



## RESEARCH PAPER

## OPEN ACCESS

# Isolation of Gram -ve Bacteria from the Root Extracts of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii*

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## Abstract

Gram -ve bacteria is highly virulent as compared to gram +ve microbes due to presence of outer membrane, lipoproteins and also due to the endotoxins they produce. Hence, their isolation and detailed studies on antimicrobial agents that can kill them is a challenging task. Earlier studies on *Proteus spp.* (*P.mirabilis*) has showed positive effect on growth of the plants like *Lycopersicon esculentum* Mill, commonly referred as Tomato plant and proven to be effective on plant development. In recent studies, we have isolated Gram -ve bacillus (*Proteus spp.*) from the root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *K. pneumoniae* from *Murray koenigii* by plating serially diluted root extract samples on MacConkey agar and MacConkey Agar w/o CV, NaCl w/ 0.5% sodium taurocholate medium. The bacterial strains isolated were identified to be Gram -ve bacilli characterised using biochemical tests and Gram staining technique. The bacilli isolated from the rootlets of *Kalanchoe pinnata* and *Euphorbia tithymaloides* were found to be motile and identified as *P.mirabilis* and in case of *Murray koenigii*, the isolated bacilli were found to be non-motile and identified as Gram -ve *K. pneumoniae*.

**Keywords:** Gram -ve; Bacteria; Roots; Extracts; *Kalanchoe pinnata*; *Euphorbia tithymaloides*; *Murray koenigii*

## Introduction

From the studies by Aibinu et al. (2007), leaf extracts of *Bryophyllum pinnatum* and *Kalanchoe crenata* are known to possess anti-microbial compounds effective against both gram positive and gram-negative bacteria and proved to be ineffective against fungal pathogens like *Candida albicans*. From the studies of Mishra et al. (2022) the anti-bacterial and antioxidant activity of hydro-alcoholic seed extracts of *Kalanchoe pinnata* and *Pongamia pinnata* is majorly due to presence of high amounts of secondary metabolites in their seed components.

*Kalanchoe daigremontiana* hydroalcoholic extracts proven to contain anti-helminthic activity on *Haemonchus contortus*. From the studies of Rivero-Pérez et al. (2022), the plant components have been found to have potent anti-biological activity majorly on larval egg hatching and proven to be effective anti-bacterial agents on the pathogens like *L. monocytogenes*. The studies on motility of the worm and larval mortality were carried out by the researcher as a part of biological control strategy. *Kalanchoe pinnata* is one of the medicinal plants commonly used for traditional medicine and known to possess various pharmacology properties including anti-inflammatory, antioxidant and cytotoxicity and whereas the cytotoxic nature of the plant flowers is due to the high flavonoid content present in them, were identified based on the studies carried out by Romero et al. (2023).

*Euphorbia tithymaloides* commonly referred as Devil's back bone is one of the medicinal plants which gained biomedical importance but often confused by other plants of the same species as a



medical plant substitution. Patil et al. (2023) has performed both macroscopic and microscopic studies on the root extracts and remaining plant parts and contributed for easy identification of the plant *Euphorbia tithymaloides* based on the biological components of the plant that can be extracted using organic solvents. *Euphorbia tithymaloides* belongs to the family Euphorbiaceae and shown to possess anti-helminthic, antifungal and anti-inflammatory properties and the plant parts are used to treat sexual transmitted diseases and skin diseases like warts and autoimmune disorder vitiligo. Leaves of the plant can be taken orally whereas root sample and whole plant can be applied externally as a treatment for ailments {OSADHI (neist.res.in)}. *Euphorbia tithymaloides* possess major composition of secondary metabolites like phenols and triterpenoids and proven be effective against (MRSA) Methicillin resistant *Staphylococcus aureus* (Anyam et al., 2024) infections.

*Murray koenigii* is well known as a traditional plant and used as spice powder and curry leaves found to contain medicinal properties that can act as an aid to cure diabetes, diarrhoea, cancer, HIV, ulcer, obesity, inflammation and skin related problems (Suthar et al., 2022; Sivakumar et al., 2023). *Murray koenigi* root extracts showed in-vitro antimicrobial activity against bacteria like *S. aureus* and fungus *Trichophyton rubrum* and can be formulated for usage as an anti-microbial agent (Malwal et al., 2011). *Murray koenigii* root extracts can be used for treatment of diarrhoea and its anti-diarrhoeal activity was studied in diarrhoea induced albino rats (Pagariya et al., 2009).

