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CHARACTERIZATION OF NOVEL SOFT ROT CAUSING GRAM-NEGATIVE BACTERIA SJCTSSP01 & ITS MANAGEMENT USING PHYTOEXTRACTS

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Keywords:

Lantana camara, Clerodendrum infortunatum, 16S rDNA sequencing, GenBank, Antibacterial activity, Soft rot of potato

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ABSTRACT: Aim: The present study focuses on studying the soft rot-causing bacteria in *Solanum tuberosum* and the activity of phytoextracts against the pathogen to prevent/control this infection. *Lantana camara* and *Clerodendrum infortunatum* has been well known for their antibacterial activity and widely used in Ayurveda, these plants were investigated for their antibacterial activity against the isolated pathogen. **Methods:** The bacteria were isolated from the infected potato sample and cultured in Potato dextrose agar (PDA) media. The isolated was characterized using morphological/biochemical properties and 16S rDNA sequencing. The pathogen was subjected to agar well diffusion assay to investigate antibacterial potential of *L. camara* and *C. infortunatum* extracts against this pathogen. **Results:** A gram-negative cocci were isolated from the infected *S. tuberosum* sample. Phylogenetic analysis using 16S rDNA sequencing, suggested no previous reports of such bacteria causing soft rot of potato. The 16S rDNA sequence was submitted to GenBank with accession ID MT491092. Phytoextracts of *L. camara* demonstrated significant antibacterial activity against the isolated bacterial strain SJCTSSP01. Prominent results were observed from solvent extracts of *L. camara which* showed an average zone of inhibition of 14 mm with petroleum ether extract and 13 mm with chloroform extract, and in the case of *C. infortunatum*, there observed zone of inhibition of 11mm. **Conclusion:** Results of this study reports a novel gram-negative bacteria that causes soft rot of potato. Phytochemicals extracts of *L. camara* exhibit a significant antagonism against the Gram-negative bacterium SJCTSSP01, with potential for field application in the control and management of this disease.

INTRODUCTION: International food gadget faces a lot of challenges including the production of crops due to climate change and various disease-causing microorganisms which acts as a hindrance in the process of food production. Nowadays, potato (*Solanum tuberosum*) stands out among the world's fundamental food crops. It also plays a major role in the food system and food security.

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Soft rot of *Solanum tuberosum* is a post-harvest bacterial disease caused by either *Erwinia* alone or by mixed infection. This disease can motive full-size economic loss during their storage intervals. Infection Enterobacteriaceae typically occurs after the crop has been harvested, generally as a result of storage or shipment conditions that support disease [1-3].

This type of potato bacterial disease can occur at the seedling stage or during storage, thus infecting the surrounding healthy potato tubers quickly and easily, which may lead to severe economic losses [4]. The pathogenic bacteria during infection produces an enzyme called pectinase. Pectinase degrades pectin, which is an important structural molecule of

the plant cell wall and is also involved in adhering plant cells together into tissues [5]. In India, the Ayurveda system of medicine has been in practice for decades. Ancient literature evidence of various plants and their parts to be used in Ayurveda, Siddha, and Unani medicine for treatment and cure of many diseases [6].

Indiscriminate use of chemical pesticides to control various pests and pathogenic microorganisms of crops plants are causing health hazard both in terrestrial and aquatic lives through their residual toxicity [7]. In comparison with synthetic drugs, antimicrobials originated from plants are not linked with side effects and have a wide therapeutic potential to cure different infectious diseases [8]. *Clerodendrum infortunatum* is a flowering shrub and it is named so because of its rather ugly leaf. It has a circular leaf. Leaves are simple, opposite; both surfaces sparsely villous-pubes elliptic, broadly elliptic. The stem is hollow, and the leaves are 6-8 inches (15-20 cm) long, borne in whorls of four on very short petioles. The inflorescence is huge, consisting of many tubular snow-white flowers in a terminal cluster. It flowers from April to August [9]. In Siddha and Ayurveda, the leaves and roots of *C. infortunatum* are used as herbal remedy for cough, asthma, diarrhoea, rheumatism, fever and skin diseases. It is also known to have hepato-protective and antimicrobial activities.

