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Pectin - Ethanolamine Graft Copolymer Hydrogel for Ointment and Transdermal Patches Consist of Tridax Procumbens L. Ethanol-Water Extract as Antiseptic and Antifungal Agent

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ABSTRACT

Currently research has been carried out for the development of transdermal drug delivery systems. Specific transdermal drug delivery system is chemically modified pectin based hydrogels. This modified gel is used to carry ethanol-water extract of Tridax procumbens L. as antiseptic, antifungal. Pectin forms gel with crosslinking agents like divalent or multivalent cations, glutaraldehyde etc. Transdermal patches and ointment with petroleum wax made from the cross-liked modified pectin gel and the leaf extract of plant in ethanol-water were loaded through diffusion method. In the current study an attempt has been made to improve the properties of the pectin gels by graft copolymerization with ethanolamine and the swelling properties were modified by varying concentration of crosslinking agent, glutaraldehyde and used for various biomedical applications,. Antiseptic and antifungal activity of loaded hydrogel were studied.

Keyword: Hydrogel, pectin modification, Tridax procumbens L. Transdermal patches, ointment.

I. INTRODUCTION

Hydrogels are three-dimensional hydrophilic polymer networks capable of imbibing large amounts of water, which have been widely used in the field of biomedicine and pharmacy [1-3], such as wound dressing, super-absorbents, drug delivery systems, etc Pectin is a complex heterogeneous polysaccharide found in primary cell wall of most plants, citrus and apple where it gives mechanical strength and flexibility due to it's interaction with other cell wall components.. The dominant structural feature of pectin is a linear chain of poly α 1-4 – galacturonic acids, with varying degree of methylation of carboxylic acid residues[4].

Pectins with low degree of methylation forms gel in presence of calcium ions whereas pectin with higher degree of methylation forms gel in acidic media with the addition of different sugars e.g., sucrose or glucose [5]. The carboxyl group of pectin is often esterified with methanol. Pectin can be classified in to high-methoxyl (>50% esterified) and low- methoxyl (<50%) pectins.The two classes have profoundly different gel forming properties [6].Typically, large concentration of sucrose (>55 wt %) are added to high-methoxyl pectins under acidified conditions (pH ~3.5) to induce gelation. Under these conditions, network formation occurs via hydrogen bonding between carboxyl or hydroxyl groups on adjacent chains and hydrophobic interactions between methyl ester groups [7-9]. It was reported that alkyl esters of pectin and pectic acid absorb bile acids, fat and cholesterol. The chemical modification of pectin (amidation, trans-eterification) is relatively easy and it modifies in a significant way physiochemical and biological properties of pectin.[3]. [10-12]. N- Alkyl pectinamides have some advantages in comparison with other alkylated derivatives of pectin. The amide bond is sufficiently resistant to hydrolysis by acids or alkali. The reaction yield of N- alkylamidees prepared by the reaction of pectin with aliphatic non branched amines are relatively high.

In this present study attempts have been made to chemically modify pectin with various concentrations of ethanolamine(1:1,1:2,1:3,1:4),this is further used for the preparation of hydrogels by crosslinking with Glutaraldehyde (GA) in acidic medium. The prepared hydrogels were characterized by FTIR, organic elemental analysis and x ray diffraction studies. Drug release studies, swelling behavior of the hydrogels have been also been done. Cytocompatibility studies of the

hydrogels have also carried out. Recommended treatments include the administration of ethanolic extract of *Tridax procumbens L*. which must be released at the infected sites, it is also useful to have dosage forms that are able to specifically release drugs, such as peptides, proteins, vermifunges and diagnostic agents .

II. MATERIALS AND METHODS

Pectin, methanol, salicylic acid and glutaraldehyde **(GA)** were obtained from Loba-Chemie Indoaustranal Co., Mumbai, India. Ethanolamine is obtained from SRL research laboratories, Mumbai, India. Hydrochloric acid 35% pure was obtained from Merck Limited, Mumbai, India. Double distilled water was used throughout the study. Commercial pectin was purified and converted in to H- form by washing with 0.1mol/1 HCl dissolved in the ethanol water mixture (1:1 v/v). Pectin subsequently was washed several times with ethanol water mixture fallowed by 96% ethanol until the chloride reaction was negative, and finally dried at 60^0C.

