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Isolation and Characterization of Endophytes from *Punica Granatum* as a Biocontrol Agents

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Abstract: **Endophytes are microorganisms which present inner parts of plant tissues, without causing any adverse effect. The purpose of this work is to investigate the potentials of phytochemical, antimicrobial, larvicidal activity and study the relatedness of endosymbionts in medicinal plant (*Punica granatum*). Phytochemical activity shows the presence of alkaloids, tannin, protein, amino acid, carbohydrates, coumarin, saponin, leucoanthrocyanin whereas emodine, Phcobatannin are absent. The antimicrobial activities of crude fungal methanol extract are tested against gram positive and gram negative bacteria by well diffusion method. It show a inhibition zone against test bacteria and in plant leaf solvent extract of ethanol, chloroform, acetone, water are examine for its antimicrobial activity against test organism. Symbiotic relationship of host- microbes revealed that has good relationship between fungi and host bacteria.**

Keywords: **Endophyte, Punica *granatum*, Symbiotic, Phytochemical, Antimicrobial.**

1. INTRODUCTION

Endophytes are endosymbionts which present inner part of the plant tissue and without causing any adverse effect. Normally certain microorganisms are capable to synthesize biological compounds which are act as a defence for the plant. Now- a- days, this type of bioactive compounds are highly useful in pharmacology, agricultural and industries. Recent research has been achieved that the natural product such as alkaloids, terpenoids, flavonoids, steroids *etc*. produced from endophytes. And also some endophytes have the ability to inhibit the contamination which referred as bioremediation. Generally microbes have the able to degrade the contamination. Numerous reports have shown that endophytic microorganism can have the capacity to control plant pathogens (Krishnamurthy and Gunamanickam, 1997), insects (Azevedo *et al.*, 2000) and nematodes (Hallmann *et al.*, 1997, 1998). Antimicrobial metabolites (Antibiotics) can be defined as low molecular – weight organic compounds made by microorganisms to protect plant from outer invade, that are active at low concentrations against other microorganisms, and are the most bioactive natural products isolated from endophytes (Strobel *et al*.,2003).

In last decade's discovery and intensive investigation of plant – associated microorganisms, termed endophytic microorganisms (endophytes) have led to the possibility of exploring the potential benefits of these promising organisms in agriculture, medicinal and pharmaceutical sectors. Endophytes can be defined in a generalist manner, as a group of microorganism that infect the internal tissues of plant without causing any immediate symptoms of infection and/ or visible manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle (De Bary (1866) first coined the term endophyte. Endophytes are ubiquitously existent in almost every plant tissue examined till date (Strobel 2002) with the increasing enormity of global health problems and the incidence of drug resistance microorganisms and new disease; it was become clear that faster and effective pursuits for drug discovery and sustainable production must be made. This cumulative crisis has already lead to the discovery and characterization of potent endophytes which can produce bioactive natural products, occasionally mimetic to their associated host plants (Eyberger *etal*. 2008; Kusuri *et al.*, 2009 ;). Endophytes are also known to produce a diverse range of biologically active secondary metabolites (Strobel and Daisy 2004; Zhang *et al* 2006; Suryanarayanana *et al*., 2009.

2. METHODS

Preparation of sample:

The healthy leaves of *Punica granatum* was crushed with different solvent (ethanol, acetone, chloroform and water) using a manual grinding. The solvent leaf extract were filtered by Whatman No1 filter paper and store for future purpose.

Qualitative Phytochemical Screening:

The results of qualitative screening of phytochemical compounds in aqueous extract of *Punica granatum* leaves revealed that the presence of alkaloids, tannin, protein, aminoacid, carbohydrates, coumarin, Phcobatannin, Saponin, Lecoanthocyanin.

