

**MOLECULAR CHARACTERIZATION OF
POST HARVEST PATHOGENS OF MANGO
(*Mangifera indica* L.) AND POMEGRANATE
(*Punica granatum* L.) AND EVALUATION OF
PLANT EXTRACTS FOR THEIR MANAGEMENT**

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PALB 8305

**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE**

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Thesis submitted to the

UNIVERSITY OF AGRICULTURAL SCIENCES, BANGALORE

In partial fulfillment of the requirements

for the award of the Degree of

MASTER OF SCIENCE (Agriculture)

in

PLANT BIOTECHNOLOGY

BENGALURU

FEBRUARY, 2021



Affectionately Dedicated to
my Beloved Father
Jambunath Saraganacharya
my Mother
Meenakshi Saraganacharya
and Sister
Swati Saraganacharya

**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES**

BANGALORE-65

CERTIFICATE

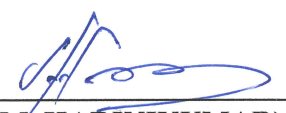
This is to certify that the thesis entitled **MOLECULAR CHARACTERIZATION OF POST HARVEST PATHOGENS OF MANGO (*Mangifera indica* L.) AND POMEGRANATE (*Punica granatum* L.) AND EVALUATION OF PLANT EXTRACTS FOR THEIR MANAGEMENT** submitted in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, Bangalore, is a *record of bonafide* research work done by **Ms. SOUMYA, J. SARAGANACHARYA**, I. D. No. **PALB 8305**, during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award for any degree, diploma, associate ship, fellowship or other similar titles.

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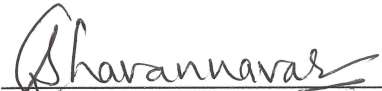

(K. M. HARINIKUMAR)
Major advisor

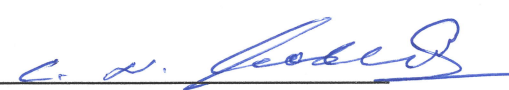
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ABSTRACT

Aspergillus rot of mango and bacterial blight of pomegranate are major diseases affecting the fruit quality and shelf life during storage. In the present investigation *Aspergillus* sp and *Xanthomonas* sp were isolated from mango and pomegranate, respectively were characterized. The bacteria isolated was gram negative, positive for starch hydrolysis, gelatin liquefaction, KOH solubility and negative for methyl red test. The surface colour of the fungal colonies were brown to black with smooth mat having white margin. Further, the 16SrRNA and ITS region sequences were used for identification of bacteria and fungus, respectively. Based on the morphological characters and sequence information, fungus isolated from mango was confirmed as *Aspergillus niger* and bacteria isolated from pomegranate as *Xanthomonas axonopodis* pv. *punicae*. The extracts from three plant species (*Tridax procumbens*, *Lantana camara* and *Eupatorium odoratum*) in different solvents were tested for inhibition efficacy against these pathogens using poisoned food technique for fungus and agar disc diffusion method for bacteria. The maximum inhibition of *Aspergillus niger* growth was observed for methanol extract of *Tridax procumbens* (78.07 %) followed by methanol extract of *Lantana camara* (67.70%) and chloroform extract of *Eupatorium odoratum* (61.10%). In case of *Xanthomonas axonopodis* pv. *punicae* also maximum zone of inhibition was observed with extracts shown maximum efficacy against fungi. The qualitative screening of selected plant extracts indicated the presence of tannins, phenol, flavonoids, terpenoids, saponins and alkaloids. The study indicates that these compounds could be the reason for potential antimicrobial activity of plant extracts.

February, 2021

Department of Plant Biotechnology
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K. M. HARINIKUMAR
Major Advisor

**ಮಾವಿನ (ಮ್ಯಾಂಗಿಫೆರಾ ಇಂಡಿಕಾ ಎಲ್.) ಮತ್ತು ದಾಳಿಂಬೆಯ
(ಫ್ಯುನಿಕಾ ಗ್ರಾನಟಮ್ ಎಲ್.) ಕಟಾವು ನಂತರದ ರೋಗಕಾರಕಗಳ ಆಣ್ವಿಕ
ಗುಣಲಕ್ಷಣಗಳು ಮತ್ತು ಅವುಗಳ ನಿರ್ವಹಣೆಗೆ ಸಸ್ಯದ ಸಾರಗಳ ಮೌಲ್ಯಮಾಪನ**

ಸೌಮ್ಯ, ಜೆ. ಸರಗನಾಚಾರ್ಯ

ಸಾರಾಂಶ

ಮಾವಿನ ಆಸ್ಪರ್ಜಿಲಸ್ ಕೊಳೆತ ಮತ್ತು ದಾಳಿಂಬೆಯ ಬ್ಯಾಕ್ಟೀರಿಯಾದ ರೋಗವು ಶೇಖರಣೆಯ ಸಮಯದಲ್ಲಿ ಹಣ್ಣಿನ ಗುಣಮಟ್ಟವನ್ನು ಕಡಿಮೆ ಮಾಡುವ ಪ್ರಮುಖ ರೋಗಗಳಾಗಿವೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಆಸ್ಪರ್ಜಿಲಸ್ ಮತ್ತು ಕ್ಸಾಂಥೋಮೊನಾಸ್ ರೋಗಕಾರಕಗಳನ್ನು ಕ್ರಮವಾಗಿ ಮಾವು ಮತ್ತು ದಾಳಿಂಬೆಗಳಿಂದ ಪ್ರತ್ಯೇಕಿಸಲಾಯಿತು, ಇವುಗಳನ್ನು ಕ್ರಮವಾಗಿ ನಿರೂಪಿಸಲಾಗಿದೆ. ಬ್ಯಾಕ್ಟೀರಿಯಾ ಗ್ರಾಂ ನಕಾರಾತ್ಮಕವಾಗಿತ್ತು. ಇದು ಪಿಷ್ಟ ಜಲವಿಚ್ಛೇದನೆ, ಜೆಲಾಟಿನ್ ದ್ರವೀಕರಣ, ಕೆಫಹೆಚ್ ಕರಗುವಿಕೆ ಮತ್ತು ಮೀಥೈಲ್ ಕೆಂಪು ಪರೀಕ್ಷೆಗೆ ನಕಾರಾತ್ಮಕ ಪರೀಕ್ಷೆಯನ್ನು ತೋರಿಸಿದೆ. ಶಿಲೀಂಧ್ರಗಳ ವಸಾಹತುಗಳ ಮೇಲ್ಮೈ ಬಣ್ಣ ಕಂದು ಬಣ್ಣದಿಂದ ಕಪ್ಪು ಬಣ್ಣದ್ದಾಗಿದ್ದು ನಯವಾದ ಚಾಪೆ ಬಿಳಿ ಅಂಚು ಹೊಂದಿತ್ತು. ಇದಲ್ಲದೆ, 16 ಎಸ್‌ಆರ್‌ಎಫ್‌ಎ ಮತ್ತು ಐಟಿಎಸ್ ಪ್ರದೇಶದ ಅನುಕ್ರಮಗಳನ್ನು ಕ್ರಮವಾಗಿ ಬ್ಯಾಕ್ಟೀರಿಯಾ ಮತ್ತು ಶಿಲೀಂಧ್ರವನ್ನು ಗುರುತಿಸಲು ಬಳಸಲಾಗುತ್ತಿತ್ತು. ರೂಪವಿಜ್ಞಾನದ ಪಾತ್ರಗಳು ಮತ್ತು ಅನುಕ್ರಮ ಮಾಹಿತಿಯ ಆಧಾರದ ಮೇಲೆ, ಮಾವಿನಿಂದ ಪ್ರತ್ಯೇಕಿಸಲ್ಪಟ್ಟ ಶಿಲೀಂಧ್ರವನ್ನು ಆಸ್ಪರ್ಜಿಲಸ್ ನೈಜರ್ ಮತ್ತು ದಾಳಿಂಬೆಯಿಂದ ಬ್ಯಾಕ್ಟೀರಿಯಾವನ್ನು ಕ್ಸಾಂಥೋಮೊನಾಸ್ ಆಕ್ಸೊನೊಪೊಡಿನ್ ಪಾಥೋವರ್ ಫ್ಯುನಿಕೇ ಎಂದು ಪ್ರತ್ಯೇಕಿಸಲಾಗಿದೆ ಎಂದು ದೃಢಪಡಿಸಲಾಯಿತು. ಸಸ್ಯಗಳ ಸಾವಯವ ದ್ರಾವಕ ಸಾರಗಳ ಎಲ್ಲಾ ಸಾಂದ್ರತೆಗಳು (ಟ್ರಿಡಾಕ್ಸ್ ಫ್ರೋಕ್ಯೂಂಬೆನ್ಸ್, ಲ್ಯಾಂಟಾನಾ ಕ್ಯಾಮರಾ ಮತ್ತು ಯುಪೋಟೋರಿಯಮ್ ಒಡರಟಮ್) ಶಿಲೀಂಧ್ರಕ್ಕೆ ವಿಷಕಾರಿ ಆಹಾರ ತಂತ್ರ ಮತ್ತು ಬ್ಯಾಕ್ಟೀರಿಯಾಗಳಿಗೆ ಅಗರ್ ಡಿಸ್ಕ್ ಪ್ರಸರಣ ವಿಧಾನದಿಂದ ಪರೀಕ್ಷಿಸಲ್ಪಟ್ಟ ಪ್ರತಿಬಂಧದ ವಿಭಿನ್ನ ವಲಯಗಳನ್ನು ಉತ್ಪಾದಿಸಿತು. ಟ್ರಿಡಾಕ್ಸ್ ಫ್ರೋಕ್ಯೂಂಬೆನ್ಸ್‌ನ ಮೆಥನಾಲ್ ಸಾರ ನಂತರ ಲ್ಯಾಂಟಾನಾ ಕ್ಯಾಮರಾದ ಮೆಥನಾಲ್ ಸಾರವು ಹಾಗು ಯುಪೋಟೋರಿಯಮ್ ಒಡರಟಮ್, ಕ್ಲೋರೋಫಾರ್ಮ್ ಸಾರವು ರೋಗಕಾರಕಗಳಾದ ಆಸ್ಪರ್ಜಿಲಸ್ ನೈಜರ್ ಮತ್ತು ಕ್ಸಾಂಥೋಮೊನಾಸ್ ಆಕ್ಸೊನೊಪೊಡಿನ್ ಪಾಥೋವರ್ ಫ್ಯುನಿಕೇ ಬೆಳವಣಿಗೆಯನ್ನು ಪ್ರತಿಬಂಧಿಸಿದವು. ಆಯ್ದ ಸಸ್ಯದ ಸಾರಗಳ ಗುಣಾತ್ಮಕ ತಪಾಸಣೆಯು ಟ್ಯಾನಿನ್ಗಳು, ಫೀನಾಲ್, ಫ್ಲೇವನಾಯ್ಡ್‌ಗಳು, ಟರ್ಪೆನಾಯ್ಡ್‌ಗಳು, ಸಪೋನಿನ್‌ಗಳು ಮತ್ತು ಆಲ್ಕಲಾಯ್ಡ್‌ಗಳ ಉಪಸ್ಥಿತಿಯನ್ನು ಸೂಚಿಸುತ್ತದೆ. ಈ ಸಂಯುಕ್ತಗಳು ಸಸ್ಯದ ಸಾರಗಳ ಸಂಭಾವ್ಯ ಅಂಟಿಮೈಕ್ರೋಬಿಯಲ್ ಚಟುವಟಿಕೆಯ ಕಾರಣವಾಗಿರಬಹುದು ಎಂದು ಅಧ್ಯಯನವು ಸೂಚಿಸುತ್ತದೆ.

ಫೆಬ್ರವರಿ, ೨೦೨೦

ಸಸ್ಯ ಜೈವಿಕ ತಂತ್ರಜ್ಞಾನ ವಿಭಾಗ
ಕೃಷಿ ವಿಶ್ವವಿದ್ಯಾನಿಲಯ, ಬೆಂಗಳೂರು

(ಕೆ. ಎಂ. ಹರಿಣಿಕುಮಾರ್)
ಪ್ರಧಾನ ಸಲಹೆಗಾರರು



Molecular characterization of Post harvest pathogens of Mango (*Mangifera indica* L.) and Pomogranate (*Punica granatum* L.) and evaluation of plant extracts for their management.



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Introduction

- India has wide range of climate and physico-geographical conditions that are suitable for cultivation of horticultural crops like fruits, vegetables, flowers, nuts, spices and plantation crops. India ranks as world's second largest fruit producer. The significant fruit crops that are consumed and exported on a larger scale are mango and pomegranate.
- Mango (*Mangifera indica* L.) belongs to the Anacardiaceae family of flowering plants. Due to its large scale cultivation, mango is called the 'King of fruits'. The most dangerous post-harvest disease in Mango is the black mould caused by *Aspergillus niger*, reducing the quality of the fruit. Pomegranate (*Punica granatum* L.) known as "Fruit of Paradise" and "vital cash crop" belongs to the Punicaceae family and is native to Iran. *Xanthomonas axonopodis* pv. *punicae* induced bacterial blight is a major disease that causes a huge loss of crop productivity in pomegranate.
- Indiscriminate use of synthetic pesticides over the years has resulted different types of hazards and toxicity. Hence Phytochemicals are used as alternative because of their eco-friendly nature. An attempt is made to screen the effect of different solvent extracts of Weeds (*T. procumbens*, *L. camara* and *E. odoratum*) against pathogen isolates.

Objectives

1. Morphological and Molecular characterization of pathogen isolates.
2. Evaluation of Plant extracts against Isolates

Materials and Methods

- Morphological characterization was done by observing the colony morphology for size, shape, colour and seen under microscope. The DNA from bacteria was extracted using alkaline lysis method and CTAB method for fungus.
- Molecular characterization of Fungus was done by amplification of ITS primers and 16S rRNA for bacteria.
- Solvent extracts of different weeds at their different concentrations were screened for antifungal activity by poison food technique and Agar disc diffusion method for bacteria.
- Qualitative phytochemical screening and Thin Layer Chromatography was done for elution of secondary metabolites present solvent extracts of different plants (*T. procumbens*, *L. camara*, *E. odoratum*).

Table 1: Botanical name, common name and plant part of Weeds used in experiment

Sl.No	Botanical name	Common name	Part used	Family
01	<i>Tridax procumbens</i>	Coat button/Ghamra	Leaf and Flowers	Asteraceae
02	<i>Lantana camara</i>	Big sage	Leaf and Flowers	Verbenaceae
03	<i>Eupatorium odoratum</i>	Siam weed	Leaves	Asteraceae

Results



Fig 1: Pure culture and microscopic view of *A. niger* and *X. axonopodis*

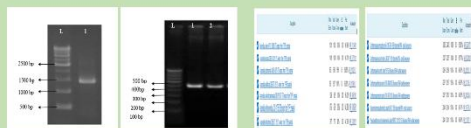


Fig 2: PCR profile and NCBI Blast result of *A. niger* and *X. axonopodis*

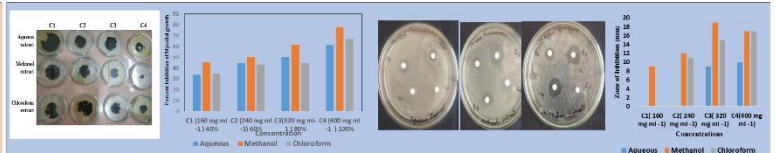


Fig 3: Effect of aqueous, methanol and chloroform extracts of *T. procumbens* against colony growth of *A. niger*

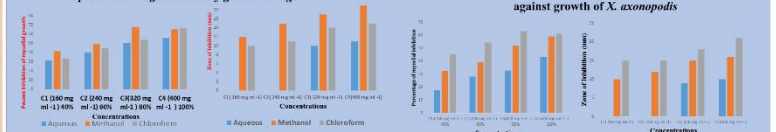


Fig 4: Effect of aqueous, methanol and chloroform extracts of *T. procumbens* against growth of *X. axonopodis*

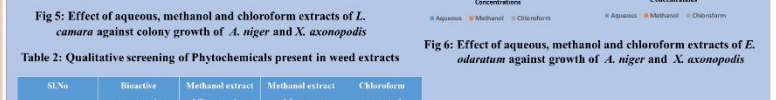


Fig 5: Effect of aqueous, methanol and chloroform extracts of *L. camara* against colony growth of *A. niger* and *X. axonopodis*



Fig 6: Effect of aqueous, methanol and chloroform extracts of *E. odoratum* against growth of *A. niger* and *X. axonopodis*

Table 2: Qualitative screening of Phytochemicals present in weed extracts

Sl.No	Bioactive compounds	Methanol extract of <i>T. procumbens</i>	Methanol extract of <i>L. camara</i>	Chloroform extract of <i>E. odoratum</i>
1	Tannins	+	+	+
2	Phenol	+	+	+
3	Flavonoids	+	+	+
4	Terpenoids	+	+	+
5	Saponins	+	+	+
6	Glycosides	+	+	+
7	Alkaloids	+	+	+
8	Anthocyanins	+	+	+
9	Steroids	+	+	+
10	Coumarins	+	+	+
11	Lavandins	+	+	+
12	Quinones	+	+	+

- A. niger*: The surface colour of the colony was Black to dark brown, the growth was rapid and there was formation of exudates. The gel eluted picture has amplified band around 1400 bp and NCBI blast result showed 98.96 % match with *A. niger*. *X. axonopodis*: The colour of the colony was yellow with 2mm in size. There was 98.81% homology match from NCBI blast results with *X. axonopodis*.
- The methanol extract of *T. procumbens* and *L. camara* has highest rate of inhibition of 78.07% and 67.7% at 400 mg ml⁻¹ and 320 mg ml⁻¹ respectively, and chloroform extract of *E. odoratum* has 63.3% of inhibition at 320 mg ml⁻¹.
- Concentration: These results were true with *X. axonopodis*. It was inferred that, as the concentration increases the rate of inhibition increases.
- The Qualitative phytochemical screening represented the presence of Tannins, Phenols, Flavonoids, Terpenoids, Alkaloids and saponins in methanol extract of *T. procumbens*, *L. camara* and Chloroform extract of *E. odoratum*.
- Thin Layer Chromatography analysis indicated the presence of more number of bands in methanol extracts of selected plants. TLC Solvents S2 (Toluene :Ethyl acetate :Methanol) in the ratio of 24:24:2 and S3 (Hexane: Ethyl acetate) in the ratio of 9:1 was more effective for elution of bands clearly with high Rf value.

Discussion

- The similar results were quoted by Ramesh Babu *et al.* (2018) where the ethanolic and crude extracts were tested for antifungal activity of *T. procumbens* against *A. niger* and proved the antimicrobial property. *Tridax procumbens* leaves revealed the presence of medicinally active constituents.
- Results of present study are in confirmation with the results of (Manenji *et al.*, 2017) who tested antifungal activity of *L. camara* plant extract against *Rhizopus stolonifer* was evaluated *in vitro* condition (poison food technique). There was an interaction between botanical plant type and concentration rate. *L. camara* at 60% concentration was most effective in controlling *R. stolonifer*, with a colony diameter of (4.3 cm) and an inhibition percentage of (48%). Hence *L. camara* can be used as effective measure to control the growth of fungus.
- The results of *in vitro* evaluation of solvent extracts of eupatorium revealed that growth of *Pycularia oryzae* was inhibited to the maximum extent by eupatorium extract extracted with acetone (91.3%) followed by methanol (85.6%), distilled water (74.5%) and petroleum ether (53.9%). The extent of inhibition of fungus was more with higher concentrations of extracts. These results were on par with findings of Sanit, 2016.
- The methanol extract of *Eupatorium odoratum* flower gave positive results for Tannin, Phytosterols or Triterpenoids, Flavonoids, Coumarins, Quinones, Cardiac Glycosides, Terpenoids, Steroids, Acids, and Phenols. Anthraquinones gave negative result in phytochemical analysis of methanol extract (Muricken and Jofeena, 2019).

Summary

- The widespread use of chemicals in agriculture has a destructive effect on soil climate and fertility and the emergence of fungicide resistance in pathogens. Hence, Plant extracts can be used as alternative replacing those chemical fertilizers.
- Methanol extracts of different tested plants are effective for inhibiting phytopathogens but under optimized concentrations.
- The presence of significant secondary metabolites in the extracts helps as a defensive tool for inhibition of phytopathogens.
- TLC screening of extracts was good for methanol solvents and out of 8 tested TLC solvents. Toluene :Ethyl acetate :Methanol in the ratio of 24:24:2 and Hexane: Ethyl acetate in the ratio of 9:1 was more effective and be used further for column chromatography.

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I INTRODUCTION

India has wide range of climate and physico-geographical conditions which are suitable for cultivation of horticultural crops like fruits, vegetables, flowers, nuts, spices and plantation crops. India ranks second in the world production of fruits (www.fao.org.in). “An apple a day keeps the doctor away” is a common adage that illustrates the importance of fruit crops to the human diet. According to ICMR (Indian Council of Medical Research) and National Institute of Nutrition, Hyderabad (NIN), the per capita consumption of fruits should be 120g /day. However in India, it is only around 46g /day indicating the scope to increase the production of fruits. India has very immense potential for the production of different fruits and also their export (Yahaya and Mardiyya, 2019).

The fruit crops that are consumed and exported on a large scale are mango and pomegranate. The cultivated area under mango is 2293 thousand hectares and production is 20,798 thousand million tonnes. The pomegranate area is 262 thousand hectares, and production is 3034 thousand million tonnes ([http://nhb.gov.in /](http://nhb.gov.in/)).

The most positive aspect of our agricultural exports is that a majority of the products in the agriculture export basket are net foreign exchange earners (Patil *et al.*, 2020). India exports mango to over 40 countries worldwide. The major importing countries of mangoes from India are UAE (61.79 %), Bangladesh (11.41 %), UK (8.92 %), and Saudi Arabia (3.79 %). India occupies top position among mango growing countries of the world and produces 40.48 per cent of the total world mango (Thakor, 2019). Pomegranate is a high value crop and its entire tree is of great economic importance. In India, Maharashtra is known as “the fruit basket” with majority of the fruits growing there and is cultivated throughout the year. India exports 51,000 ton /annum of pomegranates to various countries. Pomegranate is majorly exported to UAE (43 %), Bangladesh (16 %) and European countries (14 %), with small quantity to Saudi Arabia, Russia, Thailand, Nepal, Kuwait *etc.* ([https://www.apeda.gov.in /](https://www.apeda.gov.in/)).

Mango (*Mangifera indica* L.) belongs to family, *Anacardiaceae* and is native to South and Southeast Asia, and produces a fruit which is a large drupe. Due to its large scale

cultivation, mango is called the 'King of fruits' (Greeshma *et al.*, 2020). Because of its anti-diabetic, anti-oxidant and anti-viral properties, it ranks as the fifth most consumed fruit across the world (Shah *et al.*, 2010). Mango fruit also contains structural carbohydrates such as pectins and cellulose. The major amino acids include lysine, leucine, cysteine, valine, arginine, phenylalanine and methionine. The most important organic acids include malic and citric acids, which confer the fruit acidity (Maldonado-Celis *et al.*, 2019). Mangoes are extremely perishable and are susceptible to microbial invasion, resulting in fruit rot, texture, taste and colour changes (Onyemata and Ibrahim, 2018). A widespread post-harvest disease in mango is black mould caused by *Aspergillus niger*, which result in reducing the quality of the fruit during storage (Dhondiram and Ashok, 2017) and limits their shelf life to three to four days. It is reported that about 17-37 per cent of fresh mangoes are wasted after harvesting and marketing (Javadpour *et al.*, 2018; Duamkhanmanee, 2008).

The most adaptable subtropical minor fruit crop is pomegranate (*Punica granatum* L.) known as "fruit of paradise" and is vital cash crop. It belongs to the family, *Punicaceae* and is native to Iran. The fruit, for its attractive, juicy, sweet, acidic and refreshing arils, has a broad consumer preference. It is a good source of calcium, iron, sulphur, vitamin C, citric acid carbohydrates and minerals. With an annual pomegranate fruit export of 35,000 tonnes, India is the world's largest pomegranate producer (Pal *et al.*, 2014). Pomegranate are having up to three times more antioxidants than green tea or red wine. Antioxidants protect cells from damage, prevent diseases such as cancer and reduce inflammation and the effects of aging (Vucic *et al.*, 2019). Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is a major constraint in pomegranate production resulting huge loss. Studies on pathogen's survivability revealed that *Xanthomonas axonopodis* pv. *punicae* survives in naturally infected fruits collected from the diseased orchard for one year under laboratory conditions (25.0-38.0°C). The pathogen remains latent on the fruit and produce symptoms during post harvest stage. Temperature is one of the factor during storage for growth of inoculum (Jamadhar *et al.*, 2011). Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is a major constraint in pomegranate production resulting huge loss. First record of this disease in India was by Hingorani and Mehta from

Delhi in 1952 (Sharma *et al.*, 2017). Fruits exhibit isolated water-soaked lesions followed by necrosis with small cracks, leading to splitting of the entire fruit. Severe disease outbreaks can cause 60 to 80 per cent yield losses (Doddaraju *et al.*, 2019).

Indiscriminate use of synthetic pesticides over the years has resulted in different types of hazards and toxicity. The effects include excessive mortality, reduced reproductive potential in organisms, changes in the abundance of species and the diversity of ecosystem, reduction in the productive potential of natural resources and the development of pesticide resistance in target and non-target species. Majority of synthetic pesticides are not easily degradable and tend to enter food chains (Mulla *et al.*, 2020).

Because of their eco-friendly nature, plant-originated antimicrobial compounds may be one of the strategies for plant disease control. Phytochemicals obtained from different parts of plants are considered to be biologically active. As synthetic fungicides cause adverse effects on treated soil ecosystems, due to their non-biodegradable nature, the plant extracts with antimicrobial properties are used to inhibit growth of microorganisms on fruits from disease infection (Pal *et al.*, 2014). Along with inhibition of microbes, the biochemical properties of fruits such as total soluble solids, acidity, pectin and ascorbic acid content will be maintained. During the storage, they also increase the shelf life of fruits. Plant extract is bio-effective, biodegradable, economical and eco-friendly for use as agrochemicals. Natural plant extract compounds have antimicrobial properties that can be used as post-harvest protection for perishable products (Maqbool *et al.*, 2011).

At very low concentration, the secondary metabolites can express their effects and can easily be adsorbed (Angraeni *et al.*, 2019). The use of botanical pesticides blends well with strategies for integrated disease management to achieve and ensure the indigenous biodiversity, is protected by good crop health and natural enemies. Pesticidal plant research is growing to resolve gaps in our understanding that restrict their adoption (Isman, 2015). Commercial production of non-food cash crops, such as pesticide plants, may provide alternate sources of income for smallholders (Sibhatu *et al.*, 2015). Many plant species with pesticide properties are reported to be used for processing into new botanical

pesticides against pathogens that are rapidly growing, easy to propagate on a large scale. They also act as crop fertilizer and assist in increasing yields (Mkindi *et al.*, 2017).

As the antibacterial, antiviral, antifungal and insecticidal agents, the secondary metabolites play a significant role in the defence reactions of the plants. Hence they are used as an alternative solution to environmental problems caused by synthetic pesticides (Rioba *et al.*, 2017). The mode of antifungal activity of such extracts may be attributed to the attack of the cell wall and cytoplasm retraction in the hyphae and eventually mycelium death. Monoterpene hydrocarbons and oxygenated monoterpenes have been shown to damage cellular integrity and thus hinder respiration and transport of ions (Hashem *et al.*, 2016). Toxicity to fungal cell walls, cell membranes and cell organelles is caused by terpenes, phenols, alcohols, alkaloids, tannins and other secondary metabolites present in botanical pesticides (Yoon *et al.*, 2013).

These metabolites also inhibit the germination of spores, growth of mycelia, elongation of germ tubes, sporulation, production of essential enzymes, DNA, and protein synthesis. The plant compounds also alter the structure of hypha and mycelia, thus inhibiting the development of substances such as aflatoxin from certain fungi, such as *Aspergillus* sp. This results in a decreased pathogenicity of fungal pathogens containing mycotoxin (Martinez, 2012). Because of the lack of a peptidoglycan cell wall, gram-negative bacteria are more vulnerable to plant extracts than gram-positive bacteria. Inhibition of cellular processes such as protein synthesis, increased permeability of the plasma membrane leading to leakage of cell material and eventually death are some of the effects of botanical pesticides on bacteria (Isman, 2017).

With this backdrop, the present investigation was undertaken to screen the effect of organic solvent extracts from plants (weeds) for fungicidal and bactericidal properties to inhibit the post-harvest pathogens of mango and pomegranate with the help of phytochemicals released from plant extracts.

Objectives:

1. Isolation of post-harvest pathogens from mango and pomegranate.
2. Morphological and molecular characterization of pathogen isolates.
3. Evaluation of plant extracts against isolates

II REVIEW OF LITERATURE

A brief review of literature for the present investigation has been reviewed under the following headings.

- 2.1 Introduction to mango and pomegranate
- 2.2 *Aspergillus* rot of mango and bacterial blight of pomegranate
- 2.3 Synthetic fungicides and their effects
- 2.4 Phytochemicals as biopesticides
- 2.5 Collection, sampling and isolation of *Aspergillus niger*
- 2.6 Collection, sampling and isolation of *Xanthomonas axonopodis* pv. *punicae*
- 2.7 Morphological and cultural studies of isolate from mango
- 2.8 Molecular characterization of fungal isolate from mango
- 2.9 Morphological and cultural studies of isolate from pomegranate.
- 2.10 Molecular characterization of bacterial isolate from pomegranate.
- 2.11 Extraction of antimicrobial compounds from plant extracts
- 2.12 Evaluation of antimicrobial property of *Tridax procumbens* against pathogens
- 2.13 Evaluation of antimicrobial property of *Lantana camara* against pathogens
- 2.14 Evaluation of antimicrobial property of *Eupatorium odoratum* against pathogens
- 2.15 Statistical analysis

2.1 Introduction to mango and pomegranate

Mango (*Mangifera indica* L.) is known to the world over as a fruit with many nutritional qualities, as it is rich in minerals, fibre, vitamins and provitamins. It is also referred to as the “peach of the tropics” and is considered as exotic. Because it is sweet, refreshing and aromatic, it is commercialized throughout the world. It is the national fruit of India, Pakistan and Philippines, and it is national tree of Bangladesh. Approximately 50 % of all tropical fruits produced worldwide are mangoes. Mango is commercially grown

in more than 80 countries. Major Mango producing countries of the world are India, Brazil, and China. Thailand, Egypt, Indonesia, Mexico, Pakistan, Philippines, and Vietnam. Mango is the national fruit of India. It is also one of the most important and popular Asian fruits (Thakor, 2019).

Pomegranate (*Punica granatum* L.) is a fruit-bearing deciduous tree that belongs to the family Lythraceae. It most likely originated from Iran and Afghanistan (Bhandari, 2012). The available paleobotanical data revealed that, the pomegranate was discovered in Transcaucasia and the North Caucasus region (Vucic *et al.*, 2019). Pomegranate is a rich source of a wide variety of compounds with beneficial physiological activities, in particular with antioxidative and anti-inflammatory properties. Although most of these properties can be attributed to ellagic acid and ellagitannins, particularly punicalagins, punicalins, and gallagic acid, novel research has shown that anthocyanins and specific fatty acid profile also contribute to the reported effects. Combination of these compounds exerts synergistic effects that are markedly higher than the effect of single compounds (Abid *et al.*, 2017).

2.2 Aspergillus rot of mango and bacterial blight of pomegranate

Ripening fruits are known to be susceptible to microbial infections. *Gloeosporium mangiferae*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium species*, *Rhizopus arrhizus*, *Rhizoctonia bataticola* and *Penicillium cyclopium* have been shown to be associated with various fruit rots in mangoes. Post-harvest losses in fruits can be attributed to several factors, the most important of which is post-harvest disease. The percentage loss of fruit over the marketable period has been reported to be the highest for mango. The potential of mango as a commercial crop is markedly limited because of its high perishability, which results in considerable wastage. Mango decay caused by the plant pathogenic fungus *A. niger* is one of the most dangerous postharvest diseases, leading to the losses of fruit quality during storage. It causes severe rotting in fruits and spores of fungus spread at higher rate (Abdullah *et al.*, 2016).