A. Synthesis of modified Pectin

The reaction has been done according to method of (Sinitsya et. al. 2000). The reaction was carried out in heterogeneous medium with methanol as a solvent. . Pectin powder (5 gram) was weighed in a well equipped 250 ml three necked flask and it was suspended in 50 ml methanol. An amount of 10 ml ethanolamine was get dissolved in a 50 ml methanol and the solution was added gradually in the flask under stirring. The reaction has been carried out at 25^o C temperature until 96 hours of continuous stirring. After completion of the reaction the product was obtained in the powder form by simple filtration method. The product obtained is converted in to acid forms by washing with 0.1M HCl in an ethanol-water mixture (1:1,v/v) to convert free carboxylic group in to protonated form. Finally the sample were washed several times with 40% (v/v) ethanol until it shows the negative reaction to chloride, then treated with 80% (v/v) ethanol, filtered and dried at 60^0C. This EAMP is used for the preparation of hydrogel membranes using glutaraldehyde(GA) as crosslinking agent.

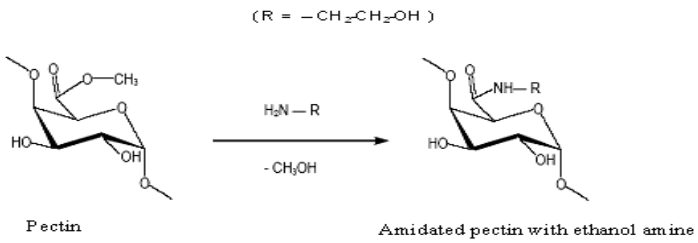


Figure 1. Amidation Reaction of Pectin with Ethanolamine (Sinitsya et. al)

B. Analysis of EAMP (Ethanolamine-Pectin copolymer) Samples

The degree of amidation (DA) the mass and molar yield of the reaction is calculated on the basis of organic elemental analysis results, according to the fallowing equations (Sinitsya et. al. 2000).

$$\frac{M_N}{M_C}\left[6+\frac{73}{100}+(K-1)\frac{M_N}{14}\right]\times 100$$

$$Y_m=\frac{M_N M_A}{14}$$

$$Y_n=\frac{DA}{73}\times 100$$

Where DA is the degree of amidation, Ym the mass yield of the reaction,i.e the relative mass of bonded amine (%) in reaction product, Yn the molar yield of the reaction i.e. the relative content of ester groups substituted by amine (%), M_N the nitrogen content (%) and M_C the carbon content (%), M_A the molar mass of amine(g mol^{-1}), 12 the carbon atomic mass (g mol^{-1}), 14 the nitrogen atomic mass (g mol^{-1}), 6 the sum of carbon in galacturonic unit, K the some carbons in amine molecule and 73 is the methylation degree(DM) of original pectin (%).

Sample	M_N (%)	Mc (%)	D_A (%)	D_M (%)	Y_M (%)	Y_N (%)
1	2.17	38.31	33	31	8.8	46

Table 1. Characterization of samples based on the results of Organic Elemental Analysis

C. Preparation of EAMP (Ethanolamine-Pectin copolymer) hydrogels crosslinked with GA (Glutaraldehyde)

The EAMP/GA hydrogel crosslinked with GA used in the study were fabricated using a casting/solvent evaporation technique. A stock viscous solution of EAMP in water (10% w/v) was prepared by dissolving 2g of EAMP in 20ml of distilled water and stirring for 2hrs at room temperature. To the dissolved EAMP solution 1ml of glutaraldehyde was added and each solution was acidified with 35% HCL solution. That solution was stirred for 30 min at room temperature to complete the crosslinking reaction. The thick crosslinked gel was finally obtained further sonicated to remove the trapped air bubbles and used for further study.

D. Swelling Study

The film was made from prepared gel by pouring it into shallow dishes (with diameter 8.5 cm) and dried in laminar flow air chamber at room temperature for 3 days. Finally, the crosslinked EAMP films were thoroughly rinsed with distilled water to remove residual GA. After drying in air, the crosslinked EAMP films (0.15 mm thickness) were cut into small disks (with diameter of ~9 mm) and used for swelling study. The swelling characteristics of test hydrogels were determined by immersing dried test samples to swell in 5 ml of a phosphate buffer solutions at pH 1.4, 5.4, 7.4, simulating gastrointestinal tract conditions [18,19] and 9.4 solutions for 24 hours. At specific time intervals, the samples were removed from the swelling medium and were carefully blotted with a piece of paper towel to absorb excess water on the surfaces. The % swelling (% Sw) of test samples were calculated from the following expression

$$\% Sw = (Ws - W_D) / W_D \times 100$$

Where `Ws' is the weight of the swollen test sample and `Wd' is the weight of the dried test sample. The sample, which had the best swelling characteristics, was subsequently selected for the salicylic acid release profile study.

