CHITOSAN-ALUMINUM MONOSTEARATE COMPOSITE SPONGES CONTAINING ASIATICOSIDE FOR PROMOTING ANGIOGENESIS IN CHRONIC WOUND

By
Miss Kotchamon Yodkhum

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy Program in Pharmaceutical Technology
Graduate School, Silpakorn University
Academic Year 2013
Copyright of Graduate School, Silpakorn University
CHITOSAN-ALUMINUM MONOSTEARATE COMPOSITE SPONGES CONTAINING ASIATICOSIDE FOR PROMOTING ANGIOGENESIS IN CHRONIC WOUND

By
Miss Kotchamon Yodkhum

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy Program in Pharmaceutical Technology
Graduate School, Silpakorn University
Academic Year 2013
Copyright of Graduate School, Silpakorn University
โครงสร้างความพรุนร่วมของไคโตซานและอะลูมินัมโมโนสเตียเรทบรรจุอะเซียติโคไซด์สำหรับกระตุ้นการสร้างเส้นเลือดฝอยในแผลเรื้อรัง

โดย
นางสาวกชมนยอดข่า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิชญาณกิจหาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2556
ลิขสิทธิ์ของบัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
The Graduate School, Silpakorn University has approved and accredited the Thesis title of “Chitosan-aluminum monostearate composite sponges containing asiaticoside for promoting angiogenesis in chronic wound” submitted by Miss Kotchamon Yodkhum as a partial fulfillment of the requirements for the degree of Doctor of Philosophy Program in Pharmaceutical Technology.

The Thesis Advisor

Associate Professor Thawatchai Phaechamud, Ph.D.

The Thesis Examination Committee

Chairman

Member

Member

Member

Member

Member
The main factors that delay wound healing of chronic wounds are low level of growth factors and high level of exudate containing high amount of tissue destructive enzymes. This study aimed to develop exudate absorbent-medical dressing that could solve these problems. Composite sponge of chitosan and aluminum monostearate (Alst) containing asiaticoside (wound healing agent) was developed.

Alst was proved to be able to dissociate in acidic aqueous medium therefore had possibility to form ionic complex with chitosan. Chitosan-Alst composite dispersions were prepared in 2% w/v lactic acid solution and evaluated an effect of chitosan to Alst ratio and mixing time on the properties of the dispersions namely pH value, viscosity and rheology, morphology and particle size of the dispersant. The pH value and viscosity of the dispersion increased by chitosan amount. Particle size of the dispersant of the system containing higher chitosan ratio was larger than that containing lower chitosan ratio, and the size increased by mixing time. Chitosan-lactate (CL) and chitosan-Alst sponges (CLA) were fabricated using lyophilization technique. The sponges were further stabilized by dehydrothermal treatment (DHT). The functional group alteration (amidation) caused by DHT was confirmed by FT-IR.

Hydrophobicity/hydrophilicity of the sponges was evaluated by contact angle measurement. Swelling behavior was studied in three buffers; pH 4, 7.4 and 10. Water sorption, erosion and asiaticoside release were evaluated in PBS pH 7.4. Alst enhanced hydrophobicity of the prepared sponges. Swelling behavior of the sponges was pH-dependent. Alst delayed swelling and decreased water sorption and erosion of the sponges which eventually sustained the drug release for 2 days. The release mechanism was analyzed as first-order kinetic. The sponges were further constructed as wound dressing. N-methyl-2-pyrrolidine (NMP) was added into the system in order to improve homogeneity of the drug and the polymer matrix. Wound dressing properties such as porosity, fluid handling ability, oxygen permeability, bio-adhesion, and asiaticoside release were evaluated. Porosity of the prepared dressings was higher than 85% v/v. Fluid absorbency and water vapor transmission rate (WVTR) of the dressings decreased when the Alst amount increased. Oxygen permeability of all the prepared dressings was apparently high. Alst reduced bio-adhesive property of the dressings. NMP increased homogeneity of asiaticoside in the matrix which induced the retardation of the substance release for 7 days. Cytotoxicity to normal human dermal fibroblast (NHDF) and normal human keratinocyte (NHEK) was investigated using MTT assay. Lactic acid and NMP exhibited dose-dependent toxicity to the both cell types. CL exhibited stimulating effect on NHDF and NHEK proliferation. Asiaticoside also promoted NHDF proliferation but with lesser effect than CL. Dressing extractions were non-toxic to both cell types. Moreover, they exhibited a stimulating effect on the cells proliferation especially for NHDF. Angiogenic activity of asiaticoside was evaluated using chick chorio-allantoic membrane (CAM) assay. The results indicated dose-dependent angiogenic activity of asiaticoside raw material. The asiaticoside-contained dressings seemed exhibit angiogenic activity. However, this might be a false positive effect due to high fluid absorption of the materials. Therefore, other evaluation methods should be performed to further confirm their angiogenic activities.
การศึกษาค่าการดูดซับน้ำ การความหนืดและพฤติกรรมการไหล และรูปร่างและขนาดอนุภาคของอนุภาคสารประกอบไอออนนิกเชิงซ้อนกับไคโตซานได้ ระบบของเหลวผสมของไคโตซานและアセียติโคサイドสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคไซด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมโนสเตียเรตสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคไซด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมโนสเตียเรตสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคไซด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมโนสเตียเรตสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคサイด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมโนสเตียเรตสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคサイด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมโนสเตียเรตสามารถแตกตัวได้ภายใต้สภาวะที่เป็นกรด ทำให้มีโอกาสในการก่อออกซิเจนในแผลเป็นพิษต่อเซลล์ทั้งสองชนิด อะเซียติโคไซด์ก็มีฤทธิ์กระตุ้นการสร้างเส้นเลือดเช่นกัน แต่ให้ผลชัดเจนน้อยกว่า สารสกัดที่ได้จากไคโตซานและอะลูมินัมโมノ
ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my thesis advisor, Assoc. Prof. Dr. Thawatchai Phaechamud for giving me the great opportunity to be a part of graduated school students. This thesis would not have been completed or written without his supervision. I would like to thank him for his kindness support, encouragement and valuable suggestion throughout my study at Faculty of Pharmacy, Silpakorn University. He has been supported me in diverse way that considerably help me to improve my skills in doing research and also for daily living. My deepest gratitude also goes to Assist. Prof. Dr. Juree Charoenteeraroon for her kindness and helpful support. She has been given me the valuable comments to my works especially for the biological evaluation parts. My sincere gratitude also goes to Assoc. Prof. Dr. Sontaya Limmatvapirat, Assist. Prof. Dr. Wiwat Pichayakorn, and Dr. Parichat Chomto for the creative guidance encouragement and valuable comments to this work.

My grateful gratitude also goes to Prof. Dr. Yasuhiko Tabata for giving me the great opportunity in doing part of my research in his laboratory; Department of Biomaterials, Field of tissue engineering, Institute for frontier medical science, Kyoto University, Kyoto, Japan. I would like to thank him for his kindness and precious support, valuable comments and sharing the great attitude in my research work and also in my living in Japan. I also would like to express my gratitude to Assist. Prof. Dr. Masaya Yamamoto for the documentary management, his kindness and patient in training me the cell culture and cell-based assays and also sharing me the valuable attitude to my work. Moreover, I would like to thank all the members of Prof. Tabata’s lab for their warm welcoming, fellowship and precious helps during my staying in Japan.

I gratefully acknowledge the Thailand Research Funds through the Golden Jubilee Ph.D. Program (Grant No. PHD/0074/2551) and Faculty of Pharmacy, Silpakorn University, for the scholarship, laboratory equipment and other facilities to conduct my research and publications.

I would like to express my love towards all of my teachers, staffs, and friends in Faculty of Pharmacy, Silpakorn University, for their support, assistance, and friendship over the years. Special thanks to my lovely sisters, Miss Jongjan Mahadlek, Miss Sureewan Duangit, and Dr. Arissarakorn Sirinararatana for helping me in everything anytime and for their generous support. Special thanks to Miss Pitikarn Karnjanapruk for her kind downloading many online papers for me. I would like to thank and express my love to all the Primpri Island members, Hist gang members, the PDGIG members and the PBIG members for their helps, precious support, encouragement, valuable friendship and memorable moments. I am very appreciated with all of you. I love you all.

Special thanks to all of my beloved family especially mom, dad and my brother for their invaluable love, encouragement, care, and always beside me, understand and support me in everything. I love you all. Finally, an apology is offered to those whom I cannot mention personally one by one here.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>English Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Thai Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>List of Abbreviation</td>
<td>xv</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1    Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2    Review of related literature</td>
<td>6</td>
</tr>
<tr>
<td>3    Materials and Methods</td>
<td>36</td>
</tr>
<tr>
<td>4    Results and Discussion</td>
<td>57</td>
</tr>
<tr>
<td>5    Conclusions</td>
<td>111</td>
</tr>
<tr>
<td>Bibliography</td>
<td>112</td>
</tr>
<tr>
<td>Appendices</td>
<td>123</td>
</tr>
<tr>
<td>Biography</td>
<td>139</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>66</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>81</td>
</tr>
<tr>
<td>9</td>
<td>81</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
</tr>
<tr>
<td>11</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>107</td>
</tr>
<tr>
<td>14</td>
<td>124</td>
</tr>
<tr>
<td>15</td>
<td>124</td>
</tr>
<tr>
<td>Tables</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>16</td>
<td>Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 50, 100 and 200 µg/mL asiaticoside solution (n=3)</td>
</tr>
<tr>
<td>17</td>
<td>Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the inter-day precision test</td>
</tr>
<tr>
<td>18</td>
<td>Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-1 intermediate precision test</td>
</tr>
<tr>
<td>19</td>
<td>Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-2 intermediate precision test</td>
</tr>
<tr>
<td>20</td>
<td>Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-3 intermediate precision test</td>
</tr>
<tr>
<td>21</td>
<td>System suitability parameters of the HPLC curve of 100 µg/mL asiaticoside solution</td>
</tr>
<tr>
<td>22</td>
<td>Raw data of percentage asiaticoside release from CL sponge</td>
</tr>
<tr>
<td>23</td>
<td>Raw data of percentage asiaticoside release from CLA05 sponge</td>
</tr>
<tr>
<td>24</td>
<td>Raw data of percentage asiaticoside release from CLA25 sponge</td>
</tr>
<tr>
<td>25</td>
<td>Raw data of percentage asiaticoside release from CLA50 sponge</td>
</tr>
<tr>
<td>26</td>
<td>Raw data of percentage asiaticoside release from CD dressing</td>
</tr>
<tr>
<td>27</td>
<td>Raw data of percentage asiaticoside release from CD05 dressing</td>
</tr>
<tr>
<td>28</td>
<td>Raw data of percentage asiaticoside release from CD25 dressing</td>
</tr>
<tr>
<td>29</td>
<td>Raw data of percentage asiaticoside release from CD50 dressing</td>
</tr>
<tr>
<td>30</td>
<td>20 degree and d-spacing of the PXRD peak of raw materials, chitosan sponge and chitosan dressings (related to the x-ray diffractogram in the section 3.1.3 and 4.1.3 of the chapter 4)</td>
</tr>
<tr>
<td>Figures</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Ionic bonding between ammonium group of chitosan and carboxylate group of the acid side chain (stearate and lactate molecule) during mixing in 2% lactic acid solution</td>
</tr>
<tr>
<td>2</td>
<td>Asiaticoside molecular structure</td>
</tr>
<tr>
<td>3</td>
<td>Chitin molecular structure</td>
</tr>
<tr>
<td>4</td>
<td>Chitosan molecular structure</td>
</tr>
<tr>
<td>5</td>
<td>Aluminum monostearate molecular structure</td>
</tr>
<tr>
<td>6</td>
<td>NMP molecular structure</td>
</tr>
<tr>
<td>7</td>
<td>Pressure ulcer model</td>
</tr>
<tr>
<td>8</td>
<td>Oxygen permeation apparatus</td>
</tr>
<tr>
<td>9</td>
<td>Four levels of angiogenesis score of micro-vessels response in CAM assay</td>
</tr>
<tr>
<td>10</td>
<td>SEM images of the untreated Alst (A) and the acid-treated Alst at various soaking times; 4 h (B), 8 h (C) and 24 h (D) at 25 and 500 magnifications</td>
</tr>
<tr>
<td>11</td>
<td>FT-IR spectra of the untreated Alst (A), the acid-treated Alst at various soaking times; 4 h (B), 8 h (C) and 24 h (D), and stearic acid (E)</td>
</tr>
<tr>
<td>12</td>
<td>Aluminum quantity of the untreated Alst and the acid-treated Alst at various acid soaking times</td>
</tr>
<tr>
<td>13</td>
<td>X-ray diffractograms of the untreated Alst (A), the acid-treated Alst at various soaking times; 5 min (B), 4 h (C), 8 h (D) and 24 h (E), and stearic acid (F)</td>
</tr>
<tr>
<td>14</td>
<td>DTG curves of stearic acid (A), 8 h-acid treated Alst (B) and untreated Alst (C)</td>
</tr>
<tr>
<td>15</td>
<td>DSC thermograms of the untreated Alst (A), the acid-treated Alst at various soaking times; 5 min (B), 4 h (C), 8 h (D), 12 h (E) and 24 h (F), and stearic acid (G)</td>
</tr>
<tr>
<td>16</td>
<td>Melting temperature range from HSM of the acid-treated Alst at various soaking times (solid circle refers to temperature that the sample begin to melt and an open circle refers to temperature that the sample melted completely)</td>
</tr>
<tr>
<td>17</td>
<td>Rheograms of the chitosan-Alst dispersions at mixing time of 5 min and 24 h where CL1, CL2 and CL4 refer to the system comprising 1, 2 and 4 %w/w chitosan, respectively</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figures</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Mean particle sizes of the chitosan-Alst composite dispersions at various mixing times</td>
<td>64</td>
</tr>
<tr>
<td>19</td>
<td>Morphology of the chitosan-Alst complex under inverted microscope of CL1 (A), CL2 (B) and CL4 (C) obtained from 5 min (1) and 24 h (2) mixing time at magnification of 200</td>
<td>65</td>
</tr>
<tr>
<td>20</td>
<td>FT-IR spectra of A = raw materials where 1=Alst and 2 = chitosan, B and C = lyophilized and DHT sponges, respectively, where 1 = CL, 2 = CLA05, 3 = CLA25 and 4 = CLA50</td>
<td>67</td>
</tr>
<tr>
<td>21</td>
<td>Possibility of changes in ionic bond between ammonium group of chitosan and carboxylate group of acid side chain (lactate and stearate molecule) where 1 indicates amidation, 2 indicates no changes and 3 indicates evaporation of lactic acid after DHT</td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>X-ray diffractograms of chitosan (A), Alst (B), and stearic acid (C) (The 2(^\theta) degree and the d-spacings were shown in appendix II)</td>
<td>70</td>
</tr>
<tr>
<td>23</td>
<td>X-ray diffractograms of the prepared sponges; CL (1), CLA25 (2), CL-DHT (3), and CLA25-DHT (4) (The 2(^\theta) degree and the d-spacings were shown in appendix II)</td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>SEM images of the prepared sponges at 200x magnification; A = lyophilized, B = DHT which 1 = CL, 2 = CLA05, 3 = CLA25 and 4 = CLA50</td>
<td>71</td>
</tr>
<tr>
<td>25</td>
<td>TG curves of 1 = Alst, 2 = chitosan, 3 = lyophilized CLA25, and 4 = CL</td>
<td>72</td>
</tr>
<tr>
<td>26</td>
<td>DTG curve of A; 1= chitosan, 2= Alst and B; 1= CL, 2= lyophilized CLA25...</td>
<td>73</td>
</tr>
<tr>
<td>27</td>
<td>DSC thermograms of the lyophilized sponges (CL and CLA25)</td>
<td>74</td>
</tr>
<tr>
<td>28</td>
<td>Morphology of chitosan powder and the lyophilized sponges (CL and CLA25) under hot- stage microscope</td>
<td>74</td>
</tr>
<tr>
<td>29</td>
<td>Contact angle of the water drop on the surface of the lyophilized (A) and DHT (B) sponges</td>
<td>75</td>
</tr>
<tr>
<td>30</td>
<td>Water sorption and erosion of the lyophilized and DHT sponges immersed in phosphate buffer pH 7.4 overnight</td>
<td>76</td>
</tr>
<tr>
<td>31</td>
<td>Digital images and swelling degree of the DHT sponges in three different buffer solutions at various immersion times</td>
<td>78</td>
</tr>
</tbody>
</table>
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>Asiaticoside release from the DHT sponges studied in phosphate buffer pH 7.4 at 37°C for 48 h (The raw data were shown in Appendix I.)</td>
<td>79</td>
</tr>
<tr>
<td>33</td>
<td>SEM images of the DHT sponges before (A) and after (B) drug release study in phosphate buffer pH 7.4</td>
<td>80</td>
</tr>
<tr>
<td>34</td>
<td>FT-IR spectra of the raw materials [A] where (a) = chitosan, (b) = Alst, (c) = lactic acid, (d) = NMP and (e) = asiaticoside, and lyophilized [B] and DHT dressings [C] where (1) = CD, (2) = CD05, (3) = CD25 and (4) = CD50</td>
<td>83</td>
</tr>
<tr>
<td>35</td>
<td>X-ray diffractograms of the raw materials [chitosan (a), Alst (b), stearic acid (c), and asiaticoside (d)] and the lyophilized [A] and DHT [B] dressings where (1), (2), (3), and (4) indicate CD, CD05, CD25, and CD50, respectively (The 20 degree and the d-spacing were shown in appendix II)</td>
<td>84</td>
</tr>
<tr>
<td>36</td>
<td>SEM images at 50x magnification of the prepared sponge dressings where A is the surface, B is the bottom and C is the cross-sectioned view</td>
<td>85</td>
</tr>
<tr>
<td>37</td>
<td>Porosity of the lyophilized and DHT sponge dressings</td>
<td>85</td>
</tr>
<tr>
<td>38</td>
<td>Mechanical strength of the lyophilized and DHT sponge dressings obtained from texture analysis in compression mode</td>
<td>86</td>
</tr>
<tr>
<td>39</td>
<td>Contact angle of the water drop on the surface of the lyophilized (A) and DHT (B) sponge dressings</td>
<td>87</td>
</tr>
<tr>
<td>40</td>
<td>Morphology of the DHT CD and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface</td>
<td>89</td>
</tr>
<tr>
<td>41</td>
<td>Morphology of the DHT CD05 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface</td>
<td>90</td>
</tr>
<tr>
<td>42</td>
<td>Morphology of the DHT CD25 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface</td>
<td>91</td>
</tr>
</tbody>
</table>
### List of Figures

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>Morphology of the DHT CD50 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface</td>
</tr>
<tr>
<td>44</td>
<td>Morphology of gauze and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of gauze coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of gauze that was contacted with the agar surface</td>
</tr>
<tr>
<td>45</td>
<td>Fluid absorbency of the DHT sponge dressings and gauze during fluid handling ability test (n=3)</td>
</tr>
<tr>
<td>46</td>
<td>Water loss of the control (pressure ulcer model), the DHT sponge dressings and gauze during fluid handling ability test (n=3)</td>
</tr>
<tr>
<td>47</td>
<td>Work of adhesion of the DHT sponge dressings and the non-adhesive plaster (positive control) where (*) indicates statistically different from the other samples when analyzed by one-way ANOVA and Post-hoc test at p value &lt; 0.05 (n=3)</td>
</tr>
<tr>
<td>48</td>
<td>Released amount of asiaticoside from the DHT sponge dressings studied by immersion method in PBS pH 7.4 at 37°C, 50 rpm (n=3) [The raw data were shown in Appendix I.]</td>
</tr>
<tr>
<td>49</td>
<td>Percentage erosion of the DHT sponge dressings after release study for 168 h in PBS pH 7.4 where (*) indicates statistically different comparing with the other samples when analyzed by one-way ANOVA and Post-hoc test at p value &lt;0.05 (n=3)</td>
</tr>
<tr>
<td>50</td>
<td>Swelling degree of the DHT sponge dressings performed in PBS pH 7.4 for 168 h (n=3)</td>
</tr>
<tr>
<td>51</td>
<td>Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to chitosan-lactate and the extraction of chitosan powder for 24 h. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (x) exhibited in chitosan powder chart meaning to the fold of the concentration. The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value &lt;0.05 (n=4)</td>
</tr>
</tbody>
</table>
List of Figures

52  Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to various concentrations of lactic acid and NMP for 24 h. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4). .......................... 102

53  Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to various concentrations of asiaticoside and asiatic acid for 24 h. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4).... 103

54  Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to various dilution concentrations of the sponge extractions (CD and CD25) for 24 h. AC refers to asiaticoside. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (x) exhibited in the charts meaning to the fold of the concentration. The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4)………………………………… 104

55  Morphology of CAM treated by PBS pH 7.4 (negative control) (A) and various amount of VEGF (positive control); 100 ng (B), 200 ng (C), and 300 ng (D) at day-9 (disc placing) and day-12 (scoring angiogenic activity) ……… 108

56  Morphology of CAM treated by various amount of asiaticoside; 40 µg (E), 80 µg (F), 160 µg (G), and 320 µg (H), at day-9 (disc placing) and day-12 (scoring angiogenic activity), the white arrows indicated responding of the blood vessels to asiaticoside-loaded disc …………………………… 109

57  Morphology of CAM treated by asiaticoside-loaded sponge dressings; CD, CD05 and CD25 at day-9 (sample placing-day) and day-12 (result observation-day)………………………………………………………….. 110

58  Calibration curve of asiaticoside ................................. 125

59  HPLC spectrum of the 100 µg/mL asiaticoside standard dissolved in PBS pH 7.4 with retention time of asiaticoside peak at 3.318 min and area under the curve of 332.45627 ………………………………………… 126
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>% w/v</td>
<td>percent weight by volume</td>
</tr>
<tr>
<td>% w/w</td>
<td>percent weight by weight</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>ε</td>
<td>epsilon</td>
</tr>
<tr>
<td>ρ</td>
<td>rho</td>
</tr>
<tr>
<td>®</td>
<td>trademark</td>
</tr>
<tr>
<td>™</td>
<td>trademark</td>
</tr>
<tr>
<td>±</td>
<td>plus per minus</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>μg</td>
<td>microgram(s)</td>
</tr>
<tr>
<td>μL</td>
<td>microliter(s)</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer(s)</td>
</tr>
<tr>
<td>2θ</td>
<td>two theta</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>Alst</td>
<td>aluminum monostearate</td>
</tr>
<tr>
<td>AR grade</td>
<td>analytical reagent grade</td>
</tr>
<tr>
<td>atm</td>
<td>atmospheric pressure</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic-fibroblast growth factor</td>
</tr>
<tr>
<td>C18</td>
<td>18 carbon atoms</td>
</tr>
<tr>
<td>CAM</td>
<td>chick chorio-allantoic membrane</td>
</tr>
<tr>
<td>CL</td>
<td>chitosan-lactate</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter(s)</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>wavenumber</td>
</tr>
<tr>
<td>cm²</td>
<td>square centimeter</td>
</tr>
<tr>
<td>CO., LTD.</td>
<td>Company Limited</td>
</tr>
<tr>
<td>D/cm²</td>
<td>dyne per square centimeter</td>
</tr>
<tr>
<td>DD</td>
<td>deacetylation degree</td>
</tr>
<tr>
<td>DHT</td>
<td>dehydrothermal treatment</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>et al.</td>
<td>and others</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>GFs</td>
<td>growth factors</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
</tr>
<tr>
<td>KeV</td>
<td>kiloelectron volt(s)</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram(s)</td>
</tr>
<tr>
<td>Log</td>
<td>logarithm</td>
</tr>
<tr>
<td>m²</td>
<td>square meter(s)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter(s)</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter(s)</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteinase enzymes</td>
</tr>
<tr>
<td>Mw</td>
<td>molecular weight</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram(s)</td>
</tr>
<tr>
<td>NHDF</td>
<td>normal human dermal fibroblast</td>
</tr>
<tr>
<td>NHEK</td>
<td>normal human epidermal keratinocyte</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer(s)</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>No.</td>
<td>number</td>
</tr>
<tr>
<td>OP</td>
<td>oxygen permeability</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer system</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>pH</td>
<td>hydrogen ion concentration</td>
</tr>
<tr>
<td>RO</td>
<td>reverse osmosis</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>V</td>
<td>volt(s)</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WVTR</td>
<td>water vapor transmission rate</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1. Statement and significance of the research problem

Chronic wound is defined as tissue injuries that have not healed within 12 weeks and often reoccur (Boateng et al., 2008). Delayed healing characteristic of chronic wound is influenced by many factors including bacterial infection, lowering of growth factors level and high exudate level. Normally, exudate has important role in all stages of wound healing in maintaining moisture and providing nutrient to the wound. However, exudate in chronic wound differs from acute wound with relatively higher level of tissue destructive proteinase enzyme, therefore more corrosive (Mustoe et al., 2006). Thereby, therapeutic approach for chronic wound should be emphasized at tissue regeneration stimulation and exudate management.

To stimulate tissue regeneration in chronic wound, many researchers have attempted notably to promote angiogenesis by deliver the angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic-fibroblast growth factor (bFGF) and/or platelet-derived growth factor (PDGF) to the wound area as reported previously (Ruel & Sellke, 2003; Bauer et al., 2005; Roy et al., 2011; Koria, 2012; Wietecha & DiPietro, 2013). This technique offers great potential and exhibits more rapid healing result than the other techniques like cell or gene delivery and mechanical stimulation (Phelps & Garcia, 2009). However, the angiogenic growth factors have limitations of their poor stability and dramatically high cost.

Besides angiogenic growth factors, natural derived substances such as asiaticoside from Centella asiatica (L.) Urban, resveratrol from Vitis spp. and ginsenoside Rg1 from Panax ginseng possess the angiogenic activities (Fan et al., 2006; Morgan & Nigam, 2013) that can be used for promoting angiogenesis in wound healing application. Asiaticoside is one of triterpene glycosides found in C. asiatica (L.) Urban. It possesses many interesting biological activities including angiogenic activity, collagen type I synthesis promotion and fibroblast proliferation stimulation that benefit for wound healing process (Shukla et al., 1999; Lu et al., 2004; Kimura et al., 2008). From aforementioned properties of asiaticoside, it is interesting to be employed as active compound in chronic wound diseases treatment.

To manage exudate level in chronic wound, the modern wound dressing such as exudate-absorbing dressing is required (Ovington, 2007). The key function of this dressing type should be removal of excess exudate while maintaining moisture at the wound area.
Foam dressings and sufficient porous scaffolds possess various properties that match for this application such as high fluid absorbency, high oxygen penetration, high water vapor transmission rate (WVTR), and maintaining moisture in the wound and providing thermal insulation (Boateng et al., 2008).

Chitosan porous structure has been popular in tissue engineering application as a tissue culture scaffold and wound dressing (Croisier & Jérôme, 2013). Chitosan itself possesses the interesting properties for wound healing application such as collagen synthesis stimulation and integrin engagement promotion. It also enhances the expression of cytokines and growth factors that can stimulate the wound healing and angiogenesis (Arca & Şenel, 2008; Muzzarelli, 2009). Chitosan dressing properties meet almost requirements of the ideal wound dressing such as biocompatible and gas exchangeable. It can keep the moist environment, protect the wound from infection of microbial organisms, and absorb the wound exudates. Moreover, it exhibits excellent fluid sorption property that suitable for medium to high exuding wound such as chronic wound.

Lyophilization is a general simple and efficient technique to prepare chitosan sponges which the solvent of the frozen chitosan solution is sublimated under reduced pressure. Normally, if acid solvent still exists in the structure after lyophilization, the sufficient neutralization process is necessary to remove the excess acid molecules in order to stabilize the chitosan structure in aqueous medium (Nwe et al., 2009). This process can be performed by soaking the lyophilized sponge in serial dilution of alkaline solution such as sodium hydroxide (NaOH) aqueous solution mixed with ethanol. Subsequently, the neutralized sponge has to be dried again by lyophilization. This process can result in structural shrinkage or loosing materials feature at nano level. Other preparation technique that can avoid the neutralization process such as supercritical fluid has been reported elsewhere (Duarte et al., 2009; Duarte et al., 2010). This method needs more special equipment and more complex preparation process than that of a simple lyophilization method. Moreover, low yield of product is produced and involves with using organic solvents.

Recent trend in wound dressing research is now concerning about incorporation of active agent(s) such like antibiotics and/or growth factors into the dressing in order to increase wound healing rate and efficacy (Boateng et al., 2008; Kim et al., 2011). However, it is rather difficult to manipulate the drug release from sponge because of its high porous character. In case of chitosan porous material, high porosity of the structure and hydrophilic nature of chitosan itself can be the main factors that promote too rapid release of the loaded active substance especially for the water-soluble substance. Therefore, reducing hydrophilicity of the chitosan might modify this polymeric material as controlled release system.