### Material and Methods

#### **Serial dilution of sample collected from root extracts of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii***

Freshly collected root lets of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* were obtained from the medicinal field area of Sree Vidyanikethan Pharmacy college and the roots of the plants were washed with distilled water of about 50ml and the sample collected was serially diluted ( $10^{-1}$  to  $10^{-9}$ ) using distilled water and  $10^{-7}$  tube of the serially diluted sample was used for plating on agar media for isolation of microbiota.

#### **Plating of microbes on MacConkey agar**

0.1 ml of serially diluted samples were plated on the MacConkey agar and MacConkey Agar w/o CV, NaCl 0.5% Sodium taurocholate to restrict the swarming motility of the *Proteus spp.* in the later and placed in incubator at 37°C for 24hrs.

#### **Characterization of microbiota isolated from root extracts of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* by using Gram staining technique**

MacConkey agar plates and MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate plates observed with microbial growth are subjected to characterization as Gram Positive and Gram negative using Gram staining Procedure explained in brief below.

Thin smear of bacterial culture is made over the slide and subjected to heat fixing. Heat fixed slides were flooded with crystal violet and incubated for 1 min. After 1min the slide is subjected to washing under running tap water and flooded with Iodine solution for 1min and similarly with acetone or ethanol for 15-30 sec and counter stained with saffranin for 1 min, subjected to washing with distilled water, air dried and observed under 20X objective lens using bright field microscope.

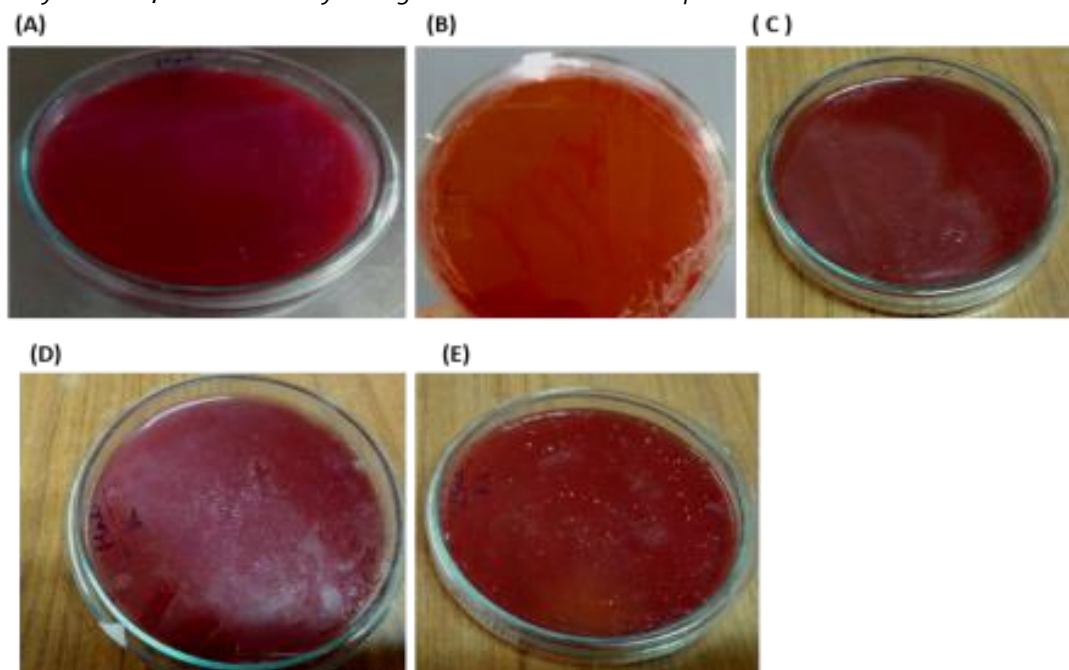
#### **Characterisation of Bacteria isolated from root extracts of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* using chromogenic substrates**

MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate medium is prepared and sterilized using Autoclave. Before to the transfer of medium to the petri plates about 0.1 to 0.5g of Ferrous sulphate and tryptophan is added to the medium under sterile conditions in the laminar air flow chamber and mixed properly. Mixed agar medium is poured in to different petri plates and allowed to solidify. To the solidified plates 0.1 ml of bacterial culture is transferred aseptically and allowed for 24 hrs incubation at 37° c in the incubator. The bacterial culture grown on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate were sub-cultured in nutrient broth and used as bacterial culture for inoculation on chromogenic substrate.