E. Drug release profile of ETE (ethanolic extract of *Tridax procumbens L.*) from EAMP hydrogel

The drug release study was carried out by using prepared cellulose membrane. The crosslinked EAMP with 1ml of GA gel was used for the drug release study. In the preparation of the drug loaded [8,9] crosslinked hydrogel, EAMP first cross-linked with 1ml concentration of glutaraldehyde with continuous stirring for 30 min at room temperature. ethanolic extract of *Tridax procumbens L.* (ETE) as model drug incorporated in the cross linked hydrogel by diffusion method. The drug ETE dissolved in ethanol and the crosslinked EAMP hydrogel kept in the ethanol solution of drug for 5hrs then finally drug loaded hydrogel washed with distilled water to remove the drug adhered to the surface of hydrogel. The release study was carried out at 37^0C.

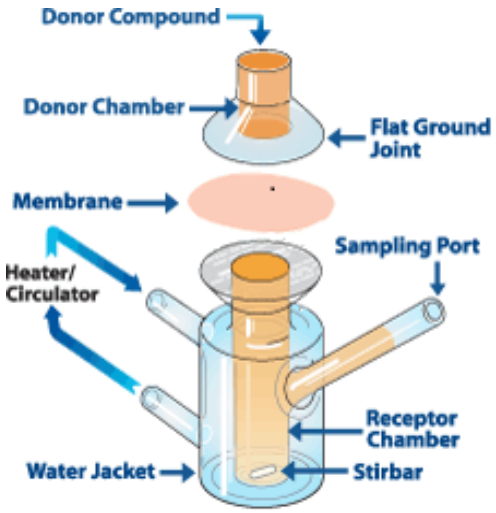


Figure 2. Frantz Diffusion Cell

F. MTT Assay

Mice were sacrificed and their spleens were removed aseptically. The cell suspension was prepared by loose potter and flushing. After centrifugation at 1000 rpm for 10 min at 250 c, erythrocytes were lysed by hypotonic solution and the cell pellets were washed twice with Dubelco minimum essential medium (DMEM). The cells were resuspended in DMEM medium and cell number were adjusted to 106 cell/ ml.

The viability of splenocytes was determined by the MTT dye technique. Hydrogel samples were cut in to 5 mm x 5mm dimensions and transferred in polystyrene petriplates. Samples were sterilized by pouring 70 % ethanol in petriplates and keeping the same under U V light in a laminar hood until the a alcohol evaporated. To the samples 20µl of of cell suspension was seeded and kept in the incubator (37o c for 1 hrs to allow the cells to adhere to the sample matrix. After cell adherence, 2mm of the DMEM medium was added to the each of the

precipitates and again incubated for 48 hrs for allowing cell proliferation. After 48 hrs of incubation, 200µl of MTT dye (4mg/ml) was added and the system as again incubated for 3 hours. After incubation the media from petriplates were discarded and 400µl of DMSO was added for the colour development. For control the cells were seeded in the petriplates. The colour was developed spectrophtometrically at 570 mm.The relative cell proliferation was measured by the fallowing formula;

$$Rp = A_{test} / A_{contrrol}$$

Where; Rp = relative cell proliferation ,
A_{test} = absorbance of the samples and
$A_{contrrol}$ = absorbance of the control.

In a similar manner, L929 cell suspension (106 cell/ml) was used to carry out the MTT assay. For blank, cells were directly seeded to the polystyrene petriplates (taken as controls) since polystyrene is a known biocompatible material.