Lantana camara is a low erect, rugged hairy; evergreen shrub belongs to the family Verbenaceae and native to tropical America. Known by several common names *viz.*, blacksage, cuasquito, angel lip, flowered sage, all over the world, it is a significant weed of which there are some 650 varieties in over 60 countries or island groups. *L. camara* has several uses, mainly as herbal medicine and in some areas as firewood and mulch. It is also used for the treatment of cancers, chickenpox, measles, asthma, ulcers, swellings, eczema, tumors, high blood pressure, bilious fevers, catarrhal infections, tetanus, rheumatism, malaria, and azoxy of abdominal viscera [10, 11]. Extracts from the lantana leaves exhibit antimicrobial, insecticidal, and nematocidal activity and also contain verbascoside, which possesses antimicrobial, immunosuppressive, and antitumor activities [12].

In this study, the bacterial pathogen causing soft rot of potato has been isolated and characterized. Antibacterial activity of 2 plant samples, *i.e.*, *L. camara* leaf and *C. infortunatum* against the isolated pathogen, was investigated.

MATERIALS AND METHODS:

Isolation of Pathogen: Infected potato samples (bacterial soft rot) were collected from the local market in Bengaluru. The sample was then chopped into small cubes of 1cm × 1cm size using a blade. It was then inoculated onto a freshly prepared sterile Potato Dextrose Agar (PDA) media. For PDA media, 2.4g of PDA agar and 2g of agar-agar were taken and dissolved in a conical flask containing about 100ml of distilled water. After inoculation of the sample onto the media it is left for incubation. After incubation, grown organism were sub-cultured onto a fresh plate containing PDA media until pure culture is obtained [13, 14].

Characterization of Pathogen: The pure culture was then studied for its colony characters such as size, form, colour, margin, elevation, opacity, motility and cell shape. The colony was also checked for its gram-positive or gram-negative nature using the standard Gram stain protocol mainly using crystal violet, gram's iodine, ethanol and safranin [15]. Further few biochemical tests were undertaken to compare the biochemical characteristics of the organism, including citrate, urease, carbohydrate and triple-sugar iron test [16-18]. For the urease test, the standard Christensen's urea agar media with phenol red as pH indicator were used [19], citrate utilization test was carried out using the common Simmons citrate agar media, carbohydrate fermentation test was carried out using fermentation tube or Durham tube to check for gas production and triple sugar iron test were carried out using majorly 3 carbohydrates in media including lactose, dextrose/glucose, and sucrose.

Molecular Sequencing: The 16s rDNA sequencing was carried out using Gene sequencing protocol involving Genomic DNA isolation from microbial samples using EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd including Lysis/Homogenization, Centrifugation, Binding (binding buffer), Washing, and Elution. Followed by this DNA isolation, PCR is carried out using the thermal cycling conditions of Denaturation, Annealing, and Extension. Then purification of PCR is done using Montage PCR

Clean up kit (Millipore) and PCR products was sequenced using the primers with the help of Terminator Cycling Sequencing Kits. Single-pass sequencing was performed on each template using 16s rRNA universal primers. The fluorescent-labelled fragments were purified using ethanol precipitation, and the samples were resuspended in distilled water and subjected to electrophoresis in a sequencer [20-22].

Phylogenetic Analysis: The obtained sequence was subjected to BLAST analysis to find homologous sequences to identify its phylogenetic origin (https://blast.ncbi.nlm.nih.gov/). The most significant match obtained through BLASTn were retrieved from NCBI GenBank Database (https:// www.ncbi.nlm.nih.gov/genbank/) and were utilized for phylogenetic tree constructions using MEGA.X software. The genus and species of the strain were confirmed based on the percentage similarity with the homologous sequence. The sequence data were submitted to the GenBank database for public access (https://www.ncbi.nlm.nih.gov/genbank/).

