

# Synthesis of crosslinked PVA/Chitosan hydrogels loaded EGF and evaluation *in vitro* characterization

## EGF yüklü çapraz bağlı PVA/Kitozan hidrojjellerin sentezi ve *in vitro* karakterizasyonunun değerlendirilmesi

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### SUMMARY


**Objective:** The aim of this study was to prepare different hydrogels using chitosan and PVA polymers and to evaluate the cytotoxicity of hydrogels through cell culture studies. In addition, FTIR spectrum measurements were aimed to determine the specific chemical groups and bonds in the structures of hydrogels and materials.

**Method:** In this study, different hydrogels were prepared and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) test was performed to evaluate the toxicity of hydrogels on mouse fibroblast cell line (L-929). In addition, specific chemical groups of materials and hydrogels used in hydrogels were determined via FTIR spectrum study.

**Results:** According to the MTT cytotoxicity results B1 (94.20±1.30 %) and D2 (82.45±1.05 %) formulations showed the greatest and lowest L-929 cell viability respectively. According to the results of the FTIR absorption spectrum, the aliphatic C = O tension band of rh-EGF was observed at 1634 cm<sup>-1</sup>.

**Conclusions:** Results showed that chitosan-PVA hydrogels containing r-EGF types were observed to increase mouse fibroblast cell viability. In the FTIR spectrum results, spectral bands showing specific chemical groups of chitosan, PVA, PEGylation agents, rh-EGF and C2 formulation were obtained.

**Keywords:** Hydrogel, cell viability, chitosan.

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### ÖZET

**Amaç:** Bu çalışmanın amacı, kitozan ve PVA polimerlerini kullanarak farklı hidrojjelleri hazırlamak ve hücre kültürü çalışmalarıyla hidrojjellerin sitotoksitesilerini değerlendirmektir. Ayrıca hidrojjellerin ve materyallerin yapılarında bulunan spesifik kimyasal grupları ve bağları belirlemek amacıyla FTIR spektrum ölçümleri yapılmıştır.

**Yöntem:** Çalışmada farklı hidrojjeller hazırlanarak hidrojjellerin fare fibroblast hücreleri (L-929) üzerindeki toksitesini değerlendirmek amacıyla 3-(4,5-dimetil-2-tiyazolil)-2,5-difenil tetrazolyum bromid (MTT) testi uygulanmıştır. Ayrıca FTIR spektrum çalışmasıyla hidrojjellerde kullanılan maddelerin ve hidrojjelin spesifik kimyasal grupları belirlenmiştir.

**Bulgular:** MTT sitotoksitesite sonuçlarına göre B1 (% 94.20 ± 1.30) ve D2 (% 82.45 ± 1.05) formülasyonları sırasıyla en yüksek ve en düşük L-929 hücre canlılığını göstermiştir. FTIR absorsiyon spektrum sonuçlarına göre rh-EGF'e ait alifatik C = O gerilim bandı 1634 cm<sup>-1</sup>'de gözlenmiştir.

**Sonuç:** Sonuçlara göre, r-EGF çeşitlerini içeren kitozan-PVA hidrojellerin fare fibroblast hücrelerinin canlılığını artırdığı gözlenmiştir. FTIR spektrum sonuçlarında kitozana, PVA'ya, PEGilasyon ajanlarına, rh-EGF'e ve C2 formülasyonuna ait spesifik kimyasal grupları gösteren bantlar elde edilmiştir.

