Research Article

Novel Thiolated *Ceasalpinia Pulcherrima*: Synthesis, Characterization and Evaluation as Mucoadhesive *In situ* Gelling Agent

Mumbai Education Trust’s Institute of Pharmacy, Savitribai Phule Pune University, Bhujbal Knowledge City, Adgaon, Nashik 422003, Maharashtra, India
*Corresponding author: nilimathombre@gmail.com

Revised: 5 June 2018; Accepted: 22 June 2018

Abstract

**Purpose:** The objective of present study was to enhance bioadhesive potential of *Ceasalpinia pulcherrima* (CP) by thiolation. **Method:** Thiolation of CP was achieved with esterification with thioglycolic acid. **Result:** Thiolated *Ceasalpinia pulcherrima* (TCP) was characterized by nuclear magnetic radiation, differential scanning calorimetry, and X-ray crystallography analysis. TCP was determined to possess 4.803mmol of thiol groups/g of polymer by Ellman’s method. Comparative evaluation of mucoadhesive property of metoclopramide containing *in situ* gel system of CP and TCP using sheep nasal mucosa revealed higher *ex vivo* bioadhesion time of TCP as compared to CP. Improved mucoadhesive property of TCP over the CP can be attributed to the formation of disulfide bond between mucus and TCP. *Ex vivo* permeation study conducted using sheep nasal showed improved drug permeation in formulation based on TCP. **Conclusion:** Thiolation of CP improves its bioadhesion and drug permeation without affecting the resultant gel property.

**Keywords:** *Ceasalpinia pulcherrima*, Thiolation, Mucoadhesion, *in situ* gel

Introduction

In the 1980s and 1990s the concept of mucoadhesion has received a significant degree of attention. The use of mucoadhesive delivery systems should thereby reduce the frequency of dosing, leading to an improved patient-compliance .(1,2) In addition, based on the intensified contact to the mucosa, various mucoadhesive polymers such as poly (acrylic acid) derivatives and chitosan are able to increase the epithelial permeability for many drugs.(3) The natural polymer is used to improve there mucoadhesion by thiolation method. Attachment of thiol group on galactomannans is by synthesis of polymer–cysteine conjugates. This modification method gives higher adhesive property than unmodified polymer. A modified property of natural polymer gives various effects on mucoadhesion.(5) Natural polymers have been used as a release modifying agent, for the development of mucoadhesive drug delivery systems.(3,4) Further advantages of thiolated polymers are their enzyme inhibitory and permeation enhancing effects, especially in combination with glutathione.(6) The polymers which are having multiple reactive functional groups to conjugate with thiol bearing ligands, biocompatible, non antigenic will be of
advantage in preparing thiomeric polymers. Seed galactomannans are polysaccharides widely distributed in nature. They have attracted considerable academic attention as well as industrial interest due to their property of forming viscous solutions in water.

*Caesalpinia pulcherrima* (CP) belongs to Leguminosae (family: Fabaceae and subfamily: Caesalpinioideae). Various pharmacological activities of *Caesalpinia pulcherrima* L. have been reported such as analgesic and anti-inflammatory, antiulcer, antimicrobial activity, antibacterial and antifungal activity, antitumor, cytotoxic activity astringent, abortifacient, emmenagogue, selective activity against DNA Repair-Deficient Yeast Mutants. (7)

Galactomannans (GM) are polysaccharides with (1→4)-β-D-mannopyranosyl residues decorated with (1→6) linked α-D-galactopyranosyl residues. GM are water soluble hydrocolloids, high molar mass, water-soluble, non-ionic polysaccharides forming highly viscous, stable aqueous solutions. GM are widely useful in the industry mainly as thickening, stabilizing agents in a range of applications in the development of edible films or coatings for food applications, gelling agents, excellent thickeners and stabilizers of emulsions, and the nontoxic nature allows their use in the textile, pharmaceutical, biomedical, cosmetics, food industries, mass-efficient aqueous thickeners, co-gellants, and nutritional supplements. Galactomannans has an advantage to form very viscous solutions at relatively low concentrations that are only slightly affected by pH, ionic strength and heat processing. These seeds of CP have a large number of commercial and industrial applications. (7)

The present study was designed with the objective to improve mucoadhesive properties of CP by synthesizing thiolated *Caesalpinia pulcherrima* (TCP). The numbers of thiol groups/g of TCP was characterized by nuclear magnetic resonance (NMR), differential scanning colorimetry (DSC), X-ray diffraction analysis (XRD).