India plays a leading role in pomegranate production with contribution of nearly 50 % of global pool. Bacterial blight of pomegranate is a major hurdle in quality production and high yield in the crop causing up to 60-80 % losses. The disease was first reported

from Delhi in 1952 (Hingorani and Mehta 1952). The pathogen infects all the aerial parts of the plant and in severe conditions, fruits show deep cracking coupled with splitting of pericarp, thereby reducing the marketability of the fruits.

Studies on disease epidemiology revealed that the disease existed throughout the year at a temperature range of 9.0-43.0°C and varying humidities (30.0-80.0%), disease development was rapid during the summer rainy months from July to September due to free water and high humidity (Jamadhar *et al.*, 2011). Postharvest damage of pomegranate by bacteria infect either the fruit through injuries or microwounds on any part of the rind of young fruits in the field. These pathogens remain latent, and cause disease after harvest (latent pathogens), the disease can originate both from wound and latent infections. Pathogen inoculum contaminating fruit surfaces can infect the fruit through rind injuries or cracks, but disease originating from latent infections established before harvest is usually more important (Teksur, 2015).

They reported that the disease was caused by *Xanthomonas axonopodis* pv. *punicae*, a gram-negative rod shaped bacterium that produces light yellow, circular, convex, and smooth colonies. Bacterial cells are capable of surviving in soil for >120 days and also survive in fallen leaves during the off-season. High temperatures and low humidity or both favour disease development (Kumar *et al.*, 2018).

2.3 Synthetic fungicides and their effects

Pesticides are a group of chemicals used for the destruction of insects, weeds, fungi, bacteria etc. They are generally called insecticides, fungicides, bactericides, herbicides or rodenticides. Pesticide residues may constitute a significant source of contamination of environmental factors such as air, water and soil. This phenomenon could become a continuous threat to the co-existence of plant and animal communities of the ecosystem. The characteristics of pesticides, such as high lipophilicity, bioaccumulation, long half-life and potential of long range transport, have increased the chances of contaminating the air, water and soil, even after many years of application (Kumar and Singh, 2017).

According to a World Health Organisation study, 80 % of all pesticides are used by developing countries (Veil, 1990). Due to lack of proper legislation, improper market regulations and ignorance shown by people, agricultural workers from developing countries are prone to experience high levels of agricultural chemicals, including Pesticides are reported to affect many aquatic and terrestrial species. Life in aquatic ecosystems such as microorganisms, invertebrates, plants and fish are badly affected by pesticides (Jayaraj *et al.*, 2016).

2.4 Phytochemicals as bio pesticides

Pest management relying heavily on synthetic pesticides has serious drawbacks in terms of handling hazards, high persistence, toxic residues in or on food, and threat to human health and the environment. Such drawbacks have brought up new challenges for developing safer chemicals for crop protection which besides active against the target pests should be safe to humans, non-target organisms and the environment. Such chemicals should also be able to counter the onset of resistance in pests to pesticides and check outbreak of secondary pests (Walia *et al.*, 2017).

As defined by the Environment Protection Agency (EPA), safer pesticide is less persistence in the environment, less toxic to non-target organisms, less likely to contaminate soil and groundwater, and allow lesser exposure to humans, domestic animals and the environment. As compared to synthetic pesticides phytochemical bio pesticides are moderately toxic, less persistent, and biodegradable. In spite of the continuous emphasis on natural pest management, the usage of phytochemical bio pesticides continues to be 1% of the total pesticide use. Nicotine, rotenone, pyrethrins and sabadilla alkaloids are the prominent example of first generation botanical pesticides. Phytochemical bio pesticides are safe to humans, animals and beneficial organisms like parasitoids, predators, pollinators (Oriyomi, 2018).

2.5 Collection, sampling and isolation of *Aspergillus niger*

Gadgile and Chavan (2010) studied on post-harvest losses in mango and they stated that majority of the post-harvest rots is caused by *Aspergillus* species. They carried an

investigation on infected mango fruit from kesar variety and isolated the pathogen responsible for post-harvest rot. The infected parts were surface sterilized with 0.1 % Mercuric chloride, washed with distilled water and isolation was carried out by following direct plate method. They reported that the major infection was due to *Aspergillus*.

Al Hindi *et al.* (2011) studied on isolation and identification of fruit spoilage fungi in different fruits. They investigated and reported that ten fruit spoilage fungi were isolated and were identified as *Fusarium oxysporum* (banana and grape), *Aspergillus japonicas* (pokhara and apricot), *A. oryzae* (orange), *A. awamori* (lemon), *A. phoenicis* (tomato), *A. tubingensis* (peach), *A. niger* (apple), *A. flavus* (mango), *A. foetidus* (kiwi) and *Rhizopus stolonifer* (date). They concluded that, *Aspergillus* spp. were widespread and the fungal polygalacturonases and xylanses are the main enzymes responsible for spoilage of fruits.

Maqsood *et al.* (2014) carried out a research work on Stem end rot majorly contributing in post-harvest losses of mango during storage. Maximum disease incidence (70 %) was recorded in Sindhura cultivar of mango. *In vitro* studies were carried out to identify fungal pathogens responsible for rotting and decaying mango fruits during storage along with isolation and testing their pathogenicity on healthy fruits. Results revealed that the isolated pathogen was *Aspergillus* accordingly with different cultivars.

Naturally infected mango and tomato fruits of different varieties showing varying degrees of rot were collected from markets and brought to laboratory. Plated plant tissues were incubated for 72 hours at 28 °C, and observed for any fungal growths. Pathogenicity test was carried out by using Koch's Postulates. It was observed that *Aspergillus niger* was the most commonly occurring fungus with 40 % frequency of occurrence (Terna *et al.*, 2015).

Abdullah *et al.* (2016) isolated and identified post-harvest rot pathogens in fruits. The infected fruit samples of apples, banana, oranges, mangos and grapes were collected. The samples were brought to laboratory and stored in refrigerator till mycological studies. The direct plating technique described by Pitt and Hocking (2009) was employed in which infected parts were directly inoculated on media supplemented with chloramphenicol as a

bacteriostatic agent. The plates were inoculated at 28 ± 1 °C for five to seven days. They concluded that *Aspergillus* was the most common genus affecting the different kinds of fruits. It appeared on 93.3 % of each of apple and grape fruits. 86.6 % of orange fruits and 80.0 % of mango fruits. *A. flavus* and *A. niger* were the dominant species on the tested fruits.

Krishnapillai and Wilson (2017) carried out a research on susceptibility of mangoes to *Aspergillus* rot in relation with sap volatile compounds. The pathogen was isolated by following Direct plating method and the isolated pure culture was confirmed by morphological and molecular work. They concluded that fruit rot can be controlled by sap production in some cultivars due to presence of antifungal compounds like terpinolene.

Ali *et al.* (2018) investigated on the fruit rot of lemon caused by *Aspergillus niger*. Survey and sampling of infected lemon fruits were done at the local market. Isolation of pathogen was done from infected portion of lemon fruits. It was cut into small pieces, surface sterilized with 5% commercial bleach for 2 minutes. They were washed twice with sterilized water and shifted to media. They concluded that the fruit rot of lemon caused by *A. niger* is one of the major postharvest diseases because it has low pH and high moisture content to make them susceptible.

The isolation, identification and pathogenicity studies were carried out on post-harvest rots in Mango fruit. Sample collection was done from the local markets in which Mangoes with similar symptoms such as dark brown to black decay spots were selected. The medium for isolation used was Potato dextrose agar. The periphery of the diseased mango tissues were cut and sterilized in 0.1% mercury chloride for 30 seconds. They were rinsed in three changes of sterile distilled water and were plated out on PDA to which they were sub-cultured on to a new set of PDA plates. The pure culture obtained were maintained in slants for further use. The isolated fungi from mango fruits were identified as *Aspergillus niger*, *Botrytis cinerea*, and *A. flavus*. (Zakawa *et al.*, 2018)

The isolation of *Aspergillus niger* from post-harvest rots of chilli fruits was done by wash off and direct transfer method. The mother culture was mentioned in slants and

stored in refrigerator for further use. The pathogenicity was checked by satisfying Koch's postulate. Hence, *A. niger* causes black rot lesions, usually on chilli fruits. Degradation enzymes produced by *A. niger* induce the softening of the flesh tissues of the infected fruits (Linh, 2019).

Zakawa *et al.* (2020) conducted a research on controlling postharvest pathogen of mango by various phytochemicals. Here they collected Mango fruit showing symptoms of deterioration. Isolation, Incidence and Identification of Fungi was done on Bush and kent varieties of Mango. The varieties showing rot symptoms were cut and sectioned fruits were surface sterilized with 0.1 % mercuric chloride for 30 seconds and rinsed with three changes of sterile distilled water. The sterilized sectioned fruits were dried between sterile filter papers and inoculated on already prepared PDA media in 9cm diameter Petri dishes and incubated at room temperature of 25 ± 20 °C for 3 days before sub-culturing on fresh sterile PDA media. Identification of the isolated fungi was carried out based on their spore and cultural characteristics on growing media. The isolate was found to be *Aspergillus niger*.

Infected pomegranate, orange, papaya, tomato, grapes, melon, mango, guava and lychees were collected in separate polythene bags. Primary observations were made by preparing slides. The isolation was carried out by direct transfer of infected parts of fruit to the already prepared media. It was concluded that the *Aspergillus* was found major dominant on different fruits like *Aspergillus brasiliensis*, *A. phoenicis*, *A. carbonarius*, *A. flavus*, *A. acidus*, *A. awamori*, *A. aculeatus*, *A. eucalypticola*, *A. oryzae* and they also produce patulin and aflatoxin (Saif *et al.*, 2020)

Affected papaya fruit of Sekaki cultivar showing signs of diseases were collected. Fungi were isolated from naturally infected fruits of papaya following a modified procedure of (Awoite *et al.*, 2013). Finally they concluded, the fungi isolated and identified through molecular identification as *Fusarium sp.*, *Lasiodiplodia theobromae*, *Aspergillus niger* and *Colletotrichum gloeosporioides* which are believed to be responsible for postharvest decay in papaya fruits (Mendy *et al.*, 2019).

Chaudhary *et al.* (2020) isolated and characterized fungi associated with deterioration of papaya fruits. Papaya fruits with typical rotting symptoms were collected and examined. They followed the direct isolation method for isolation. The pure culture obtained by single spore method. The fungi were identified on the basis of cultural and morphological characters. The fungi were *Colletotrichum gloeosporioides*, *Graphium* sp., *Fusarium pallidoroseum*, *Alternaria alternata*, *Fusarium solani*, *Aspergillus niger*, *A. flavus* and *Rhizopus microspora*. The pathogenicity of isolated fungi was proved by knife injury, cork borer injury, and pin prick injury methods on papaya fruits.

Survey and sampling of diseased pumpkin fruits were made to isolate and identify the pathogen using the methods of Fawole and Oso (2001) and Robert *et al.* (1996). The pathogenicity test was carried using the method of Chukwuka *et al.* (2010) and the severity of rot was measured and recorded after seven days of incubation. Further they concluded that, *Rhizopus stolonifer*, *Aspergillus niger*, *A. flavus*, and *Phytophthora capsici* are some of the pathogenic fungi which cause major rots of pumpkin in the studied area. (Kazi *et al.*, 2019)

Sobowale, (2019) studied on some fungi associated with rotting *Citrullus lanatus*. Rotten watermelon samples were collected. Isolation of fungi from the rotting samples was carried out using pour plate method on acidified Potato dextrose agar. Pathogenicity test was also conducted for the isolated fungi. Further they concluded that, a total of three fungi were isolated viz., *Aspergillus flavus*, *Saccharomyces cerevisiae* and *A. niger*.

Sable, (2019) carried an investigation on fruit rot caused by carbendazim resistant mutant of *Aspergillus niger* on Ber (*Zizyphus mauritiana*). The proper conduct of research was carried out by following Tissue isolation technique to get the pure culture of *A. niger*. The symptomatology and pathogenicity studies were conducted. They reported that, fruits were attacked by many pathogens at pre and post-harvest condition and spoil market quality. Among the post-harvest pathogens *A. niger* was observed to be most common.

2.6 Collection, sampling and isolation of bacteria (*Xanthomonas axonopodis*)

Golmohammadi *et al.* (2007) carried an investigation regarding the diagnosis of *Xanthomonas axonopodis* in citrus. It is considered one of the most important bacterial diseases and they isolated from areas characterized by eruptive lesions on leaves, stems and fruits, and decreases the fruit quality and yield. Each lesion along with 2 mm of the peel around it, was cut in pieces with a sterilized scalpel, comminuted in PBS buffer and left for 10-20 min at room temperature. 100 µl of the PBS extract were streaked onto plates of YPGA medium with and incubated at 25°C.

Xanthomonas arboricola pv. *juglandis* causes apical necrosis and dropping of Walnut fruit. For bacterial isolation, a 3 to 5mm piece of the margin of infected tissue was aseptically removed with a scalpel and homogenized in 60 µl of sterile distilled water. The resulting suspension was streaked on LB, yeast dextrose calcium, and modified Tween medium agar plates and incubated at 27°C for 4 days. 50 µl of the corresponding 1/10 serial dilutions in sterile distilled water were plated on LB and mTM to determine the number of bacteria in a sample. Pathogenity assay was conducted to recover the symptoms (Moragrega *et al.*, 2011).

Ibrahim *et al.* (2015) carried a research study on *Xanthomonas citri*, which is a quarantine pest and is strictly regulated by the International phytosanitary programmes. The fruits showing canker-like lesions were sampled. From each sampled fruit, one canker-like lesion with an area of 2 mm around it was peeled, cut into pieces, and then placed in a vial containing 3 ml sterile distilled water. Each vial was vortexed for 5 sec , then incubated for 10 min to allow bacteria to exude from lesions and continued to do so for many times, before being vortexed after each incubation time. Plant tissue was removed from the vial and the lesion washate was plated onto plates of Kasugamycin-cephalexin-Bravo semi-selective medium.

The bacterial blight of pomegranate, caused by *Xanthomonas axonopodis* pv. *punicae* affects the above ground plant parts and show isolated or coalesced water soaked lesions followed by necrosis with small cracks and splitting of the entire fruit. Surface sterilized samples showing one or more young lesions with typical water soaked margins

were macerated on clean surface sterilized glass slide. A loop full of macerate was streaked on to nutrient glucose agar and incubated at 28°C. Isolations from blight lesions yielded glossy yellow colonies of *X. axonopodis* pv. *punicae* with characteristic brown fuscan pigments in NGA (Sharma *et al.*, 2017).

Symptomatic leaves and fruits from Red blush grapefruit and Mexican lime with canker lesion were sampled, surface contamination was removed by using 75 % ethanol and isolations were made by crushing canker tissue from fruit in sterile tap water for 15 min. A loopful of the suspension was streaked on plates of fresh Nutrient Agar medium and incubated at 28 °C for 72 h. *Xanthomonas citri* subsp. *citri* and *Xanthomonas fuscans* subsp. *aurantifolii* are the causal agents of this disease. Pale yellow colonies from each isolation were sub-cultured onto yeast dextrose CaCO₃ suspended in sterile distilled water and stored at 4 °C for further use (Izadiyan *et al.*, 2018).

Mango bacterial black spot also called mango bacterial canker depicted the symptoms which are observed as water soaked angular spots, then bacterial exudates deposit on these necrotic portions lesions, clearly enclosed by holes. It was a Gram negative bacterium. *Xanthomonas citri* pv. *mangiferaeindicae* was isolated from an infected mango fruit through standard serial dilution and streak plate techniques. This bacterium was cultured aerobically in LB medium at 25 °C. (Liu *et al.*, 2018).

Survey and sample collection were done based on the symptoms produced in Bhagwa variety pomegranate plants and the per cent disease severity was determined using 0-6 disease severity scale given by ICAR-NRCP. Symptoms like blightening with oily ring on the leaves were selected and surface sterilized with 70 % ethanol further sterilized with 0.1 % mercuric chloride for 1 min. Followed by transferring the bits serially in sterile distilled water, then sterilized bits transferred in to eppendorf tube containing sterile water and crushed ,which allows the bacteria to diffuse in water for 10 min ,finally with the help of inoculation loop the diffused bacteria was streaked on the nutrient agar, plates incubated at 29-30 °C for 48-72 h. Appearance of yellow convex or mucoid colonies can be observed on nutrient agar after 48 hours (Harshitha *et al.*, 2018).

Ground cherry (*Physalis pubescens*) is one of the species belongs to family *Solanaceae*. A loopful of the resulting bacterial suspension was streaked on nutrient agar and incubated at 28 °C in the dark. Pale-yellow, mucoid, domed circular colonies of 1–2 mm in diameter, which are characteristic for *Xanthomonas*. Based on biochemical tests and genetic characterizations the bacteria isolates obtained in this study were identified as *Xanthomonas euvesicatoria* pv. *euvesicatoria*. Pathogenicity of these isolates was confirmed on ground cherry, tomato by artificial inoculation (Song *et al.*, 2019).

The causal organism of blight of pomegranate is *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Mehta, 1952; Vauterin *et al.*, 1995). Plants were diagnosed as infected based on typical symptoms of bacterial blight, viz., yellow water soaked lesions at early stages and corky, dark oily spots at later stages of infection based on which field survey and sampling was done. The isolation was carried by following standard serial dilution and streak plate method. Morphological characters, growth rates at different temperatures and biochemical tests were checked (Aishwarya *et al.*, 2020).

The oily spot infected samples of pomegranate were collected from Sholapur, Latur, Osmanabad and Parbhani districts of South Maharashtra, India. The pathogen (*Xanthomonas axonopodis* pv. *punicae*) was isolated, purified by single colony method and maintained aseptically on Yeast glucose calcium carbonate agar medium at 30°C for 3 to 4 days. These cultures were stored and frequently sub cultured and maintained in freeze at 4 °C. Pure cultures of *X. axonopodis* pv. *punicae* were inoculated on pomegranate cv. Ganesh, by using pinprick method (Ramdas *et al.*, 2020).

Kizheva *et al.* (2020) made an investigative study on *Xanthomonas euvesicatoria* as bacterial Spot pathogen on tomato in Bulgaria. Twenty seven strains were isolated from different regions, identified by species-specific PCR amplification. Bacterial spot caused by *X. euvesicatoria*, *X. vesicatoria*, *X. gardneri*, and *X. perforans*, are widespread and damaging bacterial disease of tomato. Irregular watery spots, surrounded by a light yellow halo and brown-black central part of the spots were the peculiar symptoms. They concluded that, this was the first report of *X. euvesicatoria* causing bacterial spot on tomato.

2.7 Morphological and cultural characteristics of isolates of *Aspergillus niger*

Aspergillus species are frequent causes of invasive fungal infections both in plants and animals. Four differential media like czapek dox agar, czapek yeast agar, malt extract agar, and czapek yeast 20 % sucrose agar were used. Standard *Aspergillus* strains for study includes *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. nidulans*, *A. terreus*. The major and remarkable macroscopic features in species identification were the colony diameter, colour (conidia and reverse), exudates and colony texture. Microscopic characteristics for the identification were conidial heads, stipes, colour and length, vesicles shape and serration, metula covering, conidia size, shape and roughness. Riddle's classic slide culture method was used for microscopic study of standard strains and isolates (Diba *et al.*, 2007).

Taxol is a diterpenoid with anticancer activities was produced in *Aspergillus niger*. The mycoflora were inoculated in Czapek yeast agar medium. Mycelium surface was velvety and smooth with regular or irregular radial furrow ornamentation. There were massive conidiospores. The spore had a head of carbon black colour and without any transudate. The back of the fungus appeared yellowish. The top of vesicle was globular or oval, blastic on the surface, with a diameter of 30–80 μ m. The conidial fructification was found to be double layered. The conidiophores grew larger when the fungus grew. The sizes of phialide were in the range of (8–12) * (2–5) μ m. The conidiophores were globular or ovular with a diameter of 20–33 μ m, and the parietes appeared very rough. The conidiospores showed obvious ridges and pitches on the surface (Zhao *et al.*, 2009).

In the early phase of cultivation the aggregation of *Aspergillus niger* conidia is dominantly affected by the pH value. First approaches to describe fungal morphology from microscopic images have been made by (Metz and Kossen, 1977). For coagulating filamentous fungi like *A. niger*, conidia aggregate in the early stage of morphologic development before germination and hyphal growth take place. A morphologically structured model for *A. niger* was proposed by (Bizukojc and Ledakowicz, 2006; Krull *et al.*, 2010)

Silva *et al.* (2011) distinguished *Aspergillus* species of the *Nigri* section from foods, grains and caves on the basis in Polyphasic Taxonomy by utilizing morphologic and

physiologic characters. They isolated the species of the *Nigri* Section on Creatine Sucrose Agar (CREA) which enabled to distinguish the *Aspergillus* species, which was characterized by the lack of sporulation and by the production of sclerotia. Scanning Electron microscopy allowed distinguishing the species into two distinct groups. They revealed that strain characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae near the apex. There was no production of sclerotia.

Kamili *et al.* (2012) studied and identified some *Aspergillus* species isolated from Dal Lake, Kashmir by traditional approach of morphological observation and culture. Potato dextrose agar or Rose-Bengal agar is only useful for identification of the molds up to genus level. The use of differential culture media like Czapek dox agar, Czapek yeast agar and Malt extract agar are used to study macroscopic and microscopic features. Macro morphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia.

Afzal *et al.* (2013) studied on the morphological identification of *Aspergillus* species from the soil of Larkana district. They used differential media like Czapek solution Agar and Malt extract agar for the identification. RHS Mini Colour Chart was used in this study to record the colony colour. Distinguishing character of *A. ficcum* was thickening of conidiophore wall. Identifying character of *A. flavus* were yellow to green or dark green colony colour and radiate heads with rough conidiophore wall. On MEA *A. flavus* showed typically columnar heads whereas *A. flavus* showed radiate heads.

The study was conducted based on macro and micro morphological characterization of different *Aspergillus* isolates. Four different media like Czapek yeast Extract Agar, Malt extract agar, Potato dextrose agar and Czapek yeast Extract Agar with 20 % sucrose were used. Colonies of *Aspergillus niger*, *A. fumigatus*, *A. parasiticus* and *A. oryzae* attained 50-60mm diameter in 10 days at 25 °C in all the four media. Colony colour from the top varied from white to cream, pale green, blue green and dark green with or without white margins in different isolates on different media. Phenotypic characterization

include variability in the pigment and texture of the colonies, growth rate at different temperatures, size and shape of the conidia (Mangal *et al.*, 2014)

Salano *et al.* (2016) studied on diversity of putatively toxigenic *Aspergillus* species in maize and soil samples in an aflatoxicosis hotspot. *Aspergillus* spp. were isolated from maize and soil samples by direct and dilution plate techniques respectively on Czapek dox agar. A total of 11 *Aspergillus* sp. were identified: *A. niger*, *A. flavus*, *A. clavatus*, *A. awamori*, *A. parasiticus*, *A. ochraceus*, *A. candidus*, *A. ustus*, *A. niveus*, *A. terreus* and *A. wentii*. Fungi isolation from maize was carried out following the procedure described by Muthomi *et al.* (2010) after surface sterilisation of kernels in 2 % NaOCl. Isolates of *A. niveus* were fertile on the top one to two thirds of the vesicle while *A. candidus* were fertile on the entire vesicle with colony diameters not exceeding 35 mm in PDA.

Clinical *Aspergillus* isolates were collected from hospitals for identification. The identification of *Aspergillus* strains isolated from various clinical samples (Broncho alveolar lavage, lung, ear, and eye tissues and wound site) included both macroscopic and microscopic characteristics. The colonies growing in Sabouraud dextrose agar were inoculated into potato dextrose agar to increase conidium and pigment productions and into Czapek-dox agar for the identification of species. For each of the growing species, the colony colour, morphology and growth characteristics at 25°C, 35°C, and 44°C were recorded. For microscopic examination, lacto phenol cotton blue was used for making a preparation, and the form and colour of conidia, the number of sterigmata, shape of vesicles and structure of conidiophores were examined. (Atalay *et al.*, 2016)

Cultural and morphological studies of black mould of onion caused by *Aspergillus niger* was carried out by (Pudake *et al.*, 2019) on different media revealed that there were variations in its colony characters, shape, growth and morphological characters. Richard's agar medium shown maximum mycelial growth (90 mm) and sporulation. Potato dextrose agar supported best mycelial growth (88.60) and sporulation of *A. niger*. Large sized conidiophore were produced on the Richard's agar and small sized conidia were noticed on tap water agar and Ashby's agar. The maximum average conidiophore length was recorded on Richard's agar while minimum setae width was observed on Ashby's agar.

The fungi were isolated from animal feed samples. Based on morphological and culture characteristics on *Aspergillus* Differentiation Media, 3 distinguished isolates were obtained. The colonial growth of Isolate 1 showed white to greenish colour and eventually became dark green to brown. Isolate 2 showed characteristic pale yellow to white hyphae, turning to black / dark brown originating from the center of the culture, while the Isolate 3 showed characteristic velvety to the woolly culture of yellow to green colour, with a white border surrounding the yellow to the greenish surface. On the reverse side of the AFPA culture plate, it also showed characteristics bright orange colour. Based on the observed characteristics, the isolates were recognized to be *A. unguis*, *A. niger*, and *A. flavus* (Usman *et al.*, 2019).

Isolation was carried out from solid waste leachates by selective enrichment technique, which were later identified as *Aspergillus fumigatus* and *A. niger*. For *A. fumigatus*, the greyish conidial coloured on PDA, white mycelium, inconspicuous to florescence, exudates were absent, uncoloured to yellowish on the reverse, red brown or green, soluble pigments were absent, sclerotia were absent in all media. While *A. niger* on the same medium coloured conidia, white mycelium, uncoloured to dull yellow or grey on the reverse, soluble pigments were absent (Ogunyemi *et al.*, 2020).

Identification of fungi based on macroscopic and micro morphological features of *Aspergillus* revealed young colonies dazzling white and the colour changes to green as the colony matures. Colony diameter ranged from 20 to 50 mm with an outer concentric ring of young white conidiophores. The hyphae were hyaline, highly branched, and septate. The conidiophores were erect and end in ovate vesicles, bearing uniseriate or biseriate sterigmata, which in turn bear conidia in basipetal succession. Conidia were green and smooth, conferring the colour to the fungal colony. The characteristic parrot green to deep green colonies with smaller conidia (3–6 µm) confirm *A. flavus* and *A. niger* (Veeraragavan *et al.*, 2020).

Papaya fruits with typical rotting symptoms were collected from fruit markets of Palanpur and identification of the pathogens, microscopic observations were carried out on the basis of cultural characters viz., colour, pattern of mycelial growth and morphological

characters of spores of all the eight pathogens and were primarily identified as *Colletotricum* sp., *Graphium* sp., *Fusarium* sp., *Alternaria* sp., *Fusarium* sp., *Aspergillus niger*, *A. flavus* (Chaudhary *et al.*, 2020).

2.8 Molecular identification of fungal isolate by amplifying internal transcribed regions

Martinez-Culebras *et al.* (2009) carried an investigative research study on Molecular characterization of the black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine. DNA extractions were performed by commercial EZNA Fungal DNA kit. Primers were derived from minisatellite which are as follows: GACGACGACGACGAC and GACAGACAGACAGACA. The reaction mixtures were performed in a thermal cycler with 3 min of denaturation at 95 °C followed by 35 cycles consisting of 30 s at 95 °C, 1 min at either 60 °C for (CAG) and 48 °C for (GACA) and 2 min at 72 °C. The ap-PCR products were separated on 1.5% agarose gels with 0.5× TBE buffer. After electrophoresis, gels were stained with ethidium bromide, and the DNA bands were visualised under UV light.

DNA of fungus was extracted using the Cetyltrimethyl ammonium bromide (CTAB) method. The fungus-specific universal primer ITS-4, 5′ TCC TCC GCT TAT TGA TAT GC-3′ were used to amplify genes encoding the ITS region and also three mycotoxin specific primers, apa-2, ver-1 and omt-1 to differentiate between mycotoxic and nontoxic *Aspergillus* isolates. PCR was performed in a total reaction volume of 25 µL which consisted of 2 µL of target DNA solution, 3 µL of the primers and 17 µL of milliQ water. The amplified PCR products were electrophoresed on a 1% agarose gel in tris-borate-EDTA (TBE buffer), visualized by staining with ethidium bromide and photographed, using a gel documentation system. *Aspergillus* strains generated PCR products ranging in size from 300 to 1185 bp (Gautam and Bhadauria, 2012)

The sequenced PCR product was aligned with other isolate sequences from NCBI Genbank for identification and for confirmation of species the BLAST program provided by the National Center for Bio-technology information (NCBI). The species of fungi from the PCR sample was identified by DNA sequencing of the Internally Transcribed Spacer

(ITS) region of rRNA gene. Segments of the entire ITS regions, including partial 5.8S rRNA and Internal Transcribed Spacer 2, complete sequence, 28S rRNA, partial sequence were amplified using the primer PGF04 5'-GGC ATC GGC C- 3'. Amplification of the ITS region of strain *Aspergillus fumigatus* isolated from soil had a size of 1703bp (Shalini and Amutha, 2014).

Culture of strain, collection of mycelium, and DNA extraction were carried out as described by Siddiquee *et al.* (2010). The Internal Transcribed Spacer (ITS) 1 and 2 regions of the rDNA gene were amplified by the Polymerase Chain Reaction (PCR) using the following two primers: forward primer, ITS1 (5'-TCC GTA GGT GAACCT GCG G-3'), and reverse primer, ITS4 (5'-TCC GCT TAT TGA TAT GC-3'). PCR reactions were carried out in a Thermo cycler. The PCR products were sequenced and submitted to the GeneBank, followed by a similarity search using BlastN. A phylogenetic tree was constructed from the evolutionary distance data using MEGA software version 4.1. Phylogenetic analysis showed that isolate was classified under the genus *Aspergillus* (Siddiquee *et al.*, 2015).

Total of 24 consecutive clinical isolates of *Aspergillus* were collected and identified using Conventional Morphology, PCR Sequencing, rep PCR and MALDI-TOF-MS. The slide with sample was loaded into the VITEK-MS machine submitted to multiple laser shots. Biotyper software (version 2.00) was used for analysing the results. Detection and analysis of rep-PCR products were performed using the DiversiLab system. The amplified fragments of various sizes and fluorescence intensities were separated and detected using a microfluidics chip with an Agilent 2100 Bioanalyzer. Sequencing of the (Internal Transcribed Spacer) ITS1 and ITS2 regions flanking 5.8S ribosomal DNA was performed for all of the isolates. PCR products were sequenced and species identification was performed by searching databases with the BLAST sequence analysis tool. The agreement of the sequence method with conventional diagnosis, rep-PCR, and MALDI-TOF-MS methods was measured using Cohen's kappa analysis (Atalay *et al.*, 2016).

Zulkifli *et al.* (2017) studied on Morphological and Molecular Diversity of *Aspergillus* from Corn Grain. Morphological and molecular identification revealed 6

species namely *A.niger*, *A.flavus*, *A. oryzae*, *A. fumigatus*, *A. clavatus* and *A. terreus*. All morphologically identified selected isolates showed 100% similarity with ITS and b-tubulin sequences except for *A. flavus* isolates which showed 99% similarity with ITS sequences. They also made Phylogenetic analysis study.

Alsohaili and Bani-Hasan (2018) made an investigative study on Morphological and Molecular Identification of Fungi Isolates from Different Environmental Sources. The DNA was extracted by using DNeasy Plant Mini Kit. Primers (ITS1and ITS4) were used to amplify ribosomal internal transcribed spacer. PCR products were purified using the QIA quick PCR purification kit. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI). Eight fungal species were identified namely *Aspergillus niger*, *A. tubingensis*, *Alternaria tenuissima* and *Fusarium oxysporum*. The results showed that the *A. niger* was the most abundant fungus obtained from all the locations and resources.

Frias De Leon *et al.* (2018) studied on the Identification of *Aspergillus tubingensis* in a primary skin infection. The isolate was examined for macro and microscopic morphology, conidia size and thermo tolerance, and a phylogenetic analysis of a benA gene segment was performed for molecular identification. DNA extraction was performed as described by (Refojo *et al.*, 2013). To identify *Aspergillus* spp., amplification of a partial DNA sequence of the benA gene was performed according to (Glass and Donaldson, 1995). The maximum likelihood analysis was performed with the MEGA 6 program using the GTR substitution model with gamma distribution. A bootstrap analysis with 1000 replicates was used to estimate the robustness of the tree branches. The isolate was associated with the reference sequence *A. tubingensis* with a bootstrap value of 97% .The maximum parsimony analysis resulted in 1077 trees. The chosen consensus tree showed a consistency index of 0.648 and a retention index of 0.687.