**Anahtar sözcükler:** Hidrojel, hücre canlılığı, kitozan.

## INTRODUCTION

Hydrogels have a three-dimensional network structure formed by cross-linking of hydrophilic molecules with covalent bonds or keeping them together with intramolecular interactions<sup>1</sup>. Hydrogels have significantly similar properties with living tissues and their biocompatibility ensures their applicability. Hydrogels can be used as a carrier for pharmaceuticals and other therapeutic molecules in pharmaceutical and biomedical engineering fields<sup>1,2</sup>. Natural and synthetic polymers are used in the preparation of hydrogel formulations<sup>2</sup>. Chitosan, is a natural polymer, widely used in the biomedical field due to its biodegradable, low immunogenicity, mucoadhesive, and nontoxicity properties<sup>3,4</sup>. Polyvinyl alcohol (PVA) is a hydrophilic synthetic polymer with a chemically inert structure. PVA is widely used in different industries such as food, biomedical and pharmaceutical industries thanks to its simple structures, adhesive properties, film-forming ability, biocompatibility, swelling abilities, and non-carcinogen properties<sup>3,5</sup>. EGF, is a polypeptide structured macromolecule, plays a crucial role in the wound healing process by accelerating epidermal cell regeneration and stimulating keratinocyte migration and cell proliferation<sup>6,7</sup>. Although EGF has widely using areas, it has stability problems in the application site. Various modifications can be performed on the EGF in order to increase the stability of EGF and thus increase its efficiencies<sup>7,8</sup>. PEGylation plays an important role in these modifications. PEGylation of EGF promoted its biological activity, stability, and solubility properties<sup>9</sup>. There are many studies in literature related to the cell viability efficiency of hydrogels including chitosan and PVA. In this study, different hydrogels were prepared and it was aimed to assess their cell viability efficiency and indicate of specific chemical groups in the formulation and materials via spectrophotometric analysis.

## MATERIAL AND METHODS

MMW chitosan (Sigma, code: 448877), HMW chitosan (Sigma, code: 419419), PVA (Sigma, code: P1763) used as polymers for hydrogel were purchased from Sigma-Aldrich (USA). rh-EGF (Sigma, code: E9644), metoksi PEG propionaldehyde (MW 5 kD, Sigma, JKA 3039), metoksi PEG propionaldehyde (MW 10 kD, Sigma, JKA 3033), were purchased Sigma Aldrich (USA). rm-EGF (Gibco, P01133) was obtained Gibco (New Zealand). Cell viability kit, (MTT), (Roche, 11465007001, Switzerland) were purchased from Roche. All materials used in these studies were in analytical grade.

### Synthesis of hydrogel formulations

In the preparation of hydrogels, the study of Sezer *et al* was used<sup>10</sup>. Chitosan and PVA were used as polymer in the preparation of hydrogel formulations. The concentration of high molecular weight chitosan and medium molecular weight chitosan in the formulations was determined as 3 % (w/v). In addition, PVA was used in formulations with a concentration of 2 % (w/v). The volumetric rate of chitosan to PVA in each formulation was determined as 4:1. The chitosan was dissolved in a 1% (v/v) acetic acid solution by stirring in a magnetic stirrer. PVA was also dissolved in the magnetic stirrer via both heating (80 °C) and stirring. Sterile bi-distilled water was used as the solvent for the preparation of formulations. After PVA was completely dissolved rh-EGF or rm-EGF (10µg/ml), PEGylated r-EGF types and crosslinking agent, glutaraldehyde (GA), was added to the PVA solution and stirring was continued for 1 h. The dissolved PVA solution was added to the chitosan solution and stirred for 4 h. The hydrogels were placed in universal bottles and stored at 4 °C. The hydrogel formulations list was stated in **Table 1**.

**Table 1:** The components of the hydrogel formulations.

Codes	Molecular weight of chitosan	rh-EGF or rm-EGF	Different molecular weight of mPEG propionaldehyde	Glutaraldehyde concentration (g/ml)
A1	MMW	rh-EGF	---	0.001
A2	MMW	rm-EGF	---	0.001
B1	MMW	rh-EGF	5kD	0.001
B2	MMW	rh-EGF	10kD	0.001
C1	MMW	rm-EGF	5kD	0.001
C2	MMW	rm-EGF	10kD	0.001
D1	MMW	---	---	0.001
D2	MMW	---	---	0.002
E1	HMW	rh-EGF	---	0.001
E2	HMW	rm-EGF	---	0.001
F1	HMW	rh-EGF	5kD	0.001
F2	HMW	rh-EGF	10kD	0.001
H1	HMW	rm-EGF	5kD	0.001
H2	HMW	rm-EGF	5kD	0.002
K1	MMW	---	---	---
K2	HMW	---	---	---

\*All hydrogel formulations contain PVA (2% w/v).

