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Cell Attachment Study on Chitosan-Aluminum Monostearate Composite Sponge Containing Seed Methanolic Extract of *Sonneratia caseolaris* (L.) Engl.

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ABSTRACT

Cork tree (*Sonneratia caseolaris* (L.) Engl.) has been historically reported as medicinal plant and food in various countries of Asia. Phenolic compounds such as gallic acid and two flavonoids, luteolin and luteolin-7-O-glucoside, are the main active substances found in cork tree which serving an interesting antioxidant and antimicrobial activities. Previous study was reported about hepatoprotective effect of cork tree extract that was interesting to be use for hepatic disease prevention. Incorporation of the cork tree extract into biodegradable polymeric drug delivery system may help prolong its activity. Chitosan has many attractive properties for applying as biomaterials especially mucoadhesive property that appropriate for attachment of the cell. Adding some additives into chitosan-based materials could improve their physical properties. However, these additives may have effect on cell attachment on chitosan. In this study, chitosan-lactate (CL) sponges and chitosan-aluminum monostearate (CLAlst) composite sponge were prepared. Attachment ability of HepG2 cells (hepatic carcinoma cell) on the prepared sponges was investigated. Moreover, effect of amount of *Sonneratia caseolaris* seed methanolic extract (CSSO) on % cell attachment on the sponges was also evaluated. The results indicated that hydrophobic substance both Alst and CSSO caused reducing of % cell attachment on the chitosan sponge due to a reducing of positive charge of chitosan in the system.

Keywords: chitosan, aluminum monostearate, *Sonneratia caseolaris*, cell attachment.

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INTRODUCTION

Cork tree (*Sonneratia caseolaris* (L.) Engl.) is the lead plant of the mangrove forest. Utilization of cork tree has been reported as medicinal plant and food in many countries of Asia. Squeezed flower juice of cork tree was used as ingredient in antidiuretic drug formulation. Moreover, its flower and leaf could be grinded and used as poultice for healing bruised wound and smallpox [1,2]. Phenolic compound, such as gallic acid, and two flavonoids, e.g. luteolin and luteolin-7-O-glucoside, are the interesting substances in cork tree which have interesting antioxidant activity [3]. Hepatoprotective effect of the cork tree extract as reported previously [4] is interesting to employ its extract as active substance in liver disease prevention and treatment. Chitosan, a positive charge natural polymer, has many attractive properties to apply as biomaterials [5]. Many researchers had developed various systems of chitosan-based materials including tissue engineering structure [6], wound dressing material [7], drug delivery system [5,8], etc. Because of a positive charge, it has excellent mucoadhesive property correlated with high cell attachment ability. Some researchers had improved mechanical properties and degradation properties of the chitosan system by adding different additives [9,10]. However, these additives could affect to cell attachment ability of the chitosan-based materials. Aluminum monostearate (Alst) is a fatty acid derivative of stearic acid. It has been reported as ingredient of vaccine formulations [11] and injectable implants [12]. From our preliminary study indicated that composite system of chitosan and Alst had better thermal stability and mechanical property than that of chitsan base. Moreover, drug release pattern from the composite system was more sustained than chitosan base as well. Therefore, this composite system was interesting to be applied as carrier of bioactive substance.

In this study, methanolic extract of cork tree seed (CSSO) was loaded into chitosan sponges system both with and without Alst. Attachment ability of HepG2 cells (hepatic cancer cell) on the prepared sponges was investigated.

MATERIALS AND METHODS

Materials

Chitosan (Aqua premier, Chonburi, Thailand) having 99.3% degree of deacetylation with molecular weight of 70 kDa was sieved through sieve No. 80 mesh before used. Aluminum monostearate (Fluka, Sigma-Aldrich.Co, Missouri, USA) and lactic acid (85% w/v; Ajax finechem, New South Wales, Australia) were purchased and were analytical grade. *Sonneratia caseolaris* methanolic extract (CSSO) was obtained from our lab by using maceration technique. Minimum Essential Medium (MEM), fetal bovine serum (FBS) and glutamine were obtained from GibcoTM, Auckland, New Zealand.

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Chitosan sponges preparations

4% w/w Chitosan oligomer powder was dissolved overnight in lactic acid solution (2% w/v) to obtain chitosan-lactate (CL) solution before filtered via cellulose fabric. Chitosan-aluminum monostearate (CLAlst) composite dispersion was prepared by dispersing Alst (2.5% w/w) in CL solution for 24 h. Thereafter, 4mL of CL solution and CLALst dispersion were filled in an aluminum foil mold having the same size of the well of 6-well plate. CL and CLAlst sponges were then fabricated using freeze drying technique. The fabricated sponges were then treated by moist heat process using autoclave sterilization (121°C, 15 psi) for 5 minute to reduce their aqueous solubility. The treated sponges were then dried in hot air oven at 65°C and sterilized by exposing UV radiation for 15 minute.

CSSO loading into the prepared sponges

CSSO was dissolved in methanol to obtain concentration of the extract of 5 mg/mL. The extract solution was gradually dropped into the sterilized sponges, CL and CLALst, to obtain various amounts of the extract; 10, 20 and 30 mg/sponge. The sponges were then left in laminar air flow hood overnight to let methanol to evaporate completely before testing with the cells.

