RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL ACTIVITY OF FOUR MEDICINAL PLANT LEAF EXTRACTS AGAINST *Pectobacterium* sp. CAUSING SOFT ROT IN POTATO

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ABSTRACT

This study was conducted to determine the antibacterial activity of the ethanol and acetone leaf extracts of *Argyreia pomacea, Coccinia grandis, Erythrina subumbrans and Gymnema sylvestre* against the causal pathogen of potato soft rot. Pathogen was isolated from soft rot potato which was identified as *Pectobacterium* sp.. The pathogenicity of the isolated *Pectobacterium* sp. was confirmed on healthy samples of potatoes. The antibacterial activity of leaf extracts was determined by agar well diffusion assay. The leaf extracts were tested at different concentrations of 200, 100, and 50 mg/mL. Based on the results, the acetone and ethanol extracts of *E. subumbrans*, the ethanol extract of *G. sylvestre*, and both extracts of *C. grandis* at 200 mg/mL possess potent antibacterial compounds against *Pectobacterium* sp.. There were no significant differences in zone of inhibition among these treatments. Therefore, further studies are necessary to isolate and identify the specific bioactive compounds in the leaves that are responsible for the observed antibacterial effects.

Keywords: *Antibacterial activity, Eco-friendly approach, Leaf extract, Pectobacterium* sp.

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1. INTRODUCTION

Potato (*Solanum tuberosum*) is a major food crop and ranks as the sixth most economically important crop worldwide, with global production reaching around 359 million tons in 2020 [1]. In Sri Lanka, potato production stood at approximately 40,748 metric tons in 2023 [2]. However, potato crops are vulnerable to several bacterial diseases, which significantly reduce both yield and quality. Among them, postharvest soft rot is one of the most destructive diseases affecting potatoes. This disease poses a major threat during the production, transit, and storage stages, often causing severe economic losses to producers. Soft rot leads to higher total losses of produce compared to any other postharvest bacterial disease [3].

Bacterial soft rot is primarily caused by pectinolytic *Pectobacterium* and *Dickeya* species, collectively known as soft rot Pectobacteriaceae (SRP). This disease remains a significant concern for the seed potato production sector in various potato-growing regions worldwide [4]. To manage bacterial diseases like soft rot, chemical pesticides have been regularly used. However, the excessive application of these chemicals has raised environmental and health concerns, leading to increased demands for alternative solutions to reduce synthetic pesticide usage [5].

As of today, there is no treatment that can stop disease progression once soft rot disease develop in a plant. Control is largely reliant on prevention, which is spread over various layers. For example, by certifying seed potatoes based on seed grade, a protocol that cannot detect latent infection of progeny tubers from symptomless plants [6]; and by the application of hygienic measures, such as washing and disinfecting machines used when planting, spraying, haulm flailing, harvesting, and grading in store [7]. At the other end of the production chain, good storage procedures are an important factor in reducing disease [6].

In response, recent research has focused on exploring alternative strategies to mitigate reliance on synthetic pesticides. One promising approach is the utilization of plants' ability to synthesize secondary metabolites, which have shown antimicrobial properties and act as natural defense mechanisms against pathogenic microorganisms [8, 9]. These natural compounds are typically less harmful to the environment, biodegradable, and less likely to contribute to bacterial resistance [10]. As a result, the development of

biodegradable pesticides has gained significant attention. Botanical pesticides, in particular, offer an eco-friendly alternative as they tend to degrade quickly, often within days or even hours [11].

Due to the growing concerns associated with synthetic pesticides and their residues, this study aimed to screen the *in vitro* antibacterial activity of selected leaf extracts against *Pectobacterium* sp., a causal agent of potato soft rot.