### Infrared absorption spectroscopy (FTIR) study

Fourier Transform Infrared Spectroscopy (FTIR) was used to indicate specific chemical groups in the formulation and materials<sup>4,5</sup>. The infrared spectrum studies of hydrogels were performed (Perkin Elmer 1600 FT-IR, England). Before starting the study, hydrogels were converted into lyophilized powder with a lyophilizer. Infrared spectrum measurements of chitosan, PVA, rh-EGF and mPEG propionaldehyde were performed with the same method.

### Cell culture

The cell viability of hydrogels was tested against the mouse fibroblast (L-929) cell line. Cells were cultured in low glucose DMEM (Dulbecco's modified eagle's medium) containing 10 % FBS (fetal bovine serum), 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin in 25 cm<sup>2</sup> polystyrene flasks<sup>11</sup>. The cells were kept at 37°C with 5 % CO<sub>2</sub>. The cells were passaged when they had reached 85-90 % confluence.

### In vitro cytotoxicity assay

*In vitro* cytotoxicity of hydrogel samples were determined by MTT cell viability assay on L-929 (ATCC® CRL-2648) cell line. The cells were seeded at a predetermined confluence per well in a

96-well plate and incubated overnight. The cells were then treated with hydrogel samples for 24 h. After the incubation period, 10 µl MTT labeling solution was added to each well. Then, the plate was incubated for 12 h. After the incubation period 100 µl of the sodium dodecyl sulfate (SDS) was added into each well and samples were incubated overnight. The spectrophotometrical absorbance of the samples were measured using a microplate ELISA reader (Epoch Biotech, USA). The wavelength to measure the absorbance of the formazan product was 550 nm. The reference wavelength determined to be 690 nm<sup>11,12</sup>.

### Statistical analysis

The results of the studies were statistically evaluated using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test.  $p < 0.05$  was considered to be indicative of significance and the results were indicated as the mean and  $\pm$  standard deviation (SD).

