incubation the obtained visible colonies (CFUs) were streaked on a fresh agar plate to obtain pure colonies. From these plates three isolates were chosen for the next primary screening process.

4.4 PRIMARY SCREENING FOR THE FERULIC ACID DEGRADING BACTERIA:

Prepare sterile modified M9 medium about 150ml (50 ml per flask). For primary screening, the isolated bacterial strains were separately inoculated and grown till growth phase in 50 ml of modified M9 medium (MM9), containing Glucose (0.25 g), $(NH4)_2SO_4$(0.1 g), $CaCl_2.6H_2O$ (0.016 g), $MgSO_4.7H2O$ (0.25), KH_2PO4 (0.015 g), and $Na_2HPO4.12H_2O$ (0.75 g) for 24 h at 30°C, and then 1% (1 ml) ferulic acid was added. A loop full of isolated bacterial strains approximately having 100-120 CFUs/ml was inoculated in each of the MM9 media with pH 7. Simultaneously, a control experiment was carried out with 1% (v/v) ferulic acid. After an additional 48 h of incubation at 30°C, potential biotransformation products were separated by acidifying with 10 N H2SO4 and extracted with equal volumes of ethyl acetate. After centrifugation (3,000 rpm for 1 min) organic layer was separated and then used for paper chromatography analysis.

4.5 PAPER CHROMATOGRAPHIC ANALYSIS:

Whatmann filter paper was taken using sterile forceps and handle it by wearing gloves. Then Fifty µl of samples were added to the chromatographic paper (Whatmann). The chromatographic tank paper was irrigated vertically with a solvent system of Butanol: Acetic acid :water(4:1:5 v/v) until the solvent travelled through a certain limit on filter paper . Then the paper was dried and diazotized para nitroaniline was sprayed to get spots. Under these conditions, vanillin appeared. And from the occurrence of spots, ferulic acid degrading microbial isolate was determined. After that, the isolate was undergone the identification process.

4.6 IDENTIFICATION OF THE ISOLATES:

4.6.1 MORPHOLOGICAL IDENTIFICATION:

a) GRAM'S STAINING:

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Isolated colony was taken with the help of an sterile inoculation loop and it was niformly spread over in the centre of a clean microscopic slide. The Bacterial cells were fixed y heating it through passing repeatedly few seconds over a flake. They were stained with a op of crystal violet solution (30 seconds), iodine (30 seconds) followed by ethyl alcohol and afranin (1 to 1.5 minutes) solution. After each staining method the cells were washed under tap ater jet and examined under compound microscope and these bacterial cells were classified as ram negative (G-) or Gram positive (G+) or Gram variable (G±) strains.

he gramnegative bacteria will accept the counter stain and becomes pink, while the ampositive bacteria remain purple colour. Results were observed and tabulated.

eagent used: Crystal violet, Grams Iodine, Alcohol, Saffranin.

MOTILITY TEST:

Mobility of the isolate was observed using SIM agar tubes, which differentiate mobile and nmotile bacteria. For motility testing a fresh 24 hours old broth culture was used. Under sterile ndition, a loopful of culture was taken and stab inoculation was done on the SIM agar tubes d incubated at 37°C for 24 hours. After incubation the results were observed and tabulated.

6.2 BIOCHEMICAL IDENTIFICATION:

INDOLE TEST:

Indole test looks for the ability of bacteria to produce the enzyme trptophanase. If the nzyme is present, it will degrade the aminoacid trptophan in the media and will produce Indole, mmonia, and pyruvic acid. Peptone broth was prepared and sterilized and inoculated with the opful of culture and incubated at 37°C for 24 hours. After incubation kovac's reagent was dded, to detect the production of indole. Formation of cherry ring indicates positive results. he result were recorded and tabulated.

eagents used: kovac's reagent

METHYL RED TEST:

Methyl red is a pH indicator, which is detect the acid-forming bacterial. Take sterile and an test tubes and pour the prepared sterile MRVP broth to each test tube and inoculate them ith the isolated and incubated the tubes at 35- degree Centigrade for 48 hours. After incubation d 3- 5 drops of methyl red to the test tubes and observe the colour change in the medium.

If a stable red color develops on the surface of the medium, after the addition of methyl d indicator, the result is said to be positive.

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If a yellow color develops on the surface of the medium, after the addition of methyl red indicator, the result is said to be negative. The results were recorded and tabulated.

Reagents used: Methyl red indicator.

c) VOGES-PROSKAUER TEST:

Voges-proskauer test performs to detect the neutral-reacting end products (acetoin) when cultivated in specific media. Take sterile and clean test tubes and pour the prepared sterile MRVP broth to each test tube and inoculate them with the isolated and incubated the tubes at 35- degree Centigrade for 48 hours. After incubation add12 drops of V-P solution I (napthol solution) and 2-3 drop of V-P solution II (40% potassium hydroxide) to each test tubes containing isolates. Then cotton plug each test tubes and shake them for 30 sec and expose the medium to oxygen. Keep them in undisturbed condition for 10-15 minutes and observe the color change in medium.

The result will be *positive*, If brown colour develops on the surface of the medium, within 15 minutes to 1 hour after the addition of V-P solution I &II.

The result will be *negative*, If no develops on the surface of the medium, within 15 minutes to 1 hour after the addition of V-P solution I &II. The results were recorded and tabulated. Reagent used: V-P solution I (napthol solution) and V-P solution II (40% potassium hydroxide)

d) CITRATE TEST:

Citrate test determines the ability of microrganisms to utilize the citrate. Some bacteria have the capability to convert the salts of organic acids, for example. Sodium citrate to alkaline carbonates. Certain bacteria use citrate as the sole carbon source. Simmon citrate agar was prepared, sterilized, and inoculated with loopful of culture and incubated at 37° C for 24 hours. Conversion of green to blue colour indicates the positive result. The results were recorded and tabulated.

e) CATALASE TEST:

The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria. Catalase has one of the highest turnover numbers of all enzymes such that one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen in a second.

Catalase production and activity can be detected by adding the substrate $H_2O_2$to an appropriately incubated (18 to 24 hour) slant culture. Organisms which produce the enzyme catalase break down the hydrogen peroxide, and the resulting O_2production produces bubbles in the reagent drop, indicating a positive test. Organism lacking the cytochrome system also lack

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the catalase enzyme and are unable to break down hydrogen peroxide, into water and $O_2$and are catalase negative.

$$2H2O \xrightarrow[\text{peroxidase}]{\text{catalase}} 2H2O + O2 \text{ (Gas bubbles}$$

Reagent used: Hydrogen peroxide.

f) OXIDASE TEST:

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. The test depends on the ability of certain bacteria to produce indophenol blue from the oxidation of dimethyl-p-phenylene and α-naphthol. In the presence of the enzyme cytochrome oxidase (gram-negative bacteria) the N, N-dimethyl-p-phenylenediamine oxalate and α- naphthol react to indophenol blue.

Oxidase disc was placed on the clean petriplate. A loopful of pure culture was placed on it. The appearance of blue colour over the oxidase disc within 30 seconds indicates positive result.

g) STARCH HYDROLYSIS:

Amylases are a class of enzymes that are capable of digesting these glycosidic linkages found in starches. Amylases can be derived from a variety of sources. Starch agar is an example of differential medium which tests the ability of an organism to produce certain alpha-amylase and oligo-1,6-glucosidase that hydrolyze starch. Starch molecules are too large to enter into the bacterial cells so some bacteria will secrete exoenzymes that will degrade starch into subunits that can be then easily utilized by the organism.

Starch agar is a simple nutritive medium with starch as the carbon source. Starch agar plates were prepared under sterilized conditions and inoculated with the bacterial culture, incubated at 37° C FOR 24 HOURS. Since no colur change occurs in the medium when organism hydrolyze starch, iodine solution is added to the plate after incubation. Iodine turns blue, purple or black (the colour depends on the concentration of iodine used) in the presence of starch. A clearing around the bacterial growth shows that the organism has hydrolyzed starch. The results were recorded and tabulated.

Reagent used: Iodine

h) GELATIN HYDROLYSIS:

Gelatin is a protein derived from the connective tissues of vertebrates, that is, collagen. The test was done to determine the ability of an organism to produce extracellular proteolytic enzymes (gelatinases) that liquefy gelatin, a component of vertebrate connective tissue. Prepare

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sterile gelatin agar plates and inoculate a heavy inoculum of an 18- to 24-hour-old isolate onto culture plates and incubate the inoculated gelatin plates at 35°C for 24 hours. Results were recorded and tabulated.

Occurrence of clear zones around the colonies indicates positive result.

No clear zone occurs indicates negative result.

i) UREASE TEST:

Urea is a nitrogen containing compound that is produced during the decarboxylation process of the aminoacid arginine in the urea cycle. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in the amide compounds such as urea and forms the alkaline end product ammonia.

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2H_2O \xrightarrow{\text{Urease}} \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Water}}{H_2O} + \underset{\text{Ammonia}}{2NH_3}$$

Christensen's urea agar plates was prepared under sterilized conditions and the bacterial culture was streaked and incubates at 37° C for 24 hours. The presence of urease is detectable when the organisms are grown in a Christensen's urea medium containing the PH indicator phenol red. As the substrate urea is split into its products, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to deep pink. This confirms the presence of urease.

The result was tabulated.

After the identification of the isolate then it was used to perform the bioconversion process.

4.7 BIOCONVERSION PROCESS:

Two sets of basal medium about 200 ml (100 ml per flask) was prepared and ten ml growth culture of screened strain was inoculated into 250 ml flask containing 100 ml basal medium. The composition of the basal medium was (g/L) 20 g glucose, 8 g yeast extract, 0.2 g K_2HPO4, 0.5 g $MgSO_4$, 1.3 mg $CaCl_2$.The pH of the media was adjusted to 6.5. and the bacterial strain along with the medium was incubated at 130 rpm at 30°C for 24 h. After incubation for the growth of organism, substrate was added to the medium. One was added with the pomegranate peel powder about 20g and another was added with the dried pomegranate peel substate, and the substrate water ratio was set at 1:3 *w/v* using sterilized basal media. After

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addition of ferulic acid rich fraction to the medium the flasks were incubated for another 48 h at 130 rpm at 30°C for bioconversion of ferulic acid into vanillin.

4.8 EXTRACTION OF BIOVANILLIN:

The bioconversion medium was centrifuged at 10,000 rpm for 10 min to remove the biomass. Collect the supernatant and acidify it to pH 2 by using 6 N HCL. Then, phenolic compounds were extracted by adding 3 volumes of ethyl acetate to the acidified supernatant. After extraction process the sample was undergone the analytic methods.

4.9 ANALYTICAL METHODS:

Qualitative analysis of vanillin production was done. For qualitative analysis of vanillin, paper chromatography method was used. Using sterile forceps, Whatmann filter paper was taken off and it was handled while putting on gloves. The chromatographic paper was then infused with 50 µl of vanillin samples (Whatmann). The butanol, acetic acid, and water (4:1:5) solvent system was used to fill the chromatographic tank and the paper was vertically placed in the tank until the solvent passed through a predetermined limit on the filter paper. The paper was then dried, and patterns were created by spraying diazotized para nitroaniline on it. In these circumstances, vanillin manifested.

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RESULT AND DISCUSSION

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5. RESULTS AND DISCUSSION:

In this present work, ferulic acid degrading bacteria was isolated from the soil sample of banana plantation and identified and used for the bioconversion process of ferulic acid to bio-vanillin using pomegranate peels as a substrate.

5.1 ENRICHMENT CULTURE:

The ferulic acid degrading bacteria was isolated from the soil sample of banana plantation and it was subjected to enrichment process. The enrichment culture is the source of ferulic acid degrading microbes which act as a inoculum for isolating the ferulic acid degrading bacteria. Kamalia Abdul Samad *et al* ., in 2020 reported that the enrichment culture was used for the isolation of ferulic acid degrading bacteria from the soil of banana plantation.

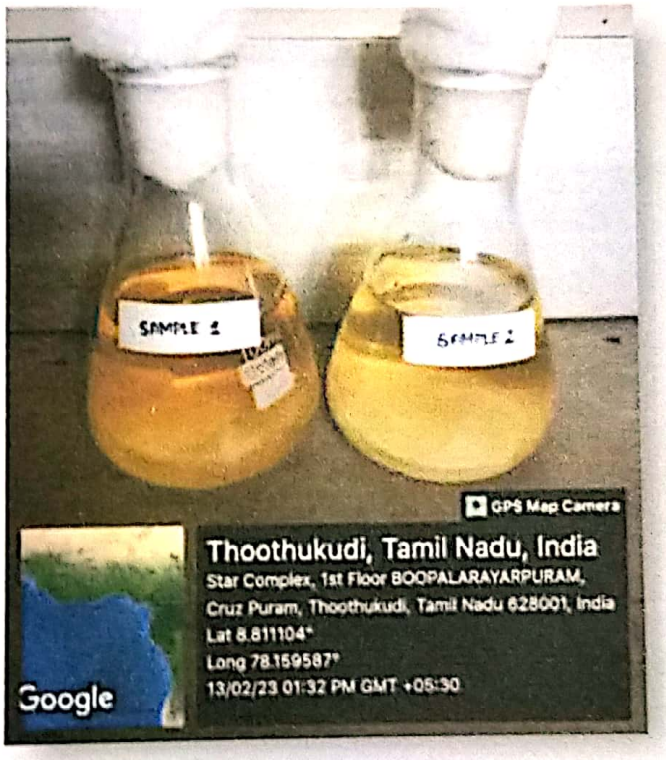


Fig 13: Enrichment culture of Sample 1 and Sample 2

5.2 ISOLATION OF FERULIC ACID DEGRADING BACTERIA:

Using enrichment culture as an inoculums, spread plate technique was performed in nutrient agar plates which was incubated at 37° C for 24 hours. From these plates 3 isolates were chosen and undergone primary screening. Renu Agrawal *et al*., (2003) isolated the ferulic acid

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degrading bacteria from the manure, paddy straw and garbage samples for the bioconversion of ferulic acid to vanillin



Fig 14 : Nutrients agar plates containing the ferulic acid degrading bacteria



Fig 15: Isolate 1

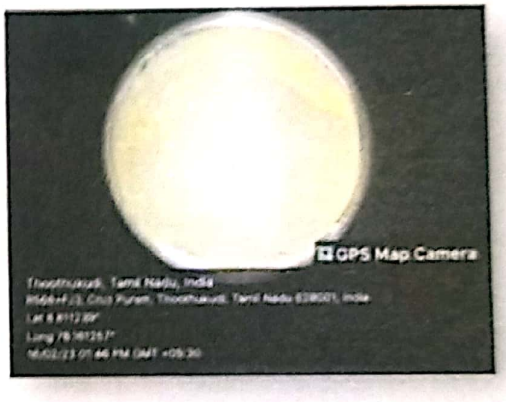


Fig 16: Isolate 2

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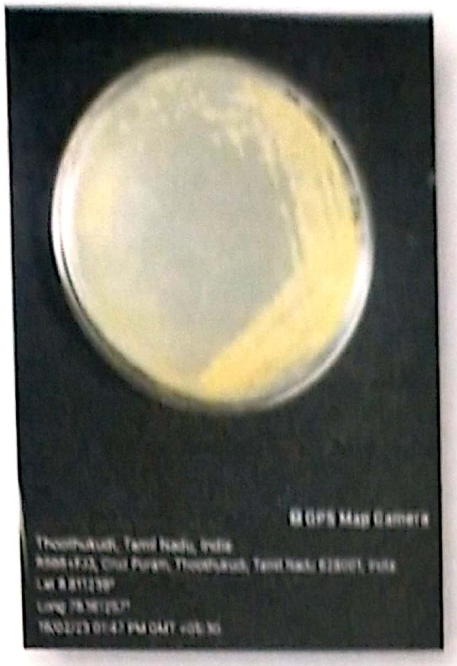


Fig 17: Isolate 3

TABLE 2 - COLONY MORPHOLOGY OF THE ISOLATES

CHARACTERISTICS	ISOLATE1	ISOLATE 2	ISOLATE 3
COLOUR	Powdery White	Glistening White	White
SHAPE	Circular	Circular	Circular
ELEVATION	Slightly raised	Raised	Raised
MARGIN	Entire	Undulate	Entire
SURFACE	Rough	Glossy	Rough
OPTICAL CHARACTERISTICS	Opaque	Translusent	Opaque

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5.3 PRIMARY SCREENING FOR THE FERULIC ACID DEGRADING BACTERIA:

The 3 bacterial isolates were inoculated in sterile M9 medium separately and incubated at 30° C for 24 hours and 1% ferulic acid was added to it and again incubated at 30° C for 48 hrs. Bushra *et al.*, in 2017 studied the production of vanillin by a novel bacterium from waste residues of rice bran oil in which M9 medium used for the screening of the ferulic acid degrading bacteria.

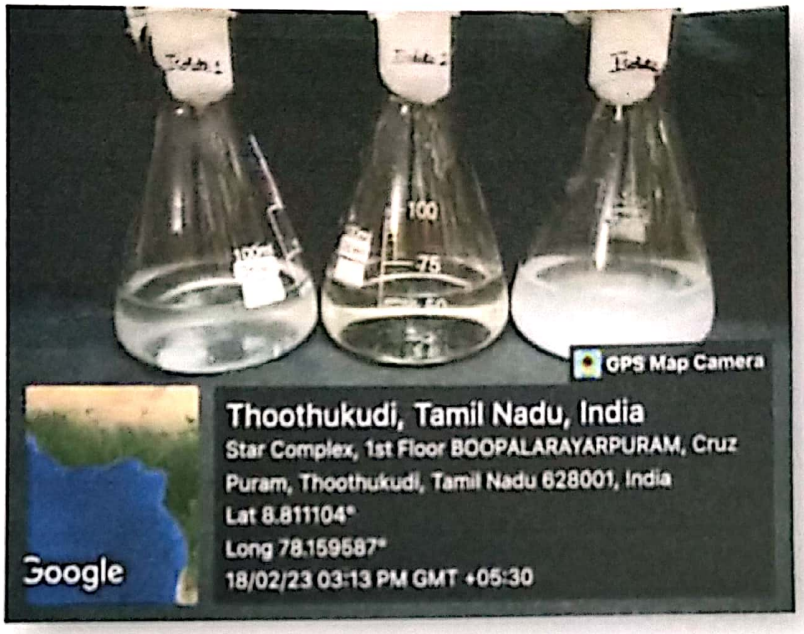


Fig 18: Primary screening of ferulic acid degrading bacteria by growing 3 isolates in M9 medium supplemented with Ferulic acid.

5.4 EXTRACTION OF BIOVANILLIN:

The M9 medium containing the 3 bacterial isolates was undergone acidification with 10 N H_2SO4 and extracted with equal volumes of ethyl acetate which was then centrifuged at 3,000 rpm for 1 min to obtain the organic layer which was used for paper chromatographic analysis. Chalikkaran *et.al.*, (2020) reported that the microbial conversion of vanillin from ferulic acid extracted from raw coir pith in which he used the extraction and acidification process.

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Fig 19: The organic layer after the acidification and extraction process

5.5 PAPER CHROMATOGRAPHIC ANALYSIS:

Using Butanol: Acetic acid : Water as a solvent system and Diazotized p-nitroaniline as a spraying agent orange yellow spots was obtained which confirmed the production of vanillin and the Retardation Factor (R_f) value was calculated. Yataro Obata *et al.*, (1962) detected the phenolic compounds by chromatography using different spraying agents included the diazotized p nitroaniline.

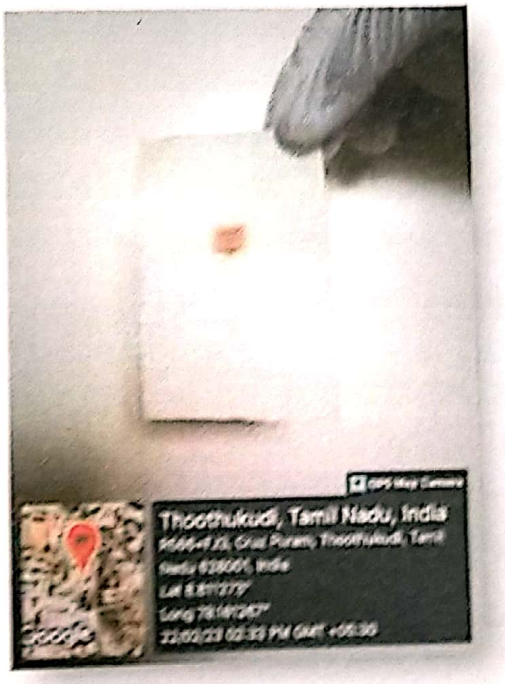


***Fig 20:* Paper chromatography done with the extract from primary screening**

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The R_f (retardation factor) value is the ratio of the solute's distance travelled to the solvent's distance travelled. The R_f value in paper chromatography was calculated by dividing the distance travelled by the component by the distance travelled by the solvent.

$$R_F = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

$$= \frac{2.6}{6.8}$$

$$= 0.38$$

The Rf value was **0.38.**

The degradation of ferulic acid by the isolate was determined by the appearance of the medium and the accumulation of bioconversion products was determined by the spot appeared on the Whatmann filter paper. In this isolate 2 was actively degraded the supplied ferulic acid compared to other two isolates. So the isolate 2 was chosen for further analysis and undergone the identification process.

5.6 IDENTIFICATION OF THE ISOLATE:

Various tests such as morphological, biochemical tests were carried out to identify the isolate. The morphological identification methods were gram staining and motility test. The biochemical identification methods were Indole test, Methyl red test, Voges-Proskauer test, Citrate test, Catalase test, Oxidase test, Starch hydrolysis, Gelatin hydrolysis, Urease test.

5.6.1 MORPHOLOGICAL AND BIOCHEMICAL IDENTIFICATION:

The isolate was identified as gram negative, non-motile, rod shaped bacterium. It can utilize citrate as their carbon source. It can produce an enzyme catalase that breakdown hydrogen peroxide to water and oxygen and do not hydrolyze Starch. It can able to produce indo phenol blue from the oxidation of dimethyl-p-phenylene and α- naphthol. Based on the morphological and biochemical characters isolate 2 may be *Enterobacter* sp. The results were tabulated.

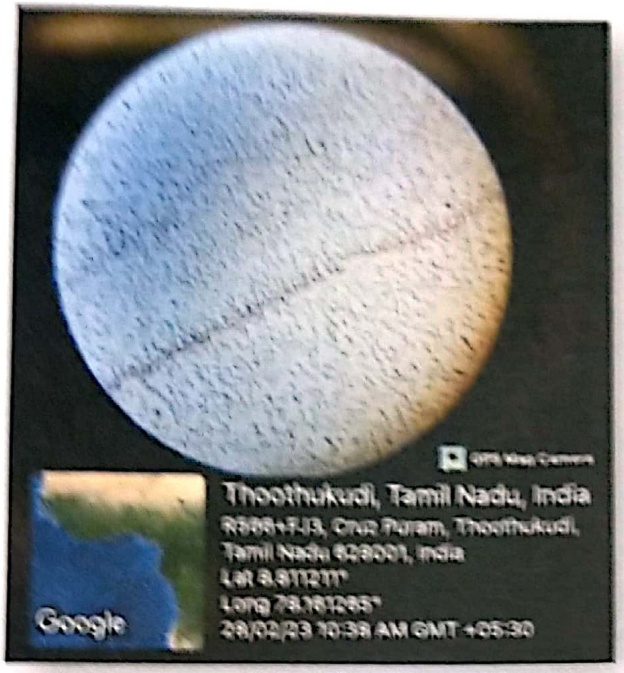
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TABLE 3 - IDENTIFICATION OF THE ISOLATE

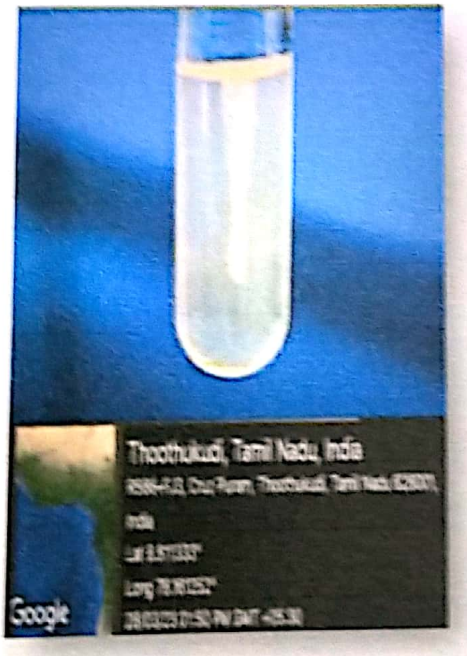
CHARACTERISTICS	ISOLATE 2
MORPHOLOGICAL CHARACTERISTICS	
GRAM'S STAINING	GRAM NEGATIVE ROD
MOTILITY TEST	MOTILE
BIOCHEMICAL CHARACTERISTICS	
INDOLE TEST	NEGATIVE
METHYL RED TEST	NEGATIVE
VOGES-PROSKAUER TEST	POSITIVE
CITRATE TEST	POSITIVE
OXIDASE TEST	POSITIVE
CATALASE TEST	POSITIVE
STARCH HYDROLYSIS	NEGATIVE
GELATIN HYDROLYSIS	NEGATIVE
UREASE TEST	NEGATIVE

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GRAM'S STAINING (Fig 21)

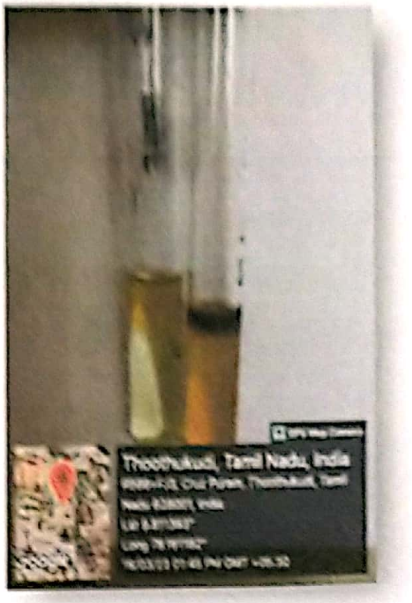


MOTILITY TEST (Fig 22)

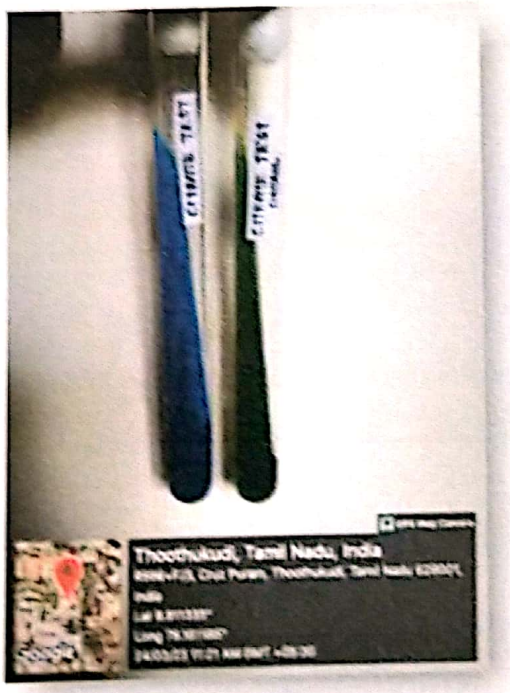


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VOGES-PROSKAUER TEST (Fig 25)



CITRATE TEST (Fig 26)

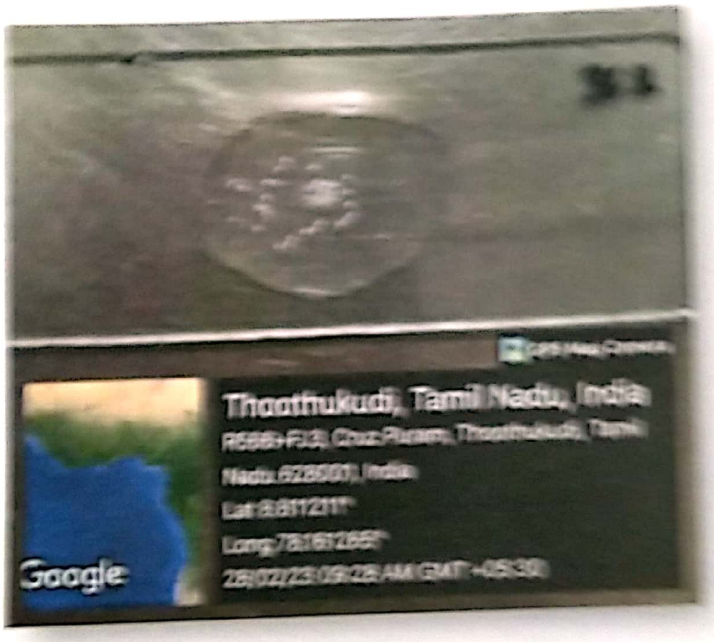


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OXIDASE TEST (Fig.27)



CATALASE TEST (Fig.28)



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5.8 BIOCONVERSION:

Two sets of sterile basal medium (100 ml per flask) was prepared and ten ml of inoculum of isolate 2 was inoculated into it and incubated at 130 rpm at 30°C for 24 h. After incubation, substrate was added to the medium. One was added with the pomegranate peel powder and another was added with the dried pomegranate peel, in substrate water ratio 1:3 *w/v*. After addition of ferulic acid rich fraction to the medium the flasks were incubated for another 48 h at 130 rpm at 30°C for bioconversion of ferulic acid into vanillin. M. Sujatha *et al.*, in 2022 performed the bioconversion process to obtain vanillin using wheat bran as a substrate. Tahir *et al.*, in 2022 demonstrated the vanillin production using different ferulic acid rich peels as a substrate which includes the pomegranate peel wastes.

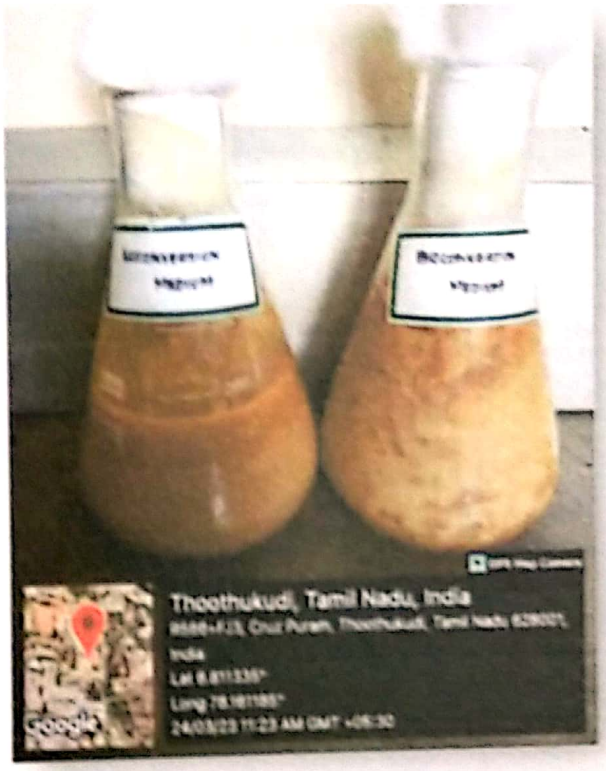


Fig 32: Bioconversion of pomegranate peel and pomegranate peel powder using isolate 2

5.9 EXTRACTION OF BIOVANILLIN:

The fermented medium was centrifuged at 10,000 rpm for 10 min and the collected supernatant was acidified with 10 N H_2SO_4. Then, phenolic compounds were extracted by

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adding 3 volumes of ethyl acetate to the acidified supernatant. Nuzrat *et.al.*, in 2017 performed the extraction and acidification process to obtain bio-vanillin from the bioconversion medium.



Fig 33: Picture showing pellet and supernatant obtained from bioconversion medium after centrifugation.

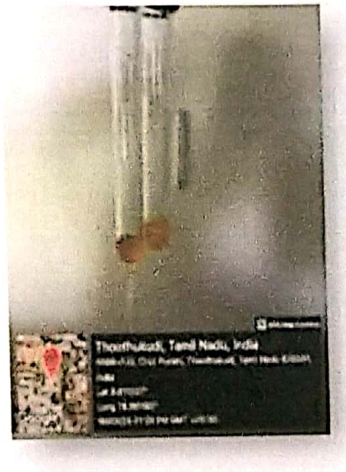


Fig 34: Supernatant collected from the bioconversion media after centrifugation

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Fig 35: Acidified supernatant

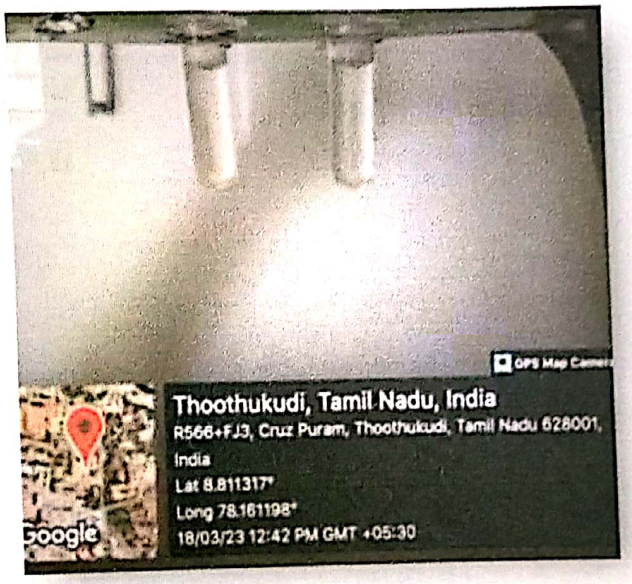


Fig 36: Organic layer obtained after extraction using ethyl acetate.

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5.10 ANALYTICAL METHODS:

For qualitative analysis of vanillin, paper chromatography method was used. Using Butanol : Acetic acid : Water as a solvent system and ferric Diazotized p-nitroaniline as a spraying agent orange yellow spots was obtained which confirmed the production of vanillin and the Retardation factor (R_f) value was calculated. J Pitelson in 2020 performed the quantitative determination of vanillin by paper chromatography. Uk Essays published a report on vanillin physiochemical properties and its production, uses in which the R_f value of vanillin was given as 0.35+/- 0.3.

The R_f (retardation factor) value is the ratio of the solute's distance travelled to the solvent's distance travelled. To calculate the R_f value in paper chromatography, divide the distance travelled by the component by the distance travelled by the solvent.

$$R_F = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

$$= \frac{3}{8.2}$$

$$= \mathbf{0.36}$$

The Rf value is about **0.36**.

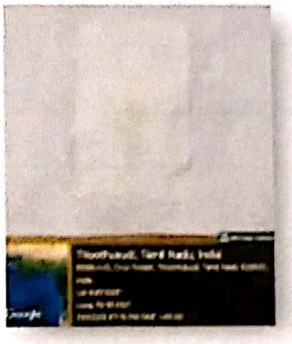


Fig 37: Paper Chromatography using Bioconversion media

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SUMMARY AND CONCLUSION

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CONCLUSION

Using chemical procedures to produce flavours includes a negative impact on the environment and poor product quality. Natural flavours are mostly preferred by most of the population as since they are preferred on the global market despite being more expensive. However, due to low concentrations of the desired product in plants, which increases the extraction and purification procedures, as well as the harvest's dependent on seasonal, climatic, and political factors, the product ion of natural flavours by direct extraction from botanical sources can no longer satisfy the high market demand As since the biotechnological methods get bloomed for the production of natural flavours in which the Vanillin is one of the finest and most widely used flavours in the world. The flavour industry is becoming more and more interested in producing vanillin from other natural sources because of the price difference between natural and synthetic vanillin and the rising consumer trend towards the healthy product. vanillin derived from vanilla pods has a fluctuating and expensive price in the market.so as an alternative method biotechnological methods were carried out for the vanillin production using natural subtrates. However, considering that the world produces three million tonnes of pomegranates annually and that the fruit's peel and seeds make up about 54% of it, this amounts to 1.62 million tonnes of trash. Because of the enormous amount of waste that is produced, it is essential to identify effective ways to valorize it by maximising the extraction of bioactive compounds from pomegranate wastes and then turning them into value-added goods.

This present study focused on the isolation and identification of ferulic acid degrading bacteria from the soil of Banana plantation for the production of bio-vanillin by bioconversion process using ferulic acid rich pomegranate peels as a substrate Naturally the soil was enriched with microorganisms which used as an medium for obtaining the ferulic acid degrading microbes which used for the bioconversion process of pomegranate peels.

From this study I conclude that the biotechnological methods pave an alternative way for the production of flavours includes vanillin using bioconversion process in which pomegranate peels were used as a substrate.

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SUMMARY

In this study the soil sample was collected from the banana plantation in Agastheeswaram, Kanyakumari and from local areas in Thoothukudi. Using enrichment culture of the soil sample the ferulic acid degrading bacteria was isolated using Nutrient agar plates in which 3 isolates were chosen for primary screening. Then primary screening of the isolates were done using M9 medium supplemented with1% ferulic acid and incubated at 37°C for 24 hours. Then acidified with 10 N H_2SO_4 and extracted with equal volumes of ethyl acetate and then centrifuged at 3,000 rpm for 1 min to obtain an organic layer. Then paper chromatographic analysis was done using Butanol: Acetic acid: Water as a solvent system and Diazotized p-nitroaniline as a spraying agent. Orange yellow spots was obtained which confirmed the production of vanillin and the Retardation Factor (R_f) value was calculated. The Rf value is about 0.38 cm. The degradation of ferulic acid by the isolate was determined by the appearance of the medium and the accumulation of bioconversion products was determined by the spot appeared on the Whatmann filter paper. In primary screening the isolate 2 actively degraded the ferulic acid compared to other two isolates. So the isolate 2 was chosen for bioconversion process and for the identification of the isolate 2 morphological, biochemical tests were carried out. The isolate 2 showed Gram negative and showed positive for Indole test, Voges-Proskauer test, Citrate test, Oxidase test, Catalase test and do not hydrolyze starch, Gelatin and negative for motility test, Methyl red test, Urease test. Based on the morphological and biochemical characters isolate 2 may be *Enterobacter* sp. Then bioconversion process was done using pomegranate peels and its powder as a substrate in basal medium with a substrate water ratio of 1:3 *w/v*. 200 ml of basal media was inoculated with 10 ml of inoculum and incubated at 130 rpm at 30°C for 24 h and after addition of pomegranate peels substrate further incubated for 48 hours at 130 rpm at 30°C for bioconversion of ferulic acid into vanillin. From the collected supernatant of the fermentation medium acidification was done with 6 N HCl and the biovanillin was extracted by adding 3 volumes of ethyl acetate to the acidified supernatant. Qualitative assessment of vanillin was performed using paper chromatography with the organic layer which was obtained from the extraction process, in which Butanol Acetic acid: Water was used as the solvent system and Diazotized p-nitroaniline as a spraying agent. Orange yellow spot was obtained which confirmed the production of vanillin and the Retardation Factor (R_f) value was calculated. The R_f value is about 0.36 cm.

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9. ANNEXURE

MEDIA

NUTRIENT AGAR (PH 6.9)

PEPTONE	7.0g
BEEF EXTRACT	3g
AGAR	15 g
DISTILLED WATER	1000 ml

NUTRIENT BROTH (PH 6.9)

PEPTONE	7.0g
BEEF EXTRACT	3g
DISTILLED WATER	1000 ml

MODIFIED M9 MEDIUM (PH 7)

GLUCOSE	0.25 g
DIAMMONIUM SULPHATE	0.1 g
CALCIUM CHLORIDE	0.016 g
MAGNESIUM SULPHATE	0.25 g
POTASSIUM DIHYDROGEN PHOSPHATE	0.015 g
DISODIUM HYDROGEN PHOSPHATE	0.075 g
DISTILLED WATER	50 ml

BASAL MEDIUM (PH 7)

GLUCOSE	20 g
YEAST EXTRACT	8 g
DIPOTASSIUM HYDROGEN PHOSPHATE	0.2 g
MAGNESIUM SULPHATE	0.5 g
CALCIUM CHLORIDE	1.3 g
DISTILLED WATER	1000 ml

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Certificate of Participation

This to certify that Ms/Mrs/Mr/Dr. Ajmal Rizwana . A.S , II . M.Sc . Microbiology , St. Mary College participated / presented his / her research paper entitled Production of Bio - vanillin from Pomegranate peels.

in the National Seminar on 'EMERGING TRENDS AND OPPORTUNITIES IN MICROBIOLOGICAL RESEARCHES' organised by PG & RESEARCH DEPARTMENT OF BOTANY, V. O. CHIDAMBARAM COLLEGE, THOOTHUKUDI on 10.03.2023.

Dr. A. Doss
Organising Secretary

Dr. C. Lakshmanan
Head of the Department

Dr. C. Veerabahu
Principal

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EVALUATION OF WOUND HEALING & BLOOD CLOTTING POTENTIAL OF SPIDER WEB, AND SYNTHESIS OF SILVER NANOPARTICLES & ITS BIOFILM ACTIVITY FROM SPIDER WEB

A DISSERTATION SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated to Manonmaniam Sundaranar University

In partial fulfilment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

MUHSINA THAHIRA M. A.

(REG. NO. 21SPMB02)

Under the Guidance of

Dr. C. Siluvai Kirubagari Anceshia, M. Sc., Ph. D.

DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS)

THOOTHUKUDI – 628001

APRIL – 2023

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DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS),

THOOTHUKUDI – 628001

APRIL – 2023

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CERTIFICATE

This is to certify this dissertation work entitled "**Evaluation of wound healing and blood clotting potential of spider web, and synthesis of silver nanoparticles & its biofilm activity from spider web**" submitted to **ST. MARY'S COLLEGE (AUTONOMOUS), Thoothukudi** affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY, Tirunelveli** in partial fulfillment for the award of "Master of Science in Microbiology" is a bonafide research carried out by **Ms. MUHSINA THAHIRA M. A.** under the guidance and supervision of **Dr. C. Siluvai Kirubagari Aneeshia,** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi, for academic year 2022-2023.

SIGNATURE OF THE GUIDE

SIGNATURE OF THE HOD

Dr. Joys Selva Mary Albert
Head
Department of Microbiology
St. Mary's College (Autonomous)
Thoothukudi

SIGNATURE OF THE DIRECTOR

Director
Self Supporting Courses
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATURE OF THE PRINCIPAL

Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

18/4/2023

SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE: 05 · 04 · 2023

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DECLARATION

I hereby declare that the Dissertation work entitled **"Evaluation of wound healing and blood clotting potential of spider web, and synthesis of silver nanoparticles and its biofilm activity from spider web"** is a bonafide record of the work completed by me during the academic year 2023 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundharanar University. I also affirm that this is an original work done by me under the supervision of **Dr. C. Siluvai Kirubagari Aneeshia,** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

C. Aneesh

SIGNATURE OF THE GUIDE

M.A. Muhsina Thahira

SIGNATURE OF THE STUDENT

PLACE: THOOTHUKUDI

DATE : 05.04.2023

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MUHSINA THAHIRA M. A.

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CONTENTS

S.NO	TITLE	PAGE NO.
1.	INTRODUCTION	1-10
2.	AIM AND OBJECTIVE	11
3.	REVIEW OF LITERATURE	12-18
4.	MATERIALS AND METHOD	19-23
5.	RESULT AND DISCUSSION	24-30
6.	CONCLUSION	31
7.	SUMMARY	32-33
8.	BIBLIOGRAPHY	

Scanned with CamScanner

LIST OF PLATES

PLATE NO.	TITLE	PAGE NO.
Plate 1.1	*Crossopriza lyoni*	4
Plate 4.1	Spider web of *Crossopriza lyoni*	19
Plate 4.2	Processed web sample	19
Plate 4.3	Spider web extract	22
Plate 5.1	Test and control blood sample (duration: 4 minutes)	26
Plate 5.2	Reaction mixture at 0 hrs	27
Plate 5.3	Reaction mixture after 48 hrs	27
Plate 5.4	Test and control tubes for biofilm inhibition	30

Scanned with CamScanner

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
Table 5.1	Effect of topical application of sample on excision wound model (Wound area mm2)	24
Table 5.2	Effect of topical application of sample on incision wound model	25

Scanned with CamScanner

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
Figure 5.1	FT-IR spectrum of the pure LDPE	28

Scanned with CamScanner

LIST OF ABBREVIATIONS

S.NO	ABBREVIATION	EXPANSION
1	NPs	Nano Particles
2	FT-IR	Frontier Transform Infrared spectroscopy
3	g	Gram
4	w/w	Weight for Weight
5	cm	Centimeter
6	mm	Millimeter
7	ml	Millilitre
8	M	Molar
9	rpm	Revolutions Per Minute
10	AgNP	Silver Nanoparticles
11	KBr	Potassium Bromide
12	µL	Micro litre
13	AgNO3	Silver nitrate
14	SEM	Standard Error of the Mean

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15	**mg**	Micro gram
16	**nm**	Nano meter

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INTRODUCTION

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1. INTRODUCTION:

Spiders have been living on Earth for about 400 million years. They are found all over the world except in Antarctica. Spiders are one of the oldest species in the world (Yunqing Gu *et al.*, 2020). About 40000 species of spiders are found in the world approximately (A. Lateef *et al.*, 2015). Spiders belong to phylum arthropoda. The four major groups of arthropods are Chelicersta (spider and scorpions), Crustacea (shrimps, lobster, and crabs), Tracheata (insects and myriapods) and the extinct trilobites (Ruppert *et al.*, 2004). Spider contains 8 legs, have chitinous coverings and two body regions, the cephalothorax and the abdomen (Glime, 2013). Spiders lack antennae, but have various has hairs penetrating their chitinous covering (A. Lateef *et al.*, 2016).

It is reported that the spider webs have existed for at least 100 million years, as witnessed in a rare find of early Cretaceous amber from Sussex, Southern England (D. Chakraborty, 2009). Spider silk are made of fiber protein which is spun by spiders. Silk is usually made by spiders to make spider webs which acts as a sticky net to catch prey or for other purposes. Some spiders do not spin web at all. The spider silk is said to be 5 to 6 times stronger than steel by weight, also stronger and more elastic than any known natural or synthetic fiber on earth. Spider silk has amazing stretching properties. They can stretch up to 140% of its length without breaking. Spider silk is waterproof and does not break even at low temperature of -40°C (Rajni Singh and Shraddha Jain, 2017). The silk is resistant to twisting and swinging, it can tolerate a great deal of energy before breaking (Jennifer Ouellette, 2012). Spider silk is secreted from the special abdominal glands of spiders. The silk quickly hardens (polymerizes) as it is spun. Spider silk is thin and soft. Spider silk is used for predation, reproduction, nesting and other life activities. The protein present in spider silk is non-toxic (Yunqing Gu *et al.*, 2020).

Types of spider silk:

All spiders produce silk for different purposes. A single type of spider can produce up to 6 different types of silk for various purposes.

The types being major ampullate (dragline) silk which is used to construct the outer covering of the web. This is the most strong and tougher type of silk, then minor ampullate silk which is stretchable and used in temporary scaffolding during construction of web, it is being used for making bandages, surgical threads. Capture spiral silk is used for the capturing

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lines of web, these are sticky and extremely tough which is used in making nets, parachutes, ropes etc. Aciniform silk is used to wrap and capture prey. Tubiliform silk is the stiffest form and is used for the protection of egg sacs, also used in making wear- resistant, light weight clothing. Piriform silk is used for the construction of a stable web, which is being used in the making of reusable bottles (Rajni Singh and Shraddha Jain, 2017).

Uses:

Spider silk has been widely exploited and said to be used as fishing lines by Polynesian fisherman, to construct water repellent hats by New Guinea tribes, in making wear- resistant shoes and cloth, strong ropes, nets, parachutes, as bullet proof vests etc., (Powers, 2013; Singh *et al.*, 2012). Spider silk has been used to make violin strings by a Japanese scientist in 2012 (Jennifer Ouellette, 2012). Silk belongs to a type of bio elastic fiber. During the time of World War II, spider silk was used as cross hair in the optical device of the sighting system of telescopes, guns (K. Thangavelu *et al.*, 2017; B. L. Thiel *et al.*, 1996; M. Heim *et al.*, 2009; J. A. Kluge *et al.*, 2008).

Spider silk possess a variety of medicinal properties such as wound healing and so on. Due to its bio compatible, biodegradable, strong nature they can be used to make artificial tendons and ligaments, tissue repair materials, sutures. They do not cause any strong immune, inflammatory and allergic responses. This property is useful in curing slow healing wounds such as diabetic ulcers. Spider cobwebs have been used for pollution monitoring in industrial and residential areas (Hose *et al.*, 2002; Ayedun *et al.*, 2013). Spiders have been also used as bio control agents in reducing populations of pests on farmlands (Maloney *et al.*, 2003). The evaluation of spider venom has been done for the treatment of cardiac arrhythmia (Novak 2001), Alzheimer's disease (Lewis and Garcia, 2003) and erectile dysfunction (Andrade *et al.*, 2008). Spider silk has been employed as additives in cosmetic such as shampoo, soaps, creams, nail varnishes to enhance the brightness, softness and toughness of the products (Vendrely and Scheibel, 2007). Due to its extremely beneficial properties spider silk is being manipulated widely in the past few decades. Since spiders are carnivores and do not like to live in groups, they are difficult to grow in large quantities (A. C. Maria *et al.*, 2018) hence by transgenic technology, spider genes are transferred to silkworms and other animals, plants and microorganisms to produce spider silk in large quantity (M. E. Alan *et al.*, 2018).

Artificial spider silk is used to make biodegradable mesh which can replace the extracellular matrix that our own cell generate and also helps in accelerating growth of new

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tissue (Rajni Singh and Shraddha Jain, 2017). Spider silk also has applicability as drug carriers, they tend to slowly release the antibiotic added to it. Dastagir *et al.*, (2020) developed artificial blood vessel using natural spiders silk as a supporting matrix. The results showed that it has mechanical properties equivalent to those of natural blood vessels, it can make cells adhere, differentiate and proliferate.

Chemical Structure:

Spider silk is a natural polypeptide, polymeric protein and is in the scleroprotein group which also encompasses collagen (in ligaments) and keratin (nails and hair). These are all proteins which provide structure. The protein in dragline silk is fibroin (Mr 200,000-300,000) which is a combination of the proteins spidroin 1 and spidroin 2. The exact composition of these proteins depends on factors including species and diet. Fibroin consists of approximately 42% glycine and 25% alanine as the major amino acids. The remaining components are mostly glutamine, serine, leucine, valine, proline, tyrosine and arginine. Spidroin 1 and spidroin 2 differ mainly in their content of proline and tyrosine.

