Anti-atherosclerotic and Anti-Biofilm properties of *Pleurotus ostreatus* Metabolites

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**Article History:**
- Received on: 14 Apr 2020
- Revised on: 27 Apr 2020
- Accepted on: 27 May 2020

**Keywords:**
- Metabolites
- Pleurotus ostreatus
- Anti-biofilm
- Drug release
- Atherosclerosis

**ABSTRACT**

To investigate the anti-biofilm and anti-atherosclerotic properties of metabolites from a mushroom, *Pleurotus ostreatus* was selected as the main objective of present study. The study involved the following series of steps to meet the objective. Coating the metal stents with fungal metabolites and vitamin-E, to study the anti-biofilm properties against biofilm producing bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*), to determine the drug release behaviour from the coated stents, and to investigate the cytotoxic assay and biocompatibility of the coated stents using MTT assay method. Anti-biofilm activity of the developed Metabolite-Vitamin E (MV\(_E\)) combinations showed significant activity against *Staphylococcus epidermidis* (0.5mg/ml) and *Pseudomonas aeruginosa* (0.75mg/ml). *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* expressed respective anti-biofilm values of 0.5mg/ml, 0.25mg/ml and 0.5mg/ml. Drug release behaviour analysis revealed controlled and sustained release of fungal metabolites from the stents coated with Metabolite-Vitamin E mixture. In *vitro* MTT assay revealed that Metabolite-Vitamin E did not inhibit the growth of L\(_{929}\) fibroblast cells; indicating the biocompatibility of the coated stents. Mushrooms producing pharmacologically significant metabolites could be exploited in treating cardiovascular diseases in human beings to a greater extent. Future analysis could be useful in identifying the significant chemical compounds present in the metabolites.

**INTRODUCTION**

Medically significant cardiovascular disease called Atherosclerosis is considered as a major complication in stent implanted patients (Moss and Ramji, 2016). The build-up of fatty cells and tissues results in complete blockage within blood vessels results in atherosclerosis further leads to heart attack, stroke and sometimes death. Angioplasty is the method to implant coronary stents to remove plaque and fatty cells within blood vessels (Hansson, 2005). In most angioplasty cases, it may leads to arterial injury (thrombosis), biofilm formation, development of vascular smooth muscle cells called as restenosis (Stefanini and Holmes, 2013). Prevention of restenosis in atherosclerosis cases were reported to be failed due to lack of essential drug concentrations at the target site (Lafont, 1998). Drugs should be delivered at the vascular site using target specific drug eluting stents (Axel *et al.*, 1997). These
stents are also known as drug-eluting stents. Different drugs like sirulimus, rapamycin, and paclitaxel was used for coating the stents (Solott et al., 1995). Another major risk associated with these stents is their high infection rate. Staphylococcus species like Staphylococcus aureus and S. epidermidis are the biofilm producing pathogens that colonize the implants in patients by adhering on their own proteins causing infection (Mack et al., 2004).

Hydroxy methyl glutaryl-CoA reductase is reported to be involved in the biosynthesis of cholesterol assimilation pathway. Pleurotus ostreatus are reported to exert positive effects in atherosclerosis by suppressing this enzyme. When the enzyme activity is retarded, the cholesterol level is reported to be reduced (Palvai and Urooj, 2014). Based on this literature survey, the enzyme inhibitor found in P. ostreatus, was named as mevinolin or lovastatin. (Guillamón et al., 2010) highlighted the presence of different anti-inflammatory compounds in P. ostreatus has the ability to prevent cardiovascular disorders. For effective and controlled release of drugs from any eluting stents a carrier is reported to be essential as per many literature surveys. Tocopherol acetate is considered as one such carrier compound used as an adjuvant in drug delivery process. The efficiency of drug releasing mechanism from the stent materials by tocopherol acetate was approved by FDA based on its biocompatible (Yang et al., 2018b) and drug permeation ability (Upston et al., 2003).

Based on these therapeutic applications of the fungal metabolites and Vitamin E as described in literature, main objectives were framed. The objective of this research is to prevent restenosis and stent associated bacterial infection using drug-eluting coronary stents. A preliminary attempt was made to use the metabolite from Pleurotus ostreatus as a significant drug to fight against atherosclerosis and biofilm formation. Metabolites were extracted, purified and mixed with vitamin-E (as carrier) and coated onto coronary metal stents. The coated stents were analysed for drug release and anti-biofilm assays.