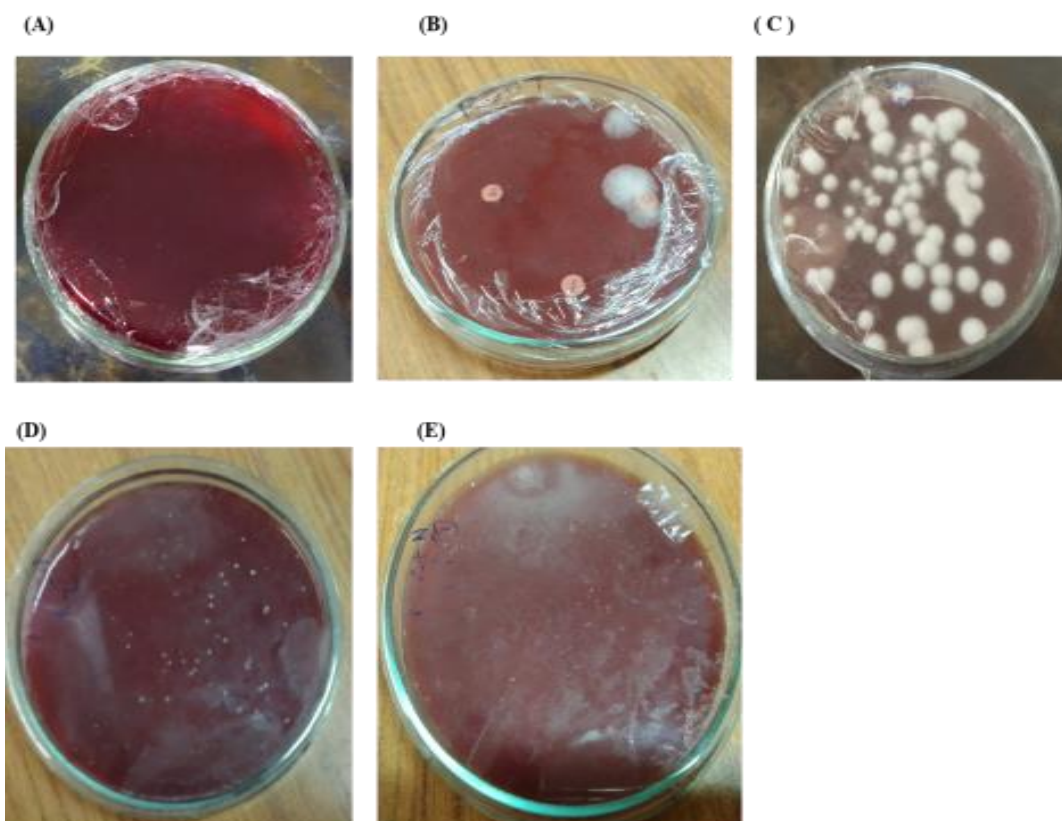
### Results

Root samples are collected from the medicinal plant garden and the gram-negative bacteria colonized on the root surface are isolated using MacConkey agar. In figure 1 the first plate is the control with absence of bacterial growth on the surface. Whereas 1B indicates the mixed sample prepared by sample from all the three plant root surfaces. In the figure we can clearly identify the

swarming motility of the *Proteus Spp.* 1C is the bacteria isolated from the root surface of *Euphorbia tithymaloides*, 1D from *Murray koenigii* and 1E from *Kalanchoe pinnata*.