Pathogenicity: To check for the pathogenicity of the organism, a confirmatory test was carried out by reinjection of the organism onto a fresh potato sample. Freshly broth culture of the isolated bacterial strain was injected into the potato sample via a sterile syringe. The sample was then transferred onto the incubator at 37 °C and left undisturbed for about a week. The sample was cut open to observe for symptoms of soft rot.

Plant Extraction: Fresh plant leaves of *Lantana camara* and *Clerodendrum infortunatum* were collected from Kerala, the leaves were air-dried in incubator for about 3 days until the samples were completely dry. The dried samples were then grounded and subjected to maceration. The plant powder 11g was mixed with 100ml of respective solvents (ethanol, chloroform, and petroleum ether) for 24 h at room temperature. The solvent was then filtered and concentrated to obtain the respective crude extract [23, 24].

Antibacterial Activity: The crude extracts were screened for their antibacterial activity against the SJCTSSP01 strain using agar well diffusion test. The crude extracts were dissolved in 100% dimethyl sulfoxide at a concentration of about 100 mg/ml from which 100µl was added into each well in the agar plate, giving a final test concentration of 10mg/well for each solvent extract. The plates were incubated at 37 °C for about 24 h and observed for the zone of inhibition [14, 23].

RESULTS:

Isolation of Pathogen: Infected plant sample (potato) was procured from local market in Bengaluru **Fig. 1A**. The potato sample was chopped into small cubes of 1cm: 1cm size and was inoculated onto a freshly prepared sterile potato dextrose agar (PDA) plate (As shown in **Fig. 1B**). Bacterial colonies that grew on the plate were sub-cultured on to fresh PDA plate till pure culture was obtained. The obtained bacterial culture demonstrated matt like colony morphology, as shown in **Fig. 1C** & **Fig. 1D** with no significant colony colour. The pure culture was maintained in PDA.

Pathogenicity of Bacterial Strain: The pathogenicity of the isolated bacterial strain was confirmed by re-inoculation of the isolated bacterial strain into a fresh potato sample and was allowed to grow for 1 week. The plant sample was observed for soft-rot symptoms after 7th day, which confirmed that the isolated bacteria are causing soft-rot-like symptoms. The observed symptoms of the infected potato are shown in **Fig. 2**. This confirmed that the isolated bacteria is able to produce soft-rot like symptoms, and hence was confirmed that it is a bacterial pathogen causing soft rot disease in potato.

Characterization of Pathogen: The pure culture was studied for its colony characteristics and biochemical properties as summarized in Table.1 & **Table 2**. The colony morphology of the pure culture demonstrated matt-like colony morphology, with irregular colony size, without any color/pigmentation. The colony was opaque, and no motility was observed. The colony was observed to be a gram's negative cocci, observed both as individual cells and cluster of cells **Fig. 3A**. Biochemical properties of the pathogen showed that in carbohydrate fermentation test its positive for glucose fermentation and positive for gas production **Fig. 3B**. The organism is citrate test positive, suggesting the presence of citrase or citrate-permease enzyme **Fig. 3C**. The organism is negative for the urease enzyme. The triple sugar iron test confirmed that the organism is able to ferment sugars and is positive for gas production.

