Herbal Composite Coated Urinary Catheters: Evaluating Its Antibacterial Activity and Biocompatibility on L929 Fibroblast Cells

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ABSTRACT: Intravascular and urinary catheters are essential components of modern medical care. Such catheters possess high risk of infection by urinary tract pathogens. Most of the pathogenic bacteria have developed resistance to modern antibiotics as a result of which we are evidencing multi drug resistance among pathogens. Thus it is necessary to develop a new antimicrobial compound from various sources. Phytochemicals from medicinal plants showing various activities have the potential of filling this need. In the present study an herbal composite was prepared using Andrographispaniculata, Terminaliachebulae and Euphorbia hirta. The herbal composite was coated on urinary catheters. Antibacterial activity of the herbal composite, phytochemical analysis of three herbs by thin layer chromatography and biocompatibility of the herbal composite coated catheters in L929 fibroblast cell lines were evaluated. Maximum zone of inhibition observed on drug carrier coated (dcc) catheter materials was found to be 33.1±1.0mm and 31.1±2.26mm against Staphylococcus aureus and Escherichia coli respectively. Uncoated catheters showed no zone of inhibition and the inhibitory zones. Herbal composite with carrier coated catheter showed higher inhibitory zones than the herbal composite coated catheters. Phenol compounds were found to be present in all the three plants; tannins were present in A. paniculata and E. hirta. Flavanoids were present in A. paniculata and T. chebula. The herbal composite showed significant cell viability percentage. From the cell viability results, it is confirmed that the developed herbal composite coated catheter is biocompatible and the developed herbal composite can be used as an alternative for commercial antibiotics.

Key Words: urinary catheters, drug resistance pathogens, herbal composite, antibacterial activity, thin layer chromatography, biocompatibility.

INTRODUCTION
Urinary tract infection (UTI) is defined as an invasion of any part of the urinary system by a bacterial or fungal pathogen. Urinary tract infection is considered as the fourth most common type of healthcare-associated infection (Magill et al., 2014). Catheter associated urinary tract infection, when left untreated, may cause infections in the kidneys (pyelonephritis) and bloodstream (septicemia), leading to sepsis or, in extreme cases, even death. A common treatment for UTI is the use of antibiotics in urinary catheters, which can break down the biofilms formed by these persistent pathogens. Most of the pathogenic bacteria have developed resistance to modern antibiotics as a result of which we are evidencing multi drug resistance among bacteria. We are running out of antibiotics and could not add any new group of antibiotics since last three decades. The WHO has reported several instances of rising resistance among commonly found nosocomial pathogens (WHO, 2016). Resistance to several antibiotics has been reported so far and it is important to note that these bacteria are also commonly found in CAUTIs. Hence their infection raises issues of resistance to the antimicrobial agents used in urinary catheter materials. As a result, it is necessary to develop a new antimicrobial compound from various sources which are not based on the existing synthetic antimicrobial agents (Shah, 2005) and one such alternative is the usage of herbal drugs.

Phytochemicals from medicinal plants showing antimicrobial activities have the potential of filling this need (Costa et al., 2008). Screening active compounds from plants has led to the discovery of new medicinal drugs which have efficient protection and treatment roles against various diseases and infections (Kumar et al. 2004; Sheeja and Kuttan 2007) and Alzheimer’s disease (Mukherjee et al. 2007). Andrographispaniculata, a member of Acanthaceae (Acanthus) family has been used for upper GI tract and upper respiratory infections, fever, herpes and other chronic diseases. Various medicinal properties like choleretic, anti diarrhoeal, immunostimulant and anti-inflammatory have been attributed to this plant in the traditional system of Indian medicine (Siripong et al. 1992). Terminaliachebula is a medium to large sized tree
belonging to the family Combretaceae and has antimicrobial, hepatoprotective, anti-inflammatory, anti-diabetic, immunomodulatory, antioxidative and adaptogenic properties (Jayaprakash and Kulkarni., 2018). *Euphorbia hirta* belong to genus *Euphorbia* and family *Euphorbiaceae*. *Euphorbia hirta* is also called as asthma herb and pill bearing spurge, possessing antimicrobial antihelmintic, antiasthmatic, sedative, antispasmodic and antifertility properties (Kumar et al., 2010). Thus in the present study an herbal composite was prepared using *Andrographispaniculata*, *Terminaliachebula* and *Euphorbia hirta* and the herbal composite was coated with urinary catheters. The antibacterial activity, the phytochemical analysis of three herbs by Thin layer chromatography and biocompatibility of the herbal composite coated catheters in L929 fibroblast cell lines.