G. Hemolysis Test

In the present work the hemolysis tests were carried out broadly on the basis of ASTM standard [19]. The test is mainly aimed at finding the extent of hemolysis caused in the presence of the sample prepared. The hemolysis percentage is defined as

$$\textbf{\% Hemolysis} = \{[OD_{test} - OD_{negetive}] / [OD_{Positive} - OD_{negetive}] \times 100$$

For this purpose goat's blood was collected in a beaker containing sodium citrate in the proportion of 3.8 g of sodium citrate per 100 ml of blood to avoid coagulation. The anti- coagulated blood was then diluted with N-saline in the proportion of 8:10. For checking the haemolysis 0.2 ml of diluted blood was added to 0.5 ml of 0.01N hydrochloric acid (HCl) followed by the dilution up to 10 ml and incubated for 60 min at 37 °C. The OD of the incubated solution was measured in an UV spectrometer at 545 nm wavelengths. Since HCl is known to cause large-scale rupture of RBC the OD count of this solution is taken as positive control referred to as $OD_{positive}$. Similarly, for negative control 0.2 ml of diluted blood was added to 10 ml of normal saline and again this was incubated for 60 min at 37 °C.

The OD of this solution is found again in an UV spectrometer at 545 nm wavelength and the OD is referred to as $OD_{negative}$. The reason for adding normal saline solution for negative control test is that this is known to cause the least RBC rupture. Having obtained the two standard OD, the OD of the material is obtained in similar lines. Sample having dimension of 5 mm X 5 mm was cut and was taken in a standard test tube containing N-saline and incubated at 37 °C for 30 min for providing temperature equilibrium. 0.2 ml of diluted blood was then added to the test tube, mixed gently and incubated for 60 min. OD of the sample is then obtained. This process is referred to as OD_{test}. The accepted norm is that if the haemolysis percentage is less than 10 the test material is taken as hemocompatible and if it is less than 5 the material is highly hemocompatible.

III. RESULTS & DISCUSSION

A. FTIR Characterization

The FTIR spectrum of EAMP hydrogel and pectin were taken in the range of 4000 – 400 cm^{-1} as KBr pellet and attenuated total reflectance (ATR for films) with the help of FTIR spectrophotometer (NEXUS – 870,Thermo Nicolet Corporation). Fig.1.A. has shown the FTIR spectra of the pure pectin. As the spectrum shown 3432 cm^{-1} due to –OH groups ,2921 cm^{-1} shows C-H stretching, 1758 cm^{-1} shows >C=O ester, 1020 cm^{-1} shown secondary alcohol(characteristic peak of -CH-OH in aliphatic cyclic alcohol C-O stretch). Fig 1 B shows the FTIR spectra of EAMP hydrogel.The carboxyl vibration region 1500-1900 cm-1 is most important for our analysis. The acid form of modified pectin has two important bands at 1674 cm-1 (amide I) and 1571cm-1 (amide II). The presence of these two bands and absence of intense carboxylate stretching bands indicates that the subsituents were bound to pectin chain by covalent amide bond, although we do not exclude the possibility that small amount of the amine salt might be found in the pectin.The conversion in to salt form lowers the carbonyl frequency and brings about the strong absorption band at 1605-1630 cm-1 belonging to the antisymmetric vibration of carboxylate anion. The intense carboxylate antisymmetric vibrations bands overlap with amide bands, which greatly complicate the analysis.

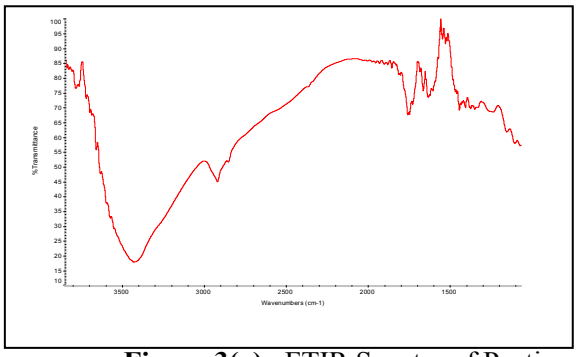


Figure 3(a). FTIR Spectra of Pectin

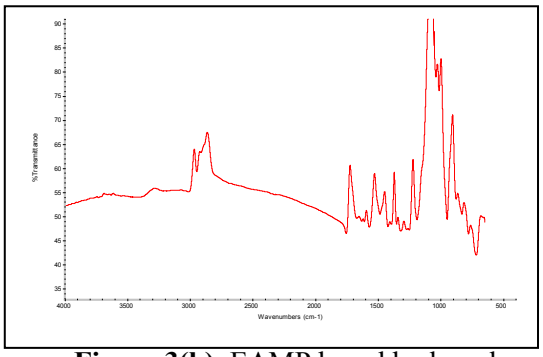


Figure 3(b). EAMP based hydrogel

Figure 3. (a), and (b) FTIR Spectra of Pectin and EAMP based hydrogel respectively.