Silk assembly:

Silk assembly *in vivo* is a remarkable process. The spider silk is spun from a structure called spinnerets present in the abdomen, usually on the underside to the rear. The number of spinnerets varies with different species but most have clusters. Spigots, a collection of nozzle like structures are present at the end of each spinneret. A single silk thread comes out of each. The silk is pulled out by gravity or the spider's hind leg. The initiation of silk assembly occurs when the silk dope is passed through the spinning duct accompanied by extraction of water, sodium and chloride (F. Vollrath *et al.*, 1998; E. K. Tillinghast *et al.*, 1984). The potassium and hydrogen ions are secreted simultaneously into the lumen of the duct which lowers the pH from 6.9 to 6.3 and it is thought to initiate protein unfolding partly by disrupting their water shell and altering coulombic forces (D. P. Knight *et al.*, 2001; F. Vollrath *et al.*, 1998; E. K. Tillinghast *et al.*, 1984; D. P. Knight *et al.*, 2000; X. Chen *et al.*, 2001).

The silk proteins are then thought to align and get packed much closer in the extensional flow-field of the drag-down tape found in the duct's distal part. As the hydrophobic polyalanine segments of the silk proteins align and are drawn closer together, they are exposed to an increasingly hydrophobic environment, which might cause them to

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convert to beta structure from alpha helical structure, which in turn results in numerous interchain hydrogen bonds formation. The latter would act as multifunctional crosslinks at nodes between the more mobile glycine-rich segments (Thomas Scheibel, 2004). Thus the silk assembly can be seen as a liquid-crystalline phase transition involving separation into polymer-rich and solvent-rich phases (D. P. Knight *et al.*, 2000). The contribution of individual silk proteins to the assembly process needs to be resolved further.

The cellar spiders also known as the daddy long legs spider, carpenter spider, daddy long-legger, vibrating spider, gyrating spider, long daddy, and skull spider is almost only ever found in those where they benefit from warm, stable temperature. These spiders spin loose and messy webs in the corner of rooms, attics, cellars, in caves and under rocks. They feed on insects and even hunt other spiders. The cellar spiders belong to the family Pholcidae. The family contains over 1800 species. The family was first described by Carl Ludwig Koch in 1850. Pholcids are thin and delicate arachnids. The body, resembling the shape of a peanut, is approximately 2–10 mm (0.08–0.39 inch) in length, and the legs may be up to 50 mm (1.97 inches) long. Pholcids are gray to brown, sometimes clear, with banding or chevron markings. Pholcus have cylindrical abdomens and eyes arranged in two lateral groups of three and two smaller median contiguous eyes. Arrangements of eight and six eyes both occur in this family. These spiders have legs roughly 4 times as large as their bodies, and their body is seperated into two segments.

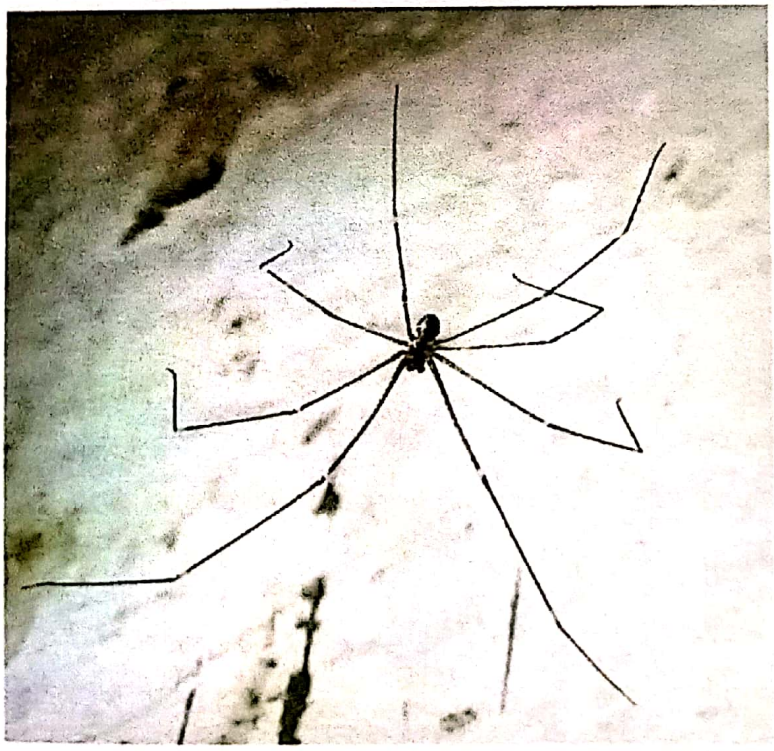


Plate 1.1 *Crossopriza lyoni*

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Scientific classification:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Chelicerata

Class: Arachnida

Order: Araneae

Infraorder: Araneomorphae

Family: Pholcidae

Genus: *Crossopriza*

Species: *C. lyoni*

Crossopriza lyoni that has adapted well in the human habitats was selected because of the high availability of spider silk which can be obtained from the environment.

Nanoparticles:

Nanoparticles are tiny materials having size ranges from 1 to 100 nanometer. Based on properties, shapes and sizes they can be classified into different types. Nanoparticles possess unique chemical and physical properties due to the high surface area and nanoscale size (Ibrahim Khan *et al.,* 2017; Pritam Kumar Dikshit *et al.,* 2021). Due to their unique characteristics, they tend to be suitable candidates for various industrial and domestic applications such as catalysis, imaging, energy based approach, environmental as well as biomedical applications. Nanoparticles have been widely utilized for promoting plant growth. Nanoparticles can be employed for drug delivery (Lee *et al.,* 2011), chemical and biological sensing (Barrak *et al.,* 2016), gas sensing (Mansha *et al.,* 2016), CO_2 capturing (Ganesh *et al.,* 2017) and other related applications. Nanoparticles are composed of three layers 1) The surface layer 2)The shell layer 3)The core (Shin *et al.,* 2016). Nanoparticles are said to have been present on Earth since its origin in the form of soil, water, volcanic dust and minerals. Humans have also started synthesizing it (M. A. Maurer-Jones *et al.,* 2013). Four major classes of nanoparticles are described based on their chemical composition 1) carbon based

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(carbon nanotube and nanofibers) 2) metal and metalloide based (Au, Ag, Cu) 3) bio-organic based 4) composite based (R. K. Pandey *et al.*, 2018)

Nanoparticles can be synthesized by various methods which are broadly classified into two classes 1) Bottom-up approach or building up approach, in which nanoparticles are synthesized from relatively simpler substances. Biological and chemical methods come under this. 2) Top-down approach, in which nanoparticles are synthesized from larger molecules, which decomposed into smaller units and these units are converted into suitable nanoparticles. Physical method comes under this approach (R. K. Das *et al.*, 2017; K. N. Thakkar *et al.*, 2010). The drawbacks of physical methods include low production rate, expensive operations and high energy consumption (Pritam Kumar Dikshit *et al.*, 2021). Even though the chemical methods are economical for large-scale production, the use of toxic chemicals and harmful by-products production limits its clinical and bio medical applications (R. Gupta *et al.*, 2018; S. Hua *et al.*, 2018). Hence the demand for reliable, non-toxic, high yielding and eco-sustainable method for synthesis of nanoparticles is increasing.

Many researchers are attracted towards green and biogenic synthesis of nanoparticles due to the feasibility and less toxic nature of the process. These processes are cost effective and environmental friendly in comparison with physical and chemical methods (Ibrahim Khan *et al.*, 2017). Metal nanoparticles are purely made of the metal precursors. Metal nanoparticles can be made from silver, gold, copper, zinc, lead and so on. Metal nanoparticles have advanced optical properties and hence find applications in many research areas. The green synthesis of metal nanoparticles is initiated by mixing noble metal salt precursors with biomaterials (M. Sriramulu *et al.*, 2020). The compound such as proteins, alkaloids, flavonoids, reducing sugars, polyphenols, etc. present in the biometrical acts as reducing and capping agents for the synthesis of nanoparticles from its metal salt precursors (P. Kuppusamy *et al.*, 2016). The reduction can be confirmed initially by visualizing the color change of the colloidal solution. The synthesis of metal NPs using plant extract mainly occurs in three stages. The first stage is marked by the reduction of metal ions (M^+ or M^{2+}) to metal atoms (M^0) and successive nucleation of the reduced metal atoms occurs. In the second stage, the coalescence of small adjacent NPs into larger size particles occurs with simultaneous increase in thermodynamics stability. At the final stage, the termination of the process takes place while giving the final shape to the nanoparticles (V. V. Makarov *et al.*, 2014; S. Si *et al.*, 2007). Gold nanoparticles have been synthesized from the biomass of wheat and oats (Parveen *et al.*, 2016) and using the microorganisms and plant extracts as reducing agent

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(Ahmed *et al.*, 2016). Most semiconductor and metallic nanoparticles have profound potential for cancer diagnosis and therapy (Ibrahim Khan *et al.*, 2017).

Silver nanoparticles are being used in wound dressings, catheter and various household products due to their antimicrobial activity (Asha Rani *et al.*, 2009). The Plasmon absorbance features of noble metal nanoparticles such as silver and gold have been exploited for a wide variety of applications including chemical sensors and biosensors (Unser *et al.*, 2015). Silver nanoparticles are most widely employed among all the synthesized nanoparticles. They contribute about 25% usage in various consumer products. Silver nanoparticles possess antibacterial, antiviral and antifungal activity. Silver nanoparticles are used in bio textiles, electrical appliances, refrigerators and other kitchen wares as they act in bulk form and their ions have the ability to inhibit bacterial growth through various mechanisms. The unique properties of silver nanoparticles such as catalytic activity and stability make them ideal (Pritam Kumar Dikshit *et al.*, 2021). The main application of AgNPs is their use in the antibacterial nanodevices, because of its Ag+ ion effect (Pritam Kumar Dikshit *et al.*, 2021). They can be used as anti-cancer agents due to their anti-proliferative effect and ability to induce cell death (K. Venugopal *et al.*, 2017). Ag nanoparticles from *Allium sativum* shown positive outcome in gastro intestinal carcinoma (S. Arivazhagan, 2004). AgNPs from *Andrographis echoioides* have been shown to inhibit the growth of MCF-2 cells and are widely used in human breast adenocarcinoma cell lines (K. Elangovan *et al.*, 2015). It is reported that the incorporation of antimicrobial nanoparticles such AgNPs into membrane matrix hinders bacterial attachment and biofilm formation (M. S. Maurer *et al.*, 2011; K. Zodrow *et al.*, 2009). Numerous studies have reported that the green synthesized nanoparticles such as Ag, Au, Pt and Pd exhibit photo catalytic activity in degradation of different dyes (A. Kalaiselvi *et al.*, 2015; S. Hemmati *et al.*, 2018; B. Kumar *et al.*, 2015; B. Kumar *et al.*, 2016; M. B. Sumi *et al.*, 2017; K. Tahir *et al.*, 2015). The usage of silver nanoparticle synthesized from *Ficus Benjamina* leaf extract for the removal of Cd (II) was studied.

The synthesized nanoparticles has to be characterized physio chemically before its application in various sectors. The analysis of size, shape, surface morphology, surface area, structure, stability, elemental and mineral decomposition, homogeneity, intensity etc. will provide information which is important for their end-use applications (Pritam Kumar Dikshit *et al.*, 2021). Size and shape are mainly analyzed using X-Ray Diffraction (XRD), Scanning Electron Microscope(SEM), Field Emission Scattering Electron Microscopy(FESEM),

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Transmission Electron Microscopy (TEM), High-Resolution Transmission Electron Microscopy (HRTEM), Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS), Condensation Particle Counter (CPC), Photon Correlation Spectroscopy (PCS), etc. For studying the surface morphology, SEM, TEM, AFM are used. Superconducting Quantum Interference Device (SQUID), Vibrating Sample Magnetometer (VSM), Electron Paramagnetic Resonance (EPR), etc. are used for the determination of magnetic properties of nanoparticles (Pritam Kumar Dikshit *et al.*, 2021). XRD, Energy Dispersive X-Ray (EDX), XPS, IR Raman, BET, and Zieta size analyzer are some of the common techniques for studying the structural properties of nanoparticles (Ibrahim Khan *et al.*, 2017). And there are other characteristic methods to study the surface area, optical property, etc. of the synthesized nanoparticles.

Wound healing:

Wound healing is a physiological process which is multi factorial and complicated. During the patch-up and recovery of wounded tissue, cellular and biochemical components as well as enzymatic pathways play vital roles (S. S. Kordestani, 2014). Cutaneous wound repair takes place in a series of coordinated and overlapping phases, the inflammatory phase, the proliferative phase and the maturation phase (J. B. McIntire, 2014).

The inflammatory phase is characterized by two portions, namely the hemostatic portion and the cellular portion. At the end of hemostasis, there is a formation of a plug of platelets and proteins at the site of traumatized tissue (A. G. Richetta *et al.*, 2011). The neutrophils and macrophages influx to the injured area is caused by the cellular portion. These cells destroy the bacteria and eliminate debris from dying cells and damaged matrix in order to proceed the repair process (L. C. Kloth *et al.*, 1990). The proliferative phase which is comprised of three sub-phases, the re-epithelialization phase, the neo-vascularization phase, the collage deposition phase. The process of epithelial renewal in response to injury is referred to as re-epithelialization (P. Savagner, 2005). The migration of micro-vessel endothelial cells to the spot for the new blood vessel networks to form is known as neo-vascularization (Cumming B.D. *et al.*, 2010). Collagen deposition provides high tensile strength and ability for the wound to heal faster (G. Broughton and R. J. Rohrich, 2005). The maturation phase involves phenotypic differentiation of the preexisting fibroblast into myofibroblasts (V. Sarrazy *et al.*, 2011), which control the synthesis and degradation of extracellular matrix (ECM) proteins (L. Mu *et al.*, 2014).

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Treatment strategies for wound healing include wound dressings, compression bandaging, debridement, negative pressure wound therapy, ultrasound, electrical stimulation, phototherapy, skin substitutes and so on. For this, natural bio materials of plant and animal origin can be used which own good medicinal properties (J.W. Yoo *et al.,* 2011). Though wound repair is a spontaneous and self-sustaining response to damaged tissue, when the tissue has been damaged so severely that it cannot heal on its own naturally, there is a need for an appropriate material to augment the wound healing process (H. M. Tahir *et al.,* 2017).

Ancient Greeks and Romans are said to have used poultices made of spider silk to treat wounds from battle and to stop bleeding. They believed that the silk had healing properties. Ancient people also used it to treat skin lesions and warts. Reports also state that the people of Carpathian Mountains also have used webs as bandages (Jennifer Ouellette, 2012). Spider webs are thought to have great clotting potential due to its fine sized fiber along with bactericidal property (F. Vollrath and D. P. Knight, 2001). The blood clotting potential of the silk may attribute to the rich vitamin k content of it (A. Lateef *et al.,* 2015). It was observed that in and around Bokaro, Jharkhand, India people were applying spider web on small cuts to stop bleeding and healing of wounds (Preeti Kumari *et al.,* 2013) The webs were also used as gauze pads to stop an injured person's bleeding hundreds of years ago (R. R. Jackson *et al.,* 1974) . Hence the use of spider silk as a natural biomaterial to heal wound would be of great value.

Anti biofilm activity:

Biofilm is a population of bacterial cells attached to living or nonliving surfaces which is surrounded by a hydrated matrix of extracellular polymeric substances (EPS) (A. D. Verderosa *et al.,* 2019). The bacterial cells enclosed in the biofilm matrix are 10–1000 times more resistant to antimicrobial agents than the planktonic ones (T. F. Mah *et al.,* 2012). The National Institutes of Health reported that the biofilm-associated microorganisms are the cause of 80% of infection in the body (M. Jamal *et al.,* 2018). Biofilms can attach to several medical devices, causing persistent infections (Z. Khatoon *et al.,* 2018). Non-conventional approaches like nanoparticles were developed to combat biofilm the antibiotic resistance of biofilms (M. A. Mohamed *et al.,* 2018). Metal nanoparticles such as AgNPs are widely used due to their antimicrobial and antibiofilm activities. AgNPs showed antimicrobial activity by disrupting the bacterial cell membrane integrity leading to the leakage of cellular content and death. AgNPs also produce reactive oxygen species that interact with cellular contents such

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as DNA, lipids, and proteins, causing malfunction in the bacteria and its death (Yun'an Qing *et al.*, 2018). AgNPs exert their antibiofilm activity by preventing the adhesion of bacterial cells to surfaces or by damaging the intermolecular forces (K. Kalishwaralal *et al.*, 2010). It was also proven that AgNPs could inhibit quorum sensing (S. Sheikh and V. Tale, 2017).

Blood clotting potential:

Blood clotting, or coagulation, is an important process that prevents excessive bleeding when a blood vessel is injured. Platelets (a type of blood cell) and proteins in the plasma (the liquid part of blood) work together to stop the bleeding by forming a clot over the injury. Typically, the body will naturally dissolve the blood clot after the injury has healed. Spider silk has been studied for its potential medical applications, including as a material to promote blood clotting. Spider silk is strong, elastic, and adhesive, which makes it a potentially useful material for this purpose. One study found that a material mimicking spider silk proteins could promote the formation of blood clots in a laboratory setting, suggesting potential for wound healing. However, further research is needed to fully understand spider silk's potential for promoting blood clotting in humans, and challenges such as the difficulty in producing large quantities of the material and ensuring its safety for human use must be addressed (Manal *et al.*, 2008).

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AIM AND OBJECTIVES

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AIM AND OBJECTIVES:

- To evaluate the cutaneous wound healing activity of the spider web of *Crossopriza lyoni.*
- To preliminarily investigate the blood clotting potential of spider web of *Crossorpriza lyoni.*
- To synthesize silver nanoparticles from the spider web of *Crossopriza lyoni.*
- To characterize the synthesized silver nanoparticles by FT-IR.
- To qualitatively analyze the anti biofilm activity of the synthesized silver nanoparticles.

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REVIEW OF LITERATURE

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3. REVIEW OF LITERATURE:

S.Arcidiacono *et al.*, (1998) purified and characterized recombinant spider silk expressed in *Escherichia coli*.

Elisabetta Bini *et al.*, (2006) developed RGD-functionalized bioengineered spider dragline silk biomaterial and illustrated the potential to bioengineer spider silk proteins into new biomaterial matrixes.

Jun Sung Kim *et al.*, (2007) investigated the antimicrobial activity of Ag nanoparticles against yeast, *Escherichia coli*, and *Staphylococcus aureus*. The result showed that yeast and *E. coli* were inhibited at the low concentration of Ag nanoparticles, whereas the growth-inhibitory effects on *S. aureus*were mild.

Amit Singh *et al.*, (2007) demonstrated spider silk as an active scaffold in the assembly of gold nanoparticles and application of the gold–silk bioconjugate in vapor sensing.

Shaobing Zhou *et al.*, (2008) prepared and characterized novel electrospun spider silk fibroin/poly(d,l-lactide) composite fiber.

Vladimir G Bogush *et al.*, (2009) developed a novel model system for design of biomaterials based on recombinant analogs of spider silk proteins.

Wen-Ru Li *et al.*, (2011) investigated the antibacterial activity and mechanism of silver nanoparticles (Ag-NPs) on *Staphylococcus aureus* ATCC 6538P.

Emiliane A Araujo *et al.*, (2012) evaluated the antimicrobial effects of silver nanoparticles (NP) obtained by a new method and tested at concentrations of 6 µg/ml and 60 µg/ml against the species *Staphylococcus aureus, Listeria innocua, Salmonella Choleraesuis, Pseudomonas aeruginosa, Escherichia coli*, and *Bacillus cereus*.

Simon Wright and Sara L Goodacre (2012) demonstrated that native web silk of *Tegenaria domestica* can inhibit the growth of the Gram positive bacterium, *Bacillus subtilis*. No significant inhibition of growth was detected against the Gram negative bacterium, *Escherichia coli*.

Mohamed E. S.Mirghani *et al.*, (2012) determined the antibacterial properties of the spider web and they were extracted using different solvents such as methanol, ethanol, acetone, and water in different conditions. Acetone extract was shown the best for antibacterial activity

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with 10 mm of diameter of inhibition zone for *Bacillus subtilis* and 9 mm of diameter of inhibition zone for *Escherichia coli*.

Markus Hofer *et al.*, (2012) used recombinant spider silk particles for controlled delivery of protein drugs.

Hannah Chung *et al.*, (2012) reviewed the recent advances in production of recombinant spider silk proteins.

Shiwei Wang *et al.*, (2013) studied a spider web strategy of type IV pili-mediated migration to build a fibre-like Psl polysaccharide matrix in *Pseudomonas aeruginosa* biofilms.

Touseef Amna *et al.*, (2013) demonstrated Zinc oxide-doped poly (urethane) spider web nanofibrous scaffold via one-step electrospinning: a novel matrix for tissue engineering.

Olena Tokareva *et al.*, (2013) reviewed the current strategies to produce recombinant spider silks.

Preeti Kumari *et al.*, (2013) reported the reduction in area of excision wound and increase in tensile strength of incision wound with the use of spider web ointment at 2.5% w/w concentration.

Hassan Roozbahani *et al.*, (2014) investigated the antibacterial activity of spider web of *Pholcus phalangioides* against food borne pathogens (*Listeria monocytogenes* and *E. coli*) which showed greater inhibitory effect against *L. monocytogenes* than *E. coli*.

Mehdi Zarei *et al.*, (2014) reported the MIC values of Ag NPs against tested food borne pathogens were in the range of 3.12-6.25 µg/mL. While *Listeria monocytogenes* showed the MIC value of 6.25 µg/mL, *Escherichia coli* O157: H7, *Salmonella typhimurium* and *Vibrio parahaemolyticus* all showed the MIC values of 3.12 µg/mL.

Anna Florczak *et al.*, (2014) studied the ability of drug-loaded functionalized spider silk spheres to serve as a carrier for targeted cancer therapy.

Muhammad Sohail Zafar *et al.*, (2014) reported the potential use of natural silk for bio-dental applications.

Kristin Schacht and Thomas Scheibel (2014) processed recombinant spider silk proteins into tailor-made materials for biomaterials applications.

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A. Lateef *et al.*, (2016) synthesised and characterised silver nanoparticles from cobweb extract and reported antibacterial activity against *E. coli*, *K. granulomatis*, *P. aeruginosa* and *S. aureus* in the range of 10-17mm.

O. I.Agapova (2017) reviewed about silk fibroin and spidroin bioengineering constructions for regenerative medicine and tissue engineering.

Roseli Salomoni *et al.*, (2017) assessed the antimicrobial activity of commercial 10 nm AgNPs on two hospital strains of *P. aeruginosa* resistant to a large number of antibiotics.

H. M. Tahir *et al.*, (2017) has evaluated the wound healing potential of silk recovered from three different by the species (i.e., *Pholcus phalangioides*, *Crossopriza lyoni*, and *Araneus diadematus*). Results showed that *Pholcus* and *Crossopriza* accelerated the skin reconstruction process and reduced wound surface area in 14 days compared to 20 days of natural untreated healing.

Esam J. Al-Kalifawi and Yasamine Jumaa Kadem (2017) demonstrated the antibacterial of different solvent(distilled water, ethanol and acetone) extracts of Al-Ankabut's home. The results of the present study shows the acetone extract has effective antibacterial activity.

Anna Karolina Kozlowska *et al.*, (2017) demonstrated the modification of silk protein by adding the nucleic acid binding domain enabled the development of a novel oligonucleotide delivery system that can be utilized to improve pharmacokinetics of RNA-based therapeutics, such as CpG-siRNA. Results showed the feasibility of using spider silk spheres as a carrier of therapeutic nucleic acids.

Sónia Troeira Henriques *et al.*, (2017) highlighted the potential of gomesin peptide obtained from Brazilian spider *Acanthos curriagomesiana*as antimicrobial and anticancer leads and the importance of selectively targeting cancer cell membranes for drug development.

Christian B Borkner *et al.*, (2017) reported the surface modification of polymeric biomaterials using recombinant spider silk proteins.

Joyce Jose *et al.*, (2017) has done a preliminary investigation on the antibacterial and blood clotting properties of Pholcidan spider webs.

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Ping Xiang *et al.*, (2018) demonstrated the *in vitro* and *in vivo* biocompatibility evaluation of electrospun recombinant spider silk protein/PCL/gelatin for small caliber vascular tissue engineering scaffolds.

J. A.Mohtar *et al.*, (2018) isolated spidroin from *Crossopriza lyoni* web. Silk processing involved the pretreatment of fibres for the shell layer removal from the surface.

Katarzyna Jastrzebska *et al.*, (2018) analysed the delivery of chemotherapeutics using spheres made of bioengineered spider silks derived from MaSp1 and MaSp2 proteins.

Yue Li *et al.*, (2018) designed nano-spider-web-like electrospun fibers of europium complexes doped polyvinyl pyrrolidone for medical suture.

Aslı Nur Özkan and Oğuzhan Doğanlar (2019) investigated the anticancer effect of spider web in human cervical cell line.

Kamil Kucharczyk *et al.*, (2019) investigated three bioengineered silks, MS1, MS2 and EMS2, and their potential to form a composite material with magnetic iron oxide nanoparticles (IONPs) and found that the composite spheres can be potentially applied for a combined cancer treatment via hyperthermia and drug delivery.

N. G. Shing Yeng and Johan Ariff Bin (2019) isolated and purified dragline silk protein from *Crossopriza lyoni*.

V. P. Ughade and S. S. Deshmukh (2019) isolated fungi such as *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp., etc. from spider web collected in different regions of Melghat forest, Maharashtra.

I. U. Haq*et al.*, (2019) reported the antibacterial activity against seven multiple MDR bacteria using web extracts of various solvents such as methanol, ethanol and acetone. Acetone extracts showed high antibacterial activity against most MDR bacteria. The zone of inhibitions were in the range of diameter from 4 to 22mm.

Ujjwala Shivaji Deshmukh and Ankita S Pansare (2019) studied the antimicrobial activity of web of spider *Stegodyphus sarasenorum* on two different bacteria. Web extracts were prepared in three different solvents Ethanol, Methanol and Acetone. Web extract in Ethanol has shown inhibition diameter 6 mm for *E. coli* and no antimicrobial activity for *S. aureus*.

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Methanol extract shown zone of inhibition 12 mm for *E. coli* and 14 mm for *S. aureus*. For Acetone it was 14 mm for *E. coli* and 18 mm for *S. aureus*.

S. Ananya Sirisha and E. Jenitta .E.P (2019), synthesized and characterized silver nanoparticles using spider web extract and reported antibacterial activity against *E.coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia, Lactobacillus acidophilus, Streptococcus mutants.*

Zafar Iqbal *et al.*, (2019) studied the antibacterial and antifungal activity of spider web extract using different solvents.

V.Muneeswaran and M.Pallikonda Rajasekaran (2019) developed automatic segmentation of gallbladder using bio-inspired algorithm based on a spider web construction model.

Anna Florczak *et al.*, (2020) reviewed silk particles as carriers of therapeutic molecules for cancer treatment.

Yunqing Gu *et al.*, (2020) analysed the mechanical properties and application of spider silk bionic material.

Sahar Salehi *et al.*, (2020) reviewed the different applications of spider silk-based materials in the field of tissue engineering *in vitro* and *in vivo*.

Alessia CG Weiss *et al.*, (2020) demonstrated the surface modification of spider silk particles to direct biomolecular corona formation.

Nayan R Maheshwari and Manojkumar Z Chopda (2020) demonstrated the enzymatic and non-enzymatic antioxidant potential of two web silk samples of spider species, *Crossoprizalyoni* (CL) and *Stegodyphus sarasinorum* (SS). The results showed that the enzymatic antioxidant potential of both web silk samples was low, but the non-enzymatic antioxidant potential of SS web silk was double than the CL web silk.

S. P.Vinay *et al.*, (2020) used hydrothermal path for the synthesis of gold nanoparticles (Au NPs) from the novel biomaterial spider cobweb. The synthesized nanoparticles were thoroughly characterized by XRD, UV–Vis, SEM, EDAX and TEM studies. Au NPs has shown the good photo catalytic degradation activity against two dyes such as Rh B and MB.

Tao Huang *et al.*, (2020) demonstrated the enhanced antibacterial activity of se nanoparticles upon coating with recombinant spider silk protein eADF4 (κ16).

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Alexander Lammers *et al.*, (2021) analyzed the volatilomes of *S. dumicola*, their nests, and capture webs in three locations in Namibia and assessed their antimicrobial potential. Volatilomes were collected using polydimethylsiloxane (PDMS) tubes and analyzed using GC/Q-TOF and showed the presence of 199 VOCs and tentatively identified 53 VOCs. More than 40% of the tentatively identified VOCs are known for their antimicrobial activity.

Behnaz Bakhshandeh *et al.*, (2021) reviewed the advances in the applications of spider silk in biomedical issues.

Mengwei Liu *et al.*, (2021). Biomimicking antibacterial opto-electro sensing sutures made of regenerated silk proteins.

Sinith Withanage *et al.*, (2021) prepared Novel antimicrobial natural polymeric hybrid hydrogels based on hyaluronic acid (HA) and spider silk (SS) using the chemical crosslinking method.

Tomasz Deptuch *et al.*, (2021) reported that MS1-type bioengineered spider silk nanoparticles do not exhibit toxicity in an *in vivo* mouse model.

Zhiyong Gong *et al.*, (2021) employed spider silks as natural optical fibers to construct biocompatible thermometers.

Di Zhang *et al.*, (2021) developed a method for the detection of Cu2+ in the environment and live cells using a synthesized spider web-like fluorescent probe. This probe has the advantages of good stability, simple operation and high selectivity, which provides a broad prospect for environmental monitoring, intracellular detection and practical application of POCT.

Seven Nazipi *et al.*, (2021) isolated an antimicrobial *Staphylococcus sciuri* with broad temperature and salt spectrum from the surface of the African social spider, *Stegodyphus dumicola*.

Sinith Withanage *et al.*, (2021) developed native spider silk-based antimicrobial hydrogels for biomedical applications. And concluded that hyaluronic acid/spider silk-based hydrogels are a promising material for collaborated antimicrobial and anti-inflammatory drug delivery systems for external use.

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Dmitry Bobylev *et al.*, (2022) developed pressure-compacted and spider silk–reinforced fibrin which demonstrates sufficient biomechanical stability as cardiac patch *in vitro*. The result showed that the combination of compacted fibrin matrices and spider silk cocoons may represent a feasible concept to generate stable and biocompatible cardiovascular patches with regenerative potential.

Sarah Lentz *et al.*, (2022) studied the structure–property relationship based on the amino acid composition of recombinant spider silk proteins for potential biomedical applications.

Fatma El-Zahraa A Abd El-Aziz *et al.*, (2022) synthesized spidroin nanoparticles (NPs) using poly (lactic-co-glycolic acid) polymer (PLGA/spidroin NPs) at different weight ratios (5: 1, 10: 1, 15: 1) and were in vitro characterized. Optimized NPs (173±3 nm) revealed considerable antibacterial effect against *Staphylococcus aureus* and *Escherichiacoli*. After 4 days of NPs application on wounds of earthworm as human skin model, macroscopical and histological examinations revealed a significant reduction in wound and re-epithelialization times.

Jun Hee Oh *et al.*, (2022) evaluated the antibacterial and antibiofilm activity of antimicrobial peptide Lycosin-II isolated from the venom of the spider *Lycosa singoriensis*. Lycosin-II showed strong antibacterial activity and biofilm inhibition effects against gram-positive and gram-negative bacteria including oxacillin-resistant *Staphylococcus aureus* and meropenem-resistant *Pseudomonas aeruginosa* isolated from patients.

VG Bogush *et al.*, (2022) developed bioadhesives based on recombinant analogues of spider web proteins.

Li Liu *et al.*, (2022) engineered circularized mRNAs for the production of spider silk proteins.

Kenrick Koh *et al.*, (2023) developed a recombinant squid suckerin-spider silk fusion protein with cell-adhesion motifs capable of thermal gelation at physiological temperatures to form hydrogels for encapsulation and subsequent release of the stem cell secretome. Freeze–thaw treatment of the protein hydrogel results in a modified porous cryogel that maintains slow degradation and sustained secretome release. Chronic wounds of diabetic mice treated with the secretome-laden cryogel display increased wound closure, presence of endothelial cells, granulation wound tissue thickness, and reduced inflammation with no fibrotic scar formation.

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METHODOLOGY

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4. MATERIALS AND METHODS:

Sample collection:

Spider webs were collected in the presence of the spider using sterile pipette to spool up the web around Thoothukudi in order to avoid sampling the wrong web for other closely related species.

Sample processing:

The collected spider webs were thoroughly washed with distilled water and soaked in distilled water overnight, allowed to air dry and stored in an air tight container.

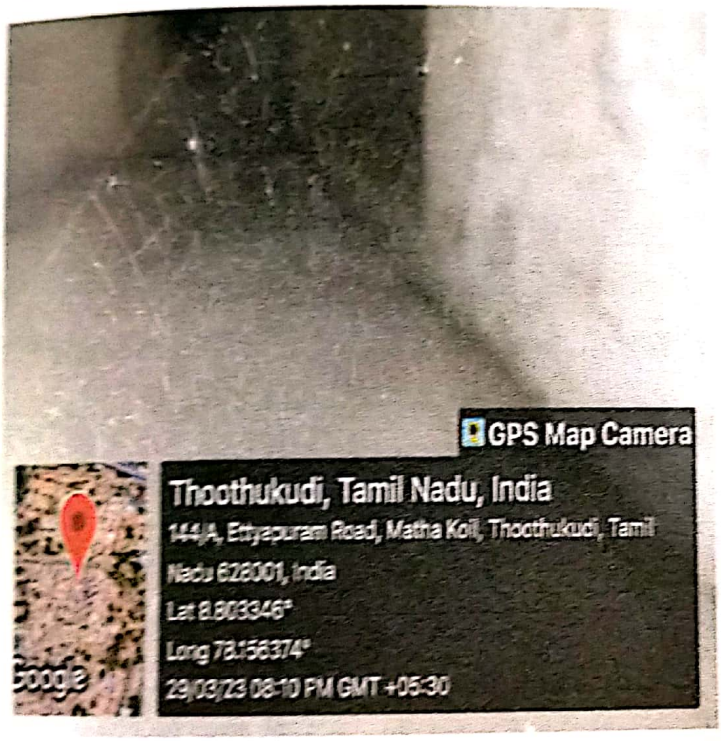


Plate 4.1: Spider web of *Crossopriza lyoni*

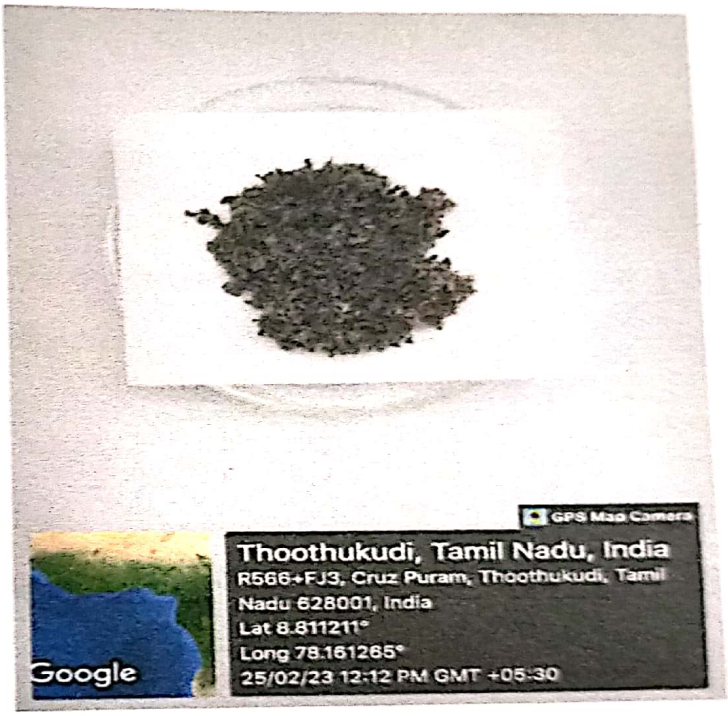


Plate 4.2: Processed web sample

Wound healing activity:

Ointment Formulation:

The spider web was mixed with 0.1% NaOH and the alkalinity of this was brought to pH 5.4 using acetic acid. Cream was formulated by using 10% extract (10g of sample was incorporated in 100gm of cream base). . A control ointment base was formulated without any drug content. The standard drug for screening the wound healing activity is Povidone iodine ointment (5%w/w) which was bought commercially.

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Animal Model:

Albino rats (150-250 gm) of either sex were procured from the animal house were used for the present study. The Albino rats were divided into three groups of six rats. Group I rats were treated with simple ointment base (control). Group II rats were treated with a reference standard Povidone iodine ointment. Group III rats were treated with 10% sample ointment respectively.

Excision wound model:

The excision wound healing activity was studied by the method described by Farahpour and Habibi, (2012). The skin area on the dorsal thoracic region of the mice was removed by using a suitable depilatory (Anne French hair removing cream) one day prior to the experiment. Alcohol (70%) was used as antiseptic for the shaved region before making the wound. The surgical procedures were carried out under sterile conditions. The experimental animals were anesthetized with anesthetic ether. After successful anesthesia mice were fixed in a dorsal posture on a surgery table. Circular, full thickness surgical wounds with diameters of 5mm, 1 cm away from the backbone were made using 5mm biopsy punch. Using this excision wound method, the epidermal, dermal, hypo-dermal and panniculus carnosus layers were removed completely. After making surgical wounds, all mice were randomly marked using a non-toxic color. The animals were divided into the following three groups of six animals each (both male and female) and were treated as given below:-

Group I - Normal control group received petroleum jelly

Group II - Standard group received Povidone iodine ointment

Group III - Drug treated group received 10% w/w of Sample.

The drugs were topically applied daily until the formation of complete epithelial layer, starting from the first day of wound excision. All the animals were monitored daily and observed for any wound fluid, evidence of infection and any other abnormalities. The diameters of the wound were measured immediately by using vernier caliper.

The wound area of each animal was measured from the first day of wounding to the days (8^{th} and 16^{th}) until the healing was complete. The wound closure was measured at regular intervals of time to see the percentage of wound closure and epithelialization time that indicates the formation of new epithelial tissue to cover the wound.

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The percentage of wound contraction was determined using the following formula:

$$\text{Percentage of wound Contraction} = \frac{\text{Initial day wound size} - \text{Specific day wound size}}{\text{Initial day wound size.}} \times 100$$

The number of days required for falling of the scar without any residual of the raw wound gave the period of epithelialization.

Incision wound model:

Animals were anaesthetized and para vertebral incisions (2.5-3.0 cm long) were made through the entire length of skin. After the incision was made, the parted skin was kept together and stitched with nylon thread at 0.5 cm apart with curved needle (No. 11). The test formulation, cream base and povidone iodine ointment were applied on wound once daily for 7 days. The sutures were removed on day 8 and wound tensile strength was measured on day 10 by using constant water flow technique.

Constant water flow technique:

On the 10thday, the animals were secured to the operation table, under light ether anesthesia. A line was drawn on normal skin on either side of wound, 3 mm away from the wound line. Two Allis forceps were firmly applied on the lines facing each other. On one side the forceps was hooked firmly to metal rod fixed to the operation table. The other forceps was connected to a leak-proof graduated polythene container through a string running over a pulley. The polythene container was connected to water reservoir placed at suitable height through a rubber tube kept occluded with a pinchcock. To measure wound tensile strength, the tube was released to allow a constant and continuous flow of water from the reservoir into the polythene container. As the weight gradually increases, it acts as a pulling force to disrupt the wound. As soon as the gapping of the wound was observed, the rubber tube was clamped and the polythene container was weighed.

And the results for excision and incision wound model was tabulated.

Blood clotting activity:

About 0.005g of washed and dried spider web was taken in a clean test tube. Another tube was kept as control without adding any agent or sample. 1ml of human blood sample

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was added to each test tube immediately after taking it from the donor. The blood in the control as well as test sample was observed for clot formation every minute for five minutes.

Synthesis of silver nanoparticles:

Step 1- Hydrolysis:

The modified method of Tszydel *et al.*, (2009) at all was used to hydrolyze the spider webs. About 0.1g of spider webs was weighed and hydrolyzed with 10 ml of 0.1M NaOH at 90°C for 1 hour.

Step 2- Centrifugation:

The hydrolyzed spider web was allowed to cool and then centrifuged at 4000 rpm for 30 minutes. The supernatant obtained was designated as spider web extract.

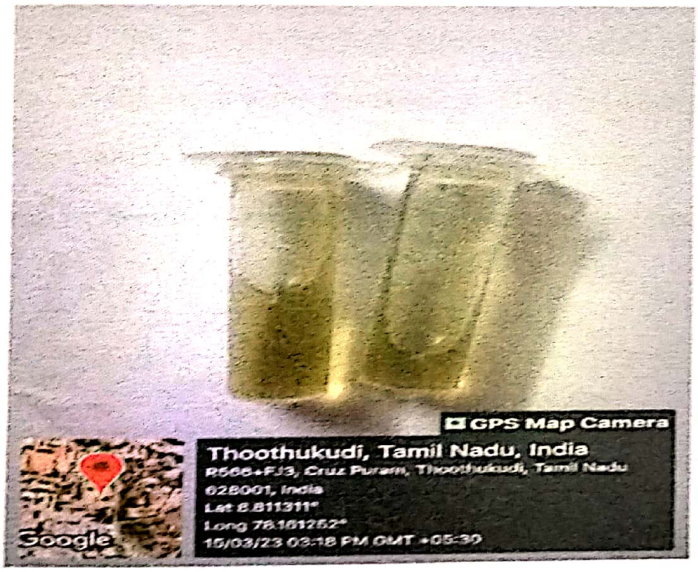


Plate 4.3: Spider web extract

Step 3- Synthesis:

The web extract was used to synthesize AgNPs as previously described (Lateef *et al.*, 2015). About 1 ml of the web extract was added to the beaker containing 40 ml of 1mM silver nitrate solution for the reduction of silver ion. The reaction was carried out in static condition at room temperature (30±2°C) for 48 hours.

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Characterization of synthesized silver nanoparticles:

Fourier-transform infrared (FTIR) spectroscopy:

Fourier-transform infrared (FTIR) spectroscopy analysis was carried out on the powder sample of AgNPs according to Bhat *et al.*, (2011). The AgNP solution was centrifuged at 10,000 rpm for 20 min. The solid residue obtained was then dried at room temperature, and the powder obtained was used for FTIR measurement using KBr pellets.

Antibiofilm assay:

Qualitative test of antibiofilm activity was done by tube adherence method. For this, Luria Bertani broth was prepared and sterilized and 2ml of the broth was taken in 5 borosilicate test tubes. A loop full of *Staphylococcus aureus* culture was inoculated in all the tubes. The sample AgNP was added in two concentrations (500µL and 1000µL) to two test tubes. 500µL of AgNO3 and 500µL of spider web extract was added to two tubes and one tube was kept as control. All the tubes were incubated at 37°C for 48 hrs. After incubation, the tubes were emptied and stained with 0.1% safranine and allowed to sit for 1 min. The excess stain was removed by washing it with distilled water for 3 times. The results were observed.

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RESULT AND DISCUSSION

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5. RESULT AND DISCUSSION:

Wound healing activity:

Wound healing activity of the spider web sample was evaluated for excision and incision wound on wistar albino rats. The albino rats were divided into three groups of six animals,

Group I - Normal control group received petroleum jelly

Group II - Standard group received Povidone iodine ointment

Group III - Drug treated group received 10% w/w of Sample.

Excision wound healing:

For excision wound healing activity, the wound area of Group I, Group II, Group III albino rats was measured on the first day, 8th day, and 16th day. Percentage of the wound contraction was calculated using the formula

$$\text{Percentage of wound contraction} = \frac{\text{Initial day wound size} - \text{Specific day wound size}}{\text{Initial day wound size.}} \times 100$$

And the results were tabulated. The results were expressed as mean ±SEM.

Table 5.1 Effect of topical application of sample on excision wound model

(Wound area mm^2)

Treatment	% wound contraction in excision wound model		
	0 day	8th day	16th day
Group I Control (simple ointment)	387.04±2.08	335.24±2.20 (13. 38%)	176.35±2.28 (54.43%)
Group II (Standard)	398.06±2.22	306.09±2.00 (23.10%)	38.14±0.65 (90.41%)
Group III (Sample 10%)	324.12±2.12	246.16±2.06 (24.05%)	112.18±0.85 (65.38%)

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The wound area was 387.04±2.08 for group I, 398.06±2.22 for group II, and 324.13±2.12 for group III on the 0thday. The wound area was then measured on the 8thday. The spider web ointment showed significant reduction in the wound area. The percentage of wound contraction on the 8th day was 24.05% which was higher than that of the standard povidone iodine ointment which was 23.10%. The control group showed 13.38% activity. On the 16thday, the percentage of wound healing for group III was 65.38%. Though it is lesser than that of the standard which is 90.41%, the spider web sample does show excision wound healing activity.

Incision wound healing:

The tensile strength of the incision wound was measured on the 10th day for the three groups of albino rats by constant water flow technique. And the results were tabulated.

Table 5.2: Effect of topical application of sample on incision wound model

Treatment	Tensile strength (g)
Group I Control (simple ointment)	138.82±0.24
Group II (Standard)	108.22±0.26
Group III (Sample 10%)	122.24±0.16

The tensile strength of the incision wound of group III was 122.24±0.16g which is higher than that of the standard which is 108.22±0.26. But it was lesser than that of the control group, which is 138.82±0.24g.

Spider webs were reported to have applied on small cuts to stop bleeding in India and healing of wounds (Preeti Kumari *et al.*, 2012). Spider webs are thought to have great clotting potential due its rich vitamin K content (Lateef *et al.*, 2015). Silk derived biomaterials which are biocompatible to the human tissues are usually used in surgery (Gellynck *et al.*, 2008).

H. M. Tahir *et al.*, (2017) demonstrated that the silk of *Pholcus phalangioides* and *C. Lyoni* caused higher rate of wound healing and reduced the epithelialization period significantly than the natural untested healing. They have also reported that the time taken by the orb web silk of *A.diadematus* to restore the wound was equal to that of povidone-iodine.

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According to Preeti Kumari *et al.*, (2013) the treatment of wound with spider silk ointment caused dramatic reduction in wound closure and re-epithelialization with a duration of 24 days as compared to 36 days of normal healing and 32 days of povidone-iodine treatment. Shahbuddin *et al.*, (2015) discovered the potency of *P. phalangioides* silk to ignite the division and subsequent migration of teeth pulp stem cells and human keratinocytes.

Hence the use of spider web with known wound healing properties would be of great significance in therapeutic treatments. The results of the present study support the use of spider web of *Crossopriza lyoni* for human and animal wound healing.

Blood clotting potential:

The blood clotting potential of the spider web was investigated by adding 1ml of freshly collected human blood sample to a test tube containing spider web sample and 1ml of blood was added to empty tube which was kept as control. The blood in both tubes were observed for clot formation every one minute.



Plate 5.1: Test and control blood sample (duration: 4 minutes)

The blood to which the spider web was added clotted within three minutes, whereas control blood took around five minutes to clot. No clots were observed in both tubes in the first two minutes. Test blood showed slight clotting in the 3rd minute. In the 4th minute, the test blood clotted completely. The control blood clotted completely in the 5th minute, slight clots were observed in the 4th minute for control.

Joyce Jose *et al.*, (2017) reported the blood clotting potential of Pholcidan spider webs. In their study, the web was shaped into thin square of 1cm and added to the tube and

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blood was added to it. They repeated the experiment three times and confirmed that the blood added to the spider web clotted within 3 minutes and the control blood took around five minutes to clot.

Manal *et al.*, (2008) also have reported the blood clotting activity of spider web by adding 0.005mg of spider web to 1ml of blood and observed the result after 5 minutes. They have concluded that after addition of spider web, the blood clotted within five minutes which may be due to the presence of fibroin protein which is a clotting agent.

Synthesis of silver nanoparticles:

Spider web extract and silver nitrate was allowed to react in a reaction vessel for 48 hours at 30±2°C. After incubation the color of the solution changed from light brown to dark reddish brown in color.

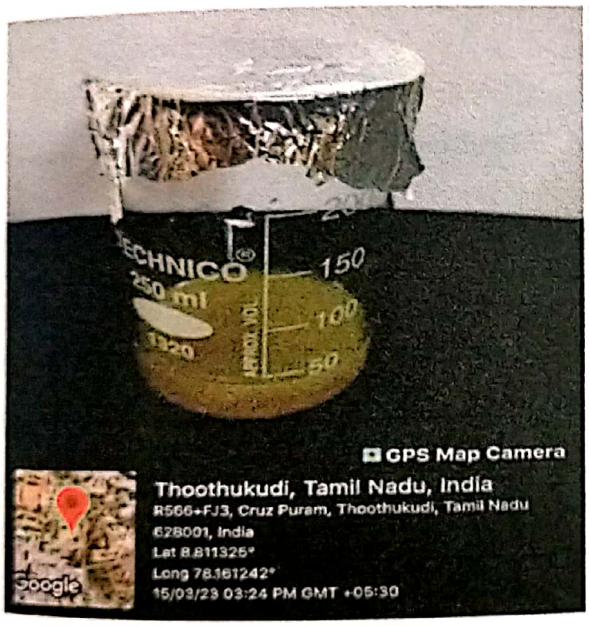


Plate 5.2: Reaction mixture at 0 hrs

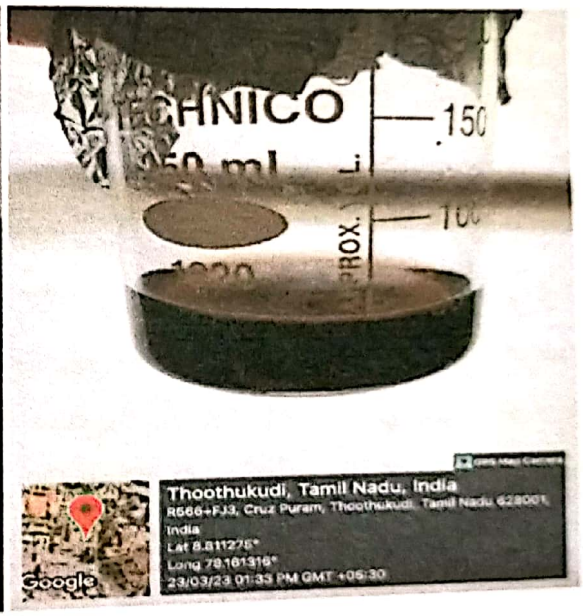


Plate 5.3 reaction mixture after 48 hrs

Characterization of the synthesized AgNPs:

Frontier Transform Infrared Spectroscopy, Scanning Electron Microscopy analysis was done to confirm the presence, size and shape of the synthesized.

Frontier Transform Infrared Spectroscopy:

FTIR spectroscopy was used to monitor the chemical functional groups on the surface of the synthesized silver nanoparticles from spider web. The obtained FT-IR spectrum of the sample is shown in Fig 5.1.