The aim of this work was to study the possible application of TCP for mucoadhesive applications by developing an *in situ* gel system employing metoclopramide hydrochloride as a model drug. Mucoadhesive properties of CP and TCP were comparatively evaluated using *ex vivo* bioadhesion studies by employing modified CP in freshly excised sheep nasal mucosa. Further, *in situ* gelling was evaluated for gelation study, gel strength, and viscosity. For the safety profile of polymer, the mucosal toxicity studies were carried out. Formulation was also subjected for *in vitro* and *in vivo* biodegradation studies.

**Methods**
**Material**
Pods of CP were collected from selected shrubs, growing in Nashik, Maharashtra, India. Metoclopramide hydrochloride was obtained as a gift sample from IPCA laboratories (India Rep Office, Andheri, India). Thioglycolic acid and l-Cysteine (LobaChemie, Mumbai, India), Ellman's reagent (5,5-dithiobis (2-nitrobenzoic acid) (DTNB) were procured from (Research-Lab fine chem industries, Mumbai, India). All other chemicals used were of analytical grades.
Synthesis of TCP
TCP was synthesized by esterification of CP with TGA (80%) in the presence of hydrochloric acid. (2) The reaction was carried out with 2 moles of thioglycolic acid for every 1 mole of hydroxyl group in CP. CP (1 g) was dissolved in sufficient amount of hot water and was added with 0.223 mL of 80% thioglycolic acid and 1 mL of 7 N HCl. These were allowed to react for 150 min at 80°C. The reaction mixture was poured into 500 mL of methanol. Precipitates of TCP so obtained were washed twice with methanol and dried at room temperature. (3-4, 8)

Characterization of TCP
Determination of thiol content
The degree of thiol group substitution was determined by quantifying the amount of thiol group on TCP and CP by Ellman’s method. (1) An accurately weighed 50 mg of TCP or CP was dissolved in 25 mL of distilled water. An aliquot of 2.5 mL of the polymer solution diluted with 2.5 mL of 0.5 M phosphate buffer (pH 8.0) was allowed to react with 5 mL of Ellman’s reagent (DTNB, 0.03%, w/v in 0.5 M phosphate buffer pH 8.0) for 2 h at room temperature, followed by the measurement of absorbance of the reaction mixture at 412nm. The numbers of thiol groups in the polymer were calculated using the standard curve obtained by reacting CP solution containing varying amount of L-Cysteine with Ellman’s reagent.

FT-IR spectroscopy
CP and TCP were subjected to FT-IR spectroscopy in a FTIR spectrophotometer (Jasco V 630) as KBr pellets in a range 4000-400cm⁻¹.

Nuclear magnetic resonance (NMR)
Nuclear magnetic resonance of the CP and TCP was carried out by using a NMR spectrometer (Bruker Avance III, 400MHz). CP and TCP in powdered form were scanned from 1 to 10ppm range under the following measurement conditions: Magnet 9.4 T superconducting Magnet; Probe-BBO 400 MHz, with Z-gradient, 2H lock; for observation of nuclei like 1H, 13C, 31P, 15N, etc. with 1H decoupling. Any of these nuclei can be fully automatically selected and optimally tuned and matched.

Scanning electron microscopy (SEM)
The surface morphology of polymer was examined by scanning electron microscopy. A small amount of powder was spread on an aluminum stub, which was placed after gold sputtering in a SEM chamber (JSM6390®, USA). Photographs were taken at acceleration voltage of 20kV electron beam.

Zeta potential measurement
CP and TCP were separately dispersed in distilled water to get stock solution of 1% (w/w). This dispersion was filled in zeta cell and zeta potential was determined using Zeta Sizer (Nano ZS90, Malvern Instruments, UK) with the help of software.

Differential scanning calorimetry (DSC)
DSC thermograms of CP and TCP were recorded using a differential scanning calorimeter (DSC, METTLER, TOLEDO, Switzerland). About 5 mg of sample was crimped in a standard aluminum pan and heated in a temperature range of 40–350°C at a heating rate of 10°C per min in nitrogen atmosphere.