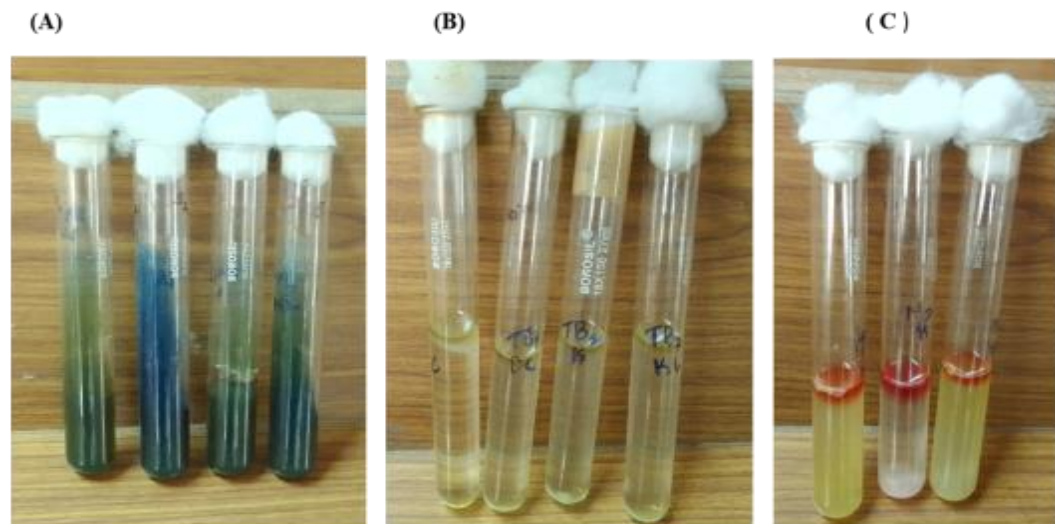
**Fig. 1.** Isolation of Gram negative bacteria on MacConkey agar (A) Control plate, (B) Gram negative bacteria showing swarming motility on MacConkey agar isolated from the mixed root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* (C) Gram negative bacteria isolated from the roots of *Euphorbia tithymaloides* (D) Gram negative bacteria isolated from the roots of *Murray koenigii* (E) Gram negative bacteria isolated from the roots of *Kalanchoe pinnata*



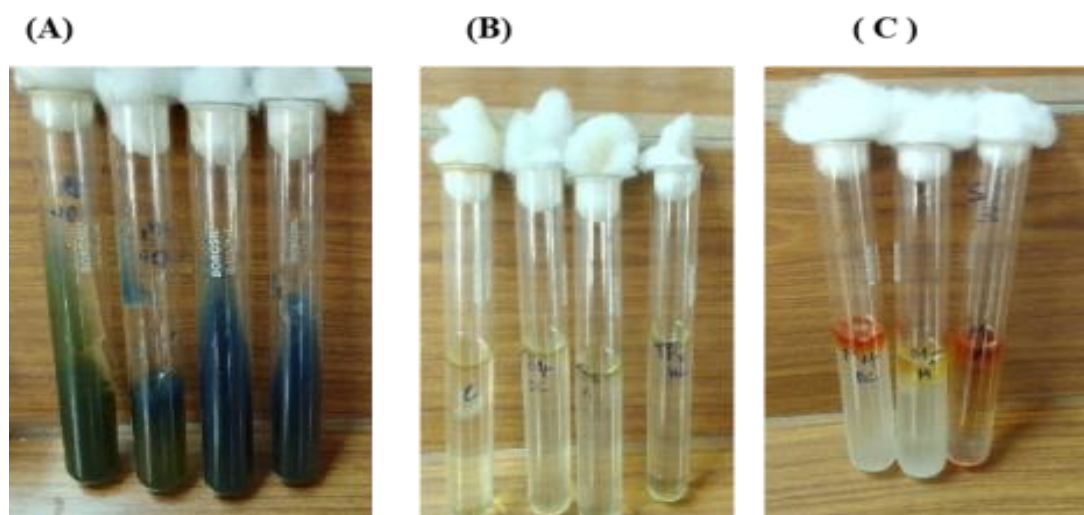
**Fig. 2.** Isolation of Gram-negative bacteria on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate (A) Control plate, (B) Gram negative bacteria isolated from the mixed root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* (C) Gram negative bacteria isolated from the roots of *Euphorbia tithymaloides* (D) Gram negative bacteria isolated from the roots of *Murray koenigii* (E) Gram negative bacteria isolated from the roots of *Kalanchoe pinnata*.

