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STARCH-CHITOSAN MODIFIED BLEND AS LONG-TERM CONTROLLED DRUG RELEASE FOR CANCER THERAPY

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ABSTRACT

The conventional drug delivery system has serious limitations. These limitations can be overcome by using the smart materials to produce an effective drug delivery system. Corn starch cross-linked chitosan using potassium persulfate as an accelerator with different ratios of corn starch/chitosan blends were prepared as smart materials for drug delivery system. The hydroxyurea drug was loaded in smart materials blends to evaluate the efficacy of new materials on therapy of Rhabdomyosarcoma cancer cell line (RD). The FT-IR results revealed that the reaction was occurred between blends materials (starch/chitosan). SEM tests illustrated that the different morphologies were obtained in blend films surface. The rate of drug release was sensitive to pH and significantly increased at pH 2.2 as compared to pH 7.4. Furthermore, the percentage of swelling was higher in acidic solution than in neutral solution. It was concluded that the starch/chitosan smart materials may be suitable for medical applications like drug delivery system to RD cell line as confirmed by the availability and morphology test of RD cell line.

Keywords: Starch, Chitosan, Drug Release, Rhabdomyosarcoma Cancer, Hydroxyurea

1. INTRODUCTION

Cancer refers to uncontrolled cell division that can attack healthy tissue (Jemal et al., 2011). Several different methods using to treatment cancer include chemotherapy, radiation therapy, surgical intervention and other techniques. Each method has its advantages and disadvantages; the choice of the appropriate method depends on the type of cancer, the location and the patient's condition. There is a highly targeted method such as radiotherapy and surgery, it can be removed the malignant tumor completely. However, when cancer spreads in multiple places of the body these methods become ineffective. Moreover, these methods need good patient health (Helleday, et al. 2008).

Chemotherapy is also widely used throughout the world to control cancer and its growth by taking medications. These drugs prevent cell division in the body, which leads to stopping tumor growth (Helleday, et al., 2008). However, these drugs not only kill cancer cells but also healthy cells. There are many harmful side effects of chemotherapy drugs such as toxicity, include loss of weight, loss of hair, the sensitivity of skin, vomiting, and the impact of immunity. There is another problem in the chemotherapy drugs that these drugs do not reach enough to the tumor tissue, which leads to the increase in the dosage. However, this leads to increased risk of healthy tissue (Wang et al., 2012). Therefore, it is necessary to develop chemotherapy drugs that have the ability to target the tumor tissue and reduce side effects.

The drug delivery system depends on two important pathways for unique performance including active and passive pathways. The passive effect is attributed to enhanced permeation retention (EPR) effect. Typically, the vessels of the tumor region contain high leakage to food and oxygen to maintain rapid growth of cancer cells. However, this technology remains specific due to there are certain tumors or certain part of the tumor that do not have such this effect. To overcome such difficulties can be used active targeting (Peer, et al., 2007). Compared to passive targeting and free drugs, active targeting can increase the chance of tumor uptake, improve therapeutic properties and reduction undesirable effect to normal cells (Ruoslahti, et al., 2012). Moreover, particles can be activated when they reach their target area, for example, their high susceptibility to pH (Muller, et al., 2004).

Some requirements for particles that are used as drug transporter should be provided such as biodegradation, biocompatibility, mechanical properties and drug compatibility (Alimohammadi, et al., 2014). Starch is used as a drug delivery system because of its unique properties that enable it to work in this field such as improving drug stability and solubility, decreasing side effects and drug toxicity, storage stability and excellent biocompatibility (Najafi, et al., 2016). But the main obstacle of starch is that it is poor in dimensional stability, mechanical properties and processability of its end products. Therefore, decided to use starch in the

compound material (Bajer and Kaczmarek, 2010, Bajer and Kaczmarek, 2010) demonstrated the process of interaction between starch and chitosan in their blend (Bajer, et al., 2010). Therefore, it is expected that a polymeric composite material will be produced delivery system for cancer therapy and that the release of the drug will be slow and controlled (Subramanian, et al., 2014).