**MATERIALS AND METHODS**

**Herbal powder procurement**

Three different medicinal herbs, *Andrographispaniculata*, *Euphorbia hirta* and *Terminaliachebula* leaf powders were commercially procured after authentication from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

**Bacterial cultures used**

About five bacterial cultures, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* isolated from the biological specimens were procured from a diagnostic laboratory at Coimbatore, Tamil Nadu, India.

All the chemicals, media used in the research were commercially procured from HiMedia, Mumbai, India. The entire research work was carried out from November 2013 to October 2014.

**Solvent extraction of medicinal herbs**

About 5 to 10g of *Andrographispaniculata*, *Terminaliachebula* and *Euphorbia hirta* plant leaves were collected and mixed into 50ml of acetone (80%). All the prepared solvent extracts were incubated for 12 hours at room temperature. This process was carried out in a controlled condition for proper extraction of bioactive compounds from each herb into the solvent. After incubation period, the extracts were filtered through whatmann No. 1 filter paper. The collected filtrate was evaporated in a room temperature under dark storage condition aseptically for the period of 12 hours. The obtained herbal concentrates were then subjected to form an herbal-composite mixture.

**Herbal composite preparation and coating of urinary catheters (Boccaccini et al., 2003)**

Antibacterial coatings on the urinary catheters were carried out using a standard slurry-dipping technique. The technique started with the preparation of stable slurry with specific amount of herbal composite in the molten polyethylene glycol (PEG). Appropriate slurry temperature (40°C) was determined by an optimization process based on a trial and error approach to achieve optimum coating thickness, uniformity and stability of composite coating as well as adequate infiltration of drug particles into coating structure. PEG (2g) with a predefined molecular weight was mixed with the extract of *Andrographispaniculata* (0.5g) in a glass vial. The mixture was heated at the range of 40 to 45°C in awater bath to obtain homogeneous slurry. The resulting slurry was homogenized in a magnetic stirrer for 5 to 10min. Under this stirring condition, the extracts of *Euphorbia hirta* and *Terminaliachebula* were added slowly to obtain herbal composite slurry mixture.

Each piece of catheter (length - 6mm) was dip coated twice with intermittent drying (suspension coating method) in the herbal-composite slurry mixture. The dip-coating procedure was carried out in sterile glass beakers on a shaker (120 rpm) for 30mins, with a drying period of about 15mins between the two coating procedures, followed by drying at room temperature. All coating steps were carried out under strict aseptic conditions. All samples were coated by a thickness of about 5mm of catheters outer diameters. After coating procedure, the catheter samples were stored at 4°C for upto 15mins. In order to increase drug loading and prevent excessive increase in catheter thickness, the coating process were repeated for replicates of each sample. Subsequently, in order to slow down the release rate of herbal drug from PEG coating and mitigate the friction effect between catheter surface and mucosa, second coating layer was formed on the catheter surface. Polyvinyl alcohol (PVA) was dissolved in DMSO to acquire a 10 w/w% solution. PEG-coated samples were submerged into PVA solution three times for 1 min each. Thereafter, these samples were stored at 0°C or in a deep freezer to implement one freeze thaw cycle and physically crosslink the samples. The coated catheters were left to dry on a clean bench for 1 week at room temperature to remove residual DMSO. The herbal composite coated catheters were mentioned as *drug coated* (dc) and the herbal composite and carrier coated catheter samples were thus mentioned as *drug-carrier coated* (dcc) materials.
Qualitative antibacterial activity of coated catheter materials (El-rehewyet et al., 2009)

The method was performed for analyzing the antibacterial activity of urinary catheter after slurry dip-coating with herbal composite and carriers (PVA). In this qualitative method the pre-measured size (length-6mm) of all sterilized materials were tested from each preparation [drug-carrier coated, herbal composite coated and uncoated catheter samples]. The materials were all rinsed twice in phosphate buffered saline (PBS) before testing to remove any surface accumulation of drug. All test materials were placed on the surface of Mueller-Hinton agar (MHA) plate which had previously been seeded with an overnight broth culture of the test organisms and incubated at 37°C for 24 to 48 hours. The experiments were carried out in triplicate. Antibacterial activity was expressed as the diameter of the zone of inhibition.