**MATERIALS AND METHODS**

The present research work was carried out in the Department of Microbiology, Sree Narayana Guru College, Coimbatore, Tamil Nadu, India. The work was done during the period of December 2019 to February 2020.

**Procurement of commercial Metal stents**

The metal stent was fabricated using SS mesh in the present study. The mesh were fabricated and designed like angioplast stent with the standard size. The developed stents were further subjected for coating with metabolite-vitamin E (MV$_E$).

**Preparation of Drug-Eluting stents coating with metabolite-vitamin E (MV$_E$)**

Coating the stents was carried out using two series of steps under aseptic conditions was conducted. Seeding is the first process to lyophilize the fungal metabolites, followed by crystallizing metabolite-vitamin E (MV$_E$) on the stent surface (Su et al., 2019).

**Seeding stent surface with fungal metabolites**

Process is composed of two steps (seeding and crystallization). For seeding, 5ml of fungal metabolite was mixed with 4ml of n-Hexane (Sigma-Aldrich). The mixture was sonicated twice with an intermittent of 10min between each cycle. Sonication was done to form a homogenous mixture of metabolite. Stent samples were dispersed into the metabolite solution in separate vials. All the vials were placed in an ultrasonic bath for 15min at 28±2°C until a metabolite seeding layer was formed on the stent surface.

**Crystallization of fungal metabolites and vitamin E on stent surface**

For crystallization, 5ml of metabolite and 1% of Vitamin-E (alpha tocopherol) as drug carrier was mixed with 5ml of ethyl acetate. The metabolite-vitamin E mixture was placed in 50ml screw-cap bottle. 25ml of hexane solution was added slowly at the rate of 1ml per minute into the metabolite mixtures using a burette. Previously seeded stents were placed inside the metabolite mixture tubes and incubated at 4±1°C. Crystallization of metabolites on the stent surface was expected after incubating for 24hours in a cold room. The stents were dried at room temperature under strict sterile conditions and stored at refrigeration temperature prior to testing.

**Anti-biofilm properties of metabolite-vitamin E (MV$_E$)**

Anti-biofilm properties of developed metabolite-vitamin E combination were determined against the test organisms (Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa) using micro-dilution method as described by La Plante et al., (2012). All the test organisms were allowed for biofilm growth on the well surface of microtitre plates. The non-adherent cells were washed from the plates using phosphate saline. Metabolite-vitamin E solution was serially diluted (two fold dilutions) and added on to the microtitre plate wells,
Table 1: In vitro Drug release analysis from fungal metabolite coated stents

| S. No | Time (hours) | Metabolite release concentration (µg) | Metabolite-Vitamin E release concentration (µg) |
|-------|--------------|--------------------------------------|-----------------------------------------------|
| 1     | 0.5          | 105                                  | 55                                           |
| 2     | 1            | 105                                  | 55                                           |
| 3     | 2            | 105                                  | 55                                           |
| 4     | 4            | 105                                  | 55                                           |
| 5     | 8            | 70                                   | 95                                           |
| 6     | 12           | 65                                   | 105                                          |
| 7     | 24           | 45                                   | 110                                          |
| 8     | 48           | 85                                   | 120                                          |
| 9     | 72           | 90                                   | 125                                          |
| 10    | 96           | 65                                   | 125                                          |
| 11    | 120          | 60                                   | 125                                          |

Table 2: Biocompatibility of the metabolite-vitamin E (MV\textsubscript{E}) coated stents – Cell viability test

| S. No | Concentration | Fibroblast cell lines – MTT Assay | Cytotoxic reactivity |
|-------|---------------|-----------------------------------|---------------------|
|       |               | *Cytotoxicity (%)                 | *Cell viability (%)  |                     |
| 1     | 5             | 17.3±2.08                         | 81.6±1.57           | No cytotoxicity     |
| 2     | 15            | 9.6±0.57                          | 90.6±0.57           | No cytotoxicity     |
| 3     | 25            | 0                                  | >99                 | No cytotoxicity     |

Sealed and incubated at sterile conditions. Anti-biofilm of the metabolite-vitamin E combination was expressed as minimal biofilm eradication concentration (LaPlante et al., 2012).

