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# Studies on Blood Clot Lysis Activity of Endophytic Fundi Isolated From *Thevetia Peruviana*

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**ABSRACT**

In the present study the blood clot lysis activity of endophytic fungi of *Thevetia peruviana* plant was studied.Total six endophytic fungi were isolated from leaves and stem of *Thevetia peruviana*. All the isolated fungal endophytes belong to three different genera viz., *Aspergillus spe, Penicillium spe* and *Fusarium spe.* The research investigation revealed that Endophytic fungal isolate *Aspegillus sp.* showed maximum blood clot lysis than *Penicillium spe* and *Fusarium spe.*

**INTRODUCTION**

Cardio-cerebrovascular disease caused by thromboembolism, arthrosclerosis, platelet adhesion and thrombus formation poses a serious threat to human health (Lei et al 2015).Thrombolytic agents such as tissue plasminogen activator, urokinase,streptokinase (SK), etc are used to dissolve the already formed clots in the blood vessels (Prasad et al 2007, Demrow et al 1995,.Briggs et al 2001). However, these drugs have certain limitations which cause serious and sometimes fatal consequences including hemorrhage, severe anaphylactic reaction, lacked specificity, etc. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted. Hence, it is a need to search the lucrative, inexpensive, easily available and safe anticoagulant and antithrombotic agent.

The significant efforts have been directed towards the discovery and development of natural products from various plant and animal sources which have antiplatelet ,anticoagulant antithrombotic and thrombolytic activities (Zhiguang, et al 2000, Rajapakse, et al 2005, .Yamamoto et al 2005, Prasad et al 2006,.Gillman et al 1995 ).In traditional culture, medicinal plants are used all over the world and they are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals (Verma KR et al 2011 ). There are evidences that consuming herbs, natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect helps in prevention of cardio vascular diseases and stroke (Bazzano LA et al 2002, Gillman MW et al 1995, Joshipura KJ et al 1999, Liu S et al 2000). A various medicinal plants such as *Ocimum sanctum, Curcuma longa, Azadirachta indica, Anacardium occidentale* (Khan et al 2011), *Alpinia conchigera, Lannea grandis, Aglaonema hookerianum* (Sultana et al 2012), *Withania somnifera, Terminalia arjuna, Moringa olifera, Asparagus racemosus* (Shahriar et al 2012), *Sida acuta* (Bahar et al

2013), *Ficus glomerata* (Kirankumar and Ramesh 2014), *Kalanchoe pinnata (*Akanda et al 2014), *Nigella sativa* (Ansari et al 2014) reported for thrombolytic activity .

*Thevetia peruviana* has inhibitory effect against HIV-1 reverse transcriptase and HIV-1 Integrase (Tewtrakul et al 2002). commonly known as yellow oleander, it is an evergreen small ornamental shrub that bears yellow, trumpet like flowers. Its fruit are deep red/black in color encasing a large seed that bears some resemblance to Chinese "lucky nut. The plant parts such as flowers, leaves, seed and root has immense medicinal properties due to the potential source of biologically active compounds. *Thevetia peruviana* plant extracts have been reported to have antimicrobial properties (Antibacterial, Antifungal and Antiparasite),Antispermatogenic, anti-termite, Antiinflammatory, Antidiarrheal etc. *Thevetia peruviana* also contains a milky sap containing a compound thevetin that is used as a heart stimulant in its natural form. This plant is particularly known for its ability to produce cardiac glycosides; flavonol glycoside from leaves. Phytochemical Analysis revealed that *Thevetia peruviana* contains a new cardiac glycoside called Digitoxigenin, Thevetin A and B, theveridoside, cerberin ,galactouronic acid, rhamnose, aucubin, ursolic acid, cardenolides, quercetin, alpha and beetaamyrin, and lupenyl acetate as prime phytoconstituents(Pragati et al 2012).However, the work on *Thevetia peruviana* on cardiac abnormalities has poorly investigated.