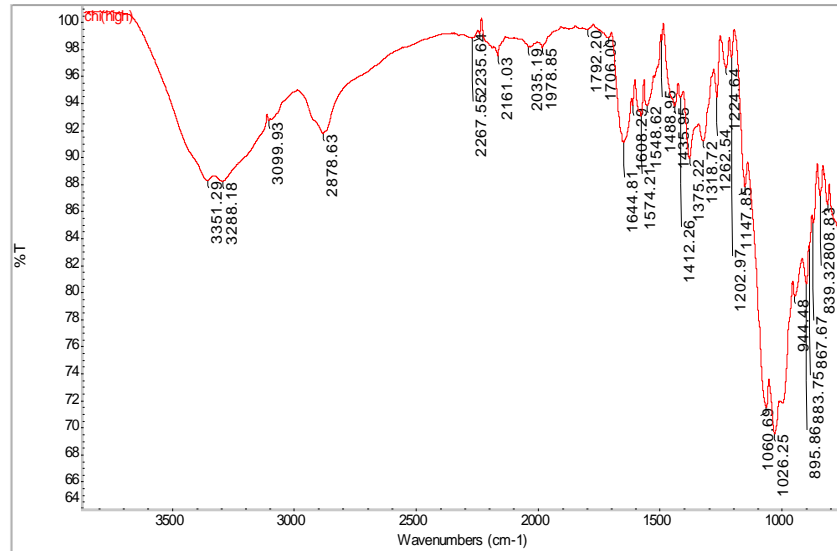
## RESULTS and DISCUSSION

### Evaluation of FTIR spectrum results

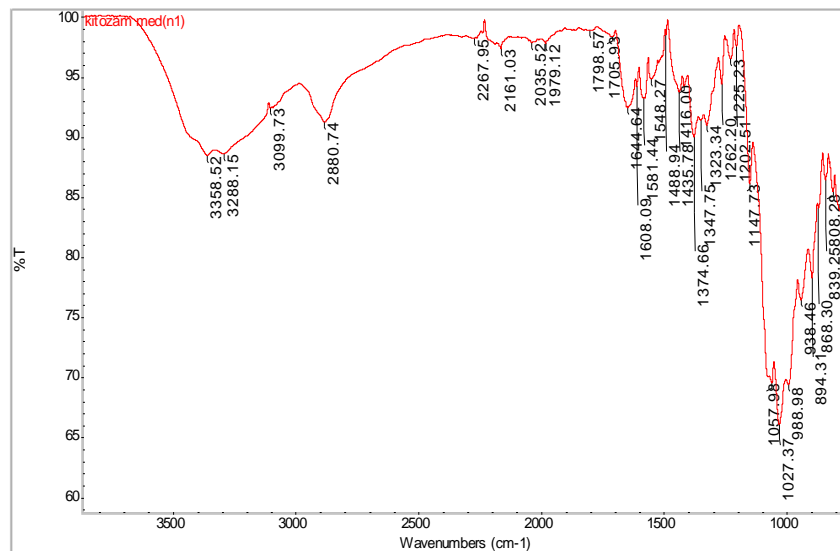
The results of IR absorption spectroscopy of chitosan were shown in **Figure 1** and **Figure 2**. Current FTIR spectrum results were quite similar

to the studies of Vino *et al.*<sup>4</sup>. According to the results, the characteristic bands of chitosan were 3358 cm<sup>-1</sup>, 3351 cm<sup>-1</sup>, and 3288 cm<sup>-1</sup> amine group N-H symmetrical stretch bands and 3099 cm<sup>-1</sup> O-H asymmetric stretch bands. The presence of N-acetyl groups was confirmed by the bands at around 1645 cm<sup>-1</sup> (C=O stretching of amide I).

1558 cm<sup>-1</sup> second amide bond corresponded to N-H bending of amide II. The CH<sub>2</sub> bending and CH<sub>3</sub> symmetrical deformation were confirmed by the presence of bands at 1412 cm<sup>-1</sup> and 1375 cm<sup>-1</sup>, respectively. Similarly, to Song *et al.* study, the absorption band at 1153 cm<sup>-1</sup> constituted to asymmetric stretching of the C-O-C bridge<sup>13</sup>.



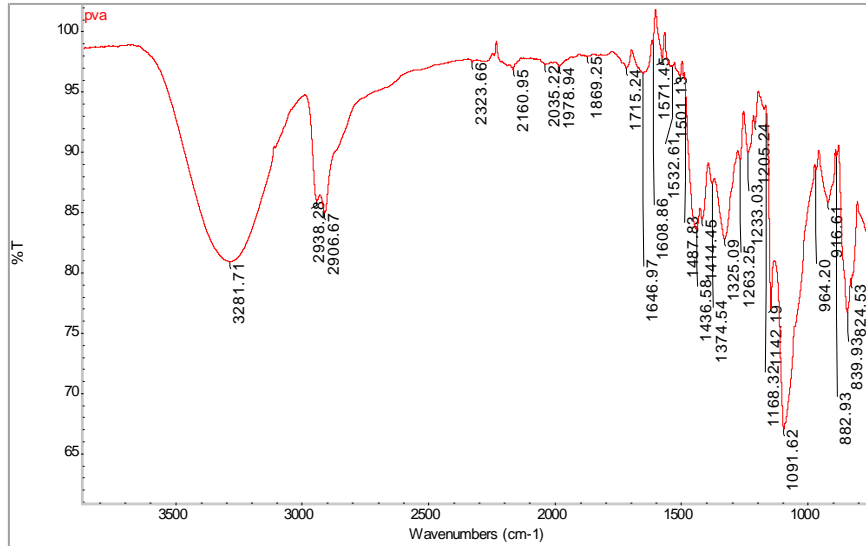
**Figure 1:** FTIR spectrum of chitosan (high molecular weight) with the characteristic signs.