2. MATERIAL AND METHODS

2.1 Preparation of leaf extracts

Leaves of the selected plant species (*Argyreia pomacea*, *Coccinia grandis*, *Erythrina subumbrans* and *Gymnema sylvestre*) were collected and dried at room temperature (28 ± 1 °C) for about two weeks until obtaining the constant weight. The dried leaves were ground to a fine powder using a laboratory grinding machine and stored in airtight bottles in the dark. Powdered leaves (100 g) was soaked in 500 mL of each solvent which were ethanol and acetone. They were left shaking for 72 h at room temperature, on an orbital shaker. The extracts were filtered separately through double-layered muslin cloth followed by Whatman No.1 filter paper and the solvent was evaporated at 40 °C using a rotary evaporator under reduced pressure. They were stored in airtight glass bottles [12].

2.2 Isolation and identification of pathogen

Potato tubers showing the typical symptoms of soft rot were collected from market and brought to laboratory for pathogen isolation. Small pieces of infected potato tuber were cut aseptically using a sterile blade along with little portion of healthy tissues. Excised samples were surface sterilized using 2% of sodium hypochlorite for 2 minutes and washed in sterile distilled water 3 times to remove traces of chemical. The surface sterilized pieces (10) were macerated with 1 or 2 mL of sterile distilled water using glass rod under aseptic conditions. A loop full of suspension was streaked on the nutrient agar (NA) medium. The streaked plates were incubated at 28 ± 1 °C for 48 h [13, 14]. The bacterial colonies developed on NA medium were observed under a microscope for the morphological studies and biochemical tests were carried out to confirm the identity of the pathogen.

2.2.1 Biochemical tests

2.2.1.1 Oxidase Test

Five drops of the Tetramethyl-P-Phenylendiamine Dihydrochlorie reagent (prepared by adding 1 g of the reagent to 100 mL of distilled water) were added to a filter paper. Then, a part of the fresh bacterial colony was transferred to the filter paper saturated with the reagent and gently crushed. The appearance of the purple colour within 10 seconds indicates a positive reaction [15].

2.2.1.2 Catalase test

A small number of organisms from a well-isolated 18- to 24-hour colony was collected using a sterilized inoculating loop and smeared on the sterilized microscope slide. One drop of 3% H_2O_2 was placed onto the organism on the microscope slide. Positive reaction is evident by immediate bubble formations [16].

2.2.1.3 KOH string test

A loopful of a colony of the organism was emulsified on the surface of a glass slide in a suspension of 3% KOH. The suspension was stirred continuously for 60 s after which the loop was gently pulled from the suspension. The test was considered positive if stringing occurred within the first 30 s of mixing the bacteria in the KOH solution [17].

2.2.1.4 Citrate utilization test

Simmons citrate medium was used by suspending 24.28 g of Simmons citrate agar in 1 L of distilled water and it was sterilized by autoclaving at 15 psi pressure (121 °C) for 15 minutes. A single isolated colony was picked and lightly streaked on the surface of the slant using a sterilized inoculating loop. Then the slants were incubated at 35 °C for 18 to 48 hours. The appearance of the visible growth of bacteria on the slant surface and the intense Prussian blue color of the medium indicate a positive reaction [18].

2.2.1.5 Salt tolerance test

The salt tolerance of bacteria at a NaCl concentration of 5% was tested using the Yeast Salt Broth (YSB) medium, which is composed of $NH_4H_2PO_4$ (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4.7H_2O$ (0.2 g), Yeast extract (5 g), and NaCl (50 g). The above materials were

dissolved in 1 L of distilled water and the pH was adjusted to 7.2. The medium was distributed in test tubes at a rate of 5 mL/tube. The tubes were autoclaved, and the medium was allowed to cool. The tubes were inoculated by adding 100 µL of the bacterial suspension at a concentration of 10^6 cfu/mL. The tubes were incubated at a temperature of 28 ± 2 °C for 72 hours, and the bacterial growth was observed [19].

2.2.1.6 Indole test

Typtone broth was used by suspending 15 g of powder in 1 L of distilled water and it was sterilized by autoclaving at 15 psi pressure (121 °C) for 15 minutes. Then 4 mL of tryptone broth was dispensed into sterilized test tube. Then it was inoculated with a small amount of a pure culture and incubated at 35 ± 2 °C for 24 to 48 hours. Then five drops of Kovács reagent were added directly into the tube. A positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent. If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy [20].