Chitosan results from nature, for example its existence in the crabs, lobsters, fungal cell walls, shrimps and in the insect exoskeletons (Dash, et al., 2011, Testing 2013). Excellent performance results from this material (biocompatibility, biodegradability, high safety and minimal toxicity with large numbers of biological activities) which gives these materials a great chance to develop them to work in wider and more useful applications (Subramanian, et al., 2014, Dash, et al., 2011). Chitosan are the drug vehicle with extensive improvement possibility and have the benefit of controlled and slow drug release, which increases drug stability and solubility, reduces toxicity and enhances efficacy (Aruna, et al., 2013).

Most kinds of sarcoma that affect the soft tissues during childhood and adolescents are under 20 years old Rhabdomyosarcoma (RMS) (Ognjanovic, et al. 2009). Because clinical trials are slow and difficult to perform, most studies on this type have been conducted in laboratories for this cancer type (Pappo, et al., 1999). There are two main types of RMS, alveolar and embryonal (Hinson, et al., 2013). Embryonal RMS contains about 15 types of different cell line and one of them RD cell line (Kang, et al., 2011).

In the present study, corn starch/chitosan blends loading hydroxyurea was produced and their physicochemical and morphological characterizations were investigated. The swelling and controlled drug (hydroxyurea) release of blends was studied at different pH value. In vitro anticancer activity was evaluated against RD cancer cells in terms of percentage cell viability through MTT and morphological cell density. It is worth mentioning here that previous studies have not explored these systems for Rhabdomyosarcoma cancer cell line therapy using hydroxyurea drug and the study of these systems at different pH media is still needed. Therefore, this is the first attempt to assess the suitability of these systems for Rhabdomyosarcoma cancer cell line therapy.

2. MATERIALS AND METHODS

2.1. Materials: The supplier of native cornstarch was Inter-scince (ST. Louis, MO – S 4180) France. The amylase and humidity content were 27% and 8–9% respectively. The mean particle size of cornstarch was 12μm. The chitosan (90% deacetylated, Mw= 161.16) was supplied by micxy reagent, Beijing, china. Hydroxyurea capsules USP 500mg supplied by Hydrea, india. Potassium persulfate ($K_2S_2O_8$) was purchased from Merck, New Jersey, United States. Poly (vinyl alcohol) (PVA) provided by Sinopharm Chemical Reagent co., china. Acetic acid glacial (CH3COOH) was purchased from Himedia, India.

2.2 Preparation of drug loaded particles: Chitosan/ corn starch blends at different concentrations (1/0, 1/3, 1/1, 3/1 and 0/1) were prepared and the compositions are reported in Table 1.

The solution of chitosan was prepared by dissolving specific weight of chitosan in 20ml of aqueous acetic acid (2%) solution at room temperature and stirring for three hours to obtain homogenous solution (Singh, 2016). The solution of starch was prepared by dissolving desired amount of starch in 20ml of aqueous acetic acid (2%) solution at room temperature and stirring for one hour until it became homogenous solution (Subramanian et al., 2014). The two solutions of starch and chitosan were mixed together and 3ml of potassium persulfate ($K_2S_3O_8$) solution (6g $K_2S_3O_8$/100ml distilled water) as a catalyst was added to mixture and heated at 60ºC for 30-45min (Testing, 2013, Aruna et al., 2013).

Then, the solution was cooled to room temperature, solid materials were collected and washed many times to remove the potassium persulfate catalyst and acetic acid. The hydroxyurea drug was then added to starch/chitosan solution and well mixed using magnetic stirrer. Moreover, the PVA solution was prepared by mixing 60mg PVA /100ml distilled water at 85-95ºC.The 3% of PVA solution (from the total starch/chitosan blends as dry weight) was added to starch/chitosan solution, dispersed the particles using ultrasonic and finally, the samples were dried at room temperature to gain the new drug (Pandey et al., 2015).

Table 1: The compositions of starch /chitosan blends.

Materials	A_o	A_1	A_2	A_3	CH
Starch (g)	2	6	4	2	-
Chitosan (g)	-	2	4	6	2
Acetic acid (%)	2	2	2	2	2
$K_2S_2O_8$((ch.+st.)*0.1)(g)	-	0.8	0.8	0.8	-
PVA (w/v) (%)	3	3	3	3	3
Hydroxyurea (%)	25	25	25	25	25

2.3 Fourier Transform Infrared Spectroscopy (FT-IR): The samples were mixed with KBr powder/sample and tested by pellet method. The conditions were 32 scans and the resolution was 4 cm^{-1} while its wave number range between 400-4000cm^{-1} (Sacithraa, et al., 2013).