Endophytic microorganisms are bacteria, fungi and actinomycetes which inhabitat the internal part of plants, causing apparently no visible changes to their hosts. Generally, endophytes show symbiotic or mutualistic relationship with their respective host plant(Jena and Tayung, 2013). Virtually all three million plant species existing on earth are the host of one or more endophyticmicroorganism(Strobel et al 2003). The endophytic microorganisms had been recognized as vital source of natural bioactive compounds with applications in many industries such as agriculture, food, medicine etc (Dasari et al 2015). Previous studies reveal that endophytes are the potential source of bioactive compounds has wild application in Agriculture, Medicine, Pharmaceutical and leather industry. Although known since long time, their importance become evident only more recently when it was shown that they play specific roles
as for instance, protecting the host-plants against insects and diseases. Some endophytes also
have the ability to improve bioremediation which leads to the improvement of soil fertility.Endophytes contribute in growth stimulation of plants by the production of phytohormone, nitrogen fixation, phosphate solubilization, and biocontrol of phytopathogens. Endophytes are the main contributor in the production of antibiotics or siderophores, by nutrient competition or by induced systemic resistance. It has been also reported that many secondary metabolites produced by plants are enhanced by microorganism present inside them as endophytes (Kumar et al., 2011,Nimnoi et al., 2010,Quecine et al., 2008,Lee et al., 2004,Wakelin et al., 2004 and, El-Tarabily et al., 2009 and Anitha *et al.,* 2013).Endophytes gains property of producing bioactive compounds, these compounds are active as anti-microbial (antibacterial, anti-fungal, anti-viral, anti-protozoal), anticancerous, anti-oxidant, immunomodulatory, antidiabitic agents etc. The main characteristic of endophyte is that they mimics the synthesis of bioactive compounds of host plant(Salini G et al 2014).As mentioned above *Thevetia peruviana*i s used as a heart stimulant ,every plant species is the host of one or more endophytes and they mimic bioactive compounds production of the host plant. Hence, the present

investigation was carried out to find the occurrence of endophytic microbes in different plant part of *Thevetia peruviana* and evaluate their blood clot activity.

## MATERIALS AND METHODS

### Collection of plant material

Fresh and healthy plant parts such as leave and stem of matured *Thevetia peruviana* were collected from premises of balaji cotton processing industry,Malegaon, Dist-Washim. All the plant parts were aseptically transported to Microbiology Research laboratory, Department of Microbiology, R.A. College, Washim in plastic bags. Thereafter,all the plant part were washed in running tap water to remove soil partials and washed with sterile distilled water.

### Isolation of microbial endophytes

The microbial endophytes from leaves and stem of *Theveti aperuviana*were isolated by adopting the method described by Hollman et al (2006).The isolation of microbial endophytes have a need of surface sterilization to remove epiphytic microorganism from the plant parts. Surface sterilization was performed by immersing all the plant parts in 70% ethanol for 3 minutes separately followed by treated with 1% of sodium hypochlorite solution for 1 minutes.Thereafter, each plants part was separately washed in sterile distilled water three times. The efficacy of surface sterilization was analyzed by inoculating 1ml final washed water of each plant part in nutrient, potato dextrose and actinomycetal a broths and incubated (Raviraj et al 2006).All the plant parts were separately crushed with fractionated sterile distilled water in mortal and pestle under aseptic condition. The Obtained plant part extracts were serially diluted.0.1ml of each dilution of each plant part separately spread on potato dextrose agar plates in aseptic condition to isolate endophytic fungi. All the potato dextrose agar plates were incubated at room temperature for 7 days. After incubation the morphologically distinct colonies on potato dextrose agar were consider as endophytic Fungi.The isolation of endophytes was confirmed by observing no growth in efficacy tested broth. All the isolated bacterial, fungal and actinomycetalendophytes were subculture on nient, potato dextrose and actinomycetal agar slants.The pure cultures of all endophytes fungi of both plant parts were preserved in refrigerator at $4^0$C and used for further investigation.

### Identification of Endophytic fungi

The identification of Endophytic fungi isolated from leaves and stem of *Thevetia peruviana* was carried out on the basis of their morphological characters. The morphology of fungal isolates was studied by macroscopic and microscopic examinations. The macroscopic examination of fungal isolates was performed by observing colony characters viz shape, size, color, margin and texture on potato dextrose agar The microscopic examination was carried out by applying wet mounting with lactophenol cotton blue on glass slide.The Identification was confirmed by comparing the characters with standard literature(Aneja., 2006).

### Evaluation of Clot lysis Activity

Anticoagulant activity of crude extracts of endophytic microbes carried by determine the lysis of blood clot. It have the need to prepare the blood clot. The preparation of blood clot and it’s lysis was done by adopting the method describe by (Al-Mamun et al 2012) with slight modifications.

**Preparation of clots**

Set of eight previously weighed micro centrifuge tubes were prepared. Each tube filled with 0.5 ml blood of healthy human volunteers and weighed. Thereafter, all the tubes were centrifuged at 2000 rpm for 5 min. After centrifugation serum was easily removed from each tube without disturbing the clot.The centrifuge tubes were then incubated at body temperature i.e.$37^0$C for 45 min in heat controlled incubator. After incubation all the tube were reweighed along with the clot.