2.3 Gram staining

Bacteria grown as colonies on plates were picked and suspended in a drop of sterile distilled water previously applied to the slide. A thin film of the sample was prepared using a sterilized inoculating loop. The smear was air-dried and heat-fixed by passing the slide over a flame two or three times. Heat-fixed smear was covered with crystal violet solution for 30 s and the stain was washed off gently with flowing water. Then the smear was covered with Gram’s iodine solution for 1 min. Then it was washed off with water and shaken off excess water. Then it was decolorized by slowly dropping a decolorizing solution (95 % alcohol) above the smear on the slide, allowing it to run down across the smear. Then it was washed with water and shaken off excess water. The slide was covered with counterstain (Safranin) for 30 s. It was washed with water and then shaken off excess water. The slide was allowed to air dry. Then the bacteria were examined under oil immersion to distinguish between Gram-positive and Gram-negative bacteria. Gram-positive bacteria appear purple as compared with Gram-negatives which appear pink [21].

2.4 Pathogenicity test

Pathogenicity test for potato was done by potato slice assay. Potato tubers were sterilized with 70 % alcohol, rinsed with sterile distilled water and aseptically cut into 1 cm thick slices. The potato slices were placed in sterile Petri dishes containing sterilized filter paper soaked with 2 mL of sterile distilled water. Tests for soft rot characteristics were done in triplicate. Potato slices were inoculated by spreading a bacterial colony on the surface using a sterile inoculating loop. Inoculated slices were maintained in moist conditions and were incubated at 27 °C for 5 days [22].

2.5 *In vitro* screening of antibacterial activity

Antibacterial activity of the crude extracts was determined by the agar well diffusion method [23]. The sterilized NA was poured into sterile 90 mm Petri plates. After solidification, 100 µL of standardized inoculum (1×10^6 cfu/mL) of bacteria was inoculated on NA plates by using a sterilized glass spreader. The wells were punched over the agar plates using a sterile cork borer of 8 mm diameter and the base of the wells was sealed with a few drops of sterilized molten agar. An aliquot of 100 µL of each extract with different concentrations was loaded into separate wells. Three different concentrations of ethanol and acetone leaf extracts (50, 100 and 200 mg/mL) were tested. Wells with solvent and antibiotic (Tetracycline - 250 ppm) were kept as negative and positive controls, respectively. Plates were incubated at 30 °C for 24 hours. The experiment followed a factorial completely randomized design with 26 treatments, and six replicates were maintained to ensure reliability. After incubation the diameter of circular inhibitory zones formed around each well were measured.

2.6 Statistical analysis

Zone of inhibition was subjected to one-way ANOVA and the mean values were compared using Tukey's multiple comparison test. Statistical analysis was done using the Minitab statistical package (V. 17.1.0., Minitab Inc.). The level of $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of pathogen

This study resulted in the isolation of 01 bacterial isolate from diseased potato tuber. Isolated colonies appeared as white, circular, convex colonies with complete, circular, opaque margins (Fig. 1A). The microscopic examination of the bacterial isolate showed the characters as listed in Table 1.