B. X-Ray Diffraction study

XRD study of the pectin and EAMP done with XRD-PW 1700, Philips, USA. Ethanolic extract of *Tridax - procumbens L* using Cu K αradiation generated at 40 Kv and 40mA. The range of diffraction angle was 10°–70oThe Figure 2d, 2c shows XRD pattern of the pectin and EAMP respectively. In x-ray diffractogram of the EAMP there shown three intense peak at 46.17° - 2θ, 22° -2θ, 39.9 °- 2θ where as in the case of pectin peak appears at 2θ. This indicates that considerable increase in the crystallinity of the EAMP based hydrogel.

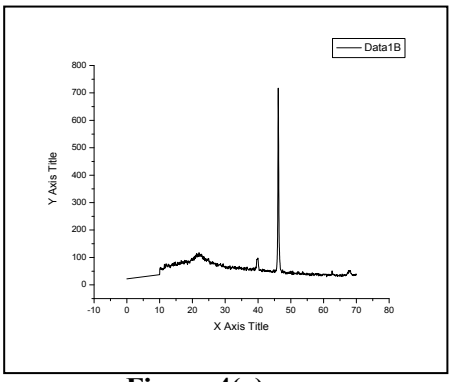


Figure 4(a)

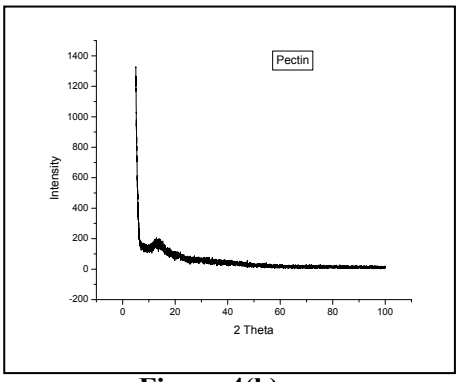


Figure 4(b)

Figure 4. (a) and (b) XRD patterns of pectin and EAMP based hydrogel resp.

C. Swelling Behavior

The EAMP was water soluble polymer so it required cross-linking by cross-linker. The EAMP hydrogel was cross-linked with 1ml of GA as a cross-linking agent respectively (acidified GA with HCl) were allow to swell in 5 ml of phosphate buffer solutions pH 1.4, 5.4, 7.4 and 9.4. The results indicates that GA crosslinked EAMP hydrogel swelled more significantly. (swelling 335%) at pH 7.4 due to a large swelling forces created by the electrostatic repulsion between the ionized acid groups as well as amide group.

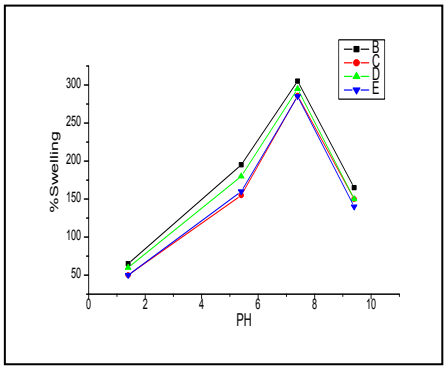
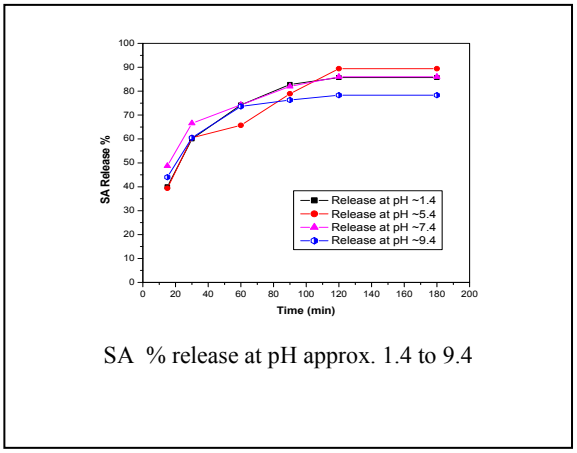


Figure 5. Swelling behavior of EAMP hydrogel (1:1, 1:2, 1:3, 1:4concentrations)