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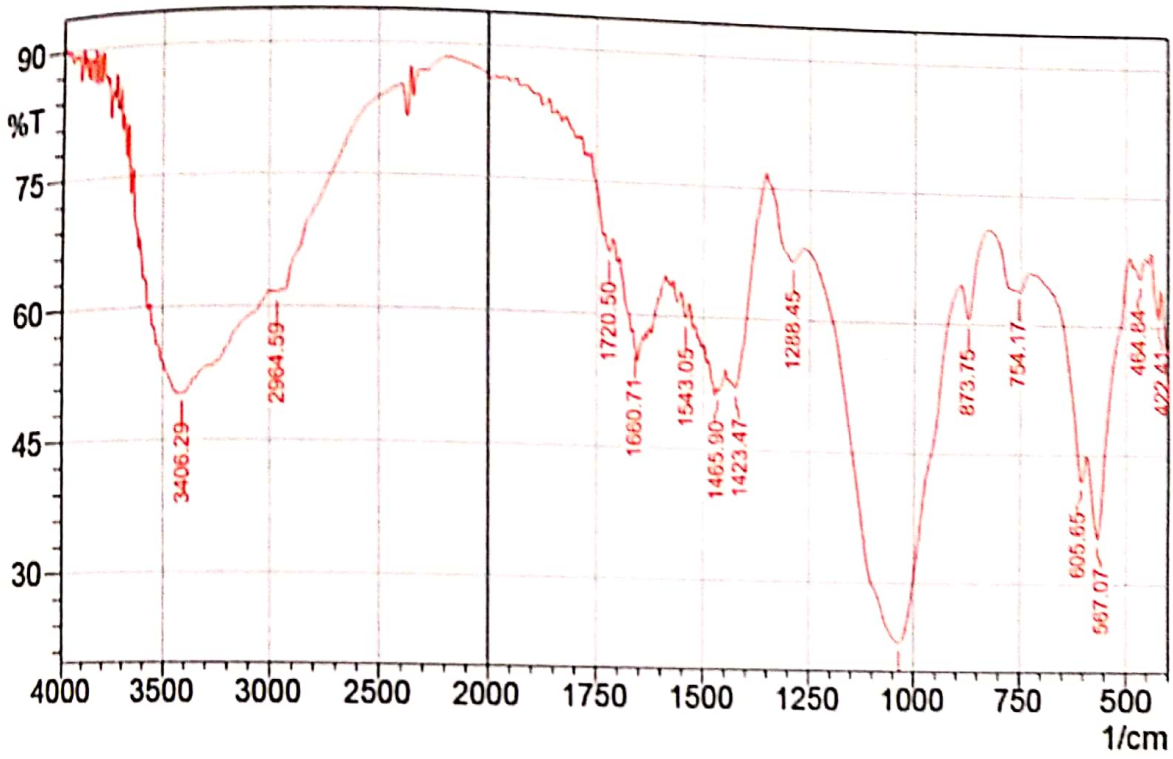


Figure 5.1: FT-IR spectrum of the pure LDPE

In the FT-IR spectrum, the band at 3406 cm^{-1} assigned to the stretching vibration of O-H bonds (S. Senthamil selvi *et al.*, 2013) due to the physically adsorbed moisture on the sample or the presence of hydroxyl group in the chemical structure of the spider web. The absorption peak located at around 2964 cm^{-1}, is due to the asymmetric stretching vibration of C-H bonds, in CH_2 and CH_3 group (V. Vanitha *et al.*, 2017). The absorption peak at 1720 cm^{-1} is related to the stretching vibration of C=O due to the presence of carboxylic group in the amino acids (M.Mobin *et al.*, 2016 and A. R. Shahmoradi *et al.*, 2023). The peaks at 1660 cm^{-1} and 1543 cm^{-1} are attributed to the stretching vibration of C=C bonds in alkenes and asymmetric stretching vibration of N-O bond in nitro compounds, respectively (M. R. Jung *et al.*, 2018 and E. Sanniyasi *et al.*, 2021). The peaks appeared at 1465 cm^{-1} and 1423 cm^{-1} are attributed to the bending vibration of C-H bonds in the aliphatic structure (H. Rajandas *et al.*, 2012). The peaks located at 1288 cm^{-1} and 1035 cm^{-1} are attributed to the stretching vibration of C-N and C-O bonds, respectively (A. A. Javidparvar *et al.*, 2023 and A. B. D. Nandiyanto *et al.*, 2019). The peaks centered at 873 $cm^{-1}$and 754 cm^{-1} are assigned to the wagging and rocking vibration of C-H bonds in the aliphatic structure (M. R. Jung *et al.*, 2018). The peaks located at 605 cm^{-1} and 567 cm^{-1} are attributed to the out of plane vibration of C–H bonds in aromatic rings (H. Rajandas *et al.*, 2012). The weak peaks at 464 cm^{-1} and 422 cm^{-1} can be

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attributed to the stretching vibration of chemical bonds formed between the Ag nanoparticles and the mentioned functional groups (M. Khan *et al.*, 2013).

A. Lateef *et al.*, 2015 reported the synthesis of silver nanoparticles from spider web extract. They reported that the AgNPs were dark brown in color and displayed maximum absorbance at wavelength of 436nm and also reported that the FTIR absorption spectrum showed distinct peaks at 3298, 2359, 2089 and 1635^{-1} cm, which indicated that the proteins were the capping and stabilization molecules and TEM analysis showed that the nanoparticles were spherical in shape with sizes ranging from 3-50 nm.

S. Ananya *et al.*, 2019 reported the synthesis of silver nanoparticles from spider web. The Silver nanoparticles obtained were subjected to UV-Visible spectroscopy in the range of 260-800nm and maximum absorbance was found to be at wavelength of 354nm which is also found to be absorption maxima for AgNPs. Silver nanoparticles were subjected to FTIR analysis where the spectrum showed distinct peaks at 3735.08 cm^{-1}, 3674.03 cm^{-1}, 3602.62cm^{-1}, 1732.36cm^{-1}, 1456.88cm^{-1}1384.28cm^{-1} and 1018.15cm^{-1}. which indicates that proteins were the capping and stabilization molecules in the biological synthesis of AgNPs and the bands at the positions are typical for N-H bonds of amines, and C=C stretch of alkenes or C=O stretch of amides. The lyophilized sample was subjected to SEM analysis, the size of the nanoparticle was 180nm and rod shaped.

Anti biofilm assay:

Tube adherence test was done for analysing the antibiofilm activity. After incubation with *S. aureus*, the tubes were stained with 0.1% safrarine and washed well with distilled water and allowed to dry. The results were observed.

The tubes in which the spider web extract, AgNO3 was added showed no activity. The tube in which 500µl of AgNO3 was added showed slight activity against biofilm, and 1000µl of AgNP inhibited the growth of biofilm completely. The biofilm produced was weakly adherent. Thus the synthesized AgNPs were able to inhibit weakly adherent biofilms.

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Plate 5.4: Test and control tubes for biofilm inhibition

The study done by Yugal Kishore Mohanta *et al.*, 2020, showed the *in vitro* anti-biofilm activity of AgNPs in a dose-dependent manner against the biofilm- forming bacteria *P. aeruginosa, E. coli,* and *S. aureus*. They reported that the treatment of *P. aeruginosa* for 24 h with AgNPs (100 µg/ml) synthesized using *G. lanceolarium* plant extract, reduced biofilm formation by >99%. Similarly, AgNPs derived from *S. anacardium* and *B. retusa* at a concentration of 50 and 60 µg/ml, respectively, reduced biofilm formation by >99%.

Kalishwaralal *et al.*, (2010) reported the anti-biofilm activity of biosynthesized AgNPs against *P. aeruginosa* and *S. epidermidis.* In that study, AgNPs of 100 nm size inhibited biofilm formation by 95-98%. Gurunathan *et al.*, (2014) also studied anti-biofilm capacity of AgNPs against four human pathogens: *P. aeruginosa, Shigella flexneri, S. aureus,* and *Streptococcus pneumonia.* They reported high levels of anti-biofilm activity by AgNPs of a particle size < 100nm.

The present study supports the usage of green synthesized silver nanoparticles from spider web against biofilm forming bacteria, which can be employed in therapeutic as well as environmental purposes.

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CONCLUSION

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6. CONCLUSION:

Spider web possess a lot of potentials which will be very much helpful for humans in many aspects. This study revealed some of the medical applications of spider web. From the results, it can be concluded that the spider web exhibit good wound healing activity in excision wound model and the tensile strength was higher than that of the standard for incision wound model. The present study also concludes that the spider web possess blood clotting activity.

The present study reported the green synthesis of silver nanoparticles from spider web extract and its potentials. The synthesized nanoparticles were characterized by Frontier Transform Infrared Spectroscopic analysis. The FT-IR spectrum showed distinct peaks, from which the presence of AgNPs and the capping and stabilization molecules were interpreted.

The present study have reported the antibiofilm activity of silver nanoparticles synthesized from the spider web extract. The AgNPs were able to inhibit biofilm growth and stand as a promising agent for the inhibition of biofilm, which is the major concern in the medical field.

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SUMMARY

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7. SUMMARY:

Spider webs were reported to have wound healing and blood clotting potential. It was used to heal wounds and stop bleeding since ancient times. The present study confirms the wound healing and blood clotting property of spider web in traditional medicines and the nanoparticles synthesized from it showed anti biofilm activity.

In this study, spider web was collected around Thoothukudi. The collected web was washed with distilled water and allowed to air dry. About 10g of web was added to 100g of petroleum jelly to make 10% w/w simple ointment and was applied to wounds on wistar albino rats. The wound area was measured at 0^{th}, 8^{th} and 16^{th}day and the percentage of wound contraction was calculated for excision wound. For incision wound, the tensile strength was measured by using constant water flow technique. The results indicated that the web possess wound healing property.

For testing the blood clotting activity, 1ml of the freshly collected human blood sample was added to each tube, one of which contained spider web and one was kept as control. The test blood sample clotted within three minutes and the control clotted around 5 minutes duration. The clotting potential of spider web may be due to the presence of fibroin (clotting agent), its rich vitamin K content and it may be the fine sized fiber.

The web was then used for the synthesis of silver nanoparticles. The spider web was hydrolyzed with NaOH at 90°C for 1 hour and then centrifuged at 4000 rpm for 30 minutes and then 1 ml of the supernatant was added to 1mM of silver nitrate and incubated at 30°C for 48 hours. The appearance of dark brown color indicated the presence of silver nanoparticles. The synthesized silver nanoparticles were characterized using Frontier Transform Infrared Spectroscopy where the spectrum showed distinct peaks at 3406cm^{-1}, 2964cm^{-1}, 1720cm^{-1}, 1660cm^{-1}, 1543cm^{-1}, 1465cm^{-1}, 1423cm^{-1}, 1288cm^{-1}, 1035cm^{-1} which indicates that proteins were the capping and stabilization molecules. And the weak peaks at 464cm^{-1}, and 422cm^{-1} can be due to the chemical bonds formed between Ag nanoparticles and the functional groups.

The synthesized AgNPs were used to evaluate the anti biofilm activity. *S. aureus* was inoculated in tubes containing Luria Bertani broth and AgNPs were added to it and incubated for 48 hours at 37°C. The contents were discarded after incubation. 0.1% safranine was used to stain the tubes for a minute and washed well with distilled water and dried. The tubes in

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which the silver nanoparticles were added inhibited the growth of weak adherent biofilm. It is clear that the AgNPs has anti biofilm activity.

This study paves the way for further attention and research to identify the bioactive compounds which are responsible for the medicinal properties of the spider web. Further studies should be undertaken to identify the exact mechanism of action by which the spider silk exert their wound healing and blood clotting effect which can be used in developing bandages, artificial tendons and ligaments, sutures etc. for safe health care services.

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Certificate of Participation

This to certify that Ms/Mrs/Mr/Dr. M.A. Muhsina Thahira, II M.Sc. Microbiology, St. Mary's College participated / presented his / her research paper entitled Green Synthesis of Silver nanoparticles from Spider web extract and its antibacterial potential in the National Seminar on 'EMERGING TRENDS AND OPPORTUNITIES IN MICROBIOLOGICAL RESEARCHES' organised by PG & RESEARCH DEPARTMENT OF BOTANY, V. O. CHIDAMBARAM COLLEGE, THOOTHUKUDI on 10.03.2023.

Dr. A. Doss
Organising Secretary

Dr. C. Lakshmanan
Head of the Department

Dr. C. Veerabahu
Principal

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NANOPARTICLES SYNTHESIS AND CHARACTERIZATION STUDY FROM *Parapenaeopsis stylifera* SHRIMP AND THEIR ANTICANCER ACTIVITY IN HUMAN COLON CANCER CELLS (HCT-116)

A PROJECT SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated to Manonmaniam Sundaranar University

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

S.RINOLAA

(REG. NO. 21SPMB03)

Under the Guidance of

Ms.R. SHYNISHA BEGAM, M.Sc., M.Phil.,

DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS),

THOOTHUKUDI – 628 001.

APRIL – 2023

CERTIFICATE

This is to certify that the project work entitled **"Nanoparticles Synthesis and Characterization Study from *Parapenaeopsis stylifera* Shrimp and their Anticancer Activity in Human Colon Cancer Cells (HCT-116)"** submitted to **St Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **Ms. RINOLAA. S** under the guidance and supervision of **Mrs. R. Shynisha Begam, M.Sc., M.Phil.,** Assistant Professor of Microbiology St Mary's College (Autonomous), Thoothukudi, for academic year 2022- 2023

SIGNATURE OF THE GUIDE

SIGNATURE OF THE HOD

Dr. Joys Selva Mary Albert
Head
Department of Microbiology
St. Mary's College (Autonomous)
Thoothukudi

SIGNATURE OF THE DIRECTOR

Director
Self Supporting Courses
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATURE OF THE PRINCIPAL

Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

18/6/2023

SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE : 05-04-2023

DECLARATION

I hereby declare that the project work entitled **"Nanoparticles Synthesis and Characterization Study from *Parapenaeopsis stylifera* Shrimp and their Anticancer Activity in Human Colon Cancer Cells (HCT-116)"** is a bonafide record of the work completed by me during the academic year 2023 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundharanar University. I also affirm that this is an original work done by me under the supervision of **Mrs. R. Shynisha Begam, M.Sc., M.Phil.,** Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

SIGNATURE OF THE GUIDE

S. Rinolaa

SIGNATURE OF THE STUDENT

PLACE: THOOTHUKUDI

DATE : 05-04-2023

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CONTENTS

S.NO	CONTENT	PAGE NO
1	INTRODUCTION	1
2	AIM AND OBJECTIVE	15
3	REVIEW OF LITERATURE	16
4	METHODOLOGY	23
5	RESULTS AND DISCUSSION	36
6	SUMMARY	46
7	CONCLUSION	47
8	BIBLIOGRAPHY	48

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
1	Parapenaeopsis stylifera	6
2	Distribution area for Parapenaeopsis stylifera	7
3	Colorectal Cancer	9
4	Dried shrimp sample	23
5	Homogenized shrimp sample	23
6 & 7	Shrimp powder mixed with Double Distilled Water	24
8	Sample after boiling	25
9	Filtration	25
10	50ml Shrimp Extract with 50ml of 1Mm $AgNO^3$	26

11	The mixture was placed in the magnetic stirrer for 1hr	26
12	Chemical synthesis of Silver Nanoparticles	27
13	UV Visible Spectrophotometer	29
14	Scanning Electron Microscope	30
15	Fortier Transform Infrared Spectroscopy (FTIR)	32
16	Mechanism of MTT Assay	34
17	Colour change after 30 minutes	36
18	Colour change after 60 minutes	36
19	UV Vis Spectroscopy graph of AgNPs with maximum absorption at 410nm	37

20	Small Nanoparticles with the size of less than 100nm	38
21	Shows highly aggromated Nanoparticles in the sample	39
22	FTIR Spectra for AgNPs which shows the presence of functional groups	40
23	Control	42
24	**20 µg/ml**	42
25	**60 µg/ml**	42
26	Determination of IC50 values from AgNPs synthesized from Parapenaeopsis	43
27	Determination of the rate of inhibition with various concentration from AgNPs synthesized from Parapenaeopsis stylifera for 24hrs of inhibition with HCT116 cells using graph	44
28	Analysis generated by the software for the determination of IC50 values	45

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
1	Taxanomic Hierarchy	6
2	Concentration vs Cell Viability	43
3	Concentration vs Cell Inhibition %	44

INTRODUCTION

Introduction:

Nanotechnology diagnoses and treats disease at this very small level. It uses nanoparticles, which are particles 100 to 10,000 times smaller than human cells. Their small size allows them to target cancer cells more effectively, which aids in diagnosis and treatment. Though science hasn't figured out how to shrink doctors, it has been able to make tools to treat cancer and other diseases smaller than a human cell. Due to their small size, nanotechnology can detect changes in very few cells. It can tell the difference between normal cells and cancer cells. Currently, scientists have limited ability to translate promising molecular discoveries into benefits for cancer patients. Nanotechnology, the science and engineering of manipulating materials at the molecular level to create devices with novel chemical, physical and/or biological properties, can provide engineered controls and tools to enable the development of new diagnostics, treatments and prevention. Nanotechnology has the potential to revolutionize cancer diagnosis and treatment. Recent scientists and engineers (approx. 1980) developed the potential for industrialization of the technology on this scale, and significant progress has been made in translating nano-based cancer treatment and diagnosis into the clinic, with several being developed. Cancer can be caught at an earlier stage, when cells begin to divide and the cancer is easier to treat. Nanotechnology could make tumors more visible in imaging tests. By coating the nanoparticles with antibodies or other materials, they can find and attach to cancer cells. The cells may be coated with a substance that signals when cancer is detected. For example, iron oxide nanoparticles bind to cancer cells and emit a strong signal that lights them up in an MRI scan. Scientists can use nanotechnology to detect cancer in blood or tissue samples. It can find DNA or cancer cell fragments that are too tiny for other tests to detect.

Nanotechnology could make cancer treatment safer and more precise. Specially designed nanoparticles deliver drugs such as chemotherapy directly to tumors. It does not release the medicine until it reaches him. This prevents the drug from affecting the healthy tissue around the tumor. This impairment causes side effects. The small size of the nanoparticles allows drug delivery to areas of the body that are normally difficult to reach. For example, the blood-brain barrier that prevents toxins from entering the brain. It also blocks certain medications. Because nanoparticles are small enough to pass through this barrier, they are useful for treating brain tumors. Clinical trials using different nanotechnology treatments are being studied by

researchers. Several of these therapies encase harmful medications in nanoparticles to make them less dangerous or to assist the medication endure the circulatory journey. Nanoparticles may one day be used to treat cancer with radiation.

Nanotechnology more precisely targets cancer cells while sparing healthy organs. Theoretically, it should have less negative effects than contemporary medical procedures like radiation and chemotherapy.There are side effects associated with current nanotechnology-based medications including diarrhoea, nausea, and weight loss. Yet, the chemotherapy medications they contain may be the cause of these issues. Researchers should learn more about the negative effects of these medicines when they evaluate them in clinical trials.

Nanomedicine is the scientific field that combines nanotechnology with drug or diagnostic molecules that enhance their ability to target specific cells or tissues. These materials can be made nano-sized and safely injected into the body. Nanotechnology is applied in healthcare which include imaging, diagnosis, or drug delivery to help medical professionals treat a variety of diseases.

Improving the ability of nanotechnologies to target specific cells or tissues is of great interest to companies producing nanomedicines. This area of research involves attaching nanoparticles onto drugs or liposomes to increase specific localisation. Since different cell types have unique properties, nanotechnology can be used to "recognise" cells of interest. This allows associated drugs and therapeutics to reach diseased tissue while avoiding healthy cells. While this is a promising area of research, very few nanomedicines exist that successfully utilise nanotechnology in this manner. This is due to ill-defined parameters associated with pairing the correct ratio or combination of nanoparticles with the drug of interest.

Companies that make nanomedicines are very interested in developing nanotechnologies' capacity to target particular cells or tissues. In this field of study, medicines or liposomes are attached with nanoparticles to improve localization. Since each type of cell has a different set of characteristics, nanotechnology can be used to "recognise" certain cells. This makes it possible for therapies and medications to target sick tissue while avoiding healthy cells. Even though this is a promising field of study, there aren't many nanomedicines that successfully apply nanotechnology in this way. This is a result of vague parameters related to matching the ideal ratio or combination of nanoparticles with the desired medication.

Balancing these competing factors is a delicate process. Although researchers have made significant progress in the field of nanomedicine over the past decade, designing and synthesizing small, stable nanoparticles capable of delivering enough drugs to treat solid tumors remains a challenge.

Nanomedicine associated nanotechnology is gaining a lot of interest from industry. This "triggered" release, in theory, could be achieved from within the body or from outside the body. Internal mechanisms include changes in the environment of tumours compared to surrounding tissue, while external stimuli includes temperature changes, light, or ultrasound. Currently, research efforts are focused on trying to understand how to release diagnostic molecules and drugs from liposomes with heat, and microbubbles using ultrasound.

Because they provide better-built, safer and cleaner, longer-lasting, and smarter goods for the medical, communications, daily life, agricultural, and other industries, nanotechnologies have had a substantial impact on practically all industries and areas of society. There are two main categories of how nanoparticles are used in common items. Firstly, by incorporating some of its special features into a pre-existing product, nanomaterials can enhance the composite products' overall performance. Secondarily, nanomaterials like nanoparticles and nanocrystals can be used directly to produce sophisticated devices with high power. Nearly all industrial areas may be impacted by the advantages of nanomaterials in the future.

Drug delivery is a recurring challenge in cancer treatment. Scientists have developed many anti-cancer drugs, but these drugs often damage healthy tissue and break down in the blood before the drugs reach the tumor site. Anticancer drugs can last longer when dissolved in certain chemical solutions, but many drugs have potentially toxic side effects. Nanoparticles are a promising type of drug delivery system. These small particles, called nanocarriers, can bind to the drug and protect it from degradation until it enters the tumor cells. However, their effectiveness as drug delivery and drug-protecting agents and their potential toxicity to patients are highly dependent on their size, composition, and chemical properties. The most widely used technique for creating stable, colloidal dispersions of AgNPs in organic or water-based solvents is chemical reduction. Boron hydride, citrate, ascorbate, and elemental hydrogen are frequently used reductants. Colloidal silver with particle sizes of several nanometers is typically produced when silver ions (Ag+) are reduced in aqueous solution. Silver atoms (Ag0) are initially created

when different complexes are reduced with Ag+ ions, and they then aggregate into oligomeric clusters. Colloidal Ag particles eventually form as a result of these clusters. Due to their distinctive physical and chemical characteristics, silver nanoparticles (AgNPs) are being employed more and more in a variety of industries, including medicine, food, health care, consumer goods, and industrial applications. They include high electrical conductivity, optical, electrical, thermal, and biological characteristics. Based on chemically reducing aqueous salts of metals, such as silver nitrate (AgNO3) in the case of silver nanoparticle production, chemical reduction is an efficient wet-chemical technique for producing zero-valent nanoparticles. At least one reducing agent is utilised to provide electrons for metal ions, reducing them to zero-valent state in order to reduce the precursor metal salt. The nanoparticles which are derived from among other things, chitin, a natural and organic polymer that forms the shell of shrimp.

Shrimp are actually crustaceans (crustaceans) with elongated bodies and a major mode of locomotion that includes swimming. Caridea and Dendrobranchiata are the most common members of this order. A narrower definition could be limited to only Caridea, smaller species of any marine group or species. In a broader sense, shrimp can be synonymous with prawn, a swimming crustacean with a long, narrow, muscular tail (abdomen), long whiskers (antennae), and stalked eyes covered with thin legs. Small crustaceans that resemble shrimp are commonly called shrimp. They swim under the belly and swim forward, although the escape response usually consists of a repetitive motion with the tail thrust back very quickly. Crabs and crayfish have strong legs, while shrimp have thin and fragile legs that are mainly used for sitting. Shrimp are common and plentiful. There are thousands of species adapted to different habitats. They can be found near the bottom of rivers and lakes, as well as on many shorelines and estuaries. To escape predators, some species jump off the seabed and sink into the sediments. They usually live from 1 to 7 years. Shrimp are mostly solitary, but can form large groups during the spawning season. One of the front claws is very extended, often exceeding half the length of the body. The modified end of this nail serves as a wide base plate to which the hinge joint is attached. It resembles an antique musket with a powder barrel that ignites when struck with a hammer. In a shrimp bite, the main function of the device is to track the prey, not to capture it.

Shrimp may be quite social and may swim and feed in vast schools, unlike their decapod relatives, crabs and lobsters. Numerous prawn species are nocturnal, spending the daytime

camouflaged among seaweed or in the cracks of coral reefs. Several species hide themselves in the sand; their protruding tentacles are the only indication of their presence. They come out at night to feed on a variety of species' eggs and larvae, small fish, worms, and smaller crustaceans.

Papenaeopsis stylifera (Milne Edwards.H, 18 37), popularly known as Kiddi shrimp, constitutes an important fishery resources along the Indian coast with wide geographical distribution. It matures in a year at a size of 65 mm (male) to 75 mm (female). Along the Indian coast, Penaeid shrimps (0.19 Mt) form a considerable proportion of crustaceans (0.4 Mt) followed by non-penaeids (0.18 Mt), stomatopods and lobsters. Among penaeids, *P. stylifera* is the most dominant species across the Indian coast (CMFRI, 2019). Especially, on the west coast of India (Maharashtra), this species contributes up to 48–50% of total penaeid landings (CMFRI, 2019). Despite its fisheries dominance and economic importance, *P. stylifera* genetic management units/genetic stocks have not been well defined and the entire stock is being treated as a single management unit.(Labrechai Mog Chowdhury *et al.*, 2021)

TAXONOMY OF *Parapenaeopsis stylifera* Shrimp

Kingdom	Animalia
Phylum	Arthropoda
Sub Phylum	Crustaceae
Class	Malacostraca
Order	Decapoda
Family	Penaeidae
Genus	Parapenaeopsi
Species	Stylifera

Table 1: Taxonomic Hierarchy



Fig 1: *Parapenaeopsis stylliferа*

Distribution

General distribution of the species is Karachi (West Pakistan), west coast of India, Palk Strait and Madras. In terms of the FAO areas code (Holthuis and Rosa, 1965) it occurs in part of sea area ISW and on some coasts of land areas 421(W), 423 and 424.Main distribution is along the west coast of India between 8°N and 21°N. Adults occur in the coastal waters up to 30–40 m depths on soft muddy bottom.

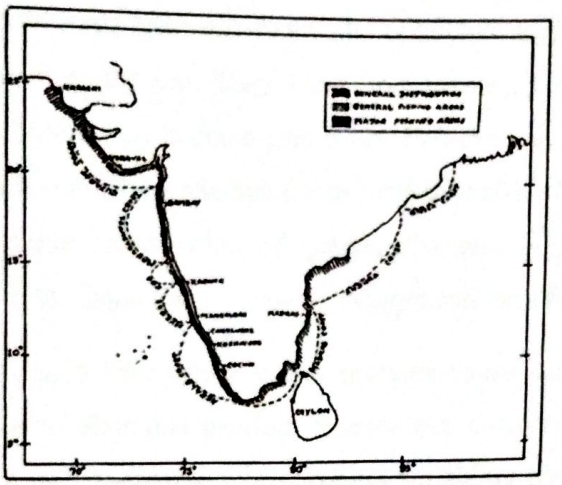


Fig 2: Distribution area for *Parapenaeopsis stylifera*

Cancer is a disease in which certain cells in the body grow out of control and spread to other parts of the body. It can form almost anywhere in the body, which is made up of trillions of cells. Normally, human cells grow and multiply (through a process called cell division) to make new cells when the body needs them. When cells become old or damaged, they die and are replaced by new cells. Sometimes this orderly process goes awry, causing abnormal or damaged cells to proliferate. These cells can form tumors, which are lumps of tissue. Tumors can be cancerous or non-cancerous (benign).Cancer is a leading cause of death worldwide .

Cancer is a leading cause of death worldwide and it can spread, or invade nearby tissue, and spread to distant parts of the body to form new tumors (a process called metastasis). Most cancers form solid tumors, but blood cancers such as leukemia usually do not. Benign tumors do not spread or invade surrounding tissue. Benign tumors usually do not grow back after being removed, while malignant tumors sometimes grow back. However, benign tumors can

sometimes be quite large. Some, such as benign brain tumors, can cause severe symptoms or be life-threatening.

The basic physical unit of cancer inheritance is caused by specific changes in genes. Genes are located on long, tightly wound strands of DNA called chromosomes. Cancer is a genetic disease. That is, because of changes in genes that control how cells work, especially how they grow and divide. Genetic changes that cause cancer can be caused by: Errors that occur during cell division. DNA damage from environmental pollutants such as chemicals in cigarette smoke and ultraviolet rays from the sun. They come from parents. The body normally removes cells with damaged DNA before they become cancerous. However, as we age, the body's ability to do this decreases. This is one of the reasons for an increased risk of cancer later in life. Each person's cancer has a unique combination of genetic changes. As the cancer grows, other changes occur. Even within the same tumor, genetic changes can vary from cell to cell.

Robert Den, M.D., said "We know which proteins cause cancer to form, grow, and spread, yet we are unable to stop this process because our current drugs cannot alter their functions. In 10 years, we will overcome this scientific stumbling block and have a profound effect on cancer and health."

Colon cancer is one of the most prevalent types of cancer. Traditional chemotherapy treatments often have harmful side effects and can lead to drug resistance, which has led to the search for alternative therapies. One promising approach is the use of nanoparticles as drug delivery systems, as they can target cancer cells specifically and reduce toxicity to healthy cells. Additionally, natural compounds from marine sources have been shown to have anticancer properties. This thesis aims to synthesize and characterize nanoparticles from shrimp and investigate their potential anticancer activity in human colon cancer cells. Colon cancer is more common in northwestern Europe and North America. In the United States, it is the third most common cancer in both men and women and the second leading cause of cancer death. It is the most treatable cancer of the gastrointestinal tract. Men and women are equally affected, and the average age of diagnosis is 6 to 70 years. Annual Incidence Rates (AARs) of colon and rectal cancer in Indian men are 4.4 and 4.1 per 100,000, respectively. The AAR for colon cancer in women is 3.9 per 100,000. For men, colorectal cancer ranks 8th and rectal cancer 9th. For women, rectal cancer is not in the top 10 cancers and colorectal cancer ranks ninth.

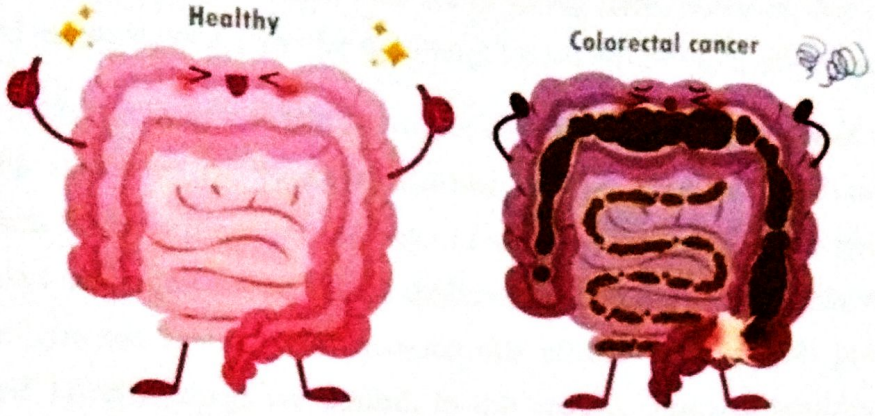


Fig 3: Colorectal Cancer

The diagnosis of middle-aged sporadic Cca is primarily due to environmental factors, and the most common hereditary Cca syndrome is familial adenomatosis, in contrast to a minority and familial group of patients under 50 years of age with a real risk genotype. Polyposis (FAP) and Lynch syndrome (Hereditary Nasal Polyposis Colorectal Cancer [HNPCC]). About 5% of all colorectal cancers are due to these two genetic syndromes, but 10–15% of unselected Cca patients carry high-risk mutations unrelated to FAP or HNPCC. The findings of epidemiologic studies show a substantial link between environmental factors and way of life and CRC. Obesity, red/processed meat, smoke, alcohol, androgen deprivation therapy, among other things, are associated with slightly elevated CRC risks. Large population studies, however, have discovered CRC protective factors such as exercise, diet (fruits and vegetables, fibre, resistant starch, fish), vitamin supplements (folate, folic acid, pyridoxine B6, calcium, vitamin D, magnesium), garlic and coffee, and medications (aspirin, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), hormonal replacement therapy in postmenopausal women, statins, bisphosphonate, and angiotensin inhibitors).

Though cancer deaths have steadily declined over the past 20 years in developed countries, cancer remains a major threat to public health in many parts of the world. Currently, the number of cancer cases is increasing in developing countries. Specifically, 55% of new cases occur in developing countries, which could increase to 60% by 2020 and 70% by 2050. Globally, cancer also imposes a significant economic and human burden. Cancer cost an estimated $1.16

trillion worldwide in 2010, or more than 2% of global GDP. However, this large number is a lower bound and does not account for significant long-term costs to families and caregivers.

Currently, the gold standard for cancer treatment is a combination of surgery, radiation therapy, and chemotherapy. However, existing methods have the disadvantages of lack of screening tests for early diagnosis and lack of tumor-specific drug delivery systems. In addition, most classical anticancer drugs cannot differentiate between cancer cells and normal cells, resulting in systemic toxicity and unwanted side effects. To solve this problem, new more selective and effective drugs are needed. In this context, bioactive peptides are increasingly considered as good drug candidates for use in cancer treatment.More and more natural peptides of animal origin have physiological functions such as immunomodulatory, antibacterial, antihypertensive, antithrombotic, antitumor, antioxidant and cholesterol. – reduced operation. However, this study focuses on bioactive peptides of animal origin that can act as anticancer agents with less toxicity to normal tissues by specifically targeting cancer cells. Also, Bioactive peptides usually consist of 2-50 amino acid residues (~102-103 Da). Therefore, they easily cross or destroy cell membranes, leading to apoptosis or necrosis. Therefore, the research and modification of bioactive peptides with antitumor activity provide new opportunities for cancer prevention and treatment.

Bioactive compounds from marine animals have been reported to have a wide range of bioactive properties. More and more recent studies focus on bioactive peptides with potential anticancer activity isolated from various marine animals such as shrimp, sponges, marine sediments, molluscs, fish and other marine organisms.

Wilson-Sanchez *et al.*,(2010), showed antimutagenic and antiproliferative activity of white shrimp lipid extract. In particular, the lipid fraction of vannamei contains compounds that reduce the mutagenicity of aflatoxin B1 and the proliferation of B-cell lymphoma cells.

In addition, Shrimp Antilipopolysaccharide Factor (SALF), an antimicrobial peptide derived from black tiger shrimp, potentiates the antitumor activity of cisplatin in vitro and inhibits the growth of HeLa cells in nude mice. This peptide also shows significant antitumor activity in human colon and liver cancer cells, even when isolated from shrimp waste.

HCT116 cell line

HCT116 is a human colorectal carcinoma cell line initiated from an adult male. HCT116 cells are used in a variety of biomedical studies involving colon cancer proliferation and corresponding inhibitors. The cell line has been utilised in tumorigenicity studies and other studies that have demonstrated the significant role Cyclin D1 plays in the activity of lithocholic acid hydroxyamide. Docetaxel, 5-FU, and flavopiridol are three drugs that can limit tumour growth in vitro when used with HCT116 cells in xenografts.

Characterization study :

UV spectroscopy or UV–visible spectrophotometry (UV–Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible regions of the electromagnetic spectrum. This method is frequently employed in a variety of practical and theoretical applications since it is reasonably affordable and simple to execute.

A simple technique that allows for quick detection and description of silver nanoparticles is UV-visible spectrophotometry. Due to the interaction between light and the moving surface electrons of silver nanoparticles, it produces a powerful absorbance band in the 400–500 nm region known as surface plasmon resonance (SPR). The concentration of sample is required to measure the absorption spectrum on a UV/Vis Spectrophotometer is the range 1x10-4 Moles. The UV region covers the wavelength range 100-400 nm and is divided into three bands: UVA (315-400 nm) UVB (280-315 nm) UVC (100-280 nm).

Scanning Electron Microscopy (SEM) examines the surfaces of substances, particles, and fibres so that minute details can be quantified and evaluated through image analysis. Industry can use SEM to address contamination issues, look into component failure, find unidentified particles, or research how chemicals interact with their substrates. Also, it can offer a plethora of knowledge to enable the study of substances, compounds, or biological samples.

SEM image interpretation is not always a straightforward process. If suitable sample preparation techniques are used and skilled microscopists conduct the analysis. SEM can be a truly powerful tool for tasks like the interpretation of surface pitting on metal components, the identification of particulates, or the exploration of physical and chemical properties of

materials.To produce high-quality, depth-of-field photographs, the SEM analysis facility will allow users to observe surface characteristics at 5 to 300,000X magnification. It is simple to determine the chemical composition of surface features when using EDS detectors mounted to each SEM.

The MTT assay measures colorimetrically the metabolic activity of cells. Several in vitro tests of a cell population's reaction to outside influences are based on measurements of cell viability and proliferation. Tetrazolium salt reduction is now recognised as a trustworthy method for assessing cell growth. Dehydrogenase enzymes help metabolically active cells reduce the yellow tetrazolium MTT (3-(4, 5 dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to produce reducing equivalents like NADH and NADPH. By using spectrophotometric techniques, the resulting intracellular purple formazan can be solubilized and measured.

Assays using tetrazolium dye can also be used to assess the cytotoxicity (loss of live cells) or cytostatic activity (change in proliferation to quiescence) of harmful substances and prospective therapeutic treatments. MTT experiments are often conducted in the dark since the MTT reagent is light-sensitive.

The cytosolic compartment of the cell mostly contains NADPH-dependent oxidoreductase enzymes, which are thought to be necessary for tetrazolium dye reduction. Consequently, the cellular metabolic activity caused by NADPH flux determines the reduction of MTT and other tetrazolium dyes. Thymocytes and splenocytes, which have modest metabolic rates, barely reduce any MTT. On the other hand, cells that divide quickly show significant rates of MTT decrease. It's crucial to remember that test conditions can change metabolic activity and, in turn, the decrease of tetrazolium dye without impacting cell viability. The amount of product will also depend on the way that tetrazolium dyes are reduced. Furthermore, evidence for the absence of enzyme catalysis in the spontaneous MTT reduction in lipidic cellular compartments/structures has been presented.

Nonetheless, even in this new paradigm, the MTT assay still evaluates a cell's reduction potential (i.e. availability of reducing compounds to drive cellular energetics). The final interpretation of cell viability remains constant as a result. The thickness of the scaffolds may have an impact on the MTT assay results when examining the viability of cells seeded on 3D fibrous scaffolds.

The intracellular reduction of MTT and the formazan concentration are both expected to be represented by the observed OD values. This has been the rationale behind using the MTT assay for almost four decades as a standard instrument to assess cell viability and proliferation, drug cytotoxicity, and metabolic and mitochondrial activities.

At various steps of the glycolytic routes to the mitochondrial electron transport chain, oxidoreductase and dehydrogenase enzymes and electron donors (mostly NADPH) can mediate the intracellular reduction of MTT. It is still debatable where formazan production occurs and how it is transported inside cells. Although the assay's widespread use to detect mitochondrial activity has been justified by the role that mitochondria play in MTT reduction, biochemical and microscopic studies have found formazan in a number of intracellular organelles. The endoplasmic reticulum, cytosolic lipid droplets, plasma membranes, nucleus, and microsomes have all been found to contain intracellular formazan granules. These findings imply that the MTT assay provides a more comprehensive measurement of mitochondrial activity. Ascorbic acid, cysteine, dihydrolipoic acid, glutathione, glutathione S-transferase, and tocopherols are additional macromolecules that can lower MTT. Many research have highlighted the MTT assay's limits and various confounding factors that must be taken into account while planning, carrying out, analysing, and interpreting the assay's results. Notwithstanding these drawbacks, the MTT test is nevertheless frequently applied and interpreted. (Mahshid Ghasemi *et al.,* 2021)

Fourier Transform Infrared Spectroscopy, also known as FTIR Analysis or FTIR Spectroscopy, is an analytical technique used to distinguish between organic, polymeric, and, in sometime situations, inorganic materials. The FTIR analysis method scans test materials and examines chemical characteristics using infrared light. The capacity to introduce and observe the energy from a certain matrix is necessary for proper FTIR analysis. Fortunately, there are a variety of sample introduction and preparation processes accessible in the lab for accurate sample analysis. Transmission was the only available mode of analysis in the early days of infrared spectroscopy. The sample had to be made translucent to the laser and infrared energy in order to be analysed by transmission. This could be done by placing the sample directly in the optical path, casting a thin film on a salt crystal, or mixing a powdered sample with salt before casting.

HATR (Horizontal Attenuated Total Reflectance) multi bounce approach for liquid and paste samples, which entails setting the sample on a crystal plate or trough in a horizontal position such that gravity operates to create close contact with the cell. It is possible to employ various crystals that will alter the sample's penetration depth. For rubber analysis, for instance, we will use a germanium crystal to reduce the impact of the highly IR absorbent components of rubber (specifically carbon black), but the zinc selenide crystal is the crystal of preference for everyday samples due to its robustness, resistance to moisture, and penetration depth. This technique also allows for us to spatially resolve differential chemistry in a very small area. (May Eid., 2022)

The surface chemistry of nanoparticles is revealed by FTIR. This method aids in determining the functional groups of both the resultant silver nanoparticles and plant extracts. It is significant to highlight that the produced silver nanoparticles are at least characterised using the FTIR approach. Sometimes, small band shifts can be seen while analysing the FTIR of phytochemicals attached to silver nanoparticles or in free form. These changes are interpreted as proof of metal reduction and nanoparticle production. According to FTIR and phytochemical investigations, alkaloids, flavonoids, tannins, terpenes, and quinones are the most significant phytochemical components responsible for the reduction and capping of silver nanoparticles.

This work aims to synthesize and characterize nanoparticles from shrimp and investigate their potential anticancer activity in human colon cancer cells(HTC116). The use of natural compounds from marine sources and the development of cost-effective synthesis methods for nanoparticles may lead to the discovery of new and effective therapies for colon cancer.

AIM AND OBJECTIVES

AIM AND OBJECTIVE

- To collect *Parapenaeopsis stylifera* Shrimp
- To prepare extract using double distilled water
- To synthesize silver nanoparticles from shrimp using chemical reduction method
- To characterize the synthesized nanoparticles using various techniques such as UV-Vis spectroscopy, FTIR, and SEM
- To execute the MTT assay
- To evaluate the cytotoxicity and potential anticancer activity of the synthesized nanoparticles in human colon cancer cells
- To investigate the mechanism of action of the nanoparticles in colon cancer cells

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Khomutov *et al.*,(2002), reported on chemical approach in which the metal ions in solution is reduced in conditions favoring the subsequent formation of small metal clusters or aggregates.

Elchiguuerra *et al.*,(2005), reported that silver nanoparticles have been tested in various fields of biological sciences viz. drug delivery, wound treatment, binding with HIV gp120 protein.

Tao *et al.*,(2006), described the reduction of various complexes with Ag+ ions leads to the formation of silver atoms (Ag0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles.

Albrecht *et al.*,(2006), stated that nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new materials at the nanoscale level.

Seery *et al.*,(2007), said that silver nanoparticles (AgNPs) are important materials that have been studied extensively and have several important applications in the field of biolabelling, sensors, antimicrobial agents and filters.

Yoosaf *et al.*,(2007), reported that metallic nanoparticles exhibit size and shape-dependent properties that are of interest for applications ranging from catalysts and sensing to optics, antibacterial activity and data storage.

Martínez-Castañón *et al.*,(2008), investigated and described different parameters that influence antibacterial effects of AgNPs. These factors include properties such as size, shape, surface chemistry, crystallinity, capping agent and dose of AgNPs.

Williams., (2008), described that the units should only be those of dimensions, rather than of any other unit of scientific measurement and it is widely agreed that nanoparticles are clusters of atoms in the size range of 1–100 nm.

Sharma *et al.*,(2008), stated that nanoparticles have been the subject of substantial research in recent years.

Wilson-Sanchez *et al.*,(2010), demonstrated the anti-mutagenic and anti-proliferative activities of lipidic extracts from white shrimp. Shrimp anti-lipopolysaccharide factor (SALF), an antimicrobial peptide from black tiger shrimp, enhances the anticancer activity of cisplatin in vitro and inhibits HeLa cell growth in nude mice. These peptides also exhibit significant anticancer activity in human colon and liver cancer cell lines, even when they are isolated from shrimp waste.

Kholoud M.M. Abou El-Nour *et al.*, (2010), presented different methods of preparation silver nanoparticles and application of these nanoparticles in different fields.

Johan van Meerloo *et al.*, (2011), said that since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells.

Petrus *et al.*, (2011), reported that among the nanoparticles, silver or its compounds have known to have high antimicrobial and broad spectrum antimicrobial activities for bacteria, fungi and virus.

S. Silambarasan *et al.*, (2013), stated that silver nanoparticles are found to play a major role in the field of nanotechnology and nanomedicine.

Preetha Devaraj *et al.*, (2013), stated that nanotechnology is currently employed as tool to explore the darkest avenues of medical sciences in several ways like imaging, sensing, targeted drug delivery, gene delivery systems, and artificial implants.

G. Suriati *et al.*, (2014), reported that chemical reduction is the most commonly used, due to its simplicity. This technique also enables variation in the molar concentration of the reactant, dispersant and feed rate of reactant in order to produce silver nanoparticles with controlled particle sizes, shapes and particle size distribution.

Ramar Manikandan *et al.*, (2015), studied on biosynthesis of silver nanoparticles (AgNPs) using ethanolic extract of rose (*Rosa indica*) petals and testing their potential antibacterial activity using selective human pathogenic microbes, anticancer activity using human colon adenocarcinoma cancer cell line HCT 15.

Rani Mata *et al.*, (2015), reported the biological synthesis of *Abutilon indicum* silver nanoparticles (AIAgNPs) using aqueous Abutilon indicum Leaf Extract (AILE).

Bonnia *et al.*, (2016), forenamed plant mediated synthesis of silver nanoparticles is gaining more importance owing its simplicity, rapid rate of synthesis of nanoparticles and ecofriendliness.

Mohamad S AlSalhi *et al.*, (2016), performed Green synthesis of silver nanoparticles using *Pimpinella anisum* seeds; antimicrobial activity and cytotoxicity on human neonatal skin stromal cells and colon cancer cells.

Miriam Buttacavoli *et al.*, (2017), demonstrated differential proteomic analysis that highlighted important pathways involved in AgNPs-EPSaer toxicity.

Subramanian Palanisamy *et al.*, (2017), performed MTT assay on human colon cancer cells.

Anu Yadav., (2018), performed antiproliferative activity of *Camellia sinensis* mediated silver nanoparticles on HT-29 human colon cancer cell line.

Wang Chengzheng *et al.*, (2018), mentioned research finding provides an insight towards the development of novel anticancer agents using biological sources.

Sandhanasamy Devanesan *et al.*, (2018), suggested that the AgNPs biosynthesized using pomegranate peel extract may be a novel, potent solution for the development of a drug for colon cancer that also has antibacterial activity.

Inmaculada de la Calle *et al.*, (2018), stated that nanoparticles exist in a number of shapes depending upon the synthesis procedures. Several techniques have been used to characterize the size, crystal structure, elemental composition and a variety of other physical properties of nanoparticles.

Stefanos Mourdikoudis *et al.*, (2018), reported that nanoscale materials often present properties different from their bulk counterparts, as their high surface-to-volume ratio results in an exponential increase of the reactivity at the molecular level. Such properties include electronic, optical and chemical properties, while the mechanical characteristics of the nanoparticles (NPs) may also differ extensively.

Kadheeja Roshni *et al.*, (2018), demonstrated the Anticancer activity of Biosynthesized Silver Nanoparticles using *Murraya koenigii* Leaf Extract against HT-29 Colon Cancer Cell Line.

Fayezeh Samari *et al.*, (2018), reported on Low-temperature biosynthesis of silver nanoparticles using mango leaf extract and its anticancer activity against colon cancer cells.

Najlaa S., Al-Radadi., (2018), demonstrated the activity of AgNPs with *Cynara scolymus L.*, extract against human cancer cell lines colon carcinoma cells (HCT-116).

Bukola Adebayo-Tayo *et al.*, (2019), said that production of nanoparticles can be achieved through physical, chemical and biological methods. Of these three methods, chemical method is the most common method which involves the use of toxic chemicals in the production of nanoparticles.

RagaaA. Hamouda *et al.*, (2019), studied on the cytotoxic activity of silver nanoparticles against colon cancer (HCT-116) cell line giving IC50 (5.369µg/ml).

Shahnaz Majeeda *et al.*, (2019), studied on Bioengineered silver nanoparticles capped with bovine serum albumin and its anticancer and apoptotic activity against breast, bone and intestinal colon cancer cell lines.

Subramanyam Deepika *et al.*, (2019), performed Screening bioactivities of *Caesalpinia pulcherrima L. swartz* and cytotoxicity of extract synthesized silver nanoparticles on HCT116 cell line.

Sang Hun Lee *et al.*, (2019), described that silver nanoparticles (AgNPs) have distinct physical and optical properties and biochemical functionality tailored by various size- and shape-controlled AgNPs. They have great potential in a variety of applications as antimicrobial agents, biomedical device coatings, drug-delivery carriers, imaging probes, diagnostic and optoelectronic platforms. The size of AgNPs can be adjusted according to a specific application—e.g., AgNPs prepared for drug delivery are mostly greater than 100 nm to accommodate for the quantity of drug to be delivered.

Xiao H *et al.*, (2019), said that silver nanoparticles induce cell death of colon cancer cells through impairing cytoskeleton and membrane nanostructure.

Fengchang Huang *et al.*,(2019), determined the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay confirmed the cytotoxic property of AgNPs against human colon adenocarcinoma cell line (HT-29) in a dose-dependent manner.

Vivek K. Chaturvedi *et al.*, (2020), approached to synthesis with the use of *Pleurotus sajor-caju* followed by microwave irritation of silver and gold nanoparticles that ensures the capping of the active phyto ingredient and further enhances the effects of nanomedicine to fight colon cancer.

Iqbal Raja *et al.*, (2020), reported on synergistic response of physicochemical reaction parameters on biogenesis of silver nanoparticles and their action against colon cancer and leishmanial cells, Artificial Cells, Nanomedicine, and Biotechnology.

Kiran P. Shejawal *et al.*, (2020), worked on synthesized nanoparticles for DPPH assay and in vitro cytotoxicity using colon cancer cell lines COLO320DM and HT29 (MTT, SRB, and Trypan blue assay).

Alaa Naseer Mohammed Ali *et al.*, (2020), studied the toxicity of silver nanoparticle on the colon cancer cell line (HT29).

Diptikanta Acharya *et al.*, (2020), studied on synthesis of silver nanoparticle (AgNPs) from marine algae *Cladophora glomerata* extract, and anticancer activity was studied against colon cancer cells HCT-116 in-vitro.

Acharya Balkrishna *et al.*, (2020), performed a comparative study on *Putranjiva roxburghii Wall.* seed extract and developed silver nanoparticles (PJSNPs) for improving bioavailability that enhance their anti-cancer activity against HCT-116 (colon carcinoma).