Figure 2A is the control plate with no bacteria on the surface whereas figure 2 B is the bacteria grown on the agar plate inoculated with the serially diluted sample collected by mixing the root washings from all the three plants. Figure 2C is the bacteria isolated from the plant *Euphorbia tithymaloides*. *Euphorbia* is well known for its medicinal properties and referred with common name Devil's back bone. Figure 2D & 2E are plates inoculated with serially diluted sample of roots of *Murray koenigii* and *Kalanchoe pinnata*

Figure 3 includes IMVIC tests performed with bacterial cultures isolated on MacConkey agar. 3A represents the citrate utilisation test and the test results are found to be positive with all the three bacterial samples. 3B represents the Indole test using tryptone broth and all the three bacterial samples tested negative for the test and 3C is the methyl red test and all the three bacterial found to be positive with the test. So, the bacteria isolated may be *Proteus spp.* especially *P. mirabilis* as it is a non-lactose fermenter.



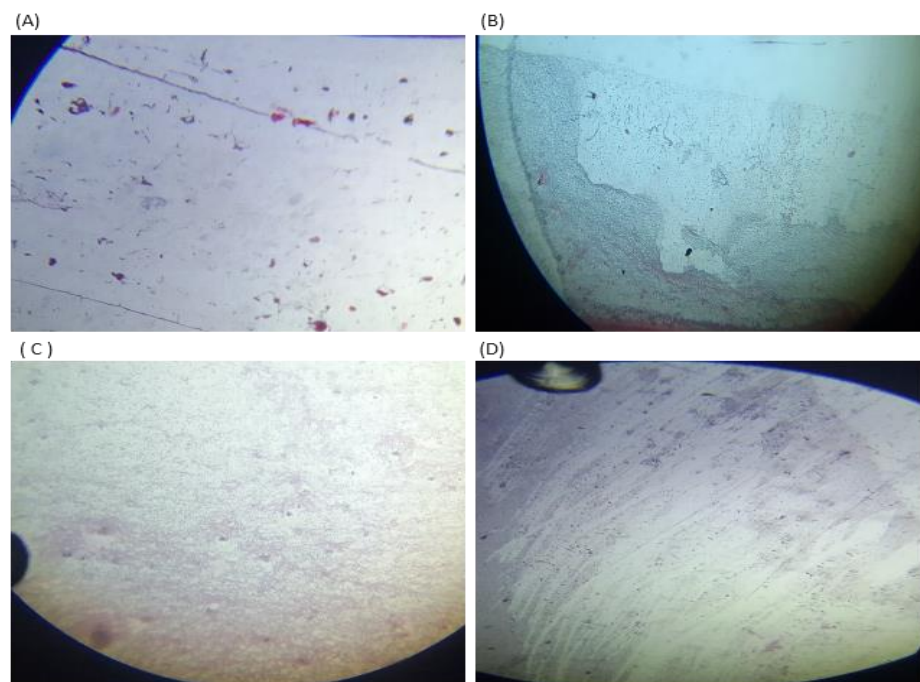
**Fig. 3** IMVIC tests with bacteria isolated on MacConkey agar plates. (A) Citrate Utilisation Test (B) Indole Test and (C) Methyl red Test



**Fig. 4.** IMVIC tests with bacteria isolated on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate plates. (A) Citrate Utilisation Test (B) Indole Test and (C) Methyl red Test

The bacteria isolated using MacConkey agar is sub cultured on to agar plates of MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate to prevent swarming motility of *Proteus spp.* and after observing definite bacterial growth the bacterial cultures are characterized using IMVIC tests. From the figure 4A, 4B, 4C the bacterial samples tested positive with citrate utilisation test and methyl red test. Root samples isolated from *Murray koenigii* has showed negative results with methyl red test and Indole test and hence confirmed to be *K. pneumoniae* a gram negative, non-motile rod-shaped bacilli but not *E. coli*. All the three bacterial samples isolated showed negative results with indole test (4C).

Characterisation of bacteria as gram Positive and gram negative were performed using Gram staining technique and the bacteria isolated from the root samples of *Kalanchoe pinnata* and *Euphorbia tithymaloides* were identified as Gram negative, motile rod shaped bacilli where from *Murray koenigi*. It is identified as gram negative, non-motile and rod shaped bacilli (MacConkey agar and MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate).