Isolation of Endophytic Fungi and Bacteria:

The leaves were surface sterilized by modified Dobranic *et al.*, after the surface sterilization leaves and flowers are cut into small pieces about 1 to 1.5 cm were evenly spaced in petri dishes containing potato dextrose agar (PDA) medium for fungal and Nutrient agar (NA) for bacterial isolation. The petri dishes were sealed using parafilm ^{TM} (Bills and Polishook, 1992). The petri dishes were monitored every day for the growth of endophytic fungal and bacterial colonies from the leaf and flower segments. The hyphal tips, which grew out from leaf segments were isolated and subcultured onto potato dextrose agar (PDA) and brought into pure culture. The isolated endophytic fungi from medicinal plants were identified down to species levels using standard monographs (Guba, 1961; Ellis, 1971; Sutton, 1980; Onions et al., 1981; Nag Raj, 1993). To prevent rapidly growing fungi inhibiting slow growing species, the former was removed frequently following isolation (Bills, 1996).The endophyts were distinguished from each other by their cultural characteristics such as colony morphology, growth rates, hyphal mat characteristics, and pigmentation of fungal colony and medium (Bills and Polihook, 1994; Frohlich *et al.*, 2000). All the endophytic isolates were documented and maintained in slant culture.

CULTURE IDENTIFICATION:

Identification of fungi:

Morphology and microscopic identification is used to identify the fungus.

Identification of bacteria:

Gram Staining:

Gram staining used to further identification of the strain. Numerous modification of the Gram stain are available however, the following method based on Skerman (1967). The smear was first air dried and gently fixed by mild flaming on the underside of the slide. The slide was flooring with iodine solution for one min and decolourizes by applying 95% ethanol drop – wise to the smear held at an angle against, Finally, it was counterstained with safranin, washed and dried. The slide was observed under oil immersion.

Spore staining test:

A differential staining technique (the Schaeffer-Fulton method) is used to distinguish between the vegetative cells and the endospores. A primary stain (malachite green) is used to stain the endospores. The vegetative cells will appear pink/red and the spores will appear green.

Motility Test:

The test carried out by transferring actively growing cultures (24 h old) to moistened agar slopes of nutrient agar incubated at 28^0 C for two days. The slope was examined daily by gently removing a loopful of the growth from the water al base of the slope using the hanging drop method. The hanging method procedure involves us of a drop of the bacterial suspension onto the depressed cavity of a microscopic slide, covered with cover slip, inverted and mounted under high power objective (x40) to observe the movement of the bacterial cells.

Acid fast stain test:

It is the differential staining techniques also called ***Ziehl-Neelsen staining*** techniques. When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat,

carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. An only decolorized cell absorbs the counter stain and takes its color and appears blue while acid-fast cells retain the red color.

Extraction of Secondary metabolites:

The extraction procedure was followed by the method of Strobel *et al.*(2004). The selected fungal species were grown in 4 liter Hopkins flask containing 1000ml of Potato Dextrose Broth (PDB) medium. The test fungal species were inoculated into the medium and incubated for 21 days at 26^{o}C. After incubation period the culture was harvested and the culture filtrate with four layered cheesecloth. In order to avoid fatty acid contamination, 0.25g of Sodium carbonate was added to the filtrate and extracted with two equal volumes of solvent Dichloromethane. The organic phase was collected and evaporation to dryness under reduced pressure at 35^{o}C. The dry solid residue was re-dissolved in methanol and store for future uses.

ANTIMICROBIAL ACTIVITY:

Antimicrobial activity of leaf extract:

Antibacterial activity of each plant leaf extracts was determined using Kirby Bayer (Bauer *et al.*, 1996) disc diffusion method. Test microorganism were selected i.e Gram positive (*Bacillus Substilis*-MTCC 441) and Gram negative (*Escherichia coli*-MTCC 443) sterile disc (6mm) were prepared with plant leaf extracts produced with different solvent ethanol, acetone, chloroform and water extract and four different fungal extract which are placed on the culture plate inoculated with the test microorganism were speeded on the petriplate. After inoculation the Petri plates were incubated at 37^{0}C for 24 hours. The zone of inhibition were measured in millimetres and compared with standard antibiotic disc kanamycin.