B. Venkatadri *et al.*, (2020), performed Green synthesis of silver nanoparticles using aqueous rhizome extract of *Zingiber officinale* and *Curcuma longa* and In-vitro anti-cancer potential on human colon carcinoma HT-29 cells.

Bilal Javed *et al.*, (2020), stated that conventional cancer treatment involves the surgical removal of the malignant parts, administration of the chemotherapeutic drugs to shrink the tumours and the radioactive killing of the growth of the abnormal cells; these methods involve many side effects including the resistance of the patient's body to receive treatment,

development of the resistance against chemotherapeutic drugs and perilous effects of the dugs on the normal or healthy cells of the body.

Li Xu *et al.*, (2020), said that from ancient times, silver and its constituents have been employed for medicinal and antimicrobial purposes. Due to their distinctive physicochemical features, silver nanoparticles (AgNPs) have been one of the most appealing nanomaterials in biomedicine. AgNPs can be made through physical, chemical, or biological processes. The primary applications of AgNPs are in the treatment of infectious diseases and cancer, as well as in the promotion of bone and wound healing, as well as in vaccine adjuvants, anti-diabetic agents, and biosensors. Due to their distinct physicochemical features in comparison to their bulk form, nanomaterials (1-100 nm materials) have gained significant attention in recent decades in a variety of sectors including biomedicine, catalysis, energy storage, and sensing.

M. Rozalen *et al.*, (2020), reported on synthesis of controlled-size silver nanoparticles for the administration of methotrexate drug and its activity in colon and lung cancer cells.

Mostafa M. El-Sheekh *et al.*, (2020), investigated on vitro cytotoxic activity of Ag-NPs on human tumor colon (HCT116) cell line and normal retina (RPE1) cell line were investigated during this study using MTT assay.

Garibo *et al.*, (2020), reported that microbial and phyto-metabolites derived NPs are produced by an environmental friendly approach and have several biomedical applications including antimicrobial and cytotoxic efects.

Kan-Sen Chou *et al.*, (2020), studied on synthesis of nanosized silver particles by chemical reduction using formaldehyde in aqueous solution.

N. Sreenivasa *et al.*, (2021), performed biogenic synthesis of silver nanoparticles using *Paenibacillus sp.* in-vitro and their antibacterial, anticancer activity assessment against human colon tumour cell line.

Essam H. Ibrahim *et al.*, (2021), described the acetone preparation of the edible plant *L. sativum* is a good antibacterial agent, good anticancer preparation at least against colon cancer as it is shown to be targeted, effective and can boost immune cells.

Sabah Ahmed Al-Zahrani *et al.*, (2021), evaluated the anticancer efficiency of marine alga *Chaetomorpha ligustica* collected from the Arabian Gulf against colon cancer cell lines HT29 and HCT116 and reported the anti-cancer potential of biosynthesized AgNPs from *C. ligustica* extract.

Vydyanath R. *et al.*, (2021), performed Anticancer Activities of Biogenic Silver Nanoparticles Targeting Apoptosis and Inflammatory Pathways in Colon Cancer Cells.

Omar Ulises Cruz-Ramírez *et al.*, (2021), studied on cytotoxic activity of the AgNP formulations was evaluated against human colon tumor HCT-15 cells.

May Eid., (2022), explained that due to the absorption of energy in the infrared region, FTIR can classify molecular signatures in composites. Stretching, bending, scissoring, and twisting are all examples of biochemical bonds that can be detected based on their molecular rotational degree and form of movement.

Saran Lotfollahzadeh *et al.*, (2022) , stated that the change of the normal colonic epithelium to a precancerous lesion and ultimately an invasive carcinoma requires an accumulation of genetic mutations either somatic (acquired) and/or germline (inherited) in an approximately 10 to 15-year period. The most important prognostic colon cancer indicator is the pathological stage at presentation. In almost all patients, a diagnostic or screening colonoscopy is required for tissue biopsy pathological confirmation of colon carcinoma.

Diptikanta Acharya *et al.*, (2022), evaluated the Systemic Mechanism of Cytotoxicity in Human Colon Cancer HCT-116 Cells of Silver Nanoparticles.

MATERIALS AND METHODS

METHODOLOGY

Collection of sample:

The shrimp species namely *Parapenaeopsis stylifera* were collected from in and around Thoothukudi, Tamilnadu, India.

Preparation of shrimp powder:

The shrimp were rinsed with distilled water to remove dirt and associated debris and shade dried for about 25-30 days. Then the dried materials were grinded to fine powder using homogenizer and stored in airtight bottles.



Fig 4: Dried shrimp sample



Fig 5: Homogenized shrimp powder

Extraction of the sample:

Five grams of finely powdered shrimp was added with 100 mL of double distilled water and boiled at 80°C for 10 min. The obtained extraction was filtered using Whatman No. 1 filter paper, and the filtrate was collected in 250 mL bottle and stored at room temperature for further usage.





Fig 6 &7: Shrimp powder mixed with Double Distilled Water



Fig 8: Sample after boiling

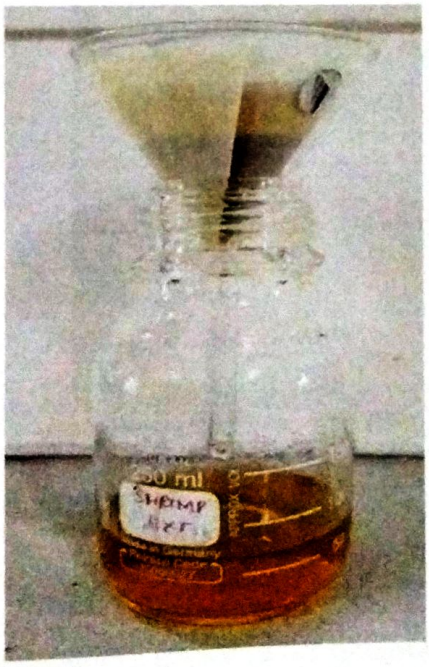


Fig 9: Filtration



Fig 10: 50 ml Shrimp Extract with 50mlof 1Mm $AgNO^3$



Fig 11: The mixture was placed in the magnetic stirrer for 1hr

Synthesis of Silver Nanoparticles:

Chemical Reduction Method

Chemical reduction is the most frequently applied method for the preparation of AgNPs as stable, colloidal dispersions in water or organic solvents. The reduction of silver ions (Ag+) in aqueous solution generally yields colloidal silver with particle diameters of several nanometers. Initially, the reduction of various complexes with Ag+ ions leads to the formation of silver atoms (Ag0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles (Nickel *et al.*, 2000; Chou and Ren, 2000; Wiley et al., 2005; Tao *et* al., 2006; Merga *et al.*, 2007).

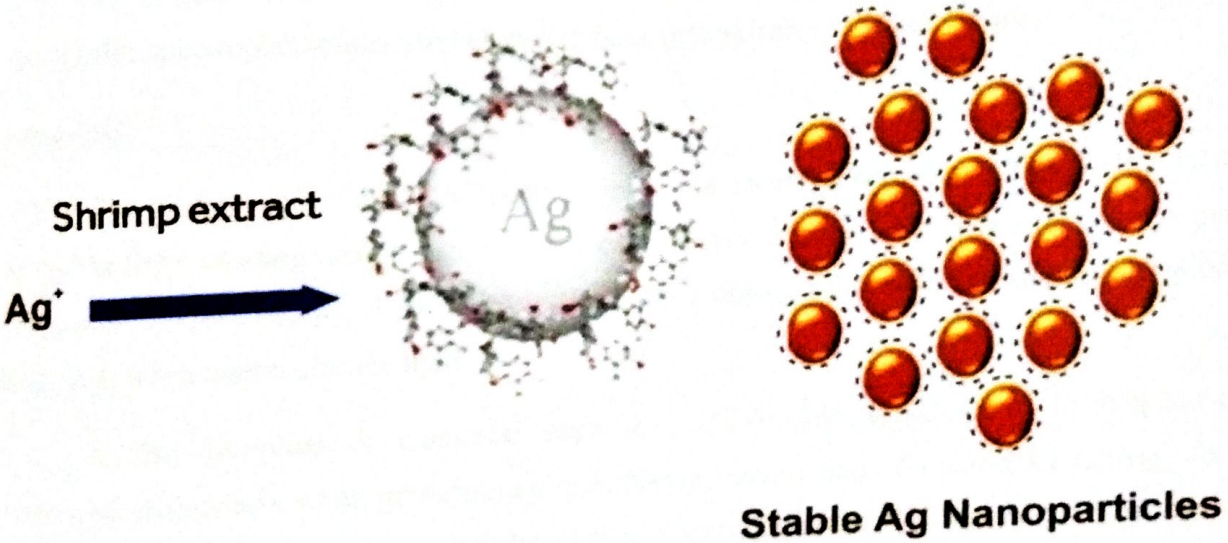


Fig 12: Chemical synthesis of Silver Nanoparticles

Colloidal silver is of particular interest because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activities (Frattini et al., 2005).

In the present investigation, we report the easy synthesis of silver nanoparticles by an environmental friendly procedure involving the in situ reduction of Ag by *Parapenaeopsis stylifera*

Silver nitrate used in this study was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. The synthesis of AgNPs was carried out using a pure aqueous extract solution of

shrimp. Take 50 mL of extract was added to 50 mL of 1 mM AgNO3 solution at room temperature, and the reduction of Ag NPs was clearly observed. For silver nanoparticles, the solution turned from yellowish to bright yellow and to dark brown. The Erlenmeyer flasks were incubated at 37 °C under agitation (200 rpm) for 24–48 h. (Kasthuri *et al.*, 2009)

Characterization of synthesized silver nanoparticles :

The following techniques are crucial for validating the synthesis of silver nanoparticles. The chemically created nanoparticles were identified using the following identifying techniques.

UV-Visible Spectrophotometer Analysis :

To validate the creation of silver nanoparticles, the colour of the sample is analysed in a UV-Visible spectrophotometer after changing from pale yellow to reddish brown.

Principle :

The fundamental principle of spectroscopy is that chemical compounds absorb ultraviolet or visible light, creating unique spectra in the process. The interaction of light and matter is the foundation of spectroscopy. A spectrum is created as a result of the excitation and de-excitation that occur when matter absorbs light.

In the discipline of analytical chemistry, UV-Visible spectroscopy is frequently employed, particularly when performing a quantitative examination of a particular analyte. For instance, UV-visible spectroscopy can be used to quantitatively analyse transition metal ions. Moreover, UV-Visible spectroscopy can be used to quantitatively analyse conjugated organic molecules. It should be mentioned that under certain circumstances, this form of spectroscopy can also be used to analyse solid and gaseous analytes.

Procedure :

Synthesized silver nanoparticles was confirmed by sampling the aqueous component of different time intervals and the absorption wavelength was noted.



Fig 13: UV Visible Spectrophotometer

Scanning Electron Microscope (SEM) :

In order to investigate the morphology of the as-prepared products, SEM images were recorded for each sample.

Principle :

Using a beam of electrons travelling at low energy to concentrate and scan specimens, the Scanning Electron Microscope (SEM) is a type of electron microscope that scans the surfaces of microorganisms. The inefficiency of the wavelength of light microscopes led to the invention of electron microscopes. Compared to a light microscope, an electron microscope's relatively short wavelengths allow for superior resolution. The Scanning Electron Microscope uses emitted electrons as opposed to transmitted electrons, which are used by the Transmission Electron Microscope. According to the Scanning Electron Microscope's operating theory, kinetic energy is used to generate signals from electron interactions. These electrons, which are utilised to observe crystalline elements and photons, are secondary electrons, backscattered electrons, and diffracted backscattered electrons. An image is created using backscattered and secondary

electrons. While the backscattered electrons reveal contrast in the composition of the specimen's elements, the secondary electrons that are emitted by the specimen serve as the principal means of identifying the morphology and topography of the specimen.

SEM is not a camera, and unlike a CCD array or film, the detector does not continuously create images. The resolution is not constrained by the diffraction limit, the fineness of lenses or mirrors, or the detector array resolution, as it is in an optical system. The SE detector is fist-sized and only senses electricity; the focusing optics may be large and coarse. Instead, the size of the electron spot—which in turn depends on both the electrons' wavelength and the electron-optical system that generates the scanning beam—determines the spatial resolution of the SEM. The size of the interaction volume, or the volume of specimen material that interacts with the electron beam, also places a limit on resolution. The size of the interaction volume, or the volume of specimen material that interacts with the electron beam, also places a limit on resolution. Despite these drawbacks, the SEM provides advantages such as the capacity to scan a sizable portion of the specimen, the ability to image bulk materials (rather than just thin films or foils), and the range of analytical modes available for analysing the specimen's composition and properties. The resolution might range from less than 1 nm to 20 nm, depending on the instrument.

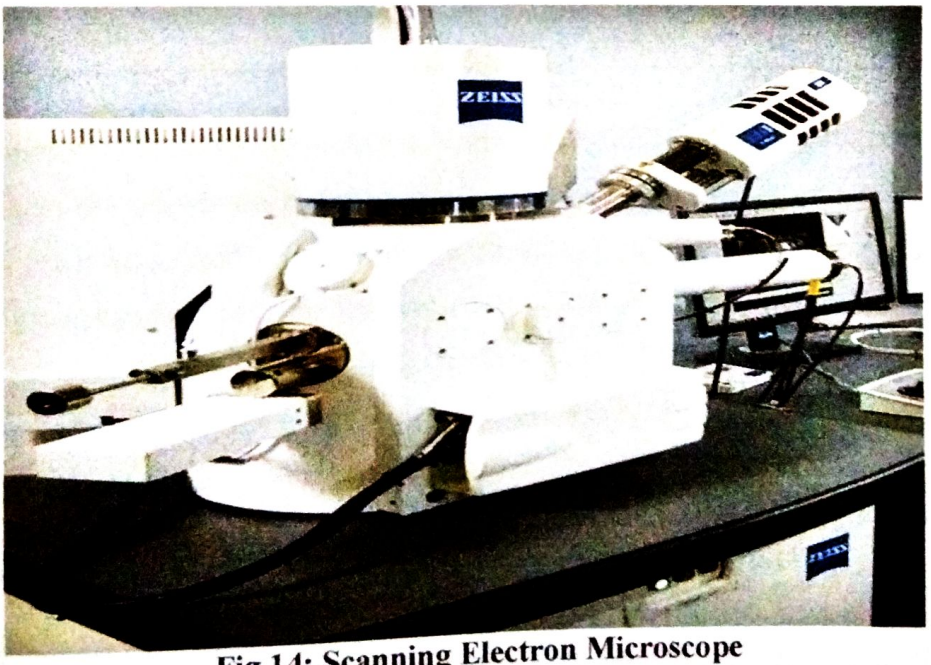


Fig 14: Scanning Electron Microscope

Fourier Transform Infrared Spectroscopy (FTIR) :

Fourier Transform Infrared (FTIR) spectral measurements were carried out to identify the functional group which is responsible for reducing and capping the silver nanoparticles. FTIR will show the structure and respective bands of the synthesized nanoparticles and the stretch of bonds.

Principle :

Fourier Transform Infrared Spectroscopy (FTIR) is a technique based on the measurement of the absorption of electromagnetic radiation with wavelengths within the mid-infrared region (4000–400 cm−1). If a molecule absorbs IR radiation, the dipole moment is somehow modified and the molecule becomes IR active. A recorded spectrum gives the position of bands related to the strength and nature of bonds, and specific functional groups, providing thus information concerning molecular structures and interactions. Feliu and co-workers studied how Pt nanostructures performed on ethanol oxidation, using a combined approach with in situ ATR-FTIR and Differential Electrochemical Mass Spectroscopy (DEMS). These techniques helped to probe adsorbates electrochemically and detect volatile reaction products. Their results were in agreement with previous findings, showing that the preferred decomposition products were related to surface structures, with COads formation on (100) domains and acetaldehyde/acetic acid formation on (111) domains.

A molecule's covalent bonds will selectively absorb radiation of specific wavelengths, which changes the vibrational energy in the bond. The type of vibration (stretching or bending) induced by the infrared radiation depends on the atoms in the bond. Because different bonds and functional groups absorb different frequencies, the transmittance pattern is different for different molecules. (Transmittance is the flipside of absorbance.) The spectrum is recorded on a graph with wavenumber (cm–1) recorded on the X-axis and transmittance recorded on the Y-axis. (Wavenumber is 1/wavelength and corresponds to the energy of the vibration of the molecular bonds). IR takes a single spectrum. FTIR uses an interferometer and performs a series of scans. IR used monochromatic light while FTIR used polychromatic light. FTIR scans up to 50 times per minute and provides better resolution.

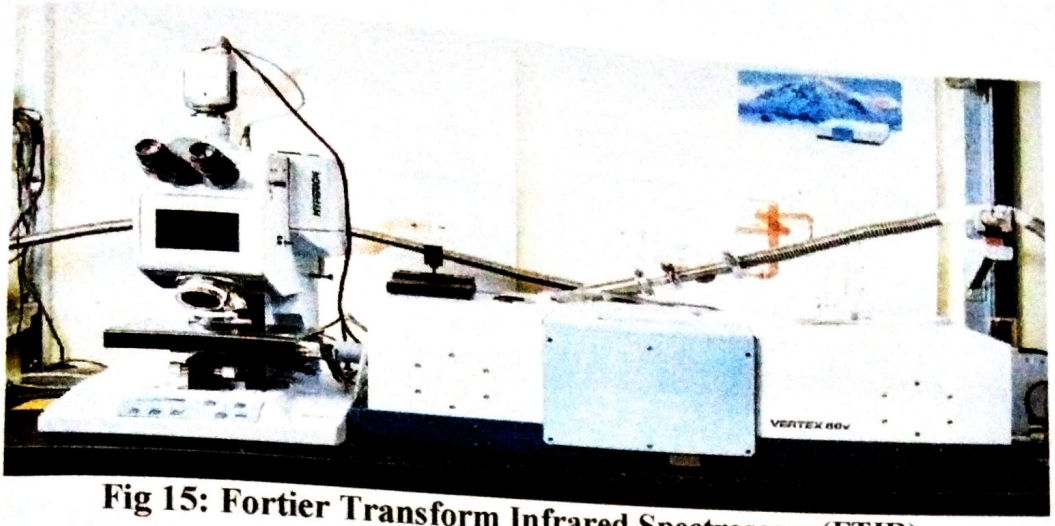


Fig 15: Fortier Transform Infrared Spectroscopy(FTIR)

Source of chemical and reagents:

Dulbecco's Modified Eagle's Medium, streptomycin, penicillin-G, L-glutamine, phosphate buffered saline, 3-(4,5 dimethylthiozol-2-yl)-2,5-diphenyltetrazoliumbromide, 2'7'diacetyl dichloro fluorescein, sodium dodecyl sulfate, trypan blue, trypsin-EDTA, ethylene diamine tetra acetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol, dimethyl sulfoxide (DMSO), and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd (India). All other chemicals used were of analytical grade, purchased from Hi media Laboratories Pvt. Ltd., India.

Extraction of the samples:

The synthesized nanoparticles were subjected to successive treatment by dissolving in 0.1% of DMSO.

Cell culture maintenance:

Colon Cancer (HCT116) cell lines were procured from the cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2 at 37°C.

MTT assay:

Principle:

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium salt made up of three aromatic rings, comprising two phenyl moieties and one thiazolyl ring, surrounding a positively charged quaternary tetrazole ring core with four nitrogen atoms. When MTT is reduced, the central tetrazole ring is broken, creating the violet-blue, water-insoluble chemical known as formazan. Because to its positive charge and lipophilic nature, the MTT reagent can pass through the mitochondrial inner membrane and cell membrane of living cells, and it is converted to formazan by metabolically active cells.

The MTT assay was created by (Mosmann *et al.,* 1983) as a result of the chromogenic characteristic of this redox chemical reaction, which offers a colorimetric-based evaluation of intracellular formazan synthesis. As a result, the assay has broad application in measuring cell metabolic activity. Although often unsupported, its utility has been used more frequently to infer secondary cell functions or states, such as viability.

MTT was used in both static and dynamic (perfusion condition) statuses of biocompatible assessment of various dentine-bonding agents (Vajrabhaya *et al.,* 2018) This assay is frequently used to detect the in vitro cytotoxic effects of medicines on cell lines or primary patient cells since for the majority of cell types the total mitochondrial activity is proportional to the number of viable cells.

The MTT assay is still commonly used and interpreted, overlooking these limitations and escaping the necessary optimization steps. Confounding factors that are frequently forgotten about include the number of cells used as a seed, the amount of MTT reagent added to the cells, the length of time the cells were incubated with MTT, the type of culture media, the removal of the cells' supernatant after MTT incubation, the wavelength at which optical density was measured, and the treatment that was being tested. It is challenging to compare the obtained OD values across different studies due to inconsistencies in these parameters.

Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light.

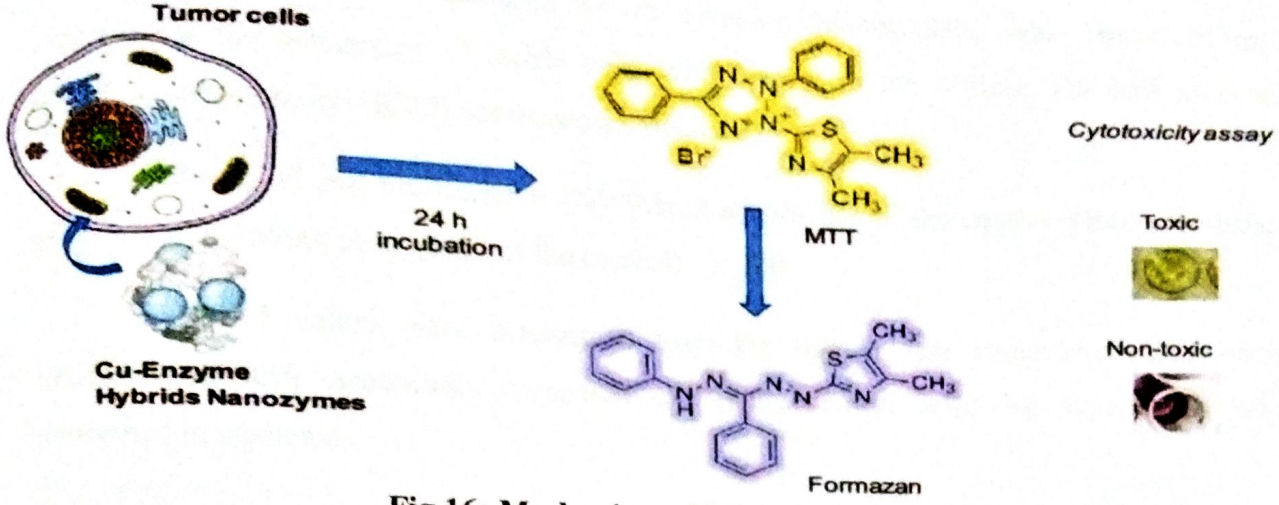


Fig 16: Mechanism of MTT Assay

The yellow 3-4,5dimethylthiozol-2-Yl)-2,5diphenyltetrazoliumbromide (MTT) is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazon, with a deep purple colour. Formazon crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate.

Reagents :

MTT stock solution:

MTT (50 mg) dye was dissolved in 10 mL of PBS. After vortexing for 1 min, it was filtered through 0.45 micro filters. The bottle was wrapped with aluminium foil to prevent light, as MTT was light sensitive. The preparation was stored at 4°C.

Procedure :

Cell viability assay, HCT116 viable cells were harvested and counted using hemocytometer diluted in DMEM medium to a density of 1 × 104 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow attachment. Then the cells were treated with the sample of different concentrations (10 to 80 µg/ml) were applied to each well. All the treated cells were incubated at 37°C in a humidified 95% air and 5% CO_2 incubator for 24 h. After

incubation, the drug-containing cells were washed with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubated for another 4 h at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC50) values were calculated.

Inhibitory of cell proliferation (%)=(Mean absorbence of the control−Mean absorbence of the sample)/(Mean absorbence of the control) X 100

The IC50 values were determined from the sample dose responsive curve where inhibition of 50% cytotoxicity compared to vehicle control cells. All experiments were performed in triplicate.

RESULT AND DISCUSSION

RESULTS AND DISCUSSION :

Visual Observation of the chemically synthesized nanoparticles

Formation of silver nanoparticles through reduction of silver nitrate into silver ion is known to be associated with colour change (Fig 18). The reaction mixture of $AgNO^3$ and the extract of *Parapenaeopsis stylifera* Shrimp turned brown indicating formation of silver nanoparticles. The intensity of the colour increases after 24 h of incubation.



Fig 17:Colur change after 30 minutes

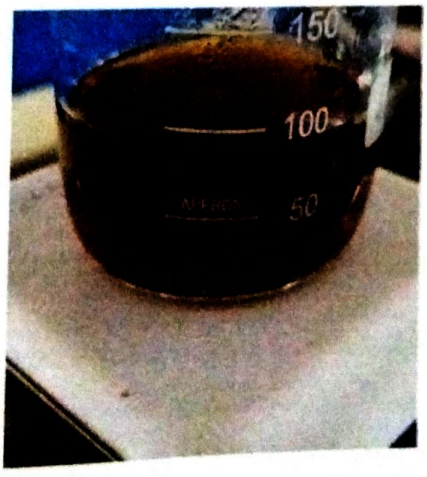


Fig 18:Colour change after 60 minutes

UV ANALYSIS :

Synthesized silver nanoparticles was confirmed by sampling the aqueous component of different time intervals and the absorption maxima was noted by UV–Vis spectrophotometer wavelength at 410 nm.

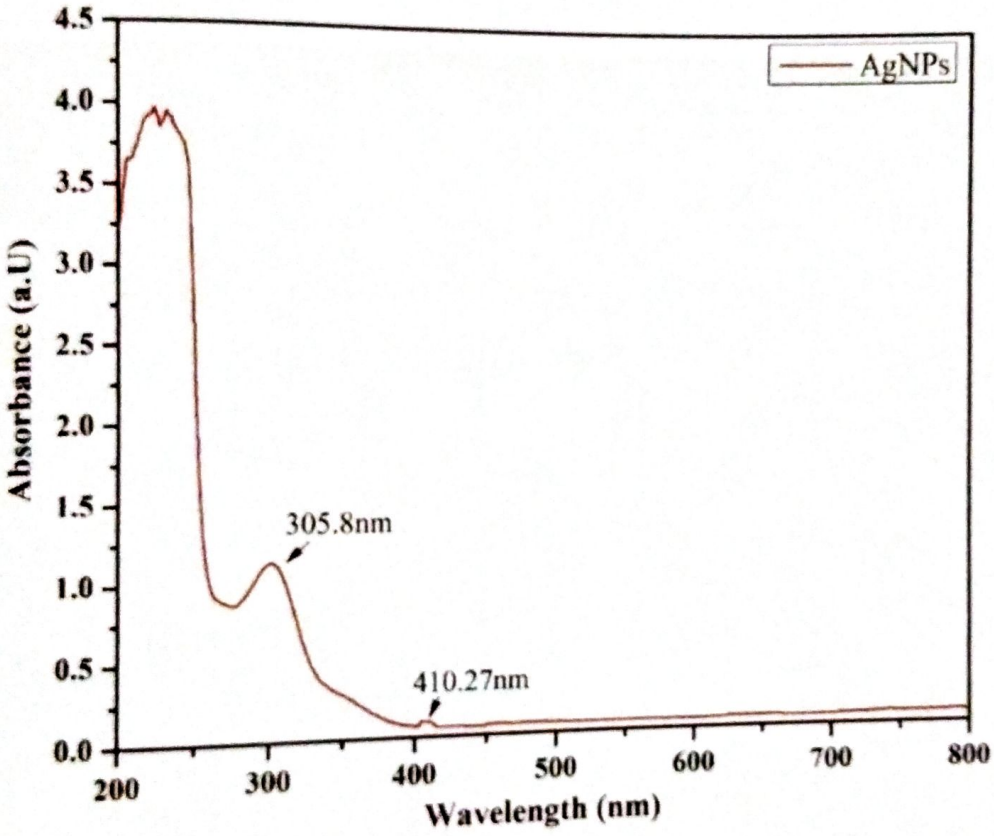


Fig 19: UV Vis Spectroscopy graph of AgNPs with maximum absorption at 410nm

The UV-visible absorbance spectroscopy is a characterization technique that shows absorbance of the material which is studied as a function of wavelength. The visible region of the spectrum is in the wavelength where the absorption was noted at 305.8nm and 410.27nm. The UV-visible spectrophotometer analysis was performed over the wavelength range 200 – 800 nm.

SEM :

In order to investigate the morphology of the as-prepared products, SEM images were recorded for each sample. Below figure shows the SEM images of Ag-NPs in two different magnifications. In Fig 20 small nanoparticles with the size of less than 100 nm are detectable. However, from Fig 21 it is clearly observed that the sample consists of highly agglomerated nanoparticles, which have formed large aggregates.

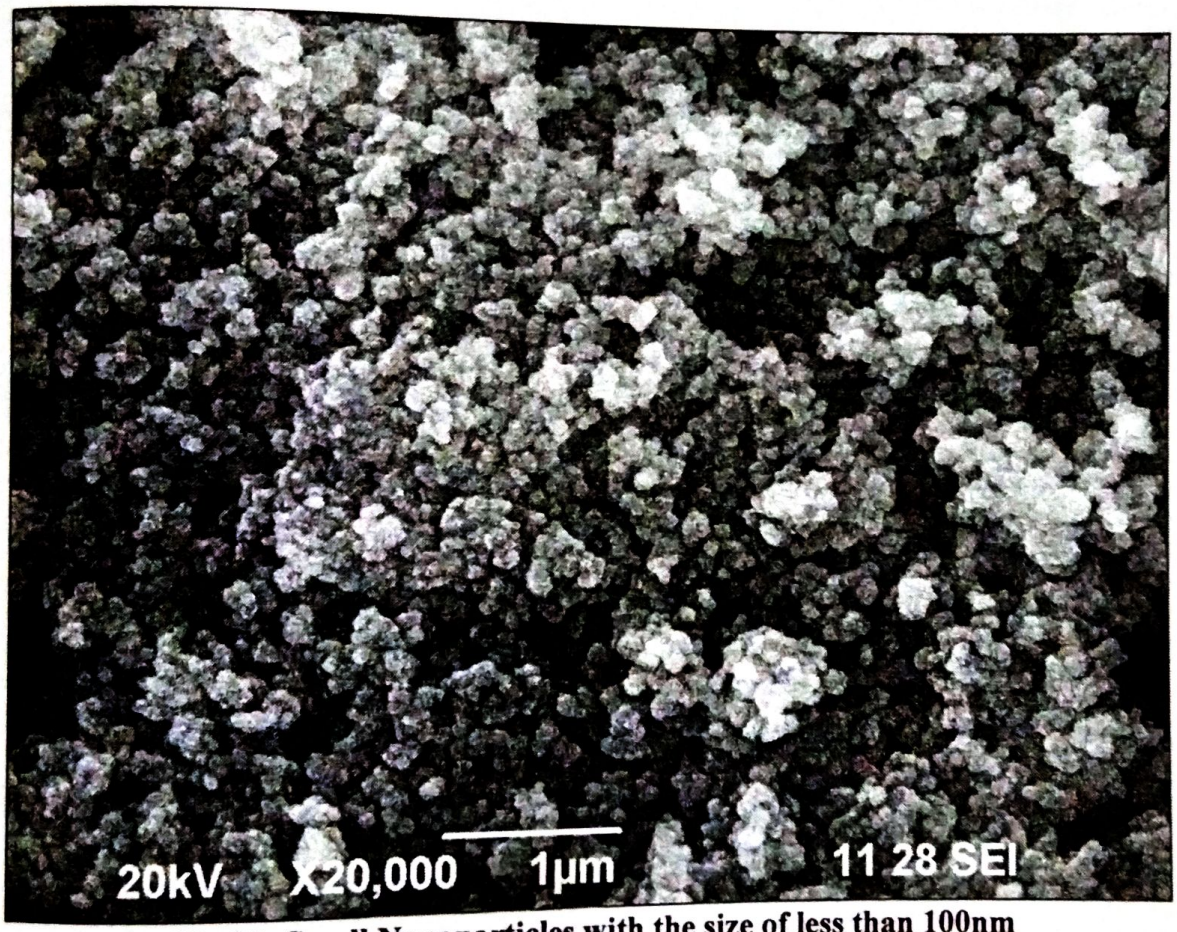


Fig 20: Small Nanoparticles with the size of less than 100nm

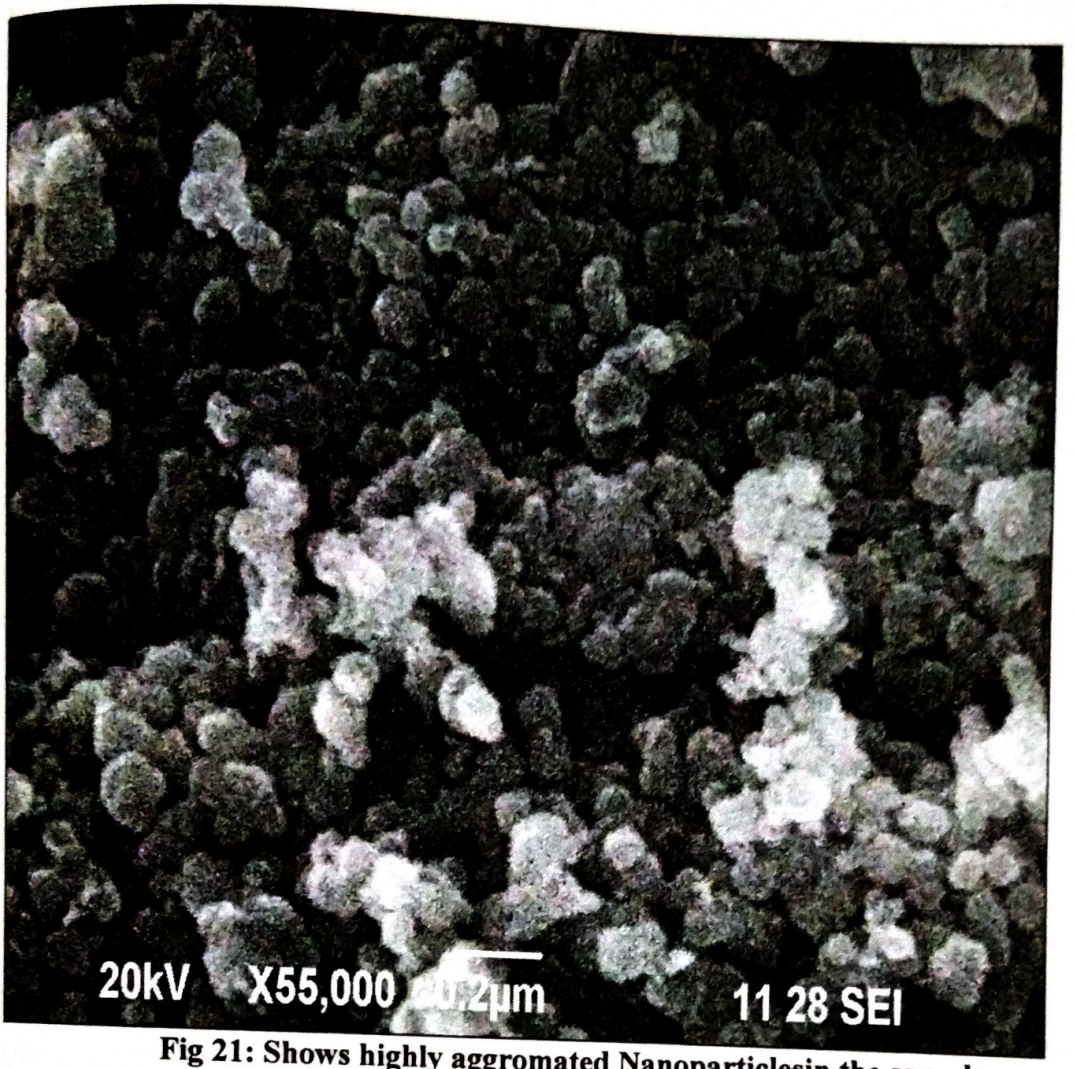


Fig 21: Shows highly aggromated Nanoparticlesin the sample

FTIR:

FTIR is used to discover functional group capping on the surface of silver nanoparticles. The peak at 3210.96 cm-1 is observed from the presence of O=H stretch of Carboxilic Acid. The band near 2113.06 cm-1shows the presence of CΞC stretching of Alkyne groups. The peak at 1772.79 cm-1 and 1753 cm-1 shows the presence of C-H bending Aromatic compounds. The presence of Nitro Compound and fluoro compounds were confirmed by the peak at 1557.52 cm-1 and 1273.72 cm-1 respectively. The peak at 1030.83 cm-1 and 799.31 cm-1 is observed due to the presence of sulfoxide and trisubstituted respectively. Such functional groups have been derived from test material.

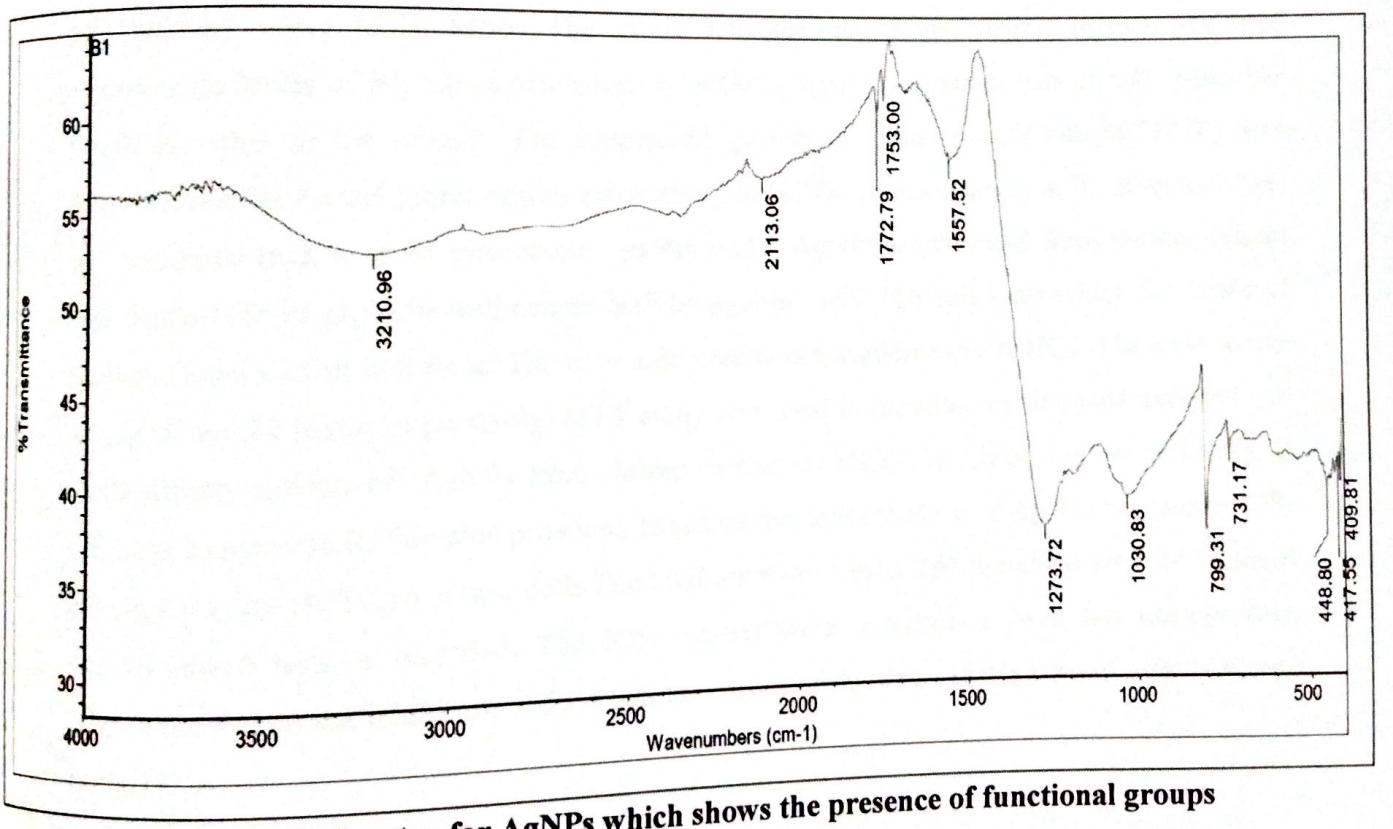


Fig 22: FTIR Spectra for AgNPs which shows the presence of functional groups

MTT ASSAY:

The MTT assay was used to assess the effects of silver nanoparticles on the viability of the human colon cancer cell lines (HCT116). The MTT Assay was performed to measure the metabolic activity and to check the cell viability and cell inhibition. MTT measures cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the sample. Results that are obtained suggest that AgNPs synthesized from *Parapenaeopsis stylifera* shrimp exhibit significant activity against colon cancer cell lines. Cells were treated for 24 hours as an increasing concentration from 10 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml and 80 mg/ml using AgNPs from shrimp extract.The result shows the Morphological changes in control and sample is treated with Colon Cancer HCT-116 cells for 24 hours. Fig.6. Shows graphic representation of cell viability using MTT assay. The silver nanoparticles from shrimp extract exhibited considerable levels of inhibition percentage of activity against the colon cancer cell describes the IC50 value of the extract. The suppressed growth of colon cancer cell(MCT116) was observed and the extract found exhibit cytotoxicity to HCT-116 cells cancer at IC 50 at 43.27 µg /ml. exhibited high level of cytotoxicity. In the study, AgNPs synthesized from shrimp extract was studied for its probable anti-cancer activity against HCT-116 cell lines which are kinds of Human Colon Cancer cell lines. The most effective concentration against HCT-116 cells where 60 µg/ml and 80 µg/ml respectively. MTT assay was used to measure significance variations in the inhibitory activity of AgNPs from shrimp extract on HCT-116 Colon Cancer cell lines. It was used to measure IC 50 value principle, based on the concentration of AgNPsthat causes 45% cell death on the HCT-116 cancer cells.The total number of cells that remained after 24 hours of the stimulated time is recorded. The IC50 values were determined from the sample dose responsive care where inhibition of 50% cytotoxicity confirmed to vehicle control cells is shown in fig.22.

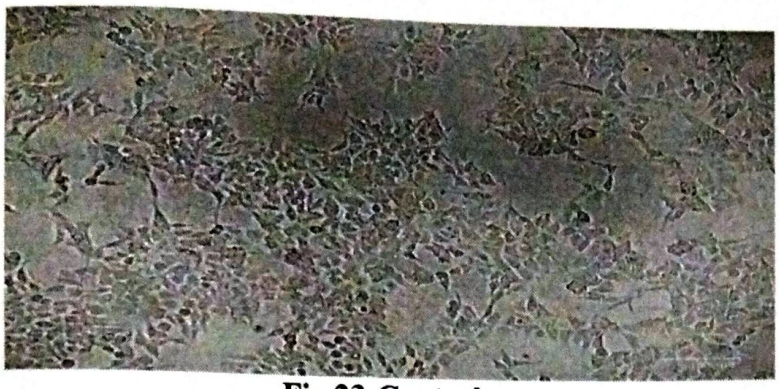


Fig 23;Control

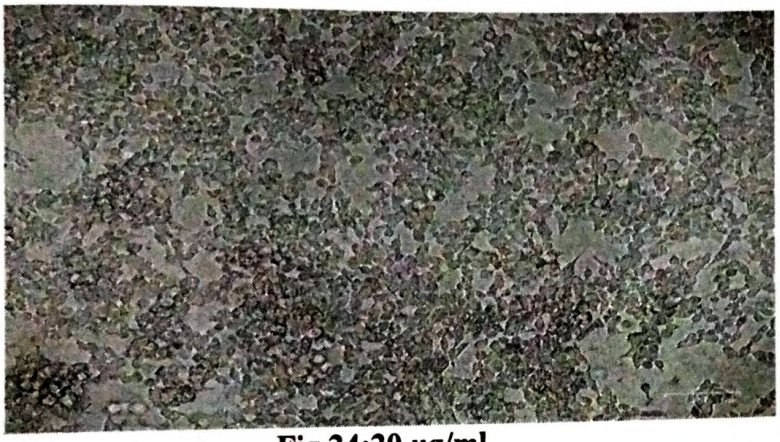


Fig 24:20 µg/ml

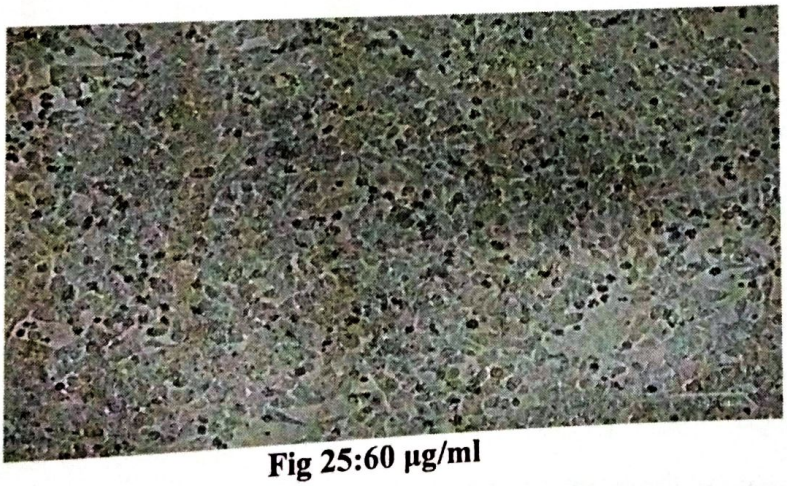


Fig 25:60 µg/ml

Photomicrograph (20x) represents morphological changes in HCT116 cells such as shrinkage, detachment, membrane bleeding and distorted shape induced bySilver Nanoparticle of treatment (10 µg, 20 µg, 40 µg and 80 µg /ml for 24hr) as compared with control. Control showed normal intact cell morphology and their images was captured by Fluorescent Microscope.

CELL VIABILITY %

	Control	10ug	20ug	40ug	60ug	80ug
	100	85.18	71.61	57.02	33.97	25.20
	100	83.95	80.07	54.57	33.36	22.95
	100	83.34	77.12	57.84	31.83	29.99
Average	100	84.16	76.27	56.48	33.05	26.05
SD	0	0.93	4.30	1.70	1.10	3.60

Table 2: Concentration vs Cell Viability

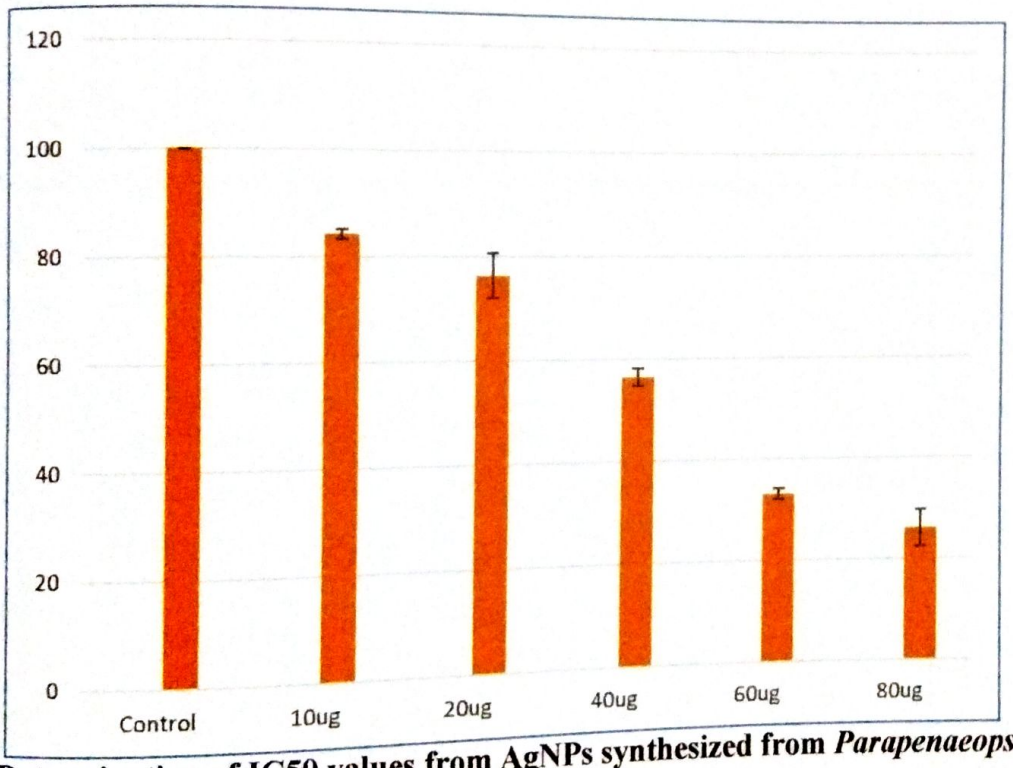


Fig 26:Determination of IC50 values from AgNPs synthesized from *Parapenaeopsis stylifera* for 24hrs of incubation with HCT116 cells using graph

CELL INHIBITION %

Control	10ug	20ug	40ug	60ug	80ug
-	14.82	28.39	42.98	66.03	74.80
-	16.05	19.93	45.43	66.64	77.05
-	16.66	22.88	42.16	68.17	70.01

Table 3:Concentration vs Cell inhibition %

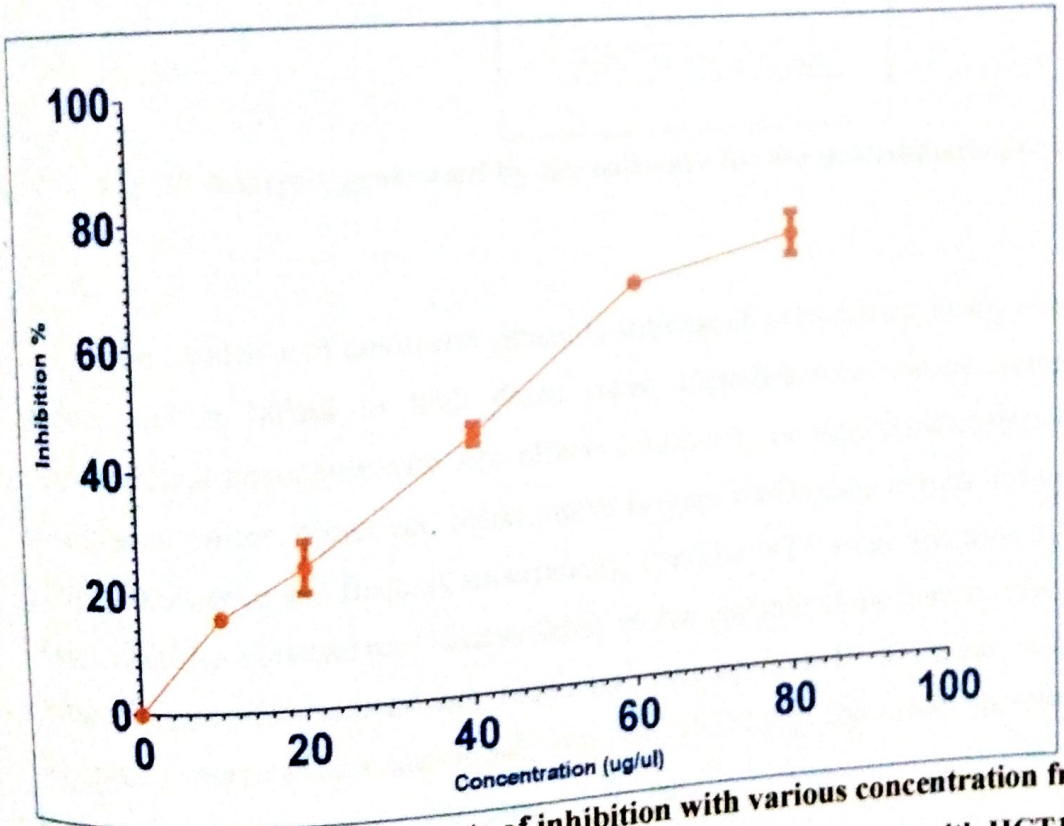


Fig 27: Determination of the rate of inhibition with various concentration from AgNPs synthesized from *Parapenaeopsis stylifera* for 24hrs of inhibition with HCT116 cells using graph

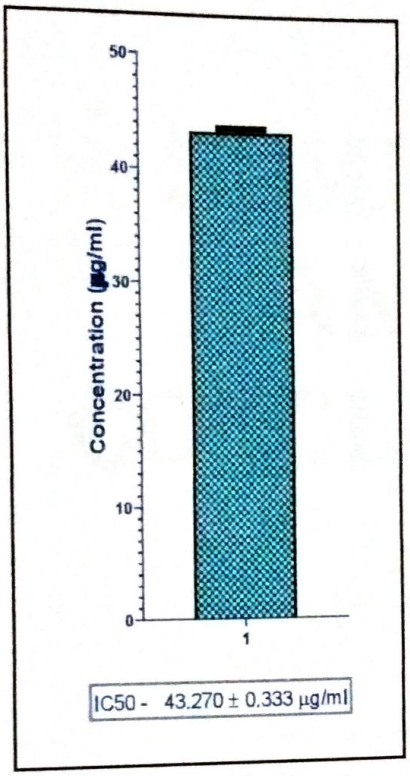


Fig 28 Analysis generated by the software for the determination of IC50 values

The incidence of colorectal cancer is anticipated to rise dramatically over the next several decades and is linked to high death rates. Unsatisfactory clinical outcomes, ineffective treatments, and treatments with side effects continue to be significant obstacles in the treatment of colorectal cancer. Moreover, colon cancer is more challenging to treat due to the development of chemoresistance and frequent recurrences. The The MTT assay was used to assess the effects of pancratistatin silver nilver Nanoparticles on the viability of the human colon cancer cell lines. Different doses of shrimp extracts (aqueous), ranging from 10–80 µg/mL, were applied against the human colorectal adenocarcinoma cell line (HCT116). The results showed that the extracts had cytotoxicity activity on colon cancer cell lines.