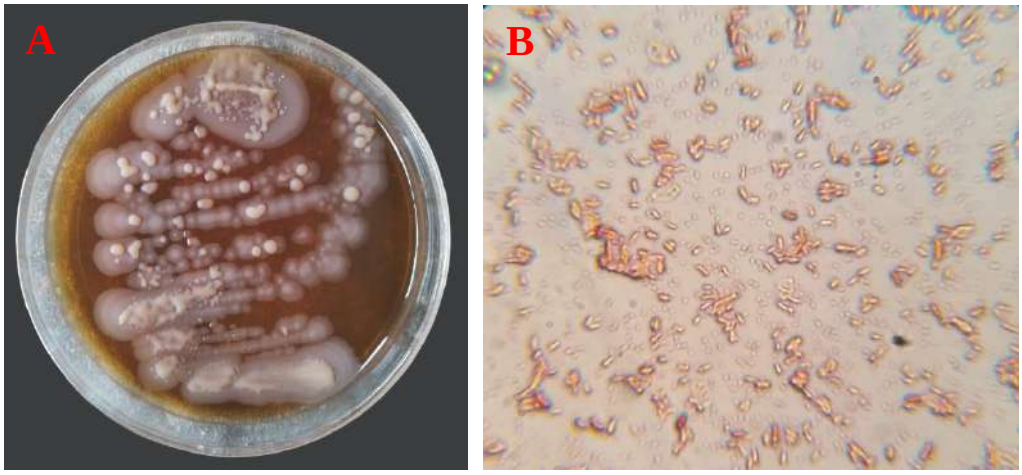

Figure 1: Morphological characteristics of *Pectobacterium* sp. recovered from soft rot of potato. (A) Upper view of the colony appearance on NA medium; (B) Gram staining of *Pectobacterium* sp. observed under a light microscope (×1000)

Table 1: Morphological and microscopic characteristics of *Pectobacterium* sp.

Character	**Notes**
Shape	Short rod
Cells aggregate	Single or pairs
Colour and the shape of the colony on NA medium	White (creamy) with a full, round, opaque

The colony characters of the isolated pathogen were matched with the characters of the genus *Pectobacterium* as similar results were recorded in previous studies [19, 24, 25]. Biochemical tests confirmed the identity of *Pectobacterium* sp. as the results matched with the result of Wasana *et al*. [24].

Table 2: Biochemical characteristics of *Pectobacterium* sp.

Biochemical test	**Result**
Oxidase test	Negative
Catalase test	Positive
Gram staining	Negative
KOH string test	Negative
Growth at 37 °C	Positive
Utilization of citrate	Positive
Salt tolerance test	Positive
Indole test	Negative

3.2 Pathogenicity test

Small water-soaked lesions developed on inoculated region of potato slice after 4-5 days. Symptoms of affected area became watery, soft, and fleshy and oozing of the tissue was observed in the region of the inoculated area (Fig. 2A). Same pathogen was found from inoculated potato upon re-isolation. Hence, the study revealed that the bacteria isolated from infected potatoes are responsible for the potato soft rot.

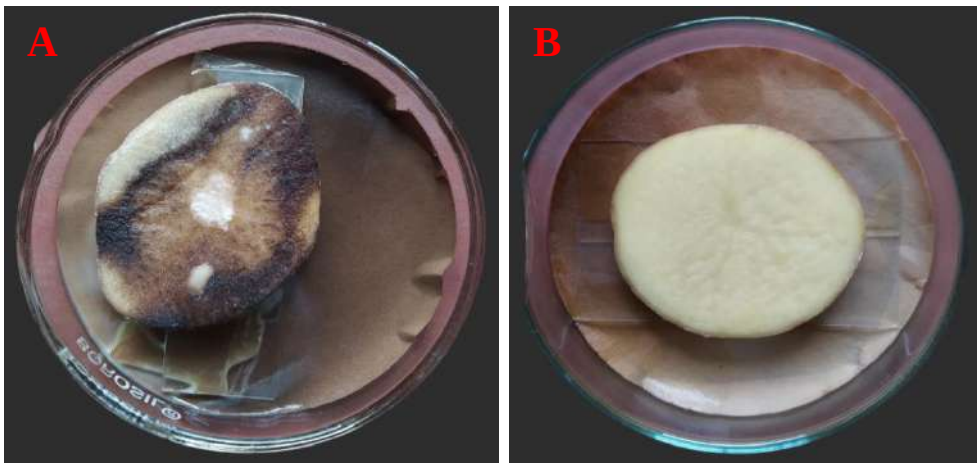

Figure 2: Pathogenicity test by potato slice method. (A) Soft rot symptoms after 4 days of inoculation; (B) Control

3.3 Antibacterial activity of leaf extracts

The zone of inhibition by different plant leaf extracts at various concentrations (50, 100, and 200 mg/mL) using different solvents (ethanol and acetone) against *Pectobacterium* sp. was shown in Table 3. Zone of inhibition was significantly different among the treatments (P = 0.000).

Table 3: Antibacterial activity of ethanol and acetone leaf extracts against the growth of *Pectobacterium* sp.