Cell attachment study

HepG2 cells were cultured in DMEM media which passage no. 40 was harvested to employ in this study. The test was performed using 6-well cell culture plate by placing the prepared sponges in the wells, thereafter, 8 mL of DMEM was added into the wells and the sponges were left to swell until they fit to the well therefore only upper surface of the sponge was attached by the cells. HepG2 cells were harvested and suspend in serum-free DMEM to obtained concentration of $1x10^6$ cell/mL. Subsequently, 1 mL of the cell suspension was added onto the surface of each sponges using micropipette. Thereafter, sponges seeded with cells were incubated at 37°C, humidified with 5% CO_2 for 48 h. After incubation, each chitosan sponges were gently rinsed using PBS pH7.4 to remove the unattached cells. Subsequently, chitosan sponges were removed from the 6-well plate and the unattached cells and the medium were collected using micropipette. Cells that attached on the bottom of the 6-well plate were collected by tripsinization and were pooled with the cells collected before. Number of the unattached cells of each well was then counted correctly under microscope using hemocytometer. Thereafter, number of the attached cells was calculated and % cell attachment on the sponge was calculated as the following equation.

$$\% \text{ attachment} = \frac{\text{No. of attached cells}}{\text{No. of seeded cells}} \times 100$$

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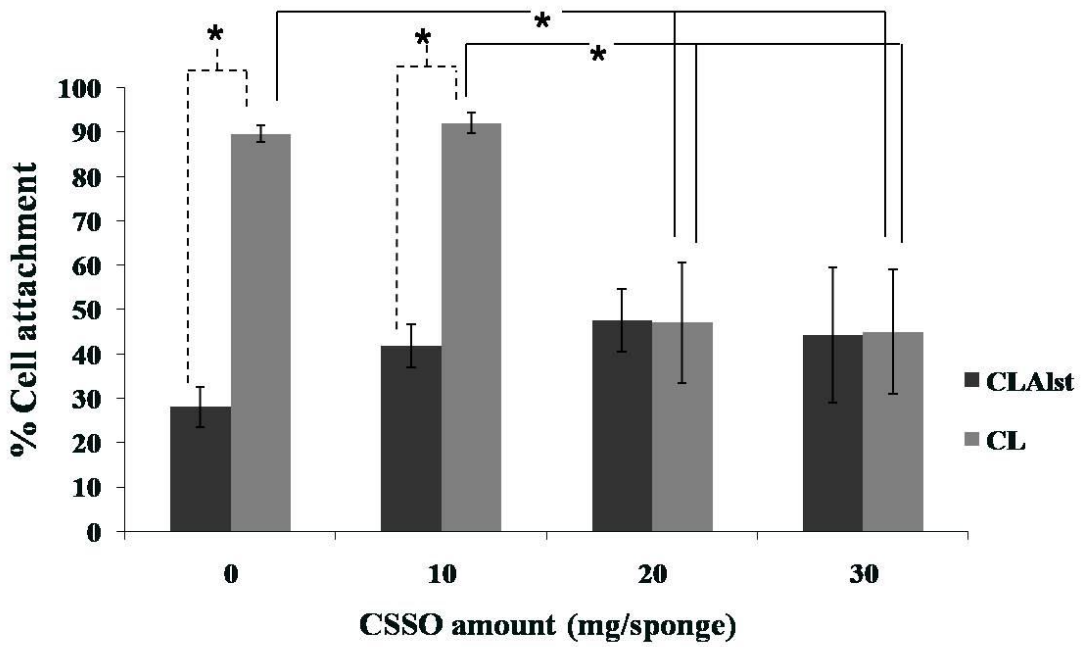
RESULTS AND DISCUSSION

For the CSSO unloaded sponge, number of the attached cells on CL sponge was approximately 60% higher than that of attached on CLAlst sponge as shown in Fig 1.



Since chitosan molecule has amino group ($-NH_2$) which could be ionized to become positive charge, it had high affinity to bind with the negative charge of polysaccharide on cell surface. For CL, its side chain is lactate (C3) molecule that easily be ionized when exposing to water whereas side chains of CLAlst were lactate and stearate (C18) molecules that randomly bound to amino groups of chitosan chain. More hydrophobicity and longer chain length of the stearate side chain might obstruct an access of water molecules; therefore ionization of the amino group was lower led to lowering of number of attached cell on CLAlst sponge. Number of the attached cells on CL sponge containing 10 mg CSSO was not different from CL sponge base. However, at higher amount of the extract, number of the attached cell was significantly reduced. In contrast, amount of CSSO had no effect on number of attached cells on CLAlst sponge system. These different results were expected to be resulted from either amount of CSSO or structural change of the sponges after loading the extract since methanol could cause shrinkage of the chitosan structure. The sponge that contained higher amount of the extract was also exposed to higher amount of methanol. Therefore, more shrinkage was observed for higher CSSO contained system. As CSSO is extracted by using methanol, it is quite hydrophobic thus higher amount of CSSO could reduce the attachment of the cells. After loading the extract, structure of CLAlst was lesser shrinkage comparing to that of CL sponge. Moreover, CLAlst sponge was more hydrophobic and denser due to stearate that contained in the system thus the attachment of the cell on the CLAlst sponge was poor anyway regardless a present of the extract. Therefore, % cell attachment on CLAlst sponge containing different amounts of CSSO was not significantly different.

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CONCLUSION

Hydrophobic substance had effect on % cell attachment on chitosan sponges due to lowering of positive charge of chitosan which lesser % cell attachment was observed for CLAlst sponge that contained fatty acid derivative. Moreover, higher amount of CSSO affected more reducing of % cell attachment on CL sponge but had no effect on CLAlst sponge. Lowering of cell attachment indicated that the CLAst system may not appropriate for using as tissue engineering structure but may advantage to apply this system as wound dressing material since it could reduce pain when removing from the wound after used.

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