**Efficacy of Metabolite-vitamin E (MV\textsubscript{E}) release for the prevention of restenosis using High performance liquid chromatography**

The efficacy of Metabolite-vitamin E (MV\textsubscript{E}) concentration released from the coated drug-eluting stents was analyzed using High performance liquid chromatography (HPLC) with a known standard. The release profile of metabolites from the biodegradable polymer matrix for a period of 120 hours was studied. The coated stent samples were placed in 10ml of saline solution (PBS) and agitated at 200rpm at room temperature. Each MV\textsubscript{E} coated stents were removed at 30 min, 1, 2, 4, 8, 12, 24, 48, 72, 96 120 hours from each respective tubes and the concentration of fungal metabolite released was determined (Ankur et al., 2007).

**Cytotoxicity assay of metabolite-vitamin E (MV\textsubscript{E})**

MTT assay is used to evaluate the in-vitro cytotoxicity of metabolite-vitamin E (MV\textsubscript{E}) coated stents. L\textsubscript{929} fibroblast cell lines were cultivated in 12-well microtitre plates to reach confluence growth. The stent samples were applied directly to the developed fibroblast monolayer. Before cell seeding, the specimens were pre-wetted in 70 % aqueous ethanol solution for 48 hours, rinsed twice with ultrapure water and immersed in 1ml DMEM fibroblast medium in 24-well plates for 2 hours in an incubator at 37°C. The specimens were then seeded with L929 fibroblast cell line at 10,000 cells per well according to routine cell-culture methods.

The plates were incubated at 37°C and 5% CO\textsubscript{2} for fifteen days. A well without drugs (control-1) and another well without cells (control-2) were included in this study. The effect of metabolite-vitamin E on fibroblast viability was evaluated using the photometric MTT assay. At each time point, samples were taken from the 24-well plates and transferred into new plates for the MTT study. About 1ml of MTT solution was added to each well and the plates were incubated for 3 hours. All the wells were rinsed and desorbed in 100ul of 70% isopropanol for the formation of purple crystals. The plates were agitated rapidly at 400rpm/min for 40min and the purple crystal dyed solution was transferred to 96-well plate and read at 550nm. The viability is expressed as a percentage (100%) of the control sample (Budman et al., 2012).
RESULTS AND DISCUSSION

Anti-biofilm properties of synergistic drugs

Antibiotic resistant characters of the biofilm producing bacteria always remain as a common problem in treatment of stent associated infections. Metabolite-Vitamin E used in the present research was analyzed for its anti-biofilm properties to overcome the resistant characters in biofilm producing organisms. Metabolite-Vitamin E combinations showed anti-biofilm activity against all the test organisms used in the research. Staphylococcus epidermidis and Pseudomonas aeruginosa exhibited anti-biofilm values of 0.75mg/ml; followed by Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus expressing 0.5mg/ml, 0.25mg/ml and 0.5mg/ml of respective anti-biofilm values for Metabolite-Vitamin E combinations.

The growth eradication concentration values were found to be significant as all the test organisms showed positive results as per the aim was framed. The eradication action of the coated stents was reported to be mainly due to the biological and pharmacological properties of fungal metabolite and the carrier Vitamin E. Guillamon et al., (2010) have focused the presence of various antioxidant and anti-inflammatory compounds of P. ostreatus; that plays a major role in treating the cardiovascular related diseases (CVD). Mevinolin or lovastatin was reported to be compound responsible for preventing CVDs. Pharmacological properties of tocopherol acetate (vitamin E) containing significant drug delivery applications were highlighted by (Yang et al., 2018a). Correlated with this, Vitamin E on the stent surface assisted the elution of fungal metabolite from the stents after placing on the titer plate. Degradation of vitamin occurs at a sustained rate when the stent was exposed on the moistened surface; which resulted in release of metabolite to release at constant rate for inhibiting the biofilm producers.