Some studies synthesized the hydrophobic chitosan for using as controlled drug delivery system. Techniques to prepare the hydrophobic chitosan could be conducted
by covalently connecting hydrophobic molecules, normally fatty acids, to free amino groups of chitosan via chemical reactions which were generally complex, takes several steps and using organic solvents and/or high temperature (Tien et al., 2003; Hu et al., 2006). According to review literature, there were two interesting information that inspired us a new simple technique to prepare hydrophobic chitosan sponges that stable in aqueous medium without using any organic solvent, surfactant, high temperature during mixing and it could avoid the neutralization of the product. The first information is an ability of metal stearates such as magnesium stearate and aluminum stearates to be dissociated into metal ion and stearate ion in dilute acidic medium by simple dispersion (The Metal Carboxylates Coalition, 2003; Phaechamud et al., 2009). This information inspired us a possibility of preparing hydrophobic chitosan sponges by simple process of mixing metal stearate with chitosan in dilute acidic aqueous solution prior to lyophilization. During mixing, we expected that negative charge stearate ion dissociated from metal stearate would form ionic bond with positive charge ammonium ion of chitosan as illustrated in Fig. 1. The second information is an effect of high temperature on amidation or transformation of water soluble ionic bond between ammonium group and carboxylate group in chitosan salt film (Zotkin et al., 2004; Mudarisova et al., 2009) and scaffolds (Kim et al., 2004) to amide bond that stable in aqueous medium. Amidation was occurred when water molecule was removed from ionic bond by high temperature (higher than 100°C). This technique may be an alternative approach to stabilize the chitosan sponge in aqueous medium without performing neutralization. Thermooxidative degradation of chitosan was found after treated the chitosan film at high temperature under air atmosphere which caused darkening of the film (Zotkin et al., 2004), therefore we decided to treat our materials under vacuum environment.

**Fig. 1** Ionic bonding between ammonium group of chitosan and carboxylate group of the acid side chain (stearate and lactate molecule) during mixing in 2% lactic acid solution
In this study, composite system of chitosan and Alst was developed as a carrier of asiaticoside in form of sponge. Possibility of Alst dissociation in lactic acid solution was studied. Effects of chitosan-Alst ratio and mixing time on physicochemical properties of the chitosan-Alst dispersions were investigated. The dispersions were further fabricated by lyophilization and stabilized by dehydrothermal treatment (DHT). Physicochemical properties of the fabricated system comparing between before and after DHT such as functional groups interaction, crystallinity, swelling behavior, hydrophobicity, mechanical strength, thermal behavior and release behavior of asiaticoside were evaluated. Finally, chitosan-Alst composite sponges containing NMP as co-solvent of asiaticoside were fabricated as wound dressing. Wound dressing properties such as morphology and pore size, porosity, fluid handling ability (WVTR and absorbency), oxygen penetration rate, bioadhesion, and asiaticoside release were investigated. Effect of the sponge’s components on cell viability was studied in normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK). Angiogenic activity of asiaticoside was studied using chick chorio-allantoic membrane (CAM) assay.

2. Objectives of this study

1. To develop chitosan-Alst composite sponge containing asiaticoside as wound dressing for chronic wound

2. To study a dissociation ability of Alst in 2% lactic acid solution

3. To evaluate the effect of chitosan/Alst ratio and mixing time on the physicochemical properties of chitosan-Alst composite dispersions

4. To evaluate the effect of DHT and Alst amount on physicochemical properties of chitosan-Alst composite sponges

5. To prepare and evaluate wound dressing properties of the chitosan-Alst sponge dressings containing N-methyl-2-pyrrolidone (NMP)

6. To study cytotoxicity of the sponges components and the sponges extractions in NHDF and NHEK

7. To evaluate angiogenic activity of asiaticoside in CAM model
3. **Hypothesis of this study**

1. Chitosan-Alst composite sponge containing asiaticoside can be successfully developed as wound dressing for chronic wound

2. Alst can be dissociated in 2% lactic acid solution

3. Chitosan to Alst ratio and mixing time influence physicochemical properties of chitosan-Alst composite dispersions

4. DHT and Alst amount have effect on physicochemical properties of chitosan-Alst composite sponges

5. The chitosan-Alst dressings containing NMP exhibits appropriate wound dressing properties

6. The sponge components and the sponge extractions are non-toxic to the NHDF and NHEK

7. Asiaticoside exhibits angiogenic activity in CAM model
CHAPTER 2
LITERATURE REVIEWS

1. Wound and wound healing
   1.1. Wound healing process
   1.2. Chronic wound
   1.3. Angiogenesis, angiogenic substances and wound healing
   1.4. Wound dressing
      1.4.1. Properties of the ideal wound dressing
      1.4.2. Classification of wound dressing
         1.4.2.1. Traditional dressings
         1.4.2.2. Modern wound dressings

2. Asiaticoside
   2.1. Physicochemical properties
   2.2. Analysis method
   2.3. Biological activities
   2.4. Wound healing and angiogenic activities
   2.5. Pharmaceutical preparations of asiaticoside

3. Chitosan
   3.1. General information
   3.2. Physicochemical and biological properties
      3.2.1. Solubility
      3.2.2. Thermal properties
      3.2.3. Biodegradation
   3.3. Biological activities
   3.4. General applications
      3.4.1. Drug delivery system
      3.4.2. Tissue regeneration and wound healing
3.5. Chitosan-based wound dressing
   3.5.1. Films/Membranes
   3.5.2. Hydrogels
   3.5.3. Nanofibers
   3.5.4. Foam/ Sponges

4. Aluminum monostearate

5. N-methyl-2-pyrrolidone
   5.1. General information
   5.2. Pharmacokinetic information
   5.3. Safety
   5.4. Pharmaceutical application

6. In vitro drug release study and drug release kinetic of porous matrix drug delivery system
   6.1. In vitro drug release study
   6.2. Drug release kinetics of the porous matrix

7. Kinetic model for drug release profile analysis
   7.1. Zero-order
   7.2. First-order
   7.3. Higuchi model
   7.4. Power law (Korsmeyer-Peppas) model

8. MicroMath® Scientist™ for Windows™

9. Angiogenesis assay
   9.1. In vitro models
      9.1.1. Endothelial cell assays
      9.1.2. Organ culture assays
   9.2. In vivo models
1. Wound and wound healing

Wound can be described as a defect or a break of the skin which is a result of physical or thermal damage or as a result of underlying physiological condition. It can be classified as acute and chronic wound based on the nature of healing process. Acute wound can heal completely within 8-12 weeks whereas chronic wound cannot heal completely within 12 weeks and often reoccur (Boateng et al., 2008).

1.1. Wound healing process

Wound healing is a complex and dynamic process co-operated by many factors including cells, inflammatory cytokines, growth factors and extracellular matrix (ECM) components. Normally, wound healing comprised of 4 overlapping stages; hemostasis, inflammation, proliferation, and remodeling (maturation) phase (Orsted et al., 2011). Detail of each stage is informed in Table 1.

Immediately after tissue injury, platelets play an important role in hemostasis. Platelets release vasoconstrictors, to constrict vessels at the wound area, together with blood clotting factors. They become activated and secrete adhesive glycoprotein, leading to platelets aggregation. They also secrete factors that activate intrinsic clotting cascades (transformation of thrombin to fibrinogen accompanying by fibrinogen transformed to fibrin) in order to form stable hemostatic plug. Moreover, platelet release PDGF to initiate subsequent healing steps.

Table 1 The 4 phases of wound healing (Orsted et al., 2011)

<table>
<thead>
<tr>
<th>Phase of healing</th>
<th>Time post injury</th>
<th>Cells involved in phase</th>
<th>Function or activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemostasis</td>
<td>Immediate</td>
<td>Platelets</td>
<td>Clotting</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Day 1-4</td>
<td>Neutrophils, Macrophages</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Day 4-21</td>
<td>Macrophages, Lymphocytes, Angiocytes, Neurocytes, Fibroblasts, Keratinocytes</td>
<td>Fill defect, Re-establish skin function, Closure</td>
</tr>
<tr>
<td>Remodeling (maturation)</td>
<td>Day 21-2 years</td>
<td>Fibrocytes</td>
<td>Develop tensile strength</td>
</tr>
</tbody>
</table>

Next step is inflammation which neutrophils and monocytes are recruited to the wound bed. Neutrophils clean up the debris and microorganisms and also release intracellular enzymes to digest fibrin matrix and surrounding tissue. Thereafter, the degradation products attract the other cells in the next step such as fibroblasts and epithelial cells. Monocytes differentiate to macrophages after they exit from the
blood vessels. Macrophages function like neutrophils by cleaning up wound area together with releasing tissue destructive enzymes (MMPs), cytokines and growth factors to generate the next step, proliferation.

During proliferation phase, many activities including angiogenesis, collagen deposition, granulation tissue formation, wound contraction and epithelialization take place. The in-growth capillaries and lymphatic vessels and collagen synthesized by fibroblasts facilitate tissue granulation.

1.2. Chronic wound

Chronic wound fails to heal due to repeated insults or underlying physiological conditions such as diabetes, malignancy, persistent infection or chronic inflammatory diseases. These conditions disrupt orderly sequence of wound healing process. Chronic wounds are often stalled in the inflammatory process and therefore produce exudate for long periods. Impaired healing of chronic wound has been found in combination with deficiency of active molecules necessary for tissue regeneration such as growth factors and high exudate level containing high amount of inflammatory cytokines and tissue destructive enzymes (Mustoe et al., 2006; Broderick, 2009). Moreover, too high exudate level can cause maceration in the wound bed and lead to bacterial infection. This is another problem of chronic wound treatment.

Growth factors (GFs), for example epidermal growth factor (EGF), PDGF, VEGF and bFGF, have effect on chemotaxis, cell proliferation induction (mitosis), angiogenesis and ECM synthesis and degradation during wound healing (Traversa & Sussman, 2001). In chronic wound, growth factors can be destroyed by MMPs presented in wound exudate with too high amount and too long time, so the GFs level are decreased (Gibson et al., 2009). Growth factors, as exogenous molecules, have been delivered to the wound bed to treat the delayed healing wound for many decades (Meyer-Ingold, 1993). This is popular in research field because it may offer great potential and exhibits rapid result. However, the growth factors have limitations of poor stability and very high cost. Moreover, at high amount they can promote unstable blood vessels growth, side effect at non-target sites and may cause a cancer in person involving with cancer risk.

Moist wound therapy is now the most accepted strategy for wound management. Moist wound healing refers to the provision and maintenance of optimal hydration of the exposed tissues in the wound as opposed to allowing or encouraging those tissues to dehydrate and dry out (Ovington, 2007). Since 1970s, various advance wound dressings have been developed in order to manage moisture of the wound. This kind of dressing possesses advantages in terms of improving healing rates and tissue restoration. Moreover, it can reduce the pain, infection and overall
health care costs. Moist wound healing dressings can be categorized into 3 kinds regard to their effects on tissue moisture levels including dressings that absorb excessive wound exudate, dressings that maintain existing levels of tissue moisture and those that add moisture to the tissues. In case of chronic wounds that have high exudate level, absorbent dressing should be employed.

1.3. Angiogenesis, angiogenic substances and wound healing

Blood circulation system is crucial for tissue survival since it brings oxygen, nutrients and other survival factors to the tissue. In the case of tissue severely damaged, blood vessels are also destroyed and may not be completely regenerated. This may delay the wound healing or tissue regeneration. Therefore, regeneration of blood vessels or therapeutic angiogenesis is needed for effectively regeneration of the damaged tissue.

Delivering angiogenic growth factors (proteins such as VEGF or bFGF) directly into the damaged tissue is popular in research field since it may offer great potential and exhibits more rapid result than the other techniques. However, angiogenic growth factors have limitations of its poor stability and very high cost. Moreover, at high amount they can promote unstable blood vessels growth, side effect at non-target sites and may cause cancer in person involving with cancer risk (Li & Li, 2003; Fischbach & Mooney, 2007).

Besides angiogenic growth factors, natural derived substances such as ginsenoside Rg1 from Panax ginseng, β-sitosterol found in Aloe vera, resveratrol from Vitis spp. and asiaticoside from C. asiatica (L.) Urban possess the angiogenic activities (Fan et al., 2006; Morgan & Nigam, 2013) that can be used as alternative agent for therapeutic angiogenesis.

1.4. Wound dressing

A variety of wound dressings are available ranging from passive adherent/non-adherent to interactive and bioactive products that contribute to the healing process. Advance wound dressings are designed to create a moist wound healing environment which allows the wound fluids and growth factors to remain in contact with wound, thus promoting autolytic debridement and accelerating wound healing. Presently it appears that no single material can produce the optimum microenvironment for all wounds or for all the stages of the wound healing process. Different type of wound dressings required for each wound types were reported previously as demonstrated in Table 2 (Watson & Hodgkin, 2005).
Table 2 Dressing for different wound types (Watson & Hodgkin, 2005)

<table>
<thead>
<tr>
<th>Wound type</th>
<th>Required dressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry necrosis, Dry slough wound</td>
<td>Hydrogel, Hydrocolloid</td>
</tr>
<tr>
<td>Wet, Loose slough wound</td>
<td>Dry hydrofibre</td>
</tr>
<tr>
<td>Granulating superficial wound</td>
<td>Foam, Hydrocolloid</td>
</tr>
<tr>
<td>Granulating cavity wound</td>
<td>Hydrofibre, Alginate</td>
</tr>
<tr>
<td>Epithelializing wound</td>
<td>Low adherent dressing, Hydrocolloid, Film</td>
</tr>
<tr>
<td>Infected wound</td>
<td>Dressing containing silver, iodine or antibiotic</td>
</tr>
</tbody>
</table>

1.4.1. Properties of the ideal wound dressing

An ideal dressing should maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms and remove excess exudates. It should also be non-toxic, non-allergenic, non-adherent and easily removed without trauma, it should be made from a readily available biomaterial that requires minimal processing, possesses antimicrobial properties and promotes wound healing (Jayakumar et al., 2011).

1.4.2. Classification of wound dressing

1.4.2.1. Traditional dressings

Cotton wool, natural or synthetic bandages and gauzes are traditional dressing have been used for long history. These dressings are dry and do not provide a moist wound environment. They may be used as primary or secondary dressings, or form part of a composite of several dressings in order to apply in a specific function (Boateng et al., 2008).

1.4.2.2. Modern wound dressings

Modern wound dressings possess essential characteristic such like keeping a moist environment around the wound to facilitate wound healing. The modern dressings are mainly classified according to the materials from which they are produced including hydrocolloids, alginates and hydrogels, and generally occur in the form of gels, thin films or membrane and foam sheets (Boateng et al., 2008). Each form of the dressing is suitable for different wound condition as described in the following details.

Fiber dressings

Nanofiber matrices have shown tremendous promise as tissue engineering scaffolds for skin substitutes. Nanofiber dressings have special
properties as compared to the conventional dressings due to their high surface area and high porosity such as high absorbency, high gas permeation, comfortability owing to its flexability, and most importantly, morphological similarity to natural extracellular matrix (ECM) in skin, which promote cell adhesion migration and proliferation (Zahedi et al., 2010).

**Hydrogel dressings**

Hydrogels are insoluble, swellable hydrophilic materials made from synthetic polymers such as poly(methacrylates) and polyvinylpyrrolidone. They are non-adherent and cool the surface of the wound, which may lead to a marked reduction in pain and therefore have high patient acceptability. Hydrogels contain significant amounts of water (70–90%) therefore promote moist healing. However, they cannot absorb much exudate, thus they are used for light to moderately exuding wounds. In addition, hydrogels have low mechanical strength and therefore difficult to handle which may affect patient compliance. Hydrogels are suitable for use at all four stages of wound healing with the exception of infected or heavily exuding wounds (Boateng et al., 2008).

**Film/Membrane dressings**

Hydrophilic polymeric membranes have a high swellability and permeability for water vapor and gasses, good fluid transport via the membrane, and a high selectivity for the transport of polar substances. These properties in combination with an adequate mechanical strength make them highly desirable for the treatment of wounds as a coverage material (Boateng et al., 2008).

**Foam dressings**

Foam dressings are porous and can maintain a moist environment together with providing thermal insulation around the wound. They are highly absorbent, absorbency being controlled by foam properties such as texture, thickness and pore size. The open pore structure also gives a high WVTR and oxygen permeability. The porous structure of the dressings, make them suitable for partial- or full-thickness wounds with minimal or moderate drainage, to highly absorbent structures for heavily exuding wounds. Due to their highly absorbency that can reduce moisture of the wound, foam dressings are not suitable for dry epithelialising wounds or dry scars (Boateng et al., 2008; Sweeney et al., 2012).
2. Asiaticoside

Asiaticoside is a major triterpene glycoside found in *C. asiatica* (Linn.) Urban. This herb has been used for long history in Ayurvedic and Chinese medicine to treat skin diseases, gastro-intestinal tract and psychotropic abnormalities (Dev, 1999; Jamil et al., 2007).

2.1. Physicochemical properties

Asiaticoside (C\textsubscript{48}H\textsubscript{78}O\textsubscript{19}) is white odorless powder with molecular weight of 959.12 and melting point in the range of 230-233°C. Its chemical structure is related to those of cholesterol, steroid and sex hormone (El-Hefnawi, 1962) as shown in Fig. 2. On hydrolysis this glycoside turned into triterpene acid; asiatic acid. Solubility of asiaticoside was studied in various solvents namely water, methanol, ethanol, n-propanol, n-butanol and a methanol-water mixture at temperature range of 278.15 to 343.15 K (Zheng & Lu, 2011). The solubility of asiaticoside in different solvents and in methanol/water mixture at various temperatures is shown in Table 3 and 4, respectively.

![Asiaticoside molecular structure](image)

**Fig. 2** Asiaticoside molecular structure

2.2. Analysis method

General method used for analysis of asiaticoside is high performance liquid chromatography (HPLC) which either isocratic or gradient mode was also reported. Previous studied (Verma et al., 1999) reported an isocratic method using reverse-phase HPLC which an octadecyl silane-packed column (C18) was employed. Water (containing 1% trifluoroacetic acid): methanol (30:70 v/v) was used as mobile phase at flow rate of 1 mL/min. Eluted asiaticoside was detected using UV detector at 220 nm. Obtained retention time of asiaticoside was approximately 5 min with percentage recovery of 97. Another study (Jain & Agrawal, 2008) reported using
HPLC in gradient mode to analyze asiaticoside in *C. asiatica* (L.) Urban extract. Mobile phase was comprised of 0.3% orthophosphoric acid buffer and acetonitrile. Column C-18 (Phenomenex-Luna 5 μ) was employed. The flow rate was set at 1.8 mL/min. Asiaticoside was detected by using UV Detector at 210 nm. Retention time of the asiaticoside peak was approximately 12 min. Other analytical methods such as TLC-densitometry technique (Sikareepaisan *et al.*, 2008) and UV spectroscopy (Wang *et al.*, 2009) have also been reported.

Table 3 Solubilities (x: mole fraction) of asiaticoside at different temperatures in five solvents, together with error limits using the 95% confidence level (Zheng & Lu, 2011)

<table>
<thead>
<tr>
<th>T/K</th>
<th>10^3 x_water</th>
<th>10^3 x_methanol</th>
<th>10^3 x_ethanol</th>
<th>10^3 x_n-propanol</th>
<th>10^3 x_n-butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>278.15</td>
<td>0.9023±0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>283.15</td>
<td>0.9037±0.0039</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>288.15</td>
<td>0.9728±0.002</td>
<td>1.0254±0.0036</td>
<td>1.0341±0.0034</td>
<td>0.9805±0.0032</td>
<td>0.9244±0.0028</td>
</tr>
<tr>
<td>293.15</td>
<td>0.9019±0.0001</td>
<td>1.1264±0.0027</td>
<td>1.1404±0.0003</td>
<td>0.9052±0.0002</td>
<td>0.9940±0.0002</td>
</tr>
<tr>
<td>303.15</td>
<td>0.9021±0.0001</td>
<td>1.2209±0.0017</td>
<td>1.2425±0.0004</td>
<td>0.9006±0.0003</td>
<td>0.9929±0.0003</td>
</tr>
<tr>
<td>308.15</td>
<td>0.9023±0.0001</td>
<td>1.3869±0.0023</td>
<td>1.4185±0.0002</td>
<td>0.9078±0.0001</td>
<td>0.9345±0.0003</td>
</tr>
<tr>
<td>313.15</td>
<td>0.9031±0.0001</td>
<td>1.6025±0.0033</td>
<td>1.6316±0.0003</td>
<td>0.9066±0.0003</td>
<td>0.9012±0.0004</td>
</tr>
<tr>
<td>318.15</td>
<td>0.9042±0.0002</td>
<td>1.8242±0.0084</td>
<td>1.8563±0.0011</td>
<td>0.9091±0.0002</td>
<td>0.9815±0.0002</td>
</tr>
<tr>
<td>323.15</td>
<td>0.9054±0.0003</td>
<td>2.1595±0.0065</td>
<td>2.1758±0.0009</td>
<td>0.9119±0.0003</td>
<td>0.9056±0.0001</td>
</tr>
<tr>
<td>328.15</td>
<td>0.9071±0.0002</td>
<td>2.3251±0.0377</td>
<td>2.192±0.0024</td>
<td>0.9143±0.0006</td>
<td>0.9093±0.0004</td>
</tr>
<tr>
<td>333.15</td>
<td>0.9102±0.0006</td>
<td>2.3712±0.0803</td>
<td>2.1860±0.0003</td>
<td>0.9090±0.0001</td>
<td>0.9099±0.0004</td>
</tr>
<tr>
<td>338.15</td>
<td></td>
<td>0.2297±0.0009</td>
<td>0.1319±0.0002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>343.15</td>
<td></td>
<td>0.0342±0.001</td>
<td>0.172±0.0006</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Solubilities (x: mole fraction) of asiaticoside in methanol + water mixture at different temperatures, together with error limits using the 95% confidence level (Zheng & Lu, 2011)

<table>
<thead>
<tr>
<th>x_w</th>
<th>298.15</th>
<th>303.15</th>
<th>308.15</th>
<th>313.15</th>
<th>318.15</th>
<th>323.15</th>
<th>328.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05882</td>
<td>0.0059±0.00002</td>
<td>0.0069±0.00002</td>
<td>0.0081±0.00002</td>
<td>0.0098±0.00002</td>
<td>0.0124±0.00004</td>
<td>0.0155±0.00005</td>
<td>0.0188±0.00026</td>
</tr>
<tr>
<td>0.0323</td>
<td>0.0108±0.00007</td>
<td>0.0144±0.00007</td>
<td>0.0181±0.00003</td>
<td>0.0204±0.00012</td>
<td>0.0217±0.00014</td>
<td>0.0247±0.00016</td>
<td>0.0256±0.00005</td>
</tr>
<tr>
<td>0.1842</td>
<td>0.0277±0.0001</td>
<td>0.0371±0.0003</td>
<td>0.0451±0.0001</td>
<td>0.0565±0.0003</td>
<td>0.0683±0.0004</td>
<td>0.0711±0.0004</td>
<td>0.0858±0.0002</td>
</tr>
<tr>
<td>0.2727</td>
<td>0.0562±0.0003</td>
<td>0.0744±0.0001</td>
<td>0.1013±0.0002</td>
<td>0.1414±0.0005</td>
<td>0.1819±0.0001</td>
<td>0.2247±0.00015</td>
<td>0.2392±0.00018</td>
</tr>
<tr>
<td>0.3600</td>
<td>0.11±0.0003</td>
<td>0.1417±0.0007</td>
<td>0.1903±0.0004</td>
<td>0.2656±0.0007</td>
<td>0.3428±0.0018</td>
<td>0.4742±0.0011</td>
<td>0.6357±0.0012</td>
</tr>
<tr>
<td>0.4576</td>
<td>0.1728±0.0003</td>
<td>0.2277±0.0003</td>
<td>0.3046±0.0008</td>
<td>0.4056±0.0015</td>
<td>0.536±0.0032</td>
<td>0.7193±0.0012</td>
<td>0.9557±0.0018</td>
</tr>
<tr>
<td>0.5676</td>
<td>0.2881±0.0002</td>
<td>0.2782±0.0005</td>
<td>0.3794±0.0001</td>
<td>0.4952±0.0015</td>
<td>0.654±0.0049</td>
<td>0.8392±0.0016</td>
<td>1.1219±0.0022</td>
</tr>
<tr>
<td>0.6923</td>
<td>0.3148±0.0004</td>
<td>0.2757±0.0005</td>
<td>0.3718±0.0011</td>
<td>0.4772±0.0003</td>
<td>0.6325±0.0032</td>
<td>0.8018±0.0015</td>
<td>1.0832±0.0017</td>
</tr>
<tr>
<td>0.8351</td>
<td>0.4526±0.0002</td>
<td>0.3214±0.0006</td>
<td>0.3251±0.0003</td>
<td>0.4265±0.0003</td>
<td>0.4651±0.0012</td>
<td>0.5786±0.0012</td>
<td>1.018±0.00017</td>
</tr>
<tr>
<td>0.8740</td>
<td>0.2855±0.0005</td>
<td>0.2509±0.0011</td>
<td>0.3447±0.0008</td>
<td>0.4505±0.0014</td>
<td>0.5690±0.0006</td>
<td>0.7601±0.0012</td>
<td>1.026±0.0007</td>
</tr>
<tr>
<td>0.9144</td>
<td>0.2444±0.0008</td>
<td>0.3074±0.0006</td>
<td>0.3933±0.0012</td>
<td>0.3146±0.0002</td>
<td>0.4768±0.0021</td>
<td>0.8839±0.0012</td>
<td>1.1095±0.0007</td>
</tr>
<tr>
<td>0.9564</td>
<td>0.3734±0.0012</td>
<td>0.4898±0.0013</td>
<td>0.5777±0.0015</td>
<td>0.746±0.0042</td>
<td>0.9924±0.0017</td>
<td>1.2111±0.0028</td>
<td>1.5552±0.0077</td>
</tr>
<tr>
<td>0.9780</td>
<td>0.5638±0.0011</td>
<td>0.6749±0.0008</td>
<td>0.8153±0.0015</td>
<td>1.0394±0.0014</td>
<td>1.2297±0.0018</td>
<td>1.4573±0.0055</td>
<td>1.9807±0.0012</td>
</tr>
</tbody>
</table>
2.3. Biological activities

Various pharmacological activities of asiaticoside have been reported previously including anxiolytic-like effect (Chen et al., 2006), antidepressant activity (Liang et al., 2008), neuroprotective effect (Xu et al., 2012), antitumor (Huang et al., 2004; Al-Saeedi et al., 2011), antipyretic and anti-inflammatory activities (Wan et al., 2012), and wound healing and angiogenic activity (Shukla et al., 1999).

2.4. Wound healing and angiogenic activities

Wound healing activity of asiaticoside has been studied in different wound healing models both of in vitro and in vivo. For gastric ulcer healing activity, effect of *C. asiatica* water extract (CE) and asiaticoside (AC) on acetic acid induced-gastric ulcers (kissing ulcers) in rats was studied by orally administration (Cheng et al., 2004). The result revealed that both CE and AC exhibited ulcer-healing activity as dose dependent manner. Moreover, they had effect on reduction of myeloperoxidase activity and promoted endothelial cell proliferation and angiogenesis at the ulcer site. Basic fibroblast growth factor (bFGF) expression was also upregulated by both CE and AC. Another study investigated inhibitory effect of CE and AC on inducible nitric oxide synthase (iNOS) during gastric ulcer healing in rat (Guo et al., 2004). The ulcer size was reduced in dose-dependent manner together with reduction of iNOS activity and protein expression at the ulcer site. This result indicated anti-inflammatory activity of the CE and AC.

Skin wound healing activity of asiaticoside was studied in both normal (punch wound) and delayed-type wound healing model (streptozotocin-induced diabetic rat). Asiaticoside solution prepared by dissolving asiaticoside in saline solution with various asiaticoside concentrations (0.05-0.2 % for normal wound and 0.2-0.4% for diabetic wound) was applied on the wound area twice daily. The obtained data demonstrated that hydroxyproline content, tensile strength, collagen content and epithelisation were significantly increased in both wound models. Moreover, in vitro angiogenesis study using CAM assay revealed that asiaticoside induced angiogenesis at concentration of 40 µg/disc (Shukla et al., 1999).

In burn wound model, the topical application of low dose asiaticoside (10^-8-10^{-12} % w/w) facilitated the burn wound repair (Kimura et al., 2008). Effects of low dose asiaticoside on burn wound healing might be due to the promotion of angiogenesis by VEGF production stimulation via the increase in monocyte chemoattractant protein-1 (MCP-1) expression in keratinocytes and the increase in IL-1β expression in macrophage.

Wound healing mechanism of asiaticoside at cytobiological and molecular biological levels had been studied in human dermal fibroblasts by using cDNA microarray technology (Lu et al., 2004). The mRNA and protein production
correlated with genes responsible for cell-cycle progression, proliferation and collagen synthesis in cell response to asiaticoside stimulation. In contrast, study performed in keloid fibroblasts revealed that asiaticoside could inhibit the collagen type I and III expression and TGF-β/Smad signaling that finally reduce the scar formation (Tang et al., 2011).

2.5. Pharmaceutical preparations of asiaticoside

Asiaticoside-loaded alginate films were prepared and evaluated their potential as wound dressing. Release of asiaticoside studied by immersion method was performed in phosphate buffer pH 7.4 containing methanol at 10 % v/v. Burst release about 30 % was found within the first hour accompanying by slower rate until reach to 90 % at 12 h. Cytotoxic to normal human dermal fibroblasts (NHDF) was evaluated using indirect method (MTT assay). The results reveal that the prepared films were non-toxic to the cells (Sikareepaisan et al., 2011).