Usman *et al.* (2019) studied on the Isolation of Aflatoxigenic *Aspergillus flavus* from Animal-Feed. Genomic DNA was extracted by the modified method as described previously (Saleemi *et al.*, 2012). The isolated genomic DNA was used as a template to amplify ITS region by polymerase chain reaction using ITS1 (forward) and ITS4 (reverse)

primers (Saleemi *et al.*, 2012; Okoth *et al.*, 2018). DNA was quantified by Nanodrop spectrometer. Later the sequences were compared to ITS nucleotide sequences (Kwak *et al.*, 2013) in the GenBank database by BLASTN at NCBI. Sequences were assembled in contigs by DNA Dragon Software. Multiple sequence alignment of closely related type was done using ClustalW and a phylogenetic tree was constructed by the Neighbour-Joining method using MEGA software (Version 7.0) with 1000 bootstrap value (Costa *et al.*, 2011; Okoth *et al.*, 2018).

Hasanin *et al.* (2019) selected lignocellulolytic fungi for isolation from agricultural soil rich with partial decayed wooden trimmings, one isolate was selected due to its selectivity towards lignin degradation. The total genomic DNA was extracted using CTAB protocol (Benito *et al.*, 1993). DNA of the fungal isolate was amplified by Polymerase Chain Reaction using ITS (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') designed for sequencing. DNA sequence with data from the reference and type strains available in public databases Gen- Bank using the BLAST program (National Centre for Biotechnology Information).

The study was undertaken for Isolation and characterisation of nitrilase producing *Aspergillus* species recovered from solid waste leachates. Samples were randomly collected from selected dump sites. The genomic DNA of the strains was extracted and purified following a standard protocol. The ITS gene was amplified by Polymerase Chain Reaction using universal ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-2 (5' TCCTCCGCTTATTGATATGC-3') primers. The ITS sequences of the strains were searched for homology with the sequences in public databases using the BLAST search program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al.*, 2007). The neighbour-Joining evolutionary model was used to construct the phylogenetic tree. The nitrilase producing strains of *A. fumigatus* and *A. niger* strain were identified (Ogunyemi *et al.*, 2020)

2.9 Morphological and biochemical characterization of bacterial isolate.

The identification of the pathogen *Xanthomonas axonopodis* pv. *glycines* which causes Bacterial Pustule of Soybean. The bacterium was gram negative, rod shaped with

round ends. The cells appeared as single, some in pairs and in clusters having single polar flagellum. The cells measured 0.4 -0.25 x 1.25- 3.00 mm in size. The biochemical features of the pathogen revealed that the bacterium liquefied the gelatin, produced the H₂S, positive for starch hydrolysis and acid produced by carbon sources but it was negative for acid production by nitrogen sources. NGA medium has luxurious growth of pathogen with maximum recovery of bacterial colonies. Circular, flattened, slightly raised, convex with yellow to bright yellow colour on SX and NA medium. 30 °C with pH of 7.0 are optimum for growth (Lambani and Jahagirdar, 2017).

A total of 75 bacteria strains were isolated containing sixty eight *Xanthomonas axonopodis* pv *manihotis*, four *Xanthomonas axonopodis* pv *cassavae* and three unidentified species. The isolates were all able to hydrolyze aesculin and showed a positive reaction to catalase, citrate and oxidase tests. They were indole, methyl-red, Voges-Proskauer and urease negative. The analysis carried out on the biochemical data clustered the *Xam* and *Xac* together at 94.5 %. The bacteria were motile and aerobic, non-fluorescent on nutrient in King's medium B. It is a gram negative rod, non-spore forming, non-pigmented colonies, motile with a single polar flagellum (Ogunjobi *et al.*, 2008).

Common bacterial blight of *Phaseolus vulgaris* is caused by the seed-borne bacteria *Xanthomonas axonopodis* pv. *phaseoli*. Semi-selective media namely *Xanthomonas campestris* pv. *phaseoli* agar and milk tween agar were used to detect the bacterium from whole bean seed extract. Colonies of the bacterium were yellow, mucoid and convex on these media and zones of hydrolysis formed around them. The pathogenicity of the strains was confirmed by stem inoculation of young bean plants. Identification of the strains was proved by using rapid methods like ELISA and PCR (Sujatha and Saigopal, 2010)

Citrus canker is one of the important diseases of citrus caused by *Xanthomonas campestris* pv *citrii* (Xcc). Morphological characteristics like colony characters like Gram staining, cell morphology, cell motility were recorded. Biochemical tests include Aesculin test, Starch hydrolysis, Tween 80 lipolysis, H₂S production, Urease production, Milk proteolysis, Gelatin liquefaction, Oxidase test. All strains were positive for aesculin test, gelatin liquefaction and tween 80 lipolysis (Suryawanshi *et al.*, 2011).

Razaghi *et al.* (2012) studied on Characterization of *Xanthomonas citri* subsp. *malvacearum* strains which causes bacterial blight of cotton. *Xanthomonas axonopodis* pv. *punicae* (Xap) cause oily spot in pomegranate. Microbial studies confirmed that, it was Gram-negative and rod-shaped bacteria with smooth, slightly raised, round, pale yellow coloured colonies. On biochemical analysis, the bacterium was positive for Potassium Hydroxide (KOH) strings (Gram-negative), catalase (aerobe), weakly positive in oxidase test, liquefied gelatin, hydrolyzed starch and casein. Observations of typical characters for genus *Xanthomonas* includes: inability to utilize Asparagine as a carbon and nitrogen source and inhibition on 0.1% triphenyltetrazolium chloride were also recorded. This *Xanthomonas* isolate was further used for pathogenicity test to confirm virulence, and re-isolated to confirm Koch's postulate. Plasmid profiling was carried out to confirm the presence of mega plasmids typical of *Xanthomonas*.

Commiphora wightii is tapped for medicinally important oleo gum resin. A bacterium responsible for this phenomena was established to be *Xanthomonas axonopodis* pv. *commiphoreae*. Bacterial isolates developed circular, creamy white colonies on NA which were initially convex turning to umbonate. Koch's postulate was satisfied resulted development of brown spots surrounded by yellow halo. The bacterial isolates were negative in Gram staining and positive for levan production, starch utilisation, catalase, urease, lecithinase, tyrosinase, gelatine liquefaction, ammonia production, H₂S production, and utilisation of citrate. In contrast isolated bacteria were negative for oxidase, NO₃ reductase, arginine hydrolase, methyl red test, production of indole, and production of reducing substances from sucrose (Samanta *et al.*, 2013).

Samples of Citrus affected with *Xanthomonas axonopodis* were collected from different nurseries. Colonies grown on YDC were yellow, convex and mucoid and differed in colony size (1 - 3 mm) with optimum temperature of 28 °C- 30 °C. It was gram negative rod shaped bacteria. There was no Growth at 4% salt concentration and were non fluorescent under UV. Cultures were further screened on the based on biochemical characterization according to Bergey's manual of Determinative Bacteriology, as *Xanthomonas* (Islam *et al.*, 2014)

Maji and Nath (2015) carried out a research study on pathological aspects of *Xanthomonas campestris* pv. *campestris* causing black rot of cabbage. Isolation and purification of the pathogen from diseased sample was done. For identification of bacterium, its morphological, cultural, and physiological and biochemical characteristics were studied following standard procedure (Pelczar *et al.*, 1957). Nutrient agar, Potato dextrose agar, Yeast extract glucose chalk agar were used. The bacterium produced straw yellow, smooth, glistening colonies with entire margin and convex elevation on NA medium and was gram-negative, short rod, mostly single and rarely in chains. The bacterium had a single polar flagellum and it was positive for catalase, hydrogen sulphide, and oxidase negative and did not produce nitrate. Tested positive for H₂S production giving a black discoloration on lead acetate paper strips.

Emitaro *et al.* (2016) studied on characterization of *Xanthomonas campestris* pv. *vesicatoria* which cause bacterial leaf spot in *Solanum scabrum*. The isolates produced yellow colonies on modified Tween B Media and Yeast Dextrose Calcium carbonate media, were rod shaped, obligately aerobic, gram negative, catalase positive, Oxidase negative and produced hydrogen sulphide gas. On the phylogenetic tree, the isolates were 100% related to *X. campestris* from NCBI sequence database.

Leaf blight and defoliation are caused by new pathovars of *Xanthomonas axonopodis* on *Schinus terebinthifolius* and *Mabea fistulifera*. Bacterial strains recovered from both hosts were typical of *Xanthomonas spp.* They were Gram-negative and formed yellow and mucilaginous colonies on YDC agar medium. They were positive for starch utilization, catalase, gelatine liquefaction, H₂S production and esculin hydrolysis. All strains produced xanthomonadin and were negative for oxidase, urease, nitrate reductase and asparaginase. They did not grow under anaerobic conditions and did not produce fluorescent pigments on King's B medium. Pathovars *X. axonopodis* pv. *schini* pv. *nov.* and *X. axonopodis* pv. *mabeae* pv. *nov.* are proposed as the causal agents (Ferraz *et al.*, 2017).

Bacterial leaf blight is a major disease of eucalypt, especially under nursery conditions. Strains pathogenic to eucalypt were Gram-negative and on YDC agar medium

colonies were yellow and mucilaginous with abundant slime formation. Bacterial strains were positive for catalase, starch utilization, H₂S production, esculin hydrolysis, xanthomonadin production, and gelatin liquefaction. They were negative for growth under anaerobic conditions, production of fluorescent pigments on King's B medium, oxidase, urease, nitrate reductase, and utilization of asparagine as a sole source of carbon and nitrogen. Based on biochemical and molecular work, the pathogen was proposed to be new pathovar *X. axonopodis* pv. *eucalyptorum* pv. nov. (Ferraz *et al.*, 2018)

The first report of sesame spot caused by *Xanthomonas campestris* pv. *sesami* was done by (Felix-Gastelum *et al.*, 2019). The symptoms were lesions on leaves, petioles, stems and capsules. Growth on yeast extract dextrose calcium carbonate agar at 27 and 32°C yielded shiny colonies with yellow pigment typical of xanthomonads. It is a Gram-negative rod-shaped bacteria were aerobic and grew on 5 % NaCl in nutrient broth. The bacterium was positive for hydrogen sulphide, lipolytic activity, catalase, levan production and liquefaction of gelatine. In contrast, it was negative for oxidase, arginine dihydrolase, nitrate reduction and starch hydrolysis. Finally, the isolates utilized glucose, arabinose, lactose, galactose, sucrose, rhamnose, glycerol, maltose and fructose.

2.10 Molecular characterization of bacterial pathogen isolate

Nunes *et al.* (2008) carried a research work on *Xanthomonas axonopodis* pv. *phaseoli* which cause Common Bacterial Blight in *Phaseolus vulgaris*. The DNA obtained by standardized protocol had high quality and the quantity was enough for the Random Amplified Polymorphic DNA reactions with random primers, and Polymerase Chain Reaction with primers of the hypersensitivity and pathogenicity gene (*hrp*).

Xanthomonad like bacteria that are associated with common bacterial blight of bean were identified on the basis of their colonial morphology, biochemical and serological properties, presence of a specific DNA fragment using PCR primers and pathogenicity on bean. The strains were further characterized using rep-PCR and restriction fragment length polymorphism (RFLP). DNA was extracted from bacterial cells using modified Murry and Thompson method (1980). Amplification reactions were conducted with two primer sets: (1) BOX A1R (50- CTACggCAAaggCgACgCTgACg- 30), (2) ERIC1R (50-

ATgTAAgCTCCTggggATTCAC30) and ERIC2 (50-AAgTAAgTgACTggggTgAgCg30) corresponding to BOX and ERIC elements, respectively (Zamani *et al.*, 2011).

Kale *et al.* (2012) carried out a study on microbial, biochemical, pathogenicity and molecular Characterization of *Xanthomonas axonopodis* pv *punicae* from Pomegranate. Plasmid profiling was carried out to confirm the presence of megaplasms typical of *Xanthomonas*. Restriction digestion of these plasmids with two restriction enzymes, viz. Hind III and Sac I revealed its size 93.64 kb and 96.18 kb, respectively. Plasmid profiling procedure can simultaneously targets pathogenicity associated loci. The plasmid DNA fingerprinting reveals higher polymorphism and is quicker than RFLP analyses of genomic DNA.

Samanta *et al.* (2013) worked and found out a novel pathover of *Xanthomonas* responsible for gumming. Isolates of this bacterium were characterised by biochemical tests, Biolog GN2 micro plate reaction, rDNA sequencing, which suggested that the pathogen belonged to the genus *Xanthomonas*. Phylogenetic analysis based on chaperone protein (*dnaK*) gene, TonB–dependent receptor (*fyuA*) gene, DNA gyrase B (*gyrB*) gene and RNA polymerase sigma factor (*rpoD*) gene sequences placed it as a member of *X. axonopodis*. Further elucidation of phylogenetic position of the test strains was achieved from a *gyrB* based tree. Test strains were confirmed to be members of *X. axonopodis*.

Xanthomonas axonopodis pv. *dieffenbachiae* is an important and destructive disease which causes bacterial blight in Anthurium. A novel molecular method was used to detect Xad in anthurium using loop-mediated isothermal amplification (LAMP) technique (Xad-LAMP). The Xad-LAMP reaction could be finished by incubating at 61–65 °C for 1 h, and the amplifcons were confirmed through gel electrophoresis, HpaII enzyme analysis, and visually inspected using SYBR Green I/calcein stain and lateral flow dipstick (LFD) assay (Jun-hai *et al.*, 2015).

Black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris* is one of the most important bacterial disease. Genomic DNA of isolates of Xcc was collected from different parts of India was extracted by CTAB method. Molecular characterization of 47

isolates of Xcc was achieved using a hrpF gene based set of primers, Dhrp Xcc F:5' - GTG GCC ATG TCG TCG ACT C -3' and Dhrp Xcc R: 5'-GGA ATA AAC TGT TTC CCC AAT G-3', designed to amplify the 3' end region of hrpF gene based PCR (Singh et al., 2014). Also, DNA polymerase chain reaction-based fingerprinting (rep-PCR) using BOX primers was done to assess the genetic diversity of these Xcc isolates (Singh et al., 2016).

Balaz et al. (2016) studied on Characterization of *Xanthomonas hortorum* pv. *pelargonii* isolated from Geranium which showed the blight symptoms. Isolates were characterized with pathogenicity, biochemical, serological, and molecular assays. Isolates reacted positive in a polymerase chain reaction using XcpM1 and XcpM2 primers specific for *X. hortorum* pv. *pelargonii*. Repetitive sequence-based PCR typing using BOX and ERIC revealed that all isolates showed two fingerprinting profiles but (GTG) 5 and REP did not reveal differences. Multilocus sequence typing of partial sequences of rpoD, dnaK, fyuA, and gyrB genes of tested isolates and sequences obtained from GenBank of *Xanthomonas* pathovar pathotype strains did not reveal genetic variability among the isolates, showing the same gene sequence pattern.

Osdaghi et al. (2016) studied on Characterization of the bacterial spot pathogen *Xanthomonas euvesicatoria* on Pepper. The putative isolates of *Xanthomonas* sp. were identified using generic and specific primers for xanthomonads. The generic primers X-ITS-F3j, X-ITS-F3k, X-ITS-F3c, X-ITS-F3d and X-ITS-R2 are capable to amplify all *Xanthomonas* species (Adriko et al., 2014), were used for the identification of the gram-negative yellow-pigmented bacterial isolates to the genus level. Species-specific primer pairs – Bs-XeF/Bs-XeR, Bs-XvF/ Bs-XvR, Bs-XgF/Bs-XgR and Bs-XpF/Bs-XpR for *X. euvesicatoria*, *X. vesicatoria*, *X. gardneri* and *X. perforans*, respectively. To determine the identity of the causal agent to the species level. Phylogenetic analysis was performed adopting the neighbour-Joining method and using Jukes Cantor model.

Chowdappa et al. (2018) studied on isolation and characterization of *Xanthomonas axonopodis* pv. *punicae* from bacterial blight of pomegranate. The genomic DNA was extracted from the bacterial pathogen by following lysis method. ITS primers 799 F (5'-AACMGGATTAGATACCCKG-3'), 1193 R (5'-ACGTCATCCCCACCTTCC-3') 3'

were used to amplify the 400 bp gene of ITS region. Genus specific primers viz., KKM-5 Forward 5'GTTGATGCTGTT CACCAGCG3', KKM-6 Reverse 5'CATTTCATTT CGCCCAAGCCC 3' were used to amplify the *gyrB* gene product of 491bp.

2.11 Extraction of antimicrobial compounds from plant extracts

The yield of crude extracts from plants obtained by maceration method using different type of solvents, were significant. The recovery percentage of extractable compounds depends on type of solvent used. Commonly used solvents are Methanol, chloroform, hexane, petroleum ether, acetone etc. The variation in the yields of extracts could be attributed to the difference in solvent polarities used which also plays a key role in increasing the solubility of phytochemical compounds (Silva *et al.*, 2014; Naima *et al.*, 2015). Therefore, this result confirmed the effect of solvent extraction and the plant organ on the yield extraction and consequently confirms the richness of this plant in polar substances (Felhi *et al.*, 2017)

The concentration of flavonoids and phenols correlates very well with the antioxidant activity as methanol extract also showed the maximum antioxidant activity. This is followed by ethyl acetate, acetone and hexane as an extraction solvent as these solvents result in the moderate extraction of flavonoids, phenols and shows moderate antioxidant activity. Chloroform and distilled water results in the least extraction of various phytochemicals, may be due to poor solubility of these phytochemicals in chloroform and distilled water and should not be the solvent of choice (Dhawan and Gupta, 2017)

2.12 Evaluation of Antimicrobial property of *Tridax procumbens* against pathogens

Tridax procumbens, a plant belonging to the daisy family, is found perennially in various tropical and subtropical regions as well as mildly temperate regions worldwide (Mundada and Shivhare, 2010). Listed as a weed and a pest plant, it has been known by several names including *Tridax* daisy in English, Jayanti veda in Sanskrit, Ghamra in Hindi, Dagadi pala in Marathi, Herbe caille in French and Thata poodu in Tamil. It inhabits waste places, road sides and hedges throughout India. Some reports from tribal areas in

India state that the leaf juice can be used to cure fresh wounds, stop bleeding and also as a hair tonic (Pai *et al.*, 2011).

Arunachalam *et al.* (2010) initiated an investigation to find a suitable alternative to synthetic antibiotics for the management of plant diseases caused by bacteria. Ethanol extracts of six weed plant species namely, *Prosopis juliflora*, *Acacia arabica*, *Achyranthes aspera*, *Calotropis gigantea*, *Ocimum canum* and *Tridax procumbens* were screened against four phytopathogens. The antibacterial activity was tested by well diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC observations reveal that, extract of *T. procumbens* having high antibacterial activity against *Xanthomonas malvacearum* (0.312 mg ml⁻¹), *X. campestris* (1.25 mg ml⁻¹), *Pseudomonas solanocearum* (2.5 mg ml⁻¹) and *P. syringae* (5 mg ml⁻¹).

Extracts of *Tridax procumbens* were prepared by using soxlet extractor. The ethanolic and aqueous extracts were tested by agar well diffusion techniques as described by (Perez *et al.* 1990) by using different concentrations of the extract (1g %, 2.5 g % and 5 g %). Aqueous extract of the leaves showed no antibacterial activity. All the strains showed 100% sensitivity to *Tridax* extract. Hence, ethanolic extract showed very good activity against *Pseudomonas aeruginosa* at a concentration of 5mg /mL. There was a statistically significant increase in the zones of inhibition as compared to some control antibiotics (Isaiarasu *et al.*, 2011).

Christudas *et al.* (2012) investigated the phytochemical screening and antibacterial activity of Petroleum Ether, Chloroform and ethanolic extracts of *Tridax procumbens*. The antibacterial activity of leaves was evaluated by Agar well diffusion method against selected bacteria. The activity of chloroform extract against *Bacillus faecalis* and *E.coli* at the concentration of 800 µg/ mL was comparable with that of standard drug ampicillin. Hence, they reported that, plants rich in tannins have antimicrobial potential because they react with proteins to form stable water soluble compounds, thereby killing bacteria by damaging cell membrane.

The essential oil of the flowers of *Tridax procumbens* was obtained by hydro-distillation and analysed by gas chromatography equipped with a flame ionization detector (GC-FID) and gas chromatography coupled with mass spectrometry (GC/MS). Twenty-six compounds were identified, which comprised 90.6% of the total constituents. The most abundant compound was (Z)-falcarinol (25.9%), followed by α -selinene (15.3%), limonene (8.3%) and zerumbone (4.3%). The oil was active against the tested Gram-positive bacteria at a concentration range of 0.14 ± 0.03 - 0.57 ± 0.05 mg/mL, while 0.67 ± 0.12 - 4.58 ± 0.41 mg/mL was effective against the studied Gram-negative bacteria. Remarkable antifungal activity was found against the tested fungi at a concentration range of 0.06 ± 0.008 - 0.10 ± 0.01 mg/mL (Joshi and Badakar, 2012).

The leaf of *Tridax procumbens* was collected, grounded and subjected to methanol for extraction with soxhlet apparatus. The extract was screened phytochemically for its chemical components. The presence of alkaloids, reducing compounds (carbohydrates), cardiac glycosides, flavonoids, saponins, terpenes and steroids was recorded. Antibacterial assay of the extract by the use of disk diffusion method revealed the extracts to have broad spectrum activity on gram positive, negative organisms respectively. The highest activity was shown in *Streptococcus typhi*, *S. flexneri* and least activity on *E.coli*. The results shows that extract has bactericidal activity (Muthusamy *et al.*, 2013).

Combination effect of the antimicrobial activities of aqueous, ethanol and petroleum ether extracts from *Zingiber officinale* and leaves of *Tridax procumbens* were evaluated using the checkerboard dilution technique. The susceptibility of the pathogenic bacteria species to the plant extracts in single and combination application were obtained using the agar well diffusion method while the minimum inhibitory concentration were determined using agar dilution method. A 12.33 ± 0.58 mm zone of inhibition was recorded for the combination of the petroleum ether extract of the two plants against *P. vulgaris*. *T. procumbens* extract alone showed 8.67 ± 0.58 mm diameter of inhibition. Also a MIC of 50 mg/ml was observed for the ethanol extract in combination (Akinbobola and Dada, 2014).

Estimation of the antimicrobial property of *Tridax procumbens*'s leaf was carried out by the use of chloroform, petroleum ether, ethyl alcohol and hexane solvents. Leaf

extract of *T. procumbens* obtained by soxhlet extractor, using the above mentioned solvents were examined against *Escherichia coli*, *Bacillus subtilis*, and *Proteus vulgaris*. The antimicrobial activity of *T. procumbens* performed by using agar well diffusion method showed a result showcasing an effective limit when as opposed to *P.vulgaris* for ethyl alcohol being used as solvent for extract. In conclusion *T. procumbens* leaf extract terminates most propitious source (Kumar *et al.*, 2015).

Disc diffusion method was used for the assay against six bacteria isolates. The results showed that highest sensitivity was observed with chloroform extract against *Streptococcus pyogenes* (34.00±0.58 mm), *E. coli* (28.00±1.15 mm) and *Klebsiella pneumoniae* (28.00±0.58 mm). Ethanol extract exhibited moderate activity, while little antibacterial activity was seen with the aqueous extract. The sensitivity of chloroform extract of *Tridax procumbens* at 80µg /mL was higher but not significantly different ($P>0.05$) from ciprofloxacin (50 µg /mL) against *E. coli*, *Proteus vulgaris*, *S. pyogenes* and *K. pneumoniae*. *T. procumbens* appear to be a source of novel broad spectrum antimicrobial agent (Oloche *et al.*, 2015).

Xanthomonas axonopodis pv. *punicae* which causes bacterial blight of pomegranate is responsible for the failure of crop upto 60-80 %. The aqueous, ethyl alcohol and acetone extracts of ten medicinally useful plants were used against it, *in vitro*. Among the different plants studied, acetone extract of *Metha spicata* (22 mm), *Allium sativum* (20 mm) and *Murraya Koenigii* (19 mm), *T. procumbens* (17mm) and *Ricinus communis* (16 mm) were found to be most effective. They reported that among them, *Mentha spicata*, *M. Koeninngi*, *A. sativum* and *T. procumbens* caused maximum inhibition of the test bacterium (Alane and Swami, 2016).

Methanolic extract of the leaves of *Tridax procumbens* was examined at different concentrations on both bacteria and fungi by disc diffusion method and agar well diffusion method. Both Gram positive and negative bacteria including *Escherichia coli*, *Vibrio cholera*, *Bacillus subtilis*, *Bacillus cereus* and *Streptococcus aureus* showed sensitivity to 250 µg /mL and 500 µg /mL plant extract. Their zone of inhibition was within 8-14 mm range in diameter for both concentrations. Several fungal species including *Candida*

albicans, *Aspergillus niger* and *A. fumigatus* were also tested for its susceptibility to methanol extract and showed within the range of 9-15 mm zone of inhibition with great potency against *C. albicans*. MIC and MFC was also determined at different concentrations to evaluate the efficacy of methanol extract (Sarkar *et al.*, 2016).

Tridax procumbens leaves were dried and extracted with various organic solvents. The leaves contained the phytochemicals such as alkaloids, carbohydrates, polyphenols and tannins respectively. Antimicrobial potentials of the extracts were determined by performing the disc diffusion techniques. Results revealed that different organic solvents extracts namely methanol, ethanol and ethyl acetate extracts documented comparatively good activity against the studied microbial strains. The methanol extract of leaves of *T. procumbens* showed comparatively better antioxidant potential. The tested plant leaf extract showed high activity against human lung cancer cells than breast cancer cell lines. 250 mg/ml plants extract showed $84 \pm 2.8\%$ toxicity against human lung cancer cells (Syed *et al.*, 2020).

2.13 Evaluation of antimicrobial property of *Lantana camara* against pathogens

The antimicrobial activity of crude methanolic and acetone extracts of *Lantana camara*, a traditional medicinal plant was determined against thirteen test bacteria and eight test fungal strains. Both the solvent extracted extracts inhibited the growth of *Streptococcus aureus* to the maximum. The fungitoxic spectrum of the test plant's leaf & stem extracts indicated maximum percentage growth inhibition at $1000 \mu\text{g mL}^{-1}$ concentration against *Alternaria alternata*. Thus, phytochemicals from *L. camara* have a broad antimicrobial spectrum and might be a novel source of antimicrobial drugs (Saraf *et al.*, 2011)

The leaf extracts of *Lantana camara* in different organic solvents (methanol, acetone, ethanol and aqueous) were assessed *in-vitro* for fungitoxic activity against phytopathogenic *Alternaria alternata*. The assessment of fungitoxicity was carried out by food poison technique using four different extracts at 200 mg /mL and their activity was recorded as radial growth and percentage inhibition. Among the four extracts, ethanol and acetone extracts showed complete inhibition of growth of fungus, while methanol extract showed 50 % inhibition and aqueous extract did not produce any inhibition of fungus.

Findings from present study confirmed that ethanol and acetone extract of *L. camara* can be used as biofungicide to control this phytopathogenic fungus (Singh and Srivastava, 2012).

Methanol, ethanol and water extracts were evaluated against four Gram positive and Gram negative bacterial isolates and two fungal strains (*Aspergillus fumigatus* and *A. flavus*). The total phenolic content was (40.859±0.017) mg gallic acid /g in the leaves of *Lantana camara*, while the total flavonoids was (53.112±0.199) mg/g dry weight. Methanol leaf extract of *L. camara* showed maximum antibacterial activity against *Streptococcus aureus* and *Pseudomonas aeruginosa* and was also effective against other bacterial strains as compared to ethanol and aqueous extracts of leaves. The methanol leaf extract of *L. camara* exhibited significant inhibition (71 %) and (66 %) against *A. fumigatus* and *A. flavus* respectively (Naz and Bano, 2013)

Ethyl acetate and methanol extracts of *Lantana camara* were used for antibacterial test and was performed using the disc diffusion method. The antibacterial activity of the extract increased linearly with increase in volume of extract (mg /mL). For Ethyl acetate extract of *L. camara*, the mean zone of inhibition ranged between 15 to 23 mm. For Chloramphenicol is a positive control, the zone of inhibition ranged from 14 to 19 mm respectively. The highest zone of inhibition observed *Klebsiella* sp. (18 to 23 mm). For methanol extract of *L. camara*, the mean zone of inhibition ranged between 18 to 25 mm. Hence methanol extract recorded more zone of inhibition compared to other extracts (Gajendiran *et al.*, 2018).

Antimicrobial Properties of Ethanolic Leaf Extract of *Lantana camara* were studied by (Kulkarni *et al.*, 2019). The preliminary phytochemical screening revealed the presence of tannins and phenolic compounds, terpenoids, phlobatannins, phytosterol, alkaloid and flavonoids in extract. The antifungal and antibacterial screening was done by agar well diffusion method. Ethanol extract showed a zone of 10mm diameter against *Aspergillus niger* at 10 mg /mL concentration. These results revealed that *L. camara* plant possesses antifungal activity against *A. niger* and *Rhizopus oryzae*.

The methanolic extracts of *Lantana camara* leaves were extracted and the *in vitro* antibacterial testing was done by using disc diffusion method against *Bacillus subtilis*, *Erwinia herbicola*, *Escherichia coli*, and *Salmonella typhi*. The methanolic extracts of leaves were tested, the extracts of *L. camara* showed the antibacterial activity against the bacterial pathogen. *L. camara* showed an appreciable antioxidant activity at 40 mg /mL concentration with an inhibition of 66.54 percent and IC₅₀ value 7.39 mg /mL. The result indicates that *L. camara* is a potential source of antibacterial and natural antioxidants (Mickymaray and Al Aboody, 2019).

The benzene, chloroform and acetone extract of *Lantana camara* for its bioactive potential was studied at various concentrations against pathogenic bacteria. The percentage yield (w/w) of these extracts was 2.6 %, 0.28 % and 6.58 % respectively and these values correspond to 1.3g, 0.14g and 3.29g of the soluble principles in fractions respectively. Chloroform extract was found to be active against 6 of the 8 tested bacteria. The inhibitory values were quite significant as compared to the control at all the concentrations used and the Critical Difference values at 5% level of significance were in the range of 0.10 to 0.18. The results suggest the antibacterial potential of the plant (Singla, R., 2019).

Phytochemical analysis and *in vitro* antifungal potential of fruits, leaves and stem of *Lantana camara* were studied. The phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins, saponins, glycosides and terpenoids in fruit, stem and leaves of *L. camara*. The *in vitro* antifungal activity of fruit, stem and leaves of *L. camara* was tested against *Colletotrichum gloeosporioides*. The results of *in vitro* experiment revealed that higher concentration of methanolic fruit extract (5 %) significantly reduced the biomass *C. gloeosporioides* up to 66 %. This effective extract of *L. camara* was partitioned with n-hexane, chloroform, ethyl acetate and n-butanol. Hence, 0.5 % concentration of n-hexane fraction of methanolic fruit extract caused the highest reduction (45 %) in the radial colony growth of the test fungus (Bashir *et al.*, 2019).

The essential oil of *Lantana camara* was extracted from dried leaf samples using the soxhlet extraction method. The oil was separated from the solvent and the bioactive

compounds were identified using gas chromatography–mass spectroscopy (GC-MS) and Fourier transform infrared spectroscopy (FTIR). Results indicated the presence of alcohols, carboxylic acids, phenols, alkanes, ketones, and primary amine compounds. GC-MS identified 43 compounds representing 95% of the total leaf essential oil components. Some of the major isolated compounds included pyrrolizine, 1-dodecanol, 1, 2-nonadecanediol, phytol, 1, 3-dioxolane, 4-undecene, 9-methyl and imidazole. This extract is highly valuable for the medical treatment of various ailments and antimicrobial property (Ayalew, 2020).

