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ISOLATION OF LACTOBACILLUS SP., FROM CAMELLIA SINENSIS AND THE BIOLOGICAL ACTIVITY OF TEA EXTRACT CONTAINING LACTOBACILLUS SP

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ABSTRACT

Tea (Camellia. sinensis) is considered as one of the essential functional foods and is highly valued for its taste, aroma and also an integral part of different culture and traditions around the globe. Through the years tea consumption is deeply associated with numerous health benefits in humans. The health promoting factor of tea is mainly contributed by the presence of bioactive compounds such as polyphenols and its antioxidant activity. In recent decades the use of probiotics for disease prevention and health benefits has considerably increased and has become a worldwide interest and indicating a new possibility for isolating probiotic strains. Lactobacillus is one of the predominant probiotic microorganisms and their health benefits to humans are proved in several studies. They used to treat several metabolic disorders and aid in maintenance of gut microbiota. The presence of lactic acid bacteria, a dominant probiotic organism in tea leaves that can exhibit probiotic properties making tea a potential safer source for isolating probiotics. The present study attempts to study the characteristics of isolated bacterial strains from leaves of C.sinensis. All isolates were studied for phenotypic characteristics and biochemical tests were done for identification. Studied for qualitative analysis of prepared crude and aqueous plant extract and observed for presence of alkaloids, flavanoids, terpenoids, steroids, glycosides, saponins and tannin. The analysis of major phytochemical constituents of C.sinensis was further confirmed by thin layer chromatography and studied antimicrobial activity against indicator pathogens. The isolates obtained were gram-positive and rod-shaped bacilli and were identified as Lactobacillus. The strainsLD1, GP1, K1 and GLK1 showed high activity and viability. The plant extracts highly showed the indication for the presence of bioactive compounds. The biochemical compounds present in the extract were further separated in TLC and the extracts showed zone of inhibition against indicator organisms for antimicrobial activity.

Keywords: Tea, Lactobacillus, Bioactive Compounds, Probiotics.

I. INTRODUCTION

Tea is proved as that second most commonly consumed drink widely around the globe besides water (Trevisanato et al. 2000). It is also one of the oldest non alcoholic beverages with caffeine content in the world. It is a most socially consumed and habitual drink by millions of people across all continents since 3000BC (Sharangi A. B et al. 2009).

Tea is considered as one of essential functional foods. A functional food consists of substances obtained from plant and animal sources containing ingredients with basic nutrition requirements along with physiological benefits. Today, tea is considered as a pleasant and enjoyable drink worldwide and its consumption varies according to the local preferences and different traditions. Tea is also served as traditional welcome, refreshment and as a digestive and it is praised for its complexity and simplicity. Tea is also considered a safe drink to innumerable people and is not capable of causing intoxication. It is an economical drink which can be afforded by people belonging to all social classes present in all countries (Weisburger et al. 1997).

Tea is a perennial shrub classified in the Theaceae family bearing single or paired white colored flowers at the axils and green colored fruits having 2-3 seeds after 5-6 years of planting. Leaf is considered as the main basis for classification of tea consisting of three chief varieties of three distinct ecotypes: C. sinensis var. assamica (the Assam-type), C. sinensis var. sinensis (the China-type) and C. assamica sub spp lasiocalyx (Cambod or Southern type). The three ecotypes show considerable variation in their ecophysiology (Carloni et al. 2013).It is a leafy evergreen crop that requires warm and humid climates with an adequate rainfall between 1150 and

1400nm throughout the year. Long hours of sunlight provide maximum yield and increasing nutrient status of tea. Therefore, a humid aerial environment is often necessary for a successful tea cultivation and good yield (Celik et al. 2006)

The primary native origin of cultivated C. sinensis plant is south – East Asia and later spread to Europe, Russia in late 17th century then progressively extended to a worldwide cultivation. About 2.7 million hectares is occupied by commercial tea cultivation worldwide and plays a pivotal role in the world economy. The three largest tea producers globally are China (560,000metric tons), India (704,000 metric tons) along with Kenya (188,000 metric tons) (Weisburger et al. 1997).

Oxidation is a process also mentioned as fermentation which naturally occurs when polyphenols present in tea are complexed when contacted with oxygen. Based on the level of fermentation of freshly plucked tea leaves they are namely of three types: fermented known as black-tea, non fermented known as green and white tea and semi fermented known as oolong- tea (Dias et al. 2013) Black tea is one of consistently consumed tea type. About 76-78% of black- tea, 20-22% of green- tea and less than 2% oolong- tea is being produced and consumed worldwide with approximately three billion kilograms of global annual tea consumption (McKay & Blumberg, 2002)

Besides being safe, enjoyable and economic aspects, Tea contains a natural source of medicinal compounds which gives protects against a vast range of diseases. Tea leaves are comprised of 700 chemical constituents and more, among which polysaccharides, flavanoids, vitamins (C, E, K), amino acids and caffeine have known to play an important role to human health. Nowadays Tea consumption practice is linked with the cell- mediated immune responses of the human body. Tea is also known to protect the cell membranes of humans from oxidative damages, normalizes blood pressure, prevents dental-caries, prevents coronary heart diseases and normalizes diabetes by able to reduce blood-glucose activity. In addition to tea contains germicidal and germistatic activities against numerous gram-positive and gram-negative bacteria such as V. cholera, Salmonella, Clostridium etc identified as human pathogenic bacteria. Tea drinking also acts a main source of some essential minerals such as manganese, which activates innumerable essential enzymes. Green-tea and black-tea infusions consist of antioxidants such as catechins possess anti- mutagenic, anti- carcinogenic and anti- tumorous properties. Epidemiological studies have also proved that tea drinking plays a integral role against human cancer (Chen et al. 1999).