Electrospun cellulose acetate fibers containing asiaticoside were prepared and evaluated the drug release. The test was conducted using two methods; immersion and transdermal diffusion through pig skin model. The test was performed using 10% v/v methanol-contained medium with two different pH values; 5.5 (acetate buffer) and 7.4 (phosphate buffer). Burst release could be observed. Asiaticoside could be released from the fibers with low percentage within 12 h and the released amount was steady at around 25% of the loaded amount (Suwantong et al., 2008). Effect of the asiaticoside-loaded fibers on fibroblast cell was investigated. The results revealed that the asiaticoside-loaded fibers could promote the cell attachment and proliferation in vitro. These materials had potential to be further developed as topical/transdermal patches or wound dressings (Suwantong et al., 2010).

Asiaticoside-loaded ultradeformable vesicles were developed as topical preparations for promoting collagen synthesis. Cytotoxicity of the system was evaluated on primary human dermal fibroblast cells by determining the extracellular lactic dehydrogenase activity. Results indicated that ultradeformable vesicles having sodium cholate molar fractions>0.2 showed a certain cytotoxicity. Study of collagen biosynthesis revealed that asiaticoside-loaded ultradeformable vesicles with a sodium cholate molar fraction of 0.2 exhibited the greatest degree of collagen biosynthesis in human fibroblasts. Ultradeformable vesicles provided the greatest in vitro skin permeation of asiaticoside showing a 10-fold increase with respect to the free drug solution and favored an increase in in vivo collagen biosynthesis (Paolino et al., 2012).
3. Chitosan

3.1. General information

Chitosan is a natural linear amino polysaccharide derived from chitin, the 2nd most abundant polymer next to cellulose. Chitin is the polymer being a main component of crustacean shell, insect exoskeleton and fungi cell wall. Chemical structure of chitin consists of 2-acetamido-2-deoxy-β-D-glucopyranose through a β(1→4) linkage as illustrated in Fig. 3. Chitosan can be produced from chitin by deacetylation the acetyl group at C-2 position for at least more than 50% (Alves & Mano, 2008). Therefore, chemical structure of chitosan randomly consists of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucopyranose residues connected through β(1→4) linkage as shown in Fig. 4. Chitosan has gained much attention in research field because it is derived from chitin which is an underutilized resource. Moreover, it is well known as highly interactive molecule because of amino groups and hydroxyl groups consisted in its structure. These functional groups allows for easily chemical modification. Deacetylation degree (DD) is the value employed to indicate number of amino groups left after deacetylation. Chitosan having higher DD exhibits higher crystallinity. Molecular weight (Mw) of chitosan can be varied in wide range and has been classified into low, medium and high but the exact range number has not accurately been defined. Furthermore, short chain chitosan or chitosan oligomer could be produced by ether chemical or enzymatic hydrolysis of the chitosan polymer chain (Zhang et al., 2010). Mw has effect on solubility and viscosity of chitosan. For the chitosan having higher Mw, it exhibits lower solubility but its solution exhibits higher viscosity. In some research fields including drug delivery and tissue engineering, low Mw chitosan and oligomer are much more useful because of their higher solubility in neutral pH than that of the higher Mw. Both of DD and Mw facilitate chitosan a wide range of physicochemical properties.

![Fig. 3 Chitin molecular structure](image)
3.2. Physicochemical and biological properties

3.2.1. Solubility

Normally, chitosan is insoluble in water and most organic solvents but can be dissolved in dilute acids such as acetic acid, formic acid and lactic acid which their pH is less than 6. The pK$_a$ value of chitosan was reported at around 6.3 (Pillai et al., 2009). In acidic environment, amino groups of chitosan are protonated and exhibited positive charge that exerts water solubility.

3.2.2. Thermal properties

Pyrolysis (a process of thermolytic degradation by chain scission and by recombination) of chitosan was studied by using simultaneous TG (thermogravimetry) and DSC (differential scanning calorimetry) in nitrogen atmosphere (Pillai et al., 2009). Result indicated that thermal degradation of chitosan followed a random scission pathway, which was initiated at weak links. TG curve of chitosan displayed the first degradation stage at the temperature higher than 200°C. Chitosan residue obtained after thermal treatment at 280°C was analyzed using FT-IR analysis. IR spectra analysis revealed a decrease of amine group and an increase of N-acetylated groups. This indicated thermal destruction of the pyranose ring and a possible thermal cross-linking of various macroradicals of the chain. Moreover, the peak of saccharide structure at 893 and 1153 cm$^{-1}$ decrease and became wide after degradation, which indicated the rupture of the $\beta$-glycosidic-linkages between the glycosamine and N-acetylglucoamine moieties. Normally, chitosan after processed into material devices or derivatization was less thermal stable than native chitosan owing to a reduction of its crystallinity as reported in the previous studies (Peniche-Covas et al., 1993; Cardenas & Miranda, 2004; Chiandotti et al., 2010). Blending chitosan with a higher thermal stable substance could improve thermal stability of the system. However, the system had higher thermal stability than the neat chitosan but lower than the pure added substance (Liao et al., 2004).
3.2.3. Biodegradation

Chitosan can be degraded by lysozyme, a non-specific protease present in all mammalian tissue. Degradation rate of chitosan is depended on DD and Mw. Previous studies reported that chitosan with lower DD (lower crystallinity) and Mw tended to be degraded more rapidly (Zhang & Neau, 2001). However, chitosan with the same DD might exhibit different degradation rate due to variation of acetamido group distribution in chitosan chain. Degradation rate of chitosan has effect on biocompatibility. Amino sugars will be produced during degradation of chitosan. If the degradation is rapidly occurred, amino sugars will be accumulated and then causes inflammatory response to body tissue (Aranaz et al., 2009).

3.3. Biological activities

Various biological activities of chitosan have been reported in the previous review (Aranaz et al., 2009). Chitosan has been claimed as biocompatible, biodegradable and non-toxic natural polymer. It possess many interesting biological activities due to its positive charge nature such as hemostatic activity, antimicrobial activity, anticholesterolemic property, analgesic effect, antioxidative effect, mucoadhesion, immunostimulating effect and permeation enhancing effect.

3.4. General applications

Chitosan has been applied using in various fields namely food, biocatalyst, wastewater treatment and medical applications (Kumar, 2000; Alves & Mano, 2008; Aranaz et al., 2009). Most applications are based on ionic interaction between cationic chitosan and other anionic molecules. Positive charge of chitosan can easily interact with various negative charge molecules such as proteins, fatty acids, lipids, negative charge polymers, etc. This makes chitosan attractive for biomedical applications such as drug delivery system and tissue regeneration.

3.4.1. Drug delivery system

Drug delivery system developed from chitosan can be prepared in different types namely tablets, hydrogels, films, micelles, micro-/nanoparticles, nanofibers and porous scaffolds. Chitosan exhibits excellent mucoadhesive property, therefore, it has been popular modified as mucoadhesive drug delivery system.

Sustained drug delivery system has gained much attention for many decades. To modify drug release from chitosan matrix, cross-linking process has been performed by using different methods. Higher degree of cross-linking and higher DD of chitosan increase compactness and hydrophobicity of the chitosan matrix that facilitate the control drug release from the matrix by controlling the swelling of the
matrix and diffusivity of the drug contained in the matrix. Cross-linking agents such as formaldehyde and glutaraldehyde were the first effective agents to be used but their remaining was blamed harmful to the body tissue. Therefore, other less harmful substances namely tri-polyphosphate (TPP), β-glycerophosphate and genipin were more acceptable in biomedical application (Berger et al., 2004).

Besides using cross-linking agents, other approach such like using hydrophobic chitosan has been studied. Techniques to prepare hydrophobic chitosan could be conducted by covalently connecting hydrophobic molecules, normally fatty acids, to free amino groups of chitosan via chemical reactions which are generally complex, takes several steps and using organic solvents and/or high temperature. For instance, fatty acid chloride was mixed with chitosan-acetate solution for 4-6 h before neutralized and precipitated with acetone. Thereafter, the precipitate was collected and washed with methanol several times to remove excess fatty acid and then dried with pure acetone to obtain the derivative powder (Tien et al., 2003). Another work prepared stearic acid grafted chitosan oligosaccharide (CSO-SA) by grafting via mediator, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Briefly, CSO was dissolved in distilled water whereas SA was dissolved in ethanol with sonicaton. Under continuous stirring, EDC was added to CSO solution and then heated up to 90°C followed by dropwise addition of the SA solution. The reaction took about 5 h at 90°C before cooled down to room temperature and stirred for further 24 h. Subsequently, the mixture was dried at 50°C in vacuum oven before dispersion in ethanol and filtered to collect the precipitate and washed again by ethanol to remove excess fatty acid. The precipitate was then dissolved in water in order to purify by further dialysis for 24 h before lyophilization to obtain the product (Hu et al., 2006). Some just blended fatty acid with chitosan solution by adding surfactant to improve wettability of the system and used high temperature during mixing (Vargas et al., 2009). However, this process did not obtain the covalent chitosan-fatty acid derivative.

3.4.2. Tissue regeneration and wound healing

Chitosan manifests several properties that are suitable for tissue regeneration and wound healing applications namely chemoattraction and activation of macrophages and neutrophils, promotion of collagen type IV synthesis, promotion of growth factors function, matrix metalloproteinase enzyme (MMP-2) inhibition, integrin entanglement stimulation, cell proliferation promotion and intrinsic antimicrobial activity. Moreover, it is chemically and enzymatically modifiable, non-toxic and biodegradable (Muzzarelli, 2009).

In tissue engineering field, chitosan has been constructed as scaffolds, films and nanofibers for using as cell culture structure. Because of positive charge
from amino groups, chitosan provide suitable environment for cell adhesion (Arca & Şenel, 2008). However, previous study reported that proliferation of endothelial cells and smooth muscle cells on chitosan film surface decreased even the tested cells could attach and spread well on the film comparing with tissue culture polystyrene. This might be a result of high positive charge of chitosan that serves strongly adhesion of the cell on the structure until reducing cell migration and then inhibiting cell growth. Moreover, chitosan is highly interactive macro-molecule that can trap some elements of the cell culture medium which later reducing the cell feeding (Denuziere et al., 1998; Chupa et al., 2000). Therefore, surface modification is needed for chitosan scaffolds that developed for using in tissue engineering field.

The adhesive nature, antifungal and bactericidal character, and permeability to oxygen of chitosan, are a very important properties associated with the treatment of wounds and burns. Chitosan is susceptible to be degraded under the influence of enzymes present in body fluids such as lysozyme and N-acetylglucosamidase. The degradation products, being chito-oligomers, are able to stimulate macrophages and positively influence collagen sedimentation, thus accelerating the wound healing process. Chitosan activates macrophages to produce and release chemokines such as interleukin-1, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (INF-γ). Moreover, it promoted production of platelet-derived growth factor (PDGF) that necessary for wound healing and angiogenesis. The oligomers act as building blocks for hyaluronan synthesis. Hyaluronan has been reported beneficial for wound healing by promotion cell motility, adhesion and proliferation of fibroblasts (Ueno et al., 2001; Muzzarelli, 2009).

3.5. Chitosan-based wound dressing

Chitosan-based wound dressings have been conducted in research works and commercially available which are mainly produced in Japan and the US (Niekraszewicz, 2005). The dressing was developed in various forms for instance non-woven fibres, gels, films and sponges for using in different purposes such as external/internal wound healing and hemostasis as demonstrated in Table 5. Because of its ability to exhibit the positive charge in physiological fluid, chitosan manifests the excellent hemostasis and mucoadhesion. Positive charge of chitosan attracts negative charge of blood components and accelerates a blood clot. Moreover, it strongly adheres to the wound bed which can reduce the volume of blood loss, therefore, reduce motility (Pusateri et al., 2003; Kozen et al., 2008). This kind of dressing has been used mainly in army to stop bleeding of the severe acute wound. However, this dressing can cause the pain during removal from the wound bed because of the highly adhesion and dressing drying out due to reduction of exudate
level. As a wound dressing, the anti-adhesive property plays a key role in determining the process of wound repair.

Besides hemostasis application, various chitosan-based dressings have been developed by placing emphasis on tissue regeneration of the wound. A large number of publications reported the development of chitosan-based dressings in different types such as fibers, hydrogels, membranes, scaffolds and sponges as reported in previous review (Jayakumar et al., 2011). Each type is appropriate for using in different wound condition. Recent trend in wound dressing research is paying more attention to incorporate active substance(s) in order to improve the wound healing rate and efficacy. Composite dressing of chitosan and other polymers or organic materials have been developed in order to optimize the dressing properties, to be employed in more specific application, and also for the growth factor(s) and drug delivery. Example of chitosan-based dressings in various forms were included in the following details.

### Table 5 Example of chitosan-based dressings available in the market (Niekraszewicz, 2005)

<table>
<thead>
<tr>
<th>Dressing</th>
<th>Utilization purpose</th>
<th>Developer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitopack C®</td>
<td>Hemostasis</td>
<td>Eisai Co</td>
</tr>
<tr>
<td>Tegasorb®</td>
<td>healing of extensive internal wounds</td>
<td>American 3M</td>
</tr>
<tr>
<td>Tegaderm®</td>
<td>healing of extensive internal wounds</td>
<td>American 3M</td>
</tr>
<tr>
<td>HemCon Bandage™</td>
<td>Hemostasis</td>
<td>HemCon Bandage™ USA</td>
</tr>
<tr>
<td>Syvek patch, RDH</td>
<td>Hemostasis</td>
<td>Marine Polymer Technologies</td>
</tr>
<tr>
<td>Clo-Sur PAD</td>
<td>Hemostasis</td>
<td>Medtronic-Scion</td>
</tr>
<tr>
<td>Chito-Seal</td>
<td>Hemostasis</td>
<td>Abbot</td>
</tr>
<tr>
<td>M-Patch and Trauma DEX</td>
<td>Hemostasis</td>
<td>Medafor</td>
</tr>
</tbody>
</table>

### 3.5.1. Films/Membranes

Chitosan-lactate (Chit-LA) and chitosan-acetate (Chit-AA) films were prepared and evaluated properties in terms of film conditions and wound healing efficacy comparing with a commercial dressing (Omiderm®: a hydrophilized polyurethane film) (Khan & Peh, 2003). The study was performed using punch biopsy wound in rat. Film condition namely transparency, flexibility, ease of removal from the wound and fluid accumulation of the chitosan dressings were not significantly different from the commercial dressing except for the adherence property which that
of chitosan films was slight lower. For the wound healing efficacy, dryness and exudation of the wound treated by the chitosan dressings were not significantly different from the commercial dressing and the untreated groups. However, the wound closure, period of epithelialization and scar formation of all the dressing treated groups were significantly different from the untreated group. This indicated that chitosan dressings could promote the wound healing with a minimal scar formation.

Chitosan film containing fucoidan was developed for dermal burn healing (Sezer et al., 2007). The result revealed that fucoidan-chitosan film treated group showed superior regenerated dermal papillary formation, re-epithelization, and closure of wounds after 14 days than that of the chitosan film treated group and fucoidan treated group. Chitosan film loaded with minocycline was developed for the treatment of severe burn wound (Aoyagi et al., 2007). The drug could be released from the film in a sustained manner. The minocycline-loaded film prepared from 83% DD chitosan exhibited excellent effect on accelerating wound healing. Antibiotic-loaded chitosan film (Noel et al., 2008) and sponges (Noel et al., 2010) were prepared for using as local antibiotic delivery at wound sites where avascular zones could prevent the delivery of antibiotics to the infected tissue. These systems were designed to be degraded in the body. Elution study revealed that the loaded drugs could be rapidly eluted from the materials with high percentage within an hour and became slower during 72 hours. These antibiotic contained materials exhibited potentially to be used as local drug delivery device for preventing musculoskeletal infections.

Chitosan–hyaluronic acid hybrid film was developed previously (Xu et al., 2007). The film could accelerate wound healing comparing to a vaseline gauze and could reduce an occurrence of re-injury when remove the dressing from the wound bed. Composite dressing films of chitosan and other polysaccharides: cornstarch or dextran, and a plasticizer: propylene glycol, were prepared and characterized (Wittaya-areekul & Prahsarn, 2006). The polysaccharides and the plasticizer could improve the mechanical properties of the chitosan film in term of water vapor and oxygen penetration, and film elasticity.

A bilayer chitosan membrane, consisting of a dense upper layer (skin layer) and a sponge-like lower layer, was developed for using as a topical delivery of silver sulfadiazine for the control of wound infections (Mi et al., 2002). The membrane showed the appropriate water vapor transmission rate, oxygen permeability and water uptake capability. Sustained release of sulfadiazine from the membrane could be modified by increasing amount of the drug. Antimicrobial assay against P. aeruginosa and S. aureus showed effective antimicrobial activity for 1 week. In vivo antimicrobial assay in rats excisional wound model confirmed that this wound
dressing was effective for long-term inhibition of the growth of the mentioned microbes at an infected site.

3.5.2. Hydrogels

Chitosan gel containing endothelial growth factor was prepared for utilization in second-degree burn wounds healing. The endothelial growth factor contained gel exhibited better and faster epithelialization compared with the other control groups (Alemdaroğlu et al., 2006).

Chitosan-polyacrylamide semi-IPN hydrogel was created for a sequential release of antibiotics (piperacillin-tazobactam) and growth factor (endothelial growth factor) which might be beneficial for the prevention of infections and to stimulate wound healing. Release of antibiotics was designed to be rapid whereas that of the growth factor was more sustained (Pulat et al., 2013). The therapeutic serum dose of piperacillin-tazobactam for topical introduction was reached at the first hour of the release. Mitogenic activity of L929 mouse fibroblast was determined to prove liberated amount of the endothelial growth factor from the gel matrix. The result indicated a presence of endothelial growth factor for 5 days.

3.5.3. Nanofibers

Biocompatible carboxyethyl chitosan/poly(vinyl alcohol) (CECS/PVA) nanofibers were prepared by electrospinning of aqueous CECS/PVA solution as wound dressing materials (Zhou et al., 2008). Effect of the fiber mats on cell viability was evaluated using mouse fibroblast (L929). Results revealed that the fiber mats was non-toxic to the cell. Moreover, cell culture results showed that fibrous mats were good in promoting the L929 cell attachment and proliferation. These indicated the potential use of this system in tissue regeneration application.

Antibacterial electrospun fiber mats of quaternary chitosan blended with PVA was prepared and evaluated antimicrobial activities against E. coli and S. aureus. The results indicated a good bactericidal activity of the materials against the tested microbes revealing their high potential as wound dressing (Ignatova et al., 2006).

3.5.4. Foam/ Sponges

Chitosan sponge containing antibiotic drug, norfloxacin, was prepared by a solvent evaporation technique for applying as wound dressing materials (Denkbas et al., 2004). Swelling behavior, norfloxacin loading, in vitro release characteristics, and antibacterial activity of the sponge were determined. Results indicated that the equilibrium-swelling ratio decreased with increasing cross-linking density. The norfloxacin release was found to be swelling controlled initially and
diffusion controlled at the extended release periods. Antibacterial activity was directly proportional to the release rate.

Chitosan scaffolds containing bFGF loaded-gelatin microparticles were prepared for using in chronic wound treatment. Results revealed that the chitosan-bFGF scaffold could accelerate the wound healing in pressure ulcer aged-mouse model. Moreover, chitosan could inhibit the neutrophil elastase activity which normally contributes to the proteolytic environment of the pressure ulcer. Therefore, chitosan itself also promoted chronic wound healing (Park et al., 2009).

Chitosan grafting lactic acid was prepared and fabricated into sponge by using lyophilization method (Lai et al., 2013). Cytotoxicity of the sponge was studied in human skin fibroblasts (HSF) and human immortal keratinocytes (Hacat) using indirect method. Sponge extraction at 25% concentration exhibited non-toxic at day-1 but serious toxic was found in the next day for the both cells. Higher concentration extractions (50 and 100%) were found serious toxic to the both cells. In release study, penicillin and erythromycin were chosen as hydrophilic and hydrophobic drug models, respectively. In case of hydrophilic drug, penicillin, it could be homogeneously blended with the hydrophilic polymer matrix. The release behavior was governed by both diffusion and erosion process, therefore, burst release was not observed for penicillin. Compared to erythromycin system, penicillin exhibits the faster release characteristics due to the faster erosion of the matrix and drug dissolution. Owing to hydrophobic nature of erythromycin, it failed to solute in polymer solution and caused the phase separation. Therefore, erythromycin was adsorbed on the surface of matrix. This exerted the burst release of the drug. Compact structure of the matrix containing erythromycin obstructed the penetration of the buffer medium into the structure and reduced the polymer matrix erosion which later delayed the drug release.

4. Aluminum monostearate

Aluminium monostearate (Al(OH)$_2$C$_{18}$H$_{35}$O$_2$) is an organic compound which is a salt of stearic acid and aluminium (Fig. 5). It is used to form gels in the packaging of pharmaceuticals, and in the preparation of colors for cosmetics. It is usually safe in commercial products, but aluminium may accumulate in the body (Wikipedia). In pharmaceutical field, aluminum monostearate is used in the formulation of penicillin G procaine with aluminum stearate suspension. It is used with peanut oil for the preparation of adjuvants for virus vaccines and ACTH peptide for injection (Hem & White, 1989).
Aluminum monostearate has been utilized as oil thickening agent in development of muscular injectable implants (Luan & Bodmeier, 2006), an excipient of suspensions formulation for prolonged release procaine penicillin and riboflavin (Wayne et al., 1949; Brzezinski et al., 1957) and an additive of vaccine formulations (Gupta & Siber, 1995). Dissociation of metal stearate such as aluminum stearate and magnesium stearate in acidic solution medium had been reported previously (The Metal Carboxylates Coalition, 2003; Phaechamud et al., 2009). They are dissociated into metal and salt form of corresponding fatty acid (stearate molecule with negative charge). This dissociation can be applied for simply blending fatty acid with chitosan by electrostatic interaction under acidic condition.

5. N-methyl-2-pyrrolidone

N-Methyl-2-pyrrolidone (NMP) is a chemical compound with 5-membered lactam structure as shown in Fig. 6. Other names for this compound are: 1-methyl-2-pyrrolidone, N-methylpyrrolidone, N-methyl-pyrrolidinone and the brand name Pharmasolve.

5.1. General information

It is a colorless to slightly yellow liquid miscible with water. It is a hygroscopic liquid with a mild amine odour. It is also miscible with most common solvents such as ethyl acetate, chloroform, benzene and lower alcohols or ketones. It also belongs to the class of dipolar aprotic solvents which includes also dimethylformamide, dimethylacetamide and dimethyl sulfoxide (Jouyban et al.,
NMP is used in the petrochemical industry, microelectronics fabrication industry, and manufacture of various compounds, including pigments, cosmetics, drugs, insecticides, herbicides, and fungicides. An increasing use of NMP is as a substitute for chlorinated hydrocarbons (Åkesson, 2001). NMP can easily solubilize medicinal agents at lower quantities compared to other common co-solvents. This may be useful when a small amount of co-solvent is required (Jouyban et al., 2010).

5.2. Pharmacokinetic information

According to report of WHO, dermal penetration through human skin has been shown to be very rapid. NMP is rapidly biotransformed by hydroxylation to 5-hydroxy-\(N\)-methyl-2-pyrrolidone, which is further oxidized to \(N\)-methylsuccinimide; this intermediate is further hydroxylated to 2-hydroxy-\(N\)-methylsuccinimide. These metabolites are all colourless. The excreted amounts of NMP metabolites in the urine after inhalation or oral intake represented about 100% and 65% of the administered doses, respectively (Åkesson, 2001).

5.3. Safety

NMP has a low potential for skin irritation and a moderate potential for eye irritation in rabbits. Repeated daily doses of 450 mg/kg body weight administered to the skin caused painful and severe hemorrhage and eschar formation in rabbits. Direct exposure to NMP for human staff using NMP in cleaning process was observed no irritation. In humans, there was no irritative effect in the respiratory system after an 8-h exposure to 50 mg/m\(^3\) (Åkesson, 2001).

According to other review (Jouyban et al., 2010), this solvent is classified as a teratogenic compound in PubChem, and twelve bioassays reported its effects on living organisms. NMP has significant cardiovascular toxicity, and the arterial pressure change induced after inter-arterial infusion is more than that caused by other solvents. The safety of NMP under various conditions and with high doses and prolonged exposure needs more detailed studies. According to available studies on animals, lower doses of NMP can cause adverse and toxic effects on males, in comparison with females. In addition, exposure to high concentrations of NMP during pregnancy in rats can cause abnormalities in fetuses. Nevertheless, no significant and robust data about human subjects are available.
5.4. Pharmaceutical application

NMP is one of the main pharmaceutical co-solvents. It acts as a very strong solubilizing agent used in parenteral and oral preparations. It is an important solvent used in the extraction, purification, and crystallization of drugs (Jouyban et al., 2010). There are commercially available of pharmaceutical preparations using NMP as a main solvent namely the controlled release gel for subcutaneous injection of leuprolide acetate (Eligard®, Sanofi-Aventis), controlled release gel for subgingival administration of doxycycline hyclate (Atridox®, Atrix Laboratories), IV solution of Florfenicol (Nuflor®, Intervet/Schering-Plough) and doxycycline gel for periodontal treatment (Doxirobe™ Gel, Pfizer). Owing to an excellent skin absorption character, NMP has been used as penetration enhancer in transdermal drug delivery system for both of hydrophilic and hydrophobic drugs from aqueous phase (Lee et al., 2005).

6. In vitro drug release study and drug release kinetic of porous matrix drug delivery system

6.1. In vitro drug release study

Generally, in vitro drug release study of the porous matrix is performed using immersion technique (Gren et al., 1996; Gren & Nyström, 1999; Prabu et al., 2008; Natu et al., 2010; Baradari et al., 2012; Canal et al., 2012; Mabrouk et al., 2013). This method can be carried out by directly immersion of the porous material in release medium. Herein, some study may fill the sample into dialysis bag or a basket before immersion. The test is performed using shaking incubator while controlling temperature and shaking rate at proper condition related to an application of the sample. Predetermined volume of release medium will be periodically collected in order to determine the drug released amount.

Another study conducted the test by using Franz diffusion flow through cell method (Steffansen & Herping, 2008). In this study, low exudate level wound (LEW) and high exudate level wound (HEW) in vitro models were developed. These models were aimed using to study drug release from foam dressing in wound producing different exudate level. Agar-anopore membrane was used as a barrier to limit amount of the release medium diffusing to the sample in LEW model. In contrast, only high-density polyethylene (HDPE) net was used to support the sample in HEW model. This let unlimited amount of the release medium diffusing to the sample. Release medium is flow through the receptor compartment with a fixed rate. Sample is placed on top of the agar-anopore membrane (LEW)/HDPE net (HEW) that cover the receptor compartment. Release medium will be periodically drawn out by an autosampler.
6.2. Drug release kinetics of the porous matrix

Drug release from the matrix can be affected by many factors including hydrophilicity/hydrophobicity of the drug and the matrix, homogeneous of the drug and the matrix, degradability of the matrix, and solubility of the drug. Drug release from the non-degraded porous matrix is driven by water penetrating into the matrix and the drug dissolving and diffusing out of the matrix (Fu & Kao, 2010). In case of hydrophilic materials, water can freely penetrate into porous structure. Therefore, drug dissolution is the main factor that controls the drug release when sink condition is presented. In contrast, hydrophobicity of the material can delay the water penetration thus water penetration rate will be the main factor that controlled drug release from hydrophobic material (Lai et al., 2013).

Initial burst release has been normally reported for the porous matrix especially for the system comprising the drug having hydrophilicity different from the matrix (Prabu et al., 2008; Noel et al., 2010; Lai et al., 2013). This occurred because of phase separation of the drug and the polymer matrix, and the drug partition to the material surface. Various drug release kinetics have been reported for different porous drug delivery systems as the following examples.

Starch-based porous matrix was developed as a carrier of dexamethasone (Duarte et al., 2009). Drug release profile of the system studied in phosphate buffer pH 7.4 was fitted well with Power law model. The exponent value \( n \) was approximately 0.6 indicating anomalous transport which demonstrated that the diffusion of the drug out of the matrix and the water uptake, and also swelling of the polymeric matrix influenced the drug release. Release kinetic of chitosan sponges containing doxycycline hyclate studied in phosphate buffer pH 7.4 was reported as first order. Curve fitting to power law model revealed release kinetic of the sponges as Fickian diffusion (Phaechamud & Charoenteeraboon, 2008). Ibuprofen-loaded calcium phosphate porous pellets were prepared and studied the drug release in phosphate buffer solution pH 7.5 (Baradari et al., 2012). Curve fitting to first order model and Hixson–Crowell equation were performed which the release profile of the pellets was fitted to Hoixson-Crowell better than the first order. This can be suggested that the release of ibuprofen is controlled by drug erosion from surface of the pellets. Bio-active glass scaffolds were loaded with gatifloxacin and studied the drug release in phosphate buffer pH 7.4 (Soundrapandian et al., 2010). The drug release profile was fit well with first order model. The exponent \( n \) of power law was lower than 0.45 indicated Fickian diffusion mechanism. Drug release profile of ciprofloxacin-loaded PVA bio-active glass scaffolds studied in phosphate buffer pH 7.4 was fitted with power law model. The exponent value indicated quasi-Fickian diffusion \( (n<0.5) \) (Mabrouk et al., 2014). This mechanism indicated that the polymer was hydrated, swollen and then the drug diffused through the swollen matrix system, which ultimately slowed down the kinetic release.
7. Kinetic model for drug release profile analysis

Qualitative and quantitative changes in a formulation may alter drug release and *in vivo* performance therefore developing tools that facilitate the product development by reducing the necessity of biological studies is always desirable (Dash *et al.*, 2010). *In vitro* drug release study has been recognized as an important element in drug development as a surrogate for the assessment of bioequivalence. It is necessary to ensure that the release of the drug from the developed system occurs in an appropriate manner. Mathematical formula employed to express the drug release results as a function of some of the dosage forms characteristics can be simplify the quantitative analysis of the values obtained from the release study (Costa & Lobo, 2001).