2.14 Evaluation of antimicrobial property of *Eupatorium odoratum* against pathogens

Methanol, ethanol, ethyl acetate, hexane and chloroform extracts of *Chromolaena odorata* were tested against clinical and phytopathogenic bacteria. Among treatments, maximum *in vitro* inhibition was scored in methanol extracts of *C. odorata* which offered inhibition zone of 8mm, 7mm, 5mm and 7mm against tested bacteria *Escherichia coli*, *Streptococcus aureus*, *Xanthomonas vesicatoria* and *Ralstonia solanacearum*, respectively. Further, ethyl-acetate and hexane extracts of *C. odorata* on TLC produced 9 spots with varied level of inhibition. The minimum inhibitory concentration value for clinical bacteria was ranged between 0.35 to 4.0 mg /mL and 0.25 to 4.0 mg /mL for phytopathogenic bacteria (Sukanya *et al.*, 2011).

The study was undertaken as an attempt of preliminary investigation of phytochemical constituents and to explore the antibacterial activity of different extracts of leaves of plant *Eupatorium odoratum* using petroleum ether, ethanol and chloroform as solvents. Various doses of extracts were screened for phytochemical constituent and evaluated for their antibacterial activities on human pathogens like *Bacillus subtilis*, *Streptococcus aureus*, *Escherichia coli* and *Salmonella typhi*. Tests for alkaloids and tannins were positive for all extracts. Tests for saponins, proteins, amino acids and anthraquinones glycoside were negative for all extracts. All extracts showed antibacterial activity at 2 mg /mL concentration. When the dose of the extract is increased, a gradual increase in antibacterial activity was observed (Nayak *et al.*, 2012)

The antifungal activity of this eupatorium plant extract extracted with different solvents was evaluated for control of blast disease in rice. The results of *in vitro* evaluation

of solvent extracts of eupatorium revealed that growth of *Pyricularia oryzae* was inhibited to the maximum extent by eupatorium extract extracted with acetone (91.3 %) followed by methanol (85.6 %), distilled water (74.5 %) and petroleum ether (53.9 %). The extent of inhibition of fungus was more with higher concentrations of extracts. The results of field trail also indicated that among different extracts, the PDI of leaf blast was the lowest in 15 % acetone extract (23.8) and the distilled water extract (5 to 15 %) indicating its effectiveness in controlling blast disease. The incidence of neck blast was also less in eupatorium extract sprayed treatments (Manjappa, 2013).

Species of the Asteraceae family and the genus *Eupatorium*, have high biological potential and are used in folk medicine to treat various diseases. The species of *Eupatorium* are rich in terpenes, phytosterols and sesquiterpene lactones, the latter being chemotaxonomic markers of the group, with broad anticancer, antiplasmodial and antimicrobial activity, making them promising for the development of new drugs. Various species of *Eupatorium* seems to hold great potential for in depth investigation for antimicrobial activities. Many species have broad folk use, with scientific confirmation of its antimicrobial properties making these plants potential sources of safer and more effective treatments (Sobrinho *et al.*, 2017)

The ethanolic and aqueous extracts of *Eupatorium odorata* were qualitatively and quantitatively screened for phytochemicals using standard methods. The inhibitory activity of the leaf extracts were evaluated against clinical pathogens: *Escherichia coli*, *Streptococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, at 100 mg /mL and 200 mg /mL concentrations. The ethanolic extract of *E. odorata* had a better percentage yield of 5.49 g, followed by aqueous extract (3.5 g). The phytochemical screening conducted on the extracts revealed the presence of flavonoid, alkaloid, saponin, cardiac glycoside, steroids, tannins and terpenoids. The test organisms were more susceptible to the aqueous and ethanolic extracts of *E. odorata* at 200 mg /mL with zones of inhibition ranging from 3.23±0.15 mm to 12.33±0.33 mm (Egbunu *et al.*, 2019).

Qualitative and quantitative phytochemical screening of *Chromolaena odorata* extracts were done by standard methods. Antibiotics susceptibility pattern of bacterial

isolates to a panel of 10 conventional antibiotics was determined by disc diffusion method. *C. odorata* methanolic extract had the highest extract yield (26.2 %). From the multidrug resistance analysis, 66.7 % of bacterial isolates tested had multidrug resistance index of 100 %. *Pseudomonas aeruginosa* was susceptible to 100 mg /mL of *C. odorata* ethanolic extract but resistant to 100 mg /mL of hot aqueous *C. odorata* extract. This study reveals the inhibitory activities of *C. odorata* extracts on multidrug resistant bacteria isolated from wounds and an indication of their potential in the treatment of wound infection (Alabi *et al.*, 2019)

Successive extracts from dried flowers of *Eupatorium odoratum* were investigated for the presence of phytochemicals. Methanol extract was used for the phytochemical and antibacterial studies. Aqueous extracts of *E. odoratum* was used for the insecticidal and larvicidal activity. As a result it was found that the plant flowers have insecticidal and larvicidal activity and a slight range of antibacterial activity. Aqueous extract exhibited the presence of enzyme polyphenol oxidase. The extract was subjected to ammonium sulphate precipitation and dialysis to precipitate proteins (Muricken and Jofeena, 2019).

Compounds were isolated from the South African weed *Chromolaena odorata* through column chromatography. Purified compounds were tested for antimicrobial activity using the p-iodonitrotetrazolium chloride (INT) colorimetric method against uropathogens. Four compounds identified as pectolinarigenin, ($\pm$) -4', 5, 7-trimethoxy flavanone, 5-hydroxy-3, 7, 4'- trimethoxyflavone and 3, 5, 7-trihydroxy-4'-methoxyflavone were isolated. Minimum inhibitory concentration (MIC) varied between 0.016 and 0.25 mg /mL. Compounds showed promising antimicrobial activity against *Escherichia coli*, *Streptococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus fumigatus* and *Cryptococcus neoformans* with MIC between 0.016 and 0.125 mg /mL comparable to gentamicin used as positive control (Omokhua *et al.*, 2020).

The sensitivity of pure isolates of *Phytophthora colocasiae* and *Fusarium oxysporum* to methanolic and ethanolic leaf extracts of *Chromolaena odorata* was evaluated using diffusion (agar and disc diffusion) and dilution (broth and agar dilution) assays. Ethanolic leaf extracts of *C. odorata* significantly increased the inhibition zone

diameter (IZD) against *F. oxysporum* when compared to methanolic leaf extracts. Ethanolic extracts of *C. odorata* were found to possess higher inhibitory effects shown by the wider inhibition zone against the pathogens using disc diffusion assay. The methanolic and ethanolic extracts of *C. odorata* showed significant higher inhibitory activities against the pathogens at MIC of 6.25 and 25 mg /mL respectively, inhibiting the growth of the pathogens completely (Okoro and Onaebi, 2020).

Qualitative phytochemical screening of *Chromolaena odorata* extracts was done by standard methods. Antibiotics susceptibility pattern of bacterial isolates to a panel of ten conventional antibiotics was determined by disc diffusion method. Antibacterial activity of *C. odorata* extracts were determined using agar diffusion method. *C. odorata* aqueous extract had higher extraction yield (24.1 %) than ethyl acetate extract. Phytochemical screening revealed the presence of saponin, flavonoids, terpenoids, steroids, tannin, glycoside and phenol of *C. odorata* extracts. *Proteus vulgaris* exhibited 100% resistance against augmentin. The highest zone of inhibition observed with the *C. odorata* ethyl acetate extract was against *Pseudomonas aeruginosa* (18.67±0.67 mm) (Alabi *et al.*, 2020).

2.15 Statistical analysis

Andriana *et al.* (2019) conducted a study on the antimicrobial property of *Tridax procumbens* against pathogens. They followed Disk diffusion method to access the antibacterial activity. The experiment was conducted under CRD and the results obtained were analysed. The statistical analysis was performed by one-way ANOVA using Minitab. The results were presented as means ± standard deviation (SD) values. Differences among treatments, controls and standard data were considered significant at $p < 0.05$ using Fisher's test.

Bashir *et al.* (2019) made an investigative research study on *Lantana camara*, its phytochemical analysis and its importance as antifungal agent. They revealed that it is source of many secondary metabolites like alkaloids, phenols, flavonoids, steroids and

hence these phytochemicals were effective in inhibiting the fungus by reducing spore germination. They carried the research work under CRD and poisoned food technique. All the data were statistically analysed by using analysis of variance (ANOVA) followed by Duncan's Multiple Range test (DMR) at $P \leq 0.05$.

Alabi, (2020) reported a study on screening the rate of inhibition of *Eupatorium odoratum* against bacterial pathogens. They revealed that, *E. odorata* aqueous extract had higher extraction yield (24.1 %) than ethyl acetate extract. Phytochemical screening revealed the presence of saponin, flavonoids, terpenoids, steroids, tannin, glycoside and phenol of *E. odorata* extracts. Analysis was performed using Statistical Package for Social Sciences (SPSS) version 20.0. Data obtained were subjected to analysis of variance and expressed as mean $\pm$ standard error. Statistical significance was determined at values ($P \leq 0.05$) and the means were separated using Duncan's Multiple Range Test.

III MATERIAL AND METHODS

The research work was carried out during 2019-20 at the Department of Plant Biotechnology, University of Agricultural Sciences, GKVK campus, Bangalore, Karnataka, India (560065). The details of the materials used and methodology are followed under the following headings.

3.1 Materials: Following materials were used for experimentation.

3.1.1 Glassware

During the course of investigation the glassware used were Petri plates, conical flasks (250 mL, 500 mL, 1000 mL and 2000 mL), funnel, test tubes, serial dilution tubes, pipettes, beakers, measuring cylinder, glass bowls, glass rod, TLC plates, TLC chamber, capillary tube, screw cap bottles (5 mL) *etc.*

3.1.2 Equipment

Standard laboratory equipment used for different experiments were autoclave, hot air oven, laminar airflow, pH meter, glass electrode, digital balance cell colorimeter, mechanical shaker, microwave oven, refrigerator, colony counter, incubator, weighing balance, single and double distillation unit, mixer (Simple grinder), centrifuge and research microscope.

3.1.3 Chemicals

Chemicals used during the course of research work were acetone, chloroform, ethanol, methanol (HI MEDIA), formaldehyde, streptomycin sulphate, spirit, sodium hypochlorite solution, mercuric chloride, dimethyl sulphoxide, calcium carbonate, ferric chloride, sulphuric acid, olive oil, Dragendroff's reagent, ammonia solution, alcohol, ethyl acetate (HI MEDIA), toluene (HI MEDIA) *etc.*

3.2 General laboratory procedure

Laboratory experiments were conducted using borosil glass wares. These glass wares were cleaned and sterilized by washing in 1N potassium permanganate and 1N

concentrated sulphuric acid. Glass wares, liquid media and solid media were autoclaved at 121.1°C, 15 psi for 15 minutes. All isolation and other studies were carried out in an aseptic condition in a laminar air flow chamber. Utensils such as, needle, spreader, forceps, scissors, spatula, inoculation loop used for inoculation were sterilized by autoclaving, followed by surface sterilized with 70 per cent (v/v) ethyl alcohol and also by holding against flame with spirit lamp.

3.2.1. Media composition and preparation

Potato dextrose agar (PDA):

Peeled potato : 200 g
Dextrose : 20 g
Agar-agar : 20 g
Distilled water : 1000 mL

Preparation: Two hundred grams of peeled potatoes were cut into small pieces and boiled in distilled water and then extract was collected by filtering through muslin cloth. Dextrose 20 g and agar 20 g each were dissolved in the potato extract and the final volume was made up to 1000 mL with distilled water and sterilized as described earlier and preserved for further use.

Potato dextrose broth (PDB):

Peeled potato : 200 g
Dextrose : 20 g
Distilled water : 1000 mL

Preparation: Two hundred grams of peeled potatoes were cut into small pieces and boiled in distilled water and then extract was collected by filtering through muslin cloth. Dextrose 20 g was dissolved in the potato extract and the final volume was made up to 1000 mL with distilled water and sterilized as described earlier and preserved for further use.

Nutrient agar (NA):

Beef extract	:	3.0 g
Peptone	:	5.0 g
Agar	:	15.0 g
Distilled water	:	1000 mL

For the preparation of NA media the above constituents were weighed and dissolved completely to form a homogeneous solution by heating in a micro-oven. Later, the pH of the media was adjusted to 6.5 by adding sodium hydroxide/hydrochloric acid and was subjected for autoclaving.

Nutrient broth (NB):

Beef extract	:	3.0 g
Peptone	:	5.0 g
Distilled water	:	1000 mL

For the preparation of NB medium the above constituents were weighed and dissolved to form a homogeneous solution by heating in a micro-oven. Later, the pH of the medium was adjusted to 6.5 by adding 0.1 N sodium hydroxide /hydrochloric acid and was subjected for autoclaving.

3.3 Sample collection of infected fruits and isolation of pathogens from infected sample fruits

Sample collection of infected or diseased fruit samples of mango and pomegranate were done at the local markets of Yelahanka near the GKVK campus, Bengaluru. Fruit sample selection was done based on the symptoms produced by pathogen on the fruits. The fruits were carried in sterile polythene bag, labelled and stored in the refrigerator. Further experiment was carried out in Biofuel laboratory, Department of Plant Biotechnology.

Aspergillus rot is major disease which cause a great damage in mango and produce the symptoms like lesions around stem end region. The centre of the lesion becomes sunken and will be covered with brownish black spores resulting in depressed mesocarp and a soft rot condition. Based on the above mentioned symptoms the samples were collected.

Xanthomonas axonopodis bacteria on pomegranate produce symptoms like spots on fruits which are dark brown in colour, irregular, slightly raised with oily appearance, sometimes are split open with L or Y shaped cracks. The sample collection of infected pomegranate fruit was done based on the above mentioned symptoms.

3.3.1 Isolation of fungal pathogen from mango

The isolation of fungus was carried out by following direct plating technique. Mango fruits showing typical symptoms of rotting were cut into small pieces along with some healthy un-rotten portion and surface sterilized with mercuric chloride (0.1 %) solution for one minute followed by three subsequent washes with distilled sterilized water to remove all the traces of mercuric chloride. The pieces were aseptically transferred to sterilized Petri plates containing 20 mL sterilized potato dextrose agar (PDA) medium and were incubated at room temperature (27 ± 2 °C). The growth of the fungus was observed periodically. The culture obtained was further purified by aerial mycelial tip technique. Pure colonies developed from the mycelial bit was transferred to PDA slant. The fungus was maintained in slants and kept in refrigerator for further research purpose.

3.3.2 Isolation of bacterial pathogen from pomegranate

Isolation of bacteria was done based on the method described by Chavan *et al.*, (2017). Enumeration of bacterial pathogen from pomegranate was done by spread plate method. The fruit samples were washed thoroughly with tap water and allowed to dry. The samples were surface sterilized with 0.1 per cent mercuric chloride solution for one minute and washed three times serially in sterile distilled water to remove the traces of mercuric chloride. The infected portion was cut into small pieces and suspended in 10 mL of sterile distilled water and squeezed gently with sterilized scalpel. When the water became slightly turbid due to bacterial cells, the suspension was serially diluted up to 10^{-5} dilutions in 9 mL

sterile water blanks. The 0.1 mL of diluted bacterial cell suspension was spread on the sterile nutrient agar petri plates. The inoculated plates were incubated at 30°C for 72 hours. The plates were observed for the development of well separated, typical, light yellow coloured bacterial colonies resembling *Xanthomonas* sp. Four colonies were chosen from each plate and purified by streak plate method. The bacterial isolate was maintained in slants and stored at -80°C in glycerol stocks for further experimental use.

3.4 Morphological identification of isolates

3.4.1 Morphological identification of pathogen isolate from mango

The pure fungal isolate from mango was sub cultured on PDA medium. The spore producing filamentous fungi were detected and identified based on morphological characteristics. Mycelial growth of filamentous fungi can be differentiated into micro and macroscopic morphological characteristics.

Under macroscopic studies, the following observations were made. Colony colour (both at the top and reverse), texture, margin, diameter, elevation and formation of exudates. Under microscopic studies, presence of conidia and conidiophores, conidia colour, structure of conidiophores, conidial shape, phialides, metulae, presence and shape of vesicles.

3.4.2 Preparation of slides for observing microscopic characters

Slides were prepared and incubated in moist chamber at 28°C for five days before observation under a light microscope. The cultures were viewed under microscope by following lacto phenol cotton blue staining procedure and it is as follows. A drop of lacto phenol cotton blue is placed on a slide. The fungal culture is carefully spread in to a thin film by using inoculating needle. A cover slip was carefully placed at the edge on the drop and slowly lowered it and observed under low to high power objectives of microscope.

Lactic acid acts as a preservative for fungi. The phenol portion kills the fungi. The cotton blue stains the fungal elements. Fungal elements were stained a deep blue and background appeared pale blue.

3.4.3 Morphological and biochemical characterization of pathogenic isolate from pomegranate

The pure bacterial isolate from pomegranate, was streaked on NA medium and was taken for identification and confirmation. The identification of bacterial isolate was done based on colony, biochemical and physiological characteristics. Colony colour, shape and appearance were recorded under colony characters. Physiological characters include growth of bacteria at different temperature. Various biochemical tests were performed for the identification of bacteria.

Gram staining

A drop of sterile distilled water was placed in the centre of glass slide. A loopful of inoculum from young culture was taken, mixed with water, and placed in the centre of slide. The suspension was spread out on slide using the of inoculation needle to make a thin suspension. The smear was dried in air and fixed through mild heating by passing the slide three to four times over the flame. The smear was then flooded with crystal violet solution for 1 min and washed gently with flow of water. Then the slide was flooded with iodine solution. After incubation at room temperature for one min, iodine solution was drained out followed by dipping in 95 per cent ethyl alcohol. After that, it was washed with water within 15 to 30 sec and blotted carefully. The smear was added with safranin solution for one min. The slide was washed gently to remove safranin and dried in air. The slide was examined under microscope at 100X power with oil immersion and data was recorded

Starch hydrolysis

Sterile starch agar plates were spot inoculated with 10 μ L overnight broth cultures of the isolates and was incubated at 28 ± 20 °C for 24-48 hours. After incubation, the plates were flooded with iodine solution. The formation of a transparent zone around the colony was taken as positive reaction for the test.

Catalase test

Catalase test was performed to study the presence of catalase enzyme in bacterial colonies. Fresh cultures of pure isolates were taken on glass slides and one drop of H₂O₂

(30 %) was added. Appearance of gas effervescence indicated the presence of catalase enzyme.

Oxidase test

Strip of Whatman's No. 1 filter paper were soaked in a freshly prepared solution of one per cent α -naphthol in 95 % ethanol (Indophenol oxidase Reagent). The colony to be tested was picked up with an inoculation loop and smeared over the moist area. A positive reaction was indicated by an intense deep-purple hue, appearing within five-ten seconds.

Gelatin liquefaction

The gelatin agar medium in the tube was inoculated with four-five drops of a 24-hour broth medium. The inoculated tubes were incubated at 28 °C for 24-48 hours. After the first incubation, the tubes were placed at 4 °C for another 24 hours. The test was considered positive if the media in the tube was liquid form due to utilization of gelatin by bacteria.

KOH test

A drop of three per cent potassium hydroxide solution was placed on a clean microscope slide. A few colonies of bacteria were emulsified to the drop of potassium hydroxide and to a dense suspension. It was continuously stirred for 60 sec and then gently pulled the loop away from the suspension and observed for any changes.

Hydrogen sulphide test

The test organism was stab inoculated on SIM agar medium. The tubes were incubated at 28 °C for 24-48 hours and observed for the formation of black precipitate on the medium.

Indole test

Sterilized test tubes containing four mL of tryptophan broth were inoculated aseptically by taking the growth from 18 to 24 h culture. The tubes were incubated at 37°C

for 24-28 hours. The 0.5 mL of Kovac's reagent was added to the broth culture and observed for the formation of cherry red / pink coloured ring at the top of the media.

Methyl red test

MRVP broth was inoculated with a pure culture of the organism and incubated at 35°-37°C for a minimum of 48 hours in ambient air. Five or six drops of methyl red reagent was added per 5 mL of broth and observed for the colour change in the broth medium.

3.4 Molecular characterization of pathogen isolates

3.4.1 Molecular characterization of pathogenic isolate from mango

Protocol for DNA extraction

For genomic DNA isolation, the cultures was grown in potato dextrose broth (pH 5.5) for 7 days at $28 \pm 1^\circ\text{C}$. DNA was extracted, using the Cetyltrimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980). Around 0.1 g of sample was picked and was placed in a mortar and homogenized with 1 mL of DNA extraction buffer (0.1 M Tris, 1.5 M NaCl, 0.01 M EDTA) and the homogenate was transferred to a 2 ml-microfuge tube. An equal volume of phenol: chloroform: isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 min at 14,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform: isoamly alcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.7 volume of isopropanol and 0.1 volume of 3 M sodium acetate pH 7.0. After 15 min of incubation at room temperature the tubes were centrifuged at 4°C for 15 min at 14,000 rpm. The DNA pellet was washed twice with 70 per cent ethanol and then very briefly with 100 per cent ethanol and air dried. The DNA was dissolved in TE Buffer. To remove RNA, 5 μL of DNase free RNase A (10 mg /mL) was added to the DNA.

After further purification, DNA was quantified spectrophotometrically (Nanodrop) and the quality was analysed in 0.9 per cent agarose gel. For ITS-PCR amplification, extracted genomic DNA was used for amplification along with 10pM of each primer and

TAQ master mix. Composition of TAQ master mix: high-fidelity DNA polymerase, 0.5mM dNTPs, 3.2mM MgCl₂, PCR enzyme buffer.

PCR amplification condition:

The internal transcribed spacer (ITS 1 and ITS 4) regions of the rDNA gene were amplified by the polymerase chain reaction (PCR) using the following two primers: forward primer, ITS1 (5'-TCC GTA GGT GAACCT GCG G-3'), and reverse primer, ITS4 (5'-TCC GCT TAT TGA TAT GC-3'). PCR reactions were carried out in a thermo cycler. The PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 minute, and extension at 70°C for 2 minutes and a final extension at 72°C for 7 minutes. The PCR product was mixed with loading buffer (8µL) containing 0.025 per cent bromophenol blue, 40 per cent w/v sucrose in water and then loaded in 2 per cent agarose gel with 0.1 per cent of ethidium bromide and the amplified product was visualized under a UV trans illuminator. The size of the bands were estimated using 500 bp DNA Marker. Then the PCR product was sent for sequencing (Biokart pvt. Ltd. Bengaluru, Karnataka) to get the sequenced results. The resulted sequence of organism were aligned and compared with the gene sequences present in the GenBank database service provider by BLASTN at NCBI (National Centre for Biological Information) .The Phylogenetic tree was constructed by using Clustal W software by using neighbour joining technique of developing tree with a maximum sequence difference of 0.75.

3.4.2 Molecular characterization of pathogen isolate from pomegranate

Isolation of genomic DNA of bacteria

The genomic DNA of the bacteria was extracted by alkaline lysis method (Sambrook *et al.*, 1989). The bacteria was grown in LB (Luria- Bertani) broth for 24 hours at 30 °C. The cells were pelleted by centrifuging at 12,000 rpm for 2 minutes. The pellet was re-suspended in 650 µL of extraction buffer (100 mM Tris HCl, pH 8.2, 100 mM EDTA and 250 mM NaCl) and incubated at 65 °C for 30 minutes. To the extract, 100 µL of 5 M potassium acetate solution was added and placed on ice for 10 minutes for precipitation of protein and carbohydrates. The supernatant was collected by centrifuging

at 12000 rpm for 8 min. The DNA was precipitated by adding equal volume of chilled isopropanol for 2 hours at -20 °C and then it was centrifuged at 12,000 rpm for 5 min. The pellet obtained was washed twice with 70 percent chilled ethanol, air dried and dissolved in 20 µL of sterile distilled water. The RNA present in the sample was removed by treating sample with 5 µL of RNase at 37 °C for 1h.

Agarose gel electrophoresis

Agarose gel (0.8 per cent) was prepared by dissolving 0.34 g of agarose in 40 mL of 1X TAE buffer and melted. The intercalating dye ethidium bromide (2.5 µL) was added and poured into the gel casting tray placing single comb at one side and allowed for solidification. Then the comb was removed and the gel was transferred to gel tank containing 1X TAE buffer. The gel loading dye was added to the known quantity of DNA and the electrophoresis was carried out at 100 V for 30 minutes. The DNA band in the gel was visualized under UV trans illuminator and documented using gel documentation unit. The concentration and purity of DNA was measured using UV spectrophotometer.

PCR (Polymerase Chain Reaction)

The two primers , forward primer (5' GGATGAGCCCGCGGCCTA 3') and reverse primer (5' CGGTGTGTACAAGGCCCGG 3') already reported for 16S rRNA sequences from the NCBI (<http://www.ncbi.nlm.nih.gov>) were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the PCR reactions. PCR was performed in 20 µL volume reaction mixture containing the following PCR components.

PCR components (20 µl)

- ✓ 2.0 µl of 1X PCR Taq. Buffer with MgCl₂ (1.5 mM)
- ✓ 2.0 µl of 10 mM dNTP's mix (200 µM)
- ✓ 0.5 µl of 16S rRNA primers (forward and reverse (0.5 µM each)
- ✓ 0.3 µl of Taq DNA Polymerase (1U Genei Bengaluru)
- ✓ 1.0 µl of Template DNA (~50 ng /µl)
- ✓ 13.7 µl of Sterile distilled water

PCR amplification was carried out with an initial denaturation at 96 °C for 4 minutes followed by 35 amplification cycles consisting of 94 °C for 1 minute, 55 °C for 30 seconds and 72 °C for 1 minute and a final extension at 72 °C for 12 minutes. Controls for PCR reactions were carried out with the same primers without providing template DNA. Then the amplified product of DNA was electrophoresed using 1 percent agarose gel as described earlier. The DNA band was visualized under UV light and documented using gel documentation unit. Amplified DNA was eluted by using gel elution kit (The Gene JET™ Gel Extraction Kit, Thermo Scientific) and got sequenced by Biokart Pvt. Ltd., Bengaluru, Karnataka. The sequence data received from the company was analysed for homology using NCBI GenBank.

Sequence analysis and homology search

Sequence results were analysed with online software of National Centre for Biotechnology Information (NCBI), USA. The BLAST (Basic Local Alignment Search Tool) search gave the partial length sequence homology with NCBI data (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul *et al.*, 1997).

Phylogenetic analysis

Phylogenetic analyses was performed to know the relationship between identified species with the species deposited in the NCBI GenBank. Phylogenetic analyses were performed using Clustal W software and phylogenetic trees were generated using the neighbour-joining algorithm.

3.5 Evaluation of Plant Extracts on Pathogen Isolates.

The experiment was carried to study the efficacy of aqueous, methanol and chloroform extracts of three different weeds (plants) for management of pathogen isolates from mango and pomegranate.

Table 1: Botanical name, common name and plant part of weeds used in experiment

Sl. No.	Scientific name	Family	Common name	Part used
01	<i>Tridax procumbens</i>	<i>Asteraceae</i>	Coat button/Ghamra	Leaf and Flowers
02	<i>Lantana camara</i>	<i>Verbenaceae</i>	Big sage	Leaf and Flowers
03	<i>Eupatorium odoratum</i>	<i>Asteraceae</i>	Siam weed	Leaves

Taxonomic Hierarchy of *Tridax procumbens*

Kingdom : Plantae
Subkingdom : Tracheobionta
Division : *Magnoliophyta*
Class : *Magnoliopsida*
Subclass : *Asteridae*
Order : *Asterales*
Family : *Asteraceae*
Genus : *Tridax*
Species : *procumbens*

Taxonomic hierarchy of *Lantana camara*

Kingdom : Plantae
Subkingdom : Viridiplantae
Superdivision : Embryophyta
Division : *Tracheophyta*
Subdivision : *Spermatophytina*
Class : *Magnoliopsida*
Superorder : *Asteranae*
Order : *Lamiales*
Family : *Verbenaceae*



Plate 1: Botanical Garden located in GKVK, from where the plant samples were collected

Genus : *Lantana*

Species : *camara*

Taxonomic Hierarchy of *Eupatorium odoratum* (*Chromolaena odorata*)

Kingdom : Plantae

Subkingdom : Viridiplantae

Superdivision : Embryophyta

Division : Tracheophyta

Subdivision : Spermatophytina

Class : Magnoliopsida

Superorder : Asteranae

Order : Asterales

Family : Asteraceae

Genus : *Chromolaena*

Species : *odorata*

3.5.1 Collection of Weed Samples

The weed samples of *Tridax procumbens*, *Lantana camara* and *Eupatorium odoratum* were collected from the botanical garden, situated in University of Agricultural sciences, GKVK Campus, Bengaluru. The samples were collected in polythene cover and brought to Biofuel Laboratory, Department of Plant Biotechnology to proceed with further research work.

3.5.2 Preparation of weed plant samples

Collected weed samples were thoroughly washed under tap water to remove dust and other impurities. The weeds were dried separately under shade with occasional shifting for about three to four weeks. The dried weeds were powdered with grinder and stored in airtight container until further use.

3.5.3 Preparation of solvent extracts from weed samples using Soxlet method

The soxlet extractor uses the solvent reflux and siphon principle to continuously extract the solid matter by pure solvent, which saves the solvent extraction efficiency works at high efficiency.

For the extraction, the selection of solvents is done with care to meet extrability and regulatory criteria. Depending upon the solubility, sterile distilled water, methanol and chloroform were selected for the extraction procedure. 100g of powder is accurately weighed and is transferred to the cup made up of 'Whatman filter paper' and placed into the extraction thimble. 500ml of each solvent, sterile distilled water, methanol and chloroform ethanol were taken in round bottom flask and heated up to their boiling point at 100, 65 and 65 degree celcius respectively. The solvent gets evaporated and moved in to the condenser where it was converted in to liquid trickled in to the extraction chamber containing the plant material. The powder was extracted for 48 hours.

At the end of the extraction process, the flask containing the plant extract was removed and extract was condensed at 50 °C in water bath for overnight. The weight of extract was measured and percentages of yield of the plant material were calculated. Extracts were further diluted to get the desired concentrations of 40 (160 mg mL⁻¹), 60 (240 mg mL⁻¹), 80 (320 mg mL⁻¹) and 100 (400 mg mL⁻¹) per cent. The extract was stored at 4 °C for further work (Sahu and Padhy, 2013).

The percentage of extraction yield was calculated by using following formula (Truong *et al.*, 2019)

$$\text{Extraction yield \%} = \frac{\text{Weight of extract}}{\text{Weight of ground plant material}} \times 100$$

The resultant plant extracts were used for phytochemical analysis, growth inhibition assay against pathogen isolates and for chromatographic analysis.

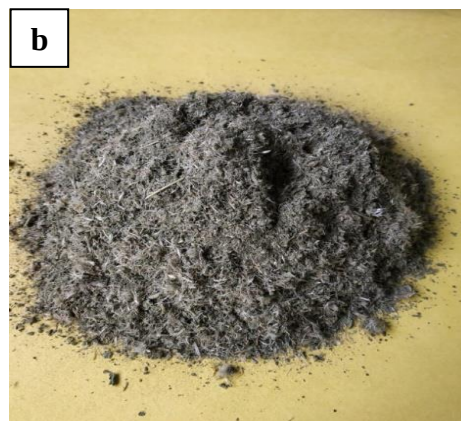


Plate 2 a: Fresh leaf sample of *Tridax procumbens*
b: Dried and powdered sample of *T. procumbens*

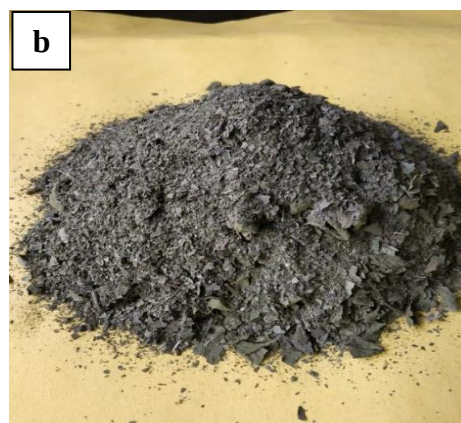


Plate 3 a: Fresh leaf sample of *Lantana camara*
b: Dried and powdered sample of *L. camara*

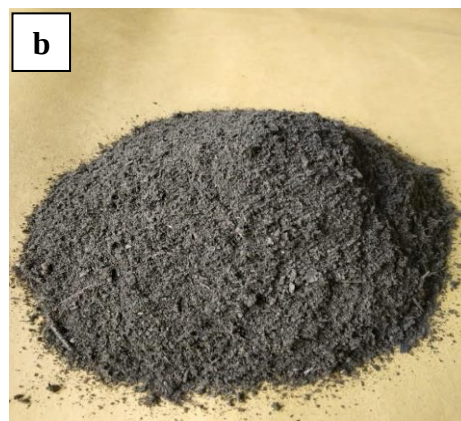


Plate 4 a: Fresh leaf sample of *Eupatorium odoratum*
b: Dried and powdered sample of *E. odoratum*

3.5.4 Screening of weed plant extracts (Phytochemicals) for fungicidal activity by poisoned food technique

The experiment was carried out in completely randomized design. The efficacy weed plant extracts were evaluated against fungal isolate under *in vitro* conditions on the PDA media using poison food technique (Nene and Thapliyal, 1993). The required quantities of individual plant extracts of different solvents (Aqueous, methanol and chloroform) with different concentrations (160 mg mL^{-1} , 240 mg mL^{-1} , 320 mg mL^{-1} and 400 mg mL^{-1}) were added separately into molten and cooled PDA. Later two mL of such poisoned medium was poured into sterile Petri plates. $10 \text{ }\mu\text{L}$ of seven day old liquid culture of the fungal isolate was spotted on to the media at the center of the Petri plates.