**Evaluation of Clot Lysis**

The blood clot lysis activity was analyze by adding 100µl of crude extract of each endophytic fugi was added in first six clot containing microcentrifuge tubes.In $7^{th}$ and $8^{th}$ clot containing microcentrifuge tubes equal volume of Asprin and sterile distilled water were added and considered as positive and negative control respectively.All the tubes were again incubated at $37^0$C for 90 minutes for clot lysis. After incubation all the microcentrifuge-tubes were taken out of the incubator and the dissolved clot with the crude endophytic extract, positive control and negative control were carefully removed from the micro centrifuge tubes. After this the microcentrifuge tubes contained undissolved clot were weighed. The blood clot lysis activity was determined by subtracting the weight blood clot with crude extract from weight of untreated blood clot and compared with positive control and negative control. The weight loss due to clot lysis activity of each applied agent was calculated in percentage.The ability of the extracts to dissolve clot in percentage of weight loss were compared with that of positive control and negative control.

**RESULTS AND DISCUSSION**

**Isolation of Endophytic Fungi**

Total 6 endophytic fungi were isolated from leaves and stem of *Thevetia peruviana*. It was also observed that fungal endophytes were predominately present leaves over stem. Among total 6 endophytic fungal isolates 4 were obtained from leave and 2 were isolated from stem. The results of present study on occurrence of microbial endophytes were found accordance with experimental findings of Ahmad et al. (2012) who has isolated 132 microbial endophytes including bacteria, fungi and actinomyces from leave, stem, root and fruit of *Digitalis lanata (wooly foxglove), Digitalis purpurea (purple foxglove), Plantagoovata (psyllium/isabgol), Dioscoreabulbifera (air potato).* Caruso *et.al.*(2000) isolated 150 fungal from the internal tissues of woody branches, shoots and leaves of different plants of *Taxus baccata* and *Taxus brevifolia*. Similarly, Jalgaonwala *et.al.*(2010) isolated 78 bacterial and 142 fungal endophytes from aerial and underground parts of various medicinal plants.

**Identification of endophytic Fungi**

The identifications of endophytic fungal isolates were confirmed by comparing their morphological characters with standard literature. Table 1 represents the identification of fungal isolates. It was observed that all the isolated fungal endophytes belong to three different genera viz., *Aspergillus, Penicillium* and *Fusarium* .The fungal endophytic isolates (FEL2, FEL4 & FES1) identified as *Aspergillus spe.*The morphological characters of (FEL1 & FES2) were found similar with *PenicilliumSpe.* And the identification of(FEL3) was confirmed as *Fusarium spe* by observing their growth on Potato dextrose agar and compared with standard literature. Similarly, Sandhu et al. (2014) isolated the fungal endophytes from *Ricinuscommunis* and these fungi were successfully identified *Aspergillus fumigates, Aspergillus japonicas,Aspergillus niger,*

*Fusarium semitectum, Curvularia pallescens, Phomahedericola, Alternaria tenuissima, Fusarium solani, Drechslera australien* and *Aspergillus repens.* Hence, these results were found accordance with the experimental findings with present investigation. Ten endophytic fungi were isolated from cocoa plant and were belonging to six genera viz *Curvularia spe*., *Fusarium spe* , *Geotrichum spe*, *Aspergillus spe*, *Gliocladium spe* and *Colletotrichum spe.* (Amin et al. 2014). These results were partially correlated with present investigation.

**Table 1 : Identification of Endophytic Fungi From Different Parts of Thevetia Peruviana**

| Sr.No. | Endophytic Fungal Isolates | Surface Colour | Shape | Texture | Possible species |
|---|---|---|---|---|---|
| **1.** | **FEL1** | Dark green | Circular | Velvety | ***Penicillium spe.*** |
| **2.** | **FEL2** | Whitewith typical black spore | Circular | Powdery and Velvety | ***Aspergillus spe.*** |
| **3.** | **FEL3** | White | Circular | Cottony | ***Fusarium spe.*** |
| **4.** | **FEL4** | Black | Circular | Cottony | ***Aspergillus spe.*** |
| **5.** | **FES1** | Black | Circular | Cottony | ***Aspergillus spe.*** |
| **6.** | **FES2** | Pinkish-White | Circular Irregular | Cottony | ***Fusarium spe.*** |

**Evaluation of Clot lysis activity**

The clot lysis activity of crude extracts of fungal endophytes were evaluated in present study.The results were presented in table 2. The clot dissolution was determined in terms of loss in weight of blood clot before and after the treatment of crud extract of fungal endophytes. The study showed that crud extract of endophytic fungi was found significant in blood clot lysis. It was observed that endophytic fungal isolates FEL4 and FES2, showed maximum clot lytic activity. FEL1, FES1, FEL2 isolates showed moderate clot lysis. Whereas, the mild clot dissolution was recorded in crud extract of FEL3 endophytic fungal isolates. The results were found significant over negative control (NC) Wheree equal volume of sterile distilled water was used instead of crud extract of microbial endophytes. However, remarkable clot lysis observed in positive control(PC). Where equal volume of Aspirin solutionr was used instead of crud extract.