Antimicrobial activity of endophytic fungal extract:

Antibacterial activity of fungal methanol extract was determined by disc diffusion method. The sterile disc were prepare with four different fungal extract which are placed on the culture plate inoculated with the test microorganism were spreaded on the petriplate. After inoculation the petriplates were incubated at 37^{0}C for 24 hours. The zone of inhibition were measured in millimetres and compared with standard antibiotic disc kanamycin.

Larvicidal activity:

Test organisms:

Larva was collected from the stagnant water bodies at Karungal with hand net. They were transported in a plastic bucket containing clean water to the laboratory. Larva was categorized based on their size as large (0.6) and small (0.2 cm) culex larvae were used for experiment as well as to maintain in the laboratory conditions.

Preparation of fungal cured for larvicidal effects:

100mg of crude residue was dissolved in 1ml of DMSO for stock solution from the stock solution concentration. 100, 200, 300, and 400 µg were prepared (Prabakar.K 1995).

Larva maintained:

A laboratory colony of *C. quinquefasciatus* was used for larvicidal activity. It was maintained at 27± 2^{o}C, 75 – 85% RH, less than 14 L: 10D photoperiod cycles. The larvae were fed with dog biscuits and yeast extract at 3:1 ratio.

Larvicidal activity:

In the present study, mosquito larvicidal activity of test fungal extracts namely *Aspergillus sp.* was carried out. Crude extract directly used against larvae in different concentration viz, 2ml, 4ml,6ml,8ml and 10ml were prepared. *C. quinquefasciatus* was used for the larvicidal activity. Twenty five larvae of early third instar were released in a 50 ml paper cup containing 5 ml of distilled water and 2ml, 4ml, 6ml, 8ml and 10ml of desired fungal extract concentration.

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Symbiotic Relationship:

Symbiotic Relationship between Endophytic fungi and their host plant leaf extract (*Punica granatum*) was studied with different solvent such as ethanol, chloroform, acetone and water. This studies was performed by agar diffusion method, in which endophytic fungi was spread on the PDA agar plate. The leaf solvent extract were placed on the centre and then kept for incubation.

3. RESULTS

Phytochemical Activity of Leaf Extract:

The results of qualitative screening of phytochemical compounds in aqueous extract of *Punica granatum* leaves revealed that the presence of alkaloids, tannin, protein, aminoacid, carbohydrates, coumarin, saponin, leucoantrocyanin whereas emodin, Phcobatannin are absent Table:1.

Table 1: Phytochemical activity of *Punica granatum*

Phytochemicals	Acetone	Ethanol	Chloroform	Water
Alkaloids	+	+	_	_
Tannin	+	+	_	_
Protein	+	+	+	_
hcobatannin	_	_	_	_
Amino acid	_	_	_	+
Carbohydrates	+	+	+	+
Coumarin	+	+	+	+
Emodin	_	_	_	_
Leucoanthocyanin	_	+	_	+
Saponin	+	+	+	+

ISOLATION AND IDENTIFICATION OF ENDOPHYTES:

Endophytic fungi:

A total of 4 different fungal species *(Aspergillus sp., Rhizomucor sp., Rhizophussp.,Trichonderma sp*.) were isolated from the leaves and flowers from *Punica granatum.*

Fig 1: Aspergillus sp. **Rhizomucors sp.** **Rhizophus sp.** **Trichoderma sp.**

Endophytic Bacteria:

Two different morphological bacterial species (Clostridium sp, *staphylococcus sp.*), were isolated from the leaves and flowers of pomegranate. These species identified through the Morphology, differential staining and biochemical characterization.

Morphological identification of bacterial species:

The bacterial species are identified through gram staining, spore staining, acid fast staining and the biochemical studies. Therefore violet stain was indicating that the test bacterium was gram positive. Clostridium sp. indicate that the green ellipses within the cells. In other hand staphylococcus sp. revealed that pink colour appears therefore it was indicating the vegetative cell.