The experiment was conducted using completely randomized design (CRD). Control was maintained without adding any plant extracts to the medium and each treatment was replicated thrice. Then such plates were incubated at $28 \pm 2^\circ\text{C}$ temperature for seven days and radial colony growth (cm) was measured. The efficacy of a plant extract was expressed as per cent inhibition of mycelial growth over control that was calculated by using Vincent's formula (1947).

The fungi toxicity of the plant extracts in terms of percent inhibition of mycelial growth was calculated using the formula:

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent inhibition of mycelium

C = Growth of mycelium in control

T = Growth of mycelium in treatment

3.5.5 Screening of plant extracts for bactericidal activity by disc diffusion method

A disc-diffusion assay was used to determine the growth inhibition of bacteria by plant extracts. A single colony from an overnight bacterial culture plate were seeded into 5 mL of an appropriate pre-warmed nutrient broth. Culture tubes are shaken at 300 rpm

and 37 °C .Using a sterile swab, cultures will be spread evenly onto pre-warmed 37 °C nutrient agar plates. Sterile filter paper discs (6 mm Whatman No.1 paper) were gently pressed onto the surface of the agar plates, and plant extracts are then pipetted onto the discs. Each plant extracts are tested at various concentration 40 (160 mg mL⁻¹), 60 (240 mg mL⁻¹), 80 (320 mg mL⁻¹) and 100 (400 mg mL⁻¹) per cent with different treatments and three replications.

The experiment was conducted by completely randomized design (CRD). Control was maintained without adding plant extracts. Plates were then inverted and incubated for approximately 24 h at 37 °C and the diameter of the inhibition zones will be measured in mm, including the diameter of the disc. The sensitivity of different solvent extracts were classified according to Ponce *et al.* (2003) is as follows:

Table 2: Classification of sensitivity of bacteria based on zone of inhibition produced

Sl. No.	Diameter(mm)	Sensitivity
1.	< 8 mm	Not sensitive
2.	9 – 14 mm	Sensitive
3.	15 – 19 mm	Very Sensitive
4.	> 20 mm	Extremely Sensitive

The sensitivity of different solvent extracts were classified also classified based on MIC (Minimum Inhibitory Concentration) values. It is the least concentration at which there is inhibition of growth of pathogen.

3.6 Resazurin assay for cell viability

Cell-based viability assays are important to determine whether cells are alive or dead. Such assays are widely used for investigating cell response to a given agent, drug or to establish relative cytotoxicity of chemicals. Living cells maintain a reducing environment within their cytoplasm and mitochondria, in which resazurin (blue and non-fluorescent) is reduced by dehydrogenase enzymes to form the red fluorescent dye resorufin.

Preparation of resazurin solution:

The 0.02 per cent of resazurin solution was prepared by dissolving two mg in 10 mL of sterile distilled water. The solution was vortexed and dissolved to make homogenous solution.

Preparation of the plates

Plates were prepared under aseptic conditions in triplicate. In a sterile 96-well plate 180 µl of diluted plant extracts were loaded and 20 µl fungal and bacterial culture was added into all the wells, controls were maintained without plant extracts. Sealed with para film and incubated at $28 \pm 2^\circ\text{C}$ for 5 days. After five days of incubation 10 µL of 0.02 per cent Resazurin solution was added into all wells and incubated for 30 min. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value (Bonnier *et al.*, 2015).

3.7 Phytochemical screening of plant extracts of different solvents

Phytoconstituents are the natural bioactive compounds found in plants. This phyto constituents work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions. Phytochemicals are basically divided into primary and secondary constituents according to their functions in plant metabolism .Primary constituents comprise common sugars, amino acid, proteins and chlorophyll while secondary constituents consist of alkaloids, terpenoids, steroids and flavonoids etc. (Praveen and Nair, 2014)

The crude products obtained in soxlet extraction technique were subjected to qualitative phytochemical evaluation according to standard procedure. (Sofowora *et al.*, 2013).

Table 3: Standard procedures for qualitative phytochemical analysis of solvent extracts of weeds

Sl. No.	Phytoconstituents	Procedure
01	Tannins (Ferric chloride Test)	2mL extract + 2mL H ₂ O + 2-3 drops FeCl ₃ (5%)
02	Phenol	2mL extract + 2mL H ₂ O + 2-3 drops FeCl ₃ (5%)
03	Flavonoids (Alkaline reagent test)	2mL extract + few drops of 20% NaOH.
04	Terpenoids (Salkowaski Test)	5mL extract + 2mL Chloroform + 2-3 drops conc. H ₂ SO ₄ .
05	Saponins (Foam Test)	5mL extract + 5mL H ₂ O + heat or Shake well
06	Glycosides (Liebermann's Test)	5mL extract + 2mL Glacial acetic acid containing a drop of FeCl ₃ Solution + 2ml conc H ₂ SO ₄
07	Alkaloids (Hager's Test)	2mL extract + few drops of Hager's reagent
08	Anthocyanins	2mL extract + 2mL HCl (2N) + NH ₃
09	Steroids (Lieberman Burchard Test)	2mL extract + 2mL CHCl ₃ + 2mL H ₂ SO ₄ (conc.) + Acetic anhydride
10	Coumarins	2mL extract + 3mL NaOH (10%)
11	Emodins	2mL extract + 2mL NH ₄ OH + 3ml Benzene
12	Quinones	2 mL extract + dilute NaOH

3.8 Thin layer chromatography purification of bioactive components present in Aqueous, methanol and chloroform extracts of weed samples.

Thin layer chromatography (TLC) was carried out to isolate the principle components that were present in extracts of different solvents. TLC studies were carried out for aqueous, methanol and chloroform extracts of weed samples. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution.



Plate 5: Soxlet apparatus used for plant extract preparation

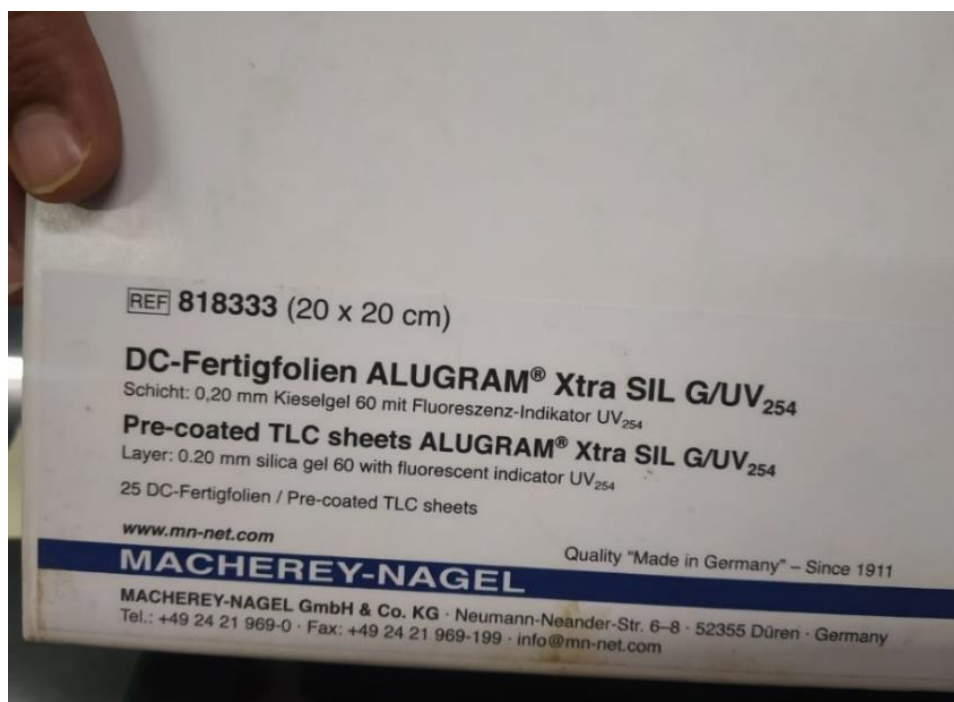


Plate 6: Pre coated Thin Layer Chromatography Sheets

TLC purification was done based on method described by Sharma and Paliwal, 2013. TLC was carried out on all the fractions using Pre-coated TLC Sheets ALUGRAM Xtra SIL G/UV₂₅₄ by using one way ascending technique. The plates were cut with scissors and marked with pencil about 1-1.5 cm from the bottom of the plate. Capillary tubes were used to uniformly apply the dissolved samples on the plates and allowed to dry. The plates were developed in a chromatographic tank using the different TLC solvent systems. The plates were dried and visualized under normal day light.

The retention factor (R_f) for each active compound was calculated for each fraction using the following formula

$$R_f (\text{Retention factor}) = \frac{\text{Distance moved by the solute/compound}}{\text{Distanced moved by the solvent (solvent front)}}$$

Table 4: List of solvent systems used for screening of phytochemicals by TLC

Sl. No.	Solvent System used	Proportion
01	Ethyl acetate : Methanol : Water (S1)	10:2:1
02	Toluene :Ethyl acetate :Methanol (S2)	24 :24:2
03	Hexane : Ethyl acetate (S3)	9:1
04	Chloroform : Ethyl acetate : Methanol : water (S4)	15 : 8 : 4 : 1
05	Ethyl acetate :Formic acid :Acetic acid : Water (S5)	100 : 11 : 11 : 26
06	Chloroform : Glacial acetic acid : Methanol : Water (S6)	5: 4: 1:2
07	Chloroform :Ethyl acetate : Formic acid (S7)	5:4:1
08	Conc HCl : Acetic acid : Water (S8)	3 :30 : 10

IV RESULTS AND DISCUSSION

The present investigation was envisaged to study the effect of Aqueous, Methanol and Chloroform extracts of *Tridax procumbens*, *Lantana camara* and *Eupatorium odoratum* against phytopathogenic fungi isolate (*Aspergillus niger*) from mango and phytopathogenic bacteria (*Xanthomonas axonopodis* pv. *punicae*) isolate from pomegranate. Results obtained from the present investigation are furnished under this chapter.

4.1 Isolation of fungal and bacterial pathogens from mango and pomegranate

Aspergillus spp. are widely distributed among the spoilage fruit fungi and also secrete several plant cell wall degrading enzymes (Al-Hindi *et al.*, 2011). Stem end rot majorly contribute in post-harvest losses of mango during storage (Maqsood *et al.*, 2014). This observation could be accounted for by the obligate saprophytic feeding habit of the fungus as well as its ability for rapid growth and sporulation on sugary substances (Terna *et al.*, 2015).

Xanthomonas axonopodis pv. *punicae* infects pomegranate fruit tissues through both natural opening as well as by injuries during storage and results in huge economic loss (Sharma *et al.*, 2017). The severity of disease is very high because of its rapid build-up of inoculum and wide spread of the disease (Chavan *et al.*, 2017).

The samples of infected fruits of mango and pomegranate which were collected from the local markets were subjected for the isolation of post-harvest pathogens. The infected parts of mango were surface sterilized and directly transferred to the potato dextrose agar (PDA) medium and the fungus was isolated based on standard plate count method. After the complete growth of fungus in the plate, based on the morphological characters the isolate was identified as *Aspergillus niger*. The results obtained by following this method of isolation in this experimental research are quite similar to the studies undertaken earlier by Maqsood *et al.* (2014).

The bacterial pathogen from pomegranate was isolated on nutrient agar (NA) medium by serial dilution and pour plate method and purification was done by streak plate method. After 48 h of incubation at 28 °C, the bacterial colonies were grown on the medium. Further bacterial strain was subjected to morphological and molecular identification and based on the results it was identified to be *Xanthomonas axonopodis*. Results are similar with the finding of Sharma *et al.* (2017) who also isolated *Xanthomonas axonopodis* from the oily spot disease infected pomegranate fruit by following similar procedure of isolation and identification.

The cultures were sub cultured on PDA slants for fungus and NA slants for bacteria kept at 28 ± 2 °C for incubation. Those slants were preserved in refrigerator at 4°C and maintained by sub-culturing once in a month in order to avoid a decline in strain viability. Such cultures were used throughout the study.

4.2 Morphological characterization of isolated pathogen from mango

During the study for isolation of pathogen from post-harvest rots produced in mango, it was noticed that *Aspergillus niger* was found to be the most prominent in all the isolation plates. This pathogen isolate grew rapidly on PDA medium maintained at temperature of 28 °C attaining the maximum diameter of 65-80 mm within four-five days. After 48 hours of inoculation at 26 ± 2 °C, the fungus produced white colonies and then due to conidial production the colonies turned black in colour (Plate no 5). Identification of fungal isolate was done on the basis of morphological and microscopic characteristics. Macroscopic features like colony colour, texture and margins, as well as microscopic features such as size of conidia, conidiophores and their arrangements were recorded and depicted in the Table 5 and 6.

Maqsood *et al.* (2014) studied on post-harvest diseases of mango and their losses. He isolated *Aspergillus niger* as the most prominent fungi during post-harvest or storage which causes huge economic losses. The morphological identification studies and the results obtained by them were similar to that of the isolated pathogen under present study.

Table 5: Macroscopic Characteristics recorded for *Aspergillus niger*

Sl. No.	Morphological features	Observation
1	Diameter of colony	25-30 mm
2	No of colonies per plate	3-8
3	Surface colour of the colony	Black to dark brown
4	Reverse colour of the colony	Colourless to light yellow
5	Colony texture	Smooth Mat
6	Colony margin	White
7	Formation of exudates	Present
8	Production of sclerotia	Absent
9	Elevations	Umbonate
10	Growth	Rapid

Table 6: Microscopic characteristics recorded for *Aspergillus niger*

Sl. No.	Microscopic Features	Observation
1	Phialides	Two series (Biserate)
2	Shape of vesicle	Globular
3	Hyphae	Branched septate
4	Fruiting body	Cleistothecia present
5	Conidia	Globose, ellipsoid
6	Phialide shape	Flask-shaped (ampulliform, with constriction)

4.3 Morphological identification and biochemical characterization for bacterial pathogen isolate of pomegranate

Xanthomonas is one of the most important plant pathogens affecting almost 268 dicot plants including economically important plants such as citrus fruits, beans, cotton, grapes and pomegranate (Pawar *et al.*, 2014). To know the basic information and properties of the target pathogen, basic microbiological and biochemical tests are important. The isolated bacterial pathogen (*Xanthomonas axonopodis* pv. *punicae*) was cultured on NA medium at 28 °C. The isolated bacteria tested positive for KOH test and negative for Gram staining indicates the gram negative nature of the bacteria. Bacterial culture was identified based on its morphology and biochemical test which were performed according to standard procedures.

Colony morphology: The *Xanthomonas axonopodis* pv. *punicae* colonies grown on NA medium were yellow, convex and mucoid and differed in colony size (1 - 3 mm). *Xanthomonas* were slow growing and difficult to isolate usually due to masking effect of other fast growing, yellow pigmented bacteria. The bacterial isolate studied was able to form smooth, slightly raised, round colonies upon 48-72 hours of incubation at 28°C.

Gram staining: Gram's staining was performed to determine Gram reaction of the isolate. Microscopic observation revealed that it is rod shaped and Gram-negative bacterium.

Growth at different temperature: Single colony of *Xanthomonas axonopodis* pv. *punicae* which were taken from mother culture by sterile loop and inoculated on the NA medium. The plates after incubation at 60 °C and 40 °C produced no growth of bacteria. The optimum temperature for *Xanthomonas axonopodis* was found 28°C- 30°C .

Growth at 4% NaCl: Single colony of *Xanthomonas axonopodis* pv. *punicae* were taken from mother culture plate by sterile loop and inoculated on the NA medium containing 4 per cent NaCl. The plates were then incubated at 28°C for 48 h. The growth of bacteria was not observed.

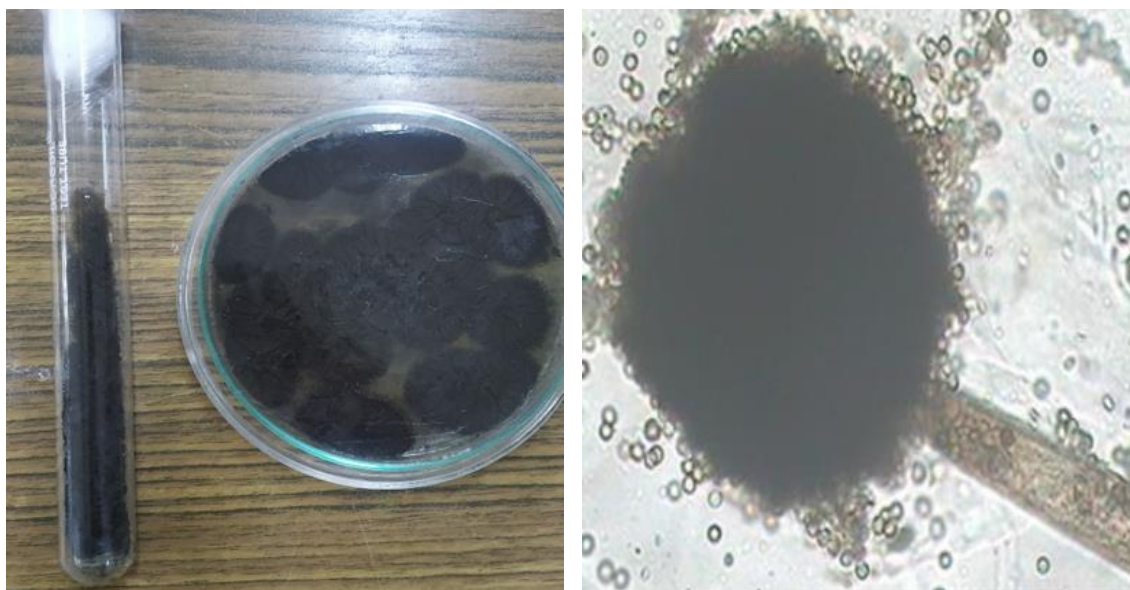


Plate 7: The isolated pure culture and microscopic view of *Aspergillus niger* from mango

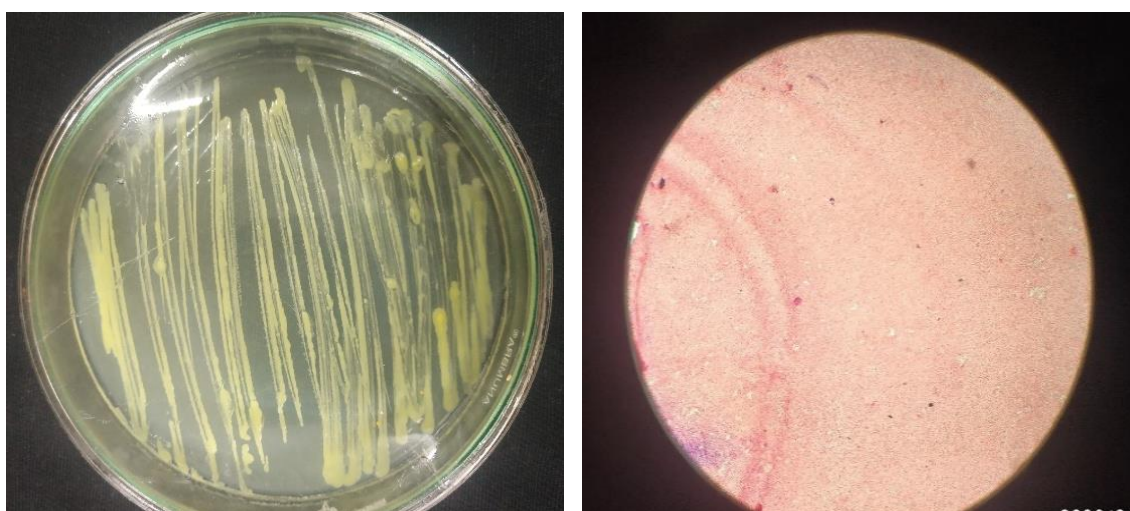


Plate 8: The isolated pure culture and microscopic view of *Xanthomonas axonopodis* pv. *punicae* from pomegranate

Table 7: Macroscopic morphological characteristics of *Xanthomonas axonopodis* isolate from pomegranate

Sl. No.	Macroscopic Features	Observation
1	Colour	Bright yellow to Yellow
2	Shape	Circular
3	Size	0.2mm
4	Appearance	Flattened, glistening
5	Growth at 60 °C, 40 °C	No growth
6	Margin	Concave
7	Opacity	Opaque
8	Consistency	Moist
9	Elevation	Elevate
10	Negative staining	Transparent rod shaped

The biochemical characteristics of bacterium causing bacterial blight of pomegranate were determined by following different tests. Fresh (48 hrs old) bacterial culture was used for all the tests. The bacteria has shown positive test for starch hydrolysis by forming clear zone around the growth of bacterium indicating the hydrolysis of starch. It has shown positive reaction for gelatin test, which indicates that the bacteria uses protein gelatin as a source of carbon and energy for its growth. Bacteria shown positive reaction for KOH solubility test indicates that the bacteria was gram negative because KOH dissolves the thin layer of peptidoglycan of the cell walls of gram negative bacteria. Bacteria showed positive reaction for hydrogen sulphide test indicates that the microbe reduces sulfur to sulfides during the process of metabolism. The pink colour of the cells after staining indicates the bacteria was gram negative. The bacteria showed positive reaction for catalase test. Catalase is a common enzyme found in nearly all living organisms exposed to oxygen which catalyse the decomposition of hydrogen peroxide to water and oxygen. The bacteria has shown positive reaction for oxidase which indicates it is aerobic.

The bacteria shown positive reaction for indole test, which indicates that tryptophan is converted into indole by bacteria. It has shown negative reaction for methyl red (MR) test indicates that bacteria do not produce sufficient acid during the fermentation of glucose and the maintenance of pH conditions.

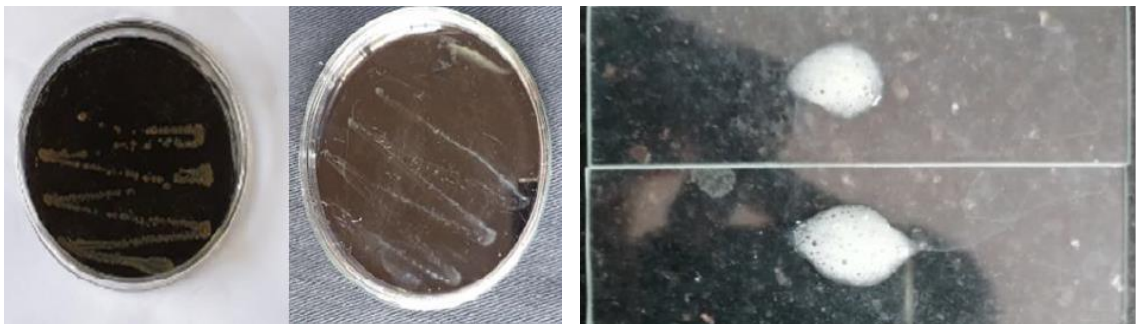
Table 8: Biochemical tests for identification of bacterial isolate causing bacterial blight of pomegranate

Sl. No.	Biochemical Test	Reaction
1	Starch hydrolysis	+
2	Gelatin liquefaction	+
3	KOH solubility	+
4	H ₂ S production	+
5	Gram staining	-
6	Catalase test	+
7	Oxidase test	+
8	Indole test	+
9	Methyl red test	-

The similar study was conducted by Chavan *et al.* (2017), where they studied on the genetic variability among the *Xanthomonas axonopodis* pv. *punicae* which causes bacterial blight of pomegranate. The results obtained for the morphological and biochemical characterization of bacteria were similar to the results for present investigation under study.

4.4 Molecular characterization of *Aspergillus niger*

Aspergillus niger is one of the major fungus which causes post-harvest loss in mango. It grows rapidly on the PDA medium (Shalini and Amutha, 2014). Along with morphological identification, molecular characterization was also carried out to confirm the type of fungus. The cells of fungal isolate, was harvested through centrifugation and



Starch Hydrolysis

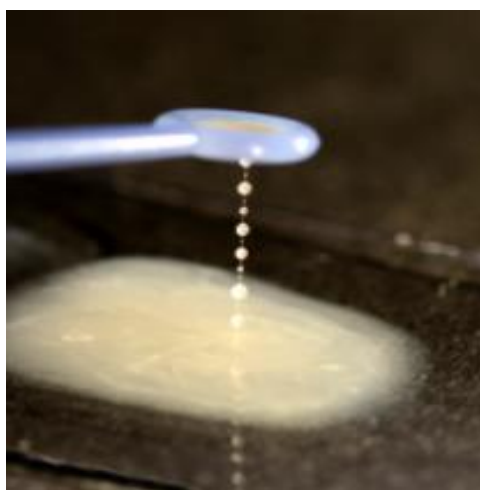
Catalase Test



Gelatin hydrolysis

Methyl red test

Oxidase test



KOH TEST

Plate 9: Biochemical characterization of *Xanthomonas axonodis* pv. *punicae*

	Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
✓	Aspergillus foetidus CBS 121.28 ITS region; from TYPE material	Aspergillus foetidus	1031	1031	100%	0.0	98.96%	601	NR_163668.1
✓	Aspergillus welwitschiae CBS 139.54 ITS region; from TYPE material	Aspergillus welwitschiae	1031	1031	100%	0.0	98.96%	596	NR_137513.1
✓	Aspergillus niger ATCC 16888 ITS region; from TYPE material	Aspergillus niger	1031	1031	100%	0.0	98.96%	576	NR_111348.1
✓	Aspergillus costaricensis CBS 115574 ITS region; from TYPE material	Aspergillus costaricensis	1014	1014	100%	0.0	98.44%	596	NR_103604.1
✓	Aspergillus piperis CBS 112811 ITS region; from TYPE material	Aspergillus piperis	1009	1009	100%	0.0	98.27%	597	NR_077191.1
✓	Aspergillus luchuensis KACC 46772 ITS region; from TYPE material	Aspergillus luchuensis	985	985	97%	0.0	98.39%	560	NR_135449.1
✓	Aspergillus brasiliensis ATCC MYA-4553 ITS region; from TYPE material	Aspergillus brasiliensis	977	977	96%	0.0	98.38%	564	NR_111414.1
✓	Aspergillus tubingensis NRRL 4875 ITS region; from TYPE material	Aspergillus tubingensis	963	963	95%	0.0	98.36%	608	NR_131293.1

Fig. 1: NCBI Blast result for *Aspergillus niger*

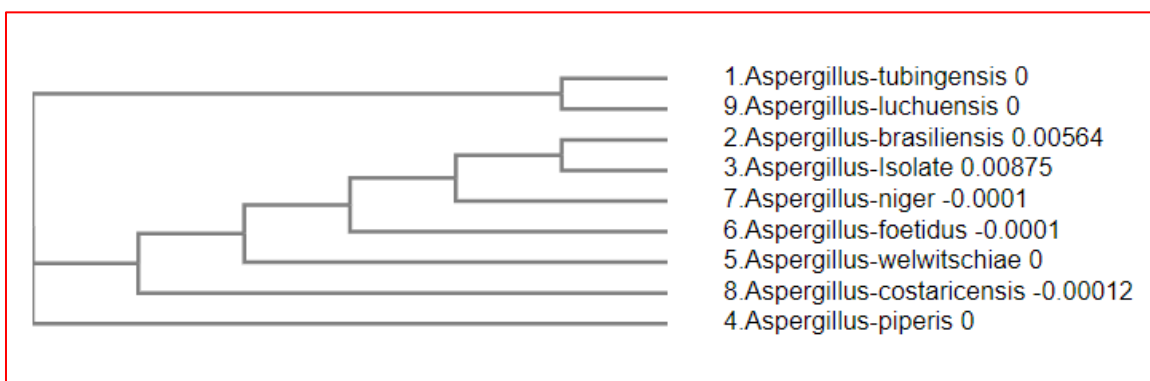


Fig. 2: Phylogenetic tree construction for *Aspergillus niger*

DNA was isolated and genomic DNA was extracted by following CTAB method and quality was assessed by gel electrophoresis and quantified using Nanodrop. Later amplification was carried using Internal Transcribed Regions (ITS1 and ITS4) primers. The PCR product was out sourced to get the sequenced results in the FASTA format. The obtained sequences were then compared against sequences on the GenBank at the NCBI website using nucleotide blast programme. The sequences of isolated fungal pathogen from mango matches up to 98.96 per cent with *Aspergillus niger*. The gel picture of amplified PCR product was viewed under gel documentation unit. The phylogenetic tree diagram was constructed with the help of nearest homolog species to that of the isolated fungal pathogen by using Clustal W software by neighbour joining method of developing tree.

Molecular characterization is a rapid and a quick procedure which requires minimal handling of pathogens. It also helps in distinguishing morphologically, similar fungal species. Several similar studies on the application of PCR technology were used for the identification and detection of fungi, by using internal transcribed spacer (ITS) markers (Shalini and Amutha, 2014). Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. Hence ITS primers were used to identify the fungus by its amplification with the template DNA.

Al-Najada *et al.* (2014) carried out a research work on isolation and classification of fungi associated with spoilage of post-harvest mango and their morphological and molecular characterization. They used ITS forward and reverse primers for the amplification of template DNA of *Aspergillus niger*. The methods of characterization and results obtained were similar to that of present study undertaken.

4.5 Molecular characterization of *Xanthomonas axonopodis* pv. *punicae*

Xanthomonas is a large genus of gram-negative, yellow-pigmented, exclusively plant-associated or pathogenic bacteria that collectively cause disease in many plant species. The broad host range of the genus contrasts with stringent host and tissue specificity for individual species and pathovars (Bogdanove *et al.*, 2012). Now pathogen

is considered as a target organism for management and phytosanitary regulations in many countries. It is difficult to detect *Xanthomonas* just on the basis of microbial and biochemical characterization. Other yellow pigmented bacteria may be isolated from the infected tissues instead of those belonging to the genus *Xanthomonas* (Kale, 2009). Molecular methods in combination with conventional methods have a great potential in rapid plant pathogen diagnosis (Kale *et al.*, 2012)

Bacterium from pomegranate infected with bacterial blight was isolated on NA medium and identified by 16S rRNA gene sequence. The genes encoding for 16S rRNA in prokaryotes are the most widely used in molecular taxonomy, because, they are 1) Sufficiently conserved, 2) Functionally constant, 3) Universally distributed and 4) Have adequate length to provide a view of evolution encompassing all living microorganisms (Madigan *et al.*, 2009).

The 16S rRNA partial gene sequence of the bacterial isolate from pomegranate showed 99.67 percent homology with *Xanthomonas axonopodis* available in the NCBI data base. Thus, the bacterium was identified as *X. axonopodis* pv. *punicae*. The phylogenetic tree was constructed with related homologues available in the NCBI GenBank with the sequences of identified organism.

Molecular confirmation of *Xanthomonas axonopodis* pv. *punicae* through ITS primer used to amplify the 400 bp gene of ITS region. The DNA of *X. axonopodis* pv. *punicae* isolates were extracted and amplified by polymerase chain reaction (PCR) by ITS forward primer 799 and reverse primer 1193. The amplified product is compared with the 100 bp molecular marker. Polymerase chain reaction (PCR) yielded an amplicon of the size of 400 bp for all the *X. axonopodis* pv. *punicae* isolates (Chowdappa *et al.*, 2018; Raghuwanshi *et al.*, 2013).

4.6 Extraction yield of extracts

Extraction yield of weeds in each solvents were determined as described in “Materials and Methods”. Extraction yields of each weed in different solvents are presented in Table below.