Extraction solvent	**Plant leaf extract**	**Inhibition zone diameter (mm) ± SD**		
		50 mg/mL	**100 mg/mL**	**200 mg/mL**
Ethanol extract	*C. grandis*	10.5^{i} ±0.55	12.667^{fg} ±0.52	16.5^{cd} ±0.55
	A. pomaceae	8^{j} ±0	8.667^{j} ±0.52	12.667^{fg} ±0.52
	G. sylvestre	10.67^{hi} ±0.52	12.67^{fg} ±0.52	16.83^{bcd} ±0.41
	E. subumbrans	11.67^{gh} ±0.52	14.67^{e} ±0.52	17.67^{ab} ±0.52
Acetone extract	*C. grandis*	8^{j} ±0	13.17^{f} ±0.75	17.17^{bc} ±0.75
	A. pomaceae	8^{j} ±0	10.67^{hi} ±0.52	16^{d} ±0.63
	G. sylvestre	8^{j} ±0	11^{hi} ±0.89	15.83^{d} ±0.41
	E. subumbrans	10.83^{hi} ±0.75	14.33^{e} ±0.52	18.33^{a} ±0.52
Positive control (Tetracycline - 250 ppm)		23.5 ± 0.54		
Control		8±0		

[#] Well diameter is 8 mm, and this value indicates the absence of an inhibition zone.

It was evident that ethanol extract of all plant species exhibited inhibition zones against *Pectobacterium* sp. across all tested concentrations except 50 and 100 mg/mL of ethanol extract of *A. pomaceae*. Among the ethanol extracts, *E. subumbrans* demonstrated the highest antibacterial activity, with a maximum inhibition zone of 17.67 ± 0.52 mm at 200 mg/mL, which was statistically comparable to that of *G. sylvestre* (16.83 ± 0.41 mm) and *C. grandis* (16.5 ± 0.55 mm) at the same concentration.

According to Utami [26], ethanol extract of *E. subumbrans* showed the most significant antibacterial activity against a sensitive strain of *Staphylococcus epidermidis*. Alkaloids, flavonoids, saponin, triterpenoids, isoflavones, and lectins have been reported to be present in the leaves of *E. subumbrans* [26, 27]. The presence of these compounds may indicate its potential antibacterial properties in this study.

Ethanolic, chloroform and ethyl acidic extracts of the aerial parts of *G. sylvestre* showed antibacterial effects against *Escherichia coli*, *Pseudomonas aeroginosa*, *Klebsella pneumoniae* and *Staphylococcus aureus* [28]. Ethanol leaf extract of *C. grandis* showed high antibacterial activity against *S. aureus*, *E. coli*, and *K. pneumonia* [29]. These studies align with the results of this study. *A. pomaceae* exhibited relatively lower inhibition zones compared to the ethanol extract of other leaves in this study.