Probiotics is defined as “the substance secreted by one organism which helps in stimulating the growth of another”. It is a live active culture when administrated in adequate amounts tends to improve the balance and composition of gut microbiota for specific effects. (FAO/WHO 2001). The GI tract is the second largest surface area of the body with more than 400m 2 of surface area and nurtures a rich flora of 500 different types of bacterial species with major health functions including immune system stimulation, protects the host body from the invading pathogens as well as aiding in digestion. Under natural circumstances, the gut microflora develops and the bacterial supplement is inessential. But in recent years due to the food and lifestyle changes which affects the access to colonization of certain type of bacteria. The usage of antibiotics, immunosuppressive therapy, irradiation and other treatments can also tend to cause alterations in the composition and can have adverse effects on the gut flora. Hence, the beneficial bacterial species introduction in to the GI tract can aid in reestablishing the gut microbial equilibrium and in preventing array of diseases (Lahteinen et al. 2010).

Several bacterial genera which are usually used in probiotic preparations are Lactobacillus, Bifidobacterium, Bacillus, Enterococcus, Streptococcus, Escherichia and few fungal strains of Saccharomyces have also been considered using for probiotics (Jin et al. 2000). But the bacterial strains with potential source of probiotics and high beneficial properties commonly belongs to the genera Lactobacillus and Bifidobacterium, since they exhibit strong anti-inflammatory properties.

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Table 1: Bacterial species used as culture for probiotic preparation (Gupta et al., 2009)

Bacterial species	Lactobacillus Sps.	Bifidobacterium Sps.	Streptococcus Sps.	Saccharomyces Sps.	Others
1.	L. acidophilus	B.bifidum	S.thermophilus	S.boulardii	Bacillus cereus
2.	L.casei (rhamnosus)	B.breve	S.salivarius subsp. thermophilus		E.coli
3.	L.fermentum	B.lactis			Enterococcus
4.	L.gasseri	B.longum			Propioni bacterium freudenreichii
5.	L.johnsonii	B.infantis			
6.	L.lactis	B.adolescentis			
7.	L.paracasei				
8.	L.plantarum				
9.	L.salivarius				
10.	L.bulgaricus				

The genus Lactobacilli is a member of the mucosal microbiota of humans and animals. It is usually found in the human adult gastrointestinal (GI) tract. This group of bacteria is required for preventing intestinal infections, for stability maintenance of gastrointestinal tract and aiding intestinal health (GU et al. 2008). They are facultative or strict fastidious anaerobes and require an acidic environment for production of lactic and other kinds of acid. LAB is generally regarded as a GRAS organism since they are non pathogenic, have not been associated with diseases and their isolated were resistant to bile and NaCl concentration and were tolerant to acidic conditions of the environment. They tend to produce bioactive agents like bacteriocins that have the ability to control pathogens growth and inhibit several gram-positive and gram negative pathogenic bacteria. Lactobacilli are also capable for antimicrobial compounds production such as bacteriocins, hydrogen peroxide and organic acids. These ideal characteristics of Lactobacilli serve as suitable criteria for a probiotic starter culture (Gupta et al. 2009).

Most of the LAB strains and taxonomically related bacteria seemed to be frequently reported to be isolated from the fermented tea leaves. Furthermore, studies have also recorded the Lactobacillus isolation from freshly collected leaves of tea. (Gharaei Fathabad et al. 2011). This study highlights some of the important existing probiotic microorganisms in tea leaves (C.sinensis) which can be considered as a safer source for isolating probiotics and an attempt made is to characterize Lactobacilli bacterial strains isolated from freshly collected tea leaves for probiotic preparation.

This study was done in order for the isolation and characterization the LAB isolates from freshly collected tea leaves for effective probiotics and to investigate tea to be potential safer source for isolating probiotic organism. Furthermore, to analyze the individual biochemical compounds of tea by phytochemical screening and the antimicrobial activity of tea extracts were examined with pathogenic organisms and its growth controlling mechanism of pathogenic organisms.

II. MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLES:

The Lactic acid bacteria strains isolated from fresh tea leaves were all collected and brought from different locations in Nilgiris including Lovedale, Gandhipettai and Ketti. These fresh tea leaves were all collected in clean, dirt free and sterile polythene bags without containing any disinfectant or detergent residue. After

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collection immediately the samples were taken to the laboratory and the bacterial isolation was performed within a day.

2.2 MEDIA:

The LAB bacteria were isolated from tea leaves collected from different locations and inoculated in freshly prepared in 70ml Nutrient broth (Hi media) conical flasks and incubated at 37˚C for 24 hours.MRS agar is a selective media for Lactobacillus spp (Table-2). The isolated distinct colonies were further subjected to sub culturing on freshly prepared MRS agar plates in order to obtain the pure culture.

Table 2: Composition of MRS media

Media used	Composition	Net Weight (in grams) per1000ml
De Man Rogosa and Sharpe (MRS media)	Protease peptone	10
	HM Peptone B	10
	Yeast extract	5
	Dextrose (Glucose)	20
	Polysorbate (Tween 80)	1
	Ammonium citrate	2
	Sodium acetate	5
	Magnesium sulphate	0.1
	Maganese sulphate	0.05
	Dipottasium hydrogen phosphate	2
Final pH (at 25˚C)6.5±0.2		

2.3 MAINTENANCE AND PREPARATION OF BACTERIAL ISOLATES:

Isolates (LD1, LD1, LD2, LD3, GP1, GLK1, GLK2 & K1) from isolated culture plate were sub cultured on MRS agar plate by quadrant streak technique and incubated at 37˚C for 24 hrs. After the incubation, the white colonies formed were chosen for single colony isolation in order to isolate the Lactobacillus species strains. In addition, the purity of the cultures was also checked by streaking on MRS agar (Hi media) and incubated at 37˚C for 24 hrs. Further single colonies cultures were subcultured on MRS agar slants for further studies.