X-ray diffraction (XRD) analysis
The X-ray diffractometry was carried out to investigate crystalline nature of the CP and
TCP. Study was carried out using an X-ray diffractometer (Bruker AXS D8 Advance). CP and TCP in powder form were scanned from 3° to 80° diffraction angle (2) range under the following measurement conditions: source, nickel filtered CuK radiation; voltage 40kV; current 35mA; step time 31.2 s; temperature range−170°C to+450°C.

**In vitro degradation**

The degradation performance of TCP was studied in various simulated fluids like simulated body fluid (SBF), simulated lungs fluid (SLF) and simulated nasal fluids. Films of TCP (0.005–1mm thickness) obtained by casting method were placed in 10mL each of simulated fluid which content in small vials. Then the vials were incubated in a shaking incubator at100rpm at 37°C for 1h. The films were withdrawn at intervals of 10, 20 and 30min, washed with distilled water, dried and subjected to degradation characteristic such as swelling degree and weight loss.(9)

**Swelling degree**

The swelling degree (SD) was characterized at 37°C. The experiments were carried out by measuring the weight gain as a function of immersion time in 20mL solution. The swelling degree was calculated according to equation given below.

\[
SD = \frac{W_t - W_o}{W_o} \times 100 \quad \text{-------- (1)}
\]

where, \(W_t\) is weight and after degrading for a predetermined time; \(W_o\) is original weight of the sample.

**Weight loss (%)**

The weight loss was calculated by comparing the dry weight (\(W_d\)) of the remaining sample after degradation for a predetermined time with the original dry weight (\(W_o\)) of the sample as in the equation. At pre-determined intervals of 0, 5, 10, 15, 20, 25, 30, 35, 40 and 60min, samples were taken out, purged with distilled water and subsequently dried until absolute desiccation, then weighed.

\[
Weight\ Loss\ (\%) = \frac{W_o - W_d}{W_o} \times 100 \quad \text{-------- (2)}
\]

where, \(W_o\) is original dry weight of the sample; \(W_d\) is dry weight of the remaining sample after degradation for a predetermined time.

**In vivo biodegradation**

The animal experiment was carried out in compliance with the protocol of institutional animal ethical committee (Reg.No. 1344/ac/10/2016-2017/CPCSEA under CPCSEA, India) for the study of in vivo biodegradation of polymer. Three male mice (Average weight of 34.28-34.35gm) were selected. Anesthesia was induced by intraperitoneal injection of pentobarbitone sodium (45 mg/kg body weight) (10). An incision (1.5cm) was inflicted laterally about the mid portion of the back. Subcutaneous pockets were formed around each incision, free film was inserted, and the wounds were closed by intermittent nylon sutures, 0.5cm apart for 3 individual male mice. Films were explanted at 10, 20, and 30 min for analysis.(6)

**Preparation of in situ gel system**

Drug loaded in situ gelling system was prepared by slowly adding a weighed amount (2.5% w/w) of each of the polymer CP and TCP into cold water separately. The mixture was slowly homogenized by using a magnetic stirrer (Remi Instruments, India). About 2.0 g of metoclopramide
hydrochloride was dissolved in resulting solution. Appropriate quantity of benzalkonium chloride was added as a preservative. The pH of formulation was adjusted between 4.5 and 5.5. The formulations were filled in glass vials (capacity10-mL), capped with rubber closures and sealed with aluminum caps. Formulations were stored at 4–8°C until used. (11-12)

**Evaluation of prepared in situ gels**

**Gelation studies**

Gelation is the process, by which the liquid phase makes a transition to gel. In brief, a 10 mL transparent vial containing a magnetic bar and each formulation were placed in a temperature water bath. The gelation point was determined when the magnetic bar stopped moving due to gelation. The consistency of formed gel was checked by visual inspection and graded as indicated in Table 1. Each preparation was tested thrice to control the repeatability of the measurement. (14)

**Table 1: Evaluation parameters of in situ gel**

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Formulation</th>
<th>Degree of gelation</th>
<th>Viscosity study (cp)</th>
<th>Gel strength (s)</th>
<th>Mucoadhesive force (dyne/cm²)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CP</td>
<td>++</td>
<td>Solution 52±0.57</td>
<td>16 ±0.96</td>
<td>4666.66</td>
<td>-15.9</td>
</tr>
<tr>
<td>2</td>
<td>TCP</td>
<td>++</td>
<td>Gel 546±0.54</td>
<td>1362±1.25</td>
<td>6533.33</td>
<td>-17.9</td>
</tr>
</tbody>
</table>