SUMMARY AND CONCLUSION

SUMMARY

Ag NPs have been the object of interest for academics for a long time because of its adjustable features. The silver nanoparticles were synthesized from /shrimp *Parapenaeopsis stylifera* by reducing silver salts by using chemical reduction method. But, additional research is needed to use the particles beyond the laboratory. Synthesizing cost-effective, evenly distributed, well-dispersed nanoparticles is always challenging as not a single synthesis method is perfect. The synthesized nanoparticles were characterized using various techniques such as UV-Vis spectroscopy, FTIR, and SEM to determine their size, shape, and chemical composition.

Synthesized silver nanoparticles was confirmed by sampling the aqueous component of different time intervals and the absorption maxi ma was detected by UV–Vis spectrophotometer wavelength at 410 nm.

SEM analysis shows the formation of silver aggregates and confirms the synthesis of silver nanoparticles.

FTIR was used to detect functional group capping on the surface of silver nanoparticles. The formation of peak and band shows the presence of presence of CΞC stretching of Alkyne groups, C-H bending Aromatic compounds, Nitro and fluoro compounds, sulfoxide.

MTT assay has been widely applied standard method that has used to evaluate cell viability. MTT assay was performed to determine the cytotoxicity and antitumour potency of the synthesized silver nanoparticles from shrimp. MTT reagents were prepared and the assay was performed. Human colon cancer cells such as HCT116 were cultured and treated with different concentrations of synthesized nanoparticles and traditional chemotherapy agents such as 5-fluorouracil (5-FU) to determine their cytotoxicity and anticancer activity. The half maximal inhibitory concentration (IC50) values were determined from the sample dose responsive curve where inhibition of 50% cytotoxicity compared to vehicle control cells. This indicates the Anticancer potency of silver nanoparticles from Shrimp *Parapenaeopsis stylifera* on colon cancer cells.

CONCLUSION

Although the anti-cancer effects of AgNPs have received a lot of attention in recent years, there are few studies on the potential effects and molecular mechanism of AgNPs in cancer.

The silver nanoparticles were synthesized by chemical reduction method from shrimp and investigated to find their potential anticancer activity in human colon cancer cells. The use of natural compounds from marine sources and the development of green and cost-effective synthesis methods for nanoparticles may lead to the discovery of new and effective therapies for cancer.

Bioactive compounds from marine animals have been reported to have a broad range of bioactive properties. Additionally, the nanoparticles are expected to have a higher selectivity for cancer cells and lower toxicity to healthy cells compared to traditional chemotherapy agents. The mechanism of action of the nanoparticles may involve induction of apoptosis, cell cycle arrest, and modulation of signaling pathways.

The first stage in ensuring that synthesised compounds have the necessary properties and that the properties of various batches can be consistently reproduced is characterization. Correlating the physicochemical characteristics of the nanoparticles' efficiency at performing a particular activity.

HCT116 is a human colon cancer cell line used in therapeutic research and drug screenings. HCT116 cells are used in a variety of biomedical studies involving colon cancer proliferation and corresponding inhibitors. MTT assay shows inhibition of 50% cytotoxicity compared to vehicle control cells. The incidence of colon cancer is anticipated to rise dramatically over the next several decades and is linked to high death rates. Unsatisfactory clinical outcomes, ineffective treatments, and treatments with side effects continue to be significant obstacles in the treatment of colorectal cancer. Moreover, colon cancer is more challenging to treat due to the development of chemoresistance and frequent recurrences.

The results of this study may contribute to the development of nanoparticle-based drug delivery systems for colon cancer treatment. Overall, this study provides a new and promising approach for the treatment of colon cancer.

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Dr. A. Doss	Dr. C. Lakshmanan	Dr. C. Vedrabahu
Organising Secretary	Head of the Department	Principal

A STUDY ON PHARAMACOLOGICAL ACTIVITIES OF *CISSUS QUADRANGULARIS*

A PROJECT SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated to Manonmaniam Sundaranar University

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

R. SELZIA

(REG. NO. 21SPMB04)

Under the Guidance of

Dr. Joys Selva Mary Albert M.Sc., M.Phil., Ph.D.



DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI – 628 001.

APRIL – 2023

CERTIFICATE

This is to certify that the project work entitled **"A study on Pharmacological activities of *Cissus quadrangularis*"** submitted to **St Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **Ms. SELZIA. R** under the guidance and supervision of Dr. Joys Selva Mary Albert M.Sc., M. Phil., Ph.D, Head and Assistant Professor of Microbiology St Mary's College (Autonomous), Thoothukudi, for academic year 2022- 2023

SIGNATURE OF THE GUIDE

SIGNATURE OF THE HOD.

Dr. Joys Selva Mary Albert
Head
Department of Microbiology
St. Mary's College (Autonomous)
Thoothukudi

SIGNATURE OF THE DIRECTOR

Director
Self Supporting Courses
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATUR OF THE PRINCIPAL

Principal
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

18/4/2023

SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE : 05.04.2023

DECLARATION

I hereby declare that the project work entitled **"A Study on Pharmacological activities of *Cissus quadrangularis*"** is a bonafide record of the work completed by me during the academic year 2023 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmanimam Sundharanar University. I also affirm that this is an original work done by me under the supervision of **Dr. Joys Selva Mary Albert M.Sc., M.Phil., Ph.D.,** Head and Assistant Professor of Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

V. Albert

SIGNATURE OF THE GUIDE

R. Selzia

SIGNATURE OF THE STUDENT

PLACE: THOOTHUKUDI

DATE : 05.04.2023

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CONTENTS

S.NO	TITLE	PAGE NUMBER
1	INTRODUCTION	1-18
2	AIM AND OBJECTIVES	19
3	EXTRACTION AND PHYTOCHEMICAL ANALYSIS	20-35
4	THIN LATER CHROMATOGRAPHY	36-39
5	ANTI OXIDANT ACTIVITY	40-43
6	ACUTE TOXICITY	44-47
7	ANTI DIABETIC ACTIVITY	48-51
8	ANTI MICROBIAL ACTIVITY	52-60
9	SUMMARY	61-62
10	BIBLIOGRAPHY	I-X
11	ANNEXURE	XI-XV

LIST OF PLATES

PLATE No.	TITLE
3.1	Sample collection of *Cissus quadrangularis*
3.2	*Cissus quadrangularis* stem powder
3.3	Soxhelet extraction of the extract
3.4	Solution after extraction
3.5	Ethanolic extract
3.6	Presence of flavonoids
3.7	Presence of carbohydrates
3.8	Presence of steroids
3.9	Presence of saponins
3.10	Absence of Protein
3.11	Absence of Anthocyanids
3.12	Absence of terpenoids
3.13	Absence of Tannins
3.14	Absence of phenolic compounds
3.15	Absence of phenolic Flavonoids
8.1	Zone of inhibition of *E.coli*
8.2	Zone of inhibition of *Staphylococcous*
8.3	Zone of inhibition of *vibrio*
8.4	Zone of inhibition of *Pseudomonas aeruginosa*
8.5	Zone of inhibition of *micrococcus*

List of Tables

Tables 3.1Phytochemicals present in *Cissus quadrangularis*

Tables 5.1 Antioxidant of ethanolic extract of *Cissus quadrangularis*

Tables 6.1 Acute toxicity effect of extract of *Cissus quadrangularis*

Tables 7.1 Anti diabetic activity of *Cissus quadrangularis* extracts with effect of single dose on Winstar albino rats

Tables 7.2 Anti diabetic activity of *Cissus quadrangularis* extracts with effect of Repeated exposure on Winstar albino rats

Tables 8.1 Antibacterial activity of *Cissus quadrangularis*

List of Figure

Figure 5.2 Effect of Ethanolic extract of *Cissus quadrangularis*

ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
+	Present
-	Absent
ANOVA	Analysis of variance
cm	Centimeter
DMSO	Dimethyl sulfoxide
DPPH	Diphenyl-1-picrylhydrazyl
g	gram
GCMS	Gas Chromatography Mass Spectrometry
hr	Hours
ha	Hectares
in	inches
IL-1β	interleukin-1β
Kg	Kilogram
L	litre
m	meter
MHA	Muller Hinton Agar

Min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimole
M.e	Methanolic extract
MTCC	Microbial Type Culture Collection and Gene Bank
NS	No sensitivity
NSAID	*Non-steroidal anti-inflammatory drugs*
OS	oral administration
C	Degree Celsius
PDA	Potato Dextrose Agar
PGE2	prostaglandin E_2
S.D	Standard deviation
Sec	Seconds
S.E.M	Standard Error of the mean
sp	Species
Vol.	Volume
Wt.	Weight.

CHAPTER 1

INTRODUCTION

Throughout, medicinal plants have a long history of use as treatments for a variety of illnesses, including asthma, gastrointestinal issues, skin conditions, respiratory and urinary issues, and hepatic and cardiovascular disease (Van Wyk and Wink, 2004; Tian *et al.*, 2014). Plant-based medications are well known for their reliability, accessibility, and affordability (Lwu *et al.*,1999). Plants can produce specific chemical or secondary metabolites that are non-nutritive but useful in defence mechanisms in order todefend themselves against pathogenic microorganisms, dangerous insects, and severe environmental changes (Prakash *et al.*, 2022). All indigenous systems of medicine, including Ayurveda, Siddha, Unani, and homoeopathy, rely on medicinal plants as their primary source of raw materials (Orient Longman 1992). The 8,000 species of medicinal plants collectively make for around 50% of all higher flowing plant species in India (Chopra RN *et al.,* 1956).

Green tea, ginger, walnuts, and other plants are examples of plants. For the activecompounds used in aspirin and toothpaste, other plants and their derivatives are regarded as key sources (Bassam A. 2012). In-depth research has been done on medicinal plants because of their vast therapeutic potential and advantages. A wide range of needs for human health are met by the complex blend of bioactive chemical (Miyazawa M. 1997). In undeveloped nations, using medicinal plants as analgesics is a long-standing custom that predates the development of modern medical sciences (Atiq *et al.*,2015) According to estimates, up to 70,000 plant species are used traditionally as medicines around the world. The effects of herbal extracts have been investigated through various tests. (F.Haq 2012) . India has a great wealth of medicinal plants and is frequently referred to as the medicinal garden of the world. As knowledge has risen, the number of novel plant-derived medications has grown at an accelerated rate. (AK Shakya 2016). There are thousands of active components found in medicinal plants that could be used to generate therapeutic drugs (Si-Yuan Pan *et al.*,2013)

The 8,000 species of medicinal plants collectively make for around 50% of all higher flowing plant species At least 70% of novel pharmaceuticals introduced in the United States in the previous 25 year are derived from natural sources, including anti-cancer therapies derived from plants like taxol . Medicinal plants' physiologically active component composition varies greatly depending on the species, soil type, and presence of microbes in

the plant (Zhao *et al.*,2011) The combination of secondary products found in plants is usually what gives plant material its advantageous therapeutic properties. The combination of secondary products in a single plant is frequently taxonomically distinct, which is compatible with the idea that a plant's medicinal activity is specific to a particular species or group of plants (Wink ,1999). Although secondary products in plants can serve a range of purposes, it's possible that their ecological role may have some relation on any potential medical benefits on people. Secondary product structures have developed to interact with molecular targets impacting the cells, tissues, and physiological function of competing microbes, plants, and animals in order to support the ecological survival of plants (Wink and Schimmer,1999). This idea of several chemicals working together in an additive or synergistic way likely stems from how secondary products function to aid in plant life. In respect to abiotic stresses such those brought on by variations in temperature, water status, light levels, UV exposure, and mineral nutrients, plants' secondary products can also operate as a protective barrier (Kaufman *et al.*,1999). The plants continue to provide mankind with new medicinal some of the beneficial properties ascribed to plants have been recognised to be flawed and medicinal plants treatment is based on the cuneiform date from around 2600 BC are from Mesopotamia among the material that were used were oils of *Commiphora species, Cedrus species, Glycyrrhiza glabra, and Papaver somniferum* are still used today for the cure of diseases ranging from colds and coughs to (fakim ,A.G. 2006).

The majority of significant medications developed over the past 50 years that have revolutionised modern medicine have been isolated from or derivatized from plants; their chemical constituents show a therapeutic balance between plant and animal drugs. The WHO supports and encourages the inclusion of herbal medicines in national health care programmes since they are widely available, reasonably priced, and time-tested, making them deemed to be far safer than contemporary synthetic medications. (P. Singh and C.L. Singh, 1981). Recently, effective treatments for some ailments that the synthetic drug industry was unable to provide have come from phytochemical and pharmacological research. These plants, which were once thought to be harmful or useless, are now recognised as important therapeutic herbs. Due to the tireless efforts of researchers, several strong medications, numerous therapeutic leads, and numerous new pharmacologically active ingredients have been produced from herbal drugs (Phillipson,M.N. 1990).

More than 200 years of traditional Chinese medicine practise include to use acupuncture, massage, breathing exercises, and food therapy. Together with conventional

Western medicine, traditional Chinese medicine has long been a crucial component of China's health care system Before Western medicines were introduced to China, many human ailments could be treated with TCM items that were both safe and effective. The Yellow Emperors Inner Classic of Materia Media (Hung Di Nei Jing; 200 BCE to 100 CE), the Divine Husband Man Classic of Materia Media (Shang Nong Ben Cao Jing; 25–220 AD), and cold-induced illnesses are notable texts in TCM (Shang han Lun ; 220 AD). China Materia Medica, which was released in 1977, is the most comprehensive guide of

Chinese herbal remedies. Of the almost 6000 medications listed, 480 are derived from plants. The Yin and Yang, which are in opposition to one another, and the five components, two independent beliefs concerning the natural law that governs good health and longevity, are the foundation of this ancient medical system, which is thought to be more than 5000 years old wu xing. (Gurib- fakim A 2006)One of the oldest sciences has evolved from medicinal plants in nations like China, Greece, Egypt, and India. Plants were frequently employed as a medicine, disinfectant, and aromatic ingredient in ancient Iran (Hamilton AC, 2004). Since humans have looked for a tool in their environment to recover from an illness, the usage of plants has been their only option for therapy (Halberstein RA, 2005). In reality, the use of medicinal plants for the treatment of diseases stretches back to the beginning of existence. Traditional medical practises are those that treat diseases at a local or regional level using natural plant materials without the aid of industrial processing, according to the World Health Organization (WHO) (Tilburt JC *et al.*, 2008). Due to its natural composition and relative lack of side effects, traditional herbal therapy has been utilised for thousands of years in both developing and developed nations (Wichtl M ,2004). 150 of the 2500 plant species present in India, out of the 21,000 species identified by the WHO as having therapeutic uses worldwide, are employed economically on a sizeable basis. India is recognised as the world's botanical garden and produces the most therapeutic herbs (Seth S.D. 2004).

Particularly in North America, the past ten years have seen a remarkable upsurge in interest in and use of medicinal plant products. According to surveys, more Americans than ever before are using plants as medicines, up from just 3% of the population in 1991 to over 37% in 1998. (Brevoort, 1998). Plant medicine sales in North America now account for around $3 billion in annual sales (Glaser, 1999). The availability of phytomedicines for sale at a variety of retail locations, the extent of their advertising in the popular media, and the recent entry of several major pharmaceutical companies into the business of producing phytomedicinal products all demonstrate that they have clearly returned to the mainstream from

their former domain of health-food and specialty stores (Brevoort, 1998; Glaser, 1999). Because they are more widely available and less expensive than contemporary medications, medicinal herbs are frequently employed in non-industrialized countries. The estimated yearly global export value of the tens of thousands of different plant species with therapeutic characteristics is $60 billion and is increasing at a rate of 6% per year. Traditional medicine is not well regulated in many nations, but the World Health Organization coordinates a network to promote safe and sensible use. The botanical herbal market has drawn criticism for having lax regulations and for selling placebo products that make medical claims but lack the evidence to back them up. Both broad concerns, including climate change and habitat degradation, and the specific threat of over-collection to satisfy market demand affect medicinal plants (Ahn, K. 2017).

All plants create chemical substances that give them an evolutionary benefit, including protecting themselves from herbivores or acting as a hormone in plant defences, like salicylic acid does. (Ahamad, A.2007). If validated by science, the content and known pharmacological activity of these phytochemicals in medicinal plants will serve as the scientific basis for their application in contemporary medicine. These phytochemicals have the potential to be used as medications. (Ahn, K. 2017). For instance, galantamine, an alkaloid approved for therapy against Alzheimer's disease, is one of nine classes of alkaloids found in daffodils (Narcissus). Alkaloids are toxic and have a bitter taste; they are concentrated in plant sections that herbivores are most likely to consume, such the stem, and they may also offer parasite protection. (Galantamine 2017).

Drugs from higher plants continue to hold a significant position in contemporary medicine, and plants continue to play a significant role in the development of new therapeutic molecules (Dev, 1997).

It is difficult to produce chemically affordable bioactive molecules (Shimomura *et al.*, 1997). Deforestation causes a rapid loss of medicinal resources, putting many priceless plants in danger of going extinct. Pharmaceutical businesses rely heavily on raw ingredients sourced from naturally occurring sources that are quickly running out. Plant tissue culture is an alternate method of propagation that is widely utilised for the commercial production of a vast variety of plant species, including many medicinal plants (George and Sherrington, 1984). Plants potentially contain many active compounds, and therefore many plants are highly toxic, and the phytotherapy in other complementary treatment modalities

carries a much higher risk of side effects and toxicity.Very dangerous and fatal side effects caused by the use of plant products have been reported. These side effects may be due to several different mechanisms. For example; direct toxic effects of the plant, allergic reactions, effects due to contamination, interactions with drugs and other plants (Ekor, M. 2013). All parts of the developing world as well as industrialised nations are seeing a rapid growth of traditional medicine.

A minimum of one complementary or alternative medical technique was employed by the population of Europe, North America, and other industrialised regions. According to reports, 75% of patients with HIV/AIDS use traditional or alternative medicine, mainly in San Francisco, London, and South Africa. (Dhal NK 2015, Aziz MA 2018, Hayta S 2014). There are three review articles in the Special Edition as well. Kigelia africana (Lam.) Benth. (Bignoniaceae), a tree species native to Africa, was reviewed by for its ethnobotanical value, phytochemistry, and pharmacological qualities (Nabatanzi, A; *et al.*,2020). Traditional medicines have made extensive use of the fruits, stem bark, roots, and leaves of this plant. It has been discovered that several phytochemical components exist, including phenolic chemicals, coumarins, terpenes, sterols, unsaturated fatty acids, and, in particular, iridoids and quinones, which have been found in all plant parts. Some of the pharmacological characteristics of this plant species that have been highlighted include antibacterial, antifungal, anti-inflammatory, and antidiabetic actions. (Faraone,I *et al* ., 2019). Plants which have one or more of its parts having substances that can be used for treatment of diseases are called medicinal (sofowora ,1982) . The medicinal values of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Hill,1952).

As plants are the foundation of traditional medicine, more than 3.3 billion people in less developed nations regularly use medicinal plants (Davidson-Hunt 2000). Several forms of seed, root, leaf, flower, or even the entire plant have been employed as therapeutic plant parts (Phillipson JD 2001). Both general hazards to medicinal plants, such as climatic changes and habitat degradation, and the specific threat of overharvesting to satisfy market demand are present (Ahn.K 2017). On clay tablets from circa 3000 BC, hundreds of medicinal herbs from ancient Sumeria are listed, including myrrh and opium. Around 800 therapeutic plants are listed in the ancient Egyptian Ebers papyrus, including cannabis, castor beans, garlic, and juniper. International organisations like the Harvest Plus programme, which has well-defined aims in addressing iron, zinc, and vitamin A shortages, have extensively endorsed plant-based approaches to managing micronutrient deficiencies In the spiritual, contemplative,

and spiritual lives of tribal people in India, medicinal plants have long played a key part. These plants have long played an important role in the spiritual, meditative, and spiritual lives of tribal people. (Mathew *et al.*,2020).

Humanity has been using medicinal herbs for millennia; it is as old as humanity itself. The variety of species used and their potential for healing are enormous. Plants that have not yet been described may have undiscovered cures. More than 50,000 higher plant species are currently thought to be used for therapeutic purposes globally (Schippmann *et al.*, 2002). This represents the largest range of biodiversity employed by people for a particular purpose and accounts for 20% of the world's vascular flora (Hamilton *et al.*, 2006). Many complex and structurally diverse chemicals can be found in plants and other natural sources. Pure secondary metabolites, essential oils, plant and microbial extracts, and newly synthesised compounds have recently attracted the attention of numerous researchers as possible antibacterial agents. (U.Mabona , *et al* 2013). The importance of medicinal plants to both individual and societal health cannot be overstated. The chemical compounds in plants that create specific physiological effects on the human body are what give them their therapeutic worth. Plants are thought to be a rich source of bioactive compounds and could serve as a substitute for other sources of insect repellents. (Yadav R, khare RK, *et al.*, 2017, Narendiran *et al.*,2016). A significant component of the worldwide medicinal market is made up of medicinal plant materials and herbal treatments made from them. Throughout the course of human history, pharmaceuticals and herbal cures have been quite effective at treating disease. Despite the fact that there is a wealth of information on their therapeutic benefits, there are no established methods for identifying plant materials based on their phytochemical, pharmacological, and therapeutic activities. The uniformity and therapeutic efficacy of medicinal plants are guaranteed by their standardisation. Since it is necessary to assess the medicinal efficacy of herbal products, i.e., to understand their pharmacological action to prove authenticity, they are tested for their identification (characterization), quality, and the quality of the extracts present.(Mukherjee K 2003) .

Traditional drug distribution techniques have gained attention from all across the world over the last ten years. According to current assessments, a sizable portion of the population in many developing nations relies on traditional healers and medicinal plants to meet their needs for primary healthcare. Although though these nations have access to modern medications, herbal medicines, or phytomedicines, have typically maintained appeal due to historical and cultural factors. The extraction of active compounds used in the production of

various medications frequently uses raw materials from medicinal plants. All contain chemicals from plants. just like in the case of laxatives, blood thinners, antibiotics, and anti-malarial medications. In addition, periwinkle, yew, and opium were used to make the active components of Taxol, vincristine, and morphine, respectively. According to the WHO, health is not just the absence of sickness or disability but also a condition of whole physical, mental, and social wellbeing. By the year 2000, the WHO wants to ensure that every person globally leads a sustainable socioeconomic productive life. (Lucy Hoareau *et al.*,1991). Medicinal plants can have a significant impact on achieving this aim. Traditional medicine is widely used in countries like China, India, Japan, Pakistan, Sri Lanka, and Thailand. In China, traditional tribal remedies account for around 40% of all pharmaceutical usage. Endod, as the plant is often known, is used as an effective molluscicide to control schistosomiasis. (Lemma,A. 1999).

In local communities all around the world, medicinal plants have long been used as a source of healing. Even yet, it is still crucial today because it serves as the primary healthcare system for 85% of the world's population (Pesic .M 2015) and a source for drug discovery because 80% of all synthetic pharmaceuticals are derived from it (Bauer and Brönstrup 2014) . Most of Africa's rural and urban populations benefit from medicinal plants as important resources and alternate sources for medical treatment (Badiaga, 2011). In fact, a number of therapies utilise the active ingredients found in medicinal plants (Kidik Pouka *et al.*, 2015). Around 80% of people around the world use traditional medicine to treat their primary health issues (Fatima *et al.*, 2015). Also, these plants offer the pharmaceutical business vital resources (Etame-Loe *et al.*, 2018). The cost of treating respiratory disorders has extended around the world, especially in Africa (Fatima *et al.*, 2015). In Cameroon, HIV kills 21% of people compared to respiratory diseases' 14% death rate (Mpondo *et al.*, 2017). Rural residents have limited access to medical facilities and traditional medications, which are usually pricy and harmful and are not always an effective way to improve people's health (Kidik Pouka *et al.*, 2015; Mpondo *et al.*, 2017). In addition, the emergence of respiratory illnesses is mostly caused by seasonal fluctuations, poverty, and pollution. Infections in the upper respiratory tract, such as colds, pharyngitis, and sinusitis, are examples of respiratory infections. Asthma, bronchitis, pneumonia, TB, and other lower respiratory tract illnesses are the opposite (Baky, 2010; Aubry, 2020). There haven't been many studies on using plants to treat respiratory infections, and the most of them have concentrated on the lower respiratory tract (Focho *et al.*, 2009; Mpondo *et al.*, 2017). Ethnobotanical studies on plants used to treat respiratory infections have primarily been conducted in markets in urban areas in the Littoral Area (Foze *et al.*, 2021).

Hence, a better understanding of upper respiratory diseases is required. The aim of this study was to discover plants and plant recipes utilised in the peri-urban and rural parts of the Littoral Area to treat upper respiratory tract affections urta.

More than 80% of the population in West Africa, like the rest of the continent, uses traditional medicine and medicinal herbs to heal themselves and their animals, according to the WHO (2002).

The world's most valuable sources of novel medicines are medicinal plants (Nalawade S.M. *et al* 2003),Chen SL., *et al* 2010),Chacko SM, 2010). 90% of the over 1300 medicinal plants used in Europe come from wild sources; in the US, about 118 of the top 150 prescription medications are derived from natural sources, (Balaunas MJ 2005). Additionally, over 25% of recommended medications in affluent nations come from wild plant species, while up to 80% of individuals in underdeveloped countries completely rely on herbal medicines for their main healthcare (Hamilton2004). According to a very conservative estimate, the pace of plant extinction today is between 100 and 1000 times greater than that expected by natural extinction, and the planet loses at least one key therapeutic candidate every two years (Pimm S, *et al.,* 1995). The World Wildlife Fund and the International Union for Conservation of Nature estimate that between 50,000 and 80,000 kinds of flowering plants are used as medicines globally. One-fifth of them, or 15,000 species, are in danger of going extinct due to habitat loss and overharvesting (Bentley *et al.,* 2010), and 20% of their wild resources have already been used up almost entirely due to rising human populations and plant consumption (Ross IA 2005).

Man has been acquainted with plants since the dawn of time and has employed them in numerous ways throughout history. Primitive man began to distinguish between plants with distinct pharmacological actions and those that were useful for medical purposes as he sought sustenance and learned to successfully deal with human pain. As the connection between man and plants grew, numerous plants began to be employed as remedies. While medical knowledge continued to advance faster than ever, the number of new medications generated from plants also rose. India has frequently been referred to as the Medicinal Garden of the globe since nature has endowed our nation with an enormous wealth of medicinal plants. the medical application of plants mentioned in the Hindu Vedas to treat various ailments The traditional medical system is currently extensively accepted and used by people all around the world. Ayurveda, Siddha, Unani, Homeopathy, Yoga, and Naturopathy are only a few of the

recognised Traditional systems of medicine that are currently practised in India (kumar N, *et al* 2015).

There are thought to be between 350,000 (Joppa L N; *et al* 2011) and almost half a million (Pimm, S. L; *et al* 2014). species of vascular plants, which account for 10% of all vascular plants (Fonnegra ,F.G.2007). and nearly half of all vascular plant species. Plants have been utilised as medicine for thousands of years and are still used today (Grover, J.K;2002). Trial and error was initially employed to identify useful plants with positive benefits, whether to treat illnesses or simply to feel better (Kunle, O.F; 2012). Traditional medicine is a term that refers to the progressive refinement of the use of these plants through many generations. Traditional medicine is defined by the official definition as "the body of knowledge, skills, and practises based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement, or treatment of physical and mental illnesses" (WHO (2000). Many kinds of seeds, roots, leaves, fruits, skins, flowers, and even the entire plant can be utilised as a source of medicine. The majority of the medicinal plants' active chemicals are employed as drugs because they have either direct or indirect therapeutic effects. Some plants create and store certain molecules in their bodies known as active compounds substances, which have physiological impacts on living things. (Phillipson,J.D., 1999). Natural and therapeutic herbs are widely available. Because they have the potential to benefit society and humanity in general, particularly in the fields of medicine and pharmacology, medicinal plants are more focused than ever. These plants' medical properties are due to their phytochemical components, which have defined pharmacological effects on the human body. Alkaloids, flavonoids, tannins, saponins, glycosides, phenolic compounds, and many more are among the most important bioactive phytochemicals (Edeogo *et al.*, 2005). A phytochemical is a naturally occurring substance found in plants including vegetables, fruits, and medicinal plants that interacts with nutrients and fibres to act against or, more particularly, to protect against diseases. According to their role in plant metabolism, phytochemicals are primarily split into two types, called primary and secondary constituents. Common sugars, amino acids, proteins, and chlorophyll are among the primary ingredients. Secondary constituents include alkaloids, flavonoids, saponins, tannins, phenolic compounds, and a variety of other substances (Krishnaiah *et al.*,2007)

The ability of plant components to stop the progression of specific diseases is another way in which they are distinguished. The toxicity and unfavourable side effects of

conventional and allopathic medications have also been significant contributors to the population's abrupt surge in demand, the growth of the herbal drug industry, and a decline in the usage of chemical treatments (Rasool Hassan BA. 2013). Many early drugs, including aspirin (from willow bark), digoxin (from foxglove), morphine (from opium poppies), quinine (from cinchona skin), and pilocarpine, were developed as a result of clinical, pharmaceutical, and chemical studies of these traditional drugs, which are primarily derived from plants (from Maranham Jaborandi). Almost 50% of the medications that are now on the market are thought to be derived in some way from medicinal plants (Yarnell E.2002, Harvey AL. 2008) . The use of phytotherapy is widespread and steadily expanding around the world. As a result, herbal medicines have become increasingly popular as a return to nature for the treatment of diseases and pains, bucking the global trend of synthetic substances. The source of medicinal herbs has always been found in nature (Frabricant DS, 2001). Isolating the active components of medicinal plants was made possible by the development of modern science, particularly during the Renaissance. This was made possible by chemical analysis and the associated equipment, such as the microscope (Reeds K.M. 1976). Since then, these active components have been synthesised by laboratory synthetic means in order to later make the medications (Atanasov A.G., *et al.* 2015) . The use of medications grew throughout time. Direct application of medicinal herbs appears to have been abandoned in modern medicine up to this point (Gertsch J. 2009).Today's medicine needs the industry generating pharmaceutical medicines, which are mostly based on the active components of plants, and consequently, these are employed as raw materials in many circumstances (Arceusz A., 2010) .

The importance of medicinal plants to the wellbeing of people and communities cannot be overstated. Plants have medical importance because they contain certain chemically active compounds that have specific physiological effects on people (Yadav R, *et al.*, 2017). Plants are thought to be a rich source of bioactive compounds and could serve as a substitute for other sources of insect repellents (Narendiran S, *et al.*,2016). Although if the world produces a lot of pharmaceuticals synthetically today, the gatherers still supply 60% of the natural resources used in pharmaceutical and herbal products. Nowadays, 25% of all medications manufactured worldwide are either directly taken from plants or contain at least one active ingredient from plants. 80% of people worldwide, according to who, depend on medications made from plants and use them for medical purposes (Haghiroalsadat F, *et al.*, 2011) . Due to its high polyphenol content and a variety of pharmacological qualities, including antioxidative, anticarcinogenic, and antiarteriosclerotic effects, experimental studies have

shown that tea has a considerable health-protective effect (Nihal Turkmen, *et al* ., 2006). Red clover, basil, dandelion, reishi mushroom, and even lemon are some of the plants that are utilised as blood purifiers to change a chronic illness by removing the metabolic poisons in the blood. Several herbs are used to balance the stomach's acid production. Antacids include herbs like marshmallow root and leaf. The herbs also supplied the healthy stomach acid required for optimal digestion. When a snake or insect bites a person, lavender, banana peels, marshmallow roots, and wormwood are used to draw the toxins from the body (Nirmal SA *et al* ., 2012). Moreover, it was more hazardous, resulting in a 30% increase in mortality, a considerable decrease in ultimate body weight, and organ malformation. All of the body's major organs are significantly damaged by excessive doses of A. scholaris; as a result, using high doses for an extended length of time should be done with caution. China has historically been the main producer of ephedra. It is a stimulant that tightens blood vessels, raises blood pressure, and quickens heartbeat. Ephedra, one of the most hazardous diet supplements, is widely used to help people lose weight. Users and doctors have reported over 800 injuries, including over 50 fatalities, to the USFDA and other state medical agencies ((Anonymous 2015). People have been studying plant species since the dawn of time in an effort to discover new medicines, which has led to the widespread use of medicinal plants that have the ability to treat a wide range of illnesses. In emerging nations, the value of therapeutic plants is becoming increasingly apparent. According to estimates, 80% of the population in India uses plants for self-care, with 60% of that population regularly using medicinal plants to treat various illnesses and roughly 40% of people using these plants in the pharmaceutical sector (Hiren BS *et al.,* 2013). According to the World Health Organization (WHO), herbal medicine is defined as finished labelled pharmaceuticals that contain active substances as aerial or subsurface components of plants or other plant material (Romila Y *et al.,* 2010). Although research into phytomedicine and nutraceuticals has grown significantly over the past three decades, there is still very little information available. While most plants chosen based on the strength of traditional knowledge have been scientifically validated and their pharmacological attributes of the plant drugs are now confirmed, many plant species having very strong ethnopharmacological values are often overlooked, creating a redundancy in the exploration of medicinal plants, having the potential to cure specific illnesses. Ethnopharmacology gives us the clue of the beneficial properties of medicinal plants (Farnsworth NR 1990, HeinrichM, *et al.,* 2010). To speed up evidence-based research in phytomedicine, the World Health Organization (WHO) has published three volumes of WHO monographs on specific medicinal plants since 1999. (WHO 2005).

Many plants are abundant sources of bioactive substances with distinct pharmacological activities that have no negative side effects (Salehi, B; *et al* 2019, Singab , A.N; 2014) . The use of low-cost medicinal plants instead of medicines to treat diabetes is widespread in underdeveloped nations where communities have long had high hopes for these plant remedies (Arumugam,G; 2013) . Developed nations are currently more likely to adopt these options. Many phytoconstituents are carried by the intricate plant matrix, which controls how these compounds interact specifically and has positive health effects (Durazzo,A; *et al* ., 2018)., Chan, C.H., *et al.*,2012). The most frequent variation in metabolism is that of glucose. According to this most typical system, the primary goal of using medicinal plants is to promote pancreatic function by either raising insulin secretion or reducing intestinal glucose uptake (Salehi,B.; *et al.*, 2019 , Arumugam,G; 2013) .

As digestive enzymes are in charge of the hydrolysis and absorption of macroelements, inhibitors that prevent them are crucial. Inhibiting the carbohydrate-digesting enzymes pancreatic -amylase (which converts polysaccharides to oligosaccharides and disaccharides) and brush border -glucosidase (which converts to monosaccharides) can help to alleviate problems with maintaining normal glycemia (Herrera, T.; 2019). Several medicinal plants with antidiabetic or antihyperglycemic properties are described in the literature and are utilised throughout the world. Recently, (Salehi.B.; *et al.*,2019). reported that 703 plants were most frequently mentioned in the literature and were -amylase and/or -glucosidase inhibitors. Plants are nature's wonder laboratories, producing a wide range of pharmaceuticals and treatments for maintaining optimal health and vitality. More than 100,000 secondary plant compounds with biological activity have been isolated from higher plants so far. The majority of these diverse compounds fall into four main chemical classes: phenolics (phenols, flavonoids, quinines, tannins, and lignins), terpenoids (monoterpenes, sesquiterpene lactones, diterpenes, saponins, and others), sulphur compounds (glucosinlates, disulphide, and acet (Chung AC 1995). India's biodiversity is renowned; as of now, 1.26 lakh plant species have been recorded (Chopra &Chopra 1955).

People have been studying plant species since the dawn of time in an effort to discover new medicines, which has led to the widespread use of medicinal plants that have the ability to treat a wide range of illnesses. In emerging nations, the value of therapeutic plants is becoming increasingly apparent. According to estimates, 80% of the population in India uses plants for self-care, with 60% of that population regularly using medicinal plants to treat various illnesses and roughly 40% of people using these plants in the pharmaceutical sector (Hiren BS, *et al*

..2013) . According to the World Health Organization (WHO), herbal medicine is defined as finished labelled pharmaceuticals that contain active substances as aerial or subsurface components of plants or other plant material (Romila Y *et al.*,2010). These pathophysiological alterations cause the emergence of epilepsy-related comorbidities. (Faure, 2016). It has been demonstrated that anxiety disorders may develop as a result of lesions occurring during epileptogenesis, particularly in the amygdala and other regions involved in emotions (Faure, 2016), much like reactive oxygen species (ROS) overproduction (Azam *et al.*, 2010). The creation of hydroperoxides and aldehydes originates from the oxidation of polyunsaturated fatty acids, which is caused by an excess of ROS. As a result of their biological activities, cell membranes are disrupted and altered in structure, which leads to cellular death (Sies and Stahl, 1995; Bonnefont-Rousselot *et al.*, 2003). Several isolated A. scholaris constituents lack reports of pharmacological properties that would support additional pharmacological research on them. Lethargy was induced in the animals by the greater dose. Moreover, it was more hazardous, resulting in a 30% increase in mortality, a considerable decrease in ultimate body weight, and organ malformation. All of the body's major organs are significantly damaged by excessive doses of A. scholaris; as a result, using high doses for an extended length of time should be done with caution. China has historically been the main producer of ephedra. It is a stimulant that tightens blood vessels, raises blood pressure, and quickens heartbeat. Ephedra is one of the most hazardous diet supplements that people use to reduce weight. Users and doctors have reported over 800 injuries, including over 50 fatalities, to the USFDA and other state medical agencies. The majority of occurrences involve heart attacks or high blood pressure that causes brain haemorrhage or stroke. (Anonymous 2015). The best place to see examples of symbiosis is in nature. The foundation for the treatment of diseases is a natural product derived from plants and animals. India has a long history of using a traditional medical system, which is widely recorded. India has a vibrant medicinal herb and spice culture. More than 2000 species have a strong potential for Ayush treatments. The majority of people in developed nations rely on Ayurvedic treatments since they are less expensive and safer because they cannot afford pharmaceutical items (Bhushan Patwardhan *et al.*,2004). Although if the world produces a lot of pharmaceuticals synthetically today, the gatherers still supply 60% of the natural resources used in pharmaceutical and herbal products. Nowadays, 25% of all medications manufactured worldwide are either directly taken from plants or contain at least one active ingredient from plants. 80% of people worldwide, according to who, depend on medications made from plants and use them for medical purposes (Haghiroalsadat.F *et al.*,2011). Due to its high polyphenol content and a variety of pharmacological qualities,

including antioxidative, anticarcinogenic, and antiarteriosclerotic effects, experimental studies have shown that tea has a considerable health-protective effect Nihal Turkmen *et al*.,2006). There are thought to be between 350,000 (Joppa LN., *et al*.,2011). to approximately 500,000 (Pimm, S.L 2014). species of vascular plants, with en percent of these being utilised as medicines (Fonnegra, F.G. 2007). Plants have been utilised as medicine for thousands of years and are still used today (Grover,J.K.; 2002) . Trial and error was initially employed to identify useful plants with positive benefits, whether to treat illnesses or simply to feel better(Kunle, O.F.; 2012).

C. quadrangularis is a shrub with four winged internodes and a thin, fleshy, fibrous, smooth stem. This plant's stem and roots have antioxidant and antibacterial properties. High concentrations of calcium and phosphorus, as well as anabolic steroids, can be found in *C. quadrangularis*. (Justin SR, *et al.*, 2011) ,(Nayar M. 1959). From ancient times, this plant's stem extracts have been utilised extensively for the early mending of fractures, gout, back discomfort, and irregular menstruation. The steroidal compounds from *C. quadrangularis* have a notable impact on the speed of callus mineralization and fracture healing. Deka DK, *et al*., 1994). *C. quadrangularis* is available as a syrup, pill, and capsule. Adults should take 1000–1500 mg per day. At least 12 to 16 weeks are needed for the maxillofacial fractures to heal. Due to recent developments in internal fixation, this period was shortened through either improved surgical methods or by blocking the physiological process of bone healing. (Singh v. *et al.*, 2011). High levels of vitamin C, vitamin A, anabolic steroids, and calcium are found in *C. quadrangularis*. The steroidal compounds from. *C. quadrangularis* had a notable impact on the mesenchymal-originated connective tissue's early regeneration, which in turn improved bone healing. (Justin SR, *et al* 2011). A member of the grape family, *Cissus quadrangularis* is a perennial plant. It is also referred as as adamant creeper, veldt grape, and winged treebine(GRIN ,ARS, USDA 2022) .(EPPO2022) The Arabian Peninsula, much of Africa, and tropical Asia are the native habitats of the species.(GRIN, ARS, USDA2022).

TRADITIONAL MEDICINE:

From ancient times, *Cissus quadrangularis* has been utilised as a medicine plant.To treat torn tendons and ligaments as well as shattered bones, cissus has been employed in a number of traditional Ayurvedic treatments.(Vibha *et al* 2017). Its name, asthisamharaka, derives from siddha medicine, where it is regarded as a tonic and analgesic and is thought to aid in the healing of fractured bones (that which prevents the destruction of bones). The Garo

tribe of Meghalaya and Bangladesh, as well as the Assamese people, have all employed *C. quadrangularis* to treat bone fractures. (Upadhyay *et al* 2011).

HISTORY OF CISSUS QUADRANGULARIS:

In Asia and Africa, cissus quadrangularis is a common vine. It is one of the Thai medicinal plants that is most frequently recommended. It has a long history of usage in Ayurvedic medicine in Africa. In the treatment of numerous disorders, the entire plant is utilised medicinally. Because it grows in many different places, Cissus quadrangularis, one of the traditional medicines that is said to have originated from Ayurveda, appears to have a wide variety of medical uses. It grows in numerous places and is used medicinally. Its traditional name, "Bone Setter," refers to its traditional use in treating bone diseases and the majority of feminine disorders (menopause, libido, and menstrual disorders). Other traditional uses relate to its purported antiulcer, antihemorrhagic, pain-relieving, and wound-healing capabilities.

There are numerous other names for the *Cissus Quadrangularis* that are used in various regions of India. Here are some of them:

CORE, CQ, CQE, CQR-300, Stemmed *Cissus Quadrangularis*, Samroi To, San Cha Khuat, Extrait de Cissus, Phet Sangkhat, Pirandai, Quadrangularis, Hadjora, Harbhanga, Harsankari, Hadjod, Mangaroli, Nalleru, Namunungwa, Phet Cha Sung Khaat, and Phet Sang Kat. Vine, Vajravalli, Veld Grape, Chaudhari, Cissus, Cissus Formula, Cissus Formulation, *C. quadrangularis*, Veldt-grape, Vitis quadrangularis, and Winged Treebine. Andison, Chadhuri (S. Kavitha *et al.*, 2015).

MORPHOLOGICAL CHRACTERISTICS AND NOMENCLATURE:

The succulent plant *Cissus quadrangularis*, often referred to as Asthisamhari, is a member of the Vitaceae family and is widespread in India's drier regions. It can be grown in plains, coastal regions, jungles, and wastelands up to 500 metres above sea level. Cuttings are used to propagate the plant (Anonymous 1992). From June through December, plant flowers(Guhabakshi DN, *et al* 2001). . Plant material is found in fragments of varied lengths, with internodes measuring 4–15 cm and having four wings.

The surface is tasteless and odourless, smooth and glabrous, buff in colour with a greenish tinge, and having an angular reddish-brown part. Petioles are 6-12mm long, and the stipules are tiny, broadly ovate, and acute. The leaves are simple, 2.5-5cm long, broadly ovate or reniform, occasionally 3-7 lobed, denticulate, glabrous, cordate, rounded, truncate, or cuneate

at the base. Flowers are arranged in spreading umbellate branches on short peduncle cymes. Calyxes can be truncate, cup-shaped, or very subtly lobed. Four ovate-oblong, short, robust petals are present. When ripe, the berry is oblong or globose, barely 6mm long, long apiculate, scarlet, and has one (very infrequently two) seeds. Ethnobotanical studies carried out by ethnobotanists in the traditional system of medicine have proven that the entire plant, including all components such as stems, leaves, and roots, possess medicinal characteristics. The current review identifies untapped potential of *Cissus quadrangularis* and may aid in bridging the gap between conventional wisdom and contemporary treatment. The quadrangular-sectioned branches of *Cissus quadrangularis* have internodes that are 1.2-1.5 cm (0.5-0.6 in) wide and 8-10 cm (3-4 in) long, growing to a height of 1.5 m (4.9 ft). A leathery edge runs along each angle. The nodes have toothed trilobe leaves that are 2–5 cm (0.8–2.0 in) broad. Each has a tendril coming out of the node's other side. Little white, yellowish, or green blooms grow in racemes; globular berries turn red when ripe. Cissus quadrangularis is an evergreen climber that expands quickly to reach heights of 5 m (16 ft) by.5 m (1.6 ft). Zone (UK) 10 hardiness. It may grow in nutrient-poor soil and is suitable for light (sandy), medium (loamy), and heavy (clay) soils. It favours well-drained soil. It may grow in highly acidic and very alkaline soils and is best on soils with a pH of acid, neutral, or basic. In the shade, it cannot grow. It can withstand drought and favours dry or moist soil. It needs a warm, tropical environment. In the months of June and July, it is propagated utilising the stem cutting techniques. The plant can be effectively propagated by taking mature stem cuttings. For the purpose of developing additional plants, stem cuttings were taken from a *Cissus quadrangularis* plant that was disease-free, healthy, and mature. It can be grown directly in prepared beds with a moderate water supply and an appropriate climbing substrate. Their mother plant's 30 cm long mature stem was cut off for replication without causing any harm. The plant consists of Various constituents such as flavanoides like quercetin, daidzein and genistein, triterpenoids like friedelin, vitamin 'C', stilbene derivatives like quadrangularin-A, resveratrol and piceatannol, iridoids like 6-0-meta-methoxy-benzozyl catapol, picroside and pallidol and phytosterols like β- sitosterol and calcium were identified as major constituents of the plant [9,10]. A and - amyrins, -sitosterol, ketosetosterol, phenols, tannins, vitamin A, carotene, calcium oxalate, 31 methyl tritiacontanoic acid, taraxeryl acetate, taraxeroliso-pentadecanoic acid, calcium ions, and phosphorus are all present in the plant stem sections. The plant's aerial parts include a brand-new asymmetric tetracyclic triterpenoid diol called 7-Oxo-Onocer-8-ene-3. Resveratrol, piceatanon, pallidol, parthenocissus, and alicyclic lipids are all found in leaves. In addition to potassium 67.5 mg, calcium 39.5 mg, zinc 3.0 mg, sodium 22.5 mg, iron 7.5 mg, lead 3.5 mg,

cadmium 0.25 mg, copper 0.5 mg, and magnesium 1.15 mg, root powder frequently offers a consistent source of these minerals (Jainu M, Devi CS .2006), (Panthong A,*et al,* 2007),(Jainu A, *et al* ., 2008), (Sen MK. 2019).

Macroscopic character:

- Aerial roots that form during rainy seasons
- The stem is subglabrous, dichotomously ranched, buff in colour with a greenish tint, fibrous, and smooth.
- Tendril can be found sporadically at nodes.
- Internodes: 4-5 cm in length and 1.2 cm in thickness.
- Depending on the species, leaves can be simple or lobed, cordate, widely ovate or eniform, serrate, detate, or even three-foliated.
- Little, greenish-white, bisexual, tetramerous, umbellate cymes with flowers are found opposite the leaves. Four to five imbricate petalsShort, complete, deciduous, cup-shaped calyx.
- Fruits are thick, globose or oblong berries that are highly acrid, dark purple to black, and succulent.One seeded plants with ellipsoid or pyriform seeds bloom and bear fruit in the post-rainy and autumnal seasons.

TAXONOMIC HIERACHY OF *CISSUS QUADRANGULARIS:*

Kingdom:	Plantae
Clade:	Tracheophytes
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Rosids
Order:	vitals
Family:	Vitaceae
Genus:	cissus
Species:	*C. quadrangularis*



Plate 1. 1 ***Cissus quadrangularis***

CHAPTER: 2

AIMS AND OBJECTIVE

- To collect and extract *Cissus quadrangularis* stem using ethanol solvent.
- To determine qualitatively the phytochemical from *Cissus quadrangularis* stem extract.
- To analyse the thin layer chromatography activity of ethanolic extract of *Cissus quadrangularis.*
- To determine the antioxidant activity of stem ethanolic extract of *Cissus quadrangularis.*
- To evaluate the acute toxicity, antidiabetic activity of stem ethanolic extract of *Cissus quadrangularis.*
- To asses antimicrobial activity of stem ethanolic extract against selected bacteria.