2.4 PHENOTYPIC IDENTIFICATION:

The isolated bacterial colonies of formed on the MRS agar plates were identified by for phenotypic characterization such as colony and cell morphology, arrangement, gram staining and biochemical tests according to the manual of Bergey's determinative of bacteriology.

2.4.1 COLONY CHARACTERISTICS:

Colony shape (both elevation and margin), size, colour as well as consistency of the colony were observed. Whether the colonies were smooth (shiny glistening surface) or rough (dull, bumpy, granular or matte surface) or mucoid (slimy or gummy appearance) and opacity of the colonies such as (transparent, translucent, or opaque) was also noted.

2.4.2 GRAM STAINING:

The isolated colonies were subjected to Gram staining in order to categorize the colonies Gram-reaction and subsequently morphology was observed by microscopy. This test is done to differentiate the isolates whether they are gram positive or gram negative and whether they are rods or cocci or coccibacilli. The procedure of gram staining is mentioned in brief as follows:

- Prepared a thin isolated culture sample smear on grease free and clean slide by heat fixing.
- The heat fixed smear was flooded with Crystal violet stain and kept for 1 minute and later rinsed the stain off with distilled water.

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- Followed by the Gram's iodine drop was flooded upon the smear and kept for 1 minute and washed with the water.
- Decolorizing agent 95% Ethyl alcohol was flooded upon the smear up to 10-20 seconds and again immediately washed with the water.
- Counter stained with safranin for up to 1 minute later wash with water.
- Air dry the smear and observe it under microscope.
- Firstly, viewed it under 10× lens for checking stain and then viewed under 100× (oil immersion)
- Note down the result observed.

2.4.3 BIOCHEMICAL TESTS:

The isolated colonies present on the primary and subcultured plates with a resemblance of Lactobacillus spp. were subjected for confirmatory identification. The isolates were chosen for biochemical tests for identification and it was performed as Indole test, Methyl red test, Vogues-Proskauer test, Simmon's citrate test and catalase test.

2.4.3.1 INDOLE TEST:

Overnight grown active cultures (LD1, GP1, K1 & MIX1) were inoculated at 1% in 5ml peptone broth tubes and were incubated at 37˚C for 24 hours and 2-3 drops of Kovac's indicator reagent was added for ring formation and was examined immediately. No cherry-red ring formation in the reagent layer on the crown of the medium was observed as positive result for Lactobacillus spp.

2.4.3.2 METHYL RED TEST:

The overnight grown active cultures (LD1, GP1, K1 & MIX1) were inoculated at 1% in 5ml MRVP broth tubes and were incubated at 37˚C for 24 - 48 hrs and 5 -6 Methyl-Red indicator reagent drops were added for colour change and was examined immediately. Formation of yellow colour was observed as a positive result for Lactobacillus spp.

2.4.3.3 VOGES-PROSKAUER TEST:

Overnight grown active cultures (LD1, GP1, K1 & MIX1) were inoculated at 1% in a 5 ml MRVP broth tubes and were incubated at 37˚C for 24 – 48 hrs. Then Barrit's reagent A (α – napthol) and Barrit's reagent B (40% KOH) was added in the ratio 3:1 and was observed for colour change. Formation of whitish yellow colour was observed as a positive result for Lactobacillus spp.

2.4.3.4 SIMMON'S CITRATE TEST:

Overnight grown active cultures (LD1, GP1, K1 & MIX1) were streaked on Simmon's citrate agar slants back and forth with an inoculum and were incubated at 37˚C for 24 – 48 hrs and colour change were noted. Formation of green to intense blue along the slant was observed as a positive result for Lactobacillus spp.

2.4.3.5 CATALASE TEST:

A loop of overnight grown culture was transferred on to a cleaned dry glass slide and 3% H2O2 drop was placed on the clean glass slide and was observed for appearance of oxygen bubbles. No formation of bubbling was observed as a positive result for Lactobacillus spp.

2.4. PHYTOCHEMICAL SCREENING:

2.4.1 COLLECTION OF SAMPLES:

The freshly collected leaves of Camellia sinensis for phytochemical screening were brought from 11 different locations in Nilgiris including Lovedale, Kerada, Halada, Achanakkal, Ketti, Kerkandi, Kekkatty, Mukratti, Odayaratti, Ketti and Ketti pallada. These fresh tea leaves were all collected in a cleaned and sterile polythene bag without any kind of disinfectant or detergent residue.

2.4.2 PREPARATION OF CRUDE EXTRACT:

The fresh plucked plant leaves were all washed under running water and were dried in shade for 15 days at room temperature. After drying the samples they were weighed and were blended with a blender and were powdered and stored in air tight plastic container. The powdered plant sample were soaked with ethanol [in ratio ethanol: plant (6:1)] in 500 ml conical flasks for 2 days with constant agitation at regular intervals and were filtered using Whatman No.1 paper. The solution was then distilled in the beaker and poured in

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petriplates to completely remove ethanol by evaporation until the filtrate were reduced to one – third of starting filtrate volume and till it provided a viscous mass. The crude extract was weighed and was all stored at 4˚C for future phytochemical analysis of the extract.