**Figure 2:** FTIR spectrum of chitosan (medium molecular weight) with the characteristic signs.

The result of IR absorption spectroscopy of PVA was shown in **Figure 3**. Similarly, the FTIR spectrum study results of the Mansur *et al.* the large bands observed between  $3500\text{ cm}^{-1}$  and  $3100\text{ cm}^{-1}$  are linked to the O-H stretching band from the

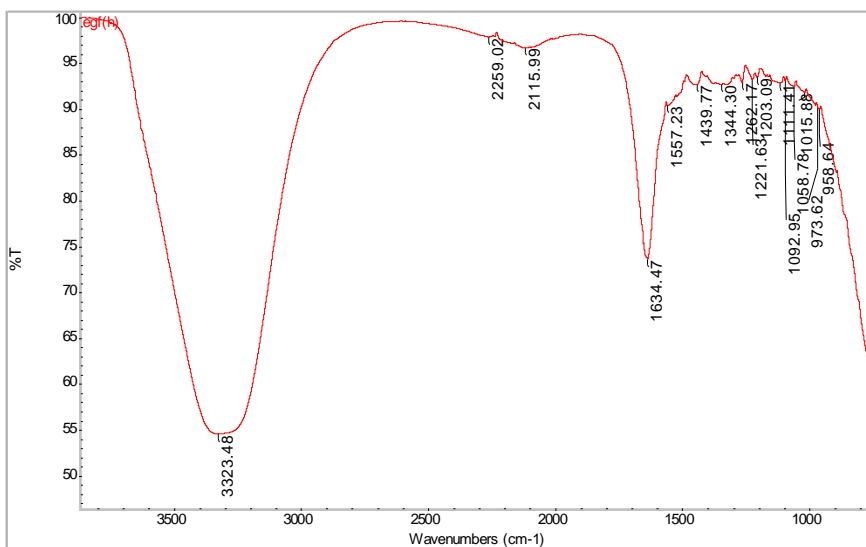
intramolecular and intermolecular hydrogen bonds<sup>5</sup>. According to the result, it was observed  $2938\text{ cm}^{-1}$  and  $2906\text{ cm}^{-1}$  bands refers to the C-H stretching from alkyl groups.



**Figure 3:** FTIR spectrum of PVA with the characteristic signs.

The FTIR absorption spectra of rh-EGF was shown in **Figure 4**. According to the results, aliphatic C=O tension band was observed at  $1634\text{ cm}^{-1}$ . In

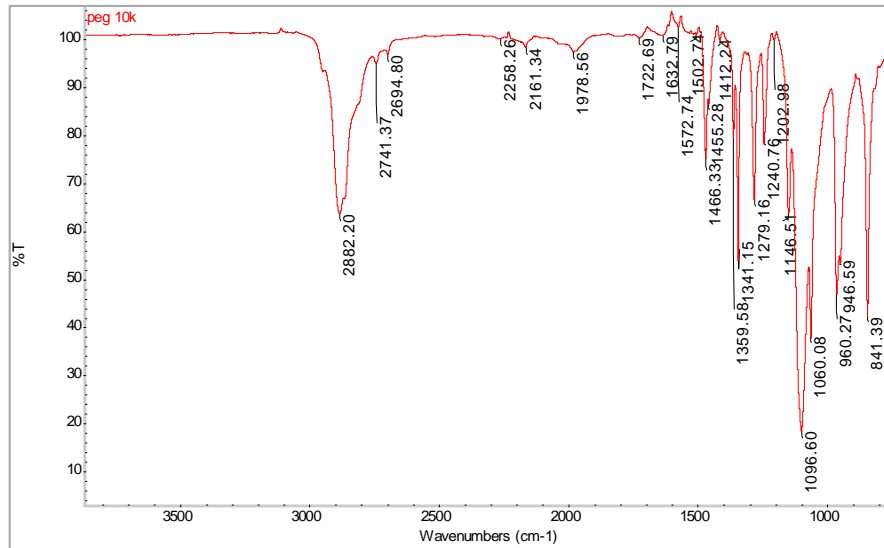
addition, tertiary C-N tension band was indicated between  $1230\text{ cm}^{-1}$  and  $1030\text{ cm}^{-1}$ . Aliphatic C-O tension band was observed at  $1620\text{ cm}^{-1}$ .