2.4. Scanning electron microscopy (SEM): The shape, surface morphology, size of corn starch and drug loaded film were examined using a scan electron microscope (SEM) at 10kV as an accelerating voltage. The dry specimens were scattering on dual twig tape stable on circular copper stubs, then painted with gold film (~15nm) to prevent electrical charge accumulation (Najafi and Baghaie, 2016).

2.5 Evaluation of in vitro drug release: The drug release study through the starch, chitosan and cross-linked films was carried out in different pH solutions at 37ºC under unstirred conditions. A known weight of the film i.e. 0.05g was put in 50ml phosphate buffer solution at different pH values (2.2 and 7.4) (A. Hinson, et al., 2013). The behavior of drug release was studied in the simulated gastric and intestinal pH conditions (Virpal, 2014). At predefined intervals of time the electrical conductivity was measured by conductivity meter (EC214, HANNA Instrument Inc., Romania) to calculate the percentage of drug in this solution. The amount of drug released through the starch, chitosan and cross-linked beads was calculated by prepared suitable calibration curve.

2.6 Swelling studies: The swelling behavior of the cross-linked films was studied at different pH solutions (7.4 and 2.2) to understand the mechanism of transfer of the drug into the cross-linked films. A specified weight of the dry sample was placed in the solution at 37ºC. At different periods of time the film was got out from the solution and the excess solution on the surface of sample was removed by tissue paper. The weight of the film was determined using sensitive balance (± 0.0001 g) (TP-214, Denver Instrument Germany) and the percentage of swelling was calculated by using the following equation (Kumari and Kundu, 2008, Singh and Kumari, 2014):

$$S = \frac{Ws - Wd}{Wd} * 100\%$$

where, Ws is the weight of sample after swelling and Wd is the dry weight of the sample.

2.7 Cytotoxicity assay: The cytotoxicity of different composites was evaluated by MTT assay. This assay depends on the change tetrazolium to formazan during interaction with mitochondrial in cultured cells, and the formazine amount produced refers to number of living cells (Sylvester, 2011).

Cells were placed into 96 well plates at 1.0 x $l0^5$ cells/ml concentration. After the incubation at 37°C for 24-48 hrs, a monolayer of RD cells was formed at 80-100% concentration. Different concentrations of drug composites (1, 10, 100, 500 and 1000µg/ml) were put into wells at a total volume of 100µl in triplicate for each well except control cells (Sun, 2015). Before MTT assay and morphological cell assessment, all samples were incubated with media for 24 hrs to facilitate hydroxyurea release into the media (Pandey, et al., 2015).

After 24 hr incubation at 37°C in 5% CO_2, The RD cells were washing by PBS solution to remove drug composites or standard anticancer drugs used that may be interacts with MTT reagents. Followed by the addition of 100µl maintenance media for all wells with 20 µl MTT reagent (Ravikumara and Madhusudhan, 2011).

The plates were incubated for 4 hrs at 37°C, under flow of 5% CO_2. The formazan particles were produced during the MTT reaction with mitochondrial enzymatic for living RD cells. After this incubation, diluted dimethylsulfoxide DMSO (1:1) in isopropanol was added that solublized formazan salt particles. By using ELISA reader, the optical density was calculated at wavelength 490-630nm (Sun, 2015, Ravikumara and Madhusudhan, 2011).

3. RESULTS AND DISCUSSION

3.1. Fourier Transform Infrared Spectroscopy (FT -IR): The results of FTIR for corn starch, chitosan and cross-linked film are shown in Figure 1. The results of FT-IR for pure starch revealed that the peak observed at 3416 is assigned to O–H group (Nnamonu et al., 2012), peaks at 2928cm^{-1} was attributed to C–H stretching bond (Subramanian et al., 2014), peaks observed at 1649cm^{-1} was assigned to the C=O stretching (Singh and Kumari, 2014), peaks in the range of 1365-1454 cm^{-1} were due to the H–C–H, C–H and O–H bending modes (Zeng et al., 2011). There were several absorbances bands at 1157, 1083, and 1018cm^{-1} which were referred to C-O stretching bond (Zeng et al., 2011, Winarti et al., 2014).