2.4.3 PREPARATION OF AQUEOUS EXTRACT:

The fresh plucked plant leaves were washed under running water and were dried in shade for 15 days at room temperature. Then after drying the samples, they were all weighed and blended with a help of a blender and were powdered. By using soxhlet extraction method, the 50g powdered plant sample were extracted with 250ml of water at 55-85˚C for 8 – 10 hrs. The sample was dried in freeze dryer and stored at 4˚C for future phytochemical analysis of extract.

2.4.4 PRELIMINARY PHYTOCHEMICAL SCREENING:

The tests done were in order to indicate the bioactive chemical constituent's presence in leaves of Camellia sinensis which are alkaloids, flavanoids, terpenoids, steroids, glycosides, saponins and tannins.

2.4.4.1 TEST FOR ALKALOIDS:

The ethanolic and aqueous extracts were evaporated and the residues were heated on top of a boiling water bath along with 2% Hydrochloric acid and after cooling the mixture, it was filtered. The crude and aqueous plant extract were treated with a few drops of 5% Sodium Hydroxide solution. Further the samples were observed for the presence of brown colour.

2.4.4.2 TEST FOR GLYCOSIDES:

2ml of glacial acetic acid was added to 0.5g and ml of crude and aqueous extract and was dissolved and mixed well. Add in a few drops of ferric chloride and concentrated sulphuric acid and reddish-brown coloration at the junction of two layers with bluish green color in the upper layer were observed.

2.4.4.3 TEST FOR TERPENOIDS:

4mg and ml of crude and aqueous plant extracts were treated along with 0.5ml of acetic anhydride and 0.5ml of chloroform. Further conc. sulphuric acid solution was slowly added and red violet color was observed for the presence of terpenoids.

2.4.4.4 TEST FOR STEROIDS:

4mg and ml of crude and aqueous plant extract were treated along with 0.5ml of acetic anhydride and 0.5ml of chloroform. Further conc. sulphuric acid solution was slowly added and green bluish color was observed for the presence of steroids.

2.4.4.5 TEST FOR FLAVANOIDS:

2g and ml of crude and aqueous extract 1ml of lead acetate solution were treated and white color was observed for the presence of flavanoids.

2.4.4.6 TEST FOR GALLIC TANNINS:

0.5g and ml of crude and aqueous extract were dissolved in 1ml of water and mixed uniformly. Further 2 drops of ferric chloride solution were added and observed for blue color for the presence of gallic tannins.

2.4.4.7 TEST FOR CATECHOLIC TANNINS:

0.5g and ml of crude and aqueous extract were dissolved in 1ml of water and was uniformly mixed. Further 2 drops of ferric chloride solution were added and green black color were observed for the presence of catecholic tannins.

2.4.4.8 TEST FOR SAPONINS:

0.5g and ml of crude and aqueous extract were treated and diluted with distilled water and was shaken for 15 minutes. The presences of saponins were indicated by the formation of 1 cm layer of foam.

2.5 THIN LAYER CHROMATOGRAPHY:

The analysis of major phytochemical constituents of crude and aqueous extract of Camellia sinensis were carried out using thin layer chromatography (TLC) using standard protocol.

Procedure:

The silica gel slurry was prepared with distilled water in the ratio 1:2.

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The slurry was then transferred to a clean dry beaker and a glass rod was used to make the slurry homogenous

Using a pipette, a small amount of slurry was added on a cleaned and dry glass slide.

The slurry then was coated on the glass slide of thickness 0.2mm and the TLC plates was moved around to form a uniform thin layer of adsorbent of 0.2 to 0.3mm thin and excess slurry was from the plate

The TLC plates were dried at room temperature

The dried plates were kept in hot air oven at 110˚C for 30 minutes to activate the silica gel.

The samples were dissolved in small vials by using volatile soluble solvent methanol

After activation of TLC plate, a small amount of medium concentration of samples were drawn with a capillary tube and spotted from 1cm above the bottom and 0.5cm from the left side of the

TLC plates

The samples were spotted on the TLC plates

The TLC plates were placed inside the chromatography chamber containing mobile phase. The mobile phase is prepared by mixing Hexane, Acetone and Chloroform in the ratio 3:1:1.

Once the solvent reached the top the TLC plates, it was later removed from the chamber and was dried in air for 1 minute.

The TLC plate is then visualized and observed for bands in UV visualizer.

2.6 AGAR WELL DIFFUSION METHOD:

It is been used for determining the antimicrobial activity and minimum inhibitory concentrations (MIC) of crude and aqueous extracts of plant Camellia sinensis against Gram (+) ve bacteria (S.aureus) and Gram (-)ve bacteria (E.coli, P. aeruginosa, S. buydoii and S. typhi). The bacterial suspensions were spread over the nutrient agar plates with 3 wells of 6mm diameter. All the wells were filled 30 of various concentrations (10µg, 50µg and 100µg) of crude and aqueous extracts. The antibiotics ampicillin and gentamicin were used as standards. The agar plates were incubated at 37˚C for 24hrs and the results were observed by diameter of the zone of inhibition.

III. RESULTS AND DISCUSSSION

3.1 COLLECTION OF SAMPLES:

The freshly plucked leaves of C. sinensis all collected from different regions of Nilgiris district, India were inoculated in freshly prepared nutrient broth present in conical flasks and incubated at 37˚C for 24 hours and observed for growth.