In vitro Drug release analysis from fungal metabolite coated stents

In vitro release study was conducted on the stent materials coated with Metabolite –Vitamin-E combination. Drugs released from crystalline Metabolite -Vitamin E (MV_E) mixture showed that the fungal metabolite release rate and release time was exponentially related. In figure-4, the lag phase exhibited initial burst effect from 0.5 h to 4 hours (55µg, 55µg, 55µg and 55µg). Followed this lag phase, increase in drug concentration was observed from 8 hours to 24 hours (95µg, 105µg and 110µg). In PBS at pH 7.0, the hydrophilic polymer, vitamin E undergoes degradation during the log phase. Due to the rate of polymer degradation, the release of drugs was facilitated at higher rate than the initial burst level concentration. During this phase the release concentration of the metabolite was remained constant (120µg, 125µg, 125µg and 125µg) from 48 hours to 120 hours indicating the sustained rate of drugs from the coated stents (Table 1).

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tration between metabolite coated and MV$_E$ coated stents. In Figure 1 the variation in release concentration between metabolite coated and MV$_E$ from the coated stents was presented. The observed variation was due to the absence of carrier, Vitamin E. The rate of degradation of the Vitamin E in the PBS was considered to be directly proportional to the rate of release of drugs. When the rate of degradation was high, then the release concentration was also found out to be high (Szymańska and Winnicka, 2015).

This drug releasing phenomenon aided by the Vitamin was correlated with its ability to prevent the formation of restenosis in atherosclerosis cases (Puranik et al., 2013). The dual role of drug-eluting stents in preventing biofilm associated infection and restenosis in coronary implanted cases were thus investigated in this study based on this polymer assisted drug releasing phenomenon.

**Cytotoxicity assay of metabolite-vitamin E (MV$_E$)**

Biocompatibility of modified metal stents is to be ensured for the patients’ safety before being implanted at the target site. Therefore, the biocompatibility of metabolite-vitamin E (MV$_E$) coated stents coated was evaluated by cytotoxicity assay (*in-vitro* cell culture model). This method has advantage over in vivo animal studies and other in vitro cell line studies. This method is very sensitive and accurate to prove the biocompatibility of any surface modified medical products. Metabolite-vitamin E coated stents did not reduce the cell viability and cell count during the study period of upto 24hours. The cell morphology, viability and numbers were compared with control samples simultaneously. The results were described below separately for each selected concentration of metabolite-vitamin E with the support of table values and graphical representations attributing for cell cytotoxicity and cell viability (Table 2).

The stents coated with selected concentrations (5, 15, 25μg/ml) of metabolites did not expressed any cytotoxic effects (Figure 2). In support to this, increase in cell viability with no significant difference in the morphology of the L929 fibroblast cells was evident after 24hours of cell culturing in the cell culture media (DMEM) when compared to control. The results revealed that the drug concentration did not inhibit the growth of cells; thus indicating the biocompatibility of the metabolite-vitamin E (MV$_E$) coated stents for the development of drug-eluting coronary stents.

**CONCLUSIONS**

Antimicrobial metabolite from *P. ostreatus* in combination with polymer, tocopherol acetate (Vitamin E) was crystallized and coated onto the stent materials to retard restenosis and biofilm formation simultaneously. Metabolite-vitamin E mixtures were used for coating the stent materials. During the study, it was found significant that the drug-eluting stents coated with Metabolite-vitamin E concentrate could able to retard the growth of biofilm forming organisms and restenosis when tested using standard assay protocols. The developed drug-eluting stents in the present study revealed the significance of the coronary implantation for patients in preventing critical cardiovascular disease due to biofilm formation and restenosis. The sustained release of metabolites from drug eluting stents fulfilled the objective of designing a medically significant coronary stents for the prevention of biofilm associated infection and atherosclerosis. The research work is appropriate for the field of Pharmacology because, the developed drug-eluting stents is considered as a novel biomedical product with a combination of drug and biodegradable polymer mixtures.

**ACKNOWLEDGEMENT**

I sincerely thank and acknowledge my guide Dr. Vijayachitra, Asst Prof in Microbiology and the management of Sree Narayan Guru College for supporting me to complete this research successfully.

**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

**Funding Support**

The authors declare that they have no funding support for this study.

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