There are number of kinetic models used for describing the overall release of drug from the dosage forms; zero order, first order, Higuchi, Power law (Korsmeyer-Peppas) model, Hixson Crowell, Baker-Lonsdale model, Weibull model, etc. Principle of some kinetic models was described as the following details.

7.1. Zero-order

Slowly release of drug from the non-degradable pharmaceutical dosage form, which assuming that area does not change and no equilibrium conditions is obtained, can be represented by an equation (1) which can be rearranged as an equation (2).

\[
Q_0 - Q_t = K_0 t \\
Q_t = Q_0 - K_0 t
\]

(1)  
(2)

Where \(Q_t\) is a liberated amount of the drug at time \(t\), \(Q_0\) is an initial drug loading, and \(K_0\) is a constant value of zero-order in a unit of concentration/time. Linear relation can be plotted between cumulative drug release and time where slope of the line represents \(K_0\). The pharmaceutical dosage forms following this profile release the same amount of drug by unit of time and it is the ideal method of drug release in order to achieve a pharmacological prolonged action. This release kinetic can be observed in various pharmaceutical dosage forms such as transdermal systems, matrix tablets, coating systems, osmotic pump (Costa & Lobo, 2001).
7.2. First-order
This model represents drug release kinetic depending on the drug concentration remaining in the system. The release of the drug which followed first order kinetics can be expressed by equation (3) which can be modified as equation (4).

\[ Q_t = Q_0 e^{-K_1 t} \]  
(3)

\[ \log Q_t = \log Q_0 - \frac{K_1 t}{2.303} \]  
(4)

Where \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of drug in the solution and \( K_1 \) is the first order release constant. Graph plotted between logarithm of cumulative drug release and time will be linear where slope of the line represents \( K_1/2.303 \). This relationship can be used to describe the drug dissolution in pharmaceutical dosage forms such as those containing water-soluble drugs in porous matrices (Costa & Lobo, 2001).

7.3. Higuchi model
This is the first model aiming for describing drug release from matrix system. It was proposed by Higuchi since 1961. This model is based on the hypotheses that (i) initial drug concentration in the matrix is much higher than drug solubility; (ii) drug diffusion takes place only in one dimension (edge effect must be negligible); (iii) drug particles are much smaller than system thickness; (iv) matrix swelling and dissolution are negligible; (v) drug diffusivity is constant; and (vi) perfect sink conditions are always attained in the release environment. The model can be represented by equation (5).

\[ f_t = \frac{Q}{A} = \frac{K_H t}{1/2} \]  
(5)

Where \( Q \) is the amount of drug released in time \( t \) per unit area \( A \), \( C \) is the drug initial concentration, \( C_s \) is the drug solubility in the matrix media and \( D \) is the diffusivity of the drug molecules (diffusion coefficient) in the matrix substance. Higuchi model can be simplified as equation (6).

\[ f_t = \frac{Q}{A} = K_H t^{1/2} \]  
(6)

Where \( K_H \) is the Higuchi dissolution constant. The data obtained were plotted as cumulative percentage drug release versus square root of time. This kinetic can be used to describe the drug dissolution from several types of modified release.
pharmaceutical dosage forms, as in the case of some transdermal systems and matrix tablets with water soluble drugs (Dash et al., 2010).

### 7.4. Power law (Korsmeyer-Peppas) model

A simple relationship described drug release from a polymeric system based on drug diffusion, polymer swelling and erosion or both phenomena was derived by Korsmeyer et al. since 1983 as shown in equation (7).

\[
\frac{M_t}{M_\infty} = K t^n
\]  

(7)

Where \(\frac{M_t}{M_\infty}\) is a fraction of drug released at time \(t\), \(K\) is the release rate constant and \(n\) is the release exponent. The \(n\) value is used to characterize different release for cylindrical shaped matrices. For the case of cylindrical tablets, \(0.45 \leq n\) corresponds to a Fickian diffusion mechanism, \(0.45 < n < 0.89\) assigns to non-Fickian transport (anomalous), \(n = 0.89\) to Case II (relaxational) transport, and \(n > 0.89\) corresponds to super case II transport. To find out the exponent of \(n\) the portion of the release curve, \(M_t/M_\infty < 0.6\) (the first 60% of drug release data) should only be used. To study the release kinetics, data obtained from in vitro drug release studies were plotted as log cumulative percentage drug release versus log time (Dash et al., 2010).

This model is generally used to analyze the release of pharmaceutical polymeric dosage forms, when the release mechanism is not well known or when more than one type of release phenomena could be involved (Costa & Lobo, 2001).

### 8. MicroMath® Scientist™ for Windows™

It is specifically designed to fit model equations to experimental data. Other programs focus on technical graphics, symbolic manipulation, matrix operations or worksheets for engineering calculations. Scientist™ incorporates all these elements, but its primary function is fitting equations to experimental data. Scientist™ can fit almost any mathematical model from the simplest linear functions to complex systems of differential equations, non-linear algebraic equations or models expressed as Laplace transforms. The Scientist Chemical Kinetic Library is a set of chemical kinetics models that can be used to simulate or analyze experimental data. The Chemical Kinetic Library includes models for zero, first and second order irreversible reactions, first order reversible reactions, and parallel first order irreversible reactions with up to three products. This program used for fitting the drug release data has been previously reported (MicroMath, 2006).
Least square fitting the experimental dissolution data (cumulative drug release > 10% and up to 80%) to the mathematical equations (power law, first order, zero order, Higuchi’s and cube root) was carried out using a nonlinear computer program, Scientist for windows, version 2.1 (MicroMath Scientific Software, Salt Lake City, UT, USA). The coefficient of determination ($r^2$) was used to indicate the degree of curve fitting. Goodness-of-fit was also evaluated using the Model Selection Criterion (MSC) (MicroMath, 2006), given below.

$$\text{MSC} = \ln \left\{ \frac{\sum_{i=1}^{n} W_i (Y_{obs} - Y_{cal})^2}{\sum_{i=1}^{n} W_i (Y_{obs} - \bar{Y}_{obs})^2} \right\} - \frac{2p}{n}$$  \hspace{1cm} (8)

Where,

- $Y_{obs}$: observed values of the $i$-th point;
- $Y_{cal}$: calculated values of the $i$-th point;
- $W_i$: weight that applies to the $i$-th point;
- $n$: number of points; and
- $p$: number of parameters

9. Angiogenesis assay

Angiogenesis is composed of many stages; growth factors liberation, endothelial cells activation, endothelial cell proliferation, migration, tubule formation, and vascular stabilization. Many techniques have been employed to evaluate substances whether they have pro- or anti-angiogenic activities. The techniques can be performed by using models from the smallest part of blood vessels; endothelial cells, and part of organ which generally use the large blood vessel such as aorta, to the whole body of animal models.

9.1. In vitro models

Formation of new blood vessels is needed co-operation of several factors; there are endothelial cells, soluble factors and extracellular matrix components. These components are also crucial components of in vitro angiogenesis assay. In vitro angiogenesis assay needed endothelial cells to be the main component representing an initial and a principle structure of new blood vessels. Whereas extracellular matrix components accommodate endothelial cells to complete function during angiogenic assay (Li et al., 2010). In vitro angiogenesis assay reported in previous study mostly use endothelial cells or part of large blood vessels as described in following details.
9.1.1. Endothelial cell assays

Angiogenic substances may have effect on angiogenesis in one or more stages of angiogenesis cascade. Three major stages involving with endothelial cells; cell proliferation, cell migration, and tube formation (cell differentiation) can be studied to evaluate angiogenic activity of substances. Examples of endothelial cell type using in angiogenesis assay obtained from both animals and human (Veeramani & Veni, 2010) are listed below.

- Human umbilical vein endothelial cells (HUVECs)
- Human mammary epithelial cells (HMEC)
- Bovine aortic endothelial cells (BAECs)
- Chicken endothelial cells (CECs)

Cell proliferation assay can be performed by direct cell count (hemocytometer method), assessment of metabolic activity of the cells (MTT assay), or measuring indirectly by cell-cycle analysis (BrdU colorimetric assay). Cell migration assay can be performed by Boyden chamber assay and scrape wound assay (wound healing model). Cell differentiation or tube formation can be assessed either in 2D or 3D assessments. The important components of this assay are endothelial cells suspension and the gel matrices of extracellular matrix component polymers (e.g. fibrin and collagen type I) or Matrigel® (a mixture of extracellular and basement protein derived from the mouse Engelbreth-Holm-Swarm sarcoma) (Goodwin, 2007).

9.1.2. Organ culture assays

Endothelial cell assay as described previously are performed by using single-cell type of endothelial cell. However, angiogenesis is naturally involved with multiple cell types; EC cells, pericytes and smooth muscle cells. Organ culture assay may provide more certain angiogenesis condition than that of endothelial angiogenesis assay. Types of organ used in this assay such as rat aortic ring, chick aortic arch, porcine carotid artery, human umbilical artery, placental vein disc, fetal mouse bone explants, and rat blood vessel, etc. The assay can be performed by culturing parts of organ or the whole organ in gel matrix (e.g. fibrin and collagen type I) for 10-14 days, thereafter, the vessel outgrowths are quantified by measuring the number and length of microvessel outgrowths from the primary explant using phase microscopy or immunohistochemical analysis (Staton et al., 2004).
9.2. In vivo models

From previous part of in vitro angiogenesis assay, the microvessel outgrowth, although, can be observed in cell culture organ assay such as aortic ring assay. However, for in vivo angiogenesis, new blood vessels are outgrowth from pre-existing micro blood vessels or capillaries but do not sprout from a large blood vessel such as aorta. Moreover, a previous study reported that a compound affecting cell proliferation, migration or differentiation in vitro may not necessarily regulate endothelial cell activity in vivo (Liekens et al., 2001). Therefore, in vivo angiogenesis assay is the vital step in drug development. There are numbers of in vivo angiogenesis model such as sponge/matrice plug assay, corneal angiogenesis assay, zebra fish, dorsal air sac model, and CAM assay (Staton et al., 2004).

CAM assay

CAM is an extraembryonic membrane formed during day 4 of egg incubation after fertilization. It is formed by fusion of chorion and allantoin. CAM is rich of blood vessels that are interesting to be used in angiogenesis assay. This assay is the most widely used technique to scan the pro- or anti-angiogenic activity of substances. CAM assay can be operated either in-ovo (performing in egg shell) or ex-ovo (performing in petri dish) methods (Ribatti et al., 1996). For in-ovo method, sterile fertilized chicken eggs are incubated at 37°C with humidifying. At 3rd day of incubation, the eggs are drilled at narrow end using 18-gauge hypodermic needle and 2-3 mL of albumen is withdrawn. At 8th-9th day of incubation, a 2-3 cm round window is open in eggshell thereafter a small disc containing the tested substance is then placed on CAM and the opening window is then closed with paraffin before returning to incubation. At 11th day of incubation, CAM is harvested and measuring angiogenic response (Zwadlo-Klarwasser et al., 2001). For ex-ovo method, the shells of the 3rd day pre incubated chicken eggs are opened and their embryos are transferred to the sterile tissue culture dish and are then returned to humidified incubator. The next 4 day, the tested substance contained in sterile disc is gently laid on surface of CAM. The embryos are then returned to incubator for 24 h before fixing in fixative solution for 2 day at room temperature. Subsequently, the fixed CAM are dissected and placed onto glass slide and dried. The dried CAMs are photographed and measured angiogenic response (Výboh et al., 2010).
CHAPTER 3
MATERIALS AND METHODS

Materials
Models for biological assays
Equipment
Methods
1. Dissociation study of aluminum monostearate (Alst)
   1.1. Sample preparation
   1.2. Physicochemical characterizations
      1.2.1. Scanning electron microscope (SEM)
      1.2.2. FT-IR spectroscopy
      1.2.3. Inductive coupled plasma-mass spectroscopy (ICP-MS)
      1.2.4. Powder x-ray diffraction (PXRD)
      1.2.5. Thermal properties analysis
         1.2.5.1. Thermogravimetry (TG) and Differential thermogravimetry (DTG)
         1.2.5.2. Differential scanning calorimetry (DSC)
         1.2.5.3. Hot stage microscope (HSM)
2. Chitosan-Alst composite dispersion
   2.1. Composite dispersions preparation
   2.2. Physicochemical characterizations
      2.2.1. Viscosity and rheology, and pH measurement
      2.2.2. Morphology under inverted microscope
      2.2.3. Particle size determination
3. Fabricated chitosan-Alst composite sponges
   3.1. Sponge preparation
   3.2. Dehydrothermal treatment (DHT)
3.3. Physicochemical characterizations
   3.3.1. Physicochemical properties
   3.3.2. Hydrophilic/hydrophobic property evaluation
   3.3.3. Water sorption and erosion study
   3.3.4. Swelling study
   3.3.5. *In vitro* asiaticoside release study (immersion method)
   3.3.6. Analysis of asiaticoside release data

4. Chitosan-Alst sponge dressings
   4.1. Sponge dressing preparation
   4.2. Physicochemical properties characterization
      4.2.1. FT-IR spectroscopy
      4.2.2. Powder x-ray diffraction (PXRD)
      4.2.3. Morphology and pore size evaluation
      4.2.4. Porosity determination
      4.2.5. Mechanical strength determination
      4.2.6. Hydrophilic/Hydrophobic property evaluation
      4.2.7. Fluid handling ability evaluation
      4.2.8. Oxygen permeability study
      4.2.9. Bio-adhesion test
   4.3. Asiaticoside release study
   4.4. Effect on cell viability studies
      4.4.1. Cell culture
      4.4.2. Sample preparation
      4.4.3. Cytotoxicity study (WST-8 method)
   4.5. Angiogenesis assay
      4.5.1. Sample disc preparation
      4.5.2. CAM assay
Materials

1. Acetic acid (CH₃COOH) (AR grade, Glacial, Lot No. K37658163, Merck KGaA, Germany)
2. Acetonitrile (HPLC grade, Batch No. 10120367, RCI Labscan LTD., Bangkok, Thailand)
3. Agarose (Low EEO, Molecular biology grade, Lot No. R36351, Research Organics Inc. Ohio, USA)
4. Aluminum monostearate (Lot No. 0001447128, Fluka, Sigma-Aldrich Co., Missouri, USA)
5. Asiatic acid (90%, Guangxi Changzhou Natural Products Development Co.Ltd, China)
6. Asiaricoside (90%, Guangxi Changzhou Natural Products Development Co.Ltd, China)
7. Chitosan (Aqua premier, Chonburi, Thailand, having 97% deacetylation degrees with 70 kDa molecular weight)
8. Dimethyl sulfoxide (DMSO) (Lot No. 453035/1, Fluka Chemie GmbH, Switzerland)
9. Disodium hydrogen orthophosphate (Na₂HPO₄) (AR grade, Batch No. AF405300, Ajax finechem Pty. LTD., New South Wales, Australia)
10. Dulbecco’s modified Eagle’s medium (DMEM) (Complete, Gibco, Invitrogen K.K., Tokyo, Japan)
11. Ethanol (Absolute, AR grade, Batch No. 10C240514, VWR international S.A.S., France)
12. Fetal bovine serum (Gibco, Invitrogen)
13. Fibroblast Basal Medium (FBM), 500 mL supplemented with 0.5 mL basic-fibroblast growth factor (bFGF), 0.5 mL insulin, 10 mL fetal bovine serum (FBS), and 0.5 mL GA-1000 (gentamicin and amphotericin B) (Lonza, Lonza Group Ltd., Tokyo, Japan)
14. Filter membrane (0.45 µm, Whatman International LTD., Maidstone, England)
15. Hydrocortisone acetate (Lot No. 025K1123, Sigma-Aldrich Chemie GmbH, Missouri, USA)
16. Keratinocyte Basal Medium (KBM), 500 mL supplemented with 2 mL bovine pituitary extract (BPE), 0.5 mL human epidermal growth factor (hEGF), 0.5 mL insulin, 0.5 mL hydrocortisone, 0.5 mL transferrin, 0.25 mL epinephrine, and 0.5 mL GA-1000 (gentamicin and amphotericin B) (Lonza, Lonza Group Ltd., Tokyo, Japan)
17. Lactic acid (Batch No. GB203609, Loba Chemie Pty. LTD., Mumbai, India)
18. Methanol (HPLC grade, Batch No. 0000019710, Avantor Performance materials Inc., Pennsylvania, USA)
19. Microcentrifuge tube (1.5 ml)
20. Micropipette tip 20, 200, 1000 µL
21. Needles 18 and 21 gauge (Nipro Corporation, Osaka, Japan)
22. N-methyl-2-pyrrolidone (Lot No. SZBB3010V, Sigma-Aldrich Co., Missouri, USA)
23. Potassium acetate (CH₃COOK) (AR grade, Batch No. 1306251669, Ajax finechem Pty. LTD., New South Wales, Australia)
24. Potassium bromide (KBr) (spectrograde, Fisher Scientific UK limited, UK)
25. Potassium chloride (KCl) (AR grade, Batch No. 0912453, Ajax finechem Pty. LTD., New South Wales, Australia)
26. Potassium dihydrogen phosphate (KH₂PO₄) (AR grade, Lot No. P5104-1-1000, QREC Chemical Co., LTD., Chonburi, Thailand)
27. Red dye (food colour, strawberry red colour, Best odour colour Co., LTD., Bangkok, Thailand)
28. Sodium acetate (CH₃COONa) (AR grade, Batch No. AF604190, Ajax finechem Pty. LTD., New South Wales, Australia)
29. Sodium bicarbonate (NaHCO₃) (AR grade, Batch No. AF310196, Ajax finechem Pty. LTD., New South Wales, Australia)
30. Sodium chloride (NaCl) (AR grade, Lot No. SG28621111, Loba Chemie Pty. LTD., Mumbai, India)
31. Sodium hydroxide (NaOH) (AR grade, Batch No. 1188-04/05, P.C. Drug Center Co., LTD., Bangkok, Thailand)
32. Stearic acid (Batch No. AF408282, Asia Pacific Specialty Chemicals LTD., New South Wales, Australia)
33. Sterile disc (6mm-diameter) (AA discs, 6.0 mm, Whatman International LTD., Maidstone, England)
34. Syringe (3 mL and 5 mL) (Nipro Corporation, Osaka, Japan)
35. Syringe filter membrane (0.45 µm, Nylon 13 mm, Fortune Scientific Co., LTD., Bangkok, Thailand)
36. Transwell permeable support (Tissue culture treated, sterile, Polystyrene plate, 75 mm insert, 100 mm dish, Corning Inc., New York, USA)
37. Vascular Endothelial Growth Factor (VEGF165, Human recombinant animal free, Merck Millipore, Massachusetts, USA)
38. Cell count reagent SF (WST-8) or Dojindo’s highly water-soluble tetrazolium salt (Nacalai Tesque, Kyoto, Japan)
Models for biological assays

1. Fertilized eggs (Suwanvajokkasikit Animal Research and Development Institute, Kasetsart University, Nakhon Pathom, Thailand)
2. Normal human dermal fibroblast (NHDF) (passage number 4-12, Department of Biomaterials, Field of Tissue engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan)
3. Normal human epidermal keratinocyte (NHEK) (passage number 3-6, Department of Biomaterials, Field of Tissue engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan)

Equipment

1. Analytical balance (CP2245 Satorious, Germany and PA214 Oharus, Oharus Corporation, USA)
2. Brookfield DVIII Ultra programmable viscometer (Brookfield Engineering Laboratories, Inc., USA)
3. Centrifuge (Sorvall® BiofugeStratos, Fisher Scientific, UK)
4. Differential scanning calorimetry (DSC) (Pyris Sapphire DSC, Standard 115V, Perkin Elmer instruments, Japan)
5. Fertilized egg incubator (Nakhonnayok Technical College, Nakhonnayok, Thailand)
6. Filter set and membrane filter 0.45 µm
7. Fourier Transform Infrared Spectrophotometer (FT-IR) (Nicolet 4700, Becthai, USA)
8. Freeze dryer (Triad™, Labconco, Missouri, USA)
9. Freezer -20°C (Sanden Intercool, Thailand), Freezer -80°C
10. Gas chromatography (Shimadzu GC-8A, Japan)
11. Goniometer (FTA 1000, First Ten Angstroms, USA)
12. High performance liquid chromatography (HPLC) (1100 series, Agilent technology, Germany)
13. Homogenizer (IKA® T25 digital ultra-turrax, IKA Works (Asia), Malaysia)
14. Hot-stage microscope (Mettler Toledo FP900 Thermosystem, Mettler Toledo AG, Switzerland and Olympus microscope)
15. HPLC column ACE® C18 (4.6 mm x 15 cm, Advanced Chromatography Technology, Scotland)
16. Inductive coupled plasma-mass spectroscopy (ICP-MS) (Model 7500 ce, Agilent, Palo Alto, CA)
17. Inverted microscope (Nikon, Eclipse TE2000-S, Japan)
18. Magnetic stirrer (IKA® RO10, IKA Works (Asia), Malaysia) and Magnetic bar
19. Micropipette (2-20 µL, 20-200 µL, 100-1000 µL, 1-5 mL) and micropipette tip
20. Oxygen permeation apparatus (Postharvest Technology Center, Kasetsart University, Thailand)
21. Particle size analyzer (Partica LA-950, HORIBA, Japan)
22. pH meter (Ultra BASIC, UB-10, Denver instrument GmbH, Goettingen, Germany)
23. Powder x-ray diffractometer (PXRD) (Miniflex II, Rigaku Corp. Tokyo, Japan)
24. Scanning electron microscope (SEM) (Maxim 200 Cam scan, Cambridge, England)
25. Shaking incubator (Shel Lab, Sheldon Manufacturing, Inc., Oregon, USA)
26. Sonication bath (Transsonic T890/H, Elma, New South Wales, Australia)
27. Texture analyzer (Charpa Techcenter, Godalming, Stable micro Systems Ltd., UK)
28. Thermogravimetric analyzer (TGA) (Pyris/TGA, PerkinElmer, USA)
29. Vacuum oven (Vaccucell 55, MMM, Germany)
30. VERSAmax microplate reader (Molecular devices Corporation, California, USA)
31. Water bath (Julabo, Japan)
Methods

1. Dissociation study of Alst

1.1. Sample preparation

Alst powder with accurate weight of 2.5 g was dispersed in 97.5 g of 2 % w/v lactic acid solution. Because Alst is a high hydrophobic substance, homogenizer was employed to promote the dispersion of Alst at the beginning for 5 minutes. After homogenization, the dispersion was continuously mixed using magnetic stirrer at room temperature. Samples obtained from various dispersing times; 5 min, 1, 4, 8, 12 and 24 h, were collected by filtering via 0.45 µm-filter paper and rinsed with 50 mL RO water for 5 times. The treated Alst samples were then dried at 50°C overnight in hot air oven and kept in desiccator for further characterizations.

1.2. Physicochemical characterizations

1.2.1. Scanning electron microscope (SEM)

Morphology of the samples was observed using SEM with 15 KeV of an accelerating voltage. The sample was strewed onto the carbon double adhesive tape that adhered on metal stub and then sputter-coated with gold. Images of the samples at magnification of 25 and 500 were taken using secondary electron image (SEI) mode.

1.2.2. FT-IR spectroscopy

Functional groups alteration of Alst residues were investigated using FT-IR spectroscopy. Each samples was grinded with KBr powder in the ratio of approximately 2:100 of sample:KBr and then compressed into pellet using KBr die kit. IR spectra were run in the wavenumber range of 4000-400 cm⁻¹ with 32 scan and 4 cm⁻¹ resolution.

1.2.3. Inductive coupled plasma-mass spectroscopy (ICP-MS)

Approximately 100 mg of each sample was accurately weighed into a beaker and then digested using nitric acid until a clear solution was observed. Concentration of aluminum (Al) in the residue of the samples was then analyzed using ICP-MS with flame mode condition (n=2).
1.2.4. Powder x-ray diffraction (PXRD)

Crystallinity of the samples was evaluated using powder x-ray diffractometer. The samples were filled and compressed into sample holder (0.2 mm-depth). The test was performed using 30 kV voltages and 15 mA currents. The Cu-Kα was used as the x-ray source which liberated the x-radiation of wavelength ($\lambda$) = 1.541841 Å. Diffractogram was run in the range of 4-60° of 20.

1.2.5. Thermal properties analysis

1.2.5.1. Thermogravimetry (TG) and Differential thermogravimetry (DTG)

Thermal degradation behavior of the samples was analyzed using TG analyzer. Sample with accurate weight of 10 mg was filled into an open porcelain crucible. The test was run in the temperature range of 30-500°C using 10°C/min heating rate under air atmosphere. Weight (%) of the sample was continuously recorded as the temperature was gradually increased. TG curve plotted between percentage of weight remaining and temperature was obtained by using the instrument software. DTG curve was plotted by using the same software. Weight remaining (%) of the sample at each degradation stage was estimated from TG curve. Temperature of initial degradation ($T_{int}$) and temperature of maximum degradation ($T_{max}$) of each degradation stage were obtained from the DTG curve.

1.2.5.2. Differential scanning calorimetry (DSC)

Melting behavior of the samples was investigated using DSC. Accurately 5 mg of the sample was weighed into an aluminum pan and sealed using a pan sealer. DSC thermogram was run in the temperature range of 30-250°C with 10°C/min heating rate under atmosphere of nitrogen gas. Sealed empty aluminum pan was used as reference.

1.2.5.3. Hot stage microscope (HSM)

Melting behavior of the samples was observed under hot stage microscope. Small amount of fine sample powder was strewed on glass slide and covered with glass cover slip. The glass slide was then inserted into the stage by placing sample powder in the center of the stage’s hole which the light from microscope can pass through. Sample was heat up in the temperature range of 30-200°C with 10°C/min heating rate. Images of the sample around its melting period were taken every 1°C using digital camera coupled with an eyepiece of the microscope and its software.
2. Chitosan-Alst composite dispersion

2.1. Composite dispersions preparation

Effects of chitosan-Alst ratio and mixing times were investigated. Chitosan-lactate (CL) solutions were prepared by dissolving various amounts of chitosan (1, 2 and 4% w/w) in 2% w/v lactic acid solution until the clear solutions were obtained. The solutions were then filtered via muslin fabric to remove the insoluble residue. The solutions were coded as CL1, CL2 and CL4, respectively. Chitosan-Alst composite dispersions (CLA) were prepared by dispersing Alst (2.5 % w/w) in each CL solutions for various mixing times; 5 min, 4, 8, 12 and 24 h.

2.2. Physicochemical characterizations

2.2.1. Viscosity and rheology, and pH measurement

Viscosity and rheological behavior of the CL solutions and CLA dispersions were studied using Brookfield DV-III Ultra programmable viscometer. Approximately 2 mL of the sample was added into sample plate reservoir. The rheology was run at room temperature using conical probe (CPE 40) in the shear rate range of 75-325 rpm with shear rate and time interval of 25 rpm and 15 sec, respectively. In the meantime, shear stress and viscosity at each data collecting point were automatically recorded by the software; Rheocalc. Rheograms were plotted between shear rate and shear stress. The pH value of the samples was measured using pH meter at room temperature (n=3).

2.2.2. Morphology under inverted microscope

Approximately 100 µL of the CLA dispersions was pipetted onto the glass slide. Drop of the dispersion was gently spread by slanting the glass slide circularly. Morphology of the dispersants was observed under inverted microscope at magnification of 200 (20× of objective lens and 10× of an eyepiece). Images of the dispersants were taken using the instrument software.

2.2.3. Particle size determination

Particle size of the dispersants was measured using particle size distribution analyzer. Approximately 200 µL of the dispersion was diluted in 5 mL RO water and stirred at 50 Hz to obtain homogeneous mixture before measurement (n=3).
3. Fabricated chitosan-Alst composite sponges

3.1. Sponge preparation

Various amounts of Alst (0.5, 2.5 and 5.0 % w/w) was dispersed in CL solution (4% w/w chitosan in 2% lactic acid solution) with continuous mixing on magnetic stirrer for 24 h. Approximately 500 mg CL solutions and CLA dispersions were filled into aluminum foil mold (cylindrical shape with 1.5 cm-diameter and 0.4 cm-depth) and then frozen at -20°C for 24 h before lyophilization using freeze dryer for 72 h. Thereafter, the dry sponges were kept in desiccator for 24 h prior to be treated with DHT. The obtained sponges without Alst and that containing 0.5, 2.5 and 5.0 % w/w Alst were coded as CL, CLA05, CLA25 and CLA50, respectively.

The asiaticoside-loaded sponges used in release study were prepared by adding asiaticoside into CL solution before mixing with Alst. Asiaticoside was added in concentration of 1200 µg/g and mixed homogeneously for 24 h using magnetic stirrer before fabrication and DHT. Therefore, each sponge contained asiaticoside approximately 600 µg. The exact weight of asiaticoside was calculated again based on the total remaining weight of the solid components after DHT.