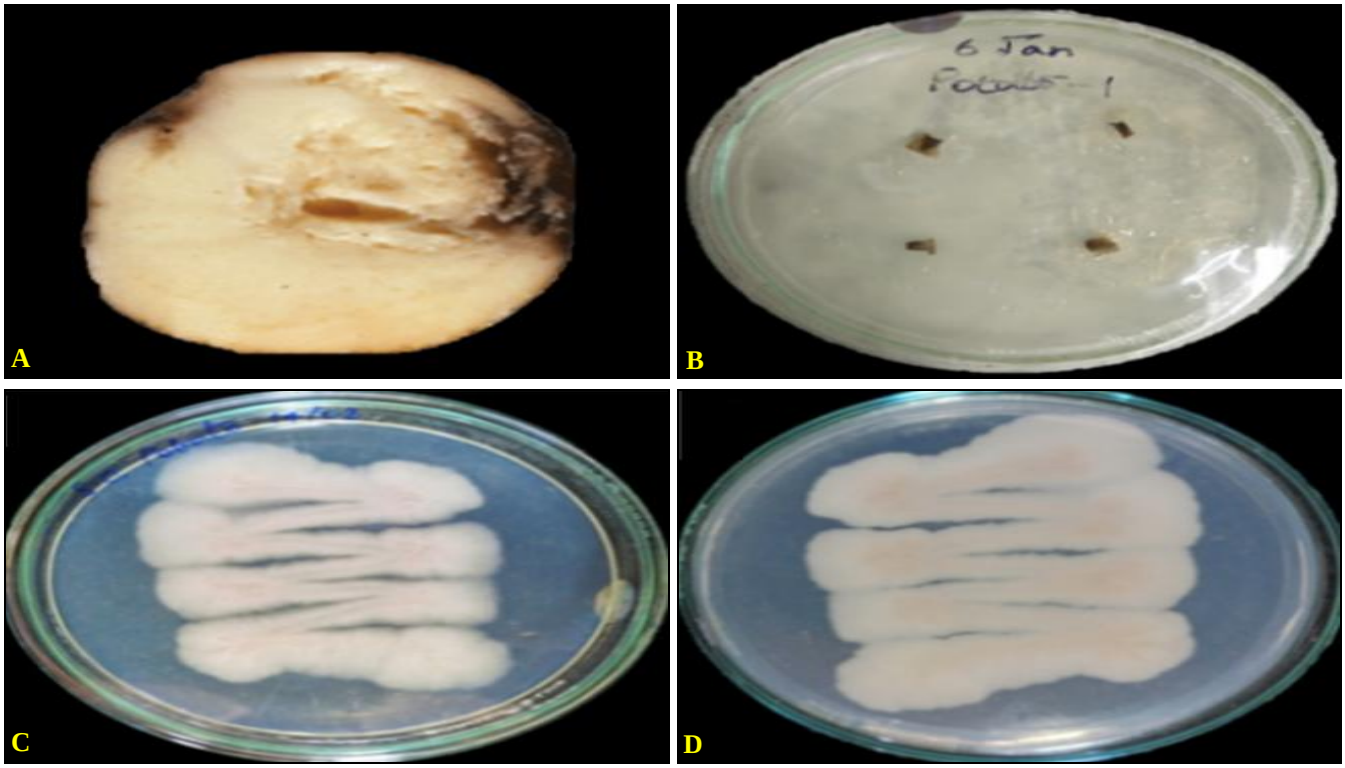

FIG. 1: ISOLATION OF PATHOGEN FROM INFECTED *SOLANUM TUBEROSUM*; A: INFECTED *SOLANUM TUBEROSUM* AS ISOLATION SOURCE; B: PLATING OF INFECTED PLANT SAMPLE ON TO POTATO DEXTROSE AGAR PLATE; C: ARIAL VIEW OF CULTURED PURE STRAIN; D: SUBSTRATE VIEW OF CULTURED PURE STRAIN