CHAPTER 3

EXTRACTION AND PHYOCHEMICAL ANALYSIS

3.1 SAMPLE COLLECTION

The plant species namely *Cissus quadrangularis* stem were collected from Nagercoil, Kanyakumari district, Tamil Nadu.



Plate 3.1 Sample collection of *Cissus quadrangularis*

3.2 PREPARATION OF PLANT POWDER

The plant were washed thoroughly with water for 2-3 times to avoid unwanted debris. The it was air dried in shade away from sunlight for 20-30 days. It was the ground to fine powder using electric mixer and stored in air tight container until further usage.

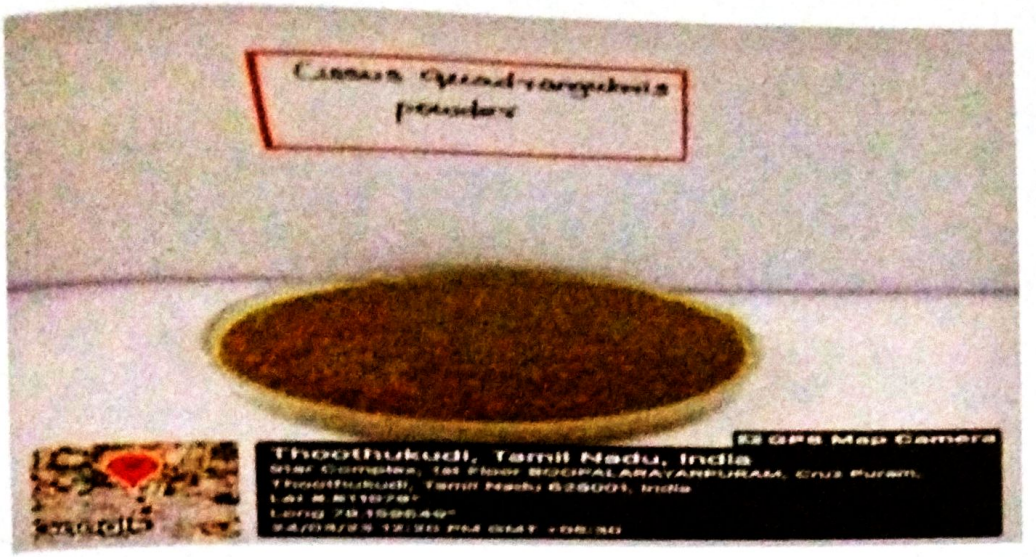


Plate 3.2 *Cissus quadrangularis* stem powder

3.3 EXTRACT OF PREPARATION:

Dried powder of the stem of *cissus quadrangularis* was weight and it was subjected to Soxhlet extraction with ethanol (99.9%) used as solvent for up to 8hrs the extraction process was continued. The extract was air dried solvent was evaporated at room temperature in a flat tray for 24- 48 hrs. After evaporation the crude extract was collected and store at 4°C for further analysis.



Plate 3.3 Soxhelet extraction of the extract



Plate 3.4 Solution after extraction

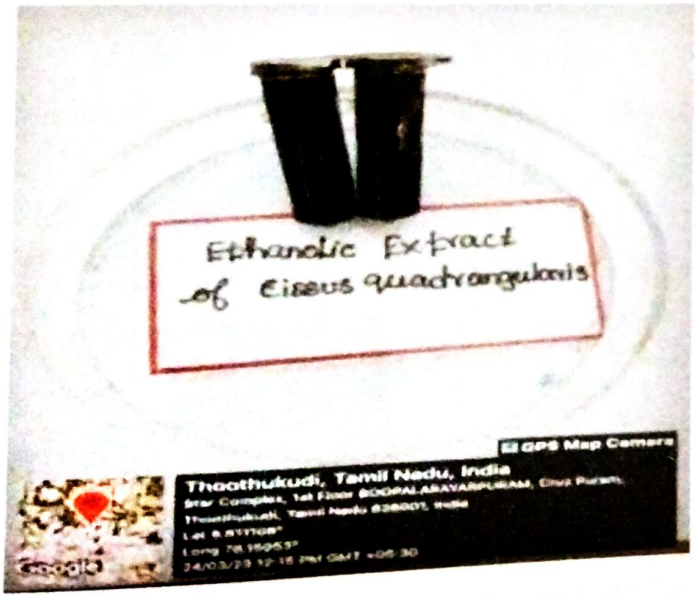


Plate 3.5 Ethanolic extract

3.4. MATERIALS AND METHODS:

Cissus quadrangularis with ethanol were subjected to various qualitative test for the identification of plants constituent present in the species

3.5 QUALITATIVE ANALYSIS OF PHYTOCHEMICAL:

3.5.1 Test for Flavonoids

To 1ml of filtrate with 2ml of dilute NaOH shows development of golden yellow.

3.5.2 Test for Carbohydrates

To 1ml of the filtrate with 5ml Benedict`s reagent and boil for 5 minutes. Bluish green colour indicates the presence of carbohydrates

3.5.3 Test for Steroids

To 1ml of the filtrate add 10 ml chloroform and 10 ml of H_2SO_4 slowly by the sides of the test tubes. Upper layer turns red and sulphuric acid layer showed yellow with green fluorescence.

3.5.4 Test for Saponins

To 1ml of filtrate with 2ml distilled water, is shaken vigorously and allowed to Strand for 10 minute . Development of foam on the surface of the mixture, lasting for 10 minutes indicates the presence of Saponins.

3.5.5 Test for Protein

To 1ml of filtrate with 5 to 6 drops of Millon`s reagent develops white precipitate which turns red on heating

3.5.6. Test for Anthocyanides

To 1ml of filtrate with 5ml of dilute HCl, the appearance of pale pink colour indicates the presence of the above compound.

3.5.7 Test for Terpenoids

To 1ml of filtrate with 2ml of chloroform and a few drops of concentrated Sulfuric acid were added along the side of the test tube. A red brown colour formed indicates the presences of Terpenoids.

3.5.8. Test for Tannin

To 1ml of filtrate with 2ml of ferric chloride gives dark green colour.

3.5.9 Test for Phenolic Flavonoids

To 1ml of filtrate with 2ml of 10% lead acetate gives brown precipitate.

3.5.10 Test for Phenolic Compounds

The extracts were treated with few drops of 10% lead acetate solution. The formation of precipitate confirmed the presence of phenolic compounds.

3.6 RESULTS AND DISCSUSSION:

The qualitative chemical test for the extract was performed. The investigation showed that *Cissus quadrangularis* stem contain flavonoids, saponins, terpenoids, carbohydrates. Flavonoids are also known as vitamin p or plants modifiers, present in high quantities. Carbohydrate is reported to have numerous role in living things, such as the storage and transport of energy and structural components. Saponins present in plants, have been suggested as possible anti – carcinogenic. The proposed mechanisms of anti – carcinogenic properties of saponins include direct cytotoxicity, immune modulatory effect , bile-acid binding and normalization of carcinogen induced cell proliferation. Terpenoids may be show cytotoxic activity against a wide range of organisms, ranging from bacteria and fungi.

TABLE 3.1 Phytochemical of the ethanol extract of *Cissus quadrangularis* stem

S.NO	Phytochemical test	*Cissus quadrangularis* stem
1.	FLAVONOIDS	+
2.	CARBONHYDRATE	+
3.	STEROIDS	+
4.	SAPONINS	+
5.	PROTEIN	-
6.	ANTHOCYANIDES	-
7.	TERPENOIDS	-
8.	TANNINS	-
9.	PHENOLIC FLAVONOIDS	-
10.	PHENOLIC COMPOUNDS	-



Plate 3.6 Presence of Flavonoids



Plate 3.7 Presence of Carbohydrate



Plate 3.8 Presence of Steroids

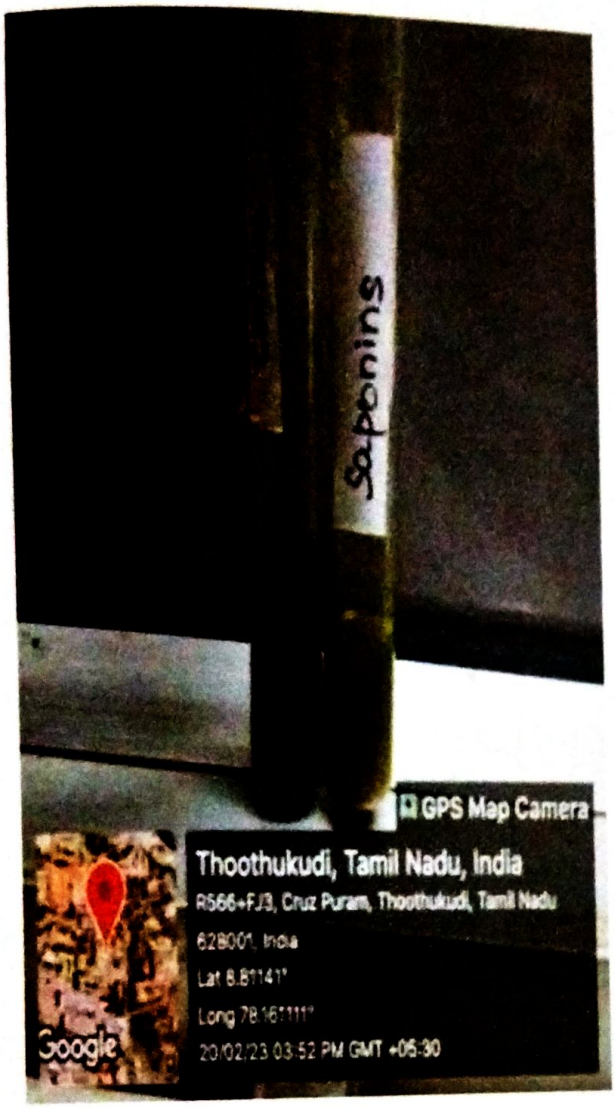


Plate 3.9 Presence of saponins

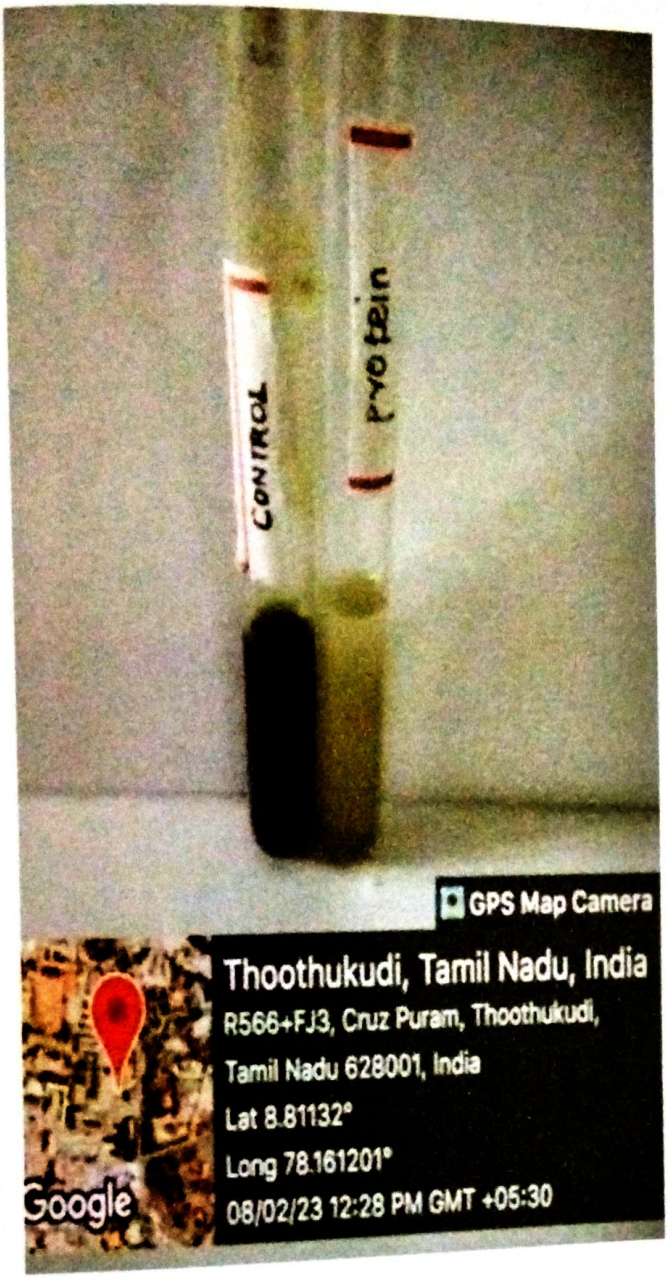


Plate 3.10 Absence of Protein

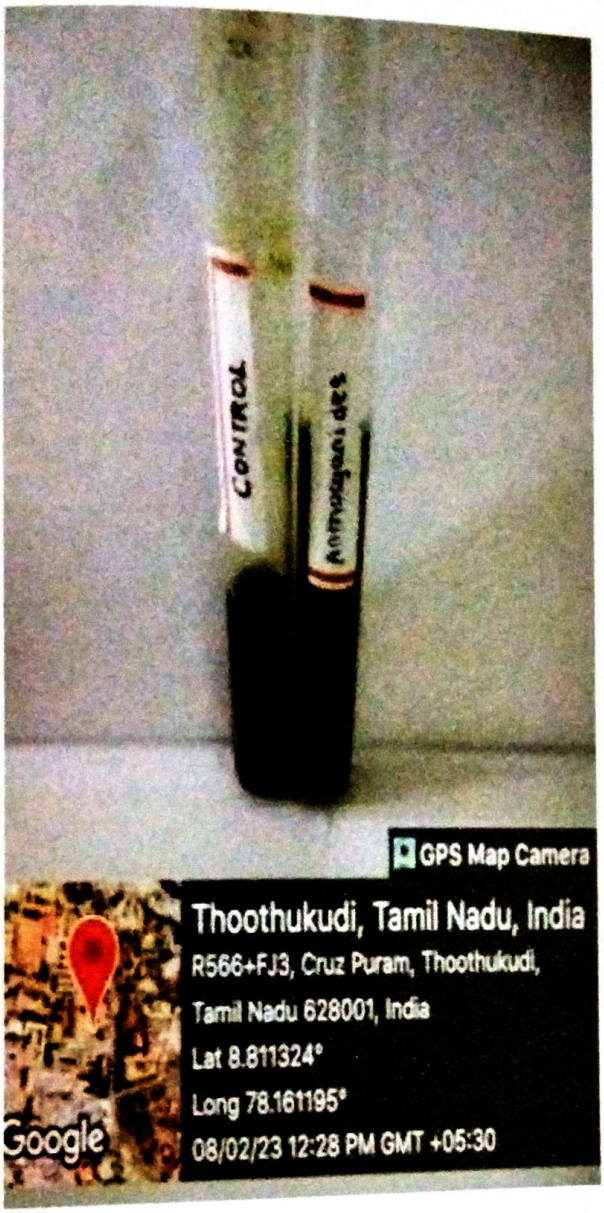


Plate 3.11 Absence of Anthocyanides



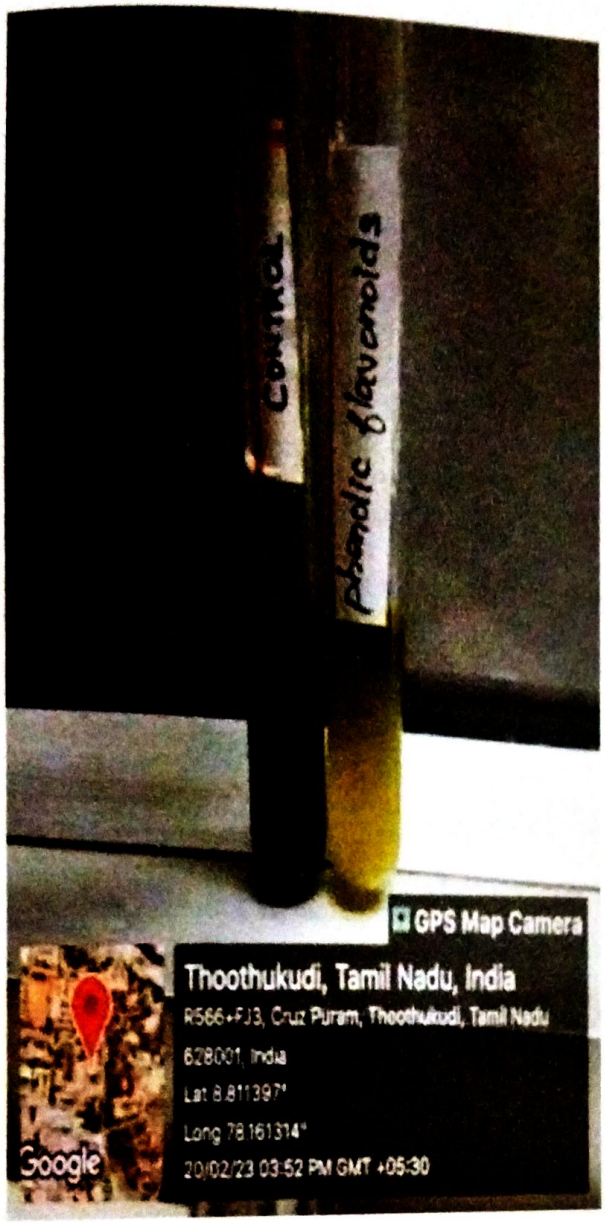
Plate 3.12 Absence of Terpenoids



Pate 3.13 Absence of Tannins



Plate 3.14Absence of phenolic Compound



P late 3.15 Absence of Phenolic Flavonoids

CHAPTER 4

THIN LAYER CHROMATOGRAPHY

4.1 INTRODUCTION:

A fundamental chromatographic method known as thin layer chromatography (TLC) is used to primarily separate non-volatile substances. TLC is typically performed on glass, aluminium foil, and plastic sheets that have been thinly coated with a variety of absorbent materials, including silica gel, cellulosic polymers, and aluminium oxides (Factor, 1991). The stationary phase is made up of the absorbent materials, whereas the mobile phase is made up of the solvent that is employed for the separation. The polarity of each phase is distinct. As the components are divided on the plane, separation by TLC is quite practical. Separation takes place due to polarity and some individuals migrating less than others (Stahl, 2013). The most practical method for producing the plant extract for TLC investigations is to extract the plant material using a mixture of alcohol and water, such as 80% ethanol (Khurram *et al.*, 2009). The solvent used has a significant impact on how the active phytochemicals are separated during TLC. The solvent solution employed has a significant impact on the movement of the active chemicals and the spot produced. The results of the trials are obtained as spots. The spots are seen via a variety of chemical operations and light imprinting techniques. The retention factor determines how many of these places there are (Rf). Retention factors vary depending on the polarity of the mobile and stationary phase and are broad characteristic values (Stoddard *et al.*, 2007). The separation of the active components in these plants is made easiest by the solvent system used here. TLC can be used to track the development of the reaction, identify the combination's constituent parts, and assess the purity of the mixture. This analytical instrument is used due to its excellent sensitivity, simplicity, separation speed, and cost-effectiveness. Depending on their polarity, different plant metabolites are extracted into various organic solvents. An alcohol-water extract's pH can be raised or lowered with a water-soluble acid or base to transform dissociated analytes into their non dissociated forms, which are then soluble in neutral organic solvents, to ensure that plant organic acids or bases are extracted into organic solvents at this stage. The organic phase can then be adjusted to the necessary volume for TLC by evaporating it under reduced pressure or under nitrogen. Due to the partitioning of

analytes into neutral solvents, the limited final volume, and the evaporation of the extract on the TLC plate prior to separation, the pH of the extract is unlikely to be fatal to bioassay microorganisms. TLC plates, or chromatoplates as they are also known, can be made in the lab, but they are most frequently bought. Among the most popular stationary phases are silica gel and alumina, though there are other options as well. Many plates contain a substance that fluoresces when exposed to short-wave UV (254 nm). TLC plates frequently use glass, aluminium, or plastic as their backing material. Glass plates are fragile and challenging to cut, yet they are chemically inert and can best endure reactive stains and heat. Both metal and plastic plates can be cut with scissors, but plastic may not tolerate the high heat needed to generate many stains and aluminium may not withstand stains that are very acidic or oxidising. When choosing the stationary phase, take into account the characteristics of your sample. Silica gel can only be applied to amino acids and hydrocarbons, as demonstrated below in. The fact that silica gel is acidic should also be noted. As a result, silica gel provides poor basic sample separation and may worsen acid-labile molecule quality. This also applies to alumina plates in acidic solutions. It's critical to understand that alumina and silica gel have different properties. Due to its simplicity, alumina does not segregate samples as thoroughly as silica gel at a given layer thickness. The stationary phase's characteristics are distinct from those of the mobile phase. Non-polar mobile phases, such heptane, are utilised, for instance, with the extremely polar material silica gel. The possibility of a combination in the mobile phase gives chemists more control over the material's bulk properties. The spots are seen after the experiment. The sheets are frequently treated with a phosphor, and dark patches form on the sheet when chemicals absorb the light impinging on a given location. Typically, this can be accomplished by simply projecting ultraviolet light onto the sheet. Spots can also be seen by chemical reactions; anisaldehyde, for instance, produces colourful adducts with numerous substances, and sulfuric acid will burn the majority of organic substances, leaving a dark spot on the sheet.

4.2 PLATE PREPARATION:

Commercially accessible TLC plates often have specified particle size ranges to increase reproducibility. They are made by combining an inert binder, such as calcium sulphate (gypsum), a little amount of water, and an adsorbent, such as silica gel. This mixture is applied as a thick slurry to a nonreactive carrier sheet, typically made of plastic, glass, or thick aluminium foil. The finished plate is heated in an oven for 30 minutes at 110 °C to dry

and activate it. For analytical purposes, the absorbent layer is typically 0.1-0.25 mm thick, while for preparative TLC, it is typically 0.5-2.0 mm thick.

4.2.1 TECHNIQUE:

- Using a capillary tube, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a Vacuum chamber. This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own

- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results.)

- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result)

4.2.2 MEASURING R_f VALUES:

However, Measurements are often taken from the plate in order to help identify the compound present. These measurements are the distance travelled by the solvent, and the distance travelled by individual sports.

When the solvent front gets close to the top of the plate, the plate is removed from the beaker and the position of the solvent is marked with another line before it has a chance to evaporate.

4.2.3 FORMULA:

The R_f value for each dye is then worked out using the Formula:

$$R_f = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

4.3 RESULT AND DISCUSSION:

Thin layer chromatography was performed foe separating organic compounds. The purpose of this experiment are to distinguish polar and non – polar solvent, to perform analysis technique using TLC and to differentiate the retention factor, R_f for different compound. TLC consists of three steps that is spotting development and visualization. If the solvent system is very non- polar components of the samples will travel further up the TLC plate than the polar components. If the solvent system is very polar then the polar component will travel further up the TLC plate than the non- polar components. TLC is widely used in analytical because of its simplicity, low cost high sensitivity and speed of separation. .The purpose of this experiment has been achieved which is the R_f value was determined for both samples, the analysis technique by using TLC was successfully performed and polarity of the solvent was able to identify. There are many uses of TLC in our daily lives. Some of the these are in food industry TLC is used in separating and studying various aspects of food items like colours, sweetness and preservation in cosmetic industry TLC is used to separate and analysis different cosmetic products and their components. R_f Values (0.65, 0.6, 0.75 and 0.80).

CHAPTER 5

ANTI OXIDANT ACTIVITY

5.1 INTRODUCTION:

An antioxidant can be define as "any substance that, when present in low concentration compared to the of an oxidisable substrate, significantly delay or inhibits the oxidation of that substrate" (Halliwell B *et al.*,1995). Dietary antioxidants like flavonoids may help lower the risk of mortality from coronary heart disease and the incidence of myocardial infarction. Antioxidants are present in various dietary natural sources such as vegetables, fruits, and drinks. (M.G. Hertog, *et al.*,1993, B.Waltenberger,A. *et al.*,2016) . Moreover, epidemiological research and meta-analyses have shown that consuming plant polyphenols over the long term may protect us from a number of diseases, including cancer, cardiovascular disease, diabetes, osteoporosis, and neurological illnesses (such as Alzheimer's disease). K.B.Pandey *et al.*,2009, D.Tewari *et al.*, 2018). Antioxidants include substances that remove or repair damaged molecules and operate as a defence mechanism that guards against oxidative damage. It can stop or slow down the oxidation brought on by free radicals, and consuming enough antioxidants is considered to guard against diseases (Celiktar *et al.*,2007). In tests on animals, a number of plant compounds have been found to have the potential to promote health. They include substances that decrease cholesterol, antioxidants, enzyme inducers, and other substances (Dragsted *et al.*,2006). The correct method must be chosen in order to accurately measure an extract's antioxidant activity (Abramovic, H. *et al.*,2017). There are numerous ways to determine this parameter (Apak .R.*et al.*,2008). but choosing one approach over another and being able to quickly compare the results with those of other authors are made difficult by the wide range of experimental settings that may be found in the literature for each method. Due to all of this, it is challenging to organise plants according to the antioxidant activity of their extracts. To determine the optimum way for sorting or selecting plant extracts according to their level of antioxidant activity, the findings of several approaches for various species should be examined using descriptive procedures of multivariate statistical techniques. Antioxidant activity in traditionally used plant species is a method of scientific validation of the medicinal plant use by Indigenous people. Plant species that are used traditionally for multiple symptoms could indicate a high level of antioxidant activity. A plant species that is used for heart disease, diarrhea, urinary issues, circulation, and blood disorder or blood reviver/ tonic could be high in antioxidant activity (L.M. McCune

2013). Oxidation is a chemical reaction can produce free radicals. Antioxidants activity are DPPH radical caused antioxidant radical by hydrogen donation (Majo *et al.*, 2008). Antioxidants are agents which scavenge the free radicals and prevent, enzymes, carbohydrates and DNA (Y Fang, *et al.*,2002). They play an important role in various fields such as medicinal field cosmetics, food industries (Nurul Aili Zakaria, *et al.*,2011). There are some synthetic antioxidant compounds such as butylated hydroxytoluence, butylated hydroxyanisole and tertiary butyl hydroquinone which are commonly used in processed foods, However, it has been suggested that these compounds have shown toxic effects like liver damage and mutagenesis (HC Grice, 1986, HP Which , 1998). Therefore, attention is being directed to harness and harvest the antioxidant principles from plant materials.

5.2 MATERIALS AND METHODS:

5.2.1 DPPH Free radical scavenging activity:

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid was purchased from Sigma-Aldrich. Phosphate buffer (pH- 7.4) and Methanol. All the chemicals and solvents used were of analytical grade. DPPH solution (0.004 %), sample extracts and standard (vitamin C) was prepared in methanol. Sample extract and standard (vitamin C) solution were prepared in different concentrations 20, 40, 60, 80 and 100 µg/ml. 0.5ml of different concentrations of standard solution or sample extracts was taken in different test tubes and then 0.5 ml of DPPH (0.004 %) solution was added and kept in dark for 30 min. and absorbance was recorded at 517 nm. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It was visually noticeable as a colour change from purple to yellow. (Majo *et al.*, 2008) The percentage inhibition activity was calculated using the formulae below:

$$\% \text{ DPPH free radical scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

5.3 RESULT AND DISCUSSION:

In the present study the scavenging activity was increasing concentration from 20-100 µg/ml but the scavenging activity of the ethanolic extract of *Cissus quadrangularis* stem showed good result than the control but more or less to standard which is summarized in Tables 5.1 and Fig. 5.1. The result indicates that the experiment reduce the radical to the corresponding hydrazine exhibiting better free radical scavenging activity near to the standard vitamin C. In the present study, ethanolic extract of *Cissus quadrangularis*

stem 31.04 % and 57.35% respectively at the concentration of 100µg/ml. so when compared to stem showed good result. This antioxidant activity may be due to the presence of flavonoids.

The effect of antioxidant on DPPH radical scavenging is through to be due to their hydrogen denoting ability (Pratheta *et al*.,2011). Phytoconstituents like tannins, flavonoids, trepenoids have been implicated as antioxidants in the scavenging radical (Badami *et al*.,003).

Ethanolic extract of cissus quadrangularis stem exhibited rapid scavenging activity but in varying rate of activity and this variation may be due to the nature of cissus quadrangularis and the concentration of the extract used.

Table 5.1 Antioxidant effect of sample-1 on DPPH.

Treatment	Dose (µg/ml)	Absorbance @517 nm	% activity against DPPH radicals
Control	----	DPPH control= 0.992	----
Vit C	100	0.162	83.66
Ethanolic extract	20	0.892	10.08
Ethanolic extract	40	0.765	22.88
Ethanolic extract	60	0.684	31.04
Ethanolic extract	80	0.556	43.95
Ethanolic extract	100	0.423	57.35

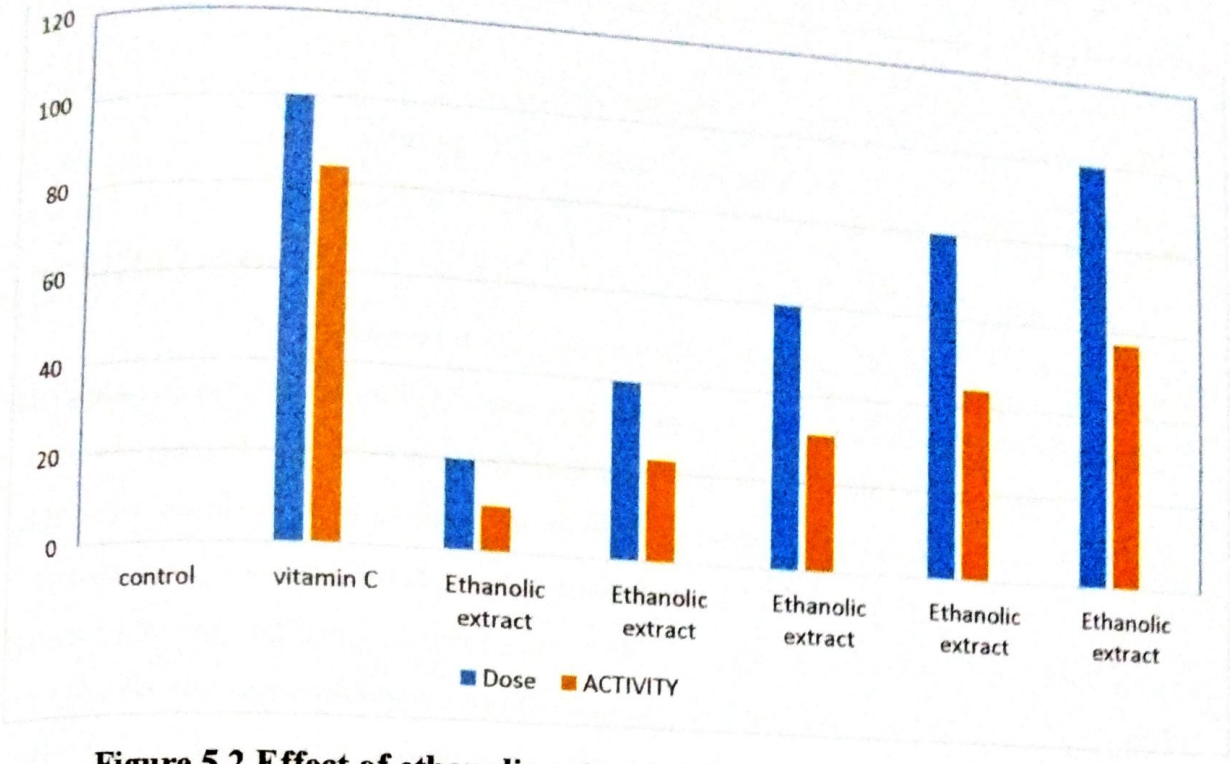


Figure 5.2 Effect of ethanolic extract of *Cissus quadrangularis* stem

5.4 CONCLUSION:

The result of the study showed that the ethanolic extract of *Cissus quadrangularis* stem and its constituents could be used as an easily accessible source of natural antioxidant and as a possible food supplement or in pharmaceutical industry. Therefore, it is suggested that further work could be done on the isolation and identification of the antioxidant component of *Cissus quadrangularis* stem and its constituents.

CHAPTER 6
ACUTE TOXICITY ANALYSIS

6.1 INTRODUCTION:

The fundamental study of poisons is toxicity. Acute toxicity was defined by the Organization for Economic Cooperation and Development (OECD) as the adverse effect that develops shortly after oral administration of a single dosage of a chemical or repeated doses given within a 24-hour period. Poison phytochemical interactions cause harm to or death to living tissues Like to science, toxicology is both an art and a science. It comprises collecting and using observational data to forecast how exposure would affect people and animals. Some plants were classified by prehistoric humans as dan The strength of secondary metabolites, the amount consumed, the duration of exposure, the plant's various parts (root, oil, leaves, stem bark, and seeds), a person's body chemistry, the climate and soil, and genetic variations within the species have all been shown to affect a given plant's toxicity (Tülay, 2012). This implies that medicinal plants should be handled carefully and toxicity research should be carried out to improve the population's awareness of the plant or plant preparation. Gerous and others as safe. To evaluate the toxicity of medicinal plants, in-vitro and in-vivo models are available. The lethal dosage 50 (LD50), which represents the dose that will kill 50% of a tested population, is used to gauge the plant's toxicity regardless of the type of extract, the sections of the plant employed, the concentration of the extract, the method of administration, and the organism in question (Amy *et al.*,2002). Moreover, histological or genetic alterations are the most pertinent markers for chronic or sub-chronic toxicity. Adverse responses to synthetic medications account for about 8% of all hospital admissions in the US. A minimum of 100,000 people every year pass away due to these toxins. It implies that prescription medicines cause at least three times as many fatalities in the US as drunk driving does. Thousands of people perish each year from allegedly "safe" over-the-counter medications. Herbal-related deaths or hospitalisations are extremely uncommon and difficult to locate. The United States National Poison Control Centers' database does not even have a section for adverse effects from plants. Therefore, the toxicity of herbal remedies must be considered in its wider perspective (Haq I. 2004). A medicine treats the signs and symptoms of a certain disease as identified by scientific pathology. Yet, the primary goal of medicinal herbs is to support the body's natural healing processes. The

gentle actions of medicinal plants typically aim to aid in the removal of excesses that have become prevalent or "support" the systems and processes that have become efficient. Only a portion of herbal medicinal approaches focus on symptom treatment. For instance, steroid anti-inflammatory medications are typically used to treat serum arthritic, but these medications have numerous unsettling side effects. The use of plants to treat these disorders facilitates excretion through the kidneys, liver, and biliary systems; modifies food metabolism; moistens dry synovia; and stimulates blood flow in the affected areas, among other effects (Philomena *et al.*,2011). The study of adverse effects that chemicals have on biological tissues is known as toxicology. It comprises knowledge of biological mechanisms, chemical reactions, interactions, and resulting alterations in tissue structure . From a human perspective, the study of toxicity has the hugely advantageous aspect that, at low doses, drugs have no effect and at high doses, they have fatal effects. As a result, there must be a range of drug concentration that falls between these two extremes and provides a graded effect. (Gokhale *et al.*,2002). Another common side effect of ingesting plant materials is neurotoxicity. When the plant chemical serves as a blocker to neurotransmission, neurotoxicity can result . A tea made from jimsom weed (Datura stramonium) has been used to treat asthama (Khan A.V., Alam M.M., 2003).Hyoscyamine and scopolamine, which are atropine-like compounds found in all parts of the plant, block acetylcholine, the main neurotransmitter of the parasympathetic nervous system.

6.2 RESULT AND DISSCUSSION:

TABLE 6.1 Result of acute toxicity of ethanolic extract *Cissus quadrangularis* stem

Parameter	1hr	2hr	3hr	4hr	24hr	8th day	14th day
Appearance	N	N	N	N	N	N	N
Activity	P	P	P	P	P	P	P
Gait	N	N	N	N	N	N	N
Reaction to stimulus							
a) Sound	++	++	++	++	++	++	++
b) Touch	++	++	++	++	++	++	++
c) Light	++	++	++	++	++	++	++
Lacrimation	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A
Piloerection	A	A	A	A	A	A	A
Stimulant	A	A	A	A	A	A	A
Depressant	A	A	A	A	A	A	A
Licking of paw	A	A	A	A	A	A	A
Convulsions	A	A	A	A	A	A	A

N- Normal; A- Absent; P- Present; +=Present Minimum; ++= Present medium

Further research is absolutely necessary to determine the effectiveness and side effects of these treatments. The same is true for the combined ethanol extract of the stem bark of Cissus quadrangularis and the fruit pulp of Aegle marmelos. (c-ECQAM). Because choosing which medicinal plants to utilise in the healthcare system should be based mostly on safety (Obici S *et al.*, 2008). Toxicological screening is done mostly on animal model in this study albino rats. Although the haematopoietic system is one of the most susceptible targets for hazardous substances and a crucial indicator of physiological and pathological condition in humans and animals, hematopoiesis and leucopoiesis were also unaffected (Harper HA, 1973), The level of dosage was increased from 50-3000mg/kg of body weight. There were no significant changes in the characteristic or activity of the model.The reaction to stimulation was positive

with both extract up to 14 days. Lacrimation, salivation, Piloerection, stimulants, depression, paw-licking, and convulsions were not observed in the study. Futher analysis on concentration and dosage of the compounds present in this species can be evaluated.

6.3 CONCLUSION:

Swiss albino mice were used in the acute toxicity testing of *C. quadrangularis* at doses of 50–3000 mg/kg extract orally. The *C. quadrangularis* was exposed once via oral inhalation of an aqueous slurry, with water serving as the vehicle for the exposure. According to the study's findings, animals in all dose groups consumed the same amount of food and water and did not experience any changes in body weight. Even at the highest dose level of 3000 mg/kg for the extract of *C. quadrangularis*, there was no mortality noted. Reports were made regarding changes in body weight, food and water intake, and cage side observations. There is no indication of any toxicity. There was no mortality recorded even at highest dose level of plant material.

CHAPTER 7

ANTIDIABETIC ACTIVITY:

7.1 INTRODUCTION:

Diabetes is a metabolic disease that affected 9.3% of adults worldwide in 2019. Its co-occurrence is suspected to increase mortality from COVID-19. The treatment of diabetes is mainly based on the long-term use of pharmacological agents, often expensive and causing unpleasant side effects (Prezor ,*et al.*,2022). The disease is classified into type 1 and type 2 which occurs due to production of insufficient insulin or destruction of βcells .It is one of the common metabolic syndrome since three are 200 million diabetic individuals due to deficiency or abnormalities in insulin action (Dheepak Jha,*et a*l., 2019). The blood glucose level in normal rats is in the range of 85-132 mg/dl (Kohn DF ,*et al.*,2002). Fruit powder of *M.cymbalaria* showed anti diabetic effect and hypolipedemic effect .The treatment was given for 15 days, after treatment a significant reduction was observed in fasting blood glucose level in diabetic treated but no hypolipidemic activity in normal rats. *Cissus quadrangularis* showed significant serum cholesterol and triglycerides .There was significant improvement in hepatic glycogen level as reported by (Deepak Jha *et al.*, 2019).

7.2 MATERIALS AND METHODS

Male Albino rats weighing 190-210g were used in the experiments. They were maintained in standard environmental conditions of temperature (25±2°C), relative humidity (55±10%) and 12 hrs dark/light cycle. They were fed with standard diet and water *ad libitum*.

7.2.1 CHEMICAL:

a) Alloxanmonohydrate Alloxan monohydrate was used as aqueous solution and given in the dose of 150mg/kg of body weight intraperitonially to induce diabetes.

b) Glibenclamide: Glibenclamide is an oral hypoglycemic agent and was obtained as gift sample from Alkem Laboratory, Mumbai. Suspension in distilled water was prepared and administered in the dose of 10 mg/kg of body weigh, orally, for standard comparison.

c) Diagnostic instrument Glucometer One Touch, Horizon, LIFESCAN, Johnson and Johnson Ltd. was used to determine the blood glucose level.

7.2.2 ANTIDIABETIC ACTIVITY:

Albino rats were divided into five groups each containing five rats and were placed in separate metabolic cages. Except control group, the animals of remaining four groups were fasted for 24 hrs. Diabetes was then induced by alloxan monohydrate (150mg/kg intraperitonially). It takes about 48hrs and after 48hours of diabetes induction the treatment was given. Group I served as control which received normal saline solution through oral route. Group II served as diabetic control. Group III received Glibenclamide (10 mg/kg) and served as reference. Group IV animals received sample I aqueous extract orally at a dose of 400 mg/kg; Blood samples were with-drawn at 0hr, 1hr, 2hrs, 3hrs,.4hrs and 24hrs, after treatment, from retro orbital plexus and were analyzed for the blood glucose level, using glucometer. This study was continued for 21 days by taking readings randomly. Blood glucose level was read from digital display of glucometer with its customized test strips. A drop of blood obtained through retro orbital plexus was placed on inserted gluco strips on glucometer. The method is widely used in clinical practice and appears to be sensitive and accurate.

7.3 RESUIL AND DISCUSSION:

Treatment with alloxan has the blood glucose level to a range of 250-270mg/dl after 5 days single dose of administration of extracts at 400mg/kg showed Significantly reduced blood glucose level at the 1st 2nd and 4nd hour after single dose administration in diabetes induced rats. Repeated dose administration with *Cissus quadrangularis* stem ethanolic extract has progressively showed to reduce the level of glucose level contingent on time over a period of 3 weeks when compared to standard the glucose range of the *Cissus quadrangularis* compared to the standard found some inhibition from 7 to 21 days.

When comparing the standard Gibenclamide showed 204±0.85% The ethanolic extract showed 275±1.80% respectively. The ethanolic extract showed activity less equal to standard in comparative manner.

Table 7.1: Effect of single dose treatment of Ethanolic extract

GROUP	TREATMENT	DOSE	SERUM GLUCOSE (mg/dL)			
			Time after glucose administration in minutes			
			0 hour	1 hour	2 hour	4 hour
I	Normal control	10ml/kg	95±0.48	95±0.48	95±0.48	95±0.48
II	Control	10ml/kg	234±0.85	234±0.85	234±0.85	234±0.85
III	Standard	10mg/Kg	204±0.85	204±0.85	204±0.85	204±0.85
IV	Ethanolic extract	400mg/Kg	280±1.18	285±1.18	282±1.12	275±1.08

Value are the mean of 3 observations ±SEM

Table 7. 2: Effect of repeated dose treatment of Sample I on glucose level in alloxan-induced diabetic rats

GROUP	TREATMENT	DOSE	SERUM GLUCOSE (mg/dL)			
			TIME AFTER GLUCOSE ADMINISTRATION IN MINUTES			
			0^{th} day	7^{th} day	14^{th} day	21^{st} day
I	**Normal control**	**10ml/kg**	**95±0.48**	**95±0.48**	**95±0.48**	**95±0.48**
II	Control	10ml/kg	234±0.85	234±0.85	234±0.85	234±0.85
III	Standard	10mg/Kg	204±0.85	154±0.16	130±1.02	92±1.08
IV	Sample I	400mg/Kg	280±1.18	270±1.16	200±1.12	180±1.08

Value are the mean of 3 observations ±SEM

CHAPTER 8

ANTIBACTERIAL ACTIVITY

8.1 INTRODUCTION:

Bacterial resistance to antibiotics is a major therapeutic problem and the rate at which new antibiotics are being produced is slowing, (Russell *et al.*,2002). Since their invention, antibiotics have significantly improved human health and are one of our most effective weapons against bacterial infections. Nevertheless, during the past few decades, these health advantages have come under threat as a result of the development of drug-resistant bacteria and the fact that many regularly used antibiotics are becoming less and less efficient against specific ailments. Investigating novel medications with lower resistance is crucial. The prevention and treatment of human diseases are significantly aided by medications made from natural sources. Traditional medicine is one of the principal healthcare systems in many poor nations (Farnsworth NR Ethno 1993),(Houghton PJ 1995). Man has long been aware of herbal treatments. Traditional medical practitioners have detailed the therapeutic usefulness of numerous indigenous herbs for a variety of illnesses. (Ramasamy S *et al.*,2009) More and more reports of medicinal plants' antimicrobial qualities are coming in from all over the world. According to the World Health Organization, 80% of the world's population uses plant extracts or their active ingredients in traditional medicines. Drugs can be used to control the dangerous microorganisms, but doing so causes a number of drug-resistant bacteria to grow, which has led to serious clinical conditions in the management of infections. Many novel antibiotics have been developed by the pharmaceutical sector, yet microorganism resistance to them has grown. Moreover, bacteria may exhibit antibacterial agent resistance via a number of methods. Certain types of bacteria have an intrinsic resistance to one or more antimicrobial agent classes. In these situations, every strain of that bacterial species displays resistance to every antibiotic class member. The development of bacterial resistance, whereby previously susceptible bacterial populations develop resistance to the antibacterial drug, is a serious problem (Alam MM et al .,1990) Knowing the mechanisms of antibacterial resistance is essential for developing methods to prevent the spread of antibiotic resistance (Asolkar LV, *et al.*, 1992)The lowering of

antibiotic inflow can limit an antibiotic's ability to enter cells. Porins, which are proteins with the ability to generate water-filled open channels that permit the passive movement of molecules across lipid bilayer membranes, are the principal regulators of flow Porins can therefore be thought of as prospective targets for bactericidal substances, particularly for Gram-negative bacteria (Bauer AW, *et al.*, 1966) Porin structural variation alters membrane permeability and serves as a means of defence against antibacterial agents (Riso JI .MC, et al 1988) One of the bacterial defence mechanisms against antibiotics is this situation, which is frequently observed in Gram-negative clinical pathogens including Acinetobacter spp. and Pseudomonas spp.(Alzoreky NS& Nakahara K 2003). Microorganisms can easily access food during harvesting, slaughtering, processing, and packing since they are naturally present in the environment (Hatab *et al.*, 2016). These microbes can withstand unfavourable packaging circumstances for food, such as low temperature, modified atmosphere packaging, hoover packaging, and traditional pasteurisation (Dimitrijevi *et al.*, 2007). Because of the substantial consumer concern over the potential harm from utilising synthetic additions to human health, the usage of these chemicals in food preservation has decreased (Gyawali and Ibrahim, 2014; Caleja *et al.*, 2016; Kalem *et al.*, 2017). preservatives (Clarke et al., 2017). Clinical microbiologists are interested in the subject of antibacterial plant extracts for two reasons. First, several of these phytochemicals are currently being tested on people, and it is extremely likely that they will find their way into the arsenal of antimicrobial medications that doctors administer (see below). According to reports, two or three antibiotics made from microbes are introduced annually on average.The pace is picking up again after slowing down in recent decades as scientists become more aware of how short an antibiotic's useful life is. It is anticipated that global investment on developing new anti-infectives (including vaccines) will increase by 60% from 1993 levels. While some of the oils used for their purported antibacterial effects have in vitro activity that has been well documented, there are few published data for many other oils (Morris et al. 1979; Ross *et al.*,1980; Yousef & Tawil 1980; Deans & Ritchie 1987; Hili *et al.*, 1997). Other investigations have focused solely on one type of oil or one type of microorganism. Although these statistics are helpful, there are methodological discrepancies across the studies that prevent direct comparisons, such as the choice of plant extract(s), test microorganism(s), and antimicrobial test method (Janssen *et al.*, 1987).

8.2 MATERIALS AND METHODS:

8.2.1 BACTERIAL STRAINS:

A total of five bacterial stain including both gram position and gram negative bacterial *Staptylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Vibrio sp. Micrococcus* were selected to assess susceptibility patterns against stem extract. The bacterial culture prior to susceptibility testing by transferring them into a separated test tube containing nutrient broth and incubated overnight at 37^0C.

TABLE 8.1 Bacterial strain:

S.NO	BACTERIAL STRAINS	Gram (+/-)
1.	*Staphylococcus aureus*	+
2.	*Micrococcus*	+
3.	*Pseudomonas aeruginosa*	-
4.	*Vibrio sp*	-
5.	*Escherichia coli*	-

8.2.2 DETERMINATION OF ANTIMICROBIAL ACTIVITY BY WELL DIFFUSION METHOD:

The antimicrobial activity was determined by well diffusion method. Muller Hinton agar with lawn culture using desire test organism. The inoculation plates were kept aside for few minutes using well cutter, three wells were made in those plates at required distance. In each step of well cutting, the well cutter was thoroughly wiped with alcohol. The extract for this study was prepared by dissolving 1g of stock powder in 1ml of DMSO. Using sterilized micropipettes 20µl extract of cissus quadrangularis, dimethyl sulfoxide (DMSO). Streptomycin (10mcg), Terramycin (10mcg) were added separately in each well of the bacterial culture plates. The Streptomycin and Terramycin were considered as Positive control and DMSO as negative control in this study. Each test was executed in triplicates.

The plate were incubated at 37^0C and 35^0C for 24 and 48 hours respectively. After incubation the inhibition of growth was analysed and result were recorded.

Antimicrobial activities were evaluated by measuring the zone of inhibition in millimetres by using graduated scale.

8.3 RESULT AND DISCUSSION:

The antimicrobial activity of the ethanolic extract of stem against human pathogens was determined by measuring the diameter or zone of inhibition expressed in millimetre (mm) (Wilkinson, 2007) and is represented in Table 8.1 showed varied in the exploitation of antimicrobial activity of zone of inhibition from 9-15 mm against all the tested microorganisms. The degree of antibiotic property depends upon the several factors such as age or the plant, duration of storage, temperature, preparation or the media and PH (Joys Albert, (2015). The stem of cissus quadrangularis showed maximum activity against the gram positive *Staphylococcus aureus* (14 mm) and *Micrococcus*(14mm) and in gram negative *E. coli* (15mm) and *Pseudomonas aeruginosa*(12mm) but it showed minimum activity against gram negative *Vibrio sp.* (9mm).When compared to the antimicrobial activity of stem extract of cissus quadrangularis the stem showed significant activity against the test microorganisms. The difference further observed in the antimicrobial effect of the cissus quadrangularis extracts studied against both gram positive and gram-negative bacteria in the present study may be due to differences m permeability barriers. In gram negative species outer membrane is fairly affect barriers for the extract and also active compounds persist in the plant (Joys Albert, 2015) The more susceptibility of gram positive bacteria to the cissus quadrangularis extract was due to the differences in their cell wall structure and their composition (Tortora GJ et al., 2001). In gram negative bacteria the outer membrane act as barrier to many environmental substances including antibiotics (Kandhasamy et al 2008 and Joys Albert, 201 5). The cell walls of gram negative bacteria which act as a barrier and prevent the entry of foreign particles may be the reason for the poor performance of the studied extracts in the Present study.