Thin layer chromatography analysis of herbal plants

The plant extracts were subjected to the analysis of phenol, flavonoids and tannins. Each plant was applied on activated TLC plates with the help of capillary tube at a 1/2 inch apart from the lower edge of TLC plate, and plate was kept in a developing chamber containing suitable solvent system for specific time until the developing solvent reaches top of the upper edge of TLC plate. Plate was taken out from developing chamber, dried and solvent front is marked by lead pencil. Compound bands/spots visualized on TLC chromatoplate by using suitable spraying reagent for the presence of specific compound. The visualized spots of the components in the chromatoplate are marked and the Rf/ value of each spot is calculated by the formula: Rf = distance travelled by the sample (cm)/distance travelled by the solvent (cm).

Biocompatibility test (Cazedeyet et al., 2009)

L29 Fibroblast cell lines were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 μg/ml streptomycin, and maintained under an atmosphere of 5% CO2 at 37°C. The sample was tested for in vitro cytotoxicity, using L292 cells by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured L292 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1x10^5 cells/ml/well (200μL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the Sample A in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (20μL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220μL) were aspirated off the wells and washed with 1X PBS (200μL). Furthermore, to dissolve formazan crystals, DMSO (100μL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability was calculated using the below formula.

\[ \text{Cell viability} = \frac{\text{Treated}}{\text{Control}} \times 100 \]

RESULTS AND DISCUSSION

Qualitative antibacterial activity of coated catheter materials

The antibacterial activity of the drug coated (dc) and drug carrier coated catheters (dcc) was evaluated by the zone of inhibition on the agar plates. Maximum zone of inhibition observed on drug carrier coated (dcc) catheter materials was 33.1±1.0mm against Staphylococcal aureus. About 31.1±2.26mm and 29.4±1.7mm inhibitory zones were observed against E. coli and S. epidermidis. K. pneumoniae and P. aeruginosa showed 26.1±1.3mm and 23.4±0.5mm of inhibitory zones. Uncoated catheters showed no zone of inhibition and the inhibitory zones of drug carrier coated catheter were found to be higher than the drug coated catheters (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Organisms</th>
<th>Zone of inhibition (mm)</th>
<th>Drug coated dc</th>
<th>Drug carrier coated urinary catheters dcc</th>
<th>Uncoated urinary catheters uc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary catheter</td>
<td>S. epidermidis</td>
<td>27.1±1.5</td>
<td>29.4±1.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>29.4±2.3</td>
<td>33.2±1.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>27.7±0.6</td>
<td>31.1±2.26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>25.0±2.6</td>
<td>26.1±1.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>23.6±0.3</td>
<td>23.4±0.5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
A. paniculata leaves were found to be active against S. aureus, E. faecalis and M. tuberculosis. Zaidan et al. (2005) have reported that the water extracts of A. paniculata possess a potential antibacterial activity towards both gram positive and gram negative bacteria. According to the results of Humnabadkar and Kareppa (2012), the aqueous extracts of A. paniculata showed maximum antibacterial activity against Staphylococcus aureus and Pseudomonas aeruginosa. Hosamani et al. (2011) have reported that the acetone and alcohol extracts of A. paniculata with higher inhibitory activity against Bacillus subtilis and Staphylococcus aureus. One of the major components responsible for the antibacterial activity is Androgrophirole. Androgrophirole showed potential antibacterial activity against most of the tested organisms. Among those, Staphylococcus aureus was found to be most sensitive with a minimal inhibitory concentration value of 100 μg/mL. It was found to be bacteriostatic. Specific inhibition of intracellular DNA biosynthesis was observed in a dose-dependent manner in S. aureus. Androgrophirole mediated inhibition of biofilm formation by S. aureus was also found.