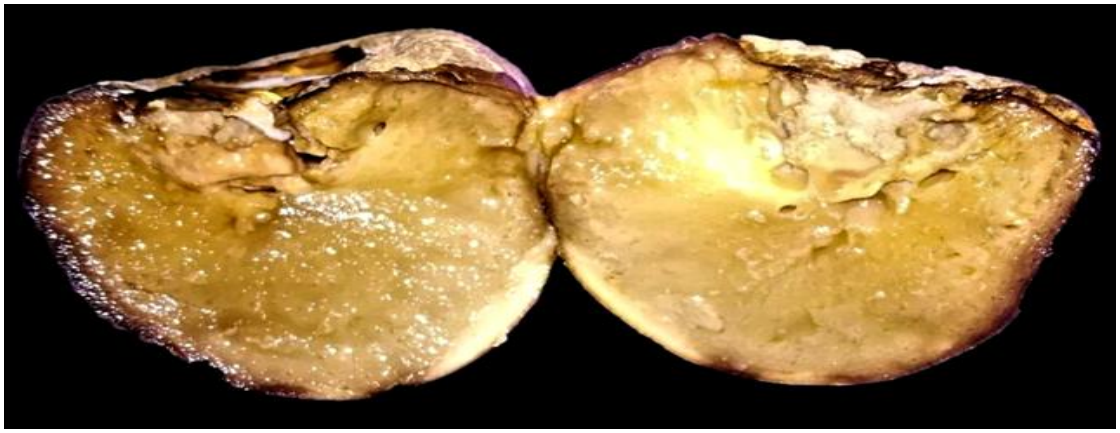

FIG. 2: PATHOGENICITY CONFIRMATION. THE ISOLATED PURE STRAIN PRODUCED SOFT-ROT LIKE SYMPTOMS UPON EXPOSING TO A HEALTHY POTATO SAMPLE

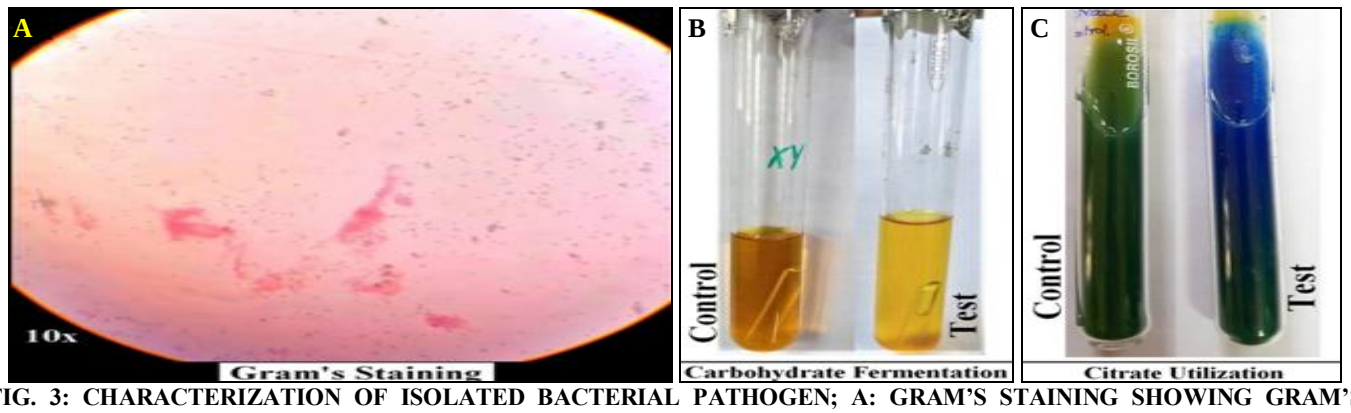

FIG. 3: CHARACTERIZATION OF ISOLATED BACTERIAL PATHOGEN; A: GRAM'S STAINING SHOWING GRAM'S NEGATIVE; B: CARBOHYDRATE FERMENTATION TEST SHOWING POSITIVE; C: CITRATE UTILIZATION TEST SHOWING POSITIVE

TABLE 1: MORPHOLOGICAL FEATURES OF ISOLATED PURE BACTERIAL PATHOGEN

S. no.	Colony Characters	Morphology
1	Size	-
2	Form	Matt
3	Pigmentation/Colour	Nil
4	Margin	Undulate
5	Elevation	Flat
6	Opacity	Opaque
7	Motility	Nil
8	Cell shape	Coccus