**Fig. 5.** Gram staining of bacteria isolated from root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigi* plated on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate (A) Mixed root sample of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigi* (B) *Euphorbia tithymaloides* (C) *Murray koenigi* (D) *Kalanchoe pinnata*

**Table 1.** Biochemical characterisation of bacterial strains *E. coli*, *Proteus mirabilis*, *Proteus vulgaris* and *Klebsiella pneumoniae* through IMVIC Tests

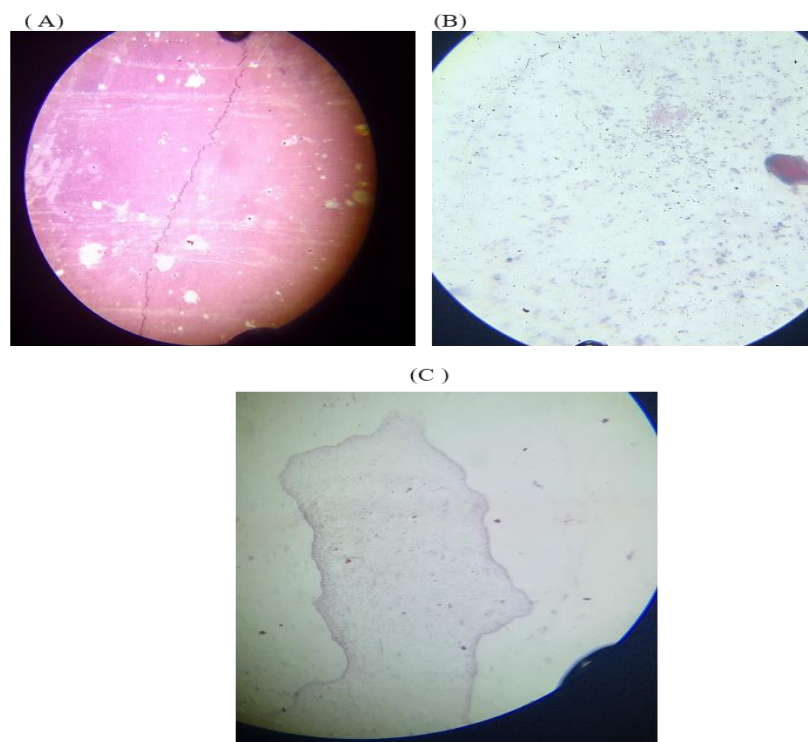
S.No.	Bacterial Strain	Indole Test	Citrate Utilisation Test	Methyl red test	Motility
1.	<i>E. coli</i>	+ve	-ve	+ve	Motile
2.	<i>Proteus mirabilis</i>	-ve	+ve	+ve	Motile
3.	<i>Proteus vulgaris</i>	+ve	+ve	+ve	Motile
4.	<i>Klebsiella pneumoniae</i>	-ve	+ve	-ve	Non-motile

Feso<sub>4</sub> and Tryptophan are used as Chromogenic substrates for the identification of *Proteus spp.* *Proteus mirabilis* and *K. pneumoniae* formed brown coloured colonies on the agar plate. *P. mirabilis* is the gram negative motile bacilli isolated and found to be present associated with the root samples of *Kalanchoe pinnata* and *Euphorbia tithymaloides* whereas *Klebsiella pneumoniae* found to be colonized on the root surface of *Murray koenigi*.