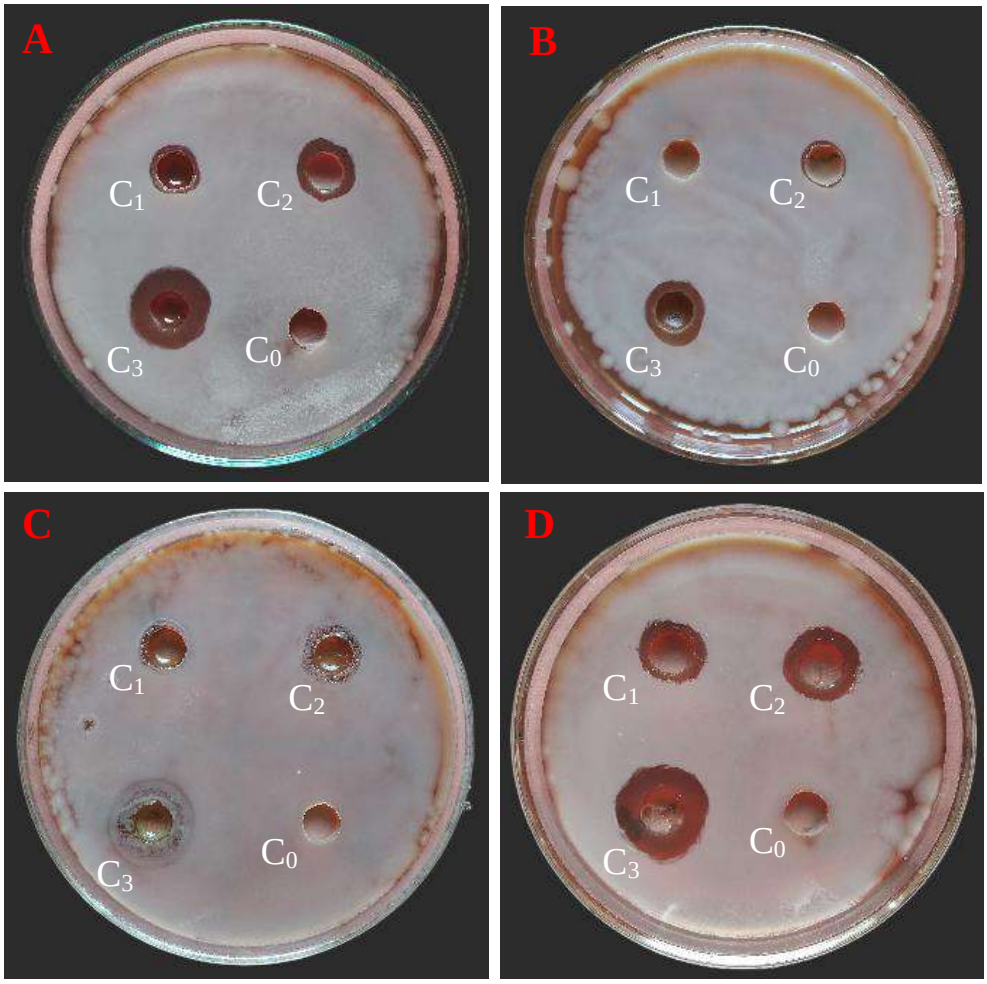

Figure 3: Effect of ethanol leaf extracts on the growth of *Pectobacterium* sp. on NA medium. (A) *C. grandis*; (B) *A. pomacea*; (C) *G. sylvestre*; (D) *E. subumbrans*. C0= Control (Ethanol); C1= 50 mg/mL; C2= 100 mg/mL; C3= 200 mg/mL.

Similarly, acetone extracts also demonstrated inhibition zones against *Pectobacterium* sp. except 50 mg/mL acetone extracts of *C. grandis*, *A. pomaceae*, and *G. sylvestre*. Acetone extract of *E. subumbrans* (18.33 ± 0.52 mm) exhibited the highest antibacterial activity at 200 mg/mL, which was statistically significant among other acetone leaf extracts.