The results of FT-IR for pure chitosan revealed that the main bands in the range from 3750cm^{-1} to 3000cm^{-1} were due to O-H groups stretching vibrations, which interfered to the N-H stretching vibration (Marchessault et al., 2006), the peaks obtained at 2920cm^{-1} is refer to C–H stretching (Subramanian , et al., 2014). The peak at 2875cm^{-1} represented H-C-H group (Singh and

Kumari, 2014). The two peaks at 1635cm-1 and 1595cm-1 were noticed and referred to amide I and amide II, respectively (Bajer and Kaczmarek, 2010; Wang et al., 2007), methyl and methylene bending vibrations groups were shown at 1421.54 cm^{-1} and 1379.1cm^{-1} respectively (Mano et al., 2003). The peak located at 1155.36cm^{-1} is related to asymmetric vibrations of C-O-C which is produced during chitosan deacetylation (Silva et al., 2012) and the band at 1083cm^{-1} was due to the glycosidic bonds (Bajer and Kaczmarek, 2010).

In the cross-linking film spectra, the significant shrinkage of the amine peak intensity at 1595cm^{-1} and it shifts to a lower value of absorption bands (Bajer and Kaczmarek, 2010). Moreover, the concentration of NH2 group was highly decrease as revealed by decrease its intensity (Subramanian et al., 2014, Kumari et al., 2016). It may be attributed to the reaction between NH_2 of the chitosan and OH^- of starch was occurred. Moreover, the small shift of NH_2 peak to a lower value indicated that the interaction of OH^- group and NH_2 was occurred (Bajer and Kaczmarek 2010).

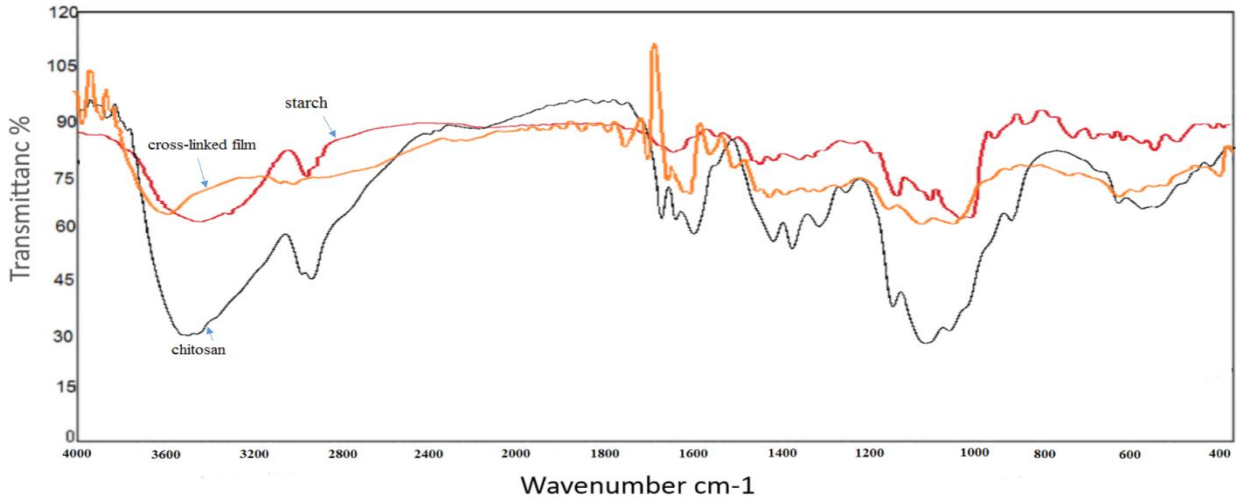


Figure1: FT-IR for corn starch, chitosan and cross-linked film.

3.2 Scan Electron Microscope (SEM): SEM images of corn starch-chitosan films were shown in Figure2. Figure2a illustrated the microstructure of (3/1) starch/chitosan film surface and it was very rough and withered large granules of starch. Whereas, the addition of 50%w/w of chitosan into the starch film matrix (Figure 2b) reduced the granules and improved the surface structure (homogeneous and smooth matrix). However, when the concentration of chitosan was further increased to (1/3) starch/chitosan, the chitosan was not fully blended with the starch as seen in Figure 2c and this is consistent with S. Othman and R. Shapi 2016.