The clot lysis in percent was also calculated in this study. It was noted that the maximum clot lysis i.e 86% was observed in FEL4 followed by 78% , 57, 50 ,43 and 14 % in treatment of crud extract of endophytic fungal isolates (FEL1), (FES1),(FEL2 ) and (FEL3 )respectively on comparing with negative control (distilled water) Where no clot dissolution was observed. In positive control (Aspirin) and 93% of clot lysis was observed. The results indicates that entophytic fungal isolates isolates found potent in blood clot lysis activity. The results of the present study found accordance with the experimental outcome of Al-Mamun et al(2012).Who has studied thrombolytic activities of some plants, namely *Tamarindus indica*(Fabaceae), *Flemingia congesta*(Fabaceae), *Lawsonia inermis*(Lythraceae), *Mesuanagassarium*(Clusiaceae, and spices, namely *Coriandrum sativum*(Apiaceae), *Curcuma longa* (Zingiberaceae), *Cinnamomumtamala*(Lauraceae), *Nigella sativa* (Ranunculaceae), *Eugenia aromaticum*(Myrtaceae), available in Bangladesh, were evaluated using an*in vitro* model. However, in the present study crud extracts of microbial endophytes were analyzed for blood clot lysis. Similarlly, Lei et al (2015) also reported the anticoagulant activity of Coumarin derivatives

of *Ainsliaeafragrans.* Coumarins are a well-known class of secondary metabolites in plantsand fungi (Murray, R.D.H, (1989), Estevez-Braun , A & Gonzalez, G.A. (1997) ,Yang, X.L. et al (2013), Sandjo, L.P. et al (2012). Coumarins are widely used in the clinic for antithrombotic therapy; for example, warfarin, which used to be a rodenticide, is now used as an anticoagulant. However, the therapeutic use of coumarin agents is severely limited by their associated adverse reactions, such as platelet disease and haemorrhages (Hayes, W. J. & Gaines, T. B. (1950),Wadelius, M. & Pirmohamed, M.(2007)).However, herbal preparations on the other hand, if taken in appropriate doses, can lead to an alternative and better option for curing various ailments. Although, toxicities of many plant extract are major concern, but due to the development of methods for lethality assay has been successfully used to biomonitor the cytotoxicity of plant materials (A.V.Krishnaraju et al (2006),D Collen (1996)). As Lei et al ( 2011) five mentioned that endophytes mimics the production of bioactive compounds of host plant. Hence, endophytes of such medicinal plant can be utilized to analyze antithrombotic, anticoagulant and clot dissolution activity by reducing toxic effect of plant materials.

Ueda et al. (2007) reported that the endophytic fungi isolated from Hibiscus leaves which produces a fibrinolytic, anticoagulant and antiplatelet agent. These results were concord with present investigation. However, the biomonitoring of crud extract of fungal endophytes for the production of fibrinolytic, anticoagulant and antiplatelet agents was not examined in the present study.

**Table 2.Effect of crude extract of endophytic fungi on lysis of blood clot.**

| Sr No | Samples of endophytic extracts | Clot lysis CL=(W1-W2)in(mg) | Percent reduction in clot Lysis(%) |
|---|---|---|---|
| 1 | FEL1 | 0.12 | 57 |
| 2 | FEL2 | 0.16 | 43 |
| 3 | FEL3 | 0.24 | 14 |
| 4 | FEL4 | 0.04 | 86 |
| | FES1 | 0.14 | 50 |
| | FES2 | 0.06 | 78 |
| 7 | NC | 0.28 | ND |
| 8 | PC (Asprin) | 0.02 | 93 |

**W1-Weight of blood clot, W2- Weight of blood clot after treatment, mg-Miligrams, ND-Not dissolved, NC Negative control, PC Positive control**

## Conclusions

The results obtained during present investigation iconclude that, The endophytic microbes,Fungi harbor the Leaves and Stem of *Thevetia peruviana*. The endophytic Fungal isolates found potent in clot lysis activity. Endophytic fungi found significant in blood clot dissolution over negative control (distilled water)

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