3.2. Dehydrothermal treatment (DHT)

Aim of applying DHT to the prepared sponges was to stabilize the samples in aqueous medium by causing amidation which is the process of removal the water molecule from ionic bond between ammonium group (-NH₃⁺) of chitosan and carboxylate group (-COO⁻) of stearate molecule therefore amide bond was created. The treatment was operated using vacuum oven at 110°C for 24 h. After the treatment, the samples were kept in desiccator for further characterizations. Weight loss after DHT was also determined to prove an effect of the process on lactic acid evaporation (n=6).

3.3. Physicochemical characterizations

3.3.1. Physicochemical properties

Functional group interaction, crystallinity and morphology of the samples were characterized by using FT-IR, PXRD and SEM respectively. Pore size of the prepared sponges was determined using image analysis program; ImageJ. At least 50 pores were determined. Thermal behavior of the CL and CLA25 was studied using TG, DSC and HSM comparing with the neat chitosan and Alst as the methods described previously (section 1.2).
3.3.2. Hydrophilic/hydrophobic property evaluation

Hydrophilic/hydrophobic property of the prepared sponges was evaluated using goniometer with sessile drop technique. Contact angle of the water drop on the sample’s surface was measured as a function of time. Sponges were cut horizontally to obtain flat surface with thickness of approximately 300 µm. Program of the software was set in video mode. Water-contained glass syringe was set in its position by adjusting distance of the needle tip at y-axis coordinate of 80 from the top of the image frame. Sample was placed on the stage and its upper surface was set at y-axis coordinate of approximately 420 from the top of the image frame. Water was gradually pumped out from the syringe with the rate of 20 µL/sec until water was dropped onto surface of the sponge. In the meantime, picture was continuously captured every second using CCD camera until the drop of water completely penetrated into sponges structure. Contact angle of the water drop on sponge surface at each second was calculated using software from First Ten Angstroms. Hydrophilicity of the sample was considered from slope of the obtained line.

3.3.3. Water sorption and erosion study

Water sorption and erosion of chitosan sponges was investigated using the method described previously (Ritthidej et al., 2002). Weight the chitosan sponges was accurately weighed and recorded as W1. Subsequently, the sponges were immersed in phosphate buffer solution pH 7.4 overnight. The wet sponges were then collected and wiped gently with filter paper to remove an excess medium before weighing. Weight of the wet sponges was recorded as W2. The dried sponges were then weighed and their weight was recorded as W3. Water sorption and erosion (% w/w) of the sponges were calculated as the equations below.

\[
\% \text{ water absorption} = \left(\frac{W2 - W3}{W3}\right) \times 100 \quad (9)
\]

\[
\% \text{ erosion} = \left(\frac{W1 - W3}{W1}\right) \times 100 \quad (10)
\]

3.3.4. Swelling study

Swelling behavior of the DHT sponges was studied in three different buffer systems including acetate buffer (pH 4.0), phosphate buffer (pH 7.4) and carbonate buffer (pH 10.0). Dry sponges were accurately weighed before immersed in the buffer medium. At predetermined times, the sponges were removed from the
medium and wiped off the excess medium by using filter paper and then weighed again. Degree of swelling was calculated as the following equation.

\[
\text{Degree of swelling} = \frac{(W_t - W_0)}{W_0} \tag{11}
\]

Where \(W_0\) and \(W_t\) are weight of the sponges before and after immersion in buffer solutions at time \(t\), respectively.

### 3.3.5. In vitro asiaticoside release study (immersion method)

Asiaticoside was loaded into sponge by directly mixing with composite dispersion before lyophilization and DHT. Each sponge contained 600 \(\mu\)g asiaticoside. Release study was performed using membrane-less method by immersing asiaticoside loaded-chitosan sponges in 6 mL release medium (phosphate buffer pH 7.4) at 37±0.5°C in shaking incubator with constant agitation rate at 50 rpm. Release medium (2 mL) was periodically collected at predetermined time up to 48 h. The same volume of fresh medium was replaced every sampling time. Released amount of asiaticoside was determined by HPLC in gradient mode using column ACE\textsuperscript{®} C18 (4.6 mm x 150 mm, Advanced Chromatography Technology, Scotland). Water-acetonitrile was employed as mobile phase with flow rate setting at 1.0 mL/min. Asiaticoside was detected by diode array detector at 210 nm (n=3). After release study, sponges were washed thrice with RO water before drying by lyophilization for further morphology study performed by SEM.

### 3.3.6. Analysis of asiaticoside release data

To predict release mechanism of the prepared sponges, cumulative percentage release of each system was fit with four mathematical equations namely first-order, zero-order, Higuchi’s and power law using Scientist\textsuperscript{™} for Windows (version 2.1). Least square regression was employed to fit the release profile data. Degree of curve fitting and goodness of fit were indicated by \(r^2\) and model selection criterion (MSC), respectively.

### 4. Chitosan-Alst sponge dressings

#### 4.1. Sponge dressing preparation

NMP was used as a co-solvent of asiaticoside in order to improve homogeneity of asiaticoside and the matrix. Briefly, 4% w/w chitosan solution (CL) was prepared by dissolving chitosan in 2% lactic acid solution with continuous
stirring overnight before filtered via muslin fabric to remove debris. Asiaticoside was dissolved in NMP and then mixed with chitosan solution. NMP was used at the concentration of 2.5 % w/w and asiaticoside content was 0.12 % w/w (1200 µg/g). Various amounts of Alst (0.5, 2.5 and 5.0% w/w) was dispersed into the previous mixture employing homogenizer at 8,000 Hz for 5 min prior to stir using magnetic stirrer continuously for 24 h at room temperature. The 20 mL obtained composite dispersion was subsequently pipetted into a flat bottom-aluminum cup (8 cm-diameter). The cup was cover with aluminum foil and wrapped vertical side with a cotton fabric prior to be frozen at -20°C overnight and then dried using freeze dryer for 72 hours. The obtained dry sponge was further treated by dehydrothermal treatment at 110°C under vacuum condition (pressure below 0.1 atm) for 24 hours using vacuum oven. The sponges dressing without Alst and that containing 0.5, 2.5 and 5.0 % w/w Alst were named CD, CD0.5, CD2.5 and CD50, respectively. Weight loss after DHT was determined (n=6).

4.2. Physicochemical properties characterization

4.2.1. FT-IR spectroscopy

Effect of NMP on functional group interaction of the prepared dressings was evaluated using FT-IR spectrophotometer. Chitosan dressings sample were cut into fine pieces using scissor. The sample powder was then grinded with KBr powder prior to compress into pellet using KBr die kit. The IR spectra were run in the wavenumber range of 400-4000 cm⁻¹ with 4 cm⁻¹ resolution and 32 scan. Chitosan and Alst raw material were also evaluated using KBr pellet method. Liquid components; NMP and lactic acid, were investigated using liquid detector accessory; smart multi-bounce HATR, of the FT-IR spectrometer.

4.2.2. Powder x-ray diffraction (PXRD)

Crystallinity of the prepared dressings and the raw materials was evaluated using powder x-ray diffractometer. The dressings were cut to obtain 2x2 cm² size and adhered to the sample holder prior to be evaluated. The diffractograms were run in the range of 4 - 60° 2θ with speed of 4° 2θ.

4.2.3. Morphology and pore size evaluation

Morphology and pore size of the prepared sponges was investigated using scanning electron microscope (SEM). The test was performed using accelerating voltage of 15 KeV in secondary electron image (SEI) mode. Pore size and shape of the sponges both horizontal plane and cross-sectioned were observed.
In order to investigate pore size of the sponge after swollen, the sponges were immersed in PBS pH 7.4 overnight and then frozen at -20°C and freeze dried again before observed under SEM. Pore size measurement was conducted by using image analyzer software; ImageJ. At least 50 pores were determined.

4.2.4. Porosity determination

Porosity ($\varepsilon$) of the chitosan sponges was measured using liquid displacement method as described previously (Yeh et al., 2009) with some modification. A measuring cylinder containing 20 mL of ethanol was weighed ($M_1$). The dried sponge was weighed ($M_s$) and then pre-immersed in ethanol in glass bottle. The sample contained glass bottle was placed into the sonication bath at temperature of approximately 30°C for an hour to assist completely penetration of ethanol into the sponges pore. Thereafter, the ethanol-impregnated sponge was then transferred into the 20 mL ethanol-contained cylinder. Ethanol volume in the cylinder was then readjusted to 20 mL before weighing ($M_2$). The sponge saturated with ethanol was removed from the cylinder, and the cylinder was weighed ($M_3$). The porosity of the sponge was calculated ($n$=3) using the following equation.

$$V_p \text{ (volume of the sponge's pore)} = \frac{(M_2 - M_3 - M_s)}{\rho_e} \quad (12)$$

$$V_s \text{ (volume of the sponge's skeleton)} = \frac{(M_1 - (M_2 - M_s))}{\rho_e} \quad (13)$$

Porosity ($\varepsilon$) = $\frac{V_p}{(V_p + V_s)} = \frac{(M_2 - M_3 - M_s)}{(M_1 - M_3)} \quad (14)$

Where $V_p$ is volume of the sponge’s pore, $V_s$ is volume of the sponge’s skeleton and $\rho_e$ is density of ethanol.

4.2.5. Mechanical strength determination

Texture analyzer was employed to evaluate the mechanical properties of the prepared sponge dressings. Mechanical strength of the sponges was evaluated using compression mode. The sponge was cut into square shape with 1x1 cm$^2$ size and approximately 3.5 mm thickness. The instrument was set up with 5 kg load cell. Cylindrical probe (3 mm-diameter (P/3), 7.07 mm$^2$ contact area, stainless) was used. The probe was set 10 mm away from the base. Sample was placed on the base by fixing the center of the sample to the center of the probe. During running a test, the probe was moved with 2 mm/sec speed until it pressed on the sponge surface and reached 6% strain. The probe was then pulled up to its origin. Stress-strain curve
was plotted by the Exponent® software. The highest stress value was recorded. At least 6 samples per formulation were analyzed.

4.2.6. Hydrophilic/Hydrophobic property evaluation

Because NMP is water miscible solvent, adding this solvent into chitosan sponge could have effect on hydrophilicity of the sponge. Effect of NMP on hydrophilic/hydrophobic property of the dressings was evaluated using the same method as described in Section 3.3.2.

4.2.7. Fluid handling ability evaluation

In vitro pressure ulcer model was prepared using the method described previously (Cullen et al., 2012) with some modifications. Agarose (0.5 % w/v) was melted in PBS pH 7.4 with 200 µL red dye added. Approximately 60 mL of the agarose solution was poured into the insert (7.5 cm diameter) of the 10 cm transwell plate. To create a rough concave surface of the agar, concave knotty aluminum foil was placed cover the agar solution before cooling. The insert was then coupled to the 10 cm plate containing 15 mL PBS pH 7.4 as illustrated in Fig. 7. This model represented the high exuding and depth wound.

The in vitro wound model and the dressing were separately weighed accurately using analytical balance. Subsequently, the sponge dressing was placed on top of the agar surface. The model was then kept in desiccator containing saturated potassium acetate solution which maintained the humidity at approximately 20% RH and then incubated at 37±0.5°C. The model coupled with the dressing was taken out for weighing every 4 h up to 24 h. Moreover, at each weighing point, the dressing and the model were decoupled and weighed. To determine the water vapor transmission rate (WVTR), the linear relation plotted between time and weight of the model plate coupled with the dressing was created. The WVTR was obtained from slope of the straight line. Fluid absorbency was determined after 24 h of the test by calculation the weight difference between that of the wet dressing (after 24 h) and dry dressing (before test). Three dressings of each sample were evaluated (n=3).
4.2.8. Oxygen permeability (OP) study

This test was modified from the ASTM1434-82 standard method and the method described previously (Ayranci & Tunc, 2003). Apparatus was set as demonstrated in Fig. 8. Sponge (6 cm-diameter) was inserted between two gas diffusion chambers and fixed with the clamp. Pure nitrogen gas and oxygen gas was separately flushed into the glass chamber with flow rate of 100 mL/min via plastic tube connected to the gas input port. The gas output tubes were left open-ended to let the gas freely diffuse and exchange with air. The gases were flown to fulfill their chambers for 30 min prior to check the oxygen content. Mixed gas from the nitrogen gas chamber was periodically drawn out every 30 min using needle and syringe and determined oxygen content by using gas chromatography (GC) until it reach equilibrium. Three samples per formulation were evaluated (n=3).

**GC condition**

Column: 1.93 m, PorapakQ 80/100, Shimadzu
Carrier gas: Helium, pressure 200 kPa
Temperatures: Injection port 150°C, Column 70°C, Detector 70°C
Detector: Thermal Conductivity Detector (TCD)
Recorder: Plotter Chart Speed 5 mm/min, Shimadzu C-R8A
Sample size: 1 mL
Analysis time: 4 min
Fig. 8 Oxygen permeation apparatus

Area under the curve obtained from GC analysis was employed to calculate percentage of oxygen comparing with that of 2 % v/v standard oxygen gas. Percentage oxygen was transformed to mass of the gas permeated via the dressing within 1 h using the following equation.

\[ M = \frac{c \times v \times t \times M_w \text{ of } O_2}{100 \times V} \]  \hspace{1cm} (15)

Where  
\( M \) = mass of \( O_2 \) permeated through the sample per hour (g/h)  
\( c \) = oxygen content obtained from GC analysis (% v/v)  
\( t = O_2 \) penetrating time (min) = 60 min  
\( M_w \) of \( O_2 \) = 32  
\( V \) = volume of \( O_2 \) at 1 atm = 24451.28 mL

Oxygen permeability (OP) of the dressings was calculated as the following equation (Ayranci & Tunc, 2003).

\[ \text{OP} = \frac{M \times d}{A \times \Delta P} \]  \hspace{1cm} (16)

Where;  
\( \text{OP} = \text{oxygen permeability (g.m/m}^2\text{.h.atm)} \)  
\( M \) = mass of \( O_2 \) penetrating via the sample per hour (g/h)  
\( d \) = sample thickness (m)  
\( A = \) penetrating area (m\(^2\)) = 0.00126 m\(^2\) (of the 4 cm diameter-chamber)  
\( \Delta P = \) the difference in \( O_2 \) pressure between the two side of the dressing = 1 atm
4.2.9. Bio-adhesion test

Bio-adhesion of the sample was investigated using texture analyzer in compression mode. Load cell was set at 5 kg. Sponge sample was cut into cylindrical shape with 10 mm diameter before adhered to the 10 mm-cylindrical probe using double adhesive tape. Dorsal porcine skin was shaved and cleaned prior to be cut in size of 3x5 cm² and then placed onto the plastic pad. The pad was then placed on the base of the machine at the position that fixed the location of the probe at middle area of the skin. Before run a test, the skin was mounted with 0.5 mL PBS pH 7.4 for a minute and then wiped with tissue paper and the sponge was also dipped into PBS pH 7.4 for 15 sec. The probe was set at 10 mm away from the skin surface. During the test, the probe was moved down with speed of 1 mm/sec until press on the skin with 15 g force then the probe was retained at this position for 2 min before return to the origin point. Force was recorded from the beginning of the probe movement until returning to the origin. Area under the curve plotted between force and distance, representing the resistance between sample and the skin during probe withdrawing, was calculated by Exponent® software. Non-adhesive plaster was also evaluated as a control group (n=3).

4.3. Asiaticoside release study

Release behavior of asiaticoside from sponge dressing containing NMP was conducted using immersion method. Sponges were cut into square shape with size of 1.5 cm x 1.5 cm before weighing an accurate weight using analytical balance. Subsequently, each sponge sample was put into 100 mL-glass bottle containing 15 mL PBS pH 7.4 as a release medium. The bottles were then placed in shaking incubator that maintaining temperature and shaking rate at 37±0.5°C and 50 rpm, respectively. At predetermined time, 1.5 mL of the release medium was drawn out and replaced with the same volume of the fresh buffer solution. Asiaticoside amount contained in sampling medium was determined using HPLC technique as the method described previously in section 3.3.5. The test was performed using 3 sample of each dressing formulation (n=3). Release profile analysis was performed using the method described in section 3.3.6.

Swelling behavior of the sponge in PBS pH 7.4 at the same time corresponding with the release study was determined using the same method described previously in section 3.3.4.
4.4. Effect on cell viability studies

4.4.1. Cell culture

Normal human dermal fibroblasts (NHDF) and normal human keratinocytes (NHEK) were routinely culture in standard cell culture media; fibroblast basal medium (FBM) supplemented with 5% fetal bovine serum for NHDF and keratinocyte basal medium (KBM) for NHEK. Cell culture medium was changed every 3-4 days. For subculture, trypsination was performed when the cell number was higher than 90% confluent.

4.4.2. Sample preparation

Pure substances (lactic acid, NMP, CL, asiaticoside and asiatic acid) were dissolved in cell culture medium at concentration range covering its content in 10 mg-sponge immersed in 1 mL cell culture medium. DMSO was used as a co-solvent for asiaticoside at concentration of 0.5% v/v. During the test, complete Dulbecco’s modified eagle medium (DMEM) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin was used as the extraction medium for NHDF and the KBM was used for NHEK.

Sponges (selected formula) and chitosan powder extractions were prepared by immersing of each sample in cell culture medium with final concentration of 10 mg/mL in 24 wells-cell culture plate at 37°C for 24 h. Cell culture medium was collected and filled into 2 mL micro-tube before centrifuged at 5,000 rpm for 1 min to obtain the clear supernatants. The obtained supernatants were 1x dilution. Thereafter, the supernatant were serially 2-folds diluted with cell culture medium to obtain 0.5x, 0.25x and 0.125x dilution extractions.

4.4.3. Cytotoxicity study (WST-8 method)

Effect of pure substances and sponges extractions on the cell viability was performed using MTT assay method (WST-8). After trypsination, the cells were suspended in cell culture medium at concentration of 50,000 cells/mL. Subsequently, 100 µL cell suspensions were pipetted into each well of 96 wells-plate and incubated for 24 h. After 24 h, cell culture medium was aspirated and replaced with 100 µL sample solution before returned to incubator for another 24 h. Thereafter, sample solution was aspirated and the cells were washed twice with 100 µL PBS pH 7.4 and then 100 µL cell counting reagent (WST-8) was added into each well. The cells were incubated at 37°C for 4 h prior to detect a UV absorbance (Abs) by microplate reader at 450 nm with reference wavelength at 650 nm. Percentage of cell viability was calculated by using the following equation. Cell
culture medium was used as negative control and DMSO (100 µL/mL) was used as positive control.

\[
\text{Cell viability (\%)} = \left( \frac{\text{Abs of sample}}{\text{Abs of control}} \right) \times 100
\]

(14)

4.5. Angiogenesis assay

In this study, the in ovo method was performed because it is cheap, easy and not wasteful to be used for screening the angiogenic activity of several substances at the same time.

4.5.1. Sample disc preparation

Prior to load asiaticoside, 20 µL of 3 mg/mL hydrocortisone acetate dissolved in methanol was loaded into the cellulose discs (6 mm-diameter). Hydrocortisone acetate was used in order to reduce an inflammatory induced-angiogenesis. Stock solution of asiaticoside was prepared by dissolving the substance in mixture of methanol and DMSO (1:1) to obtain a concentration of 8 mg/mL. The stock solution was further 2-fold diluted to 4 concentrations using the same solvent. Subsequently, 20 µL of each dilution was pipetted to drop into cellulose disc and left in laminar hood until methanol was completely evaporated. This step was performed twice to obtain the disc containing asiaticoside 40, 80, 160 and 320 µg/disc. PBS pH 7.4 was employed as a negative control. VEGF, a positive control, was dispersed in DMEM prior to load in the sterile disc with the amount of 100, 200 and 300 ng/disc. The discs were left to dry in laminar hood overnight. The sponge dressings were cut into square shape (0.5 x 0.5 cm² size and 0.35 cm thickness).

4.5.2. CAM assay

This study was approved by the ethics committee for the use of laboratory animals of Faculty of Pharmacy, Silpakorn University as attached in Appendix II. To investigate angiogenic activity of asiaticoside, in ovo method was performed as the method described previously (Zwadlo-Klarwasser et al., 2001) with some modifications. Fertilized chicken eggs were incubated in humidified incubator at 37°C with wide-end up. During incubation, the eggs were moved by adjusting slope of the egg tray back and forth for 60° every 2 hours. At day-4 of incubation, the eggs were wiped with 70% ethanol before drilled a small hole at the narrow-end by using 18 gauge-needles and then drawn out 3-4 mL of albumen by using 21 gauge-needles. The hole was subsequently sealed with melted candle and the eggs were returned to incubate for another 3 days. At day-8, the plane of the egg tray was set stationary at horizontal plane for allowing CAM and yolk sac drop away from
eggshell to create an air sac at the wide-end. At day-9, the eggs were wiped with 70% ethanol and small round window (2 cm diameter) over the air sac at wide-end was created using forceps. Thereafter, 0.5 mL PBS pH 7.4 was gradually dropped onto the shell membrane to wet the membrane before sucked out. The membrane was then gently peeled off from the CAM surface using forceps. Subsequently, sample disc was place onto the CAM surface near the medium blood vessels. The open window was then covered with parafilm and the egg was returned to incubate for another 3 days before observing the result. Approximately 10 embryos per sample were used (n=10). Average score of angiogenic activity was calculated from the scored embryos by exclusion of those that failed to be score due to being dead or the CAM was folded. Angiogenic activity of the tested substances was scored as the following 4 levels (Fig. 9) (Ponce & Kleinmann, 2003):

Score 0 = no blood vessel response to the tested substance
Score 1 = little response is observed; some small or medium microvessels incline to the tested substance
Score 2 = moderate response is observed; the medium or large microvessels certainly incline to the tested substance
Score 3 = strong response is observed; the large microvessels certainly incline to the tested substance and exhibit spoke wheel pattern

Fig. 9 Four levels of angiogenesis score of micro-vessels response in CAM assay (Ponce & Kleinmann, 2003)
CHAPTER 4
RESULTS AND DISCUSSION

1. Dissociation study of Alst

1.1. SEM

SEM images of the untreated Alst and the acid-treated Alst at various times are demonstrated in Fig. 10. Untreated Alst powder was more fine and bulky than that of the acid-treated Alst. Particles of the acid-treated Alst were larger as the soaking time was longer. Moreover, character of the particles was changed from fine bulky powder into waxy clusters. These results indicated that the acid-treated Alst particles were gradually dissociated into stearic acid and aggregated during soaking in dilute acid solution.

![Fig. 10 SEM images of the untreated Alst (A) and the acid-treated Alst at various soaking times; 4 h (B), 8 h (C) and 24 h (D) at 25 and 500 magnifications](image)

1.2. FT-IR spectroscopy

FT-IR spectra of the untreated Alst, the acid-treated Alst and stearic acid were illustrated in Fig. 11. IR spectra of the untreated Alst exhibited dominant absorption peaks of –C=O stretching at 1590 cm\(^{-1}\) and 1470 cm\(^{-1}\) wavenumbers. These absorption peaks are identical peaks of the metal carboxylate stretching (Deamer et al., 1967; Phaechamud et al., 2009).
For the acid-treated Alst, new absorption peaks in the IR spectra gradually appeared as the soaking time was increased. The new absorption peaks located at 1704 cm\(^{-1}\), 1425 cm\(^{-1}\), 1302 cm\(^{-1}\) and 724 cm\(^{-1}\) which assigned to \(-\text{C}=\text{O}\) stretching of carboxyl group of stearic acid, methylene deformation, methylene wagging, and methylene rocking vibration, respectively (Shoeb et al., 1999; Inoue et al., 2004), were found after soaking in the acid solution for 8 h and their intensity was increased by soaking time. These peaks were also found in the IR spectra of stearic acid. Moreover, intensity of the broad peak around the wavenumber range of 3700-3200 cm\(^{-1}\) assigning to \(-\text{OH}\) groups of Alst was gradually decreased with mixing time. Therefore, these spectra changes indicated the occurrence of stearic acid dissociated from Alst during soaking in acid solution. However, dominant peaks of the metal carboxylates at 1590 cm\(^{-1}\) and 1470 cm\(^{-1}\) were still observed in the IR spectra of the acid-treated Alst even at 24 h soaking time. This result revealed that Alst was not totally dissociated into stearic acid within 24 hours when exposed to the tested dilute acidic medium. This result could be confirmed by ICP-MS.

![FT-IR spectra](image)

**Fig. 11** FT-IR spectra of the untreated Alst (A), the acid-treated Alst at various soaking times; 4 h (B), 8 h (C) and 24 h (D), and stearic acid (E)

### 1.3. ICP-MS

ICP is the technique employed to detect elements quantity in any materials by providing the drastic high temperature (approximately 5500°C) via inductively coupled plasma (the medium) to break bond in substance molecules and generate metal ions that have high electrical conductivity. Thereafter, these metal ions are separated by the difference of their electrical conductivity and detected by the MS (Wolf, 2005).
Quantity of aluminum in the Alst samples was determined by using ICP-MS. Aluminum content in the untreated Alst and the acid-treated Alst was demonstrated in Fig. 12. Untreated Alst contained aluminum approximately 3.91 g/kg. Aluminum content in the acid-treated Alst was apparently decreased to 1.19 g/kg at 8 h of soaking time whereas decreased with slower rate than that of early 8 h and about 0.57 g/kg of aluminum content was left at 24 h soaking time.

![Aluminum quantity of the untreated Alst and the acid-treated Alst at various acid soaking times](image)

Fig. 12 Aluminum quantity of the untreated Alst and the acid-treated Alst at various acid soaking times

1.4. PXRD

Diffractograms of the untreated Alst, stearic acid, and the acid-treated Alst are shown in Fig. 13. Diffractogram of the untreated Alst exhibited amorphous pattern whereas that of the pure stearic acid obviously exhibited crystalline pattern with many sharp and strong intensity at 6.6°, 11°, 21.4° and 23.7° 2θ. The highest intensity was appeared at 6.6° 2θ and followed by 21.4°, 23.7° and 11°, respectively. Crystallinity of the Alst sample was gradually increased as the treated time was increased. For the treated Alst, the sharp and strong intensities were also found at the same angle of the diffractogram of the pure stearic acid but with different intensities. The highest intensity of the diffractogram of Alst was found at 21.5° 2θ and followed by 23.8° and 6.7° 2θ, respectively. In crystal structure of pure stearic acid, the d-spacings of 4.1 Å (2θ = 21.6°) correspond the [110] planes (Moniruzzaman & Sundararajan, 2004). Because Alst was not totally dissociated, the differences of the diffractogram pattern might be caused by the Alst residue that interfering crystalline structure of the dissociated stearic acid. Previous study reported that the blend system of carbamate and pure stearic acid exhibited a major change in diffractogram comparing to that of
pure stearic acid. For the blends, the intensity at $21.6^\circ$ 2$\theta$ was significantly increased (Moniruzzaman & Sundararajan, 2004).

![X-ray diffractograms of the untreated Alst (A), the acid-treated Alst at various soaking times; 5 min (B), 4 h (C), 8 h (D) and 24 h (E), and stearic acid (F)](image)

**Fig. 13** X-ray diffractograms of the untreated Alst (A), the acid-treated Alst at various soaking times; 5 min (B), 4 h (C), 8 h (D) and 24 h (E), and stearic acid (F)

### 1.5. Thermal properties

#### 1.5.1. TG and DTG

DTG curves of stearic acid, untreated Alst and 8h-acid-treated Alst are illustrated in Fig. 14. DTG curve of the untreated Alst exhibited two stages of degradation with temperature of maximum degradation rate ($T_{\text{max}}$) of 318°C and 403°C and weight loss of 15 % and 45 %, respectively. After 8 h of soaking in dilute acid solution, DTG curve of the acid-treated Alst exhibited single stage of degradation with $T_{\text{max}}$ of 284°C and 41% weight loss. This was near to that of stearic acid which found at 304°C with 88 % weight loss. These results indicated that Alst was dissociated into stearic acid when soaking in acid solution. However, weight loss of the acid treated Alst was approximately 55 % after treated for 24 h suggesting that Alst was not totally dissociated into stearic acid within 24 h.
1.5.2. DSC

DSC thermograms of the samples are demonstrated in Fig. 15. DSC thermogram of the untreated Alst and 5 min-acid-treated Alst were not clearly exhibited endo- or exothermic peak. Generally, melting point of Alst is in the temperature range of 200-225°C (Rowe et al., 2009). After soaking in acid solution for 4 h, broad endothermic peaks appeared in the temperature range of 55-60°C and around 100°C which assigned to melting point of stearic acid and moisture evaporation, respectively. Stronger and sharper of the endothermic peak at 55-60°C indicated an increment of stearic acid with the soaking time.

Fig. 14 DTG curves of stearic acid (A), 8 h-acid-treated Alst (B) and untreated Alst (C)

Fig. 15 DSC thermograms of the untreated Alst (A), the acid-treated Alst at various soaking times; 5 min (B), 4 h (C), 8 h (D), 12 h (E) and 24 h (F), and stearic acid (G)
1.5.3. HSM

Melting behavior of the untreated Alst, stearic acid and the acid-treated Alst was detected under HSM in the temperature range that covered their melting point. Alst was gradually melted at the temperature range of 160-173 °C whereas the pure stearic acid was suddenly melted when the temperature was reached to 57 °C. The melting temperature range of Alst was narrowed down and decreased to the temperature about 60 °C as the soaking time was increased as shown in Fig. 16.