Fig 3.1: Inoculation of fresh leaves of C.sinensis in to nutrient broth

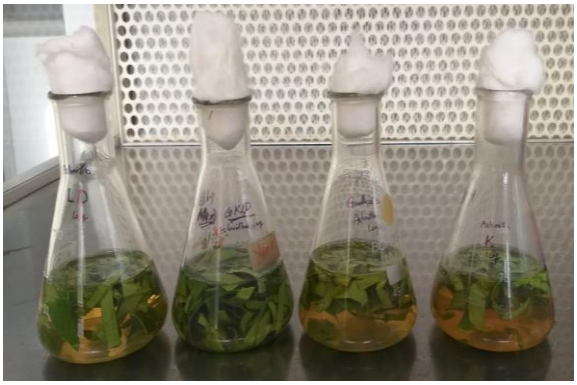

Fig 3.2: Growth of organism in nutrient broth

3.2 MAINTENANCE AND PREPARATION OF BACTERIAL ISOLATES:

The bacterial isolates (LD1, LD2, LD3, LD4, GP1, GLK1, GLK2 & K1) obtained from isolated culture plates were all sub cultured on MRS agar plates for 37˚C for 24 hrs to check the purity of the colonies and were stored for future use.

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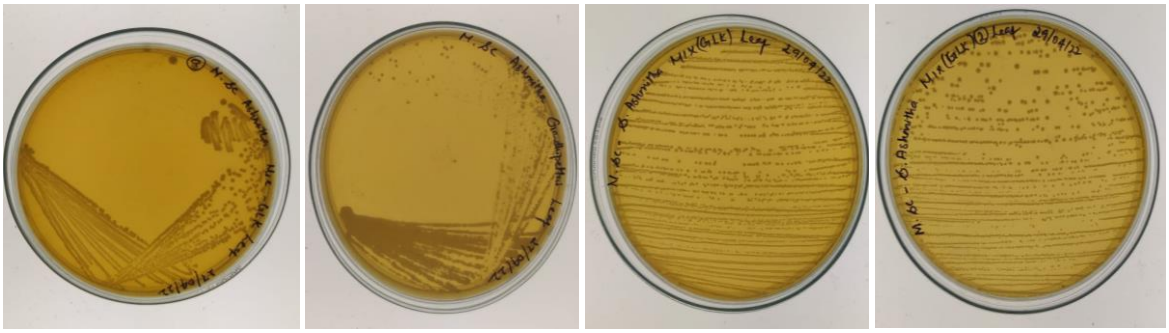

Fig 3.2.1: Colonies streaked on MRS agar for subculturing (Representative photo)

3.3: PHENOTYPIC CHARACTERIZATION:

The 8 bacterial isolates designated as LD1, LD2, LD3, LD4, GP1, GLK1, GLK2 & K1 isolated from C.sinensis were studied for colony morphology, arrangement and characteristics. (Table 4.1) provides the colony characteristics.

Table 3.1: Colony characteristics of the culture

Colony	Shape	Elevation	Optical features	Margin	Pigmentation	Texture	Size
LD1	circular	convex	opaque	entire	Creamy white	smooth	Medium
LD2	circular	convex	opaque	entire	Creamy white	smooth	Medium
LD3	circular	convex	opaque	entire	Creamy white	smooth	Medium
LD4	circular	convex	opaque	entire	Creamy white	smooth	Medium
GP1	circular	Raised	opaque	entire	Creamy white	smooth	Small
GLK1	circular	convex	opaque	entire	Cream	smooth	Medium
GLK2	circular	convex	opaque	entire	Cream	smooth	Medium
K1	circular	Raised	opaque	entire	Creamy white	smooth	Small

3.3.1 GRAM STAINING:

The microscopic characterisation of the cultures was observed to be Gram positive and rod shaped. A study by Prabhurajeshwar et al., (2017) observed similar results that Lactobacillus is a gram positive and rod-shaped bacilli were observed.

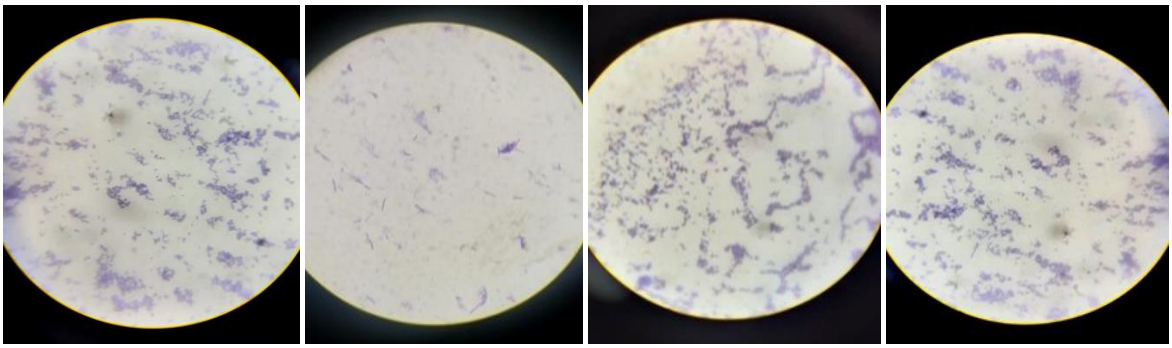

Fig 3.3.1: Microscopic view of the cultures after gram staining

3.3.2 BIOCHEMICAL TESTS:

The isolated colonies present on the primary and subcultured plates with the resemblance Lactobacillus spp isolates subjected to biochemical tests for identification. The identification performed on Indole, Methyl red, Voges-proskauer, Simmon's citrate and Catalase test. The isolates obtained were Indole, Methyl red, Voges - Proskauer, Catalase negative and Simmon's-Citrate positive. The results were similar with the results of