Plate 8.1 Zone of inhibition of E. coli



Plate 8.2 Zone of inhibition of Staphylococcus aureus



Plate 8.3 zone of inhibition of vibrio

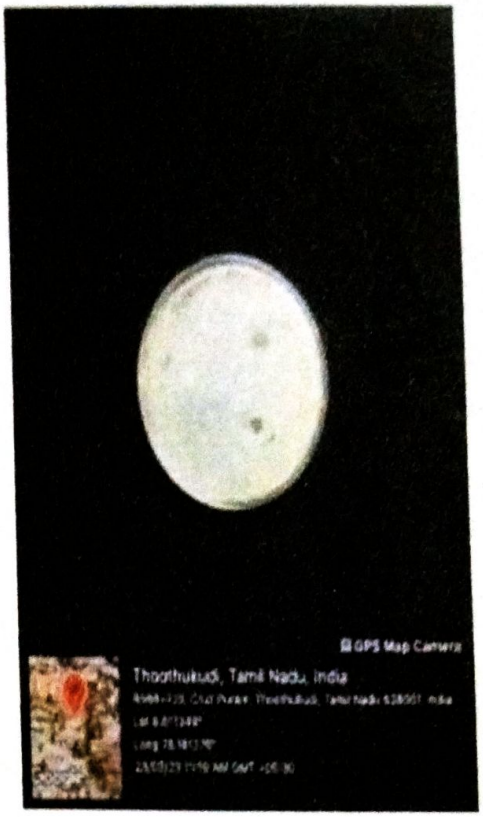


Plate 8.4 Zone of inhibition Micrococcus

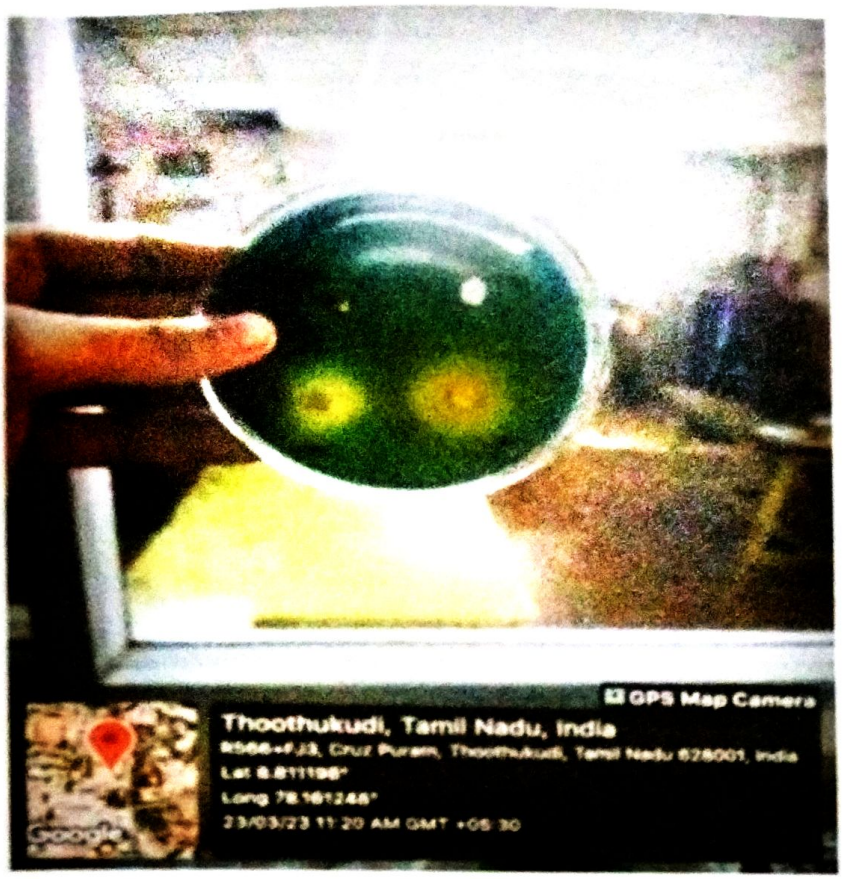


Plate 8.5 Zone of inhibition of Pseudomonas aeruginosa

Tables 8.2 Antibacterial activity of ethanolic extract of stem of cissus quadrangularis

S.NO	Human pathogens	Zone of inhibition (mm)	Zone of inhibition (mm)	Zone of inhibition (mm)
1.	Escherichia coli	Position control (Terramycin) 19mm	Negation control DMSO	Stem 15mm
2.	Staphylococcus aureus	19mm	DMSO	14mm
3.	Vibrio	(streptomycin) 12mm	DMSO	9mm
4.	Pseudomonas aeruginosa	20mm	DMSO	12mm
5.	Micrococcus	12mm	DMSO	14mm

8.6 CONCLUSION:

The extracts of *Cissus quadrangularis* have high yield of chemical constituents which inhibit the bacterial colonies. The ethanolic extract showed higher range and broad spectrum of antibacterial activity. Maximum zone of inhibition was observed in *E.coli* with 15mm with ethanol extract of Cissus quadrangularis and minimum inhibition was 8mm with respectively. However further studies are required to evaluate the potential effectiveness of the antibacterial agents from plant species.

CHAPTER 09

SUMMARY

Herbal medicine are used in treatment of varied diseases and these type of drugs are sometimes improperly used. Therefore, these herbal drugs which serve as an alternate of conventional chemical drugs needs detailed investigation *in vivo* and *in vitro* under modern medicine. Compared to synthetic drug natural drugs have place in therapy recently. Their effect, low cost, easy availability, native species specification and scarce toxicity has earned them an place in the scientific research. Based on the test evidences currently tremendous interest in the potential properties and bio active components isolation on secondary metabolites are into concern. All these new approach will replace synthetic drugs with eco friendly natural substance with less to no side effects (Joys Selva Mary Albert 2018). These extracts of medicinal plants can be used as whole but in case of conventional drugs only the active ingredients is separated and used.

The dissertation entitled "A STUDY OF PHARMACOLOGICAL ACTIVITIES OF *Cissus quadrangularis*" embodies the native plant variety of Tamil Nadu especially the southern region. The ethanolic extracts were analysis phytochemically by various qualitative methods which reported the presence of flavonoids, saponins, in both and presence of carbohydrates, in ethanolic extract. The ethanolic extract was observed as a consortium of may chemical components.

Thin layer chromatography (TLC) is an affinity -based method used to separated compounds in a mixture. TLC is a highly versatile separation method that is widely used for both qualitative and quantitative sample analysis

The ethanolic extract showed comparatively good result in DPPH radical scavenging activity thus contributing to the antioxidant activity or potential of the extract

The toxicity analysis was done to examine the lethal dosage up to which the animal model can stay alive. The results intrepeted that there was no mortality or changes in behavior in the rat model with dose 2000mg/kg of body weight. The reaction to stimulus was normal and so 400mg/kg was selected as the optimal dose for all other *in vivo* and *invitro* test in the study.

The plant has the potential of wound healing ability and preferred due to its wide spread ability, nontoxicity. Absence of side effects and effectiveness crude preparations. *Cissus quadrangularis* ethanolic extract has progressively showed to reduce the level of glucose level contingent on time over a period of 3 weeks. Ethanolic extract showed lesser reduction level. Thus the plant can be used as a anti diabetic drug and utilized under safe doses for type I and type 2 diabetes.

The extracts of *Cissus quadrangularis* showed bactericidal and bacteriostatic activity. The ethanolic extract showed maximum zone of inhibition against *E.coli* within(15mm).

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ANNEXURE:

ANNEX 1

DEFINITIONS

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.

Delayed death means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

Dose is the amount of test substance administered. Dose is expressed as weight of test substance per unit weight of test animal (e.g. mg/kg).

GHS: Globally Harmonised Classification System for Chemical Substances and Mixtures. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physical–chemical properties) and ILO (hazard communication) and coordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

Impending death: when moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor (See the Humane Endpoint Guidance Document (9) for more details).

LD50 (median lethal oral dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Limit dose refers to a dose at an upper limitation on testing (2000 or 5000 mg/kg).

Moribund status: being in a state of dying or inability to survive, even if treated (See the Humane Endpoint Guidance Document (9) for more details).

Predictable death: presence of clinical signs indicative of death at a known time in the future before the planned end of the experimen ; for example: inability to reach water or food. (See the Humane Endpoint Guidance Document (9) for more details)

ANNEX 2

PROCEDURE TO BE FOLLOWED FOR EACH OF THE STARTING DOSES
GENERAL REMARKS

For each starting dose, the respective testing schemes as included in this Annex outline the procedure to be followed.

Annex 2 a: Starting dose is 5 mg/kg bw

Annex 2 b: Starting dose is 50 mg/kg bw

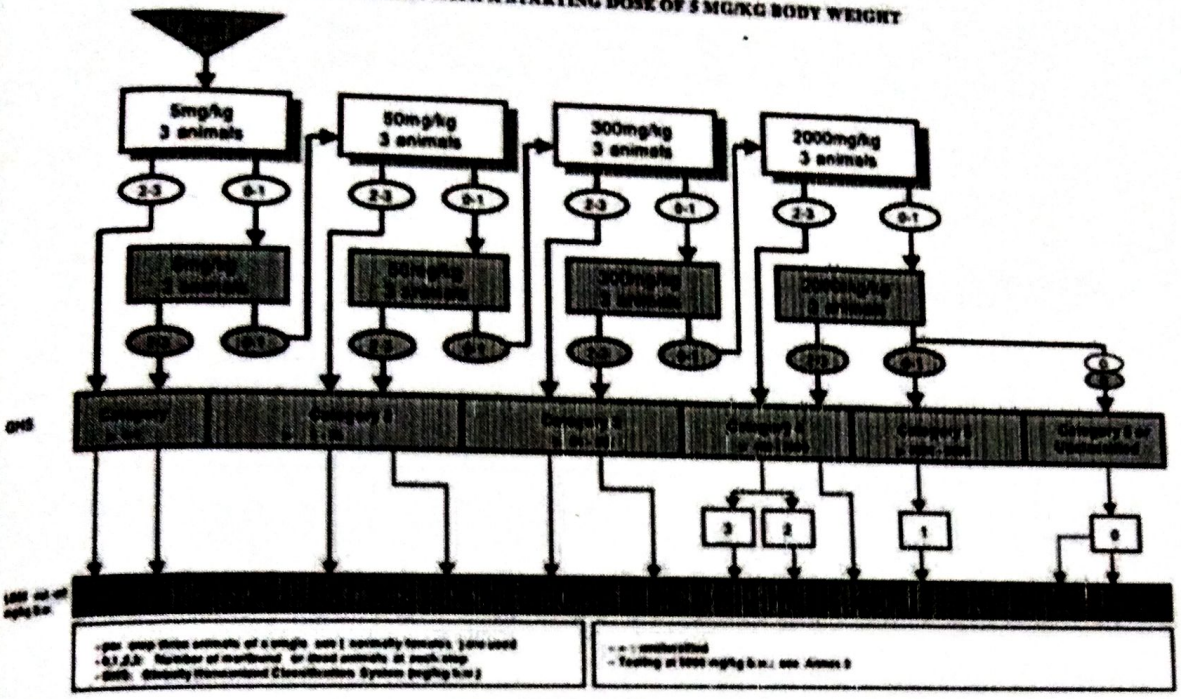
Annex 2 c: Starting dose is: 300 mg/kg bw

Annex 2 d: Starting dose is: 2000 mg/kg bw

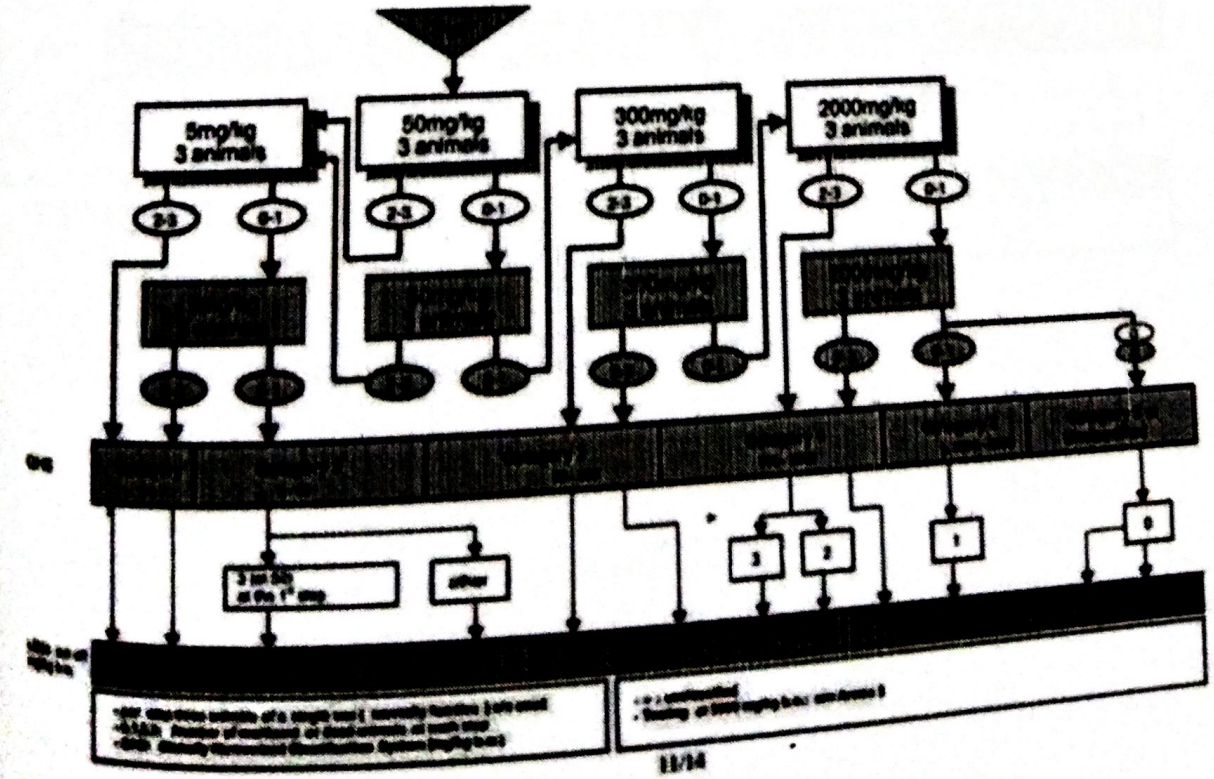
Depending on the number of humanely killed or dead animals, the test procedure follows the indicated arrows.

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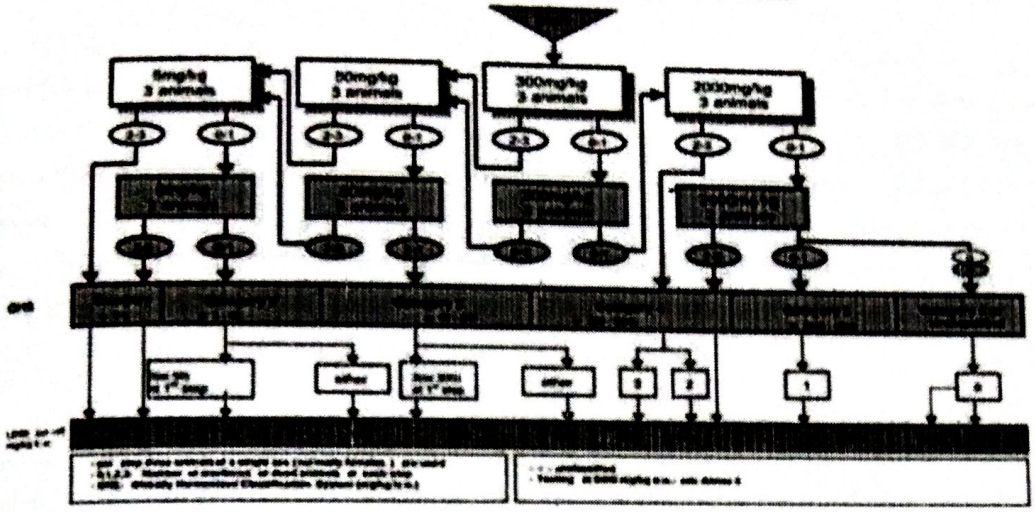
ANNEX 2a: TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT



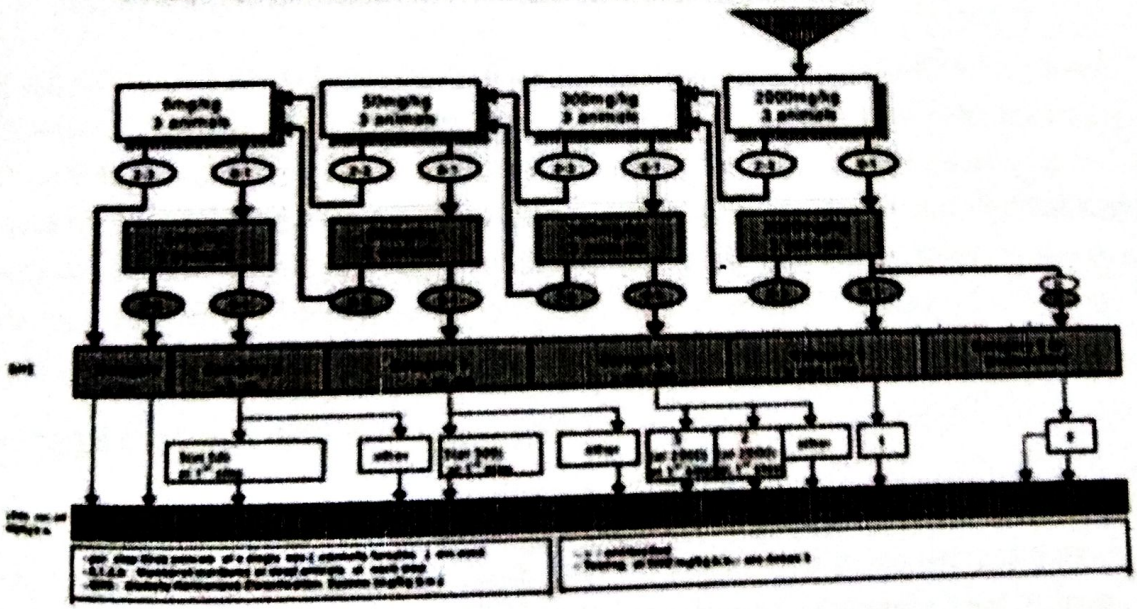
ANNEX 2b: TEST PROCEDURE WITH A STARTING DOSE OF 50 MG/KG BODY WEIGHT



ANNEX 3: TEST PROCEDURE WITH A STARTING DOSE OF 300 MG/KG BODY WEIGHT



ANNEX 3A: TEST PROCEDURE WITH A STARTING DOSE OF 2000 MG/KG BODY WEIGHT



ANNEX 3

CRITERIA FOR CLASSIFICATION OF TEST SUBSTANCES WITH EXPECTED LD50 VALUES EXCEEDING 2000 MG/KG WITHOUT THE NEED FOR TESTING

Criteria for hazard Category 5 are intended to enable the identification of test substances which are of relatively low acute toxicity hazard but which, under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD50 in the range of 2000-5000 mg/kg or equivalent doses for other routes. The test substance should be classified in the hazard category defined by: 2000mg/kg<LD50<5000mg/kg (Category 5 in the GHS) in the following cases:

If directed to this category by any of the testing schemes of Annex 2a-2d, based on mortality incidences;

if reliable evidence is already available that indicates the LD50 to be in the range of Category 5 values, or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.

Through extrapolation, estimation or measurement of data if assignment to a more hazardous category is not warranted, andreliable information is available indicating significant toxic effects in humans, orany mortality is observed when tested up to Category 4 values by the oral route,where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance, or where expert judgement confirms reliable information indicating the potential for significant acute effects from the other animal studies.

TESTING AT DOSES ABOVE 2000 MG/KG

Recognising the need to protect animal welfare, testing of animals in Category 5 (5000 mg/kg) ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health (10). No further testing should be conducted at higher dose levels.

When testing is required a dose of 5000mg/kg, only one step (i.e. three animals) is required. If the first animal dosed dies , then dosing procedes at 2000mg/kg in accordance with the flow charts in Annex 2. If the first animal survives, two further animals are dosed. If only one of the three animal dies , the LD50 value is expected to exceed 5000mg/kg. If both animals die, then dosing proceeds at 2000mg/kg.



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Certificate of Participation

This to certify that Ms/Mrs/Mr/Dr. Selzia . R , II . M.Sc Microbiology , St . Mary's College participated / presented his / her research paper entitled Phytochemical screening of ethanolic extract of Cissus quadrangularis.

in the National Seminar on 'EMERGING TRENDS AND OPPORTUNITIES IN MICROBIOLOGICAL RESEARCHES organised by PG & RESEARCH DEPARTMENT OF BOTANY, V. O. CHIDAMBARAM COLLEGE, THOOTHUKUDI o 10.03.2023.

Dr. A. Doss

Dr. C. Lakshmanan

Dr. C. Veerabah

SCREENING OF ELEMENT CONCENTRATION IN MARINE MACROPHYTE OF TUTICORIN COAST, SOUTHEAST COAST OF INDIA

A PROJECT SUBMITTED TO

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

Affiliated to Manonmaniam Sundaranar University

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

N. VINCY

(REG. NO. 21SPMB05)

Under the Guidance of

Dr. T.P. Kumari Pushpa Rani M.Sc., M.Phil., Ph.D

DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS),

THOOTHUKUDI – 628 001.

APRIL – 2023

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DEPARTMENT OF MICROBIOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS),

THOOTHUKUDI – 628 001.

APRIL – 2023

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CERTIFICATE

This is to certify that the project work entitled **"Screening of element concentration in marine macrophyte of Tuticorin Coast, Southeast Coast of India"** submitted to **St. Mary's College (Autonomous), Thoothukudi** affiliated to **Manonmaniam Sundaranar University, Tirunelveli** for the partial fulfillment for the award of Master of Science in Microbiology is a bonafide research carried out by **Ms. VINCY. N** under the guidance and supervision of Dr. T. P. Kumari Pushpa Rani M.Sc., M. Phil., Ph.D, Assistant Professor of Microbiology, St. Mary's College (Autonomous), Thoothukudi, for academic year 2022 - 2023.

SIGNATURE OF THE GUIDE

Dr. Joys Selva Mary Albert
Head
Department of Microbiology
St. Mary's College (Autonomous)
Thoothukudi

SIGNATURE OF THE HOD

SIGNATURE OF THE DIRECTOR

Self Supporting Courses
St. Mary's College (Autonomous)
Thoothukudi - 628 001.

SIGNATUR OF THE PRINCIPAL

Principal
St. Mary's College (Autonomc.
Thoothukudi - 628 001.

18/4/2023

SIGNATURE OF THE EXAMINER

PLACE: THOOTHUKUDI

DATE : 05.04.2023

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DECLARATION

I hereby declare that the project work entitled **"Screening of element concentration in marine macrophyte of Tuticorin Coast, Southeast Coast of India"** is a bonafide record of the work completed by me during the academic year 2023 in St. Mary's College (Autonomous), Thoothukudi and submitted as a partial fulfillment of requirements for the award of the Degree of Master of Science in Microbiology prescribed by the Manonmaniam Sundharanar University. I also affirm that this is an original work done by me under the supervision of **Dr. T. P. Kumari Pushpa Rani M.Sc., M.Phil., Ph.D** Assistant Professor, Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi.

SIGNATURE OF THE GUIDE

N. Vincy.

SIGNATURE OF THE STUDENT

PLACE: THOOTHUKUDI

DATE : 05.04.2023

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ACKNOWLEDGEMENT

In the name of GOD the most beneficent and the mostgracious, first and foremost all praise to be GOD for giving me the opportunity, patience, help and guidance for the completion of this dissertation.

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To my Parents, and my friends, thank you for bringing me up to be who Am today. My success symbolize and reflects on the undivided support and love from all of you.

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Last but not the least I express my hearty thanks to all my family members.

VINCY. N

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DEDICATED TO
MY BELOVED
PARENTS



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CONTENTS

S.NO	TITLE	PAGE
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	10
3	AIM AND OBJECTIVES	26
4	METHODOLOGY	27
5	RESULT AND DISCUSSION	32
6	SUMMARY AND CONCLUSION	36
7	BIBLIOGRAPHY	37

Scanned with CamScanner

LIST OF FIGURES

Figure No.	Title	Page No.
Figure 1	Seaweed sample collection area (Manapad)	27
Figure 2	Seaweed sample collection area (Tharuvaikulam)	28
Figure 3	Fresh Seaweed sample	29
Figure 4	Dried seaweed sample	29
Figure 5	Analysis of heavy metal by using ICP-MS	30
Figure 6	Inductively Coupled Plasma Mass spectrometry(ICP-MS)	31

LIST OF TABLES

Table No.	Title	Page No.
Table 1	Analysis of heavy metals (ppm)	32

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LIST OF ABBREVIATIONS

m	-	meter
µg	-	microgram
mg	-	milligram
kg	-	kilogram
g	-	gram
Ca	-	Calcium
Mo	-	Molybdenum
Fe	-	Iron
Zn	-	Zinc
Cu	-	Copper
Pb	-	Lead
Mn	-	Manganese
Cr	-	Chromium
Ni	-	Nickel
Co	-	Cobalt
Hg	-	Mercury
As	-	Arsenic
Cd	-	Cadmium
ICP-MS-		Inductively Coupled Plasma Mass Spectrometry
FW	-	Fresh Weight
DW	-	Dry Weight
AAS	-	Atomic Absorption Spectroscopy
N	-	North
E	-	East

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INTRODUCTION

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INTRODUCTION

Macroalgae are aquatic organisms found in nearly all marine ecosystems (Maehre *et al.*, 2014). Many heavy metals have been found in relatively high concentrations in marine macrophytes. Algae are a diverse group of photosynthetic organisms ranging from unicellular (microalgae) to multicellular (microalgae) forms inhabiting freshwater and marine environments (Bharathiraja *et al.*, 2015). Macroalgae, commonly known as seaweeds, are fast growing organisms that resemble plants, with some species able to grow up to 60 m in length (Mc Hugh *et al.*, 2003). Various types of seaweeds are known worldwide by name, such as Kombu (*Laminaria spp.*), Wakame (*Undaria pinnatifida),* and Nori (*Porphyra spp.*). These seaweeds are used in the food industry (Kato *et al.*, 2016: Jimenez-Escrig *et al.*, 2011). Seaweeds are classified mainly into three major classes, viz., Chlorophyceae, Phaephyceae, and Rhodophyceae, on the basis of pigmentation colour, morphological, and anatomical characteristics. (Manivannan *et al.*, 2009; Pramanick-Mitra *et al.*, 2014).

Seaweeds or macroalgae plants like organisms usually refer to the several species of multicellular marine algae; Red algae (Rhodophyta), Brown algae (Phaeophyta), and Green algae (Chlorophyta). The red algae are multicellular and Brown algae are multicellular and Green algae largely unicellular. Seaweeds any of the Red, Green, or Brown marine algae that grow along seashores. Green and red algae are both members of the plantae kingdom, whereas brown algae are members of the Chromista Kigdom. The terms "Macro algae" and "seaweeds" are commonly used to describe these organisms found in the marine environment. Red algae, as well as in calcified green Halimeda. In the *Rhodophyticcoraclina officinalis*, the processes of inorganic carbon and nutrient uptake and assimilation are affected by elevated CO_2 due to the changes in enzyme activity (Hofmann *et al.*, 2012a). Generally, a seaweeds absorbs the nutrients via their blades and uses it for photosynthesis and metabolism. Seaweeds in marine ecosystem have a highly effective method for storing considerable amount of carbon (Filbee *et al.*, 2020, Chen *et al.*, 2020, Krause *et al.*, 2016). Furthermore, the building of artificial reefs has been reported to have a significant role in the carbon sequestration of marine forests by providing seaweeds. (Sondak, *et al.*, 2015, Chung *et al.*, 2013). Among the nutrients, iron is necessary for the phototrophs, such as seaweeds,

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to produce chlorophyll (Liu *et al.*, 2004, Behnke *et al.*, 2020). The red algae find considerable use in modern European also used to make other products such as agar, carrageenans, and food additives. In general, algae are widely distributed in the aquatic environment and easy to collect, identify, and the bioaccumulation of trace metals occur in higher degrees. Macro algae are important primary producers; they serve as food for a variety of organisms; and they are considered efficient and reliable indicator organisms for metal pollution in the environment (Misheer *et al.*, 2006; Han *et al.*, 2008; Wallenstein *et al.*, 2009). All the fundamental for bioindicators (Campanella *et al.*, 2001, Condi, 2002). Seaweeds could as their cell wall contains a high mineral concentration. Anionic carboxyl is found in polysaccharides and proteins. (Machado *et al.*, 2002).Moreover, seaweed that is harvested in the nation is used to make a variety of products, including food, medicine, cosmetics, fertilizers, biofuels, and items that reduce environmental pollution (Hoq *et al.*, 2016). As primary producers in the marine environment, macroalgae are crucial components of food chains. Moreover, particularly in Asian nations, they were employed as a natural source of food and medicine. The seaweeds (macroalgae) are widely dispersed across the oceans and seas, extending from the surface of the water to great depths, or connected to different types of substrates like sand, mud, rocks, shells, and coral (Apaydn *et al.*, 2010).

Macroalgae, the cell wall comprises a variety of polysaccharides and protein, some containing sulphate, anionic carboxyl, and phosphate groups that have a very high capacity for metals retention (Deniaud *et al.*, 2017). Elements are accumulated by marine macroalgae and then transferred up the trophic chain by herbivores and detritivores. As a result, they are an important bioindicator of marine environmental pollution caused by elements (Villares *et al.*, 2001). Due to their capacity for accumulation, several seaweeds have been used as markers of heavy metal pollution. Prior the implication of those species as bioindicators, thorough biological research of eligible species and the levels of heavy metals in their tissues should be conducted. Seaweeds are fundamental components of coastal ecosystem and are responsible for much the coastal primary production community structure and ecosystem function (Hurd *et al.*2014; Schiel and foster 2015).Some seaweeds are recognized to concentration metals up to levels many times larger than those found in the surrounding waters (Farias *et al.*, 2002). The waste deposition has resulted in high levels of total copper

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in the seawater, with 20µg L^{-1} currently and 200µg L^{-1} at the discharge point in the past (Castilla 1996; Henriquez – Castillo *et al.*, 2015).

Sargassum, a marine macro algal genus in the phaeophyceae family, is found in both tropical and temperate oceans. It is a member of the Sargassaceae family and the Fucales order. It is a large, economically significant, and ecologically dominant brown algae found throughout the tropics. It has been discovered to be the most diverse genus among Phaeophyta in Indian and represented by 38 species. *Sargassum* is found along the southern coasts of Tamil Nadu, India, and many other parts of Asia, and it is said to be used as animal feed, food ingredients, and fertilizer. *Sargassum wightii* contains a high concentration of flavonoids, which supports its antioxidant activity (Meenakshi *et al.*, 2009).Brown seaweed *Sargassum wightii,* which contains significant amounts of quaternary compounds such fucoidan and fucoxanthin, polyphenols (phloroglucinol, phlorotannins, and phenoic acids etc.) and Flavonoids (flavonols and flavonol glycosides). A soluble polysaccharide is fucoidan. Responsible for a variety of biological processes include anticancer, anticoagulant, antithrombotic and antiviral action too. An important biological function of the presence of sulphate groups in various amount is what cause fucoidan (Wijesibghe and jeon 2012; Borazjani *et al*, 2017). The polysaccharide composition of brown seaweeds has been shown to make them effective in metal biosorption. Brown seaweeds' cell walls include an amorphous matrix of acid polysaccharides linked to one another by proteins, which is embedded in a fibrillar compartment made primarily of cellulose microfibrils (Andrade *et al.*, 2004). *Sargassum wightii* is a brown alga rich in sulfated polysaccharides, which have a variety of biological properties such as free radical scavenging and antioxidant effects. Cyclosporin-induced hepatotoxicity has been linked to increased levels of lipid peroxidation, 8-hydroxy-2-deoxyguanosine, and protein carbonyls, as well as a decrease in ATPase activities and lipid profile changes, as well as histopathological changes. Concurrent treatment with sulfated polysaccharides, on the other hand, has been shown to restore the cyclosporine-induced abnormality (Jossephine *et al.*, 2008). Seaweeds will be used to monitor and restore the environment. As a result, some seaweed is used as biomonitors to study environmental pollution. *Sargassum wightii* is seaweed that accumulates metals and is used as a bioindicator for heavy metal pollution. *Sargassum wightii* biomass packed column was employed to study the copper removal as a function of bed height, flow rate and inlet

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copper (II) concentrations. Among these, brown seaweeds are proved efficient in metal biosorption (Davis *et al.*, 2003) because of their polysaccharide content.

Ulva lactuca is a common macroalgae that can be found in sheltered harbors up to 10 meters deep, at all intertidal levels, and in northern regions. *Ulva lactuca* is a plant that thrives on rocky or sandy coastal and estuarine beaches (Aslan *et al.*, 2010).*Ulva lactuca* was the marine algae that were most prevalent along the coastline among seaweeds. It can act as a good bioindicator for pollution in the marine environment since it can reflect the amounts of trace elements (Saleh, 2015). *Ulva lactuca*, a marine microalga, was used to remove cadmium ions from aqueous solutions and to research how the treatment process is impacted by temperature, time, and initial metal concentration, pH, and biomass amounts. The Ulva genus contains useful elements that can be used in the chemical, pharmaceutical, cosmetic, food, and energy industries (Bruhn *et al.*, 2011). A member of the phylum Chlorophyte is the green seaweed *Ulva lactuca*, also known as sea lettuce (Wichard *et al.*, 2015). *Ulva lactuca*is found in littoral and sublittoral zones (Rodriguez *et al.*, 2015).

The surface of seaweeds provides a suitable substrate for the settlement of microorganisms and also secretes various organic substances that serve as nutrients for bacterial multiplication and the formation of microbial biofilms (Steinberg *et al.*, 2002; Staufenberger *et al.*, 2008; Singh, 2013). Alphaproteobacteria and gammaproteobacteria have a worldwide distribution in oceanic and coastal water (Venter *et al.*, 2004; Rusch *et al.*, 2007). *Actinomycetes* in the ocean have been shown to produce a diverse ecology. There are many different species of marine *actinomycetes*, which are extensively found in sediments, seawater, and aquatic animals. Nowadays, sponges, ascidians, corals, and brown algae are the main marine invertebrate groups that these *actinomycetes* are separated from the potential for this subject is even more appealing considering that just 1% of microorganisms can currently be isolated using conventional culturing methods (Subramani *et al.*, 2012; Jan *et al.*, 2010). In addition, rare actinomycetes including *Actinoalloteichus*, *Agromyces*, *Agrococcus*, *Amycolatopsis*, *Nonomuraea*, *Phycicoccus*, *Saccharothrix*, and *Serinicoccus* were discovered from these marine organisms (Valliapppan *et al.*, 2014). Actinobacteria are abundant in the marine environment, particularly in sediments, but they also coexist with a variety of marine organisms, including fishes, sponges, macroalgae, corals, and tunicates (Abdelmohsen *et al.*, 2014). Endophytes

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develop secondary metabolites during this symbiotic relationship with the host that increase the host's fitness and tolerance to environmental stressors in exchange for the host providing them with nourishment and protection (Dinesh *et al.*, 2017). Although many different species of actinobacteria, both endophytic and epiphytic, are known to be hosted by macroalgae, little has been done to evaluate their biotechnological potential, with the majority of the bioactivity screenings focusing on fungus and other epiphytic microorganisms (Egan *et al.*, 2008). Nonetheless, several research showed that actinobacteria isolated from macroalgae can produce bioactive substances, such as antibiotics, anticancer, and anti-inflammatory substances (Brana et al., 2015; Uzair *et al.*, 2018). Every marine biological process depends on marine microorganisms, which is why there is growing interest in understanding their populations and functions. Examples of these creatures include bacteria, fungi, other unicellular organisms, diatoms, spores, and larvae of marine animals (Zozaya *et al.*, 2016). Algae and bacteria interact in a wide variety of symbiotic ways, mostly known as mutualism, commensalism, and parasitism (Ramanan *et al.*, 2016; Fuents *et al.*, 2016).

Heavy metals occur naturally in the environment and are necessary for survival, but they can be dangerous when they accumulate in organisms. Mercury, cadmium, arsenic, chromium, nickel, copper, and lead are some of the most common heavy metals that pollute the environment (Hazrat *et al.*, 2019).

The brown algae concentrated primarily Cr, Co, Cu, Cd, As, and Fe, whereas the green algae concentrated primarily Zn and Pb. The metal bioaccumulation behavior of *Padinacrassa, Sargassum thunbergii*, *Caulerpara cemosa*, and *Asparago psistaxiformis* was found to be similar using cluster analysis, principal component analysis, and the metal pollution index. The overall hazard index (HI) of seaweed consumption to adults was less than one, while the HI of *Sargassum oligocystum*, *Turbinaria ornate*, *Sargassum polycystum*, and *Sargassum thunbergii* consumption to children was greater than one, indicating a moderate or high risk to children.

Seaweeds are excellent at filtering metals such as zinc, iron, copper, cadmium, and nickel, as well as some potential carcinogens, from seawater. They remove toxic materials from the environment and accumulate in body cells, which can be 4,000 - 20,000 times

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greater than the surrounding water. Marine algae contain nearby all of the development of plants, animal, and people , including trace elements like iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) , and carbon monoxide (Co), nickel (Ni). They are typically ingested by algae from the nearby saltwater in excess of what necessary for metabolism (Kalesh *et al.*, 2006).

Heavy metal pollution has emerged due to anthropogenic activity which is the prime cause of pollution, primarily due to mining the metal, smelting, foundries, and other industries that are metal-based, leaching of metals from different sources such as landfills, waste dumps, excretion, livestock and chicken manure, runoffs, automobiles and roadworks. Heavy metal use in the agricultural field has been the secondary source of heavy metal pollution, such as the use of pesticides, insecticides, fertilisers, and more. Natural causes can also increase heavy metal pollution such as volcanic activity, metal corrosion, metal evaporation from soil and water and sediment re-suspension, soil erosion, geological weathering.

Metals cannot be broken down and are nonbiodegradable. Organisms may detoxify metal ions by hiding the active element within a protein or depositing them in intracellular granules in an insoluble form to be excreted in the organism's faeces or for long-term storage. When the heavy metals are swallowed or inhaled into our bodies, they bio accumulate in our system. Thus they are classified as dangerous. This bioaccumulation causes biological and physiological complications. Some heavy metals are necessary for life and are called essential elements which are required for a variety of biochemical and physiological functions (Masindi *et al.*, 2018,C.H. Walker *et al.*, 2012,Y.E. Martin *et al.*, 2012, P. K. Gautam 2016 ,P.B. Tchounwou *et al.*, 2012).

Metal toxicity causes the formation of free radicals which causes DNA damage, alteration of sulphydryl homeostasis, and lipid peroxidation. Alterations have also been noted in metal-mediated calcium homeostasis due to membrane damage which causes a variety of calcium dependent systems to be activated including endonucleases. Free radical formation has mostly been investigated for iron, copper, nickel, chromium and cadmium. The last three metals are recognised for the carcinogenic properties. Iron, copper, vanadium, chromium and cobalt follow the Fenton reaction of the superoxide and the hydroxyl radical (M. Valko *et al.*, 2005).

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The heavy metals mercury (Hg), arsenic (As), lead (Pb), nickel (Ni), cadmium (Cd), copper (Cu), zinc (Zn), cause toxicity by entering the biological system. (Garty 2001, Banuelos *et al.*, 2015, Sharma *et al.*, 2015).These heavy metals have existed in the earth crust since its formation. Because of the astounding increase in the use of heavy metals, there is an imminent surge of metallic substances in both the terrestrial and aquatic environment (Tchounwou *et al.*, 2012). Acid rain dissolves industrial pollutants and heavy metals in the soil, contaminating both surface and subsurface waters (i.e. rivers, lakes, and seas) (Gao & Liu 2017).The increasing of heavy metals disturbing the environment especially in aquatic systems. Pollutants and heavy metals released through industrial effluents mostly damage aquatic ecosystems, which pose a risk to both human and ecological health (Rai, 2010) (Lourie *et al.*, 2010). Most of the investigation has been made on microalgae and macrophytic algae they are considered valuable indicators because they have accumulation activity. Trace elements like manganese (Mn), copper (Cu), lead (Pb), and zinc (Zn) present in the very low quantities and are considered as the important micronutrients for proper plants growth. Other elements such as copper (Cu), molybdenum (Mo), nickel (Ni), chorine (Cl), and zinc (Zn) are important for the plant growth in less concentrations. (R. Reeves John wiley, 2000). Metals such as magnesium (Mg), copper (Cu) and Iron (Fe) are essential elements for living organisms because of their important role as coenzyme, vitamins and in several metabolic paths (Chlorophyll a and b synthesis, electron transfer during photosynthesis, nitrogen fixation, seed maturation, stimulation of germination and growth). But other metals such as aluminium (Al), cadmium (Cd), mercury (Hg),chromium(Cr) and lead (Pb) are also important for plant metabolism , but due to their potential toxicity it may harm to the plants if it accumulated by them (Rubio *et al.*, 2017;Lami *et al.*, 2015). Transition metals, metalloids, lanthanides, and actinides are among the group of elements with metallic characteristic that make up heavy metals (Singh*et al.*, 2011).

Heavy metals have a variety of effects on both human and animal bodies. Heavy metal build up in the fatty tissue around the organ will have an effects on the central nervous system, affecting its functions. In terms of the arsenic metalloid, inorganic arsenic is recognised as a genotoxic type that influences the emergence of cancer of the liver, bladder, and lungs (Rose *et al.*, 2007). Due to its high toxicity and water solubility, the non –

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essential metal cadmium (Cd) is both a substantial pollutant and a considerable environmental risk (Pinto *et al.*, 2004; Benavides *et al.*, 2005). "Toxic metals, which include "heavy metals," are individual metals and metal compounds that have been shown to have a negative impact on people health. Many of these exist in trace amounts metals are required for life to exist. However, they become toxic in larger quantities. They may accumulate in biological systems and become a serious health risk (OSHA2004). Heavy metal contamination can have devastating effects on the recipient environments ecologicalbalance and a variety of aquatic organisms (Farombi *et al.*, 2007; Vosyliene and Jankaite, 2006; Ashraj, 2005). Different toxic mechanisms of heavy metals increase our knowledge on their harmful effects on the body organs, leading to better management of animal and human poisonings.

Chromium (Cr) is a naturally occurring heavy metal in industrial processes that can be found in the earth's crust and seawater (Tchounwou *et al.*, 2012). Chromium has several oxidation statesranging from 2 to + 6, with trivalent and hexavalent elements the most common stable forms (Shekhawat *et al.*, 2015). Cr (VI) is linked to a number of diseases and pathologies, whereas Cr (III) is needed in trace amounts for natural lipid and protein formation. Metabolism, as well as a cofactor for insulin action (Achmad *et al* 2017). Heavy metal cadmium (Cd), which is extremely dangerous, has recently spread throughout the entire world. Cadmium is released into the environment through a variety of human activities, including mining, smelting, the production of nickel and cadmium batteries, metal plating, pigments, plastic stabilizers, the discharge of sewage sludge, and the usage of phosphate fertilizers (Nordberge *et al.*, 2018). A severe risk to the health of living things is posed by the toxicity and bioaccumulation tendency of heavy metals in the environment. Across the world, macro algae have been extensively employed to measure heavy metal pollution in freshwater and marine habitats (Conti and Cecchetti, 2003).

Lead is a non-biodegradable metal that occurs in nature in relatively small amounts. Because of human activities such as manufacturing, mining, and the use of fossil fuels, atmospheric lead levels are steadily rising. When exposed to levels higher than the optimum, lead is toxic to the human body. Children are more vulnerable to lead poisoning; when they come into contact with lead-contaminated dust, the severity of the poisoning increases (Loh *et al.*, 2016). Lead (Pb) - (II) ion has been discovered to be one of the most dangerous heavy

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metals, capable of causing harm to animals and plants. Lead (Pb) - (II) ions enter the food chain primarily through drinking water, and aquatic creatures bio accumulate them (Oladoye 2022). Heavy metals occur naturally in the environment and are necessary for survival, but they can be dangerous when they accumulate in organisms. Mercury, cadmium, arsenic, chromium, nickel, copper, and lead are some of the most common heavy metals that pollute the environment (Hazrat *et al.*, 2019).

Chromium is a carcinogenic and toxic metal. It exists in two stable oxidation states in the environment: chromium (III) and chromium (IV) (VI). Chromium (III) is less dangerous chromium (VI). During industrial operations, they can interconvert to each other. However, conversion of chromium (VI) to chromium (III) is less harmful to the environment because the latter is lower in toxicity. Chromium is used in a variety of industries that endanger regional climates. In comparison to natural chromium emissions from the environment, the ferrochrome industry emits the most (Coetzee *et al.*, 2020, Kimbrough *et al.*, 1999).

Copper is widely recognized as an essential micronutrient for all living organisms. It participates in normal physiological functions of plants such as chlorophyll formation, photosynthesis, and carbohydrate and protein metabolism. Copper deficiency alters critical metabolic processes, and excessive exposure is toxic (Schwartz *et al.*, 2003).

Zinc is widely used in a variety of industries, including galvanization, paint, batteries, smelting, fertilizers and pesticides, fossil fuel combustion, pigment, polymer stabilizers, and so on, and zinc is abundant in effluent from these processes (Paramar *et al.*, 2013). Too much zinc, on the other hand, can result in serious health issues like stomach pains, rashes, vomiting, nausea, and anaemia (Fu *et al.*,2011).Symptoms of zinc toxicosis include vomiting, diarrhea, bloody urine, icterus (yellow mucus membrane), liver failure, renal failure, and anemia (Duruibe *et al.*, 2007).

Iron poisoning typically occure from ingestion excess irons those results in acute toxicity. Mild symptoms which occure within hours include vomiting, diarrhea, abdominal pain and drowsiness (Manoguetta *et al.*, 2005).

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REVIEW OF LITERATURE

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REVIEW OF LITERATURE

Agarwal *et al.*, (2022) reported that heavy metals are primarily discharged in the lower Gangetic delta from a variety of anthropogenic sources. Because of their non-conservative nature, they are a major source of concern. Heavy metals Zn, Cu, and Pb were found in *Enteromorpha compressa* estuarine water and thallus body tissue. The Bioaccumulation Factors (BAF) calculated for all selected heavy metals show that Pb has the highest value, followed by Zn and Cu. The overall result suggests that seaweed could be a potential bio-purifier in coastal areas that are heavily impacted by anthropogenic activities.

Poeloengasih *et al.*, (2022)reported that the current study was carried out to reduce heavy metal residues in *Ulva sp*. Collected from Sepanjang beach, Gunungkidul, Indonesia, using various processing treatments, namely, washing with citric acid and blanching at various times and seaweed-to-solution ratios. Inductive Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine the number of heavy metal residues (As, Cd, Pb, and Hg), and visual green colour loss was measured using a colour spectrophotometer.

KJ Lee *et al.*, (2022)assessed that the concentrations of heavy metals and arsenic (As) species in 11 distinct types of seaweed gathered from Korea's largest coastal cities. The concentrations of heavy metals in the seaweed fell within the following ranges: cadmium (0.023-0.232 mg/kg fresh weight [fw]), and lead (0.025-0.222 mg/kg fw), with the majority of them complying with international standards for edible seaweeds. However there was a lot of total As present—1.020 to 20.525 mg/kg fw. In particular, when it comes to *Sargassum* seaweed.

Rivas *et al.*, (2022)studied to show that controlled outdoor cultures can be used successfully to remove excess heavy metal accumulation in *Gracilaria chilensis*, an important commercial seaweed farming model. We developed a simple and effective heavy metal depuration protocol that used seawater and tap water removal to reduce the concentration of 10 heavy metals, including As, Cu, and Cd but not Zn, from biomass after 7 days of culture. At 7 days, the percentage of heavy metal depuration ranged from 32 to 92%.

Filippini *et al.*, (2021)reported that seaweed consumption has lately increased in the West despite being a staple in Asia for centuries. Superfoods, which are known for their

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nutritional value and abundance in important components, can also absorb significant amounts of heavy metals and iodine from the environment and turn into health hazards. The study's objective was to use ICP-MS to determine the presence of iodine and arsenic as well as 20 heavy metals in seaweeds distributed across Italy (total and inorganic fraction). The Rhodophyta contained the greatest amount of the elements. Asian seaweed has 1.16 mg/kg of cadmium. The largest concentrations of iron, zinc, and magnesium were found in the Asian seaweed.

Khandaker *et al.,* (2021)analysed and evaluated the amounts of 17 elements in seaweed sourced locally, as well as any possible carcinogenic and noncarcinogenic concerns. The materials were homogenised, pulverised, and measured with the ICP-OES method. It has been discovered that the concentrations of the necessary elements K, Ca, Mg, Zn, and Na are often a little bit higher than those of a number of potentially harmful metals, namely Cd, Pb, Ag, and As, with Ni, Cr-VI, and Si being the exceptions. According to statistical research, all of the latter share a common origin and are more concentrated in the marine ecosystem.

Chowdhury *et al.,* (2021)reported that an atomic absorption spectrometer to examine the minerals (Ca, Mg, Fe, Zn, Mn, and Cu) and heavy metals (Pb and Cd) present in seaweeds taken from Bangladesh's coastal waters (AAS). The findings demonstrated that the amounts of minerals and heavy metals varied between species. The highest Ca content was found in Phaeophyta (18565 mg/kg-dw), which was 19 times greater than the Chlorophyta value of 950 mg/kg-dw. This is so because the ability of various seaweed groups to absorb minerals is influenced by environmental factors such as salinity, pH, light intensity, the availability of minerals in seawater, interactions between elements, growth and metabolic processes, etc.

Costa *et al.,* (2020)examined that the main similarities and gaps in studies examining the effects of heavy metals on benthic marine seaweeds; this paper presents a comprehensive review. Worldwide research on the genera *Sargassum, Ulva,* and *Enteromorpha* was extensive. These results underline the significance of increased efforts to establish a succinct baseline of knowledge using comparable criteria in order to carry out global assessments of the effects of heavy metals on photosynthetic organisms.

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Monteiro *et al.*, (2019)reported that toxic substances like cadmium (Cd), lead (Pb), mercury (Hg), inorganic arsenic (iAs), or even some micronutrients, particularly iodine (I), for which seaweed has been shown to have an antioxidant role. In addition, the European Commission asked for the gathering of monitoring data on their levels in an effort to improve risk evaluations and possibly establish upper limits on European legislation. This research attempted to measure the quantities of these components in several seaweed species.

Madkour *et al.*, (2019)reported that by using Flame Atomic Absorption Spectrometry, the average contents of Zn, Cd, Cu, Co, Fe, Mn, and Ni in the ashes of *Caulerpa racemosa, Cystoseira myrica, Digenea simplex, Hormophysa cuneiformis, Padina pavonica*, and *Sargassum cinereum* were measured seasonally (AAS). The species were collected from three different locations along the northern Red Sea coast. The highest Fe average (575.88 g/g dry weight) was recorded by *Cystoseira myrica.* The results showed that the studied macroalgae have significantly different abilities to accumulate certain heavy metals under local conditions, and they can be used as a good target for monitoring metal pollution in marine waters.