![Diagram showing melting temperature range from HSM of the acid-treated Alst at various soaking times](image)

**Fig. 16** Melting temperature range from HSM of the acid-treated Alst at various soaking times (solid circle refers to temperature that the sample begin to melt and an open circle refers to temperature that the sample melted completely)

All of above results suggested that dissociation of Alst soaked in 2% lactic acid solution was nearly equilibrated within 8 h of mixing. Dissociation of the metal carboxylates was reported as reversible process and has dissociation constant (pKₐ). However, the pKₐ value of aluminum stearates has not been reported due to its low solubility in aqueous medium (The Metal Carboxylates Coalition, 2002).

2. Chitosan-Alst composite dispersions

2.1. Viscosity and rheology, and pH value

Rheograms of the CLA dispersions at mixing time of 5 min and 24 h are demonstrated in Fig. 17. Shear stress (D/cm²) refers to an applied force whereas shear rate refers to gradient of velocity. Rheograms of all samples exhibited Newtonian flow behavior. The sample that needs higher shear stress to evoke the same shear rate as the others meaning this sample has higher viscosity than the others.
Therefore, the viscosity of CLA dispersions containing 4% w/w chitosan was higher than that of system containing 2% w/w and 1% w/w chitosan, respectively. Slightly shifting of shear stress to lower value at the same shear rate when mixing time was increased indicated a viscosity lowering of the dispersion by mixing time. Viscosity lowering of the dispersions during mixing might be a result of chitosan network formation interruption. During mixing, –COO\(^-\) of stearates, that dissociated from Alst, bound to –NH\(_3^+\) of chitosan via electrostatic interaction to form chitosan-stearate complex. This complex could be further bound to stearic acid via hydrophobic interaction to create larger complex cluster by mixing time. This larger complex could more interrupt network formation of chitosan and eventually led to viscosity lowering of the system.

![Rheograms of the chitosan-Alst dispersions at mixing time of 5 min and 24 h where CL1, CL2 and CL4 refer to the system comprising 1, 2 and 4 %w/w chitosan, respectively](image)

**Fig. 17** Rheograms of the chitosan-Alst dispersions at mixing time of 5 min and 24 h where CL1, CL2 and CL4 refer to the system comprising 1, 2 and 4 %w/w chitosan, respectively

The pH value of the CL1, CL2, and CL4 was 2.97±0.01, 3.45±0.01 and 4.90±0.01, respectively. Alst significant enhanced the pH value of the prepared dispersion especially for the CL1 and CL2. The pH value of CL1, CL2 and CL4 at 24h-mixing time was 3.29±0.01, 3.64±0.01 and 5.01±0.02, respectively. Al(OH)\(_3\) that gradually occurred during dissociation of Alst molecules after immersed in acidic aqueous medium might be a cause of the pH value increment. Dissociation of metal carboxylates generally depends on pH and temperature. The lower pH and the higher temperature, the higher dissociation of the substances (MorningStar Consulting Inc. on behalf of The Metal Carboxylates Coalition, 2002; Phaechamud *et al.*, 2009).
Therefore, Alst could dissociate in the lower chitosan concentration system which having lower pH value easier than that of the higher chitosan concentration system.

### 2.2. Particle size and morphology under inverted microscope

Particle sizes of the chitosan dispersions at various mixing times are demonstrated in Fig. 18. For CL1 and CL2, the dispersant particle size significantly increased as mixing time was increased upto 12 h of mixing. However, trend of particle size increment was not clearly demonstrated for the CL4. This phenomenon could be confirmed by morphology observation under inverted microscope as shown in Fig. 19. Result of this study corresponded with that reported in the previous work that in the system comprised low ratio of chitosan to fatty acid, a larger complex size was obtained (Vargas et al., 2009). The lower chitosan concentration system containing higher fatty acid ratio could form larger complex size when mixing time was increased as mentioned previously that chitosan-fatty acid complex could be larger by mixing time which rendering by hydrophobic interaction.

According to acid neutralizing capacity of metal carboxylates, it has been employed as acid acceptors in plastic manufacturing process (Schwarzenbach et al., 2009). In this study, lactic acid which was used as solvent for chitosan could be neutralized by ammonium groups of chitosan. Moreover, aluminium ions that dissociated from Alst could also neutralize the lactic acid. In contrast, the positive charge chitosan could be neutralized by anionic stearate molecule.

![Fig. 18 Mean particle sizes of the chitosan-Alst composite dispersions at various mixing times](image)
According to all above results, the system comprised 4% w/w chitosan exhibited the most homogeneity therefore interesting to be further developed as sponge material, and 24 h is the most appropriate mixing time.

3. Fabricated chitosan-Alst composite sponges

3.1. Physicochemical properties

3.1.1. Weight loss after DHT

Aim of using DHT in this study was to remove water molecule between ionic bond of ammonium group of chitosan and carboxylate group of the acid side chains. Therefore, the DHT sponges could lose their weight due to water loss. However, boiling point of substance is reduced when the pressure is reduced. Boiling point of lactic is 79.6°C at 1 mbar (US Environmental Protection Agency, 2003). In this study, DHT was performed at 110°C under pressure of less than 0.1 mbar thus lactic acid has possibility to evaporate from the chitosan sponges during DHT. Therefore, weight loss of the treated sponges was also due to lactic acid evaporation. Weight loss after DHT was determined to confirm this possibility. Net content of lactic acid was about 33.33, 30.77, 23.53, and 18.18 % w/w in CL, CLA05, CLA25 and CLA5 sponges, respectively. Highest percentage water loss was found in CL system at 15.70 % and the value was lower in the CLA sponges inversely with Alst amount as demonstrated in Table 6.
Table 6 Weight loss of the prepared sponges after DHT (110°C, 24h, vacuum condition)

<table>
<thead>
<tr>
<th>Sponges</th>
<th>% Weight loss (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>15.70 ± 0.34</td>
</tr>
<tr>
<td>CLA05</td>
<td>14.20 ± 0.67</td>
</tr>
<tr>
<td>CLA25</td>
<td>9.38 ± 0.69</td>
</tr>
<tr>
<td>CLA50</td>
<td>5.98 ± 0.29</td>
</tr>
</tbody>
</table>

3.1.2. FT-IR spectroscopy

FT-IR spectroscopy was employed to detect an interaction between amino group of chitosan and carboxylate group of the lactate and stearate side chain molecules. Fig. 20 exhibits IR spectra of the raw materials (A), the lyophilized (B) and the DHT (C) sponges in range of 1200 – 1900 cm\(^{-1}\) wavenumber. This region indicates amide I (–C=O), amide II (–NH–) and free amino group of chitosan, and carbonyl (–C=O) stretching of the carboxylic acid. IR spectra of Alst (Fig. 20A(1)) exhibited strong absorption peak at 1590 and 1471 cm\(^{-1}\) which assigned to –C=O stretching of the metal carboxylates (Phaechamud et al., 2009). Chitosan spectrum (Fig. 20A(2)) exhibited overlapping broad absorption peak of amide I (–C=O) and free amino group (–NH\(_2\)) in range of 1644 and 1597 cm\(^{-1}\) (Pawlak & Mucha, 2003).

The spectra of lyophilized CL sponge (Fig. 20B) exhibited absorption peaks at 1726 cm\(^{-1}\) that assigned to carbonyl stretching (–C=O) of carboxyl group of unionized lactic acid, and 1590 cm\(^{-1}\) that assigned to –C=O stretching of carboxylate salt of lactic acid that bound to –NH\(_3\)+ group of chitosan. Sharper peaks than that of lyophilized CL sponge at around 1587 and 1465 cm\(^{-1}\) was found in spectra of the lyophilized CLA sponges which belonging to –C=O stretching of stearate side chain dissociated from Alst. Their intensity increased by an increment of Alst amount.

The –C=O stretching peak at 1587 cm\(^{-1}\) was less intensified whereas broad peak of amide I band at around 1628 cm\(^{-1}\) was more intensified after DHT especially in CLA25 and CLA50 systems (Fig. 20C). This indicated amidation or a transformation of ionic bond between –NH\(_3\)+ group of chitosan and –COO\(^-\) of stearate side chain to amide bond. However, this transformation was not clearly observed in spectra of CL and CLA05 sponges. Previous work reported that a boiling point of lactic acid at pressure of 1 mbar is approximately 79ºC (US Environmental Protection Agency, 2003). Therefore, we expected that lactic acid might partially evaporate during DHT (110 ºC, 24 h) under vacuum condition (pressure less than 0.1 mbar) and free amino group was left instead. Possibility of this event is demonstrated in Fig. 21.
Fig. 20 FT-IR spectra of A = raw materials where 1 = Alst and 2 = chitosan, B and C = lyophilized and DHT sponges, respectively, where 1 = CL, 2 = CLA05, 3 = CLA25 and 4 = CLA50
Fig. 21 Possibility of changes in ionic bond between ammonium group of chitosan and carboxylate group of acid side chain (lactate and stearate molecule) where 1 indicates amidation, 2 indicates no changes and 3 indicates evaporation of lactic acid after DHT.

3.1.3. PXRD

X-ray diffractograms of the raw materials (chitosan, Alst and stearic acid) are illustrated in Fig. 22. Chitosan diffractogram exhibited identical peaks at 10.47, 20.02, 22.51, and 29.18 °2θ that were identified as 020, 110, 120 and 130 crystal reflections (Zhang et al., 2005), respectively. The highest intensity belonged to the 120 reflection located at 22.51 °2θ. The 110 peak located around 20 °2θ corresponds to the crystallinity of chitosan (Pacheco et al., 2011) which the more intensity of this peak indicates the higher crystallinity of the chitosan. Alst diffractogram exhibited dominant peaks at 6.67, 11.38, 19.55, and 22.87 °2θ whereas that of stearic acid also exhibited the dominant peaks at the same 2θ region but with drastically higher intensity than that of Alst peaks.

X-ray diffractograms of the prepared sponge (CL and CLA25) are displayed in Fig. 23. The diffractogram of the lyophilized CL (1) exhibited amorphous pattern with broad peak around 18.62 °2θ whereas that of the lyophilized CLA25 (2) exhibited more crystallinity pattern with broad peak around 20.95 and 23.70 °2θ. After treated by DHT, the diffractogram of CL (3) still exhibited...
amorphous pattern with broad peak around 20.96 °2θ that located in the same region of 110 reflection peak of the chitosan raw material but with very low intensity comparing to the raw material. The explicit transition was observed in the diffractogram of the CLA25-DHT (4). The sharp peak appeared at 21.30 °2θ indicating an increment of the material crystallinity. Any other peaks belonging to stearic acid was not found in the CLA25-DHT diffractogram indicating that stearic acid was not occurred after DHT. Previous study reported that DD influenced chitosan crystallinity. The chitosan with very high or very low DD exhibited the highest crystallinity where the medium DD (near to 50%) exhibited the lowest crystallinity (Zhang et al., 2005). According to IR spectra analysis, amidation was occurred after treating the CLA sponge by using DHT. Amidation after DHT reduced DD of chitosan in the prepared sponge therefore this might be a cause of crystallinity increment of this system. The similar results about the increment of chitosan-acid salt film crystallinity after the heat treatment was reported previously (Ritthidej et al., 2002). This previous study treated different chitosan-acid salt films with moist heat treatment (at 60°C with 75 % RH) for 24, 48, 120, or 360 h. The result from XRD and FT-IR evaluation indicated that amidation was occurred in the treated films by the time after the moist heat treatment. This was a result of self-protonation of the acid molecule during heating process which was later reacted with the amine nucleophile to form amide bond and then eliminated water molecule as a byproduct. Amidation occurred by the heat treatment caused a reduction of hydrophilic group of chitosan therefore reduced the water sorption and dissolution of the chitosan films (Ritthidej et al., 2002).
Fig. 22 X-ray diffractograms of chitosan (A), Alst (B), and stearic acid (C) (The 2θ degree and the d-spacings are shown in appendix II)

Fig. 23 X-ray diffractograms of the prepared sponges; CL (1), CLA25 (2), CL-DHT (3), and CLA25-DHT (4) (The 2θ degree and the d-spacings are shown in appendix II)
3.1.4. SEM

SEM images of the lyophilized and DHT sponges are demonstrated in Fig. 24. Morphology of almost system exhibited homogeneous porous pattern with irregular pore shape except for that of CLA50 that did not exhibit porous but sheet-like structure. Pore’s wall of CL was smooth whereas that of CLA05 and CLA25 were partly covered with cluster of the non-dissociated Alst and stearates. Pore’s wall of the CLA50 was completely covered with the non-dissociated Alst and stearates. Pore size of the CL, CLA05 and CLA25 determined by using image analysis program was in the range of 100–220 micron. Pore size of the CL was smaller than that of CLA sponges which the pore size was increased by Alst amount. Effect of Alst on pore size enhancement might be due to steric effect of the Alst that interfering network formation of chitosan chain therefore the higher the Alst amount, the lower the chitosan network formation.

![SEM images of chitosan sponges at 200x magnification](image)

**Fig. 24** SEM images of chitosan sponges at 200x magnification; A = lyophilized, B = DHT which 1 = CL, 2 = CLA05, 3 = CLA25 and 4 = CLA5

3.1.5. Thermal properties (TG, DSC, HSM)

TG curves plotted between weight (%) and temperature (°C) and the DTG curves of the samples are shown in Figs. 25 and 26, respectively. Initial temperature of the degradation ($T_{int}$) and temperature of the maximum reaction velocity ($T_{max}$) are shown in Table. 7. The first thermal event of all substances, except for that of Alst, was exhibited in the temperature ranges of 30-125°C with 2 - 4 % weight loss. This attributed to water loss from polymer network. Thermal degradation stage of chitosan were reported previously (Peniche-Covas et al., 1993; Wanjun et al., 2005). First stage belonging to water loss is in the ranges of 25-140°C. The second degradation stage of deacetylation and depolymerization process appears in higher temperature range; 200-400°C. Second stage of the thermal
degradation of chitosan in this study appeared in the range of 300-420°C expecting to be only depolymerization process because chitosan used in this study has high deacetylation degree (97%). For DTG curve of chitosan, unsmooth pattern was observed in the ranges of 120-300°C expecting as the degradation event of impurities. The $T_{\text{max}}$ of the second event was found at 335.46°C with 30% weight loss.

Comparing with chitosan, TG curve of CL exhibited two degradation stages at lower temperature range with $T_{\text{max}}$ of 203.31°C and 331.73°C, respectively. The first stage of degradation displayed a cleavage of lactate side chain from chitosan molecules whereas the second stage assigned to the depolymerization of chitosan chain via scission of C-O-C bond at β(1,4)-linkage (Wanjun et al., 2005). Lower degradation temperature of CL indicated that it was less thermal stable than chitosan raw material. The similar result has been reported previously that acid derivatives of chitosan were less stable than chitosan itself which more thermal stability was observed for the longer side chain system (Liao et al., 2004). Two stages of thermal event around 318°C and 403°C were observed for TG curve of Alst. The first and second stages were expected to be a breakage of aluminum and stearate molecule, and a cleavage of stearate chain, respectively. Comparing to the CL, TG curve of CLA25 exhibited a small shifting of $T_{\text{int}}$ and $T_{\text{max}}$ to a higher temperature at approximately 216°C and 335°C which indicated that the CLA25 was more thermal stable than the CL but lower than chitosan. Lessening of the thermal stability of the CL and the CLA25 was due to a reduction of crystallinity of their structure after fabrication. Protecting effect of Alst on thermal properties of chitosan in the CLA25 system might be an effect of long chain of stearate ($C_{18}$) molecule that could protect chitosan backbone better than that of lactate ($C_3$) molecule according to effect of the side chain length as mentioned previously.

![Fig. 25 TG curve of 1 = Alst, 2 = Chitosan, 3 = lyophilized CLA25, and 4 = CL](image-url)
**Figure 26** DTG curve of A; 1= Chitosan, 2= Alst and B; 1= CL, 2= lyophilized CLA25

**Table 7** Weight loss (%), initial temperature of the degradation ($T_{int}$) and temperature of the maximum reaction velocity ($T_{max}$) of the single substances and the prepared sponges

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak No.</th>
<th>$T_{int}$ (°C)</th>
<th>$T_{max}$ (°C)</th>
<th>Weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>1</td>
<td>45.00</td>
<td>68.58</td>
<td>4.396</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>321.87</td>
<td>335.46</td>
<td>30.011</td>
</tr>
<tr>
<td>Alst</td>
<td>1</td>
<td>277.27</td>
<td>318.56</td>
<td>15.142</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>381.81</td>
<td>403.26</td>
<td>44.872</td>
</tr>
<tr>
<td>CL</td>
<td>1</td>
<td>50.00</td>
<td>59.15</td>
<td>2.729</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>164.77</td>
<td>203.41</td>
<td>18.894</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>309.09</td>
<td>331.73</td>
<td>45.997</td>
</tr>
<tr>
<td>CLA25</td>
<td>1</td>
<td>41.66</td>
<td>61.22</td>
<td>3.061</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>182.95</td>
<td>216.51</td>
<td>16.84</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>311.36</td>
<td>335.66</td>
<td>45.958</td>
</tr>
</tbody>
</table>
DSC thermograms of chitosan sponge; CL and CLA25 are illustrated in Fig. 27. Small-broad exothermic peak was observed around 162°C in thermogram of the CL. The exothermic peak indicated an occurrence of new product obtained via thermal cross-linking process of chitosan molecules (Wanjun et al., 2005). For the CLA25, tiny endothermic peaks were exhibited around 59°C and 69°C expecting as a melting temperature of stearic acid.

![Fig. 27 DSC thermograms of the lyophilized sponges (CL and CLA25)](image)

According to HSM results as demonstrated in Fig. 28, chitosan powder was gradually burnt while its color was darkened during heating. CL powder was slowly shrunk together with darkening of the color. Melting of stearate on chitosan backbone in CLA25 was observed since 59°C whereas chitosan backbone was gradually shrunk and darkened.

![Fig. 28 Morphology of chitosan powder and the lyophilized sponges (CL and CLA25) under hot-stage microscope](image)
3.2. Hydrophilic/hydrophobic property

Determining the hydrophilicity of the porous materials by measuring static contact angle (Kim et al., 2003; Kosorn et al., 2012) or a reduction of the contact angle as a function of time (Prabu et al., 2008) has been reported previously. This method reported contact angle as a point of time but in this study the contact angle was measured continuously every seconds in order to observe the trend of contact angle reduction by the time.

The graphs plotted between contact angle and water penetration time are illustrated in Fig. 29. Contact angle was gradually reduced as water gradually penetrated into sponge structure. Penetration speed of water drop was controlled by hydrophilic/ hydrophobic properties of the materials. Generally after water is dropped onto the surface of nonporous material, hydrophilicity of the substrates will cause spreading of water. This leads to an increment of the drop base area accompanying by reduction of the drop height which eventually reduces contact angle. However, the tested material in this study was porous therefore another factor that could influence the decrement of contact angle was water penetration. Comparing between the lyophilized sponge (Fig. 29A), fastest reduced contact angle was observed from the CL system therefore it was more hydrophilic than the others. For CLA systems, contact angle reduction was slower in the system containing higher Alst amount which indicated that Alst enhanced hydrophobicity of the materials. After DHT (Fig. 29B), CLA sponges exhibited higher contact angle value than that of their lyophilized samples due to the transformation of the hydrophilic ionic bond into hydrophobic amide bond. However, faster contact angle reduction of the CL sponge was observed after DHT indicating more hydrophilic of the system which might be a result of lactic acid partially evaporation that left the hydrophilic free amino group in the chitosan structure.

Fig. 29 Contact angle of the water drop on the surface of the lyophilized (A) and DHT (B) sponges
3.3. Water sorption and erosion

Lyophilized CL sponges were rapidly dissolved after soaking in phosphate buffer pH 7.4 and CLA05 was also completely dissolved but with slower rate than that of CL, whereas CLA25 and CLA50 were still left some residue after soaking overnight. Since CL and CLA05 were completely dissolved, their water sorption could not be determined. Water sorption (%) of CLA25 and CLA50 were approximately 1,036 and 692 %, respectively, as shown in Fig. 30. The chitosan sponges both CL and CLA systems was changed after DHT to water insoluble but could swell in aqueous medium. DHT-CL sponge was swollen and exhibited gel-like structure with transparent yellow color after immersion in phosphate buffer pH 7.4 with mean percentage water sorption of 3,303 % w/w. The DHT-CLA sponges were opaque and their percentage water sorption was lesser than that of the DHT-CL. The value of the DHT-CLA05 was 1,692 % w/w and reduced when the Alst amount increased.

Lower percent of erosion of the prepared sponges was exhibited in the system containing Alst comparing to that of CL sponge as shown in Fig. 30. As the amount of Alst increased, lower percent of erosion was demonstrated. However, for the DHT-sponges, percent of erosion of the CLA50 was higher than that of the CLA25 which might be due to the lower strength of its structure.

![Fig. 30 Water sorption and erosion of the lyophilized and DHT sponges immersed in phosphate buffer pH 7.4 overnight](image-url)
3.4. Swelling behavior

An initial stage of polymer dissolution is water sorption but in a permanent system or a cross-linked matrix, swelling will replace at the point dissolution discontinue (Ferapontov et al., 2013). In case of a permanent porous matrix, water can freely diffuse into the matrix via the pores. Mesh size of the structure relates the swelling ratio which is the factor that modulates drug release from the matrix (Berger et al., 2004). Swelling of hydrophilic chitosan materials is evoked by hydrogen bond formation between water molecules and hydrogen bonding groups of chitosan namely hydroxyl group (−OH) and free amino group (−NH₂) (Yao et al., 1998). Moreover, chitosan exhibits pH sensitive swelling property due to ionic interaction at the free amino groups. In acidic environment where pH value is less than 6.5, free amino group of chitosan can be protonated and exhibits positive charge that generates charge repulsive force between the polymer chain that eventually causes swelling of the structure. On the contrary at pH value more than 6.5, amino group of chitosan is not protonated but swelling of the structure still occurs by hydrogen bonding with the lower degree than that of the charge repulsive force (Park et al., 2006).

Swelling behaviors of CL and CLA sponges are demonstrated in Fig. 31. Sponges drastically swelled in acidic condition (pH 4.0). This was a result of protonation of the free amino groups (−NH₂) in acidic medium as described previously. In mild basic environment of phosphate buffer pH 7.4 which was higher than pKₐ value of the amino group of chitosan (pKₐ ~ 6.5), sponge could also swell but with slower rate and approximately three times lower swelling degree than that of in acidic environment. In strong basic environment of carbonate buffer pH 10.0, swelling ratio of all sponges was the lowest comparing with the other medium. In this condition, ammonium ion of chitosan was neutralized to unionized amino group. Therefore, swelling degree of the sponge was gradually decreased by immersion time especially for CL sponge.

DHT had effect on transformation of water-soluble ionic bond between ammonium group and carboxylate group of the acid side chain into water insoluble amide bond. However, pH dependent swelling of all the prepared sponges could still be observed. This revealed that some free amino groups were still left in the structure which meant the partial transformation of ionic bond into amide bond.

Alst addition apparently influenced swelling behavior of chitosan in all buffer systems. The system comprising higher Alst amount exhibited lower degree of swelling and took longer time to reach equilibrium swelling. Hydrophobic nature and long carbon chain of the stearate (C₁₈) side chain could retard the water diffusion into sponges structure, therefore delayed the swelling rate. Lower degree of equilibrium swelling of the sponges containing higher Alst amounts might be a result of more rigidity of the structure caused by steric effect of the high amount and long stearate side chain.
Fig. 31 Digital images and swelling degree of the DHT sponges in three different buffer solutions at various immersion times
3.5. *In vitro* asiaticoside release

Asiaticoside is the main triterpene glycoside found in water extract of *C. asiatica* (Linn.) Urban. It possesses interesting wound healing activities (Shukla *et al.*, 1999). The mechanism studied at cytobiological and molecular biological levels in human skin fibroblast revealed that asiaticoside stimulated mRNA and protein production correlated with genes responsible for cell-cycle progression, proliferation and collagen synthesis (Lu *et al.*, 2004). Asiaticoside releasing profiles of the prepared sponges are demonstrated in Fig. 32. Burst release at higher than 80% was observed from CL sponge within 30 min. Released asiaticoside amount was drastically decreased to less than 25% in CLA sponges and the percentage release was reverse variation with Alst amount. These profiles were concurrent with the swelling study results. As described previously, the swelling of the permanent porous matrix is a main factor for controlling the drug release. Therefore, the CL sponge which exhibited the highest degree and fastest rate of swelling exhibited the fastest asiaticoside release. In case of CLA sponges, hydrophobicity and wax cluster of stearate side chain could obstruct the water diffusion into sponge structure with amount dependent, which influenced on swelling and eventually delayed the drug release.

![Asiaticoside release from the DHT sponges studied in phosphate buffer pH 7.4 at 37°C for 48 h (The raw data are shown in Appendix I.)](image-url)
SEM images of the sponges before and after release study are demonstrated in Fig. 33(A) and 33(B), respectively. All sponges displayed interconnected porous structure with pore size of approximately in range of 100-140 micron for the sponge before release study and were larger to approximately in range of 160-220 micron after release study. Knotty waxy cluster of stearate was still homogeneously dispersed cover the chitosan wall in CLA sponges which indicated stability of the DHT materials in aqueous medium.

![SEM images of the DHT sponges before (A) and after (B) drug release study in phosphate buffer pH 7.4](image)

**Fig. 33** SEM images of the DHT sponges before (A) and after (B) drug release study in phosphate buffer pH 7.4

### 3.6. Release profile analysis

Results of release profile analysis are demonstrated in Table 8. Drug release from CLA sponges fitted well with first-order model. This indicated that drug release from CLA sponges was proportional to an amount of the drug remaining in the sponges. Normally for the water soluble drug contained-porous matrix, first-order kinetic is found as the main release mechanism (Costa & Lobo, 2001). Exponent (n) obtained from power law model of all CLA sponges was less than or equal to 0.45 indicating Fickian diffusion as the main mechanism of drug release from these systems (Siepmann & Peppas, 2001). This clearly explained that only diffusion impelled the drug release from CLA which did not include the matrix degradation. Solute liberated from the non-degraded polymer matrix is mainly driven by diffusion (Fu & Kao, 2010) where concentration gradient of the solute, which controlled by remaining concentration of the solute, is the main factor that controls rate of water diffusion into the matrix. According to swelling behavior study, all sponges reached the equilibrium swelling state in phosphate buffer pH 7.4 at approximately 1 h then their structure was stable from this point. After equilibrium swelling, therefore, remaining concentration of the drug was the main factor that controlled the drug release from the sponges. In case of CL sponge, the apparent burst release was found at the first point of sampling (30 min).
Table 8 Degree of curve fitting ($r^2$) and goodness of fit (MSC) from curve fitting of \textit{in vitro} asiaticoside release profiles of the CLA sponges

<table>
<thead>
<tr>
<th>Sponges</th>
<th>first-order</th>
<th>zero-order</th>
<th>Higuchi's power law</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>MSC</td>
<td>$r^2$</td>
</tr>
<tr>
<td>CLA05</td>
<td>0.9944</td>
<td>4.7375</td>
<td>0.7905</td>
</tr>
<tr>
<td>CLA25</td>
<td>0.9983</td>
<td>5.9187</td>
<td>0.8358</td>
</tr>
<tr>
<td>CLA50</td>
<td>0.9970</td>
<td>5.3844</td>
<td>0.9014</td>
</tr>
</tbody>
</table>

The prepared CLA sponges were high porous, stable in aqueous medium after DHT, able to absorb high amount of fluid, and could release asiaticoside in a sustained manner therefore they were interesting to be further developed as wound dressing. However, burst release still be observed which indicate low homogeneity of asiaticoside in the polymer composite matrix. Therefore, in the next step we added NMP as a co-solvent of asiaticoside in order to improve homogeneity of the substance in the matrix.

4. Chitosan-Alst sponge dressings

4.1. Physicochemical properties

4.1.1. Weight loss after DHT

Comparing to the result in section 3.1.1, NMP-contained sponges exhibited the same trend but higher weight loss than that of sponge without NMP as shown in Table 9. Weight loss of the CD, CD05, CD25 and CD50 were 19.25 % w/w, 19.40 % w/w, 14.90% w/w and 7.82 % w/w, respectively. Therefore, it can be concluded that NMP was also partially evaporated during DHT.