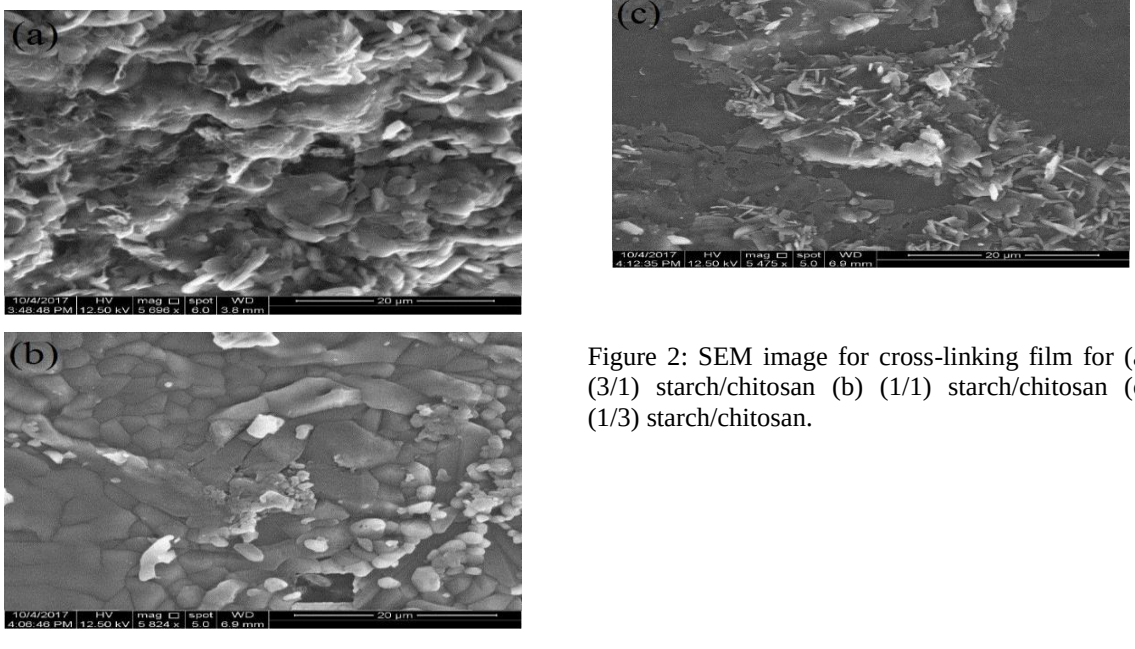


Figure 2: SEM image for cross-linking film for (a) (3/1) starch/chitosan (b) (1/1) starch/chitosan (c) (1/3) starch/chitosan.

3.3 In Vitro Drug Release Studies: Drug release profiles of A_o, A_1, A_2, A_3, CH blends in acid and neutral media are shown in Figure 3 and 4. It was observed that the release characteristics of chitosan-starch film depend on both composition of film and pH of release medium (Virpal, 2014). The release behaviors of hydroxyurea were shown similar initial release behavior. The release of the drug was fast in the first hour in both acid and neutral solution, while it was moderate release over 9 hours and finally, a nearly constant release of the hyd -roxyurea was observed for remaining 25 hours. The drug release rate was maximum for first hour and it may attribute to present some of the drug on the outer surface of the films and it was released when the films were immersed in the fluid media (Raizaday et al., 2015).

Figure 3 shows that the quantity of drug released at pH 7.4 was decreased with increasing chitosan amount in the blend. This may be due to the formation of a dense matrix as a result of increasing chitosan in the blends which led to reduce the swelling of the films (Kumari and Kundu, 2008). Therefore, the solvent penetration was reduced and hence reduced the amount of drug released. This result is consistent with (Wang et al., 2007). Therefore, the drug release at pH 7.4 is largely depending on solvent diffusion through the blends. The reason may be the protonation of amino groups in chitosan is lacked at pH 7.4 (Kumari et al., 2016, Lim et al., 2013).