Komariyah *et al.*, (2019)studied that both freshwater and seawater include metals and minerals. Heavy metals, such as lead (Pb) and other heavy metals, when present in high concentrations in marine waters, can be hazardous to marine life, both animals and plants. A harmful heavy element like lead (Pb) may be present in seaweed in high concentrations, which may slow the growth of the algae. The goal of this study is to compare the lead levels in seaweed Eucheuma cottonii with the requirements of the Indonesian National Standard (SNI) in order to demonstrate the differences in lead levels that have accumulated there.

Ardiyansyah *et al.*, (2019)reported that every country's main problem is the contamination of poisonous chemical substances, one of which is cadmium (Cd). *Gracilaria* sp is a biota capable of accumulating heavy metals in the form of cations, anions, or organic compounds via adsorption on the surface of talus. The average Cd content in fishpond water is 0.0144 mg/l, which exceeds the 0.01 mg/l threshold established by Research Regulation Number 82 (2001). The Cd content in sediment and Gracilaria sp. Is 0.0024 mg/kg, which is less than the 0.2 mg/kg threshold established by BSN (2009).

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Cadar *et al.*, (2019)Studied seaweed is a natural treasure that has been thoroughly studied for therapeutic purposes. It is well known that seaweed is an indicator of heavy metal accumulation. Kelp must meet certain conditions regarding the accumulation of heavy metals before it can be used for medical purposes. This paper investigates the heavy metal content of algae.

Nordberg *et al.*, (2018)reported that all human diets contain trace amounts of cadmium (Cd). In order to take preventive action, it is essential to determine the harmful effect that manifests at the lowest exposure level, or the critical effect, when assessing the danger to human health. Overexposure to Cd may result in cancer, effects on the kidneys, lungs, liver, skeleton, and reproductive system. Our research also demonstrates that renal effects, as measured by sensitive biomarkers like urinary beta-2-microglobulin, calcium, and N-acetyl-beta-D-glucosaminidase content, occur at lower cumulative Cd exposures than those that result in skeletal effects as well as at lower exposures than previously thought, such as by the World Health Organization.

Nguyen *et al.*, (2018)studied that the patterns of elemental concentration and heavy metal accumulation in the edible seaweed Gracilaria fisheri were studied in ponds located in three provinces of Southern Thailand. The amounts of elements and heavy metals found in seaweed were found to be in the following ranges: Calcium (Ca) 9.22-10.01, magnesium (Mg) 11.40-13.40, potassium (K) 1.85-23.35, sodium (Na) 1.60-4.96, copper (Cu) 1.65-2.91, manganese (Mn) 557.05-746.75, zinc (Zn) 30.15-36.60, iron (Fe) 172.95-841.23, nickel (Ni) 6.03-826, chromium (Cr) 1.43-2.

Ginneken *et al.*,(2018) studied that *Caulerpa sertlatioides* (Cuba), *Caulerpa brachypus* (Bali, Indonesia), *Undaria pinnatifida* (West-Donegal, Ireland), and *Ulva lactuca* (Ireland) are four different species of marine seaweeds that have been examined for heavy metal accumulation (Easters-Scheldt, the Netherlands). Inductively Coupled Plasma Spectroscopy (ICP)-techniques were used to determine the presence of heavy in fresh seaweed fronds under mechanical pressure of 10 bar from seaweed biomass in the laboratory (Al, As, Cd, Co, Cr, Cu, Fe, Mo, Ni, Pb & Zn).

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Chen *et al.*, (2018)reported that the distribution of 10 metals and metalloids in 295 dried seaweeds (brown and red) was examined in this study, and the hazard index was used to calculate the potential health risk (HI). By descending order of mean values, the elements in seaweeds are Al > Mn > As > Cu > Cr > Ni > Cd > Se > Pb > Hg. Red seaweeds had considerably greater concentrations of Cd, Cu, Mn, and Ni than brown seaweeds did (P 0.01). Ni-Cr content in seaweeds demonstrated somewhat positive relationships ($r = 0.59$, P 0.01), according to correlation analysis.

Rubio *et al.*, (2017)report that twenty metal concentration levels in edible seaweed (*Chondrus, Eisenia, Gelidium, Himanthalia, Laminaria, Palmaria, Porphyra,* and *Undaria*) .Higher levels of hazardous and trace components were found in red seaweed. *Porphyria* has the potential to be a bioindicator for metals. In comparison to organic cultivation (0.13 mg/kg), the mean Cd level from conventional cultivation (0.28 mg/kg) was two points higher. Seaweed (4 g/day) ingestion helps to increase dietary intake of metals, primarily Mg and Cr. Al, Cd, and Pb intakes were 0.064, 0.001, and 0.0003 mg/day on average, respectively. Based on the findings, this study concludes that intake of seaweed does not pose a severe health risk for exposure to the toxic metals Al, Cd, and Pb, but other toxic metals should be closely monitored.

Porcia *et al.*, (2017) suggested heavy metals are significant pollutants continuously released into the biosphere, both naturally and anthropogenically. Conceptually, metal speciation, bioavailability, and associated toxicity in marine organisms depend on a wide array of abiotic and biotic factors. To cope with heavy metal excess, several mechanisms exist in tolerant seaweed species. In contrast, an excessive heavy metal presence can inhibit photosynthesis, reduce pigment concentration and growth, induce cation losses, and disrupt gametophyte development in non-tolerant seaweed species.

Kato *et al.*, (2016)studied that the purpose of this study was to track the levels of metals, such as Cd, Pb, Cu, and Zn, in commercially significant and often consumed brown, red, and green algae species that were taken from the Thondi coast in southeast India. The green seaweed *Chaetomorpha linum* has greater metal concentrations than the other seaweed samples evaluated (Cd 8.51 mg kg1, Pb 5.24 mg kg1, Cu 15.38 mg kg1, and Zn 22.34 mg kg1). Between and across the various species of seaweed samples under study, the metal

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contents differed significantly (P 0.05). It was discovered that various concentrations of the metals Cd, Pb, Cu, and Zn were found in the seaweed species.

Sharma *et al.*, (2016)reported that physiological reactions to greater doses of cadmium (Cd) and their capacity for Cd accumulation were examined in four different rapeseed (*Brassica napus* L.) In soil under greenhouse conditions, plants were subjected for 30 days to Cd doses of 0, 50, 100, and 150 mg/kg. Of all the examined cultivars, cadmium was accumulated higher in the roots than the shoots, with Zhong Zhuang having the highest cadmium levels. The highest translocation factor after the lowest Cd dose was for CZL (0.8) and Benefit (0.6). Several treatments and cultivars have different K and Ca contents.Several Cd dosages (0, 50, 100, and 150 mg Cd/kg) in soil were applied to plants for 30 days in a greenhouse environment.

Cavalier *et al.*, (2016)studied that the higher-level taxonomy of euglenozoans has to be significantly improved in light of the discovery of numerous new species as well as developments in ultrastructure and sequence phylogeny (including here the first site-heterogeneous). The pellicular-strip-adorned Euglenoida, the anaerobic Postgaardia that relies on surface bacteria and has specially modified feeding mechanisms, and the newly discovered subphylum Glycomonada are all examples of this. Two new infraphyla, Entosiphona and Dipilida, both of which have three feeding rods, make up the Euglenoida. The superclass Rigimonada, which comprises the Dipilida, has a longitudinal stiff strip.

Henriquez *et al.*, (2015) reported in the past years, coastal waters have been exposed to an increase in copper concentration due to anthropogenic inputs. In this work, microcosm experiments were carried out to address the changes on picophytoplankton community composition of the disturbed area, when challenged to copper additions. Flow cytometry data showed that 25 μg L−1 of copper addition exerted a positive effect in the growth kinetics on part of the eukaryotic picophytoplankton communities, independently of the site. Members of the Prasinophyceae class, a Coscinodiscophyceae diatom, as well as Phaeocystis, respond in a short time to the environmental disturbance, making them excellent candidates for further studies to evaluate phytoplanktonic species as sentinels for copper disturbances in coastal marine ecosystems.

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Bharathiraja *et al.*, (2015)reported that in terms of both food security and environmental concerns, algae constitute the most sustainable fuel source. Algal energy is becoming more popular as interest in inefficient and unsustainable biofuels made from agricultural crops grows. Progressively more emphasis has been paid to CO2 mitigation and rapid biomass buildup that also successfully accomplishes bioremediation.

Benfares *et al.*, (2015)suggested that the purpose of this study was to look into and assess the concentrations of trace metals (Cd, Pb, Cr, and Hg) in the *Cystoseira compressa* algae from the coastal city of Bou Ismael (Algeria). The concentrations of heavy metals and the level of pollution in the region were determined using physicochemical analyses of seawater, sediments, and algae in wastewater from the three sites. Overall, the findings supported the heavy metal bioaccumulation of algae from the genus *Cystoseira,* as well as their utility as biomarkers of pollution in coastal areas.

Shekhawat *et al.,* (2015)studied that on previously conducted studies, this article reviews the toxicity of chromium and its effects on living things. The environment contains chromium, a hazardous heavy metal, in oxidation states ranging from -2 to +6. Trivalent and hexavalent chromium, however, are the most stable forms. Compared to hexavalent chromium, trivalent chromium has less cellular absorption. Ingestion, skin contact, and inhalation are the three most frequent ways that humans are exposed to chromium. The main health risks posed by chromium.

Chakraborty *et al.*, (2014)reported that algal species, which are common along many nations' coastlines, reflect the environmental conditions of the coastal waters and could be effective biomonitoring tools for anthropogenic pollution. Ten elements (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) of potential environmental significance have their heavy metal concentrations measured in saltwater. The highest concentrations of heavy metals, including Cd (125 lg g-1), Pb (2,425 lg g-1), and Zn (7,974 lg g-1), were found in the finer sediment fractions (250 lm) of Port Pirie. Spatial differences in heavy metal concentration in both seawaters and sediments were observed at all sites.

Zou *et al.,* (2014)studied that heavy metal transfer into aquatic creatures, which causes numerous unexpected changes in nutrition and primary and/or secondary metabolism,

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is a severe challenge for aquaculture in heavy metal-polluted coastal areas. By characterising biochemical and metabolic responses using both 1H NMR and GC-MS techniques under acute (47 M, 1 day) and chronic stress (8 M, 7 days), the dual role of the heavy metal copper (Cu) in the metabolism of the photosynthetic organism *Sargassum fusiforme* was evaluated in the present study.

Azza Khaled *et al.*, (2014)reported that Flame Atomic Absorption Spectrometry was used to map the distribution of Cd, Cu, Fe, Ni, Pb, and Zn concentration in two species of seagrass and six species of marine macroalgae, including *Cystoseira sp., Gelidium crinale, Laurencia obtusa, Gracilaria verrucosa, Jania rubens,* and *Enteromorpha compressa.* According to the measured levels of heavy metals, distinct species exhibited varying levels of metal buildup. Across all of the stations that were examined, El-Boussit station generally recorded the highest concentrations. Parmar *et al.*, (2013) suggested that cadmium is a potentially toxic heavy metal that reduces plant productivity by interfering with photochemistry.

Sadhasivam *et al.*, (2012) reported that heavy metals typically reach aquatic environments through air deposition, geological matrix erosion, or anthropogenic activity brought on by industrial effluents, home sewage, and mining wastes. The present study analysed the concentration of seven elements (Cd, Cu, Mn, Ni, Pb, Zi and Hg) in eight seaweeds such as *Centrocerousclavutum, Sargassum wightii, Colpemenia sinosa, Spyridia hypnoides, Valoniopsis pachynema, Ulva reticulata, Gelidialla acerosa & Turbinaria ornata* and two seagrasses such as *Syringodium isoetifolium & Cymodocea serrulata* collected from three stations along southeast coast of India.

Tchounwou *et al.*, (2012)studied that heavy metals are naturally occurring substances with a density at least five times larger than that of water and a high atomic weight. Concerns about their possible impacts on human health and the environment have been raised as a result of their widespread distribution in the environment as a result of their numerous industrial, residential, agricultural, medical, and technical applications. Arsenic, cadmium, chromium, lead, and mercury are among the priority metals that are important for public health because of their high level of toxicity.

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Karthick *et al.*, (2012) reported that Mn, Pb, Zn, Cd, Cu, and Cr concentrations were determined in six seaweed samples from Wandoor, south Andaman Island (*Acetabularia calyculus, Corallina sp., Galaxura marginata, Sargassum duplicatum, Sargassum swartzi, and Dictyota bartayresiana*). Metals were extracted and quantified using atomic absorption spectrometry from sample homogenates. Heavy metal concentration levels in the current study were determined in the following order: Mn > Pb > Cd. It was discovered that Zn was only found in the free floating brown seaweed *Sargassum swartzi*. The concentration of metals was significantly different (p 0.05) with respect to different species around the study area, according to one-way analysis of variance (ANOVA).

Singh *et al.*, (2011) reported that the earth's crust naturally contains heavy metals, but uncontrolled human activity has significantly changed their geochemical cycles and biological equilibrium. This causes a buildup of metals in plant portions that contain secondary metabolites, which gives rise to a specific pharmacological activity. Humans can have negative health effects from prolonged exposure to heavy metals as cadmium, copper, lead, nickel, and zinc. Many biotechnological applications of molecular knowledge of plant metal accumulation also exist, however their long-term impacts may not yet be known.

Bhatnagar *et al.*, (2010) studied that biological activity can take place in ocean in our planet. Marine source have a lots of biological activity such as anti-cancer, anti-tumor, anti-microtubule, anti- proliferative, cytotoxic, photo protective as well as antibiotic and antifouling. Also the marine environment have a largely untapped novel microbes, including bacteria, fungus, *actinomycetes*, microalgae – *cyanobacteria*, and diatoms. These microbes are make a bioactive secondary metabolites. Marine microbes have general aspects of biotechnology application,bioactive substances, biophysical and biochemical properties and chemical structure it have been conducted by the research.

Rai PK (2010) reported that since lakes or reservoirs are frequently utilized for drinking, bathing, irrigation, and aquaculture, aquatic pollution poses a severe issue to the scientific community globally. From 2004 to 2005, a tropical lake receiving discharges from a thermal power station, a coal mine, and a chlor-alkali company was sampled at four different locations for nine metals and a number of physicochemical characteristics. A significant percentage reduction in metal concentrations was seen during field

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phytoremediation tests employing aquatic macrophytes. The decrease in percentage for various metals ranged from 25% to 67.90% in Belwadah (with *Eichhornia crassipes* and *Lemna minor*), 25% to 77.14% at Dongia nala (with *E. crassipes*, *L. minor*, and *Azolla pinnata*), and 25% to 71.42% at the Ash Pond Site of G.B. Pant Sagar (with L. minor and *Azolla. pinnata*).

Apaydin *et al.*, (2010) studied that seaweed (*Ulva lactuca*) from eight different districts of Istanbul, Turkey, was gathered with the intention of examining the concentrations of harmful and important trace elements using energy-dispersive X-ray fluorescence spectrometry (EDXRF). The samples have been examined by means of two annular radioactive sources and an Ultra-LEGe detector. Within the ranges of 0.8-1.8%, 1.1-2.1%, 0.6-2.3%, 0.2-3.4%, and 0.9-2.7%, respectively, are the metal concentrations in the seaweed samples used in this investigation. The findings showed that these seaweeds do indeed contain some important elements but no harmful ones.

Aslam *et al.*, (2010) studied that concentrations of six different elements (Ca, K, Fe, Ba, Ti and Sr) were measured in twelve lichen species from two geographically different Traregions. The incident beam and fluorescence X-rays emitted from the target were detected and analyzed with an Ultra-LEGe detector. Five of the six elements (Ca, K, Fe, Ba and Ti) are available in the twelve lichen species living in 12 different habitats. But, Sr is the only available element in some lichens living in some certain localities.

Qari *et al.*, (2010) reported that a comparison of the levels of heavy metals in forty species of red seaweed from 26 genera and three different coastal regions of Karachi (Buleji, Paradise Point, and Nathia Gali). The concentration of the metals Mg> Fe > Mn, Zn > Cu reveals a clear tendency in the majority of seaweed species. At Buleji, Paradise Point, and Nathia Gali, there was a significant variance in the metals' accumulation. Buleji had higher concentrations of Mg, Ni, Zn, Cr, Pb, Co, and Cd than Paradise Point and Nathia Gali did. In comparison to Buleji and Paradise Point, Mn and Cu accumulations were higher at Paradise Point, and Fe concentrations were higher at Nathia Gali.

Rodriguez *et al.*, (2010) studied to be the process of metal bioaccumulation in marine food chains is poorly understood due to a lack of data on metal concentration. As a result,

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we wanted to describe the spatial and temporal variation of heavy metal concentrations in seaweeds and seagrasses. Seasonal collections were made at Estero Banderitas in November 2004, February, and April 2005. Our findings revealed that iron, copper, and magnesium were the most abundant metals in seagrasses, red algae, and green algae. Furthermore, our findings suggest that heavy metals may be regularly incorporated into the diets of many herbivorous animals, with serious consequences for their health.

Lourie *et al.,* (2010) studied that artificial multimetal aqueous solutions, the biosorption of copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd), cobalt (Co), and nickel (Ni) was investigated as a function of metal content in solution and amount and size of bark particles utilized for sorption. It was discovered that Ca2+ concentration had no effect on the uptake of metal from solutions with a high heavy metal content. In terms of metal uptake, *Chlorella sp.*When bark was treated with algae, sorption of Co, Zn, Ni, and Cd from a solution containing high amounts of calcium and heavy metals increased by about 50%. Cu and Pb were completely absorbed into the water at low metal and calcium concentrations. A significantly high 50–60% of other metals were taken up from solutions with low metal and Ca concentration. Low pH 3.0 had no effect on metal sorption from solutions containing a lot of metal.

Kim *et al.,* (2009) reported that seaweed aquaculture is still a young sector in the US. Cultured seaweeds may accumulate pollutants like heavy metals because a large number of seaweed farms are situated in urbanised estuaries. To biomonitor potentially hazardous metals, *Gracilaria tikvahiae*, a warm-temperate red algal species, and *Saccharina latissima*, a cold-water brown algal species, were grown at three locations in the estuaries of Long Island Sound (LIS) and New York (NY). For human consumption, metal concentrations were nearly always below all national and international regulatory limits.

Wallenstein *et al.*, (2009) studied that to provide baseline levels of a selected pool of elements for intertidal algae found on the rocky shores of Island, and. Coastal shallow water hydrothermally active sites are uncommon worldwide. As a result, information on macroalgal communities found in such specific environments is a valuable resource for future research, not only for comparing to sites that have been artificially enriched with

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heavy metals (polluted), but also for assessing the impact of increased temperature and acidity on such communities.

Besada *et al.*, (2009) reported that the concentrations of six common heavy metals (Cd, Pb, Hg, Cu, Zn, total As, and inorganic As) were measured in this study's analysis of the concentrations of the first topic (Cu and Zn by flame AAS; Cd, Pb and total As by electrothermal AAS; total mercury by the cold vapour technique; and inorganic As by flame-hydride generation). Participation in recurrent intercalibration exercises between the IAEA (International Atomic Energy Agency) and QUASIMEME (Quality Assurance of Information in Maritime Environmental Monitoring in Europe) was used to determine accuracy.

Meenakshi *et al.*, (2009) studied that seeks to assess two seaweeds, *Ulva lactuca and Sargassum wightii,* for their total flavonoid content and in vitro antioxidant activity. *S. wightii* had higher levels of total flavonoid content and methanolic extract antioxidant activity, measuring 2.020.07 mg GAE/g and 1.160.11 mg GAE/g, respectively. The FTIR study demonstrates the existence of polyphenolic signals at various wavelengths. The estimated moderate antioxidant activity of flavanoids was compared to the industry standard of gallic acid.

Rose. M (2007) reported that in order to offer information for dietary exposure estimations and to support consumer guidance, this study assessed the concentration of total and inorganic forms of arsenic in seaweed that is available for retail sale for eating. Only one of the nine samples of hijiki seaweed that were examined had inorganic arsenic, which can lead to liver cancer, at levels between 67 and 96 mg/kg. The limit of detection for the method utilized was determined to be less than 0.3mg/kg inorganic arsenic in all other species of seaweed. The UK Food Standards Agency (FSA) advised people not to eat hijiki seaweed since doing so could considerably increase dietary exposure to inorganic arsenic.

Farombi *et al.*, (2007) reported that as a Bulk Scientific Atomic Absorption Spectrophotometer was used to measure the levels of Zn, Cu, Cd, As, and Pb in the kidney, liver, and gills. It was also measured the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione (GSH) concentration,

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and malondialdehyde (MDA) production. The heart, kidneys, liver, and gills all had heavy metal concentrations that varied from 0.25 to 8.96 parts per million (ppm), 0.69 to 19.05 ppm, 2.10 to 19.75 ppm, and 1.95 to 20.35 ppm. GSH content rose by 81%, 83%, and 53%, respectively, in the liver, kidney, and heart, but fell by 44% in the gills. The work thus offers a justification for the use of oxidative stress indicators in aquatic pollution biomonitoring.

Misheer *et al.*, (2006) studied that the elements intake by the marine macroalgae *Caulerpa racemosa* (Sea Grapes), an edible seaweed (chlorophyta, green alga). The sum of the concentrations of Mn, Fe, As, B, Ti, Zn, and Hg. The *C. racemosa* has a high ability to accumulate titanium, boron, and arsenic but a low ability to absorb iron. At Treasure Beach near Durban Metropolis in the fall, a typical *C. racemosa* sample contained Mn (5.2+/-ppm), Fe (0.21+/-0.01 ppm), As (8.5+/-0. Ppm), B (1090+/-ppb), Ti (159+/-ppb), Zn (3.8+/-0.1 ppb), and Hg (189+/-ppb). .

Gautam *et al.*, (2005) examined that a number of meteorological factors related to the hurricane at various times, including its evolution, intensification, and landfall. Based on the classification into several stages, analysis of the hurricane's surface latent heat flux (SLHF) and precipitation rate (PR) is done

Pinto *et al.*, (2004) reported that exogenous silicon supply's impacts on rice development metrics and the level of arsenic deposition were the subject of this study's planned investigation. The Saline Agricultural Research Centre's wire house, which is home to the Institute of Soil and Environmental Science, served as the site of the experiment. Treatments included in the study were control, (100 M) and (200 M) concentrations of arsenic, (5 mM) concentration of silicon, and (both (5 mM Silicon + 100 M) concentration of silicon plus (200 M) concentration of arsenic). The findings showed that in a solution containing 5 mM Si, the largest amounts of fresh shoot weight, dry shoot weight, fresh root weight, and dry root weight were found. The most tillers were likewise seen in the (5 mM Si) solution, however silicon administration was unable to reduce the genotype's high arsenic levels.

McHugh (2003) studied that pharmaceutical companies are currently paying increased attention to the bioactive compounds found in seaweeds for the development of

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new drugs. Scientists are paying close attention to seaweeds because of their bioactive chemicals, which have unusual features such being anti-viral, anti-tumor, anti-inflammatory, and anti-lipedemic. The key topics covered in this review were metabolites, seaweed kinds, and characteristics. Focus is placed on the primary metabolite and characteristics of seaweeds.

Conti *et al.*, (2003) investigated as potential biomonitors of heavy metal contamination in coastal marine environments. Cd, Cr, Cu, Pb, and Zn concentrations were measured in the green algae *Ulva lactucaL.* and the brown algae *Padina pavonica (L.).* The results of this study were also compared to those obtained previously from uncontaminated sites in the Sicilian Sea, Italy. Metal accumulations were high in the species studied, with concentration factors (CFs) exceeding 10,000 in comparison to concentrations in marine waters. Metal concentrations measured in this area could be used as a baseline for intraspecific comparisons within the Tyrrhenian Sea, a body of water about which little is known.

T.A Davis *et al.,* (2003) reported that the passive removal of toxic heavy metals such as Cd(2+), Cu(2+), Zn(2+), Pb(2+), Cr(3+), and Hg(2+) by inexpensive biomaterials, termed biosorption. A detailed description of the macromolecular conformation of the alginate biopolymer is offered in order to explain the heavy metal selectivity displayed by the brown algae. The role of cellular structure, storage polysaccharides, cell wall and extracellular polysaccharides is evaluated in terms of their potential for metal sequestration. Binding mechanisms are discussed, including the key functional groups involved and the ion-exchange process. The sorption behavior (i.e., capacity, affinity) of brown algae with various heavy metals is summarized and their relative performance is evaluated.

Farias *et al.*, (2002) studied that eleven distinct types of Antarctic algae were tested for their capacity to accumulate various metals and metalloids from the region's aquatic environment. As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Se, Sr, V, and Zn were the elements that were quantified. The samples were digested using an improved microwave-assisted digestion method, and the elements were identified using inductively coupled plasma optical emission spectrometry. At concentrations of up to 3095 g g 1 for Fe, Monostroma hariotii and Phaeurus antarcticus had the highest amounts of trace elements.

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Villareses *et al.*, (2002) studied that seasonal variation in the contents of various metals (Al, Cr, Cu, Fe, Mn, Ni, and Zn) in two macroalgae genera, *Ulva* and *Enteromorpha*, was studied at 22 sites on Spain's northwest coast. The seasonal variation in the different metals in both seaweeds followed a similar pattern and appeared to be caused by dilution during periods of maximum growth and concentration during periods of slow growth.

Banuelos *et al.*, (2002) reported that heavy metals, such as cobalt, copper, manganese, molybdenum, and zinc, are essential in trace amounts for growth by plants and other living organisms. However, in excessive amounts these heavy metals have deleterious effects. Like other organisms, plants possess a variety of detoxification mechanisms to counter the harmful effects of heavy metals. Phytoremediation is an emerging technology that uses plants and their associated rhizospheric microorganisms

Misra. S, (2001) studied that seeds from self-fertilized transgenic plants were germinated on toxic levels of cadmium-containing media and scored for tolerance/ susceptibility to this heavy metal. Transformed seedlings' root and shoot growth was unaffected by up to 100 M CdCl2, whereas control seedlings showed severe inhibition of root and shoot growth and chlorosis of leaves. These experiments show that agriculturally important plants like *Brassica napus* can be genetically engineered for heavy metal tolerance/sequestration and, eventually, heavy metal partitioning in non-consumed plant tissues.

Campella *et al.*, (2001) reported that the contents of Cd, Cr, Cu, Pb, and Zn were assessed in specimens of four marine organisms: the brown alga *Padina pavonica (L.)* Thivy, the two gastropod molluscs *Monodonta turbinata* Born and Patella *caerulea L.*, and the seagrass *Posidonia oceanica(L.)* Delile. A region that has been largely unaffected by human activity. The four species gave a rather clear-cut image of the bioavailable metal burdens in the various sites of the chosen area. The station where the neighbourhood harbour is located showed a strong pattern of rising metal contents. Discussion is held regarding the implications of the observed accumulation patterns in biomonitoring, particularly in relation to Posidonia oceanica's various tissues.

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Reeves: John wiley (2000) studied that the utilisation of nickel hyperaccumulator plants is a brand-new method for recovering nickel from popular rock types. Before, the concept of phytomining nickel was put out, but it was never carried out. Since the mining industry wants to reduce the pollution and high energy consumption linked to metal extraction and because mining has a negative reputation due to its effects on the environment, this status may soon change. The mining industry's reputation will improve if CO2 sequestration and metal extraction work together. Additional benefits include the ability to produce nickel hyperaccumulator plants and recover nickel on unproductive soils.

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AIM AND OBJECTIVES

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AIM AND OBJECTIVES

- Collection of seaweeds from Tuticorin coastal region.
- Preparation and process of seaweeds to heavy metal analysis.
- Heavy metal analysis by using Inductively Coupled Plasma- Mass Spectrometry method.

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MATERIALS AND METHODS

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MATERIALS AND METHODS

Study area

The study site selected for the sampling area with the geographical platform of Manapad (8°22'39" N and 7°83'8" E) and Tharuvaikulam (8° 53.5845' N and 78° 10.2297' E) Tuticorin Coastal region, Gulf of Mannar, Southeast Coast of India (Fig. 1 and Fig. 2). The sites varied in distance from one another ranging from 77.9 km to 80 km. The study area is situated on the distal end of Gulf of Mannar Biosphere Reserve. The rocky shore of Manapad inhabits an astonishing biodiversity, representing nearly almost all the invertebrate phyla and urochordates. Hard rocky bottom of this area greatly supports the algal diversity and provide suitable shelter and feeding ground for grazers. Field surveys were undertaken to the selected sampling stations of the Manapad coastal region. The substratum of study (Tharuvaikulam) consist of either sand, slit, rocks covered with mud or coral stones.

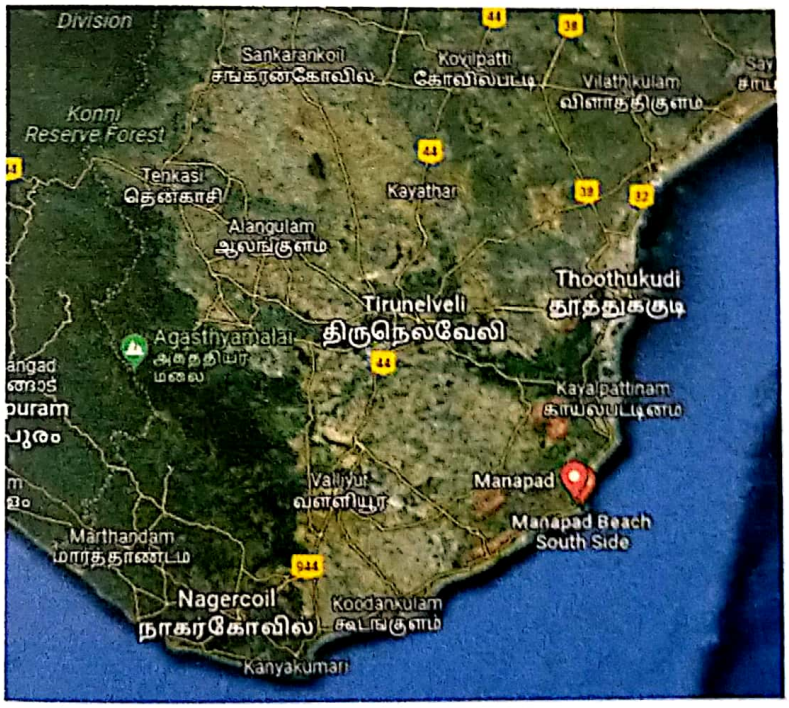


Figure 1: Seaweed sample collection area (Manapad)

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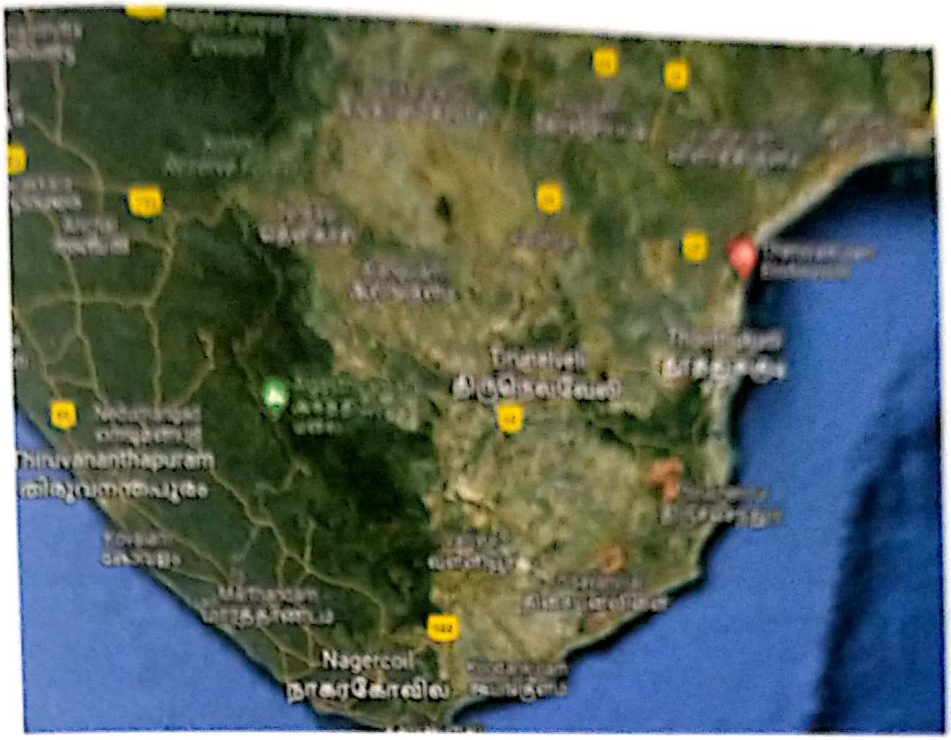


Figure 2: Seaweed sample collection area (Tharuvaikulam)

Collection and processing of seaweed samples

The fresh, undamaged seaweed samples were collected from Manapad and Tharuvaikulam, Tuticorin Coastal region, Gulf of Mannar, Southeast Coast of India.The collected seaweed samples were identified by using standard floras (Aleem, 1993), such as brown algae (*Sargassum wightii)* (Manapad) and green algae (*Ulva lactuca*) (Tharuvaikulam). The collected seaweed samples were washed with running tap water, stored in an icebox, and transferred to the lab (Fig.3). In the laboratory, the algae samples were soaked in double-distilled water and washed twice for removal of surface contaminations. Finally, the washed samples were dried at room temperature in dark conditions for 20 days (Fig.4). After complete drying, the seaweed samples were ground to a powder with the help of a homogenizer. The samples were carefully packaged and wrapped with black cloth to protect them from sunlight after receiving the thin powder by sifting and stored at 4°C for later use.

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Figure 3: Fresh Seaweed samples



Figure 4: Dried seaweed samples

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Sample preparation and Digestion

The heavy metal concentration in seaweed samples were analyzed according to the methodology of AOAC (2015). Approximately 0.50 ± 0.05 g (wet weight) of each seaweed samples were weighed in the digestion vessel and 5 mL of 65% nitric acid (Suprapur, Merck, Kenilworth, NJ, USA) and 1 ml of hydrogen peroxide were added and allowed to stand in a fume cupboard for 1 h at room temperature for pre-digestion. Then the samples were placed in a microwave digestor (MARS 6, CEM Corp., Matthews, NC, USA) for digestion. The temperature was set to 180°C at 1000 W with a 20° C ramp from the ambient temperature. The final hold was set at 180° C for 20 min followed by cooling until the temperature was below 90° C. The samples digest were then transferred into separate polypropylene tubes and brought to a 50 ml using MilliQ water (Arium lab water systems, Sartorius, Gottingen, Germany).

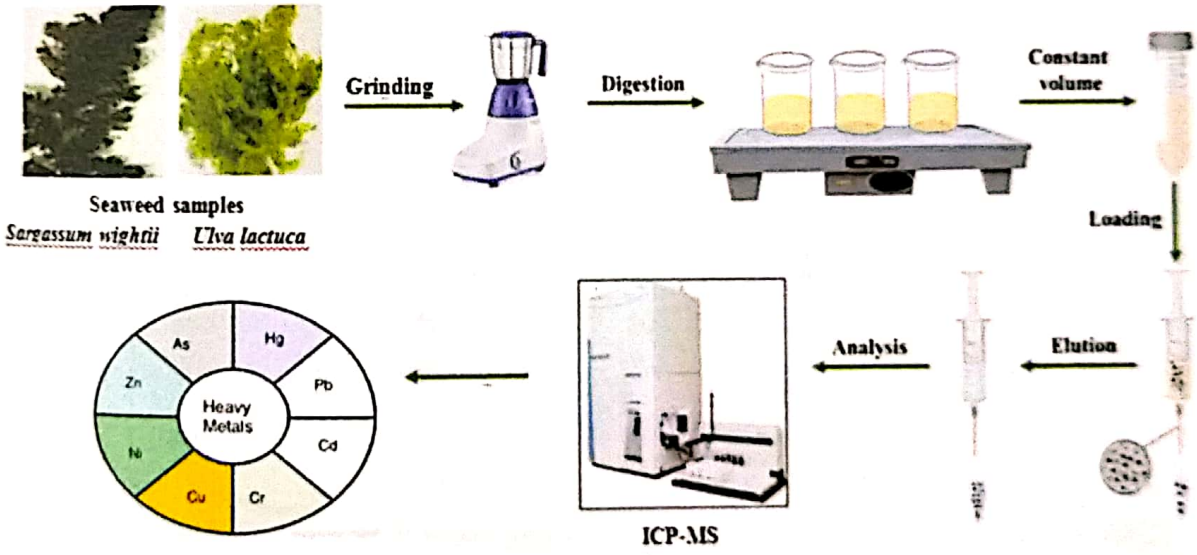


Figure 5: Analysis of heavy metals by using ICP-MS

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Instrumentation- ICP MS

Inductively coupled plasma mass spectrometry (iCAP RQ, Thermo Fisher Scientific, Bremen, Germany) was used to determine the concentrations of trace elements viz. [chromium (Cr), copper (Cu), zinc (Zn), Iron (Fe) and lead (Pb)]. Multi-element standard (ICP multi-element standard XVI; Merck, Darmstadt, Germany) was used for the analysis. The instrument was calibrated using a serially diluted multi-element standard from 0.1–25 µg/L. Yttrium in a 10 µg/L concentration was used as an internal standard in all standards and samples. Two blank samples were run to check for background contaminant that may arise from reagents and apparatus during each analysis. The blank value was subtracted from the analytic value. Twenty blank samples containing 10% of nitric acid were run in ICP-MS.

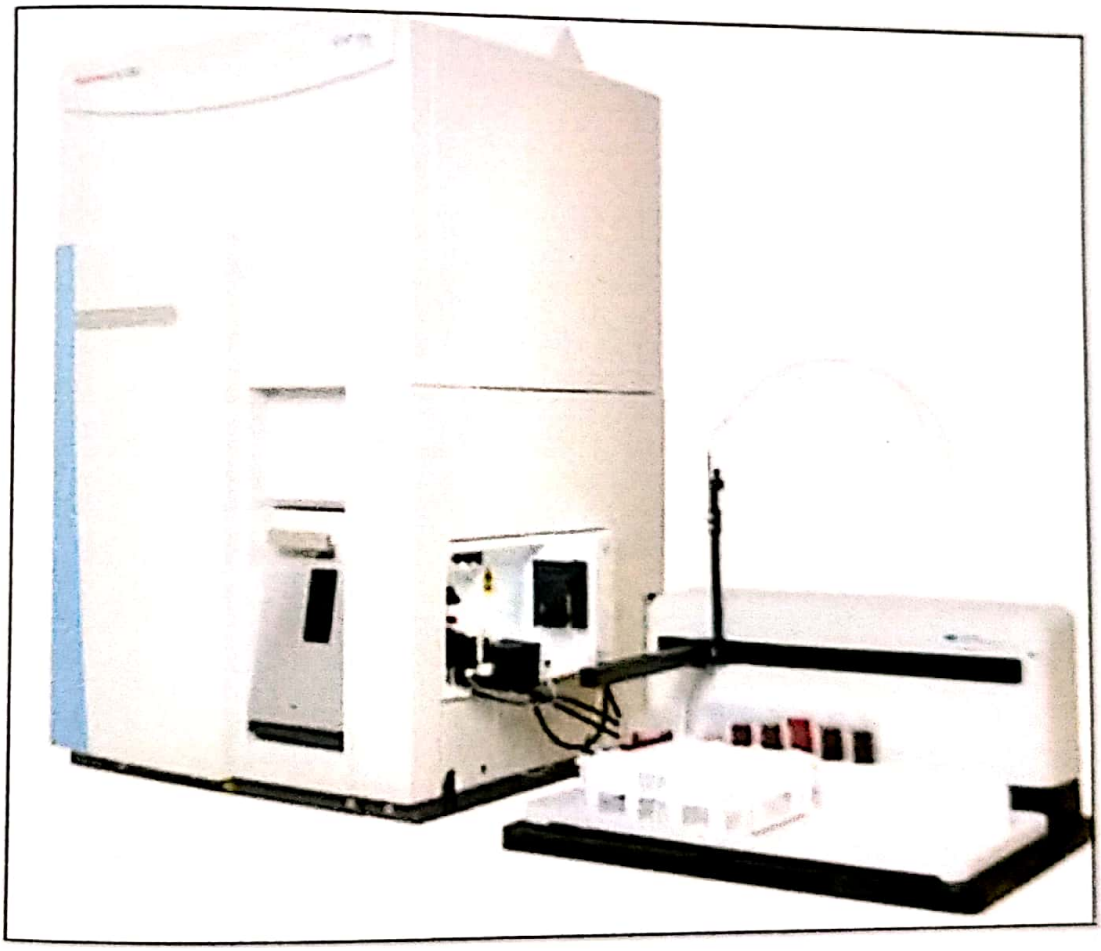


Figure 6: Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

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RESULT AND DISCUSSION

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RESULT AND DISCUSSION

Heavy metal concentration in seaweeds by ICP- MS analysis

Concentrations of the metals (Fe, Zn, Cu, Cr and Pb) were analysed in the collected seaweed samples like *Sargassum wightii* from Manapad and *Ulva lactuca* from Tharuvaikulam, Tuticorin Coast, Gulf of Mannar, and Southeast Coast of India. The distribution of metal concentrations in seaweed samples of *Sargassum wightii* and *Ulva lactuca* is given in table 1.

Table: 1 Analysis of heavy metals

Seaweed samples	Location	Heavy metals	Concentration (ppm)
Sargassum wightii	Manapad	Iron (Fe)	12.071
		Zinc (Zn)	5.113
		Copper (Cu)	2.107
		Chromium (Cr)	1.051
		Lead (Pb)	0.816
Ulva lactuca	Tharuvaikulam	Iron (Fe)	15.06
		Zinc (Zn)	6.115
		Copper (Cu)	4.617
		Lead (Pb)	2.011
		Chromium (Cr)	1.714

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Heavy metal content was determined in seaweeds such as *Sargassum wightii, Ulva lactuca.* ICP-MS analysis of dried and powdered seaweeds indicates that marine seaweeds contain heavy metal such as Iron (Fe), Zinc (Zn), Copper (Cu), Chromium (Cr), and Lead (Pb), is present in *Sargassum wightii,*and *Ulva lactuca.*

In the present study, the higher concentration of iron (Fe) in *Ulva lactuca* is 15.06 ppm when compared to *Sargassum wightii* 12.071ppm. The concentration of zinc (Zn) content is more in *Ulva lactuca* is 6.115 ppm and in *Sargassum wightii* the concentration of zinc (Zn) is 5.113ppm. The concentration of copper (Cu) content in *Ulva lactuca* is 4.617ppm and in *Sargassum wightii* the concentration of copper (Cu) is 2.107 ppm. The concentration of chromium (Cr) in *Ulva lactuca* is 1.714 ppm and in *Sargassum wightii* the concentration of chromium (Cr) is 1.051ppm. The concentration of Lead (Pb) in *Ulva lactuca* 2.011 ppm and in *Sargassum wightii* the concentration of lead (Pb) is 0.816 ppm.

In *Sargassum wightii,* the element of iron (Fe) showed the highest concentration, while the concentration of lead (Pb) is relatively low. From highest to lowest, the concentration of all the heavy metals in the *Sargassum wightii* was Fe (12.071) > Zn (5.113) > Cu (2.107) > Cr (1.051) > Pb (0.816). In *Ulva lactuca,* the element iron (Fe) showed the highest concentration, while the concentration of chromium (Cr) is relatively low. From highest to lowest, the concentration of all the heavy metals in the *Ulva lactuca* was Fe (15.06) > Zn (6.115) > Cu (4.617) >Pb (2.011) > Cr (1.714).

The minimum concentration of the Chromium (Cr) which ranged from 2.20 to 11.00 $\mu g\ g^{-1}$ DW was in the *padina boryna* species and maximum concentration were in the *Sargassum boveamum* and *Cystoseria myrica* species.(Ahmed I. Rushed).When compared to two other algal plants, distinct Chlorophyta species were discovered to have relatively high amounts of heavy metals. According to this, the Chlorophyta group has a propensity to absorb more metals than the other two categories. *Caulerpa racemosa* is the organism that takes in the most heavy metals, followed by *Halimeda tuna, Caldophora koiei, Entomorpha compressa,* and *Rhizocloniumko-chiamum.*They all do, however, exhibit significant amounts of Iron (Fe) and Copper (Cu), and they are picky when it comes to other heavy metals. The concentrations of heavy metals are lower in marine brown seaweeds (Phaeophyta) than in

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green plants (Chlorophyta). Several species of Phaeophyta division selectively collect heavy metals, excluding Iron (Fe) and Copper (Cu).The minimum concentration of the Copper (Cu) was observed in the *padina boryana* species and maximum concentration was in the *Sargassum boveanum.*

The concentration of Iron (Fe) ranged from 13.81 to 60.2 µg g^{-1} DW with a minimum concentration in *Turbinaria elatensis* species and a maximum concentration in the *Sargassum boveamum* species. The high lead (Pb) content of seaweed can be influenced by environmental factors such as sediment, water, and the high absorption capacity of seaweed (bioaccumulation).Seaweed (*Gracilaria sp.*) is bioaccumulation formed by the transfer of tropical contaminants among species in lipophilic conditions (chemicals that accumulate in fat) and move to another level(Neff, 2004; Sudarmaji, J Mukono, 2006; He and Chen, 2014) (Christopher S. and Paul J. Harrison, 1994; He and Chen, 2014).The highest Iron(Fe) level was found in green algae *Enteromorpha compressa* collected from the Romel station during the summer, followed by that collected from the El-Boussit station during the same season (3865.96, 1284.11 g/g dry weight). *Laurencia obtusa*, a red algae, had a relatively high Fe concentration in summer (467.58 g/g dry weight) at the Alam El-Roum station, while *Cystoseira crinita* (brown algae) and *Gelidium crinale* (red algae) had their highest values in autumn (467.58 and 410.94 g/g dry weight) at respectively. *Cystoseira crinita*, on the other hand, had the lowest Fe values in spring at both the Romila and Mina-Hashish stations.Iron was the most abundant trace element in the samples examined. The lowest and highest iron levels in marine algae species were 99 g/g in *Corallina elongate* from Sinop and 3,949 g/g in *Antithamnioncruciatum* from Sinop, respectively.

Iron contents in marine algae species have been reported in the literature to range from 32.9-103 g/g (Ruperez 2002), 20-1,040 g/g in commercially available edible marine algae (van Netten *et al.* 2000), and 357-8,821 g/g (Topcuoglu *et al.* 2003).Iron in the diet is critical for lowering the incidence of anemia. Iron deficiency occurs when there is a high demand for iron.

Metal variation in species from different sampling sites may be related to factors such as tidal range, temperature, salinity, dissolved nutrients, type of tissue, age of algae, nutritional history, and geological structure of the study area (Abdallah, 2008).

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Lead contents in marine algae species have been reported in the literature ranging from 1.67-4.82 g/g (Conti and Cecchetti 2003), 0.02-24.7 g/g (Sawidis *et al.* 2001), 0.1-9.1 g/g (Topcuoglu *et al.* 2003), and 0.01- 0.64 g/g (van Netten *et al.* 2000).Lead (Pb), a heavy metal, is harmful even at low amounts and has unknown functions in living things.High levels of lead (Pb) in the study area's algae could be attributed to the use of fossil fuels and oil pollution (Dadolahi *et al.*, 2011). Furthermore, Lead (Pb) contamination from sediment particles appears to be theoretically possible (Giusti, 2001)

The brown algae of *Sargassum swartzi* contained Zinc (Zn) exclusively. By activating dehydrogenases and protein-synthesis enzymes in this species, the concentration of zinc may be caused by regulated (Besada 2009).

Copper (Cu) concentrations in *Ulva spp.* differed significantly across seasons and sampling sites ($p \leq 0.05$).

The lowest chromium content in marine algae species was 0.50 g/g in *Porphyra umbilicalis* collected from Rize and *Ulva lactuca* collected from Trabzon, while *Antithamnion cruciatum* collected from Sinop had the highest chromium content of 11.6 g/g. In the literature, chromium contents in marine algae species have been reported in the 0.06 - 2.3 µg/g. Chromium is a mineral that is required by humans and has been linked to carbohydrate, lipid, and protein metabolism (RDA 1989).

According to this study, seaweeds have higher amounts of a few heavy metals than other metals, such as lead and chromium. The amount of heavy metals present in seaweed production is below the safety level set by the national food agency and the environment ministry, according to safety guidelines. Thus, the seaweed that was discovered close to harbours and locations for the disposal of industrial waste is dangerous and unfit for ingestion based on this investigation. Because they are present in the environment in combinations rather than independently, heavy metals end up in humans through the food chain. Given their position at the base of the food chain, macroalgae are likely the primary source of metals for many creatures, including fish and invertebrates that consume them. Consequently, research on heavy metal concentrations in macroalgal species may assist comprehend the environmental load of potentially harmful metal emissions from anthropogenic activities as well as the transfer of elements from abiotic compartments (water and sediments) to humans.

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SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

The present study estimated the concentrations of metals such as iron, zinc, copper, chromium, and lead from the marine seaweeds (*Sargassum wightii* and *Ulva lactuca)* collected from Tharuvaikulam and Manapad areas, the Tuticorin Coast, the Gulf of Mannar, and the Southeast Coast of India. The results showed that total metal contents in *Sargassum wightii* and *Ulva lactuca* along the studied area recorded their highest levels for Tharuvaikulam (*Sargassum wightii)*, while the lowest value was recorded for the Manapad (*Ulva lactuca)*. This study showed seaweeds are contaminated with high concentrations of a few heavy metals such as chromium and lead when compared to other metals. Based on the safety standards, the concentration of heavy metals in seaweed production passes the safety threshold rules issued by the national food agency and the environment ministry. Thus, based on this research, the seaweed that was found in areas near places of industrial waste disposal and harbours is unsafe and not suitable for consumption. Heavy metals do not exist separately but as mixtures in the environment, and they reach humans through the food chain. Since macroalgae are located at the base of the food web, they are probably the main source of metals for many animals like invertebrates and fish that feed on them. Therefore, the investigation of heavy metal concentrations in the macroalgal species may provide useful information on the transfer of potentially toxic elements from abiotic compartments (water and sediments) to humans, as well as help in understanding the environmental load of potentially toxic metal releases from anthropogenic activities. Further studies can be conducted extensively to identify other minerals and potentially toxic heavy metals in seaweeds for healthy safety management.

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This to certify that Ms/Mrs/Mr/Dr. Vincy. N , II M.Sc Microbiology , St. Mary's College participated / presented his / her research paper entitled Heavy metal analysis of Sargassum sp., in the National Seminar on 'EMERGING TRENDS AND OPPORTUNITIES IN MICROBIOLOGICAL RESEARCHES' organised by PG & RESEARCH DEPARTMENT OF BOTANY, V. O. CHIDAMBARAM COLLEGE, THOOTHUKUDI on 10.03.2023.

Dr. A. Doss
Organising Secretary

Dr. C. Lakshmanan
Head of the Department

Dr. C. Veerabahu
Principal

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