Table 9 Weight loss of the lyophilized sponge dressings after DHT (110°C, 24h, vacuum condition)

<table>
<thead>
<tr>
<th>Sponges</th>
<th>% Weight loss (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>19.52 ± 1.29</td>
</tr>
<tr>
<td>CD05</td>
<td>19.40 ± 1.87</td>
</tr>
<tr>
<td>CD25</td>
<td>14.90 ± 1.01</td>
</tr>
<tr>
<td>CD50</td>
<td>7.82 ± 0.88</td>
</tr>
</tbody>
</table>
4.1.2. FT-IR spectroscopy

FT-IR spectra in wavenumber range of 2100–1300 cm\(^{-1}\) of the raw materials and the chitosan dressings are illustrated in Fig. 34. IR spectrum of chitosan (A (a)) exhibited amide I and amide II band at 1644 and 1597 cm\(^{-1}\), respectively. The Alst spectrum (A (b)) exhibited dominant peak of metal carboxylate at 1589 and 1471 cm\(^{-1}\) which belonging to carboxylate stretching. The spectrum of lactic acid (A (c)) exhibited broad peak around 1716 cm\(^{-1}\) which is the region of –C=O stretching of carboxyl group. The NMP spectrum (A (d)) displayed strong broad peak in wavenumber range of 1700-1600 cm\(^{-1}\) which assigned to –C=O stretching of the lactam ring. Normally, plain five-membered lactam ring exhibits strong –C=O stretching peak in the wave number range of 1750-1700 cm\(^{-1}\) (Lin-Vien et al., 1991). For NMP structure, nitrogen atom at position-1 of the ring is substituted by methyl group which might be a cause of the shifting of the –C=O stretching to the lower wavenumber region. The spectrum of asiaticoside (A (e)) exhibited small peak at 1734 and 1644 cm\(^{-1}\) that ascribed to –C=O stretching of asiatic acid structure (Sondari et al., 2011).

FT-IR spectra of the lyophilized dressings (Fig. 34B) displayed broad absorption band around 1800-1500 cm\(^{-1}\) with sharp peak at approximately 1628 cm\(^{-1}\). This band was expected belonging to the lactam ring of NMP that overlapping with –C=O stretching of carboxylate group of the acid molecule that bound to ammonium group of chitosan (ionic bond) around 1590 cm\(^{-1}\). The –C=O stretching band of Alst appeared at 1588 and 1470 cm\(^{-1}\) which its intensity increased by Alst amount. Small broad peak around 1731 cm\(^{-1}\) was expected ascribing to carbonyl stretching (–C=O) of carboxyl group of unionized lactic acid. Asiaticoside peak did not seem to appear in the spectra of the lyophilized dressings since a very low content of asiaticoside was incorporated into the matrix. After DHT, a minor change was found in spectrum of the CD sponge. The sharp peak at 1629 cm\(^{-1}\) was shifted lower to 1622 cm\(^{-1}\). In case of the CD05, CD25 and CD50, intensity of the metal carboxylate dominant peaks at 1588 and 1470 cm\(^{-1}\) were dramatically lessened. The new sharp peak at 1644 cm\(^{-1}\) displayed in their spectra which was expected as the –C=O stretching of the amide bond that occurred due to the effect of DHT. Lessening of the small broad peak at 1730 cm\(^{-1}\) and changing of the peak around 1644-1628 cm\(^{-1}\) could indicate evaporation of the lactic acid and NMP during DHT. NMP is non-ionic therefore it cannot form ionic bond with other components in this prepared system. However, its structure composed of nitrogen molecule and –C=O that could form hydrogen bond with water molecule, and hydroxyl and amino group of chitosan. Therefore, IR spectrum around 3500-3000 cm\(^{-1}\) was broadened due to hydrogen bonding (data not shown).
Fig. 34 FT-IR spectra of the raw materials [A] where (a) = chitosan, (b) = Alst, (c) = lactic acid, (d) = NMP and (e) = asiaticoside, and lyophilized [B] and DHT dressings [C] where (1) = CD, (2) = CD05, (3) = CD25 and (4) = CD50

4.1.3. PXRD

X-ray diffractograms of the raw materials (chitosan (a), Alst (b), stearic acid (c), and asiaticoside (d)) are shown in Fig. 35. Chitosan diffractogram exhibited identical peaks at 10.47, 20.02, 22.51, and 29.18 °2θ whereas that of Alst and stearic acid exhibited dominant peaks at 6.67, 11.38, 19.55, and 22.87 °2θ with different intensities as described previously in section 3.1.3. X-ray diffractogram of asiaticoside exhibited several small sharp peaks in range of 4-25 °2θ. High intensity peaks were observed at 4.19, 13.33, 13.91, 16.71, and 19.76 °2θ.

X-ray diffractograms of the lyophilized dressings are illustrated in Fig. 35 [A]. The CD diffractogram (1) exhibited amorphous pattern with the broad peak around 20.01 °2θ. No identical peak of the asiaticoside was observed in any dressing diffractograms due to very low amount of the substance was incorporated into the polymer composite matrix. Adding Alst apparently enhanced crystallinity pattern of the diffractogram of the prepared dressings. Sharp peak with higher intensity appeared at 21.39 °2θ for the CD05 (2), 20.99 °2θ for the CD25 (3), and 6.12, 20.70 and 22.43 for the CD50 (4). After DHT (Fig. 35 [B]), the diffractogram pattern of the CD0 and CD05 was still amorphous whereas that of the CD25 and CD50 apparently exhibited sharper peak at around 21.00 °2θ indicating higher
crystallinity of the materials due to amidation caused by DHT. This result corresponded with the NMP free-sponge (section 3.1.3) suggesting that NMP had no effect on amidation during DHT.

![X-ray diffractograms of the raw materials](image)

**Fig. 35** X-ray diffractograms of the raw materials [chitosan (a), Alst (b), stearic acid (c), and asiaticoside (d)] and the lyophilized [A] and DHT [B] dressings where (1), (2), (3), and (4) indicate CD, CD05, CD25, and CD50, respectively (The 2θ degree and the d-spacing were shown in appendix II)

4.1.4. SEM

SEM images displaying morphology of the chitosan dressings surface, bottom and cross-sectioned are illustrated in Fig. 36. There were open pores on surface and bottom of all sponges but with different morphology. On the surface, pores were not as much as found on the bottom and cross-sectioned. Small clusters of Alst/stearate were homogeneously dispersed on chitosan structure of the Alst-contained sponges. Comparing between cross-sectioned of the sponges, the system containing higher Alst amount exhibited more irregular pore shape. This might be a result of steric hindrance effect of the stearate molecule that blocked intra-/inter-molecular bonding of chitosan chain. Denser structure could be observed from the sponge containing higher Alst amount due to the cluster of Alst/stearate that existed in the pores.
4.1.5. Porosity

All sponges have porosity higher than 85% v/v as shown in Fig. 37. The CD50 seems to have the lowest porosity comparing with the others when observed by SEM images. However, it was not statistically different between their porosity when measured by liquid displacement method. This indicated effectiveness of the prepared dispersions for fabrication, and the fabrication technique used for preparing the sponge dressing.
4.1.6. Mechanical strength

Strength of the Alst-contained sponges was higher than that of the CD sponge and the value increased by Alst amount (Fig. 38). This corresponded with the SEM image observation that the system containing higher Alst amount were more compact than that of containing lower Alst amount. The previous research work found that chitosan-fatty acid composite films exhibited high mechanical strength (high modulus of elasticity or the stress to strain ratio) but low flexibility (low % elongation at break) (Srinivasa et al., 2007(a)). Strength of all sponges was increased after DHT expecting as a result of lower moisture that led to lower flexibility of the structure. The previous research reported an effect of moisture on mechanical strength of chitosan films that the mechanical strength of the material was reduced when exposed to high moisture (Srinivasa et al., 2007(b)).

![Graph showing mechanical strength of lyophilized and DHT sponge dressings](image)

**Fig. 38** Mechanical strength of the lyophilized and DHT sponge dressings obtained from texture analysis in compression mode

4.1.7. Hydrophilic/hydrophobic properties

Hydrophilicity of the sponge dressing was evaluated by measuring contact angle of the water drop on the sponge surface as a function of time. Reduction by the time of contact angle of the water drop on a porous material is a result of water penetration into sponges structure and water drop spreading on the material surface. Hydrophilicity of the material is the main factor that speeds these
process as described previously in section 3.2. Contact angle reduction by the time of chitosan sponges with NMP (Fig. 39) exhibited the same trend with chitosan sponge without NMP (section 3.2). CD exhibited faster contact angle reduction than the CD05, CD25 and CD50, respectively. Therefore, hydrophilicity of the sponge dressings were ranged as CD > CD05 > CD25 > CD50. After DHT, hydrophobicity of the Alst contained-sponge was increased due to amidation whereas that of CD decreased because of lactic acid evaporation during DHT that left hydrophilic free amino groups of chitosan instead.

Comparing with the lyophilized sponges without NMP, hydrophobicity of the sponge containing NMP was higher especially in the Alst-contained systems. This signified that NMP at the material surface was evaporated. Moreover, NMP has been used as a co-solvent for the low water soluble substances (Jouyban et al., 2010). Therefore, NMP facilitated homogeneity of hydrophilic chitosan and hydrophobic Alst during mixing.

Fig. 39 Contact angle of the water drop on the surface of the lyophilized (A) and DHT (B) sponge dressings
4.1.8. Fluid handling ability

Morphology of the chitosan dressings (CD, CD05, CD25 and CD50) and gauze covering on the in vitro pressure ulcer model at various times is illustrated in Figs. 40-44, respectively. The sponge dressings gradually swelled by the time which their swelling speed could be sorted from the fastest to the lowest by the following order; CD, CD05, CD25 and CD50. Their swelling pattern corresponded with their fluid absorbency as demonstrated in Fig. 45. CD exhibited the fastest speed and the highest fluid absorbency which was facilitated by high hydrophilicity of the system. Fluid absorbency of the Alst contained-sponges was drastically low comparing with the CD because of their higher hydrophilicity. The CD50 exhibited the lowest fluid absorbency whereas those of the CD05 and CD25 were not different. Gauze did not swell but was more moistened by the time. Its fluid absorbency was drastically lower than the CD. It could absorb fluid faster than the Alst contained-sponges at the first 8 h. Thereafter, its absorbency gradually increased with slower rate than the CD05 and CD25 but still higher than the CD50. The CD and the gauze were fully soaked with fluid whereas the Alst contained-sponges were rather dry. The CD texture was jelly-like while that of the Alst contained-sponges gradually softened like a rubber.

Morphology of the agar surface drastically changed after contacted with the CD and also the gauze with lesser damage. This was a result of the rapid fluid absorption of the materials. Whereas that of contacted with the CD05, CD25 and CD50 were almost the same with the beginning. The previous report revealed that absorbent dressing such as alginate dressings had high fluid absorbability. This could cause burning sensation to the patient when applied to the wound due to a rapid movement of the fluid from the wound into the dressing (Sweeney et al., 2012).

Water loss of the chitosan dressings, gauze and the pressure ulcer model (control) is demonstrated in Fig. 46. WVTR obtained from the slope of the straight line plotted between contacting time and water loss is shown in Table 10. The pressure ulcer model exhibited the highest WVTR (1,648 g/m²/day) and followed by the CD (1,416 g/m²/day), gauze (1,304 g/m²/day), CD05=CD25 (1,144 g/m²/day), and CD50 (1,024 g/m²/day), respectively. High WVTR of the CD was due to its high hydrophilicity that led to the rapid swelling therefore enlarging pore size of the structure and also the area of evaporation. In case of the Alst contained-sponges, hydrophobicity of Alst delayed the fluid absorption therefore water was gradually evaporated. The gauze did not swell but it had a larger net size than the chitosan sponges therefore it exhibited high WVTR.

Normally moisture at the wound bed should be preserved by an absorbent dressing at an appropriate level that should not too high until causing maceration in the wound bed but should not too low until causing dryness to the wound bed. Even though CD and gauze exhibited high WVTR but they handled too
much fluid therefore could cause maceration at the wound bed. On the contrary, the Alst-contained dressings absorbed lower fluid amount than CD and were rather dry therefore they might provide more appropriate moisture to the wound bed than CD and gauze.

**Fig. 40** Morphology of the DHT CD and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface.
Fig. 41 Morphology of the DHT CD05 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface.
**Fig. 42** Morphology of the DHT CD25 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface.
Fig. 43 Morphology of the DHT CD50 and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of the dressing coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of the dressing that was contacted with the agar surface.
Fig. 44 Morphology of gauze and the pressure ulcer model at various times during fluid handling ability study where the first row demonstrates bottom view of gauze coupled with the model, the middle row demonstrates agar surface of the model, and the last row demonstrates surface of gauze that was contacted with the agar surface.
**Fig. 45** Fluid absorbency of the DHT sponge dressings and gauze during fluid handling ability test (n=3)

**Fig. 46** Water loss of the control (pressure ulcer model), the DHT sponge dressings and gauze during fluid handling ability test (n=3)
Table 10 WVTR of the control (pressure ulcer model), the sponge dressings and gauze (n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>WVTR (g/m²/day) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,648.00 ± 55.43</td>
</tr>
<tr>
<td>Gauze</td>
<td>1,304.00 ± 84.29</td>
</tr>
<tr>
<td>CD</td>
<td>1,416.00 ± 63.50</td>
</tr>
<tr>
<td>CD05</td>
<td>1,144.00 ± 77.15</td>
</tr>
<tr>
<td>CD25</td>
<td>1,144.00 ± 113.42</td>
</tr>
<tr>
<td>CD50</td>
<td>1,024.00 ± 60.40</td>
</tr>
</tbody>
</table>

4.1.9. Oxygen permeability (OP)

Oxygen (O₂) content (% v/v) and OP of the dressings; CD0, CD05 and CD25, are demonstrated in Table 11. CD50 was excluded from this test because it was fragile and difficult to set in the apparatus. The O₂ content in air was 19.35 % v/v. The O₂ content permeated through the dressing at 30 min and 1 h was near 20 % v/v. These indicated equilibrium condition of the oxygen permeation. Statistical analysis indicated no difference of the O₂ content between that of air and permeated through all the samples. The content value at 1 h was employed to calculate OP of the samples. The OP of all dressings were higher than 3.5 g.m/m².h.atm and were not significantly different. These results corresponded with porosity test and SEM images that the sponge structure was very high porous therefore oxygen gas could freely permeate through their unique structure. High oxygen permeation through the chitosan porous materials due to a high porosity has also been reported previously (Mi et al., 2001; Mi et al., 2002). However, the non-porous chitosan film exhibited low OP (Caner et al., 1998).

Table 11 Oxygen content in ambient air and percentage oxygen penetrated through the sponge dressings at 1 h and OP of the chitosan dressings (n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>O₂ content (% v/v) mean ± SD</th>
<th>OP (g.m/m².h.atm) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>19.35 ± 2.89</td>
<td>3.69 ± 0.14</td>
</tr>
<tr>
<td>CD0</td>
<td>20.78 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>CD05</td>
<td>21.61 ± 4.84</td>
<td>3.90 ± 0.70</td>
</tr>
<tr>
<td>CD25</td>
<td>18.65 ± 2.56</td>
<td>3.54 ± 0.43</td>
</tr>
</tbody>
</table>
4.1.10. Bio-adhesion

Bio-adhesion of the chitosan dressings was evaluated using texture analyzer in compression mode. Porcine skin was employed as a biological model. Work of adhesion indicates force that required for pulling the sample from the skin. High value of the work of adhesion indicates high bio-adhesion of the tested material. Fig. 47 shows that the CD exhibited highest bio-adhesive property followed by the CD05. Work of adhesion of the CD and CD05 was significantly higher than that of the CD25, CD50 and the non-adhesive plaster whereas that of the last three samples were not significantly different. Chitosan has excellent bio-adhesive property due to amino groups in it structure that exhibit the positive charge in physiological fluid (Pusateri et al., 2003 ). The positive charge attracts the negative charge of the blood components and accelerates blood clot. Moreover, it strongly adheres to the wound bed which can reduce the volume of blood loss, therefore, reduce motility (Pusateri et al., 2003 ; Kozen et al., 2008 ). However, the high bio-adhesion can cause pain during the dressing removal from the wound bed if the wound bed is dried. In this study, amino groups of chitosan in Alst-contained sponges were protected by stearate molecules therefore their positive charge was less exhibited. The low bio-adhesion could benefit for reducing the pain during the dressing change.

![Work of adhesion of the DHT sponge dressings and the non-adhesive plaster (positive control) where (*) indicates statistically different from the other samples when analyzed by one-way ANOVA and Post-hoc test at p value < 0.05 (n=3)](image)

**Fig. 47** Work of adhesion of the DHT sponge dressings and the non-adhesive plaster (positive control) where (*) indicates statistically different from the other samples when analyzed by one-way ANOVA and Post-hoc test at p value < 0.05 (n=3)
4.2. *In vitro* asiaticoside release from sponge dressings

Asiaticoside release study from the sponge dressings was performed to investigate an effect of NMP on the drug release characteristic comparing with the sponges without NMP in the previous section (section 3.5). Release profile of asiaticoside from the sponge dressings are demonstrated in Fig. 48. The test was conducted in PBS pH 7.4 at 37°C for 7 days. The CD exhibited the fastest drug release that near to 80% within 8 h followed by gradual drug release during 7 days. The Alst contained dressings released asiaticoside with more sustained release pattern than that of CD. This asiaticoside release pattern corresponded with the result in section 3.5 that Alst prolonged asiaticoside release by retardation of the water penetration into sponge structure. For the Alst contained system, NMP loaded-sponge dressings exhibited more sustained release pattern comparing with the release profile of the sponge without NMP in section 3.5. The sponges without NMP could release the drug completely within 2 days whereas the NMP contained system could gradually release the drug longer than 7 days. This could be the role of NMP that enhanced homogeneity of asiaticoside and the matrix components. The previous study revealed that the more homogeneity of the drug and the matrix, the more sustainable drug release characteristic of the system (Prabu et al., 2008; Noel et al., 2010; Lai et al., 2013). Percentages erosion of the dressings measured after release study for 168 h are displayed in Fig. 49. The CD significantly exhibited the highest percentage of erosion at 38 % w/w followed by the CD05 and CD25 at approximately 20 % w/w and the least was CD50 with 16 % w/w.

Swelling degree of the sponge dressings immersed in PBS pH 7.4 is demonstrated in Fig. 50. CD was suddenly swollen after immersion in PBS pH 7.4 and reached equilibrium swelling at 90 min with swelling degree of 28.5 % w/w. Thereafter, the swelling degree decreased due to the degradation of the structure. The Alst-contained dressing (CD05, CD25 and CD50) gradually swollen where the CD05 and CD25 reached equilibrium swelling at 24 h with swelling degree of 11.7 and 7.9 % w/w, respectively, and subsequently degraded gradually. The CD50 reached equilibrium swelling since 48 h with swelling degree of 5.7 % w/w followed by the matrix degradation.
Fig. 48 Released amount of asiaticoside from the DHT sponge dressings studied by immersion method in PBS pH 7.4 at 37°C, 50 rpm (n=3) [The raw data were shown in Appendix I.]

Fig. 49 Percentage erosion of the DHT sponge dressings after release study for 168 h in PBS pH 7.4 where (*) indicates statistically different comparing with the other samples when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=3)
Asiaticoside release data were fit with mathematical models using Scientist program. Degree of curve fitting ($r^2$) and goodness of fit (MSC) obtained from curve fitting are displayed in Table 12. The data of CD was excluded from the analysis because the drug release was too fast therefore it was not fit with the models. Release profile of the CD05 was fit well to first-order model with $r^2$ and MSC of 0.9709 and 3.2030, respectively. The exponent ($n$) from power law was 0.39 indicating Fickian diffusion as the main mechanism of drug release. For that of the system containing higher Alst amount were fit well with Higuchi’s model. The $n$ value from power law of the CD25 was 0.45 which also indicating Fickian diffusion as same as the CD05. In case of the CD50, the $n$ value was 0.52 which indicating non-Fickian diffusion. This mean relaxation of the polymer chain in the material structure was the main factor that controls the drug release (Martínez-Ruvalcaba et al., 2009).

Comparing between the $n$ values from power law of the dressings, the value increased when Alst amount increased. This indicated that as the Alst amount was increased, release behavior of the loaded asiaticoside was controlled by relaxation behavior of the dressing structure. The CD05 could swell faster than the CD25 and the CD50, respectively, after immersion in the medium buffer solution. At equilibrium point of swelling, drug release was controlled by drug dissolution and diffusion out of the polymer matrix, and also by the polymer matrix degradation. The release data employed to fit the mathematical models were the value in range of

![Fig. 50](image_url)

**Fig. 50** Swelling degree of the DHT sponge dressings performed in PBS pH 7.4 for 168 h (n=3)
12 h to 168 h. This indicated that the CD05 and the CD25 had reached equilibrium swelling already before or in early point of this data range which was corresponded with swelling behavior study. In case of the CD50 that using the same data range with the others in curve fitting, the n value indicated that relaxation of the structure was the main factor the control drug release from the system. These suggested that the CD50 did not reach equilibrium swelling within 12 h and might be gradually swollen and degraded with slow rate during the test. According to the data of zero-order, $r^2$ and MSC increased when the Alst amount increased. This indicated that release mechanism tended to be more zero-order as the Alst amount increased.

<table>
<thead>
<tr>
<th>Sponge</th>
<th>first-order</th>
<th>zero-order</th>
<th>Higuchi's</th>
<th>power law</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>MSC</td>
<td>$r^2$</td>
<td>MSC</td>
</tr>
<tr>
<td>CD05</td>
<td>0.9709</td>
<td>3.2030</td>
<td>0.6269</td>
<td>0.5860</td>
</tr>
<tr>
<td>CD25</td>
<td>0.9518</td>
<td>2.6986</td>
<td>0.7883</td>
<td>1.1526</td>
</tr>
<tr>
<td>CD50</td>
<td>0.9854</td>
<td>3.8628</td>
<td>0.9703</td>
<td>3.1544</td>
</tr>
</tbody>
</table>

**4.3. Effect on cell viability**

Cell viabilities of the NHDF and NHEK after 24 h exposure to the pure substances are illustrated in Figs. 51-53 and those of exposed to the sponge dressing extractions are illustrated in Fig. 54. The cell viability was determined employing WST-8 assay. Cell viability of the group treated by the negative control (cell culture medium) was assumed as 100 %. According to the Fig. 51, the positive control (DMSO 100 µL/mL) significantly exhibited negative effect on the cell viability where the effect was more evident for the NHDF than the NHEK. Chitosan-lactate (CL) was toxic to the NHDF at a concentration higher than 5 mg/mL whereas non-toxic to the NHEK at the concentration up to 6.67 mg/mL. CL at the concentration not more than 3.33 mg/mL significantly promoted cell proliferation for the both cell types. In case of chitosan powder extraction, it had little effect on NHDF cell viability but it promoted cell proliferation of the NHEK.

Cell proliferation stimulating effects of chitosan were reported as the following details. The activity of chitosan may be due to its function in similar way to hyaluronan or it may accelerate fibroblast proliferation indirectly by forming polyelectrolyte complexes with serum components such as heparin. Moreover, it may potentiate growth factors such as PDGF in serum (Howling et al., 2001). In this study, CL could easily exhibit a positive charge in cell culture medium therefore could interact with the serum components and growth factors which eventually potentiated the function of these molecules.
The similar results to NHDF were reported in the previous studies as informed in the following details. Effect of the water soluble chitosan; carboxymethyl chitosan, on normal skin fibroblasts and keloid fibroblast were studied using MTT method (Chen et al., 2002). The results revealed that the carboxymethyl chitosan could promote the normal fibroblast proliferation when cultured with the serum-supplemented DMEM but inhibited the keloid fibroblast proliferation. Moreover, it could inhibit collagen type I synthesis from the keloid fibroblast. Another work investigated an effect of water-soluble chitosan chloride on normal human skin fibroblast and human keratinocyte using methyl-[³H]-thymidine cell proliferation assay (Howling et al., 2001). The results indicated that the chitosan chloride promoted the fibroblast proliferation but inhibited the keratinocyte proliferation. The fibroblast proliferation promoting effect was found when using cell culture medium supplemented with serum. In contrast, the water insoluble chitosan was not exhibit cell proliferation promoting effect on mouse skin fibroblast (Okamoto et al., 2002). Moreover, it inhibited fibroblast proliferation even when using the cell culture medium supplemented with serum (Mori et al., 1997). However, it was found to promote HUVECs cell migration (Okamoto et al., 2002) and increase interleukin-8 (IL-8) production from fibroblast. The IL-8 is reported to be a potent activator of angiogenesis (Mori et al., 1997).

![Fig. 51 Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to chitosan-lactate and the extraction of chitosan powder for 24 h. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (x) exhibited in chitosan powder chart meaning to the fold of the concentration. The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4).](image-url)
The contradictory results to this study of the effect of chitosan on keratinocyte cell viability were reported in the previous studies. Effect of different Mw chitosan on human keratinocyte cell line; HaCaT, was investigated previously (Wiegand et al., 2010). According to the result, Mw-dependent negative effect of chitosan on the HaCaT cell viability was observed. The high Mw chitosan (120 kDa) exhibited more negative effect on the cell viability than the chitosan oligosaccharide (5 kDa). Another study reported an effect of chitosan chloride on the HaCaT and the normal human keratinocytes (Howling et al., 2001). Dose-dependent toxicity of the chitosan chloride on the both types of keratinocyte was demonstrated. However, chitosan and cell type used in these previous studies were different from those of employed in this study. Therefore, further study should be investigated to confirm the effect of chitosan on keratinocyte.

Effect of lactic acid and NMP on cell viability of NHDF and NHEK is demonstrated in Fig. 52. Lactic acid was toxic to the NHDF at the concentration higher than 1.67 mg/mL but at the concentration range used in this study; 0.15 – 10.00 mg/mL, had less toxicity to the NHEK. IC\textsubscript{50} value of lactic acid to NHDF was 2.42 mg/mL. NMP was non-toxic to the NHDF at the concentration not more than 12.5 mg/mL with 87.44 % cell viability but when increasing the concentration to 25
mg/mL the cell viability drastically decreased to 35.40%. IC\textsubscript{50} of NMP for the NHDF in this study was approximately 20.58 mg/mL. Toxicity of NMP to the NHEK was observed at the concentration higher than 3.12 mg/mL with IC\textsubscript{50} of 20.69 mg/mL.

![Fig. 53 Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to various concentrations of asiaticoside and asiatic acid for 24 h. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4).](image)

According to Fig. 53, asiaticoside with concentration up to 720 µg/mL was non-toxic to the NHDF and NHEK. Previous study reported IC\textsubscript{90} value of asiaticoside for fibroblast at more than 400 µg/mL (Coldren \textit{et al.}, 2003). Asiaticoside at some concentrations significantly promoted NHDF proliferation. Fibroblast proliferation stimulating effect of asiaticoside has been reported both \textit{in vitro} and \textit{in vivo} (Maquart \textit{et al.}, 1990; Shukla \textit{et al.}, 1999; Lee \textit{et al.}, 2012). Mechanism of asiaticoside on fibroblast activities in molecular and gene expression levels was studied previously (Lu \textit{et al.}, 2004). The result indicated that asiaticoside influenced the genes responsible for cell-cycle progression and proliferation, and also that of correlating with extracellular matrix synthesis. Asiaticoside can be hydrolyzed to asiatic acid. Asiatic acid exhibited toxicity to the both cells with IC\textsubscript{50} value of 21.74 % and 13.12 % for NHDF and NHEK, respectively. Cytotoxicity of asiatic acid on cancer cells has been reported previously (Lee \textit{et al.}, 2002; Park \textit{et al.}, 2005).
Fig. 54 Cell viability of NHDF (left) and NHEK (right) measured by WST-8 assay after exposure to various dilution concentrations of chitosan sponges (CD and CD25) for 24 h. AC refers to asiaticoside. PC refers to the positive control (DMSO 100 µL in 1 mL cell culture medium) and NC refers to the negative control (cell culture medium). The (x) exhibited in the charts meaning to the fold of the concentration. The (*) indicates statistically different from the NC when analyzed by one-way ANOVA and Post-hoc test at p value <0.05 (n=4).

Effects of the extraction of the dressing on the cell viability of NHDF and NHEK are demonstrated in Fig. 54. Among the Alst-contained sponge dressings, CD25 exhibited the most interesting properties that appropriate for employing as the absorbent-medical dressing therefore it was chosen to test cytotoxicity whereas CD was evaluated as the comparing group. All extractions (10 mg/mL) were non-toxic to the both cell types. Moreover, the extraction also promoted cell viability especially for the NHDF with the average cell viability in the range of 80-170 % of the negative control. The activity of the extraction was expected to be due to the CL and asiaticoside that dissolved from the sponges. The higher cell viability was observed in the CD system because the CD could be degraded more rapid than the CD25.
4.4. Angiogenesis assay

Angiogenesis plays an important role during wound healing process in order to bring nutrients and oxygen to the regenerating tissue (Cheng, Guo et al., 2004). Angiogenic activity of asiaticoside was evaluated in CAM model. Various amounts of asiaticoside in range of 40 – 320 µg were loaded in cellulose disc and placed on CAM of the 9-days incubated chick embryo. Angiogenesis score was analyzed three days later by scoring the morphology and orientation of the blood vessels surrounding the substance-loaded disc. Digital images demonstrated CAM morphology at day-9 (disc placing day) and day-12 (scoring day) of the control groups; PBS pH 7.4 and VEGF (VEGF\textsubscript{165}), and asiaticoside are illustrated in Figs. 55 and 56, respectively.