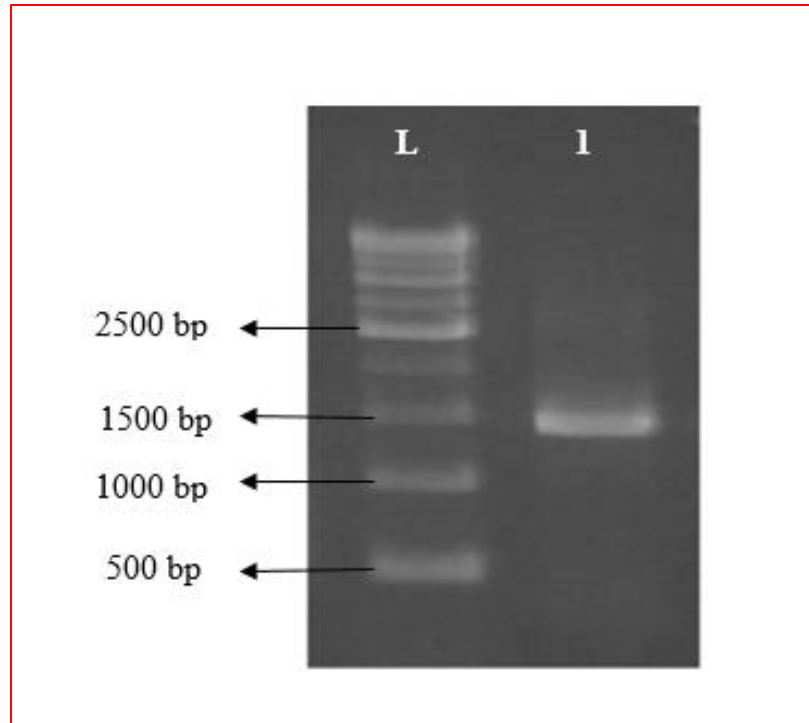


Plate 10: PCR profile of *Aspergillus niger* isolate amplified using ITS primers

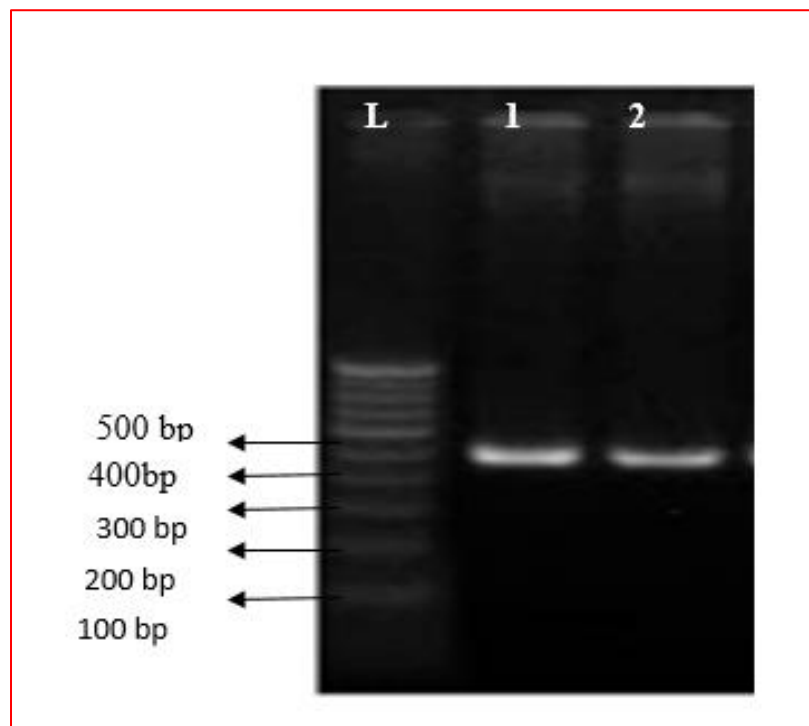


Plate 11: PCR profile of *Xanthomonas axonopodis* isolate amplified using 16S rRNA primers

	Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
✓	Xanthomonas axonopodis strain LMG 538 16S ribosomal RNA, partial sequence	Xanthomonas axonopodis	2745	2745	100%	0.0	99.67%	1502	NR_026317.1
✓	Xanthomonas euvesicatoria strain DSM 19128 16S ribosomal RNA, partial sequence	Xanthomonas euvesicatoria	2695	2695	100%	0.0	99.07%	1535	NR_104773.1
✓	Xanthomonas arboricola strain LMG 747 16S ribosomal RNA, partial sequence	Xanthomonas arboricola	2689	2689	100%	0.0	99.00%	1502	NR_125714.1
✓	Xanthomonas campestris strain ATCC 33913 16S ribosomal RNA, complete sequence	Xanthomonas campestris	2689	2689	100%	0.0	99.00%	1547	NR_074936.1
✓	Xanthomonas hortorum strain LMG 733 16S ribosomal RNA, partial sequence	Xanthomonas hortorum	2689	2689	100%	0.0	99.00%	1502	NR_026386.1
✓	Xanthomonas vasicola strain LMG 736 16S ribosomal RNA, partial sequence	Xanthomonas vasicola	2689	2689	100%	0.0	99.00%	1502	NR_026383.1
✓	Xanthomonas euvesicatoria pv. citrumelonis strain LMG 9325 16S ribosomal RNA, partial se...	Xanthomonas euvesicatoria pv. citru...	2689	2689	99%	0.0	99.07%	1499	NR_104965.1
✓	Xanthomonas fuscans strain LMG 826 16S ribosomal RNA, partial sequence	Xanthomonas fuscans	2689	2689	99%	0.0	99.07%	1499	NR_104958.1

Fig. 3: NCBI Blast result for *Xanthomonas axonopodis* pv. *punicae*

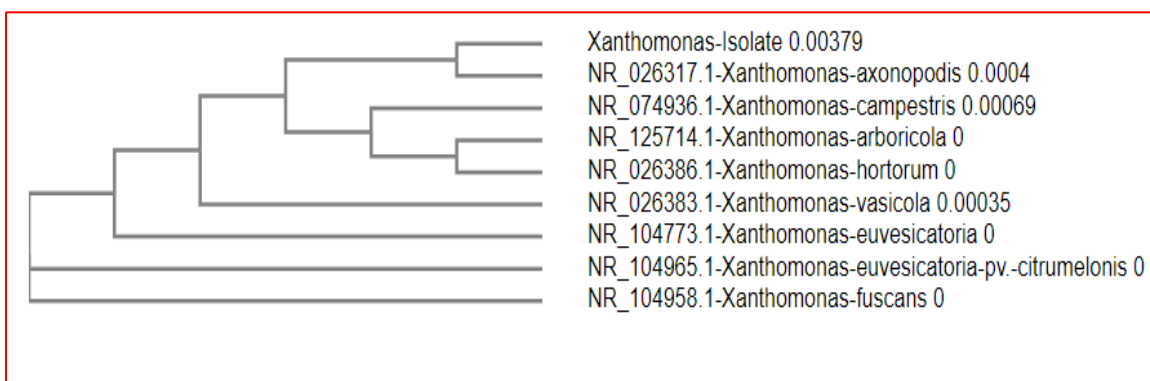


Fig. 4: Phylogenetic tree construction for *Xanthomonas axonopodis* pv. *punicae*

Table 9: Effect of different solvents on per cent extraction yield from dry weight of weeds

Weeds	Extraction yield (%) in solvents		
	Aqueous	Methanol	Chloroform
<i>T.procumbens</i>	15	28	20
<i>L.camara</i>	17	35	25
<i>E.odaratum</i>	19	31	26

Extraction is the main process by which bioactive compounds may be obtained from biomass materials. The objective of extraction process is to maximize the amount of target compounds and to obtain the highest biological activity of these extracts (Pourmorad *et al.*, 2006). Extraction yield and biological activity of the resulting extract is not only affected by the extraction technique but also by the extraction solvent (Ajanal *et al.*, 2012).

Due to the variety of bioactive compounds contained in plant materials and their differing solubility properties in different solvents, the optimal solvent for extraction depends on the particular plant materials, and the compounds that are to be isolated (Mahdi-Pour *et al.*, 2012). Among solvents tested, methanol resulted in the highest extraction yield, followed by chloroform and aqueous in all the selected indicating that the extraction efficiency favors the highly polar solvents.

Extraction yield of *Tridax procumbens*

The maximum extraction yield of *T.procumbens* was reported in methanol extract (28 %) and was followed by chloroform extract (20 %). The results were minimum in aqueous extract at the rate of 15 per cent. Hence methanol extract is a good polar solvent to extract maximum bioactive components. These results are in line with Das *et al.* (2017) who extracted air dried powdered leaves of *Tridax procumbens* with different solvents chloroform, hexane, petroleum ether, ethanol and methanol to yield crude extracts (Per cent yield of the crude extracts) 11.50 – 25 per cent w/w.

Extraction yield of *Lantana camara*

In case of *Lantana camara* leaves methanol exhibited (35 %) maximum extraction yield whereas minimum 17 per cent extraction yield was observed in chloroform. The results corroborate with findings of Patel (2013) who prepared *L. camara* leaves extracts by using polar to non-polar solvents like water, methanol, chloroform and petroleum ether. Water, methanol, chloroform and petroleum ether extracts of *L. camara* leaves showed extraction yields of 45.21, 20.21, 5.34 and 2.56 per cent respectively. These results are in line with Pawar *et al.* (2013) who found that the solvent methanol soluble extractive values (Appearance- greenish brown) 32 % w/w and water soluble extractive value brown 13.23 per cent w/w.

Extraction yield of *Eupatorium odoratum*

E. odoratum weed extracts exhibited maximum extraction yield in methanol (31 %) followed by 26 per cent in chloroform and lowest 19 per cent in aqueous extract. Egbunu *et al.* (2019) reported that the ethanol extract had significant percentage yield (5.49 g) after the extraction, while the aqueous extract had a yield of 3.5 g. The results were in line with Sobrinho *et al.* (2017) who reported 27.05 per cent alcohol soluble extractive of *E. odoratum* whereas water soluble extractive was 34.90 per cent, hexane soluble extractive was 29.65 per cent, acetone soluble extractive was 13.48 per cent and methanol soluble extractive was 29.83 per cent.

4.7 Screening of solvent extracts of selected weeds against *Aspergillus niger* and *Xanthomonas axonopodis* pv. *punicae* by Resazurin assay for cell viability

Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxido-reductases within viable cells. Resorufin is further reduced to hydroresorufin (uncolored and non-fluorescent). A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk (McNicholl *et al.*, 2007; Bonnier *et al.*, 2015).

The effectiveness of this modified resazurin assay has been demonstrated with weed extracts against *Aspergillus niger* and *Xanthomonas axonopodis* pv. *punicae* and the results were furnished in table 10 and table 11 respectively. The visual observations of resazurin assay for cell viability was revealed for aqueous, methanol and chloroform Extract of selected weeds at different concentrations at 40 per cent (160 mg ml⁻¹), 60 per cent (240 mg ml⁻¹), 80 per cent (320 mg ml⁻¹) and 100 per cent (400 mg ml⁻¹) against *A.niger*. It was revealed that, methanol and chloroform extracts of *Tridax procumbens* remain blue at 100 and 80 % conc. respectively. The methanol extract of *L.camara* remain blue at 80 and 100 per cent. The chloroform extract of *Eupatorium odoratum* remain blue at 100 per cent concentration. It indicates that cell death occurred at that concentration of weed extracts.

In case of *Xanthomonas axonopodis* pv. *punicae*, the methanol extract of *Tridax procumbens* remain blue at 80 and 100 per cent and chloroform extract at 80 per cent. The methanol extract of *Lantana camara* remain blue at 80 and 100 per cent. The aqueous and chloroform extracts of *Eupatorium odoratum* remain blue at 100 per cent

In current study, the methanol and chloroform extracts of weeds extracts are found to show fungicidal and bactericidal activity. The results are supported by Webster *et al.* (2010) they reported antimycobacterial effect of different of 5 medicinal plants on *Mycobacterium tuberculosis* by microplate resazurin assay. Hussain *et al.* (2011) who tested essential oils of lamiaceae by resazurin assay method against some phytopathogenic bacteria they found that this method was effective, sensitive and quick against essential/volatile oils. This provided the reproducible and accurate results and allowed direct comparison of the antifungal and antibacterial activity of the tested extracts.

4.8 Screening of solvent extracts of selected weeds for fungicidal activity (*Aspergillus niger*) by poisoned food technique

In present investigation, solvent extracts of selected weeds were evaluated *in vitro* for their efficacy against *Aspergillus niger* through poisoned food technique at 40, 60, 80 and 100 per cent concentrations. All the below mentioned experiments were carried out by using CRD and controls were maintained without adding any plant extract to the media.

Table 10: *In vitro* evaluation of plant extracts against *Aspergillus niger* by resazurin assay method

Solvent extracts	Cell viability at different concentration			
	40 %	60 %	80 %	100 %
<i>Tridax procumbens</i>				
Aqueous	pink	pink	pink	pink
Methanol	pink	pink	pink	blue
Chloroform	pink	pink	blue	pink
<i>Lantana camara</i>				
Aqueous	pink	pink	pink	pink
Methanol	pink	pink	blue	blue
Chloroform	pink	pink	pink	pink
<i>Eupatorium odoratum</i>				
Aqueous	pink	pink	pink	blue
Methanol	pink	pink	pink	pink
Chloroform	pink	pink	pink	blue

Pink/ colorless – Live

Purple/ blue - Dead

Table 11: *In vitro* evaluation of plant extracts against by *Xanthomonas axonopodis* pv. *punicae* by resazurin assay method

Solvent extracts	Cell viability at different concentration			
	40 %	60 %	80 %	100 %
<i>Tridax procumbens</i>				
Aqueous	pink	pink	pink	pink
Methanol	pink	pink	blue	blue
Chloroform	pink	pink	blue	pink
<i>Lantana camara</i>				
Aqueous	pink	pink	pink	pink
Methanol	pink	pink	pink	blue
Chloroform	pink	pink	pink	pink
<i>Eupatorium odoratum</i>				
Aqueous	pink	pink	pink	blue
Methanol	pink	pink	blue	pink
Chloroform	pink	pink	blue	blue

Pink/ colorless – Live

Purple/ blue - Dead

4.8.1 Evaluation of antimicrobial activity of extracts *Tridax procumbens* against *Aspergillus niger*

The aqueous, methanol and chloroform extracts were prepared and their potential to inhibit *Aspergillus niger* were calculated by using Poison food technique. The data obtained was analyzed and results are mentioned in table 12.

Table 12: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against colony growth of *Aspergillus niger*

Colony Diameter (cm)					
	Control	Aqueous	Methanol	Chloroform	MEAN
C1	9.00 (00)	5.93 (34.03)	4.90 (45.50)	5.87 (34.77)	5.57
C2	9.00 (00)	4.97 (44.77)	4.47 (50.37)	5.10 (43.30)	4.84
C3	9.00 (00)	4.47 (50.37)	3.47 (61.47)	4.97 (44.77)	4.30
C4	9.00 (00)	3.47 (61.47)	1.97 (78.07)	2.97 (66.97)	2.80
MEAN		4.71	3.70	4.73	
F test @ 1%		**	**	**	
		TRT	CONC	T*C	
SEM		0.651	0.340	1.101	
SED		0.910	0.450	1.581	
CD @ 1%		2.412	1.323	4.181	

(Figure in parenthesis are the percentage values of inhibition)

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

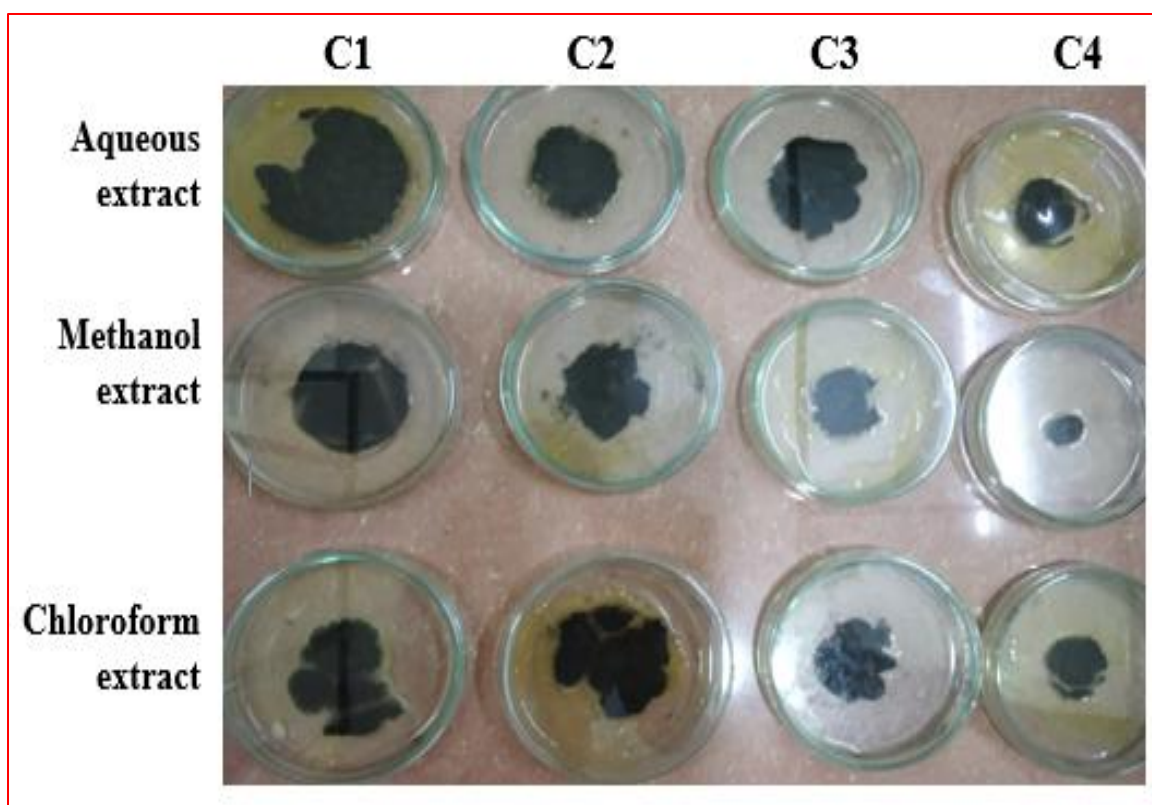


Plate 12: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against colony growth of *Aspergillus niger*

Legend: C1:160 mg mL⁻¹

C2: 240 mg mL⁻¹

C3: 320 mg mL⁻¹

C4: 400 mg mL⁻¹

The result of antifungal screening of *Tridax procumbens* revealed that all the solvent extracts showed prominent antifungal activity as shown in Table 12. The experiment was carried out by using CRD and control was maintained without adding any plant extract to the medium. It was shown that the highest inhibition of growth of fungus was shown by methanol extract of *T. procumbens* at 400 mg ml⁻¹ concentration with 78 per cent of growth inhibition. The fungal range of inhibition was from 4.9 – 1.9 cm in diameter. This was followed by chloroform and aqueous extracts which showed 67 and 62 percent inhibition at 400 mg ml⁻¹. The lowest antifungal activity was shown by aqueous extract at 160 mg ml⁻¹ concentration at the rate of 34%. Since the growth is inhibited at all concentrations the MFC value is taken at that concentration where there is maximum rate of inhibition (400 mg ml⁻¹). It was observed that the plant extracts inhibited the growth of fungus with increasing concentrations. It can be concluded that methanol extract of *T. procumbens* can be used to control the fungus effectively.

The similar results were quoted by Ramesh Babu *et al.* (2018) where the ethanolic and crude extracts were tested for antifungal activity of *Tridax procumbens* against *Aspergillus niger* and proved the antimicrobial property of *Tridax procumbens* leaves and revealed the presence of medicinally active constituents.

The phytochemical screening *Tridax procumbens* leaves showed that the presence of tannin, saponins, flavonoids, terpenoids, triterpenoids steroids, glycosides, anthroquinones, polyphenol and protein. The antimicrobial activity of plant extracts was detected by the indication of zone around the disc and reported the convincing phytochemicals and antimicrobial evidence for the relevance of *T. procumbens* leaves (Syed *et al.*, 2020).

Methanolic extract of leaves of *Tridax procumbens* was examined at different concentrations on both bacteria and fungi by disc diffusion method and agar well diffusion method. Bacteria showed sensitivity to 250 µg /mL and 500 µg /mL plant extract and *Candida albicans*, *Aspergillus niger* and *A. fumigatus* were also tested for its susceptibility to methanol extract and showed significant value within the range of 9-15mm zone of inhibition (Sarkar *et al.*, 2016)

4.8.2 Evaluation of antimicrobial activity of *Lantana camara* against *Aspergillus niger*

The aqueous, methanol and chloroform extracts of *Lantana camara* were prepared and their potential to inhibit *Aspergillus niger* were calculated by using Poison food technique. The data obtained was analyzed and results are mentioned in table 13.

Table 13: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against colony growth of *Aspergillus niger*

Colony diameter (cm)					
	Control	Aqueous	Methanol	Chloroform	MEAN
C1	8.9 (00)	6.17 (31.46)	5.27 (41.46)	5.97 (33.66)	5.80 (35.53)
C2	8.8 (00)	5.37 (40.36)	4.57 (49.2)	4.97 (44.76)	4.97 (44.7)
C3	8.8 (00)	4.47 (50.36)	2.9 (67.7)	4.17 (53.66)	4.00 (55.53)
C4	8.8 (00)	3.97 (55.86)	3.1 (65.5)	3.00 (66.6)	3.14 (65.01)
MEAN		4.99 (44.51)	3.92 (56.45)	4.53 (49.67)	
F test @ 1%		**	**	**	
		TRT	CONC	T*C	
SEM		0.642	0.351	1.11	
SED		0.908	0.497	1.572	
CD @1%		2.415	1.322	4.183	

(Figure in parenthesis are the percentage values of inhibition)

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

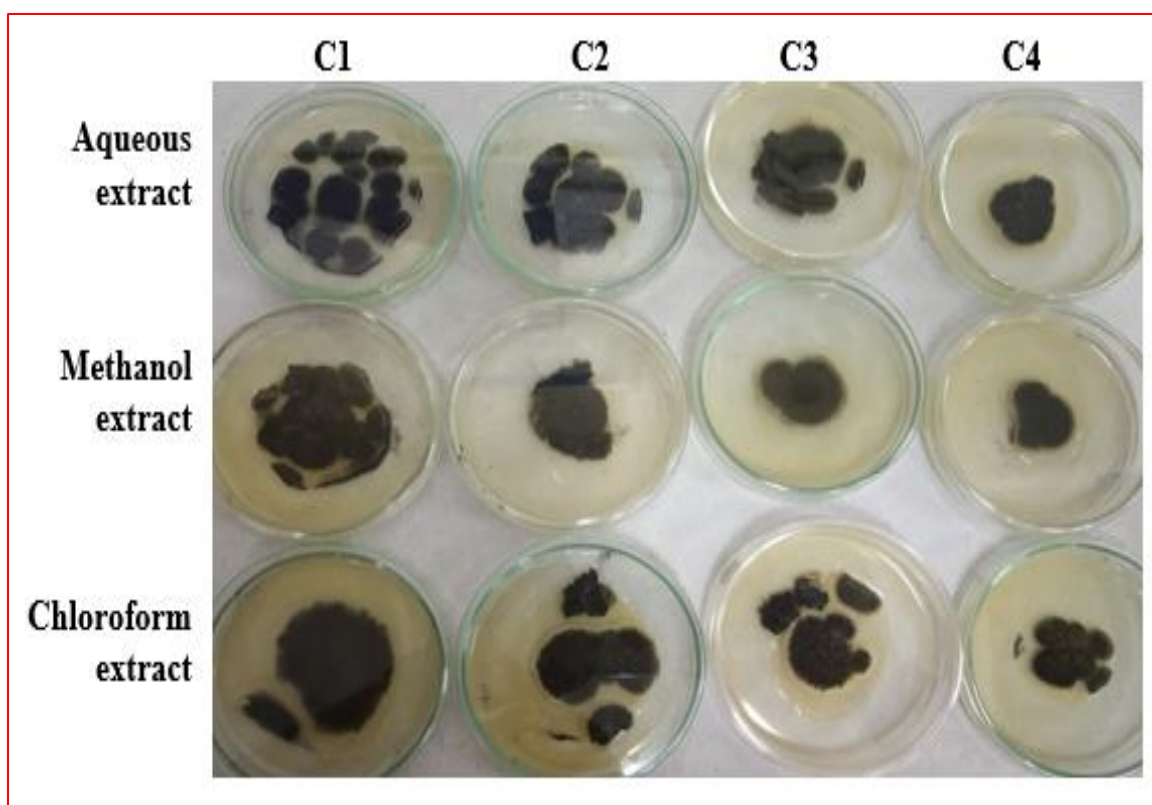


Plate 13: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against colony growth of *Aspergillus niger*

Legend: C1:160 mg mL⁻¹

C2: 240 mg mL⁻¹

C3: 320 mg mL⁻¹

C4: 400 mg mL⁻¹

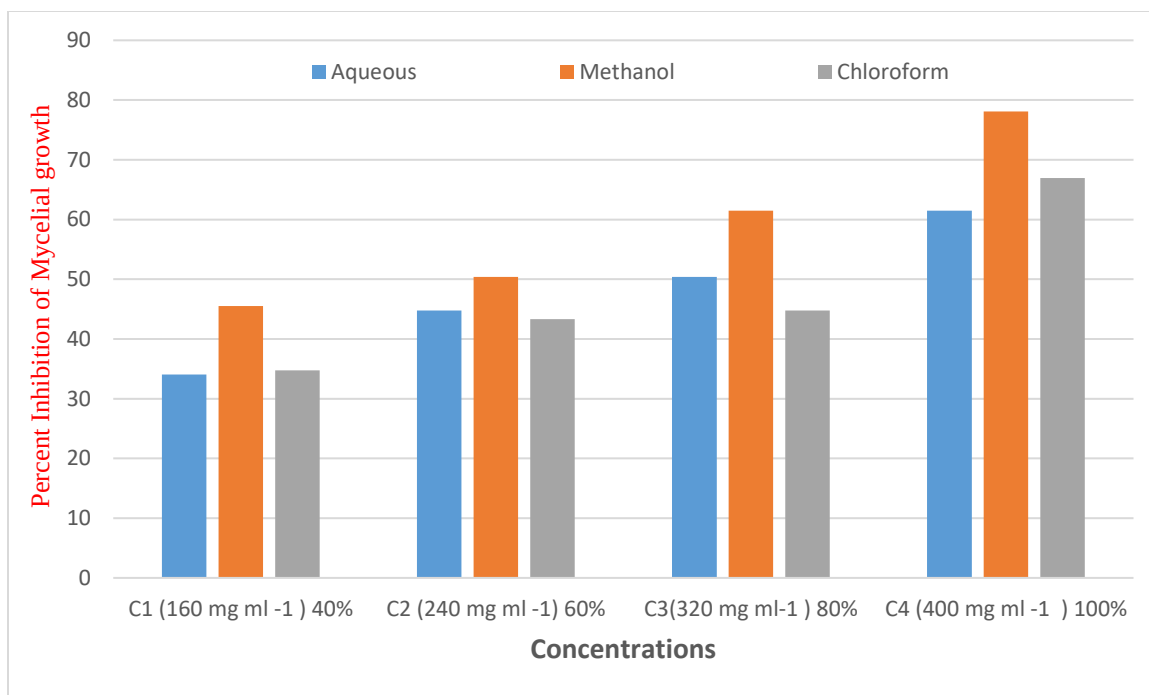


Fig. 5: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against colony growth *Aspergillus niger*

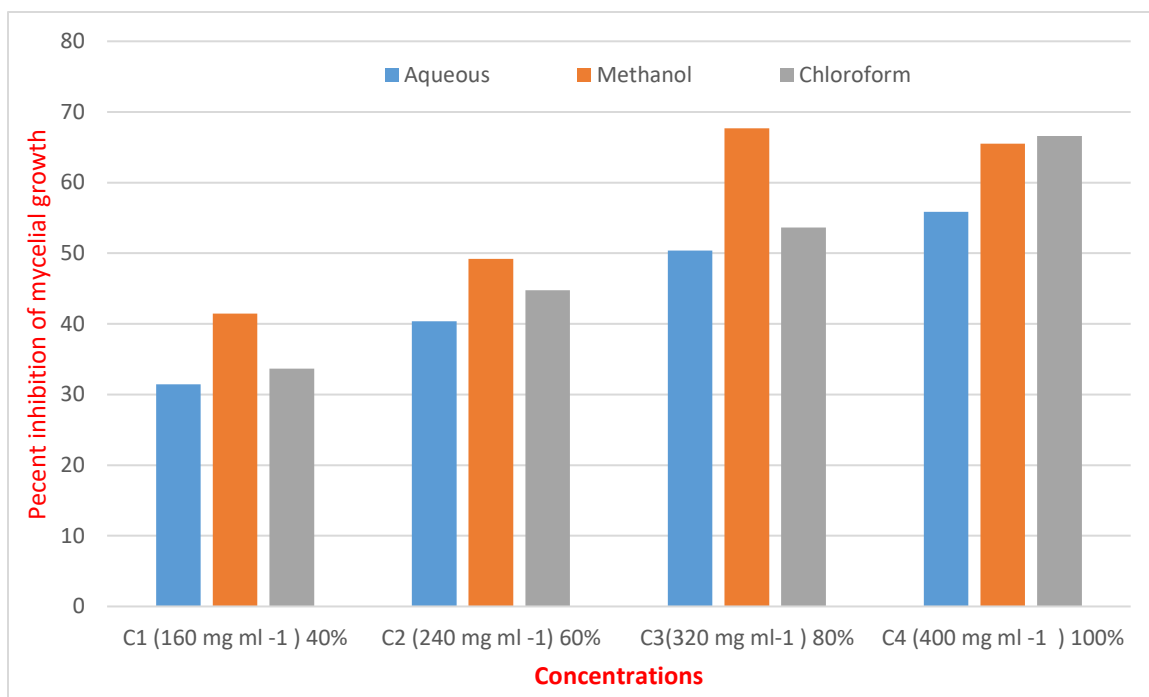


Fig. 6: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against colony growth of *Aspergillus niger*

Results showed that, 320 mg ml⁻¹ concentration of methanolic extract exhibited maximum inhibition (67.7 %) with colony diameter 2.9 cm. While other concentrations also significantly reduced the test fungal growth up to 56-66 per cent. This was followed by chloroform extract which showed maximum inhibition of 66 per cent and the fungal zone of inhibition ranges from 5.9 – 3 cm. Aqueous extract showed the least fungal growth inhibition of 31 per cent with 6 cm colony diameter at 160 mg ml⁻¹ concentration. Since the growth is inhibited at all concentrations of aqueous and chloroform extract, the MFC value for both extract is 400 mg ml⁻¹. The minimum fungicidal concentration (MFC) for methanol extract is 320 mg ml⁻¹. It was observed that the plant extracts inhibited the growth of fungus with increasing concentrations. These results revealed that methanolic extract of *Lantana camara* plant possesses antifungal activity against *Aspergillus niger*.

Biofungicidal or biocontrol activity of *Lantana camara* against phytopathogenic *Alternaria alternata* was tested by (Kulkarni *et al.*, 2019). The methanol, acetone, ethanol and aqueous extracts of *Lantana camara* were assessed *in-vitro* for fungitoxic activity by food poison technique at 200 mg /mL concentration and their activity was recorded as radial growth and percentage inhibition. The ethanol and acetone extracts showed complete inhibition of growth of fungus, while methanol extract showed 50 per cent inhibition and aqueous extract did not produce any inhibition of fungus. Findings from present study confirmed that ethanol and acetone extract of *L. camara* can be used as biofungicide.

Results of present study are in confirmation with the results of (Manenji *et al.*, 2017) who tested antifungal activity of *Lantana camara* plant extract against *Rhizopus stolonifer* was evaluated *in vitro* condition (poison food technique). There was an interaction between botanical plant type and concentration rate. *L. camara* at 60 per cent concentration was most effective in controlling *R. stolonifer*, with a colony diameter of (4.3 cm) and an inhibition percentage of (48%). Hence *L. camara* can be used as effective measure to control the growth of fungus.