**Viscosity measurements**

Viscosities of formulations before and after gelation were measured by using Brookfield DVE viscometer using spindle number-3 at 100 rpm shear rate. The viscosity was recorded with increasing temperature in the range of 20–30°C.(13)

**Gel strength determination**

It is expressed in terms of time (in seconds) required by a 35g piston for penetration of 5 cm distance, through the 50g gel formulation. Test was performed using gel strength apparatus modified at laboratory. CP and TCP solutions (50g) were placed in 100 mL measuring cylinders and gelation was induced by means of temperature. The piston (weight: 35g) was then placed on to the gel. The gel strength was measured as the time (seconds) required moving the piston 5 cm down through the gel.(13)

**Ex vivo bioadhesive strength**

The mucoadhesive potential of each formulation was determined by measuring the force required to detach the formulation from nasal mucosal tissue using a modified method as published in the literature. (15) In brief, nasal tissues were carefully removed from the nasal cavity of sheep obtained from the local slaughter house. Tissues were immediately used after separation. At the time of testing, a section of nasal tissue was secured (keeping the mucosal side out) to the upper probe using a cyanoacrylate adhesive. The surface area of each exposed mucosal membrane was 4.2 cm.(1) At room temperature, fixed amount of samples of each formulation were placed on the lower probe. Probe with mucosal tissue was lowered until the tissue contacted the surface of the sample. Immediately, a slight
force was applied for 2 min to ensure intimate contact between the tissue and the sample. The mucoadhesive force, expressed as the detachment stress in dyne/cm², was determined from the minimal weights that detached the tissues from the surface of each formulation using the following equation.

\[
\text{Detachment stress} = \frac{m \times g}{A} \quad \text{(3)}
\]

Where, \( m \) is the weight added to the balance in grams; \( g \) is the acceleration due to gravity taken as 980 cm/s² and \( A \) is the surface area of sheep nasal mucosa.\(^{16}\)

**Ex vivo permeation studies**

Fresh nasal tissues were carefully removed from the nasal cavity of sheep obtained from the local slaughter house. Tissue sample was sandwiched in Franz diffusion cells (capacity 16 mL) displaying a permeation area of 3.14 cm². Phosphate buffer (pH 6.6) was added to the acceptor chamber. The temperature was maintained at 37°C. After a pre-incubation time of 20 min, formulation was placed in the donor chamber. At predetermined time points, 1-mL samples was withdrawn from the acceptor compartment, replacing the sampled volume with phosphate buffer after each sampling, for a period of 5 h. The samples withdrawn were filtered and used for analysis. Blank samples (without drug) were run simultaneously throughout the experiment to check for any interference. The amount of permeated drug was determined using a UV–visible spectrophotometer at 310 nm. Permeability coefficient (\( P \)) was calculated by the following equation:

\[
P = \frac{dQ/dT}{C_0 \times A} \quad \text{(4)}
\]

Where, \( dQ/dT \) is the flux or permeability rate (mg/h); \( C_0 \) is the initial concentration in donor compartment; and \( A \) is the effective surface area of nasal mucosa.\(^{(9,16)}\)

**Mucosal toxicity studies**

Being sensitive than other mucosa, nasal mucosa was used to study mucosal toxicity. Mucosa incubated in phosphate buffer solution (pH 6.6) after collection it was compared with tissue incubated in the diffusion chamber with gel formulation (TCP). Tissue was fixed in 10% buffered formalin (pH 7.2), routinely processed and embedded in paraffin. Paraffin sections (7 μm) were cut on glass slides and stained with hematoxylin and eosin. Sections were examined under a light microscope, to detect any damage to the tissue during *ex vivo* permeation study.\(^{(14)}\)

**Result and discussion**

**Synthesis of TCP**

The covalent attachment of CP to thioglycolic acid was achieved by ester bonds formation between hydroxyl group of beta-galactane moieties of CP and carboxyl group of thioglycolic acid (Figure 1).

After complete drying, product appeared as off-white odourless powder and was found to be soluble in water.