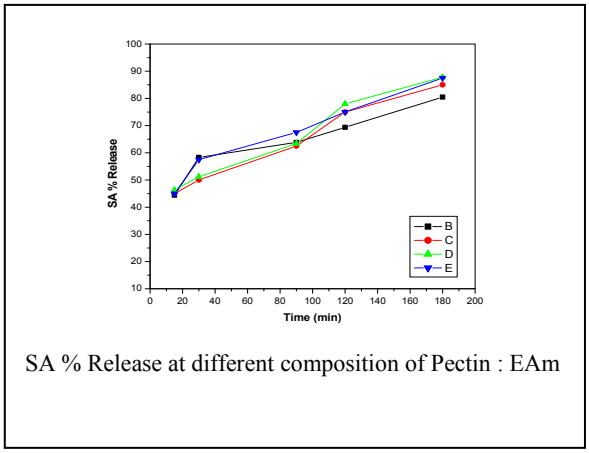
D. Drug Release Studies

The EAMP cross-linked with 1 ml of cross-linking agent (GA) which is further acididified with HCL showed more % swelling, so it was selected for drug release studies. Fig 3A and 3 B shows the ETE release profiles from the GA crosslinked EAMP hydrogel at deferent pH ~ 1.4, 5.4,7.4 ,9.4 and drug release behavior of hydrogels at different concentration(1:1,1:2,1:3 and 1:4) which is prepared by varying concentration of ethanolamine. It is evident from the graph 3, A at pH 5.4, the amount of ETE release increased significantly (approximately 89%) and at pH 7.4 the amount of ETE release is increases considerably (~85%) because the swelling of the hydrogel network increases at neutral pH. After 5 hrs of release at pH 7.4 the cumulative ETE release still maintained at approximately 85%. This due to some ETE molecules may be cross linked with hydrogel network directly by GA, and those cannot be released unless polymer matrices are degraded. Fig. 3. B. shows that release pattern of ETE increases considerably with increasing concentration of ethanolamine. It it is evident from the graph that ETE release is approximately 85% at concentration 1:2 1:3,1:4, and at 1:1, the release of ETE is relatively low (approximately 72%). Thus it clearly suggests our view that SA release increases with the increasing concentration of ethanolamine.



SA % release at pH approx. 1.4 to 9.4

Figure 6(a)



SA % Release at different composition of Pectin : EAm

Figure 6(b)

Figure 6. Drug release profile from the hydrogels,6 (a) drug release profile of EAMP hydrogel at defferent pH (1.4, 5.4, 7.4 and 9.4),6 (b) drug release profile of EAMP hydrogel at defferent concentrations (1:1, 1:2, 1:3 and 1:4).

E. Drug Release Kinetics from the Hydrogel

The release kinetics of the drug from hydrogels indicates that hydrogels of 1:1 and1:4 concentration both follows Higuchian kinetics, thus both are diffusion-controlled. The hydrogels which are prepared in 1:2 and 1:3 concentrations follows zero order release kinetics.

Diffusion systems may release drug following Higuchian or Fickian kinetics. The rate of release of a drug dispersed as a solid in an inert matrix has been described by Higuchi [32–34]. In this model, it is assumed that solid drug dissolves from the surface layer of the device first; when this layer becomes exhausted of the drug, the next layer begins to be depleted by dissolution and diffusion through the matrix to the external solution. In this fashion, the interface between the region containing dispersed drug and that containing dispersed drug moves into the interior as a front. For the purposes of data treatment the above model is depicted by the following equation;

M ¼ kt1=2

Where M is the mass of the drug released per unit area, k is a constant, so that a plot of amount of drug released versus the square root of time, t, should be linear if the release of the drug from the matrix is diffusion controlled. The release of drug from a diffusion system can also be described by Fick's first law of diffusion [35, 36]:

J ¼ _D

where J is the flux of drug across a membrane in the direction of decreasing concentration (amount/area-time), D is the diffusion coefficient of the drug in the membrane (area/time) and dCm/dx is the change in concentration of drug in the membrane over a distance x. Drug release pattern from Patches B and C also followed zero-order kinetics

indicating that the patches can be used for controlled drug delivery system. Fig, 7, Drug release kinetics from the EAMP hydrogels, Figure 7.(a),(b),(c) and (d).