4.8.3 Evaluation of antimicrobial activity of *Eupatorium odoratum* against *Aspergillus niger*

The aqueous, methanol and chloroform extracts of *Eupatorium odoratum* were prepared and their potential to inhibit *A. niger* were calculated by using poison food technique. The data obtained was analyzed and results are mentioned in table 14.

Table 14: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against colony growth of *Aspergillus niger*

Colony diameter (cm)					
	Control	Aqueous	Methanol	Chloroform	MEAN
C1	9.00 (00)	7.40 (17.70)	6.07 (32.57)	4.90 (45.50)	6.12 (31.92)
C2	8.81 (00)	6.47 (28.07)	5.47 (39.20)	4.10 (54.40)	5.34 (40.56)
C3	8.90 (00)	6.03 (32.93)	4.30 (52.20)	3.30 (63.33)	4.73 (47.37)
C4	9.00 (00)	5.10 (43.30)	3.67 (59.20)	3.50 (61.1)	3.81 (57.62)
MEAN		6.25 (30.50)	4.88 (45.79)	3.88 (56.81)	
F test @ 1 %		**	**	**	
		TRT	CONC	T*C	
SEM		0.652	0.360	0.639	
SED		0.907	0.501	1.580	
CD @ 1%		2.421	1.318	4.191	

(Figure in parenthesis are the percentage values of inhibition)

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

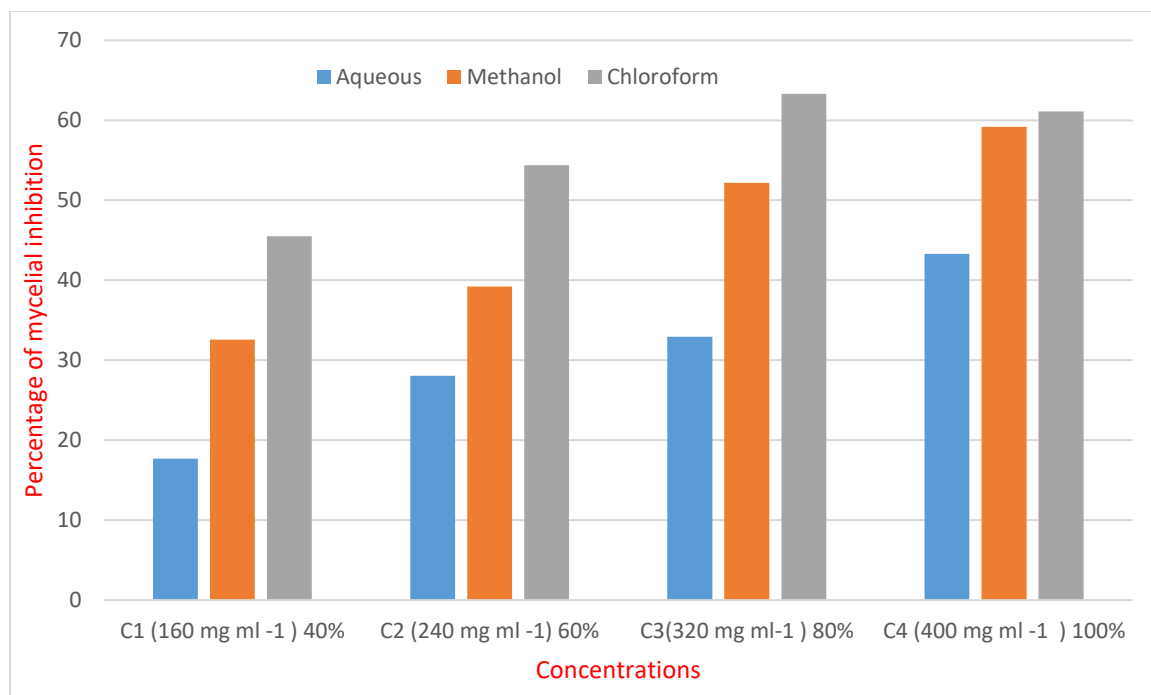


Fig. 7: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against colony growth of *Aspergillus niger*

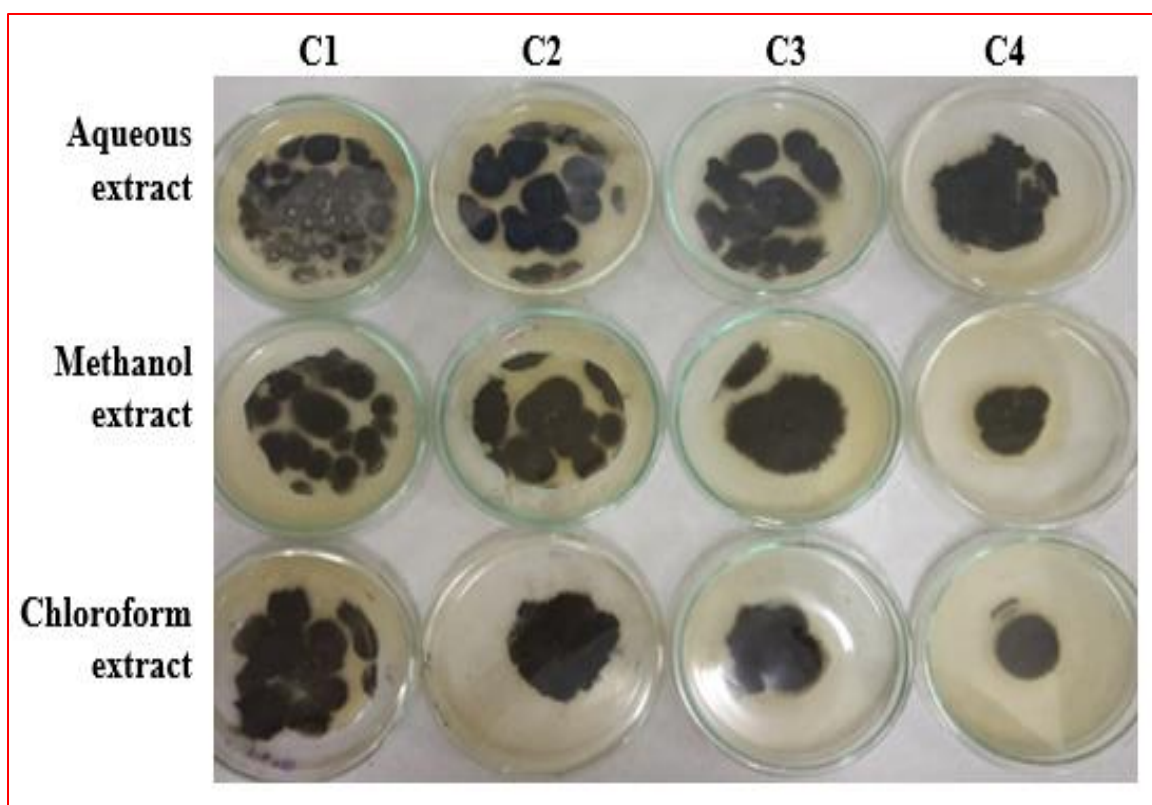


Plate 14: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against colony growth of *Aspergillus niger*

Legend: C1:160 mg mL⁻¹

C2: 240 mg mL⁻¹

C3: 320 mg mL⁻¹

C4: 400 mg mL⁻¹

Plants contain thousands of constituents which are valuable sources of new and biologically active molecules possessing antimicrobial property (Gurjar *et al.*, 2012). The *Eupatorium odoratum* leaf extract has diverse biological activities like inhibition of the growth of some bacteria to enhancement of haemostasis and blood coagulation, anti-inflammatory, astringent, diuretic, and hepatotropic activities. Phytochemical analysis revealed the presence of many secondary metabolites (Muricken and Jofeena, 2019).

In the present study, it was observed from table 14 and analysis of obtained data revealed that, the chloroform extract of *Eupatorium odoratum* showed the maximum growth of inhibition of fungus at the rate of 63.3 per cent with radial colony diameter of 3.3 cm at 320 mg mL⁻¹ concentration. This was followed by methanol extract which showed maximum growth of inhibition ranging from 32 – 59 per cent with radial colony diameter ranging from 3.6 – 6 cm at different concentrations. Aqueous extract showed the least rate of inhibition of growth of fungus ranging from 17 – 43 per cent with radial colony diameter of 5- 7.1 cm. Hence, chloroform extract of *E .odaratum* exhibited stronger antifungal activity compared to methanol and aqueous extract. It was inferred that the growth of fungus was inhibited with increasing concentration. Since the growth is inhibited at all concentrations the MFC (Minimum Fungicidal Concentration) value for aqueous and methanol extract is 400 mg mL⁻¹ and for chloroform it is 320 mg mL⁻¹.

The antifungal activity of this eupatorium plant extract exactacted with different solvents was evaluated for control of blast disease in rice by following poisoned food technique by Manjappa (2013). The results of *in vitro* evaluation of solvent extracts of eupatorium revealed that growth of *Pyricularia oryzae* was inhibited to the maximum extent by eupatorium extract extracted with acetone (91.3%) followed by methanol (85.6%), distilled water (74.5%) and petroleum ether (53.9%). The extent of inhibition of fungus was more with higher concentrations of extracts.Thse results were on par with findings of (Sanit, 2016)

4.9 Screening of plant extracts against *Xanthomonas axonopodis* pv. *punicae* isolated from pomegranate

In present investigation, solvent extracts of selected weeds were evaluated *in vitro* for their efficacy against *X. axonopodis* through disc paper method at 40, 60, 80 and 100 per cent concentrations. All the below mentioned experiments were carried by using CRD and control were maintained without adding plant extracts to the solvent.

4.9.1 Evaluation of antimicrobial activity of *Tridax procumbens* against growth of *Xanthomonas axonopodis* pv. *punicae*

The aqueous, methanol and chloroform extracts of *Tridax procumbens* were prepared and their potential to inhibit *Xanthomonas axonopodis* pv. *punicae* were calculated by using disc diffusion technique. The data obtained was analyzed and results are mentioned in table 15.

Table 15: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against growth of *Xanthomonas axonopodis* pv. *punicae*

	Aqueous	Methanol	Chloroform	MEAN
C1(160 mg ml ⁻¹)	0.00	9.00	0.00	3.00
C2(240 mg ml ⁻¹)	0.00	12.00	11.00	7.67
C3(320 mg ml ⁻¹)	9.00	19.00	15.00	13.67
C4(400 mg ml ⁻¹)	10.00	17.00	17.00	15.67
MEAN	4.75	14.50	10.75	
F test @ 1%	**	**	**	
	TRT	CONC	T*C	
SEM	0.642	0.352	1.112	
SED	0.908	0.497	1.573	
CD @ 1%	2.415	1.323	4.183	

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

The Effect of aqueous, methanol and chloroform extracts of *Tridax procumbens* on inhibition of growth of *Xanthomonas axonopodis* pv. *punicae* were examined *in vitro* by Kirby-Bauer disc diffusion method (Oloche and Nongo, 2015). The zone of inhibition of methanol extract was highest with 19 mm at 320 mg ml⁻¹ concentration. The range of zone of inhibition for chloroform and aqueous extract lies from 11-17 mm and 9-10 mm respectively. The methanol extract showed highest antimicrobial potential while aqueous extract showed the least. The MIC of methanol extract is assessed to be according to the agar dilution method of Kariba stated with modifications. Results show that bacteria is sensitive to methanol extract of plant. MIC value for methanol extracts were assessed to be 320 mg mL⁻¹ followed by aqueous and chloroform extracts was found to be 400 mg ml⁻¹ each. It was noticed that the zone of inhibition increased with increased concentrations. Hence methanol extract of *T.procumbens* can be used to inhibit the growth of *X. axonopodis* pv. *punicae* effectively.

Similar study was undertaken by Syed *et al.*, (2020) and revealed that the methanol and ethyl acetate extract showed good activity against selected pathogenic bacteria. Antimicrobial potentials of the extracts were determined by performing the disc diffusion techniques. Results revealed that different organic solvents extracts namely methanol, ethanol and ethyl acetate extracts documented comparatively good activity against the studied microbial strains. The methanol extract of leaves of *Tridax procumbens* showed combatively better antioxidant potential. This study indicated that *T. procumbens* had potent inhibitory effects on XO inhibitory, antioxidant, and antibacterial activities. These results are in line with the findings of (Andriana, *et al.*, 2019 and Das *et al.*, 2017).

4.9.2 Evaluation of antimicrobial activity of *Lantana camara* against growth of *Xanthomonas axonopodis* pv. *punicae*

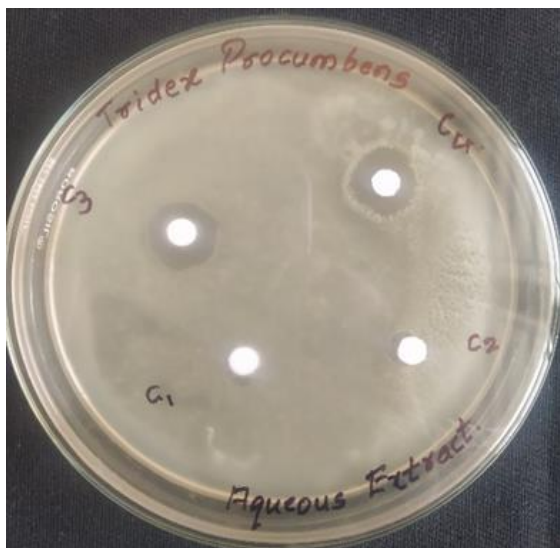
The aqueous, methanol and chloroform extracts of *L. camara* were prepared and their potential to inhibit *Xanthomonas axonopodis* pv. *punicae* were calculated by using disc diffusion technique. The data obtained was analyzed and results are mentioned in table 16.

Table 16: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against growth of *Xanthomonas axonopodis* pv. *punicae*

	Aqueous	Methanol	Chloroform	MEAN
C1(160 mg ml ⁻¹)	0.00	12.00	10.00	7.33
C2(240 mg ml ⁻¹)	0.00	15.00	11.00	8.67
C3(320 mg ml ⁻¹)	10.00	17.00	14.00	13.67
C4(400 mg ml ⁻¹)	11.00	19.00	15.00	15.00
MEAN	5.25	15.75	12.50	
F test @ 1%	**	**	**	
	TRT	CONC	T*C	
SEM	0.651	0.346	1.101	
SED	0.899	0.553	1.323	
CD @ 1%	2.301	1.327	4.221	

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

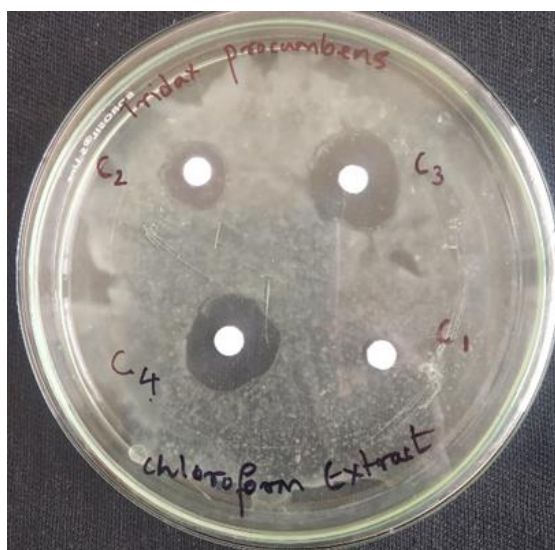
Data presented in the table 16 showed that methanol extracts of *Lantana camara* at varied concentrations were effective for inhibition of *Xanthomonas axonopodis* pv. *punicae*. Maximum inhibition zone was reported for methanol extract (19 mm) at 400 mg mL⁻¹ concentration. Sensitivity of the test organisms was observed to be highest in the methanol extract and least in aqueous extract. The sensitivity of methanol extract was significant with zone of inhibition of 19 mm at 400 mg mL⁻¹. This was followed by chloroform and aqueous extract of *L.camara* showing sensitivity range from 10-15 mm and 10-11 mm respectively. The bacteria was resistant to aqueous extract at 160 mg mL⁻¹ and 240 mg mL⁻¹ concentrations. The MIC values for aqueous extracts were found to be 320 mg mL⁻¹, and 400 mg mL⁻¹ for both methanol and chloroform extracts. The zone of inhibition was observed to be increased with increasing concentrations. Hence all the



Aqueous extract of *Tridax procumbens*



Methanol extract of *Tridax procumbens*



Chloroform extract of *Tridax procumbens*

Plate 15: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against growth of *Xanthomonas axonopodis* pv. *punicae*

Legend: C1: 160 mg mL⁻¹
 C2: 240 mg mL⁻¹
 C3: 320 mg mL⁻¹
 C4: 400 mg mL⁻¹



Aqueous extract of *Lantana camara*



Methanol extract of *Lantana camara*



Chloroform extract of *Lantana camara*

Plate 16: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against growth of *Xanthomonas axonopodis* pv. *Punicae*

Legend: C1:160 mg mL⁻¹
 C2: 240 mg mL⁻¹
 C3: 320 mg mL⁻¹
 C4: 400 mg mL⁻¹

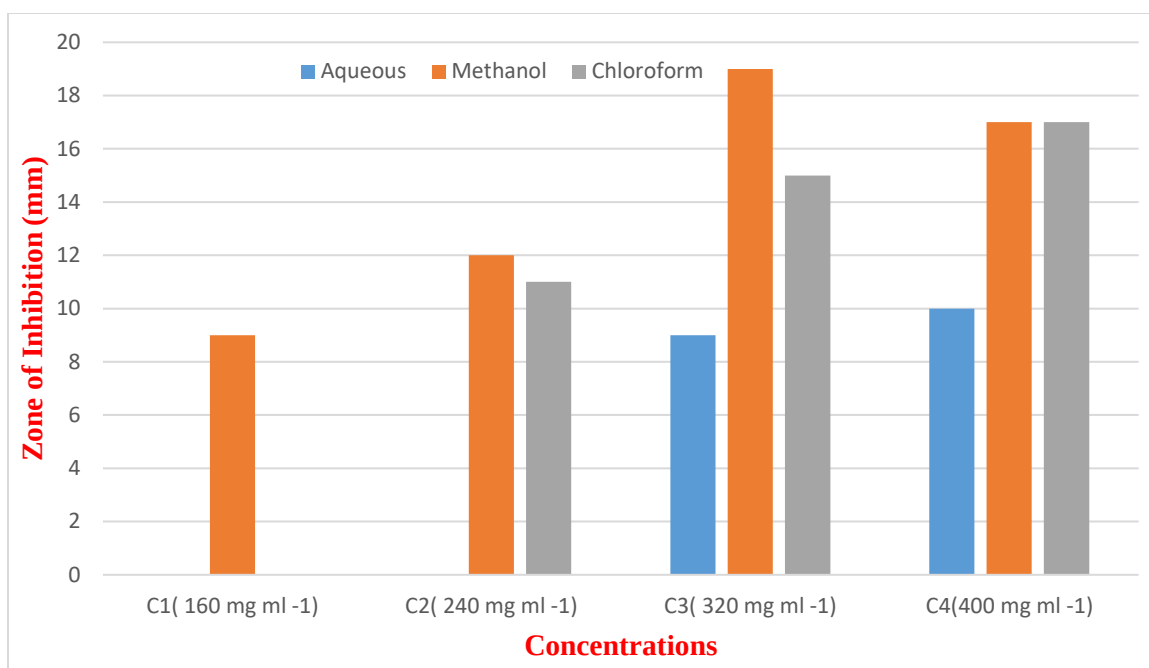


Fig. 8: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Tridax procumbens* against growth of *Xanthomonas axonopodis* pv. *punicae*

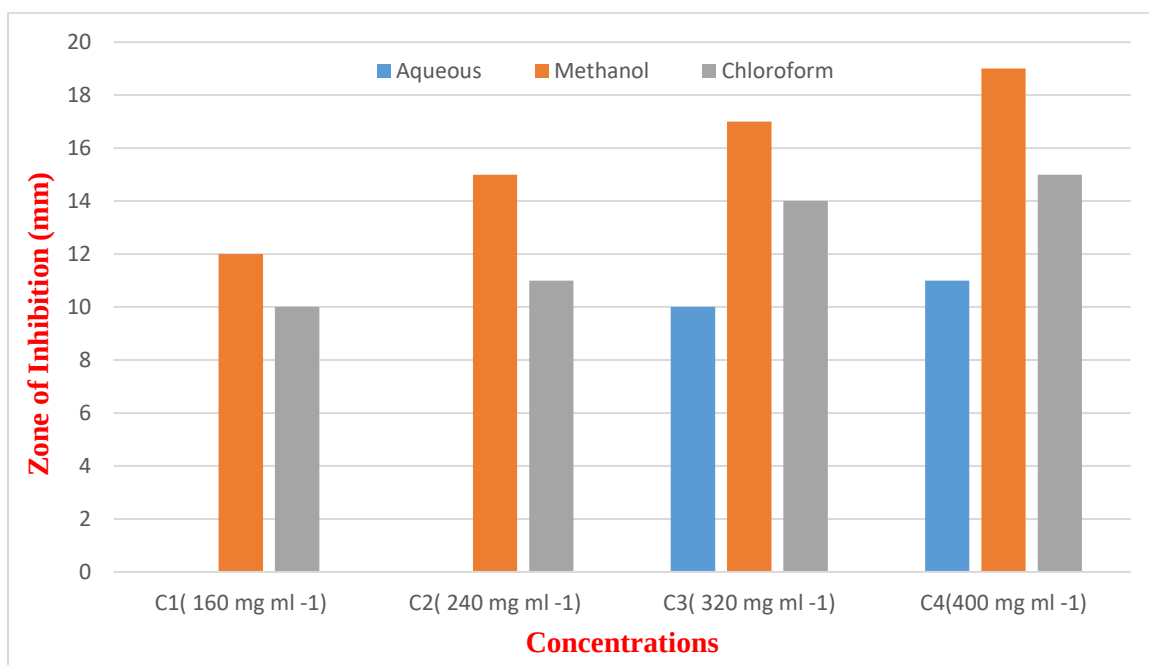


Fig. 9: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Lantana camara* against growth of *Xanthomonas axonopodis* pv. *punicae*

interactions between the treatments and concentrations were significant at 1 per cent level of significance.

Results of present study are in confirmation with the results of Muthulakshmi and Neelanarayanan, (2019) who found that the different organic solvents gave significant results against bacterial pathogens. The leaves extracts were qualitatively ascertained for the presence of phytochemicals such as alkaloids, phenols, tannins, saponins, terpenoids, flavonoids, by adopting standard methods. The antibacterial activities of *Lantana camara* leaves extracts were tested against pathogenic bacteria by using the agar disc diffusion method. The minimum inhibitory concentration of the leaf extracts on *Pseudomonas aeruginosa* were 12 mg /mL for the methanolic extract and 48 mg /mL for the ethanolic extract. The minimum inhibitory concentration on *Streptococcus* spp. was 8 mg /mL for the methanolic extract and 4 mg /mL for ethanolic extract, for *S. aureus* it was 25 mg/ml for the methanolic extract and 7 mg /mL for the ethanolic extract. Therefore, *L. camara* leaves contain bioactive compounds believe to have a bactericidal effect at various concentrations species wise. These results are also in line with the findings of (Shah *et al.*, 2018).

4.9.3 Evaluation of antimicrobial activity of *Eupatorium odoratum* against growth of *Xanthomonas axonopodis* pv. *punicae*

The aqueous, methanol and chloroform extracts of *E. odoratum* were prepared and their potential to inhibit *Xanthomonas axonopodis* were calculated by using Agar disc diffusion technique. The experiment was undertaken by using CRD (Completely Randomized Design). The data obtained was analyzed and results are mentioned in table 17.

From the table 17 the sensitivity of chloroform extract was found to be having highest antimicrobial property by producing 21 mm zone of inhibition at 400 mg mL⁻¹ concentration. Hence the bacteria is sensitive to chloroform extract of *Eupatorium odoratum*. The bacteria is resistant to aqueous extract at both 160 mg mL⁻¹ and 240 mg mL⁻¹ concentration. This was followed by methanol extract which showed a range of inhibition from 10-16 mm. The MIC value for aqueous extract is 320 mg mL⁻¹ and 400

mg mL⁻¹ for both methanol and chloroform extract since the zone of inhibition increases with increasing concentration. The range of zone of inhibition produced for aqueous extract is 9-10 mm. Hence *E. odoratum* can be effectively used for the inhibition bacteria. The data was statistically computed and confirmed that all the interactions between the treatment and concentration were significant at 1 per cent level of significance

Table 17: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against growth of *Xanthomonas axonopodis* pv. *punicae*

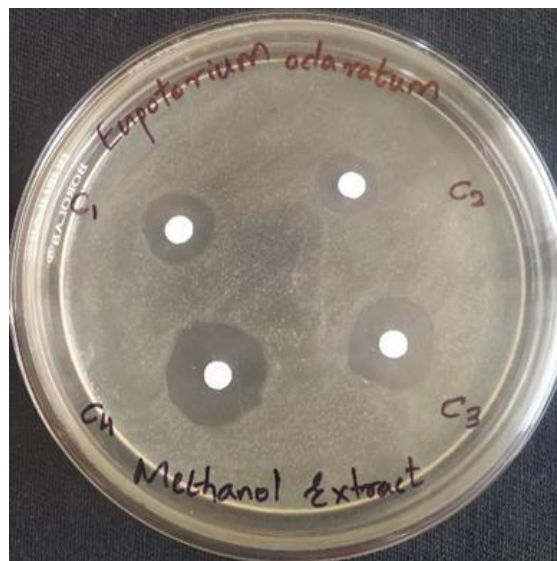
	Aqueous	Methanol	Chloroform	MEAN
C1(160 mg ml ⁻¹)	0.00	10.00	15.00	8.33
C2(240 mg ml ⁻¹)	0.00	12.00	15.00	9.00
C3(320 mg ml ⁻¹)	9.00	15.00	18.00	14.00
C4(400 mg ml ⁻¹)	10.00	16.00	21.00	15.67
MEAN	4.75	13.25	17.25	
F test	**	**	**	
	TRT	CONC	T*C	
SEM	0.650	0.359	1.112	
SED	0.981	0.510	1.584	
CD	2.450	1.318	4.183	

(Legend C4: 400 mg ml⁻¹, C3: 320 mg ml⁻¹, C2: 240 mg ml⁻¹, C1:160 mg ml⁻¹)

The obtained results are in line with Alabi *et al.*, (2019) where qualitative and quantitative phytochemical screening of *Chromolaena odorata* extracts were done by standard methods. Antibiotics susceptibility pattern of bacterial isolates to a panel of ten conventional antibiotics was determined by disc diffusion method. *C. odorata* methanol extract had the highest extract yield (26.2%). From the multidrug resistance analysis, 66.7% of bacterial isolates tested had multidrug resistance index (MDRI) of 100%.



Aqueous extract of *Eupatorium odoratum*



Methanol extract of *Eupatorium odoratum*



Chloroform extract of *Eupatorium odoratum*

Plate 17: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against growth of *Xanthomonas axonopodis* pv. *punicae*

Legend: C1: 160 mg mL⁻¹
 C2: 240 mg mL⁻¹
 C3: 320 mg mL⁻¹
 C4: 400 mg mL⁻¹

Pseudomonas aeruginosa was susceptible to 100 mg /mL of *C. odorata* ethanolic extract but resistant (0.00 ± 0.00) to 100 mg /mL of hot aqueous *C. odorata* extract. The results of the findings can also be compared with the results obtained by (Egbunu *et al.*, 2019; Sobrinho *et al.*, 2017).

4.10 Qualitative phytochemical screening of solvent extracts of plants (weeds)

Qualitative phytochemical analysis of plants confirms the presence of various biologically active secondary metabolites and the present investigation was carried out to screen the presence of secondary metabolites. Chemical tests were carried out using standard procedures to identify the constituents as described by Sofowara (1993) and as also explained previously under “Materials and Methods”. Since methanol extract of *Tridax procumbens* and *Lantana camara*, chloroform extract of *Eupatorium odoratum* showed good antimicrobial property for the tested pathogens, Observations were taken for those extracts for presence or absence of phytochemicals, were noted and are presented in Table 18.

Results presented in the table 18 reveals the presence of secondary metabolites for the tested extracts of weeds. The methanol extract of *Tridax procumbens* showed the presence of tannins, phenol, flavonoids, terpenoids, saponins, glycosides, alkaloids, steroids and coumarins but extracts showed absence for anthocyanines, emodins and quinones. Similarly, methanol extract of *Lantana camara* showed the presence for all tested secondary metabolites except steroids and coumarins. Similarly, all the tested secondary metabolites were present in chloroform extract of *Eupatorium odoratum* except glycosides, anthocyanin and coumarins. Hence due to the presence of these bioactive principles concludes, the pathogen growth inhibition.

The active principles were extracted from *Tridax procumbens* and screened for various phytochemicals. The selected leaves showed the presence of phytochemicals such as, glycosides, flavonoids, saponins, steroids, alkaloids, carbohydrates, polyphenols and tannins. The methanol extract showed the presence of flavonoids, alkaloids, carbohydrates, polyphenols and tannins. The ethanol extract showed the presence of glycosides, flavonoids, carbohydrates, polyphenols and tannins. Water extract showed the presence of

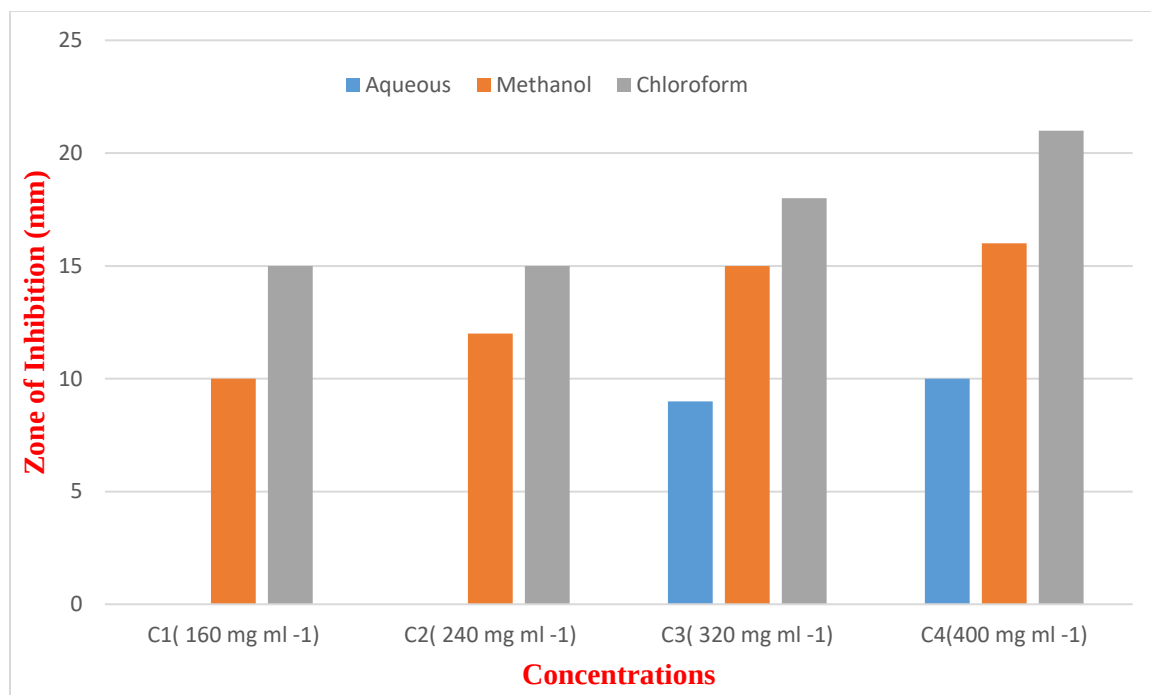


Fig. 10: Effect of different concentrations of aqueous, methanol and chloroform extracts of *Eupatorium odoratum* against growth of *Xanthomonas axonopodis* pv. *punicae*

glycosides, flavonoids, saponins and polyphenols (Syed *et al.*, 2020; Das *et al.*, 2017; Sarkar *et al.*, 2016).