The average yield of this synthesis amounted to 48% of the utilized amount of CP. Thioglycolic acid is soluble in water and methanol. Precipitation with methanol from an aqueous solution and subsequent washing by keeping the precipitate overnight was found to be sufficient purification method for TCP. TCP was found to contain 4.803 mmol of thiol
groups/gas determined by quantifying the amount of thiol group by Ellman’s method.

**FT-IR**

FT-IR spectra of CP and TCP showed (Figure 2) alcohol (O-H), phenols, H-bonded 3332.99 cm\(^{-1}\), alkanes (stretch) (C-H) at 2916.37 cm\(^{-1}\), alkene (C=C) 1635.64 cm\(^{-1}\), alkanes (C-H)(bend) 1381.03 cm\(^{-1}\), alcohols, ethers, esters, carboxylic acids, anhydrides 1033.85 cm\(^{-1}\), aromatic (C-H) 802.39 cm\(^{-1}\), and alkenes(C-H) 671.23 cm\(^{-1}\). FT-IR spectrum of TCP showed all the peaks found in CP but additional peaks at 2330.01 cm\(^{-1}\) due to –SH stretch.

**Nuclear magnetic resonance (NMR)**

In order to explore possible modification of CP, we compared the \(^1\)H NMR spectra of CP and TCP (Figure 3). As illustrated in Figure 3, the majority of CP and TCP chemical shifts were between 1 and 8 ppm. NMR spectra of CP (Figure 3) showed and multiplets at 3.90 (O-CH\(_2\)), 4.71 (-O-CH\(_2\)-OH), (O-CH), 4.9 (-O-), multiplets at 3.47, 3.73 due to CH (OH). NMR spectra of TCP (Figure 3) showed singlets similar to CP but additional singlets at 3.25 due to (SH). This result confirmed thiolation of CP developed as TCP.(15)

---

**Figure 1: Synthesis of TCP from CP**
Scanning electron microscopy (SEM)
Scanning electron microscopic images showed surface morphology of CP and TCP. The shape of CP and TCP was found to be round (Figure 4). Both CP and TCP had smooth surfaces without crack, hole and fracture.

Zeta potential measurements
CP and TCP (Figure 5) had zeta potential values of -15.9mV and -17.9mV respectively indicating anionic nature in distilled water (Table 1). The result showed that zeta potential was negative and it was shifted to higher negative value after thiolation. This might be due to the presence of large number of -OH groups as anionic structure.

Differential scanning calorimetry (DSC)
Figure 6 showed the DSC thermograms of CP and TCP. The DSC curve of CP showed one sharp endotherm at 250.88°C with heat of fusion of -97.61J/g and one sharp exothermat143.58°C with heat of fusion 81.23J/g. DSC thermogram of TCP showed an endotherm at119.75°C with heat of fusion of–160.45 J/g. Thus, a decrease in the endothermic transition temperature and heat of fusion of CP was observed on thiolation of CP.
**X-ray diffractometry (XRD)**

X-ray diffraction spectra of CP and TCP are shown in (Figure 7). X-ray diffractogram of CP is typical of amorphous material with characteristic peaks appearing at 19.00°(2θ) while the diffractogram of TCP showed characteristic peak at 20.01°(2θ). The peak intensity of TCP is slightly greater than the CP. No marked changes are observed on thiolation.

![Figure 5: Zeta potential of CP and TCP](image)

**In vitro degradation**

Figure 8 showed the swelling ability of films in different simulated fluids within 35min. The swelling degree was found to be larger in SBF than that in SNF/SLF. The swelling behavior of films was similar in SNF and SLF. The weight losses of films in SBF, SLF and SNF at 10, 20, 30 and 35min are shown in Figure 8 for samples with same polymer content, the weight loss speed in SBF was slower than SLF and SNF. The rapid weight loss was mainly caused by the breaking down of CP molecules.

![Figure 6: DSC thermogram of CP and TCP](image)

![Figure 7: XRD spectra of CP and TCP](image)
In vivo degradation
In the in vivo study, the rate of degradation was found to be rapid maintaining 30% weight at the end of 20 min. The films showed complete degradation by the end of 30 min. The complete films were not recovered at the end of 40 min, due to foreign body response. As a result of the in vivo implantation the typical response results in the accumulation of cells such as macrophages around the foreign body. Free radicals, acidic products or enzymes produced by these cells during the foreign body response may accelerate degradation.