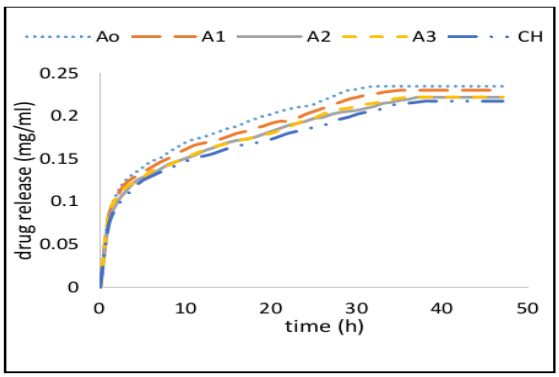


Figure 3: Drug Release of A_o, A_1, A_2, A_3, CH at pH =7.4.

In acid media, it was observed that the rate of drug release was increased proportionally as boosting in the release time. The reason of raise in drug release with raising in time may be attributed to the hydration of starch in acid media and the other reason is protonation of amino groups of chitosan to give highest swelling of starch/ chitosan blends as compared with neutral media (Raizaday et al., 2015, Bakain et al., 2015). The mechanism of the drug release through the composites films was the penetration of media in to drug vehicle, dissolve the drug and then it is diffusion out drug vehicle through the same path. Thus, the drug release takes place from the compact matrix due to increase in swelling of the beads and penetration of the solvent over a period of time (Kumari et al., 2016).

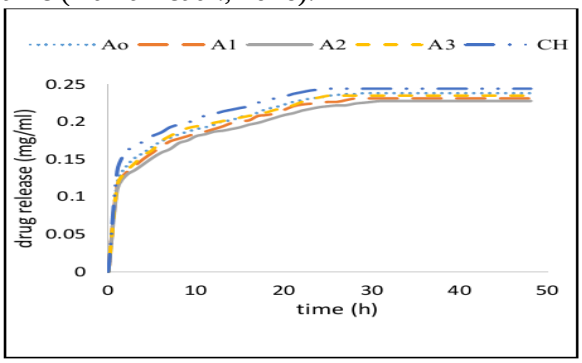


Figure 4: Drug Release of A_o, A_1, A_2, A_3, CH at pH =7.4.

The results indicated that the release of hydroxyurea from chitosan/starch film is pH dependant, the hydroxyurea released faster in acidic environment than neutral environment as a consequence of starch hydrolysis and protonation of amino groups in acidic environment. This result is consistent with (Lim et al., 2013, Jaimes et al., 2014). From our observation the acidic environment effect on starch/chitosan blends was higher than neutral environment because the chitosan is very sensitive to acidic environment and the protonation of amino groups in chitosan occurred in acidic environ-m ent.

3.4 Swelling Studies: It is not feasible to record the swelling data of pure starch. The chitosan/ starch blends have better strength as compared to pure starch and they are capable to longer time in swelling media. Therefore, the swelling studies are performed only on cross-linked chitosan-starch films and chitosan film (Singh and Kumari, 2014). When the composite films are put in the swelling media, the solution starts to diffuse inside the films and consequently try to swell (Kumari and Kundu, 2008).

The results indicated that the percentage of swelling in pH =7.4 increased with passage of (Figure 5) time. The rate of swelling of biodegradable cross-linked films increases linearly for first four hours followed by almost a constant swelling for rest of the studied time period. The results revealed that the percentage of swelling is

also influenced by the content of chitosan and starch in the film. The swelling percentage of the cross-linked films was lower with increase the concentration of chitosan in the blend and this may be due to the formation of a dense matrix in pH=7.4. Similar results were found by (Hinson et al., 2013, Singh, 2016).

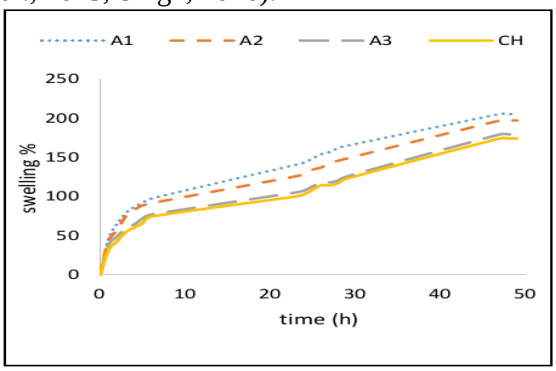


Figure 5: Swelling behavior in pH =7.4 for corn starch/chitosan composite and pure chitosan.