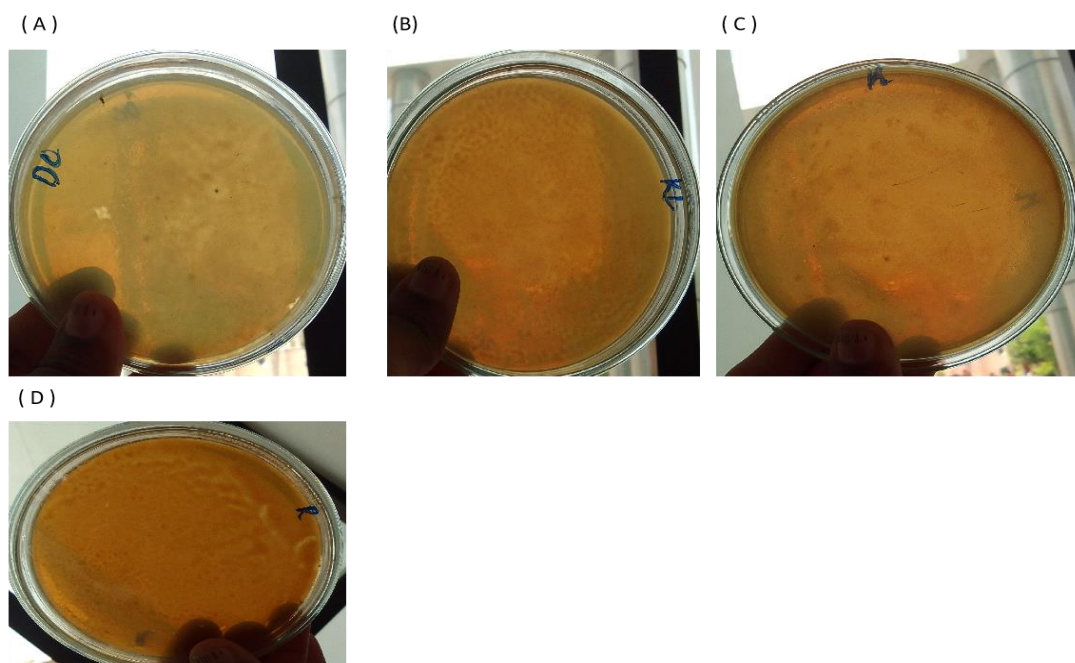
#### Discussion and Conclusion

*Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigi* plants are normally used as treatment for ailments in the medical field. *P. mirabilis* proved to be effective for promoting the plant growth in Tomato (*Lycopersicon esculentum* Mill) plant base on the studies carried out by Amaresan et al., (2021) and isolation, studies on the microbiota associated with the root surface of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigi* can add scientific value to the literature.

Leaves, Bark and roots of *Murray koenigi* proven to possess anthelmintic, anti-inflammatory, antiseptic and anti-diarrhoeal activities and contains astringent, aromatic and bitter taste (Pagariya et al., 2009). Present findings on *Murray koenigi* root microbiota scientifically proved the association of *K. pneumoniae* bacteria with the root. *K. pneumoniae* is a non-motile gram negative rod shaped bacilli and induces pneumonia in the humans.



**Fig. 6.** Gram staining of bacteria isolated from root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* plated on MacConkey Agar. (A) *Euphorbia tithymaloides* (B) *Murray koenigii* (C) *Kalanchoe pinnata*



**Fig. 7.** Characterisation of Gram -ve bacteria on root samples of *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii* on MacConkey Agar w/o CV, NaCl w/ 0.5% Sodium Taurocholate using chromogenic substrates Feso<sub>4</sub> and Tryptophan (A) *Euphorbia tithymaloides* (Devil's choke) (B) *Kalanchoe pinnata* (Kalanchoe) and (C) *Murray koenigii* (Karvepaku) and (D) Mixed root sample of three plants *Kalanchoe pinnata*, *Euphorbia tithymaloides* and *Murray koenigii*

*Euphorbia tithymaloides* is a medicinal plant used for the treatment of fever, inflammation and tumors. The plant possesses various pharmacological properties like anti-inflammatory, anti-tumor, anti-diabetic, anti-cancer, antileishmanial, anti-malarial, anti-helminthic, anti-microbial, antioxidant, anti-ulcerogenic, and cytotoxicity (Srivastava et al., 2019; Mavundza et al., 2022). *Proteus mirabilis* is one of the microbiota found to be associated with the root layers and may be responsible for the better plant growth.

*Kalanchoe pinnata* is generally used for the treatment of bacterial, fungal, and viral infections and as well as asthma, kidney stones, inflammatory problems, and ulcers. *Kalanchoe pinnata* is well known to possess various pharmacological properties like anti-inflammatory (Nascimento, 2018), immunosuppressant, nephroprotective (Harlalka et al., 2007), antihypertensive (Ghasi et al., 2011), antimicrobial (Okwu et al., 2011), anti-leishmanial (Muzitano et al., 2011), healing (Nayak et al., 2010), anti-helminthic (Majaz et al., 2011), analgesic, anticonvulsant (Nguelefack et al., 2006), antitumor (Mahata et al., 2012; Majaz et al., 2011; Supratman et al., 2001) and contain various flavanoids and flavanol chemical compounds associated with it. Further research on the medicinal plants and the associated microbiota with the plants can help in formulating better medicines or drugs to treat the ailments for the better survival of the mankind.

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#### Author Contributions

EB, MDS, VN and STB conceived the concept, wrote and approved the manuscript.

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#### Competing interest

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Not applicable.



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