The use of natural products with therapeutic properties is as ancient as human civilisation and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale, 1984). Most of the drugs used today are acquired from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO, 1992), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural (Yue-ZhongShu, 1998). Almost all of the synthetic drugs cause side effects and also most of the microbes developed resistant against the synthetic drugs. To alleviate this problem, antimicrobial compounds from potential plants should be explored. T. chebula is routinely used as traditional medicine by tribals of Tamil Nadu to cure several ailments such as fever, cough, diarrhea, gastroenteritis, skin diseases, candidiasis, urinary tract infection and wound infections (Dash and Bhagwan, 1991). Plant fruits appear to have evolved complex antibiotic compounds to cure various diseases like cancer, cardiovascular, digestive and pathogenic bacteria. Antibacterial activity of T. chebula extracts against several bacterial strains have been reported (Chattopadhyay et al., 2007; Bag et al., 2009). It is effective in inhibiting Helicobacter pylori (Malkzadeh et al, 2001). The extract showed a broad spectrum of antibacterial activity against gram-positive and gram-negative bacteria. This was supported by an earlier study on an alcoholic extract that exhibited greater activity than the aqueous and hexane extracts against bacteria, with no cellular toxicity (Ahmad et al., 1998). The broad spectrum of antibacterial activity was reported for T. chebula (Phadke and Kulkarni, 1989) and T. arjuna (Singh et al., 2008). The ethanol extract at a concentration of 1 mg/disc showed maximum inhibition against S. epidermidis, followed by B. subtilis.Gupta et al. (2002) reported that a T. pallida fruit methanolic extract showed maximum activity against gram-negative bacteria, while that of T. bellerica showed the highest inhibition zones against P. aeruginosa and E. coli (Ghosh et al., 2008). Two possibilities that may account for the higher antibacterial activity of alcoholic extracts are the nature of biological active components (alkaloids, flavonoids, essential oil, tarpemoids, tannins, etc.), which may be enhanced in the presence of ethanol; and the stronger extraction capacity of ethanol that may have yielded a greater number of active constituents responsible for antibacterial activity (Ghosh et al., 2008).

Traditionally, Euphorbia hirta plant extracts are used in sore and wound healing, as ear drop for boils in the ear and treatment of stents could be effective and might be accounted as a promising lead for new antibacterial drug development.
Thin layer chromatography analysis of herbal plants

Three types of phytochemical compounds analyzed in the present research to identify the bioactive compounds from the herbal plants using TLC were presented in Table 2. For phenol, ethyl acetate was used as mobile phase with ferric chloride as spraying agent. Green colour spots were observed. For tannin, methanol was used as mobile phase with ferric chloride as spraying agent. Grey colour spots were observed. For flavonoids, ethyl acetate was used as mobile phase with aluminium chloride as spraying agent. Orange colour spots were observed. The Rf values were evaluated by comparing with the standards. The Rf value for phenol, tannin and flavonoids were calculated as 0.78, 0.83 and 0.86 respectively. Phenol compounds were found to be present in all the three plants (Andrographispaniculata, Terminaliachebula and Euphorbia hirta). Tannins were present in Andrographispaniculata and Euphorbia hirta. Flavonoids were present in Andrographispaniculata and Terminaliachebula.

Table 2: Thin layer chromatography analysis of herbal plants

<table>
<thead>
<tr>
<th>S. No</th>
<th>Phytoconstituents</th>
<th>Mobile phase</th>
<th>Spraying agent</th>
<th>Plant extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Andrographispaniculata</td>
</tr>
<tr>
<td>1.</td>
<td>Phenol</td>
<td>Ethyl acetate</td>
<td>10% Ferric chloride</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Tannin</td>
<td>Methanol</td>
<td>10% Ferric chloride</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Flavonoid</td>
<td>Ethyl acetate</td>
<td>11% Aluminium chloride</td>
<td>+</td>
</tr>
</tbody>
</table>

Andrographispaniculata, has been used for upper GI tract and upper respiratory infections, fever, herpes and other chronic diseases. Various medicinal properties like choleric, antiarthroheal, immunostimulant and anti-inflammatory have been attributed to this plant in the traditional system of Indian medicine (Siripong et al. 1992). The primary medicinal component of A. paniculata is andrographolide, which is a diterpene lactone. Andrographolide has been reported for its anti-cancer (SheejaandKuttan, 2007), anti-HIV (Calabrese et al, 2000) and cardio-protective and hepatoprotective properties among others. Other active components include 14-deoxy-11, 12-dihydroandro-grapholide (andrographolide D), homo-andrographolide, andrographosterin and stigasterol (Siriponget al. 1992). Xu et al. (2006) have investigated the antimicrobial activity using A. paniculata (methanolic and aqueous) extracts and authentic andrographolide against nine human bacterial pathogens. Their results indicated methanolic extracts of A. paniculata to be active against only two of the pathogens. Phytochemical screening of A. paniculata showed the presence of Flavonoids, Alkaloids, Steroids, Saponins, Tannins and Phenolic compounds.