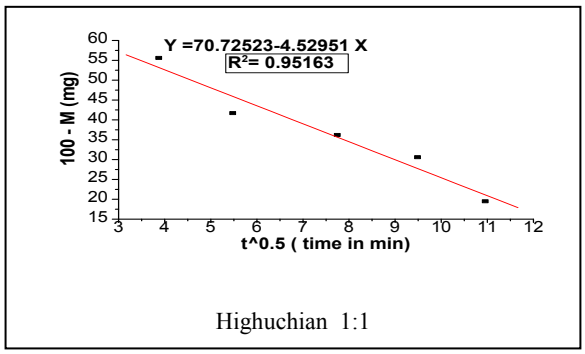


Figure 7(a). Drug release kinetics from the hydrogel (1:1)

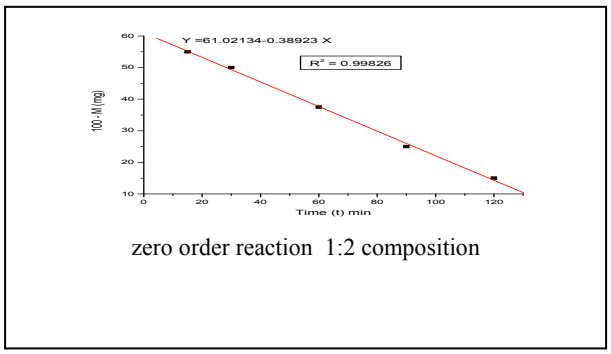


Figure 7(b). Drug release kinetics from the hydrogel (1:2)

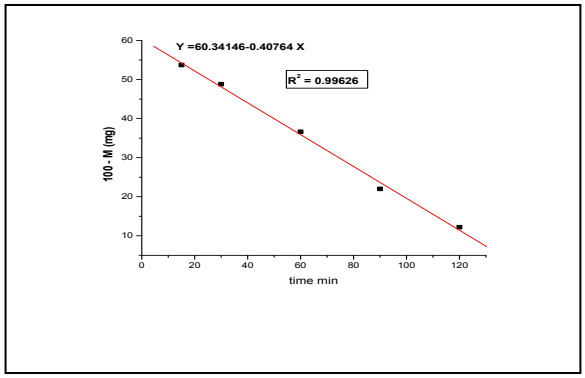


Figure 7(c). Drug release kinetics from the hydrogel (1:3)

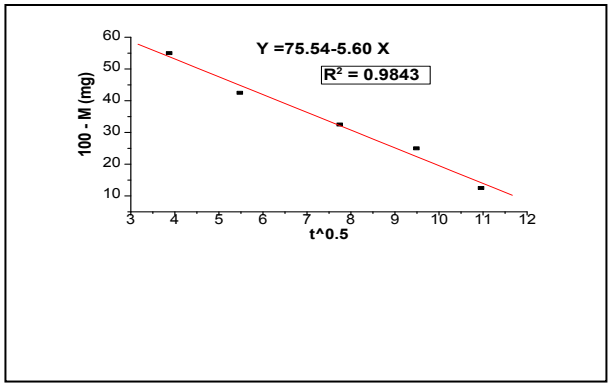


Figure 7(d). Drug release kinetics from the hydrogel (1:4)

F. Hemolysis Study

Hemocompatibility results of the EAMP hydrogels reveals that all the hydrogel samples have value less than 5, hence it clearly indicates that all the samples in defferent compositions (1:1, 1; 2, 1:3 and 1:4) are highly hemocompatible.

S.No.	Hydrogel compositions	OD of the sample	Hemolysis	Remarks
1	1:1	.0110	3.33	Highly hemocompatible
2	1:2	.0122	3.69	-
3	1:3	.0131	3.96	-
4	1:4	.0143	4.33	-

Table 2. Hemolysis result of EAMP Hydrogel in defferent concentrations (1:1, 1:2, 1:3, 1:4)

IV. CONCLUSION

In this work EAMP has been prepared by the amino de-alkoxylation (aminolysis) of pectin with various concentrations of ethanolamine. The structural changes in the modified pectin have been investigated using FTIR spectroscopy. Vibration bands of amide bands (amide I, amide II) and N- alkyl groups have been observed in FTIR spectra of EAMP and assigned. The data obtained were in good agreement with other analytical methods (organic elemental analysis). The amidation with primary amines permits various functional groups to be attached to pectin macromolecule, which influences the physical and chemical properties of pectin derivatives and their possible application. Crosslinking between pectin

macromolecule can occur when another part of amine radical carries such active groups like chlorine, oxyrane rings or other amino groups. Finally such bioactive molecules as protein, peptides, enzymes or drugs can be immobilized on the surface of pectin surface via amidation. We think that EAMP based hydrogel could be more interesting for colon targeted drug delivery systems.

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