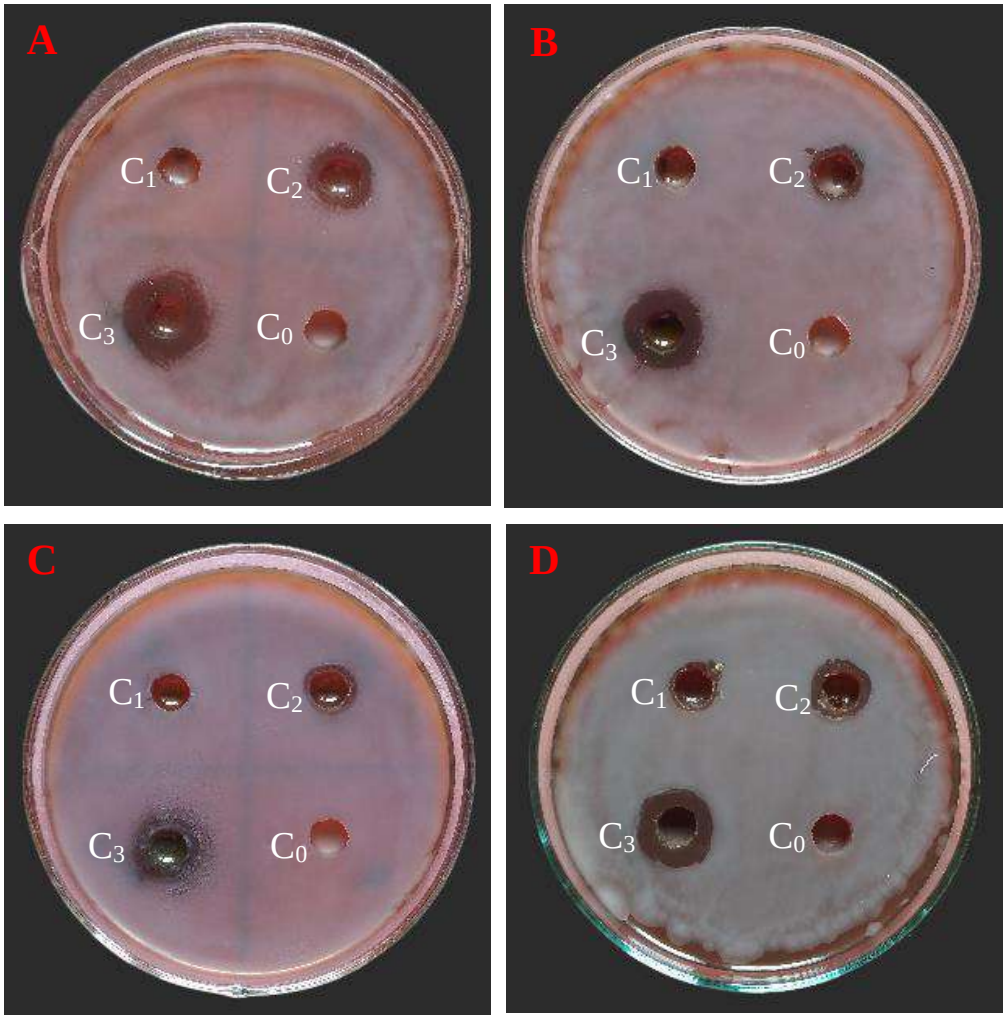

Figure 4: Effect of acetone leaf extracts on the growth of *Pectobacterium* sp. on NA medium. (A) *C. grandis*; (B) *A. pomacea*; (C) *G. sylvestre*; (D) *E. subumbrans*. C0= Control (Acetone); C1= 50 mg/mL; C2= 100 mg/mL; C3= 200 mg/mL.

Acetone extract of *C. grandis* (200 mg/mL) showed the inhibition zone of 17.17 ± 0.75 mm while the ethanol extract of *C. grandis* (200 mg/mL) showed the inhibition zone of 16.5 ± 0.55 mm. Mahendrarajah *et al*. [30] stated that *E. coli* showed higher susceptibility to *C. grandis* in ethanol and acetone extract which might be due to the

presence of alkaloids, saponins, flavonoids and tannins in both ethanol and acetone extracts of *C. grandis*, which is consistent according to the results of this study.

Acetone extract of *E. subumbrans* (18.33 ± 0.52 mm) tends to exhibit slightly larger zone of inhibition compared to ethanol extract of *E. subumbrans* (17.67 ± 0.52 mm) at the concentration of 200 mg/mL. However, the difference in efficacy between the two different solvent extracts may vary depending on the solubility of specific antibacterial compounds in respective solvents. These findings suggest that the ethanol and acetone leaf extract of *E. subumbrans* possess potential antibacterial properties against *Pectobacterium* sp.

CONCLUSION

E. subumbrans displayed consistent and considerable inhibition at all concentrations tested for both solvent extracts against *Pectobacterium* sp.. Based on the results, it can be concluded that the acetone and ethanol extracts of *E. subumbrans*, the ethanol extract of *G. sylvestre*, and both extracts of *C. grandis* at a concentration of 200 mg/mL possess potent antibacterial compounds against *Pectobacterium* sp., demonstrating superior efficacy compared to the other tested plant leaf extracts. Therefore, further investigation into the specific bioactive compounds responsible for this activity could lead to the development of effective natural antibacterial treatments for plant pathogen.

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