Characterization of in situ gel system
Gelation studies
The thermal gelation characteristic of CP solution was studied by measuring gel formation temperature (GFT), which was in the range of 25–30°C. Gelation characteristics was assessed on a ordinal scale ranging between − (no gelation), ++ (immediate gelation remains for few hours), +++ (immediate gelation remains for longer duration). Both CP and TCP showed that immediate gelation remains for few hours (Table 1). Gelation characteristic of CP did not affect on thiolation. Rapid gelation favours the inter diffusion process between the polymer and mucus layer providing stronger adhesion.

Viscosity measurement
Apparent viscosity values were measured for liquid formulation and gel using Brookfield viscometer DVE with spindle no.3 at 100 rpm. The results showed (Table 1) marked increase in viscosity for both polymers after sol to gel transition. TCP based gel is more viscous than CP based gel.

Gel strength measurement
In the development of an in situ gelling system, the gel strength is important in finding the condition, which allows easy administration as liquid and enhance
residence time at administration site. Optimal *in situ* gel must have suitable gel strength so as to be administered easily and can be retained at mucosal surface for longer time after administration. In TCP gels marked increase in gel strength was observed. The gel strength values between 25 and 50s were considered sufficient. The gel strength less than 35s may not retain its integrity and may erode rapidly while gels having strength greater than 64s are too stiff and may cause discomfort to the mucosal surfaces. The TCP showed the gel strength values in the range 36–46s (Table 1) which are acceptable (17).

**Ex vivo** bioadhesion studies

*Ex vivo* bioadhesion was studied by the method reported by using drug loaded *in situ* gelling system based on CP and TCP. The mucoadhesion force is an important parameter in studying bioadhesion. The mucoadhesion force for CP and TCP was found as 4666.66 dynes/cm² and 6533.33 dynes/cm² respectively (Table 1). TCP showed almost 1.5-folds improved mucoadhesive property as compared to CP. The mucoadhesive property of CP can be explained by the fact that secondary hydroxyl (OH) groups are principle source of mucoadhesion. The improved mucoadhesive property of TCP can be attributed to the formation of disulfide bond between the SH groups of thiolated polymers and mucus via thiol /disulfide exchange reactions. More over polymers with charge density can serve as good mucoadhesive agents. It has also been reported that anionic polymers are more effective bioadhesive than cationic or non ionic polymers (A zeta potential study revealed anionic nature of CP and TCP).

**Ex- vivo permeation study**

Formulation of TCP was further subjected to *ex vivo* permeation studies on the basis of their physical parameters and drug release study using the sheep nasal mucosa. The permeation profile of TCP formulation was shown in Figure 9. The percent drug permeated after 270 min was found to be 89.45% from CP formulation and 98.87% from TCP formulation.

![Figure 10: Histopathological evaluation of sections of sheep nasal mucosa used in mucosal toxicity (A) Formulation treated mucosa; (B) Untreated mucosa](image)

**Mucosal toxicity study**

Photomicrographs of the nasal mucosal tissue after the permeation studies were observed for histopathological changes in comparison with the phosphate buffer treated mucosa (Figure 10). The section of mucosa treated with formulation showed very slight degeneration of nasal epithelium along with slight erosion. This might be result of mucoadhesive and permeability enhancing property of TCP in the formulation. There was no sign of remarkable destructive effect of formulation on the treated nasal mucosa.

**Conclusion**

CP has been proved in many articles as a promising *in situ* gelling for the mucosal route of administration. In this study, thiolation of CP was carried out to enhance bioadhesive property of CP. Thiolation of
CP did not alter its gelling ability. Moreover TCP has permeation enhancing effect without causing mucosal toxicity. The in situ gel system with TCP exhibits better texture and ideal gelling properties along with good in-vitro degradation. The present study gives an extensive characterization of a TCP and should therefore facilitate the development of new drug delivery systems providing a greatly prolonged residence time on various mucosal tissues like nasal, rectal, ocular, vaginal etc.

Acknowledgments
Authors are thankful to M.E.T.’s Bhujbal Knowledge City, Institute of Pharmacy, Adgaon, Nashik, Maharashtra, India for providing necessary facilities and funds to carry out this work.

References