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Extraction of secondary metabolites in endophytic fungi:

Secondary metabolites from the 21 days old culture *Aspergillus sp.*, *Rhizomucor sp.*, *Rhizophussp.*,*Trichonderma sp.* were screened by the followed by (Strobel *et. al.*, 1996). The culture filtrate was extract with the dichloromethane and the solvent was then removed by evaporated under vacuum. The solid residue there by obtain was re- dissolved by methanol.

Antimicrobial Activity of extracts:

Among The punica granatum leaf solvent extract of ethanol, chloroform, acetone and water ethanol extract showed maximum activity of 5mm zone inhibition Table:2, Fig:2. Among The four different fungal methanol extract methanol extract showed maximum activity of 4mm zone inhibition Table:3, Fig:3.

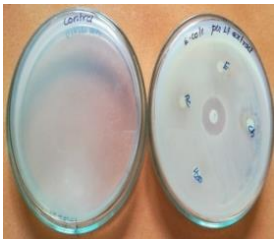
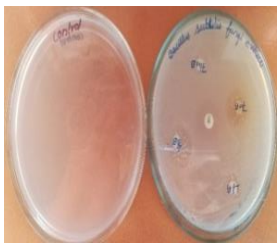


Fig 2: plant extract with plant extract with *E. Coli*



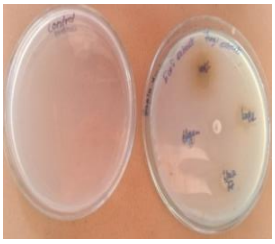


Fig 3: fungal extract in *B. Substiis* fungal extract in *E. Coli*

B. Subtilis

Table 2: Antimicrobial activity of different solvent extract of *Punica granatum* leaves against test organism.

SI. No	Microorganism	Zone of inhibition(mm)				
		Ethanol	Acetone	Chloroform	Water	Kanamycin
1.	Escherichia coli	5	4	4	3	10
2.	Bacillus subtitles	5	5	5	3	11

Table 3: Antimicrobial activity of different fungal methanol extract against test organism

SI. No	Microorganism	Zone of inhibition(mm)				
		Aspergillus sp.	***Rhizomucor sp.***	***Rhizophus sp.***	***Trichoderma. sp***	Kanamycin
1.	Escherichia coli	5	4	5	3	10
2.	Bacillus subtitles	5	5	5	3	11

Symbiotic relationship between endophytic fungi and host plant leaf extract:

Symbiotic relationship between endophytic fungi (*Aspergillus*sp.and Rhizophus sp) result of this study, fungi are freely grown on the plates. Here extract disc will not inhibit by endophytic fungi. Therefore, it revealed that highly symbiotic relationship between entophytic fungi and their host plant Fig:4.

Fig 4: Aspergillus sp. With leaf extract Rhizophus sp. Wth plant leaf extract

Mosquito larvicidal Activity:

Larvicidal Effect of the Culture Filtrate Aspergillus *sp.*

Crude extract directly used against larvae in different concentration viz, 2ml, 4ml,6ml,8ml and 10ml were prepared. *C. quinquefasciatus* was used for the larvicidal activity.

Twenty five larvae of early third instar were released in a 50 ml paper cup containing 5 ml of distilled water and 2ml, 4ml, 6ml, 8ml and 10ml of desired fungal extract concentration.

The toxicity of the late third instar larvae of *C. quinquefasciatus* to fungal extract of was noted and the result was presented in Table 4.

Table 4: Laricidal activity of Dicholoromethane extract of Aspergillus sp. against C. quinquefasciatus

Concentration (ml)	No of Larvae	Motility
2	5	0
4	5	2
6	5	4
8	5	5
10	5	5
Control	5	0

4. DISCUSSION

Tens of thousands of natural products have been described, but in a world where we are not even closed to documenting all the extent species, there are almost certainly many more thousands of compounds waiting to be discovered. Likewise, Plant endophytes are takes place a major role to producing bioactive products which are potential applications in agriculture, medicine and food industry. In the past two decades, these bioactive compounds are useful as antimicrobial and insecticidal activity. Therefore, this work is mainly concentrated to separate the secondary metabolites of endophytes and to analyse its activities.