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Prabhurajeshwar et al., (2017) biochemical characteristics of L.fermentum. The results of the biochemical properties of cultures of isolated bacterial strains are listed in (Table 4.2)

Table 3.2: Results obtained after biochemical tests of Lactobacillus spp

Bacterial isolates	Indole test	MR test	VP test	Simmon's citrate test	Catalase test
LD1	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve
GP1	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve
K1	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve
GLK1	(-)ve	(-)ve	(-)ve	(+)ve	(-)ve

Fig 3.5: The results of Indole test of bacterial isolates (LD1, GP1, K1 & GLK1)

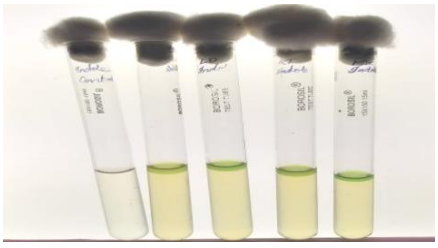

Fig 3.6: The results of Methyl red test of bacterial isolates (LD1, GP1, K1 & GLK1)

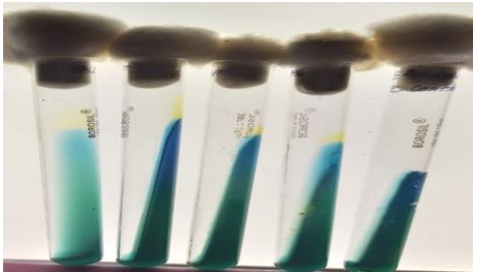

Fig 3.7: The results of Voges-Proskauer test Of bacterial isolates (LD1, GP1, K1 & GLK1)

Fig 3.8: The results of Simmon's citrate test Of bacterial isolates (LD1, GP1, K1 & GLK1)

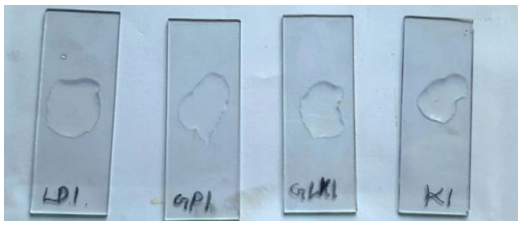

Fig 3.9: The results of catalase test of bacterial isolates (LD1, GP1, K1 & GLK1)

3.4. PHYTOCHEMICAL SCREENING:

3.4.1 CRUDE ETANOLIC EXTRACTION:

A study by Shah et al., (2018) reported that the plant Camellia sinensis has high antioxidant properties. The results obtained concluded that ethanol extract of Camellia sinensis possesses significant antioxidant activity and the concentration of phytochemicals was higher in leaves as compared to root and stem. The fresh leaves of C.sinensis were washed and shade dried at room temperature. The samples were weighed, powdered and soaked in ethanol for 2 days further filtered. After the evaporation at 50˚C for 48 hours, the crude extract amount obtained was calculated in (Table 4.3)

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Fig 3.4.1: The powdered sample of C.sinensis soaked in ethanol solvent for crude extraction

Table 3.3: Calculation and summary of total yield obtained

Sample	Amount of sample taken for extraction (g)	Solvent used for solubility	Weight of the tube (g) [w1]	Weight of the tube with sample(g) [w2]	Amount of extract obtained (g) [W= w2 - w1]
Tea (C.sinensis)	50g	Ethanol	13g	24.6g	11.5g

3.4.2 AQUEOUS EXTRACTION:

The leaves of C.sinensis were placed in Soxhlet apparatus with water used as solvent based on polarity in order to separate different chemical constituents at 55-85°C for 8 -10 hrs. As previous documented by Pradhan et al., (2021) The acetone extract of leaves of Camellia sinensis and Camellia assamica were observed to have most effectual antimicrobial activity since polar solvents have the ability for bioactive compounds extraction which are involved in anti-cancer, anti-microbial and anti-oxidant activities. The amount of aqueous extract obtained was calculated in (Table 4.3). The sample was freeze dried and was stored at 4°C for phytochemical analysis.

Fig 3.4.2: The aqueous extraction of C.sinensis in Soxhlet apparatus

Table 3.4: Calculation and summary of total yield obtained

Sample	Amount of sample taken for extraction (g)	Solvent used for solubility	Weight of the tube (g) [w1]	Weight of the tube with sample(g) [w2]	Amount of extract obtained (ml) [W= w2 - w1]
Tea (C.sinensis)	25g	Water	13g	63.1g	50ml

3.4.3 PHYTOCHEMICAL ANALYSIS OF CRUDE EXTRACT:

The phytochemicals present in C.sinensis were examined by the methodology described by Shah et al., (2018). He reported that among the phytochemicals the concentration of tannins were found to be significantly higher

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in leaves compared to stem and roots of tea plant. The chemical constituent's alkaloids, flavanoids, terpenoids, glycosides, steroids, saponins as well as tannins in crude extract of Camellia sinensis were observed and the results were noted (Table 3.5)

Table 3.5: The results of photochemical analysis of crude extract

Phytochemical tests	Results
Alkaloids	(+)ve
Glycosides	(-)ve
Terpenoids	(-)ve
Steroids	(+)ve
Flavanoids	Slightly present
Gallic tannins	(-)ve
Catecholic Tannins	(+)ve
Saponins	(+)ve