TABLE 2: BIOCHEMICAL PROPERTIES OF ISOLATED PURE BACTERIAL PATHOGEN

S. no.	Test	Result
1	Citrate	Positive
2	Urease	Positive
3	Carbohydrate	Positive
4	Triple sugar	Positive
5	Gram's staining	Negative

Molecular Sequencing of Pathogen: The organism was subjected to molecular sequencing analysis to identify the genus and species. The details of the sequencing analysis are summarized in **Table 3**. The sequence length of 1271bp was obtained from the sequencing process, which was cross verified with the NCBI database using the BLASTn tool. However, there was no significant match in the database, which correlated with the observed biochemical and morphological characteristics of the isolated organism. Hence, the organism was named as 'Soft rot causing bacterium SJCTSSP01' with a strain name being SJCTSSP01. The phylogenetic tree analysis of the obtained sequence was performed using MEGA-X software, as shown in **Fig. 4**.

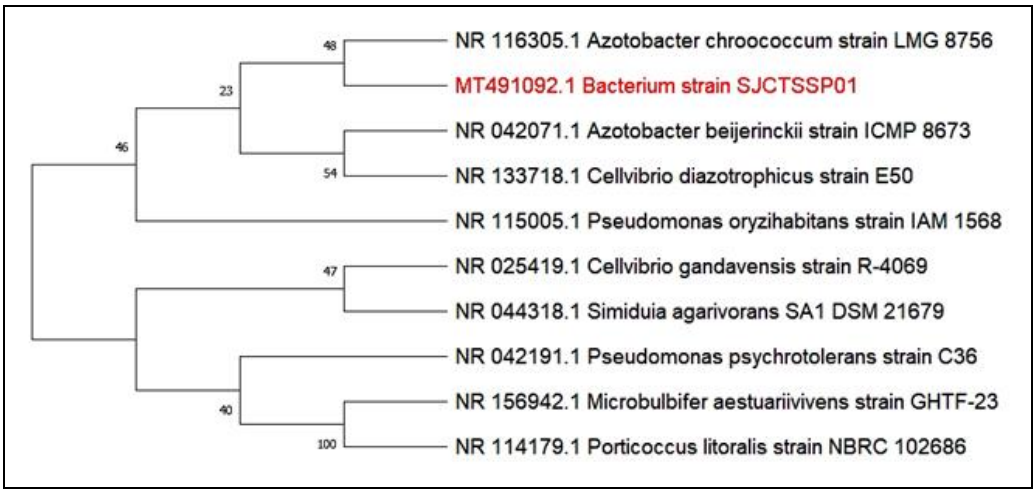

FIG. 4: PHYLOGENETIC TREE ANALYSIS OF 16s rRNA SEQUENCE OF THE ISOLATED PATHOGEN

TABLE 3: SEQUENCING ANALYSIS OF 16s rRNA OF ISOLATED PATHOGEN

GenBank Submission ID	MT491092
Target Gene	16s rRNA
No.of Base Pairs	1271 bp
Organism	Unknown bacterium
Strain	SJCTSSP01

FASTA Gene Sequence

> Soft Rot Causing Bacterium SJCTSSP01

GGATCTTCGGACCTCACGCTATTAGATGAGCCTAGGTCGGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTCGTAAAGCACTTTAAGTTGGG
AGGAAGGGCTCATAGCGAATACCTGTGAGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGTCTGGCTAGAGTGCGGTAGAGGGTAGTGGAATTTCCAGTGTAGCGGT
GAAATGCGTAGATATTGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGACACTGACACTGAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGATCCTTGAGATCTTAGTG
GCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATG
GATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGT
AACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCA
AGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATC
GCTAGTAATCGTGAATCAGAACGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGT
TGCTCCAGAAGTAGCTAGTCTAACCCTCGGGAGGACGGTTACCACGGA

The sequence with the specified details was submitted to NCBI GenBank database with an accession ID MT491092. Based on the obtained sequence and biochemical properties, it is identified that the isolated pathogen is a novel species that causes soft rot in potato.