**Figure 4:** FTIR spectrum of rh-EGF with the characteristic signs.

IR absorption spectrum result of mPEG propionaldehyde 10 kD was shown in **Figure 5**. According to the results, aliphatic C-H tension band was observed at 2882  $\text{cm}^{-1}$ . Moreover, the C-O tension band was shown at 1096  $\text{cm}^{-1}$ . The bands

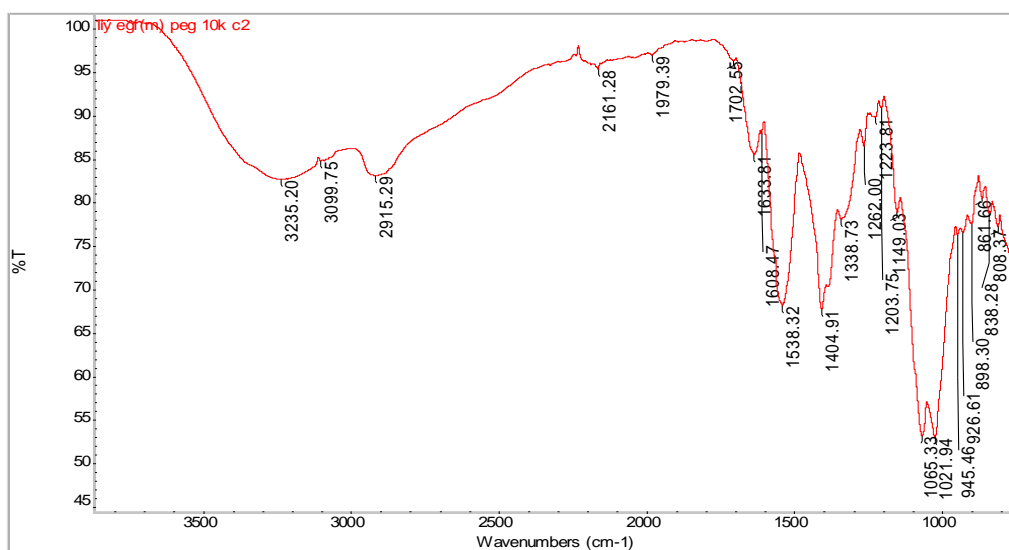
between 1260  $\text{cm}^{-1}$  and 1050  $\text{cm}^{-1}$  were linked to the C-O-C ether stretching band refers to tertiary C-N tension. A tension band of C-O was observed at 1620  $\text{cm}^{-1}$ .



**Figure 5:** FTIR spectrum of mPEG propionaldehyde 10 kD with the characteristic signs.

The C2 formulation was prepared using medium molecular weight chitosan, PVA and GA. It also contained rm-EGF and mPEG propionaldehyde 10 kD. The result of IR absorption spectroscopy of C2 formulation was shown in **Figure 6**. According to the results, there was a wide band of O-H stress that forms the intramolecular and intermolecular

hydrogen bonds between 3500  $\text{cm}^{-1}$  and 3000  $\text{cm}^{-1}$ . Aliphatic C-H tension band was observed at 2915  $\text{cm}^{-1}$ . Moreover, the presence of aliphatic C = O tension band at 1608  $\text{cm}^{-1}$  and O-C tension band at 1065  $\text{cm}^{-1}$  indicated that there was an ester group in the formulation.