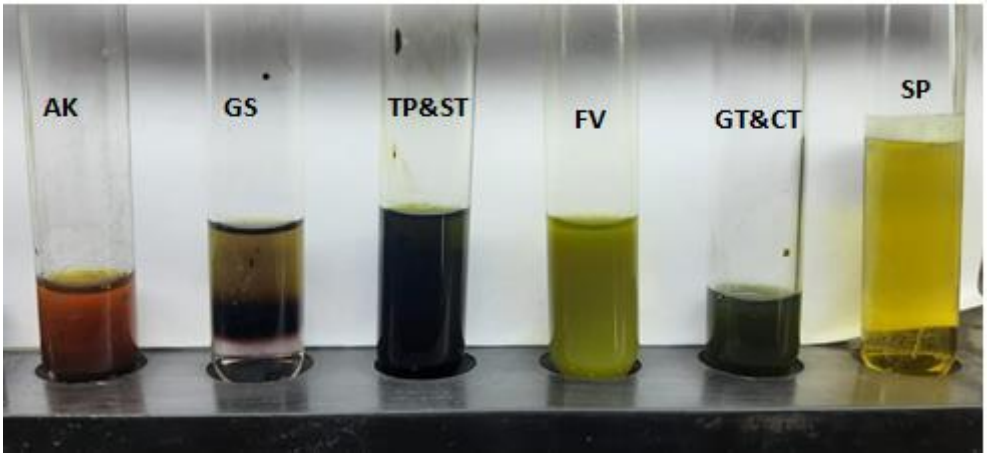

Fig 3.4.3: The test results of photochemical analysis

3.4.4 PHYTOCHEMICAL ANALYSIS OF AQUEOUS EXTRACT:

A study by Pradhan et al., (2021) observed that the aqueous leaf extracts of Camellia plant varieties possess biological compounds revealed strong antimicrobial activity against pathogenic strains and was to detect the chemical constituent's alkaloids, flavanoids, terpenoids, glycosides, steroids, saponins as well as tannins in aqueous extract of Camellia sinensis were observed and the results were noted (Table 4.6)

Table 3.6: The results of photochemical analysis of crude extract

Phytochemical tests	Results
Alkaloids	(+)ve
Glycosides	(+)ve
Terpenoids	(+)ve
Steroids	(-)ve
Flavanoids	(+)ve
Gallic tannins	(-)ve
Catecholic Tannins	(+)ve
Saponins	(+)ve

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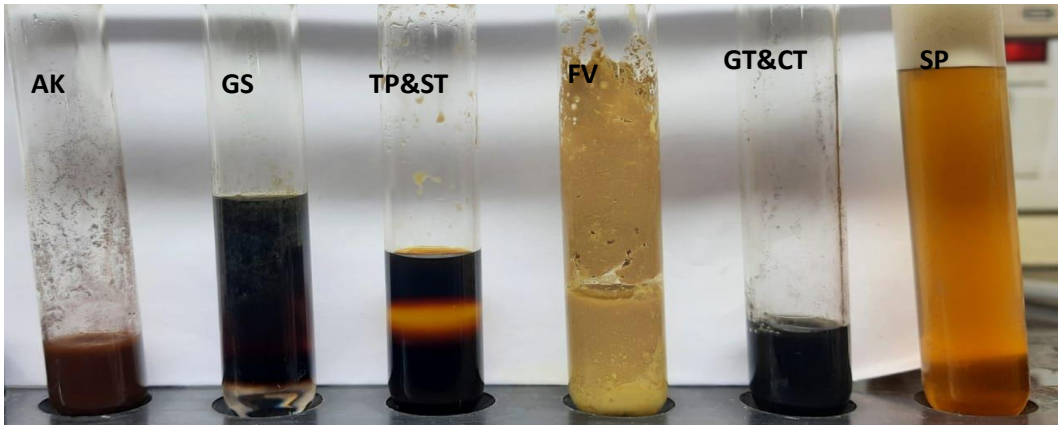

Fig 3.4.4: The test results of photochemical analysis

3.5 THIN LAYER CHROMATOGRAPHY:

The major phytochemical constituents analysis of C.sinensis was further affirmed by thin layer chromatography using the silica gel plates which were precoated using standard protocol (Pradhan et al., 2021) The purpose of this study was in order to separate bioactive compounds. The samples were spotted on TLC plates and were placed in a chromatography chamber containing mobile phase. The developed plates were then observed under the ultraviolet light (254 -336nm).

Fig 3.5.1: The plant samples were spotted on TLC plates

Fig 3.5.2: The TLC plates were placed in the chromatography chamber

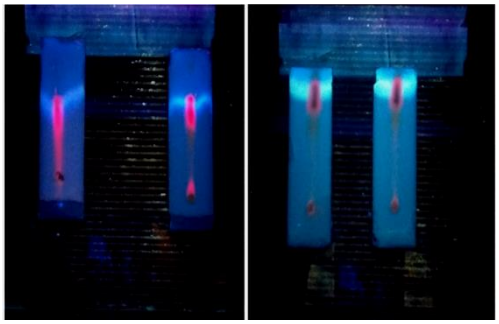

Fig 3.5.3: The developed TLC plates

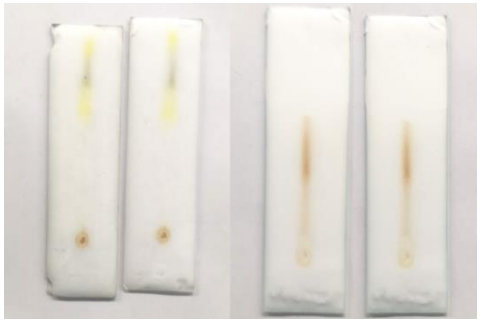

Fig 3.5.4: The developed TLC plate observed under UV light

3.6 AGAR-WELL DIFFUSION METHOD:

The study was conducted to detect the presence of antimicrobial activity of crude and aqueous extract of plant C.Sinensis by well diffusion method. A study by (Unban et al 2021) reported that C.sinensis has high antioxidant properties and can prevent the invasion of pathogenic organisms in humans and observed their antimicrobial activity against the pathogenic organisms S.aureus, E.coli, P.aeruginosa, S.buydoii and S.typhi. The bacterial suspensions were spread over the nutrient agar plates with 3 wells of 6mm diameter and were filled 30µl each