Terminaliachebula has antimicrobial, hepatoprotective, anti-inflammatory, antidiabetic, immunomodulatory, antioxidative and adaptogenic properties (Jayaprakash and Kulkarni., 2018). Terminaliachebula contains different types of phytochemicals such as glycosides, alkaloids, flavonoids, phenolic compounds, saponin, steroids, quinine and tannin (Jayaprakash and Kulkarni., 2018). Terminaliachebula also possess antimicrobial, hepatoprotective, anti-inflammatory, antidiabetic, immunomodulatory, antioxidative and adaptogenic properties. The chemical constituents of the ethanolic and methanolic leaf extracts of the plant were relatively similar in the presence of proteins, fats and oils, gums and mucilages as primary metabolites and alkaloids, flavonoids, terpenoids, tannin, phenol, steroid, glycoside, saponin, coumarin as secondary plant metabolites. E. hirta possess 28 18 15 anti-anaphylactic, antioxidant, anticancer, anti-oxidant, antiplacenta aggregation and anti-inflammatory, aatoxin inhibition, antifertility, antiplasmodial, antiamaemic, larvicidal, and insect repellent activities (Lanhers et al., 1991). The bioactive compound present in E. hirta are flavanoids, triterpenoids, alkanes, and alkaloids (Kumar et al., 2010). Thus in the present study the anti-bacterial activity of the synthesized herbal composite was due to the presence of various bioactive compounds.

Biocompatibility of the coated catheters in L929 fibroblast cell lines

Table 3: Cell viability of the developed herbal composites

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Cell Viability* (%)</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>98 ± 0.00</td>
</tr>
<tr>
<td>40</td>
<td>96 ± 0.00</td>
</tr>
<tr>
<td>60</td>
<td>95 ± 0.01</td>
</tr>
</tbody>
</table>
Table 3 represents the cell viability of the herbal composite. The herbal coated catheters showed significant cell viability percentage. Cell viability for 20µg/ml was found to be 98% and 96% for 40µg/ml. About 95 ± 0.01%, 92 ± 0.02% and 89 ± 0.02% was observed on 60, 80 and 100µg/ml. Thus from the cell viability results, it is confirmed that the developed herbal composite coated catheter is biocompatible.

The inhibition effect of andrographolide on TNFα and the cell cytotoxicity was evaluated by Hus et al., 2006. The inhibition effect of cytotoxicity effect of Andrographolide on TNFα was measured by in vitro L929 cell proliferation/cytotoxicity assay on microtiter plates. The results showed that the inhibition percentage was increased as andrographolide concentration increases, and the IC50 was found to be 60 LM as TNFα antagonist. No cytotoxicity was found at this concentration. Terminalia chebula extracts were found to be effective in decreasing the ammonia accumulation in the media, thereby reducing its toxic effect on cells. DPPH assay further confirmed the free-radical scavenging ability of the extracts which increased with the increase in concentration of each extract. Cell proliferation/apoptosis, cytoskeletal structure, and ECM production were further evaluated by live-dead assay and phalloidin/cytokeratin staining, respectively. The cytoskeletal structure and ECM secretion of the cells treated with extracts showed higher cellular activity in comparison to control. Inspite, these extracts of T. chebula on both types of skin cells and optimized concentration in which it could be used as a bioactive component for wound healing applications by increasing cell proliferation and decreasing free-radical production without affecting the normal cellular matrix (Dolly et al., 2014). Cytotoxicity of the Euphorbia hirta plant extract was investigated by Abbas et al., 2015. L929, the results indicated that extract did not show any significant toxicity up to 200µg/ml for 48 h. Several studies were conducted on cytotoxicity assay and the plant extract showed no cytotoxicity on L929 (Mouse fibroblast) cell lines (Ali, 2017; Luisa et al., 2014; Basaket al., 2016). In accordance with the siliar works our synthesised herbal composite also showed significant cell viability values. Thus, our developed herbal composite due to its biocompatible nature can be used in catheter coating and in treatment of UTI.

Conclusion

Thus the three herbs were selected and herbal composite was prepared. Synergistic activity of the herbal composite and biocompatibility of the herbal composite coated catheters were evaluated. The herbal composite coated catheters showed strong inhibitory zones. The herbal composite-carrier coated catheters was found to be higher than the herbal composite coated catheters. Uncoated catheters showed no zone of inhibition. Phenol compounds were found to be present in all the three plants (Andrographispaniculata, Terminalia chebula and Euphorbia hirta) tannins were present in Andrographispaniculata and Euphorbia hirta. Flavanoids were present in Andrographispaniculata and Terminalia chebula. The herbal coated catheters showed significant cell viability percentage. From the cell viability results, it is confirmed that the developed herbal composite coated catheter is biocompatible and the developed herbal composite can be used as an alternatives for commercial antibiotics.

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