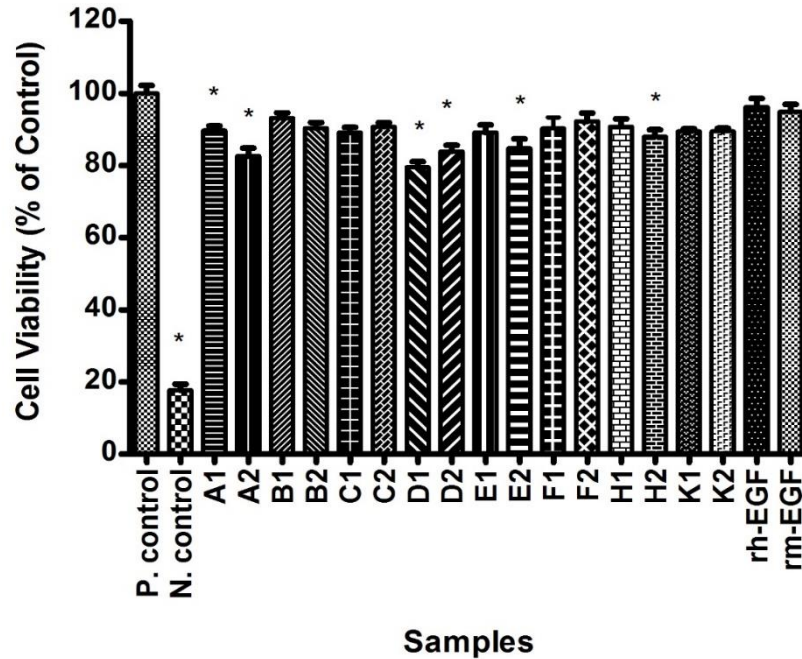


**Figure 6:** FTIR spectrum of C2 formulation with the characteristic signs.

### Assessment of MTT cell viability results

MTT cell viability results of hydrogels were shown in **Figure 7**. A formulation is accepted cytotoxic when the cell viability rate is under 70 %<sup>12</sup>. The cell viability results indicated that the formulations were safe for the cytotoxicity perspective on L-929 cell line. According to the results, there were substantial differences to the cytotoxicity results between the control group and rh-EGF free

formulations containing only chitosan and PVA ( $p < 0.05$ ). The cell viability rate of control (untreated) group was determined to be 100 %. MTT cell viability results showed that hydrogels including rh-EGF, rm-EGF and PEGylation agent performed a crucial contribution to cell viability. According to the MTT cytotoxicity results B1 (94.20±1.30 %) formulation showed the greatest L-929 cell viability. F2 formulation (93.20±2.35 %) also showed high L-929 cell viability effect.



**Figure 7:** Cytotoxicity results of formulations in L-929 cell line.

\*Positive control group and negative control group were untreated. Positive control group included DMEM and L-929 cells, negative control group contained only DMEM.

The cytotoxicity study results showed that there were significant differences ( $p < 0.05$ ) between control group (100 %) and compared to the hydrogel formulations such as D1 (79.60±1.20 %), D2 (82.45±1.05 %). According to the results, the absence of EGF and PEGylation agents in D1 and D2 formulations could be explained as the reason for having lower cell viability compared to the control group. The results showed that, cell viability of A1 formulation containing rh-EGF was 87.82±0.85 % and A2 formulation containing rm-EGF was 82.64±1.56 %. Results showed that B2 formulation including medium molecular weight chitosan and F2 formulation with high molecular weight chitosan had close cell viability results. It

could be concluded from these results, the molecular weight of chitosan in the formulations did not make a significant difference in cell viability. Moreover, cell viability activity of rh-EGF and rm-EGF were 95.30±1.30 % and 93.80±0.95 % respectively.

### CONCLUSION

In this study, chitosan-PVA hydrogels were prepared and cytotoxicity of hydrogels was evaluated. Moreover, FTIR was used to indicate of specific chemical groups in the formulation and materials. Cytotoxicity results promoted the crucial effects of hydrogels on cell viability. According to the results, r-EGF types and PEGylation agents in

hydrogels have been observed to increase cell viability. In conclusion, it can be said that chitosan-PVA hydrogels including r-EGF types and PEGylation agents shed light on further cell viability studies.

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