Antibiotic Susceptibility Test: The isolated pathogen was subjected to an antibiotic susceptibility test (AST) against plant extracts of two test plants *i.e.*, *Lantana camara* and *Clerodendrum infortunatum*. Using agar well diffusion assay, the pathogen was tested for its susceptibility against the three different extracts (ethanol, chloroform & petroleum ether) of the 2 test plants. Among the two plants, the *L. camara* extracts showed significant antibacterial activity against this isolated test pathogen **Fig. 5**. The results of the AST analysis are tabulated in **Table 4**. It was observed that the highest activity was demonstrated by *L. camara* petroleum ether extract with 14mm zone-of-inhibition, suggesting that the non-polar compounds present in the plant is acting as antibacterial agents.

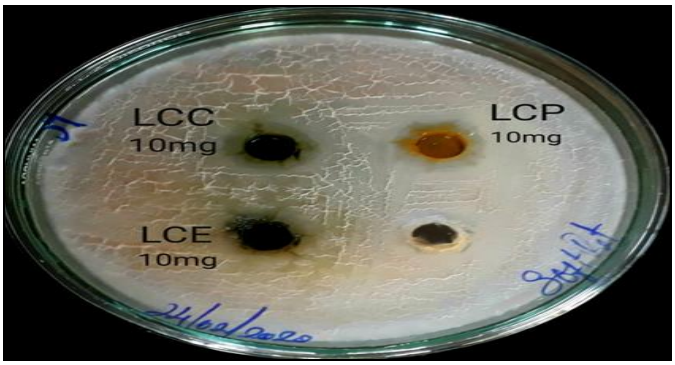

FIG. 5: ANTIBACTERIAL ACTIVITY OF *LANTANA CAMARA* EXTRACTS ON ISOLATED BACTERIUM SJCTSSP01. [LCE: Ethanol extract; LCC: Chloroform extract; LCP: Petroleum ether extract.]

TABLE 4: ANTIBACTERIAL ACTIVITY OF TEST PLANTS ON ISOLATED PATHOGEN SJCTSSP01

	Ethanol extract	Chloroform extract	Pet. Ether extract
	Zone of Inhibition (mm)		
Lantana camara	-	13	14
Clerodendrum infortunatum	11	-	-

DISCUSSION: In this study, a novel gram-negative pathogenic bacteria SJCTSSP01 was isolated and confirmed that it causes soft rot disease in a fresh potato sample. A literature review has revealed no such reports in the earlier incidence of a gram-negative cocci bacteria causing soft rot disease and hence this is a novel report in this plant disease investigation.

Clerodendrum infortunatum and *Lantana camara* were examined for their antibacterial activity against the isolated pathogen SJCTSSP01. The antibacterial activity was observed through agar well diffusion assay [25]. In this study, prominent results were observed in the case of *Lantana camara*, which showed an average zone of inhibition of 14 mm with petroleum ether extract and 13 mm with chloroform extract [14]. The use of phytoextract of *Lantana camara* suggests being efficient against soft rot bacteria, which could be characterized further and thus, finding its way into the arsenal of lucrative antimicrobial drugs [26].

Identification of effective plant extracts against the soft rot bacterium SJCTSSP01 of potato has the potential to help farmers to control the potato soft rot disease during the storage period. The use of herbal extracts to control plant diseases is an environment-friendly approach and an effective alternative to toxic chemical pesticides [13].

CONCLUSION: Based on the results of this study, it can be concluded that the petroleum ether extract of *Lantana camara* has potential antibacterial activity against the novel gram-negative cocci bacterium (SJCTSSP01) isolated in this study. Hence crude extract of *Lantana camara* may be utilized as a biofertilizer and biocontrol agent to control and manage the soft rot of potato disease. The current study brings out the use of herbal remedies to control the soft rot disease in potato. Further research is in progress to identify the active compounds present in leaf extracts by phytochemical analysis [14].

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CONFLICTS OF INTEREST: No known conflict of interest for this work.

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