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of distinct concentrations (10µg, 50µg and 100µg) of crude as well as aqueous extracts and were incubated at 37˚C for 24hrs. The antibiotics ampicillin and gentamicin were used as standards. The zone of inhibition was observed and results were obtained in diameter of the zone of inhibition in (Table 3.7 and 3.8)

Table 3.7: the zone of inhibition of crude extract of C.sinensis measured in centimeters

Concentrations of crude extract / Test organism	10 µg/ml	50 µg/ml	100 µg/ml	Antibiotic (standard)
Escherchia coli	0.6cm	1cm	1.3cm	1.2cm
Pseudomonas aeruginosa	1cm	1.3cm	1.5cm	1.5cm
Salmonella typhi	0.6cm	1cm	1.5cm	1.2cm
Staphylococcus aureus	0.8cm	1cm	1.5cm	1.2cm
Shigella buydoii	1cm	1.2cm	1.5cm	1.6cm

Table 3.8: the zone of inhibition of aqueous extract of C.sinensis measured in centimeters

Concentrations of aqueous extract / Test organism	10 µg/ml	50 µg/ml	100 µg/ml	Antibiotic (standard)
Escherchia coli	0.6cm	1cm	1.3cm	1.2cm
Pseudomonas aeruginosa	1cm	1.3cm	1.5cm	1.5cm
Salmonella typhi	0.6cm	1cm	1.5cm	1.2cm
Staphylococcus aureus	0.8cm	1cm	1.5cm	1.2cm
Shigella buydoii	1cm	1.2cm	1.5cm	1.6cm

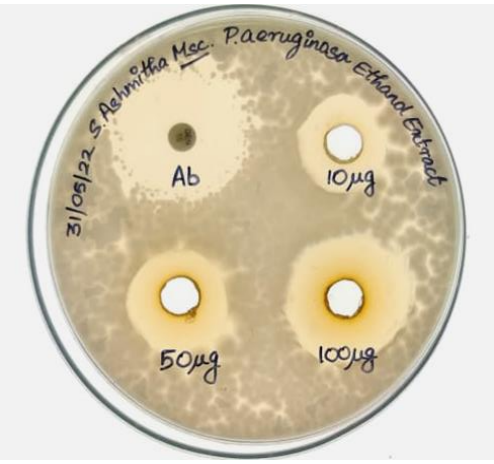

Fig 3.6.1: The zon inhibition of test sample against *E.coli*; Ab- Gentamicin as standard against *P.aeruginosa*;

Fig 3.6.2: The zone of inhibition of test sample Ab- Gentamicin as standard

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Fig 3.6.3: The zone of inhibition of test sample against S.typhi; Ab- Gentamicin as standard

Fig 3.6.4: The zone of inhibition of test sample against S.aureus; Ab- Ampicillin as standard

Fig 3.6.5: The zone of inhibition of test sample against *S.buydoii*; Ab- Gentamicin as standard

The antimicrobial activity of crude extract of C.sinensis against indicator organisms

Fig 3.6.6: Inhibitory activity of test sample against *E.coli*; Ab- Gentamicin as standard

Fig 3.6.7: Inhibitory activity of test sample against P.aeruginosa; Ab- Gentamicin as standard

Fig 3.6.8: Inhibitory activity of test sample against S.typhi; Ab- Gentamicin as standard

Fig 3.6.9: Inhibitory activity of test sample against S.aureus; Ab- Ampicillin as standard

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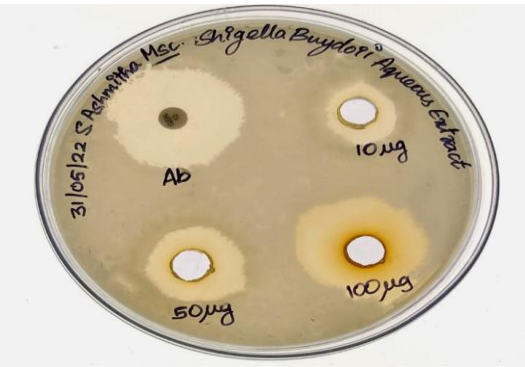

Fig 3.6.10: Inhibitory activity of test sample against S.buydoii; Ab- Gentamicin as standard

IV. CONCLUSION

The emerging demand for probiotic supplements having health benefits beyond nutrition has provided ample opportunity to explore the possibilities for isolating new probiotic strains from various sources. Tea being one of the ancient medicinal beverages with major source of providing health benefits in humans. The microflora present in leaves of tea is indigenous and includes the strains of lactic acid bacteria. In this study the LAB isolated from tea leaves are studied for colony characteristics, morphology, microscopic and phenotypic characteristics were studied with required chemical and selective media. The extracts of the plant were screened for phytochemical studies and bioactive compounds were analyzed. The analyses of bioactive compounds were further confirmed by spotting the plant extracts in TLC and observed under UV light. The antimicrobial property of extracts was studied against standard microorganisms and they were capable of inhibitory activity indicating a zone of inhibition. The results obtained collectively indicated that the lactobacilli isolates obtained from the tea samples have the most promising properties and have high capable for potential probiotics in food and medical industries and tea may be considered as a source for safer isolation of probiotics.

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