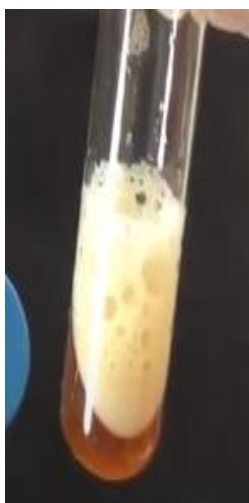
Table 18: Qualitative screening of Phytochemicals present in weed extracts

Sl. No.	Bioactive compounds	Methanol extract of <i>T. procumbens</i>	Methanol extract of <i>L. camara</i>	Chloroform extract of <i>E. odoratum</i>
1	Tannins	+	+	+
2	Phenol	+	+	+
3	Flavonoids	+	+	+
4	Terpenoids	+	+	+
5	Saponins	+	+	+
6	Glycosides	+	+	-
7	Alkaloids	+	+	+
8	Anthocyanins	-	+	-
9	Steroids	+	-	+
10	Coumarins	+	-	-
11	Emodins	-	+	+
12	Quinones	-	+	+

Present (+)

Absent (-)

The preliminary phyto-chemical screening of leaf extracts of *Lantana camara* revealed that the presence of ten phytoconstituents in aqueous extracts such as alkaloids, phenols, flavonoids, steroids, saponins, proteins, tannins, resins, gums and amino acids, and also the same phytoconstituents were present in Ethanol extracts of *L. camara* including terpenoids. The methanol extracts of *L. camara* shown 11 phytoconstituents like alkaloids, phenols, flavonoids, steroids, terpenoids, saponins, proteins, tannins, oils, resins and aminoacids. But, the petroleum ether extracts of *L. camara* revealed only five



Saponins



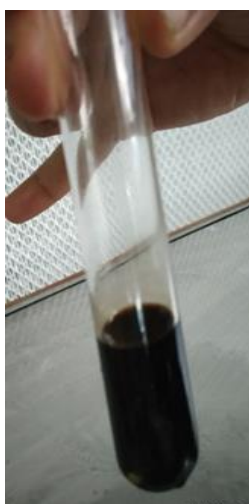
Coumarins



Emodins



Glycosides



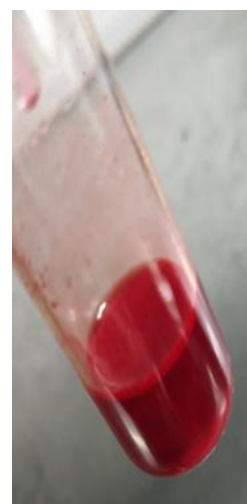
Phenols



Quinones



Alkaloids



Steroids



Tannins



Flavinoids



Terpinoids

Plate 18: Phytochemical screening of plant extracts

compounds such as alkaloids, phenols, flavonoids, steroids and resins. It indicated the presence of effective phyto-chemical compounds in the extract of methanol. The presence of flavonoids, saponins and tannins might be a major active secondary metabolite in the leaf extract (Muthulakshmi and Neelananarayanan, 2019; Shah *et al.*, 2018; Naz and Bano, 2013)

The methanol extract of *Eupatorium odoratum* flower gave positive results for tannin, phytosterols or triterpenoids, flavanoids, coumarins, quinones, cardiac glycosides, terpenoids, steroids, acids, and phenols. Anthraquinones gave negative result in phytochemical analysis of methanol extract. Similar to leaves, flowers are also rich source of secondary plant metabolites. Qualitative analysis showed that *E. odoratum* flower is rich in quinones. (Muricken and Jofeena, 2019).

Flavonoids, cardiac glycosides, steroids, terpenoids were present in all solvent extracts of *Eupatorium odoratum*. Tannins and alkaloids were present in all solvent extracts except n-hexane, while saponins were present in ethyl acetate, methanolic and residue extracts but absent in n-hexane and chloroform extracts. Anthraquinone and reducing sugars were absent in all extracts of *Chromoleana odorata* samples (Abubakar *et al.*, 2020).

4.11 TLC Screening for Aqueous, Methanol, and Chloroform Extracts of selected plants (weeds)

TLC (Thin Layer Chromatography) is an adsorption chromatography which is versatile, speedy and sensitive where samples are separated based on the interaction between a thin layers of adsorbent attached on the plate. (Ingle, 2017). Phytochemical analysis can be performed by various qualitative methods and TLC profiling was carried out using various solvent system of varying polarity. The presence of various phytoconstituents are detected by thin layer chromatography technique. Data obtained from TLC analysis helps in selection of appropriate solvent system for separation of pure compounds by column chromatography. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract (John *et al.*, 2017).

Thin layer chromatography is usually done for a better identification of the bioactive compounds. Different R_f values of the compounds provides an idea about their polarity that may also help in selecting a particular solvent system for further isolation of any compound from the plant extracts using chromatographic and spectroscopic techniques. Compound showing high R_f value in less polar solvent system have low polarity while those with a low R_f value have high polarity¹² (Sonam *et al.*, 2017). In general, the adsorptivity of compounds and the eluting power of solvents increases with polarity (De *et al.*, 2010).

4.11.1 TLC Screening for aqueous, methanol, chloroform extracts of *Tridax procumbens*.

Total of eight TLC solvent systems were used in present investigation to know most suitable solvent system for separation of compounds for aqueous, methanol and chloroform extracts of *Tridax procumbens*. The R_f values and colour of separated bands in different solvent are summarized in Table 19.

From table 19, it was inferred that phytochemical constituents give different R_f values in different solvent system. It can be observed from the above table that methanol and chloroform extracts of *Tridax procumbens* eluted more number of bands when compared with that of aqueous extract. The separation and the number of bands obtained depend on the type of solvents used. Out of 8 tested TLC solvent, Solvent 2 (Toluene :Ethyl acetate :Methanol) in the ratio of 24 :24:2 eluted more number of bands clearly visible bands which are of yellow, green, brown, black, blue and orange colour. This was followed by S7 (Chloroform: Ethyl acetate: Formic acid) in the ratio of 5:4:1.

The qualitative analysis was made by using TLC. The compounds in the extract have been proceeds for the separation of by thin layer chromatography. For *Tridax procumbens* extract chloroform: ethyl acetate: methanol: butanol (30:15:30:25) was used and principle pigments were separated. The three bands were separated with R_f values of 0.41 (Green spot), 0.53 (Red spot) and 0.78 (Brown spot) (Kamble and Dahake, 2015)



Plate 19: Set up for Thin Layer Chromatography

Table 19: Effect of different TLC solvents for aqueous, methanol and chloroform extracts of *Tridax procumbens* and profiling

Solvents	Number of differentiated Spots / Bands			Corresponding Rf Values			Colour of bands
	A	M	C	A	M	C	
S1	2	4	3	0.45,0.65	0.45,0.60, 0.67,0.69	0.56,0.76,0.89	Yellow, Green, Brown, Black, Blue, Orange
S2	4	11	10	0.59,0.66, 0.76, 0.79	0.98,1,0.96,0.94, 0.93,0.83,0.88, 0.76,0.72,0.67, 0.71,	0.98,0.96,0.94, 0.93,0.83, 0.88, 0.76,0.76,0.67,	
S3	3	5	4	0.83,0.92, 0.95	0.76,0.80,0.83, 0.95,1	0.97,0.95, 0.92,0.83	
S4	2	2	3	1,0.88,	1,0.94	1,0.91,0.85	
S5	3	4	4	0.94,0.91, 0.88	0.97,0.94, 0.88,0.82	0.94,0.91 ,0.85,0.82	
S6	2	2	2	0.35,0.42	0.47,0.52	0.5,0.47	
S7	4	6	7	1,0.97,0.71, 0.62,0.6	0.37,0.4,0.42, 0.6,0.62,0.65	0.6,0.6,0.8,0.94, 0.82,0.8,0.71	
S8	2	3	3	0.23,0.34	0.48,0.34,0.27	0.34,0.27,0.23	

(A: Aqueous Extract, M: Methanol Extract, C: Chloroform Extract, S1 –S8 TLC Solvents)

4.11.2 TLC Screening for aqueous, methanol, chloroform extracts of *Lantana camara*

Total of eight solvent systems were used in present investigation to know most suitable solvent system for separation of compounds for aqueous, methanol and chloroform extracts of *Lantana camara*. The Rf values and colour of separated bands in different solvent are summarized in Table 20.

Table 20: Effect of different TLC solvents for aqueous, methanol and chloroform extracts of *Lantana camara* and profiling

Solvents	Number of differentiated Spots / Bands			Corresponding Rf Values			Colour of bands
	A	M	C	A	M	C	
S1	1	1	1	0.54,	0.52,	0.52,	Yellow, Green, Brown, Black, Blue, Orange
S2	4	13	12	0.31,0.41, 0.46,0.51	0.28,0.31,0.35, 0.36,0.41,0.53, 0.58,0.68,0.73, 0.76,0.78,0.88, 0.91,0.96,0.95, 0.9	0.3,0.21,0.33, 0.38,0.4,0.51, 0.56,0.66,0.73, 0.75,0.76,0.86, 0.88,0.95,0.93, 0.91	
S3	4	5	5	0.11,0.19, 0.23,0.26	0.11,0.14,0.19, 0.21,0.23,	0.19,0.21,0.23, 0.26,0.28	
S4	2	3	3	0.76,0.97,	0.76,0.79,1	0.73,0.76,0.97,	
S5	3	2	2	0.5,0.79,0.44	0.44,0.64	0.52,0.73	
S6	2	2	2	0.11,0.23	0.14,0.23	0.11,0.23	
S7	4	5	5	0.94,1,0.91, 0.85,	0.4,0.57,0.65, 0.74,1	0.62,0.71,0.8, 0.97,0.6	
S8	3	3	3	0.11,0.18, 0.23	0.11,0.18,0.23	0.13,0.16,0.20	

(A: Aqueous Extract, M: Methanol Extract, C: Chloroform Extract, S1 –S8 TLC Solvents)

From the above table it can be inferred that, the bioactive components present in aqueous, methanol and chloroform extracts of *L. camara* eluted different number of bands based on their polarity and the TLC solvents itself. The methanol and chloroform extracts eluted more number of bands compared to the aqueous. Out of eight tested TLC solvents, S2 (Toluene: Ethyl acetate: Methanol) in the ratio of 24:24:2 was more effective having highest length of solvent front. The bands obtained in S2 are having greater corresponding Rf values indicating that eluted bands (Bioactive compounds) are less polar. Followed by this S7 (Chloroform: Ethyl acetate: Formic acid) in the ratio of 5:4:1 and S3 (Hexane: Ethyl

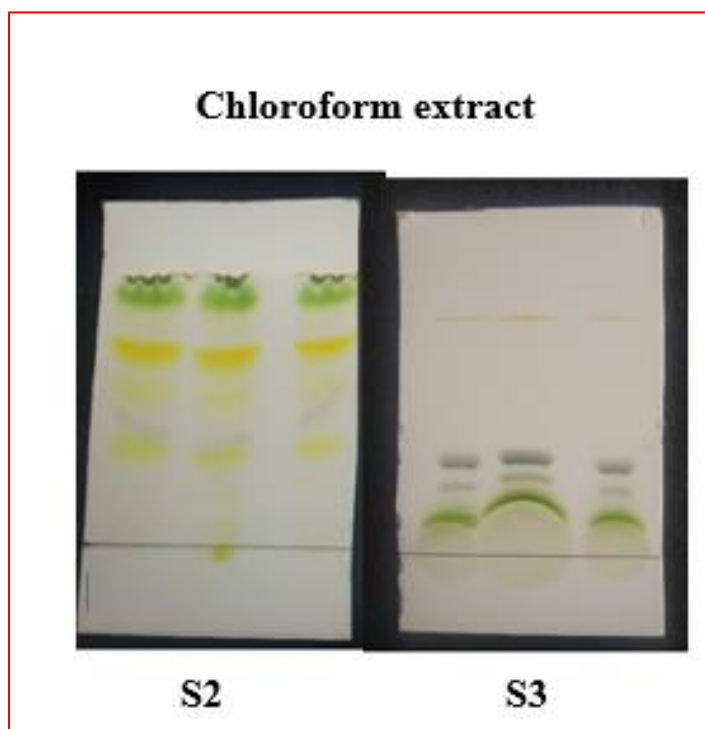
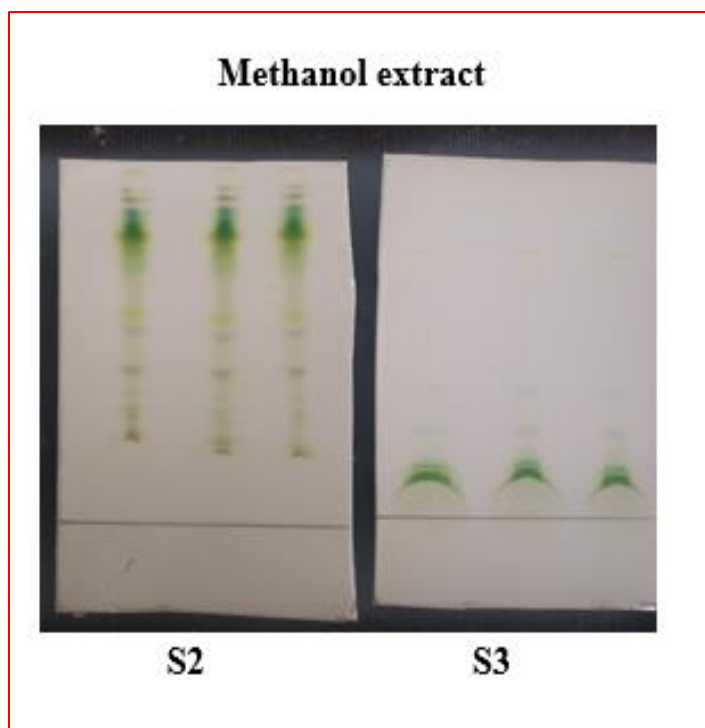


Plate 20: TLC profiling of methanol and chloroform extract of *Tridax procumbens* under S2 and S3 Solvents

Legend: S2: Toluene: Ethyl Acetate: Methanol (24:24:2)

S3: Hexane: Ethyl acetate (9:1)

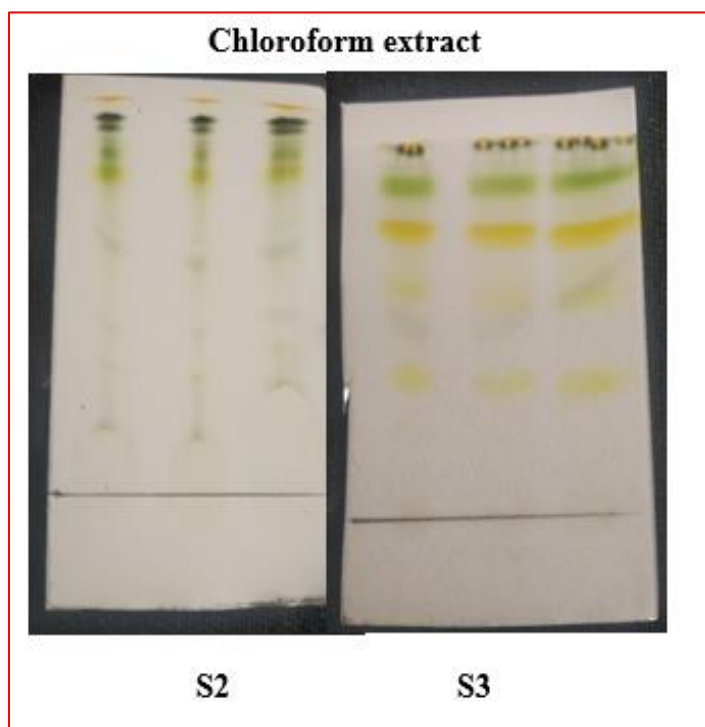
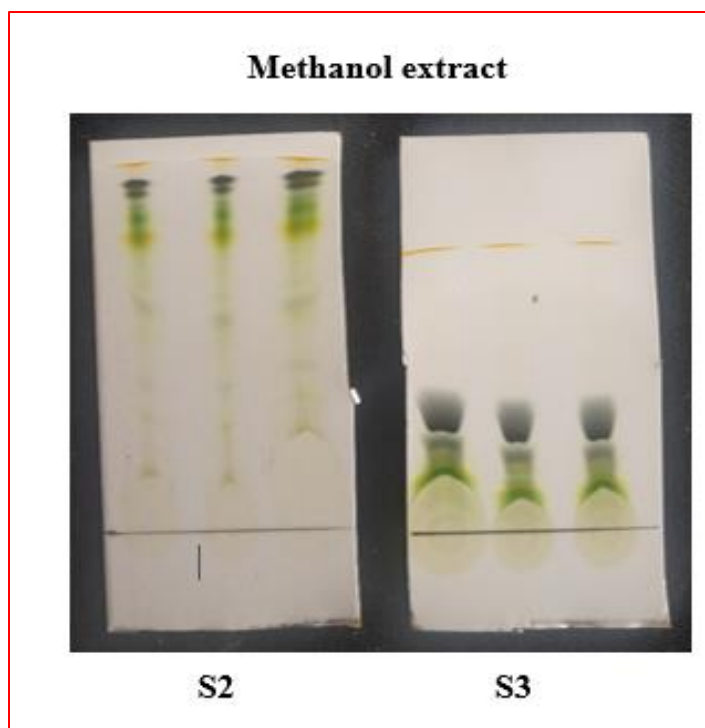


Plate 21: TLC profiling of Methanol and chloroform extract of *Lantana camara* under S2 and S3 Solvents

Legend: S2: Toluene: Ethyl Acetate: Methanol (24:24:2)

S3: Hexane: Ethyl acetate (9:1)

acetate) in the ratio of 9: 1 eluted the bands clearly. Hence above three solvents can be used for column chromatography to identify the phytochemical present.

Dichloromethane and methanol extracts of *L. camara* were subjected to thin layer chromatography for the separation of polar and non-polar compounds (Venkatachalam *et al.*, 2011). For the dichloromethane extract, the mobile phase was the mixture n-hexane: dichloromethane (1:1); and for the methanolic extract the mobile phase was the mixture, chloroform: methanol: water (7.5:1.5:0.5). TLC plates were examined in an ultraviolet (UV) chamber at 254nm. UV active compounds, component-1 (R_f value = 0.123 (colouration violet, the compound would be glycoside), and component-2 (R_f = 0.343 (colouration blue, the compound would be phenolic acid) were observed in the dichloromethane extract. Three spots having R_f values of 0.569 (colouration yellow the compound would be flavonoid), 0.868 (colouration light blue, the compound would be phenolic acid), and 0.96 (colouration yellow, the compound would be flavonoid) in the methanolic extract at 254nm.

4.11.3 TLC Screening for aqueous, methanol, chloroform extracts of *Eupatorium odoratum*

Total of eight TLC solvent systems were used in present investigation to know most suitable solvent system for separation of compounds for aqueous, methanol and chloroform extracts of *Lantana camara*. The R_f values and colour of separated bands in different solvent are summarized in Table 21.

It was noticed from Table 21 that aqueous, methanol and chloroform extracts of *Eupatorium odoratum* eluted bands in all the TLC solvents tested based on their polarity. The aqueous extract eluted less number of bands compared to methanol and chloroform extracts. TLC solvent S2 (Toluene :Ethyl acetate :Methanol) in the ratio of 24:24:2 was more effective in which there was more than 10 bands and shown clearly under visible light with Yellow, Green, Brown, Black, Blue, Orange colours .In S4 (Chloroform : Ethyl acetate : Methanol : water) in the ratio of 15 : 8 : 4 : 1 there was almost no band observed. In S8 (Conc Hcl: Acetic acid: Water) in the ratio of 3:30: 10 eluted bands of high polarity having less R_f value.

Table 21: Effect of different TLC solvents for aqueous, methanol and chloroform extracts of *Eupatorium odoratum* and profiling.

Solvents	Number of differentiated Spots / Bands			Corresponding Rf Values			Colour of bands
	A	M	C	A	M	C	
S1	1	1	1	0.89	0.80	0.89	Yellow, Green, Brown, Black, Blue, Orange
S2	6	11	11	0.28,0.3,0.33, 0.35,0.41,0.45	0.26,0.31,0.35, 0.5,0.58,0.75, 0.76,0.83,0.86, 0.9,0.98	0.25,0.31,0.33, 0.36,0.51,0.56, 0.65,0.73,0.83, 0.85,0.86	
S3	4	5	5	0.11,0.14,0.28, 0.38	0.11,0.16,0.26, 0.35,0.28	0.11,0.14,0.16, 0.19,0.21	
S4	1	1	1	0.97	1	0.97	
S5	2	2	2	0.55,0.67	0.52,0.70	0.55,0.61	
S6	2	2	2	0.11,0.19	0.11,0.23	0.11,0.21	
S7	5	6	6	0.48,0.97,0.68, 0.77	0.71,0.77,0.82, 0.91	0.28,0.34,0.57, 0.65,0.85,0.91	
S8	4	3	4	0.,0.16,0.23, 0.11,0.16,0.18	0.11,0.16,0.23	0.11,0.16,0.20	

(Aqueous Extract, M: Methanol Extract, C: Chloroform Extract, S1 –S8 TLC Solvents)

Separation of the TLC spots was conducted in different solvent system individually using ethyl acetate, hexane and methanol as mobile phase. The range of Rf values are reported to be 0.3 to 0.9 for terpenes, 0.05-0.85 for phenolic compounds, and 0.5 to 0.9 for flavonoids in *Chromolaena odorata* extracts (Talukdar *et al.* 2010). Rf values for these groups of compounds vary with different mobile phases. Leaf extracts of *C. odorata* contain alkaloids, phenols, flavonoids, saponins, cardenolides, anthraquinones and tannins. Alkaloid is a secondary metabolite present in all the weed plants. Alkaloids are complex compounds found occurring naturally in plants, which are toxic to insects and plant parasitic nematodes. The alkaloids present in *Chromolaena odorata* have shown

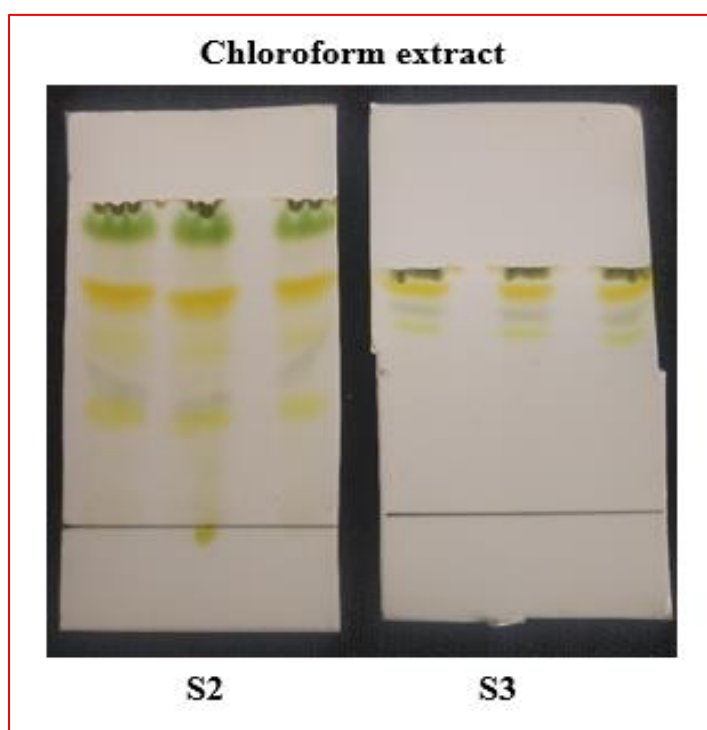
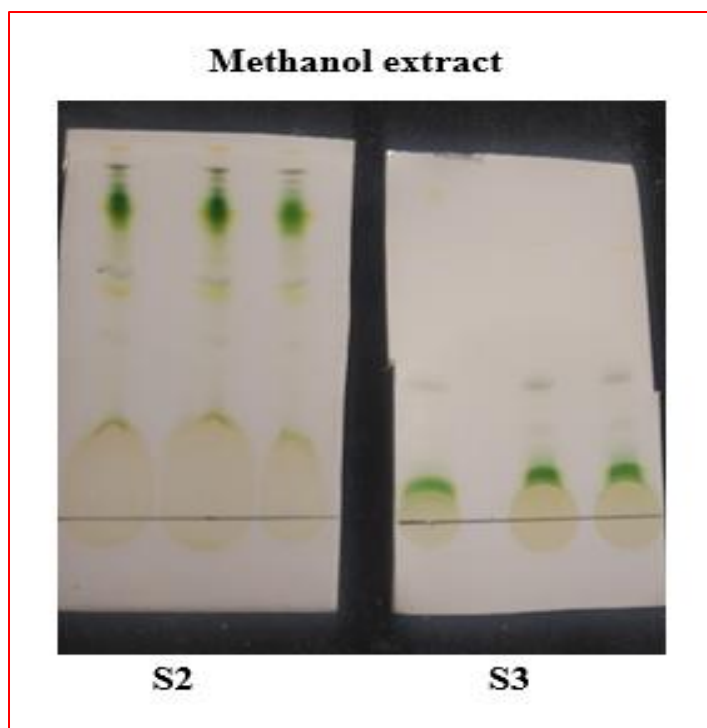


Plate 22: TLC profiling of Methanol and chloroform extract of *Eupatorium odoratum* under S2 and S3 Solvents

Legend: S2: Toluene: Ethyl Acetate: Methanol (24:24:2)

S3: Hexane: Ethyl acetate (9:1)

nematostatic and nematocidal effects on plant-parasitic nematodes (Thoden *et al.* 2009). TLC is regarded as a highly sensitive technique for identifying volatile organic compounds isolated from natural products. It is a powerful tool for compound separation for compound class categories on the basis of their R_f values using some solvent systems of various polarity (Jumaat *et al.*, 2017).

V SUMMARY AND CONCLUSION

The investigation dealing with the post-harvest pathogens of mango and pomegranate causing huge economic losses and the screening the effect of solvent extracts (Aqueous, methanol and chloroform) of tested weeds (*Tridax procumbens*, *Lantana camara* and *Eupatorium odoratum*) on the isolated pathogens for fungicidal and bactericidal activity , qualitative estimation of phytochemicals ,their results are summarized in this chapter.

Morphological characterization of microorganism which includes both microscopic and macroscopic features is the first step of approach regarding the identification of targeted microorganism. Hence colony characters and microscopic view of the isolated fungi and bacteria give a idea for their classification. The colour of the colony of obtained fungus isolated from mango was black to brown with each colony having size of 25- 30 mm in diameter. The phialides were biserrate and there was no formation of sclerotia. Hence the fungus was molecularly characterized and confirmed by PCR amplification of ITS (Internal Transcribed Spacers) primers and the organism was confirmed as *Aspergillus niger* by using NCBI Blast tool.

Similarly, morphological characterization of bacteria revealed yellow colour colonies having 2-3 mm diameter and microscopic observation confirmed the bacteria was gram negative and rod shaped. The bacteria was positive for KOH test, Catalase test, oxidase test, gelatin test, indole test, starch hydrolysis and showed negative reaction for methyl red test. Molecular techniques are rapid, more sensitive, specific and efficient compared to the conventional method which work at the molecular level. Because of the complexity of DNA–DNA hybridization, 16S rRNA gene sequencing was used as a tool to identify bacteria at the species level which are ubiquitous and conserved across the bacterial species. The bacteria was identified by comparing already present gene sequences at NCBI and isolate bacteria was confirmed as *Xanthomonas axonopodis*.

Weeds by their very nature, are resistant to pathogen attack because of conserved phytoconstituents (Secondary metabolites) which help in plant defence mechanism. Hence the selected weeds (*Tridax procumbens*, *Lantana camara* and *Eupatorium odoratum*) were screened to inhibit the growth of bacteria and fungus under *in vitro* conditions by using poisoned food technique for fungus and agar disc diffusion method for bacteria by isolating secondary metabolites through their solvent extracts. For both the methods methanol extracts of *T. procumbens* and *L. camara*, Chloroform extract of *E. odoratum* has maximum rate of inhibition of fungus and bacteria at their higher concentrations.

Aqueous, Methanol and Chloroform extracts of selected weeds have prominent antifungal activity against *Aspergillus niger*. Comparatively Methanol extract of *T. procumbens* and *L. camara*, Chloroform extract of *E. odoratum* at their highest concentration (400 mg ml^{-1}) were more efficient to inhibit the growth of fungus. The same results were true in case of bacteria (*Xanthomonas axonopodis* pv. *punicae*) for screening of bactericidal property of weeds against them. Least rate of inhibition was observed for aqueous extract of weeds. It was inferred that the rate of inhibition increased with increased concentration of solvent extracts of selected weeds. Resazurin assay method was good diagnostic tool to understand the death of cells due to presence of weed extracts of tested concentrations by colour change. It was noticed the colour change from pink to purple occurred at higher concentrations like 320 mg ml^{-1} and 400 mg /ml .

The dissolution of dried powder of weeds in distilled water, methanol and chloroform help to isolate phytoconstituents (secondary metabolites) present. Hence qualitative phytochemical screening resulted in presence of tannins, phenols, flavinoids, terpinoids, saponins and alkaloids were present in all the solvent extracts of selected weeds. But there was absence of glycosides, anthocyanins, coumarins, Emodins and quinones in tested extracts. Hence it indicates qualitatively the presence of secondary metabolites in the tested plant extracts which are the major tools for defence system in plant system. Therefore it indicates that the presence of these bioactive compounds are the source of inhibition for growth of isolated bacteria and fungus.

Thin layer chromatography which was carried out to purify and isolate the bioactive components present, act as efficient tool for understanding number of bioactive compounds present and also their polarity. The separation of bands (phytoconstituents) depends on the polarity of TLC solvent and their polarity itself. It was inferred that the band with higher R_f value were less polar and their movement is more. 8 TLC solvents were selected to standardize the best solvent which separate more number of band with clarity. Toluene: Ethyl Acetate: Methanol in the ratio of 24:24:2 was highly efficient in this manner. Hence this solvent can be used in column chromatography for further work.

Future line of work

- The green house and field conditions evaluation of weed extracts needs to be established for stability and reproducibility of the results obtained.
- *In vitro* screening of many more weed extracts having prominent antimicrobial property as a potent biopesticide can be performed.
- Dose optimization is necessary.
- The standardized TLC Solvent, Toluene: Ethyl Acetate: Methanol in the ratio of 24:24:2 should be used for column chromatography for elution of Secondary metabolites.

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APPENDIX-I

DNA Extraction Reagents & Preparation of reagents

i) Cetyl-Trimethyl Ammonium Bromide (CTAB) / NaCl solution:

NaCl of 4.1 g was dissolved in 80 ml of water to which 10 g of CTAB was added slowly, in order to increase the rate of dissolving it was heated to 65°C. Finally the volume was made up to 100 ml by adding sterile water.

ii) NaCl (5M)

The stock solution of NaCl was prepared by dissolving 29.2 g salt in 80 ml water. volume was adjusted to 100 ml with water and later it was sterilized by autoclaving and stored at room temperature.

iii) Tris base (1M pH 8.0)

The stock solution of Tris base was prepared by dissolving 11 g of Tris base in 80 ml of water then the pH was adjusted to 8.0 with concentrated HCl. and then it was sterilized by autoclaving and stored at room temperature.

iv) EDTA (0.5M)

EDTA of 18.61 g was added in 80 ml water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets and then it was sterilized by autoclaving and stored at room temperature.

v) Chloroform: Isoamyl alcohol (24:1 v/v)

Chloroform of 96 ml was mixed with 4 ml of isoamyl alcohol and stored under refrigerator conditions.

vi) Phenol: chloroform:Isoamyl alcohol (24:24:1v/v)

Equilibrated phenol of 50 ml was mixed with 48 ml of chloroform and 2 ml of isoamyl alcohol and stored at 4 °C.

vii) TE (Tris EDTA pH 8.0) buffer

Tris base (IM, pH 8.0) of 1 ml was mixed with 0.2 ml of EDTA (0.5 M pH 8.0) and finally the volume was adjusted to 100ml with sterile water and finally this solution was sterilized by autoclaving.

viii) RNAase

RNAase of 10 mg was dissolved in 1 ml of Tris EDTA buffer (pH 7.8) and it was heated to near boiling point. This was finally stored under freezing temperature.

ix) TBE (5X. pH 8.0)

Tris base of 27 g and 13.75 g of boric acid were dissolved in 200 ml of sterile water. to which 10 ml of EDTA (0.5 M) was added and finally volume was adjusted to 500 ml by adding sterile water.

x) Ethidium Bromide (100mg / ml)

Ethidium bromide of 1 g was added to 100 ml of sterile water and was stirred using magnetic stirrer until the dissolution of dye. Later the container was wrapped in aluminum foil and stored at room temperature.

xi) 70 Per cent ethanol

Absolute alcohol of 70 ml was mixed with 30 ml of distilled water and it was stored at room temperature