It was observed that the swelling percentage of film in pH 2.2 solution was higher than pH 7.4 solution (Figure 6). It was concluded that the films in pH less than 6 have new structure which the protonation was occurred in acid media for amino and imine groups in films surface and therefore, it followed relaxation of the polymeric chains (Lim et al., 2013). Initially, amino and imine groups at the film surface were protonized and it leads to separate the hydrogen bonds of amino and imine groups and this lead to diffuse the solution inside the film faster as compared to pH 7.4 to separate the hydrogen bonding inside the film network. Further, the percentage of swelling significantly faster at pH 2.2 as compared to pH 7.4 (Kumari and Kundu, 2008).

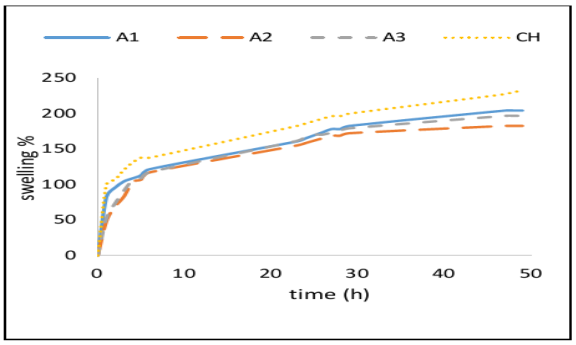
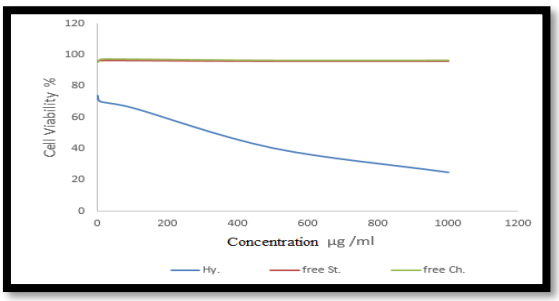


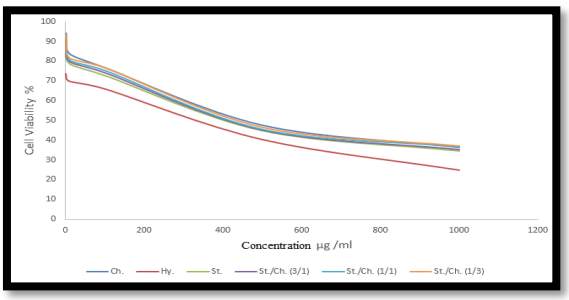
Figure 6: Swelling behavior in pH 2.2 for native chitosan and its composite.

3.5 In Vitro Anticancer Evaluations Using RD Cell Line: The viability assays provide report on cell killing and metabolic activities. The MTT assay was applied in this work because it is effective and quick process for examining mitochondrial activity (Sabudin et al., 2012).

After loading hydroxyurea drug onto pure starch, pure chitosan and starch/chitosan film, the toxicity of the drug was examined via incubation with RD cell line. The MTT assay was performed to show the toxicity of the drug vehicles (pure corn starch and pure chitosan) (Sun, 2015). Figure 7a showed the cells treated with pure corn starch, pure chitosan and pure hydroxyurea, which gave a good idea about the health of cells in pure starch and pure chitosan as biocompatible materials. The most-dead cells can be seen at pure hydroxyurea, which means they can be used as an effective agent to treat this type of cancer cell (Pandey et al., 2015), while cells health in the pure starch and pure chitosan did not affect and remind without change. This meant that pure corn starch and pure chitosan have not toxicity onto RD cancer cells.
On the other hand, the results of curing the cancer cells with drug/starch, drug/chitosan and drug/starch-chitosan (St:Ch) blend with different percentage of St:Ch revealed that the cancer cells were effectively killed in a certain concentration range and its behaviors were similar to behavior the pure hydroxyurea drug. The cell viability with using drug/starch, drug/chitosan and drug/starch-chitosan blend with different percentage of St:Ch can be inferred by the number of dead cells, as show in Figure7b. Similar results were found by (Pandey et al., 2015).