Endophytes can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry. In addition, it has been shown that they have the potential to remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilisation and nitrogen fixation. There is increasing interest in developing the potential biotechnological applications of endophytes for improving phytoremediation and the sustainable production of nonfood crops for biomass and biofuel production. (Robert P. Ryan *et al.*, 2007)

The present investigation, Endophytes are isolated from plant (punica granatum) by following standardize protocols. Total four different fungal sp. (*Aspergillus sp., Rhizomucor sp., Rhizophussp.,Trichoderma sp)* and two different bacterial sp.(Clostridium sp, *staphylococcus sp.*)

The phytochemical analysis of plant leaf extract, it shows the presence of alkaloids, tannin, protein, aminoacid, carbohydrates, caumarin, saponin, leucoanthocyanin, and the absence of emodin and Phcobatannin. Recently, a number of the studies have been reported on the phytochemistry of medicinal plants, particularly on vegetative part like leaves and stems. Herbs that have tannins as their componds are used for treating intestinal disorders such as diarrhea and dysentery thus exhibiting antibacterial activity. Tannins are widely used in traditional medicine in treating wounds and to arrest bleeding. Some of these bioactive compounds which are synthesized as secondary metabolites as the plant grows also serve to protect the plant against microbial attacks and microbial attacks and prepedation by animals.

The antimicrobial activity of crude fungal methanol extracts are tested against representative Gram positive and Gram negative bacteria by well diffusion method. Fungal extract shows maximum inhibition zone 4mm against *E. Coli.* Theendophytic fungi in plant tissues opened up new possibilities in the search for metabolically active compounds. Cuomo *et al.,* (1995) examined a large number of terrestrial and marine fungal isolates and found a higher number of anti-microbially active species among marine isolates. According to Dreyfuss and Chapela (1994), about 4000 secondary metabolites of fungal origin have been described as biologically active.

In the present study has plan to produce biocontrol agent such as larvicidal, bacteriocidal insecticidal etc., The antimicrobial activity of *punica granatum* leaf solvent extract of ethanol, chloroform, acetone and water was examined for its antimicrobial activity against test microorganism. Among the extract, ethanol extract showed maximum activity of 5mm zone inhibition. Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacology study leading to the synthesis of more potent drugs and safe use.

Symbiotic relationship shows the result of good relationship between fungi and host plant. In symbiotically conferred stress tolerance, the endophytic fungi were considered to act as a type of biological trigger that activated the defence systems of a host (Rodriguez and Redman, 2008).

5. CONCLUSION

Endophytes are present inside of plant tissue and it produces some bioactive compound which is pharmacologically active substance with low toxic effect. The present investigation, examine the isolation of endophytes from plant *punica granatum.* Hereby to analyse the phytochemical activity in plant leaves and fungal methanol extract. The result of phytochemical activity shows the presence of alkaloids, tannin, protein, amino acid, carbohydrates, coumarin, saponin, leucoanthrocyanin whereas emodine, Phcobatannin are absent. Finally, from my studies I conclude both the endophytic fungal extracts and plant extracts act as an efficient biocontrol agent. And have the ability to control mosquito larvae and insects, also effective against antibacterial and larvicidal activity. It was believed that both endophytes and plants are mutualise, they are beneficial each other. Plant extract also effective Endophytic enzymes are believed to be highly benefits in agriculture, industry and human health field, through the enzyme activity studies. Further studies are still needed to identify the compounds responsible for the antimicrobial activity. The symbiotic relationship also revealed through this studies. Through this study I conclude there is no notable harm between endophytes and their host plant. The high potential of microorganisms is need to study.

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