(a)



(b)

Figure 7: MTT assay of (a) The effect of pure hydroxyurea, pure corn starch and pure chitosan on toxicity of RD cell line (b) The effect of pure hydroxyurea drug and its blends with chitosan, starch and crosslinking film with different percentage of St:Ch on toxicity of RD cell line

The death of RD cells for different vehicles such as pure starch, drug/starch-chitosan (1/1) vehicles and free hydroxyurea drug was estimated. The results showed that the slow rate of dead cell was obtained in drug/starch-chitosan (1/1) vehicles. Moreover, the cytotoxic effects of films increased with an increase in concentration of drug (Figure 8). The toxicity was increased as the concentration of drug raised from 1 to 1000µg/ml (Ravikumara and Madhusudhan, 2011).

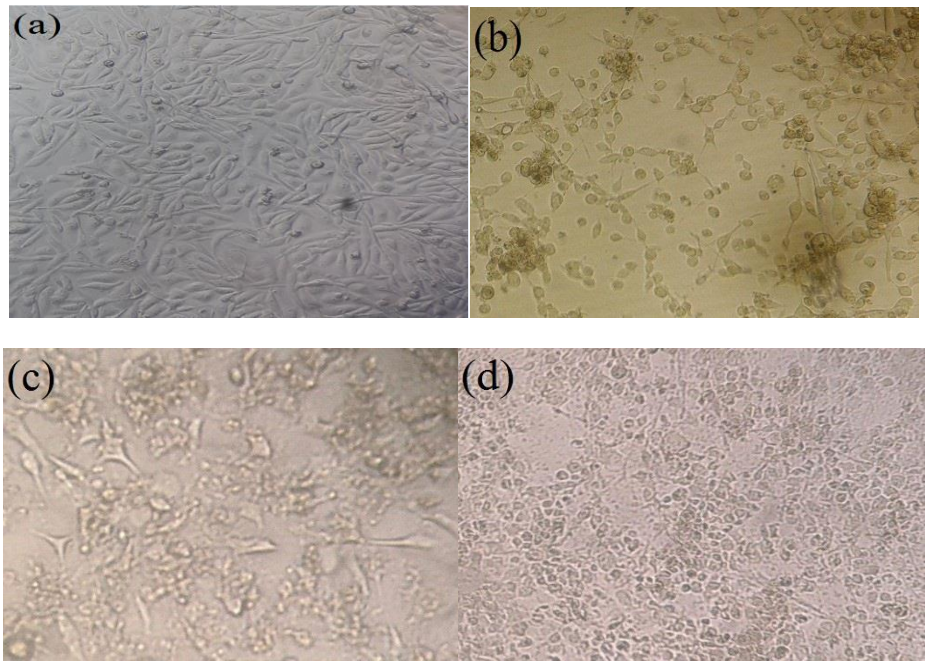


Figure 8: Morphology of RD cell density (a) without any drug (b) after treatment with pure starch (c) after treatment with crosslinking film in 100 µg/ml (d) after treatment with free hydroxyurea drug.

4. Conclusions:

The starch/chitosan vehicles were successfully prepared using potassium persulfate ($K_2S_2O_8$) as a catalyst to product biocompatible composite films. The hydroxyurea was loaded these vehicles and the products were used as an anticancer drug. FT-IR spectra results revealed that the amine peak intensity was significantly decreased, and it has small shifts to a lower value of absorption bands. It was concluded that the reaction between the NH^{+3} groups of the chitosan and OH^- groups of starch were occurred. The SEM results illustrated that the roughness of films surface was increased with an increase percentage of starch. In addition, the results indicated that the release of hydroxyurea drug from chitosan/starch film is pH dependant and the hydroxyurea drug released faster in acidic environment than neutral environment and the drug release at pH 7.4 decreased with an increasing in chitosan amount in the blends. Moreover, the results revealed that the percentage of swelling is also influenced by the content of chitosan and starch in the film. The swelling percentage of the chitosan/starch films was low with increase the concentration of chitosan at pH7. The pure starch and pure chitosan have not toxicity effect on RD cells and remind without change while drug/starch, drug/chitosan and drug/starch-chitosan (St:Ch) blend with different percentage of St:Ch have toxicity effect and they effectively killed cancer cells in a certain concentration. It was concluded that the corn starch/chitosan smart materials may be suitable for medical applications like drug delivery system to RD cell line as confirmed by the availability and morphology of RD cell line.

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