Micro-blood vessels on the day-12 CAM were larger and more branching than that of the day-9 CAM. Moreover, their orientation and position were changed during 3 days after disc placing because of the movement of the growing embryo. The embryo movement sometimes caused folding of the CAM around the disc placing area therefore some embryos could not be scored. The surrounding blood vessels of the negative control (PBS) disc did not evidently incline to the disc therefore their score was indicated as zero. Average angiogenesis score of all VEGF groups was less than 1 as shown in Table 13. The surrounding blood vessels of the VEGF-loaded disc were not clearly inclined to the disc regardless the VEGF amount. This indicated that the VEGF was not effective in this test despite its nature of exhibiting the strong angiogenic activity. This might be because of lacking the spatio-temporal gradient of the VEGF release from the cellulose disc. Naturally, spatio-temporal gradient is necessary for angiogenic activity of VEGF. There are seven different isoforms of the VEGF active form (VEGF-A), which respectively consists of 121, 145, 148, 165, 183, 189, or 206 amino acid (Hoeben et al., 2004). Two isoforms; the 121 and 165, released from the inflammatory response cells during tissue regeneration process are mainly responsible for angiogenesis (Gabhann et al., 2007). Spatio-temporal gradient of the VEGF is created by the difference of extracellular matrix protein-binding affinity of the VEGF isoforms. The VEGF\textsubscript{121} that lacks heparin-binding protein acts as the endothelial cell stimulator in the early phase whereas the VEGF\textsubscript{165} that comprises heparin-binding protein is reserved in the extracellular matrix. In the later stage, the VEGF\textsubscript{165} molecules will be gradually liberated by ECM degradation due to tissue destructive enzymes to function in the later stage of angiogenesis cascade; cell proliferation, migration and survival phase (Ferrara et al., 2003). Angiogenic activity of VEGF\textsubscript{121} could be successfully evaluated using CAM model which the VEGF\textsubscript{121} was loaded in the modified-fibrin gel that could sustain the VEGF\textsubscript{121} release effectively (Ehrbar et al., 2004). Therefore, the result from the present study indicated that cellulose disc was not appropriate for using as the carrier of the VEGF in this assay.
Asiaticoside exhibited dose-dependent angiogenic activity (Fig. 56). Angiogenesis scores of asiaticoside are shown in Table 13. Mean angiogenesis score of 40, 80, 160 and 320 µg/disc asiaticoside was 1.25, 1.25, 1.83, and 2.12, respectively. Due to its low aqueous solubility, asiaticoside in this study was loaded into the cellulose disc by dissolving in DMSO/methanol mixture which eventually after methanol evaporation DMSO was left in the disc together with asiaticoside. The DMSO dank-asiaticoside-loaded discs could sustain the asiaticoside release therefore its angiogenic activity could be observed in this study. Angiogenic activity of asiaticoside has been reported both from in vitro study in CAM model (Shukla et al., 1999) and in vivo studies in rat and mice (Cheng et al., 2004; Kimura et al., 2008). Angiogenesis promotion mechanism of low dose asiaticoside in burn wound was suggested as a result of enhancing monocyte chemoattractant protein-1 (MCP-1) production from keratinocyte and inducing interleukin-1β (IL-1β) production from macrophage that increasing VEGF production (Kimura et al., 2008) in the wound.

Digital images demonstrating the CAM morphology and blood vessel response to the prepared asiaticoside-loaded dressings; CD0, CD05 and CD25, at sample placing-day (day-9) and result observation-day (day-12) are shown in Fig. 57. At day-12, the CD0 size was lager due to swelling and partly sunk into the CAM tissue. The CD05 and CD25 size were also larger due to swelling but only laid on the CAM surface. As can be seen from the Fig. 57, small blood vessels seemed sprouting around the CD0 which indicated angiogenic activity of the CD0. The mean angiogenesis score was approximately 2.67 as shown in Table 13. However, the high hydrophilicity and positive charge of the CD0 exerted excellent mucoadhesions and fluid sorption of the matrix that could highly adhere the CAM tissue therefore this could be a false positive angiogenic activity of the CD0. The CD05 and CD25 also could swell but they did not have apparent effect on CAM morphology because of its low hydrophilicity. Some of the nearby small blood vessels seemed inclining into the material body which might be due to the released asiaticoside from the dressings. The mean angiogenesis score of the CD05 and CD25 were 1.00 and 1.17, respectively. Due to a false positive result, this assay may not suitable for the high swellable and high fluid absorbable sample.
Table 13 Summary table of CAM assay of PBS pH 7.4 (negative control), VEGF (positive control), various amount of asiaticoside and asiaticoside-loaded sponge dressings detailing the total number of the used embryos, the dead embryos, the unable to be evaluated embryos (N/E), evaluable embryos and average angiogenesis score.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total embryo</th>
<th>Dead embryo</th>
<th>N/E</th>
<th>Evaluable embryo</th>
<th>Average score (total score / evaluable embryos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS pH 7.4</td>
<td>10</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>0.00 (0/6)</td>
</tr>
<tr>
<td>VEGF 100 ng</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>0.43 (3/7)</td>
</tr>
<tr>
<td>VEGF 200 ng</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>0.17 (1/6)</td>
</tr>
<tr>
<td>VEGF 300 ng</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>0.20 (1/5)</td>
</tr>
<tr>
<td>Asiaticoside 40 µg</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>1.25 (10/8)</td>
</tr>
<tr>
<td>Asiaticoside 80 µg</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>1.25 (10/8)</td>
</tr>
<tr>
<td>Asiaticoside 160 µg</td>
<td>10</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>1.83 (11/6)</td>
</tr>
<tr>
<td>Asiaticoside 320 µg</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>2.12 (17/8)</td>
</tr>
<tr>
<td>CD0</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>2.67 (16/6)</td>
</tr>
<tr>
<td>CD05</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>1.00 (7/7)</td>
</tr>
<tr>
<td>CD25</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>1.17 (7/6)</td>
</tr>
</tbody>
</table>
Fig. 55 Morphology of CAM treated by PBS pH 7.4 (negative control) (A) and various amount of VEGF (positive control); 100 ng (B), 200 ng (C), and 300 ng (D) at day-9 (disc placing) and day-12 (scoring angiogenic activity)
Fig. 56 Morphology of CAM treated by various amount of asiaticoside; 40 µg (E), 80 µg (F), 160 µg (G), and 320 µg (H), at day-9 (disc placing) and day-12 (scoring angiogenic activity), the white arrows indicated responding of the blood vessels to asiaticoside-loaded disc
**Fig. 57** Morphology of CAM treated by asiaticoside-loaded sponge dressings; CD, CD05 and CD25 at day-9 (sample placing-day) and day-12 (result observation-day)
CHAPTER 5
CONCLUSIONS

Alst dissociated in dilute acidic solution could be blended with chitosan in acidic aqueous medium without using high temperature or surfactants. CLA dispersion was successfully prepared by simple mixing at room temperature which chitosan to Alst ratio and mixing time influenced the size of the CLA ionic complexes. The most appropriate chitosan concentration and mixing time were 4% w/w and 24 h, respectively. Chitosan-Alst composite sponges were fabricated by lyophilization. Stability of the sponge in aqueous medium could be improved by DHT performing at 110°C for 24h under vacuum condition. DHT partially caused amidation by removal of water molecule from ionic bond of ammonium group of chitosan and carboxylate group of the acid side chain (lactate or stearate) therefore left amide bond instead. Alst enhanced hydrophobicity of the prepared sponges therefore reduced swelling and water sorption of the sponges and eventually facilitated the sustainable drug release from the matrix for 2 days. Chitosan-Alst sponges containing NMP as a solvent for asiaticoside was prepared as wound dressing. NMP enhanced homogeneity of asiaticoside into the polymer matrix therefore could extend drug release form 2 days to 7 days. Hydrophobicity of Alst affected the wound dressing properties whereas NMP had no effect on amidation caused by DHT. Fluid handling ability of the dressings such as WVTR and fluid absorbency were decreased by Alst amount with amount-dependent manner. All the prepared sponges exhibited apparently high oxygen permeability regardless the amount of Alst. Alst reduced bio-adhesive property of the prepared dressings which could be expected that the dressing will not cause pain during dressing removal from the wound bed. Cytotoxicity test in NHDF and NHEK indicated that the prepared sponge dressings were non-toxic to the both cell types. Moreover, CL, asiaticoside and the sponge dressing extractions exhibited cell proliferation stimulating effect especially for NHDF. Asiaticoside exhibited dose-dependent angiogenic activity in CAM model. The asiaticoside-loaded dressings also exhibited angiogenic activity in CAM model. However, the activity might be a false positive result due to the high fluid absorptivity of the materials. Therefore, the further study should be performed in order to confirm the angiogenic activity of the prepared sponge dressings. All results indicated that the prepared sponge dressings modified by Alst with DHT especially the CD25 had potential to be further utilized as an absorbent medical dressing for chronic wound healing application. However, in vivo wound healing evaluation should be further investigated.


The Metal Carboxylates Coalition. (2003). *Catagory development and justification, and proposed test plan for aluminum stearates. U.S. High Production Volume*
(HPV) Chemical Challenge Program  Retrieved From:  

The Metal Carboxylates Coalition (2003). Category development and justification, and  
proposed test plan for aluminum stearates. U.S. High Production Volume  

chitosan: hydrophobic matrices for controlled drug release. Journal of  

Traversa, B. & Sussman, G. (2001). The role of growth factors, cytokines and proteases  


Veeramani, P. V. & Veni, G. (2010). An essential review on current techniques used in  
2379-2387.

high performance liquid chromatography of asiaticoside in Centella asiatica.  
Phytochemical Analysis, 10: 191-193.

effect of leptin in the quail chorioallantoic membrane. Acta Veterinaria Brno,  
79: 13-17.

Wan, J. Y., Gong, X., Jiang, R., Zhang, Z. & Zhang, L. (2012). Antipyretic and  
anti-inflammatory effects of asiaticoside in lipopolysaccharide-treated rat  
through up-regulation of heme oxygenase-1. Phytotherapy Research, online  
access (DOI:10.1002/ptr.4838).

Wang, C. X., Han, W., Fan, L. & Wang, C. L. (2009). Enzymatic pretreatment and  
microwave extraction of asiaticoside from Centella asiatica. Journal of  
Biomedical Science and Engineering, 2: 526-531.


1110-1112.

effects of chitosans on the human keratinocyte cell line HaCaT. Skin  
Pharmacology and Physiology, 23: 164-170.

wound angiogenesis. Advance in Wound Care, 2(3): 81-86.

Wikipedia. Aluminum monostearate. Retrieved From:  
http://en.wikipedia.org/wiki/Aluminium_monostearate

chitosan polysaccharides composite wound dressings. International Journal of  
Pharmaceutics, 313: 123-128.


Appendix I

HPLC and Drug release data
Appendix I
HPLC and Drug release data

1. Mobile phase ratio

Table 14 Mobile phase ratio in HPLC gradient mode

<table>
<thead>
<tr>
<th>Time</th>
<th>% Acetonitrile</th>
<th>% RO water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

2. Calibration curve of asiaticoside

Table 15 Area under the HPLC curve of the various asiaticoside concentrations solution

<table>
<thead>
<tr>
<th>Asiaticoside concentration (µg/mL)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>35.02</td>
<td>35.95</td>
<td>35.57</td>
<td></td>
<td></td>
<td></td>
<td>35.51</td>
<td>0.47</td>
</tr>
<tr>
<td>25</td>
<td>78.05</td>
<td>77.72</td>
<td>77.31</td>
<td></td>
<td></td>
<td></td>
<td>77.69</td>
<td>0.37</td>
</tr>
<tr>
<td>50</td>
<td>154.52</td>
<td>154.52</td>
<td>153.80</td>
<td>154.24</td>
<td>154.09</td>
<td>154.04</td>
<td>154.20</td>
<td>0.28</td>
</tr>
<tr>
<td>80</td>
<td>258.46</td>
<td>258.62</td>
<td>258.82</td>
<td>257.95</td>
<td>258.56</td>
<td>259.04</td>
<td>258.58</td>
<td>0.37</td>
</tr>
<tr>
<td>100</td>
<td>325.79</td>
<td>325.78</td>
<td>327.52</td>
<td>326.36</td>
<td>327.30</td>
<td>327.60</td>
<td>326.73</td>
<td>0.85</td>
</tr>
<tr>
<td>120</td>
<td>391.13</td>
<td>391.78</td>
<td>390.61</td>
<td>391.85</td>
<td>392.10</td>
<td>390.70</td>
<td>391.36</td>
<td>0.63</td>
</tr>
<tr>
<td>200</td>
<td>667.74</td>
<td>668.53</td>
<td>667.33</td>
<td>668.23</td>
<td>671.03</td>
<td>671.07</td>
<td>669.00</td>
<td>1.65</td>
</tr>
<tr>
<td>400</td>
<td>1404.71</td>
<td>1401.78</td>
<td>1394.80</td>
<td></td>
<td></td>
<td></td>
<td>1400.43</td>
<td>5.09</td>
</tr>
</tbody>
</table>
Fig. 58 Calibration curve of asiaticoside

\[ y = 3.5243x - 20.843 \]

\[ R^2 = 0.9995 \]
Fig. 59 HPLC spectrum of the 100 µg/mL asiaticoside standard dissolved in PBS pH 7.4 with retention time of asiaticoside peak at 3.318 min and area under the curve of 332.45627

3. Method validation

3.1. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. The accuracy were recommended to be performed at the 80, 100 and 120 % levels of label claim. The accuracy of this method was determined by calculation of the % recovery of asiaticoside. The 50, 100 and 200 µg/mL of asiaticoside were estimated by measuring the peak area and fitting the value to the equation of the calibration curve (n=3). The recommendation of % recovery is 100 ± 2% (Center for Drug Evaluation and Research, 1994; European Medicines Agency, 1995).
Table 16 Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 50, 100 and 200 µg/mL asiaticoside solution (n=3)

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>area</th>
<th>Calculated asiaticoside concentration (µg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>154.52</td>
<td>154.52</td>
<td>153.80</td>
</tr>
<tr>
<td>100</td>
<td>325.79</td>
<td>325.78</td>
<td>327.52</td>
</tr>
<tr>
<td>200</td>
<td>667.74</td>
<td>668.53</td>
<td>667.33</td>
</tr>
</tbody>
</table>

3.2. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability (inter-day precision), intermediate precision (intra-day precision) and reproducibility. Repeatability of this method was conducted by estimating the correspondence responses six times on the same day with 100 µg/mL of asiaticoside. The intermediate precision of this method was carried out by estimating the correspondence responses six times in the next day with 100 µg/mL of asiaticoside. The RSD is an estimate of sample analysis precision. The recommendation of RSD is ≤ 2.0 % (Center for Drug Evaluation and Research, 1994; European Medicines Agency, 1995).

Repeatability (intra-day precision)

Table 17 Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the inter-day precision test

<table>
<thead>
<tr>
<th>Injection number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mean</th>
<th>SD</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>325.79</td>
<td>325.78</td>
<td>327.52</td>
<td>326.36</td>
<td>327.30</td>
<td>327.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>98.35</td>
<td>98.35</td>
<td>98.85</td>
<td>98.52</td>
<td>98.78</td>
<td>98.87</td>
<td>98.6210</td>
<td>0.2419</td>
<td>0.25</td>
<td>98.62</td>
</tr>
</tbody>
</table>
Intermediate precision (inter-day precision)

Day 1

Table 18 Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-1 intermediate precision test

<table>
<thead>
<tr>
<th>Injection number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mean</th>
<th>SD</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>325.79</td>
<td>325.78</td>
<td>327.52</td>
<td>326.36</td>
<td>327.30</td>
<td>327.60</td>
<td>mean</td>
<td>SD</td>
<td>% RSD</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Concentration (µg/mL)</td>
<td>98.35</td>
<td>98.35</td>
<td>98.85</td>
<td>98.52</td>
<td>98.78</td>
<td>98.87</td>
<td>98.6210</td>
<td>0.2419</td>
<td>0.25</td>
<td>98.62</td>
</tr>
</tbody>
</table>

Day 2

Table 19 Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-2 intermediate precision test

<table>
<thead>
<tr>
<th>Injection number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mean</th>
<th>SD</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>336.74</td>
<td>335.44</td>
<td>333.78</td>
<td>335.67</td>
<td>333.97</td>
<td>334.92</td>
<td>mean</td>
<td>SD</td>
<td>% RSD</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Concentration (µg/mL)</td>
<td>101.46</td>
<td>101.09</td>
<td>100.62</td>
<td>101.16</td>
<td>100.68</td>
<td>100.95</td>
<td>100.99</td>
<td>0.31</td>
<td>0.31</td>
<td>100.99</td>
</tr>
</tbody>
</table>

Day 3

Table 20 Area under the curve, calculated asiaticoside concentration, %RSD and % recovery of the 100 µg/mL asiaticoside solution (n=6) of the day-3 intermediate precision test

<table>
<thead>
<tr>
<th>Injection number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mean</th>
<th>SD</th>
<th>% RSD</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>333.38</td>
<td>333.05</td>
<td>332.46</td>
<td>332.58</td>
<td>332.95</td>
<td>331.68</td>
<td>mean</td>
<td>SD</td>
<td>% RSD</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Concentration (µg/mL)</td>
<td>100.51</td>
<td>100.41</td>
<td>100.25</td>
<td>100.28</td>
<td>100.39</td>
<td>100.03</td>
<td>100.31</td>
<td>0.17</td>
<td>0.17</td>
<td>100.31</td>
</tr>
</tbody>
</table>

3.3. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample (European Medicines Agency, 1995). The eight concentrations of asiaticoside solution were prepared in PBS pH 7.4 at concentration of 12.5, 25, 50, 80, 100, 120, 200, and 400 µg/mL. The linearity was plotted between peak areas
versus concentration of asiaticoside. The recommendation of $r^2$ is $\geq 0.999$ (Center for Drug Evaluation and Research, 1994). The result was demonstrated as the Fig. 58 with the $r^2$ of the obtained line of 0.9995.

### 3.4. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity (Center for Drug Evaluation and Research, 1994; European Medicines Agency, 1995). In this method, the interval between 12.5 and 400 µg/mL was analyzed.

### 3.5. System suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated (Center for Drug Evaluation and Research, 1994).

The system suitability test was determined to check various parameters of this procedure such as resolution (Rs), tailing factor (T), symmetry, plate number (N), retention time (tr), and repeatability (RSD). The recommendation of Rs, T, N, and RSD is $> 2$, $< 2$, $> 2000$ and $< 1 \%$, respectively.

#### Table 21 System suitability parameters of the HPLC curve of 100 µg/mL asiaticoside solution

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>average</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs</td>
<td>6.97</td>
<td>6.77</td>
<td>7.13</td>
<td>6.97</td>
<td>6.85</td>
<td>6.89</td>
<td>6.930</td>
<td>0.124</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>0.520</td>
<td>0.520</td>
<td>0.518</td>
<td>0.517</td>
<td>0.520</td>
<td>0.520</td>
<td>0.519</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>Symmetry</td>
<td>0.452</td>
<td>0.449</td>
<td>0.444</td>
<td>0.461</td>
<td>0.456</td>
<td>0.452</td>
<td>0.452</td>
<td>0.006</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td>3.254</td>
<td>3.012</td>
<td>3.347</td>
<td>3.174</td>
<td>3.092</td>
<td>3.077</td>
<td>3.159</td>
<td>124</td>
<td>-</td>
</tr>
<tr>
<td>tr</td>
<td>3.312</td>
<td>3.303</td>
<td>3.318</td>
<td>3.311</td>
<td>3.307</td>
<td>3.300</td>
<td>3.309</td>
<td>0.007</td>
<td>-</td>
</tr>
<tr>
<td>Area</td>
<td>333.38</td>
<td>333.05</td>
<td>332.46</td>
<td>332.58</td>
<td>332.95</td>
<td>331.68</td>
<td>332.683</td>
<td>0.593</td>
<td>-</td>
</tr>
<tr>
<td>conc. (µg/mL)</td>
<td>100.51</td>
<td>100.42</td>
<td>100.25</td>
<td>100.28</td>
<td>100.39</td>
<td>100.03</td>
<td>100.31</td>
<td>0.17</td>
<td>0.17</td>
</tr>
</tbody>
</table>
4. Release profile of asiaticoside

4.1. Raw data of the section 3.5. (Chapter 4): \textit{In vitro} asiaticoside release study

Table 22 Raw data of percentage asiaticoside release from CL sponge

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>73.62</td>
<td>73.31</td>
<td>63.65</td>
<td>70.19</td>
<td>5.67</td>
</tr>
<tr>
<td>1</td>
<td>81.23</td>
<td>81.53</td>
<td>76.40</td>
<td>79.72</td>
<td>2.88</td>
</tr>
<tr>
<td>4</td>
<td>84.26</td>
<td>84.88</td>
<td>84.17</td>
<td>84.44</td>
<td>0.39</td>
</tr>
<tr>
<td>8</td>
<td>87.08</td>
<td>87.80</td>
<td>87.55</td>
<td>87.48</td>
<td>0.37</td>
</tr>
<tr>
<td>12</td>
<td>87.62</td>
<td>87.67</td>
<td>86.25</td>
<td>87.18</td>
<td>0.81</td>
</tr>
<tr>
<td>16</td>
<td>90.78</td>
<td>90.50</td>
<td>88.88</td>
<td>90.06</td>
<td>1.02</td>
</tr>
<tr>
<td>24</td>
<td>93.02</td>
<td>92.80</td>
<td>91.81</td>
<td>92.55</td>
<td>0.65</td>
</tr>
<tr>
<td>36</td>
<td>95.02</td>
<td>95.04</td>
<td>94.06</td>
<td>94.71</td>
<td>0.56</td>
</tr>
<tr>
<td>48</td>
<td>96.76</td>
<td>97.89</td>
<td>96.35</td>
<td>97.00</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 23 Raw data of percentage asiaticoside release from CLA05 sponge

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>11.23</td>
<td>13.68</td>
<td>18.07</td>
<td>14.32</td>
<td>3.47</td>
</tr>
<tr>
<td>1</td>
<td>16.52</td>
<td>20.06</td>
<td>25.49</td>
<td>20.69</td>
<td>4.52</td>
</tr>
<tr>
<td>4</td>
<td>33.08</td>
<td>36.06</td>
<td>43.19</td>
<td>37.44</td>
<td>5.20</td>
</tr>
<tr>
<td>8</td>
<td>47.13</td>
<td>50.04</td>
<td>59.75</td>
<td>52.30</td>
<td>6.61</td>
</tr>
<tr>
<td>12</td>
<td>61.60</td>
<td>63.68</td>
<td>73.57</td>
<td>66.28</td>
<td>6.40</td>
</tr>
<tr>
<td>16</td>
<td>71.09</td>
<td>72.91</td>
<td>82.07</td>
<td>75.36</td>
<td>5.89</td>
</tr>
<tr>
<td>24</td>
<td>81.27</td>
<td>82.29</td>
<td>88.66</td>
<td>84.07</td>
<td>4.01</td>
</tr>
<tr>
<td>36</td>
<td>89.22</td>
<td>89.37</td>
<td>93.00</td>
<td>90.53</td>
<td>2.14</td>
</tr>
<tr>
<td>48</td>
<td>93.27</td>
<td>93.73</td>
<td>95.78</td>
<td>94.26</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Table 24 Raw data of percentage asiaticoside release from CLA25 sponge

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>15.22</td>
<td>14.03</td>
<td>13.76</td>
<td>14.34</td>
<td>0.77</td>
</tr>
<tr>
<td>1</td>
<td>20.89</td>
<td>20.01</td>
<td>19.71</td>
<td>20.20</td>
<td>0.61</td>
</tr>
<tr>
<td>4</td>
<td>33.70</td>
<td>33.81</td>
<td>31.82</td>
<td>33.11</td>
<td>1.12</td>
</tr>
<tr>
<td>8</td>
<td>49.09</td>
<td>49.31</td>
<td>47.25</td>
<td>48.55</td>
<td>1.13</td>
</tr>
<tr>
<td>12</td>
<td>61.96</td>
<td>62.09</td>
<td>61.14</td>
<td>61.73</td>
<td>0.51</td>
</tr>
<tr>
<td>16</td>
<td>71.22</td>
<td>71.62</td>
<td>70.96</td>
<td>71.27</td>
<td>0.33</td>
</tr>
<tr>
<td>24</td>
<td>83.06</td>
<td>82.37</td>
<td>83.28</td>
<td>82.90</td>
<td>0.48</td>
</tr>
<tr>
<td>36</td>
<td>90.60</td>
<td>90.31</td>
<td>90.86</td>
<td>90.59</td>
<td>0.28</td>
</tr>
<tr>
<td>48</td>
<td>95.10</td>
<td>95.05</td>
<td>94.13</td>
<td>94.76</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Table 25 Raw data of percentage asiaticoside release from CLA50 sponge

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>AVG</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>10.54</td>
<td>13.28</td>
<td>12.17</td>
<td>11.99</td>
<td>1.38</td>
</tr>
<tr>
<td>1</td>
<td>14.52</td>
<td>19.08</td>
<td>17.09</td>
<td>16.90</td>
<td>2.28</td>
</tr>
<tr>
<td>4</td>
<td>22.46</td>
<td>29.54</td>
<td>26.61</td>
<td>26.20</td>
<td>3.56</td>
</tr>
<tr>
<td>8</td>
<td>33.30</td>
<td>43.47</td>
<td>37.19</td>
<td>37.99</td>
<td>5.13</td>
</tr>
<tr>
<td>12</td>
<td>43.16</td>
<td>55.81</td>
<td>47.52</td>
<td>48.83</td>
<td>6.42</td>
</tr>
<tr>
<td>16</td>
<td>51.91</td>
<td>65.62</td>
<td>56.71</td>
<td>58.08</td>
<td>6.96</td>
</tr>
<tr>
<td>24</td>
<td>64.10</td>
<td>76.97</td>
<td>68.20</td>
<td>69.76</td>
<td>6.57</td>
</tr>
<tr>
<td>36</td>
<td>79.56</td>
<td>86.54</td>
<td>79.86</td>
<td>81.99</td>
<td>3.95</td>
</tr>
<tr>
<td>48</td>
<td>85.30</td>
<td>89.56</td>
<td>83.91</td>
<td>86.26</td>
<td>2.95</td>
</tr>
</tbody>
</table>

4.2. Raw data of the section 4.2. (Chapter 4): *In vitro* asiaticoside release study from chitosan dressings (NMP contained sponges)

Table 26 Raw data of percentage asiaticoside release from CD dressing

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>20.45</td>
<td>15.98</td>
<td>21.37</td>
<td>19.27</td>
<td>2.88</td>
</tr>
<tr>
<td>1</td>
<td>34.04</td>
<td>26.18</td>
<td>33.06</td>
<td>31.09</td>
<td>4.28</td>
</tr>
<tr>
<td>4</td>
<td>81.17</td>
<td>69.55</td>
<td>83.41</td>
<td>78.04</td>
<td>7.44</td>
</tr>
<tr>
<td>8</td>
<td>88.92</td>
<td>82.64</td>
<td>88.31</td>
<td>86.63</td>
<td>3.46</td>
</tr>
<tr>
<td>12</td>
<td>91.32</td>
<td>86.22</td>
<td>90.49</td>
<td>89.34</td>
<td>2.73</td>
</tr>
<tr>
<td>16</td>
<td>92.48</td>
<td>87.03</td>
<td>91.17</td>
<td>90.23</td>
<td>2.84</td>
</tr>
<tr>
<td>24</td>
<td>93.40</td>
<td>88.24</td>
<td>92.28</td>
<td>91.31</td>
<td>2.72</td>
</tr>
<tr>
<td>36</td>
<td>93.86</td>
<td>89.06</td>
<td>92.86</td>
<td>91.93</td>
<td>2.53</td>
</tr>
<tr>
<td>48</td>
<td>96.27</td>
<td>92.91</td>
<td>95.95</td>
<td>95.04</td>
<td>1.86</td>
</tr>
<tr>
<td>72</td>
<td>98.19</td>
<td>94.15</td>
<td>97.64</td>
<td>96.66</td>
<td>2.19</td>
</tr>
<tr>
<td>96</td>
<td>98.66</td>
<td>94.96</td>
<td>96.19</td>
<td>96.60</td>
<td>1.89</td>
</tr>
<tr>
<td>120</td>
<td>99.72</td>
<td>94.42</td>
<td>96.37</td>
<td>96.84</td>
<td>2.68</td>
</tr>
<tr>
<td>144</td>
<td>100.31</td>
<td>96.36</td>
<td>97.72</td>
<td>98.13</td>
<td>2.00</td>
</tr>
<tr>
<td>168</td>
<td>99.12</td>
<td>96.85</td>
<td>96.20</td>
<td>97.39</td>
<td>1.53</td>
</tr>
</tbody>
</table>
Table 27 Raw data of percentage asiaticoside release from CD05 dressing

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1.5</td>
<td>16.27</td>
<td>18.14</td>
<td>14.38</td>
<td>16.26</td>
<td>1.88</td>
</tr>
<tr>
<td>3</td>
<td>21.28</td>
<td>21.42</td>
<td>18.28</td>
<td>20.33</td>
<td>1.77</td>
</tr>
<tr>
<td>6</td>
<td>28.14</td>
<td>28.24</td>
<td>23.62</td>
<td>26.67</td>
<td>2.64</td>
</tr>
<tr>
<td>12</td>
<td>37.08</td>
<td>36.65</td>
<td>30.93</td>
<td>34.89</td>
<td>3.43</td>
</tr>
<tr>
<td>24</td>
<td>49.18</td>
<td>47.56</td>
<td>40.65</td>
<td>45.80</td>
<td>4.53</td>
</tr>
<tr>
<td>36</td>
<td>60.29</td>
<td>59.04</td>
<td>49.90</td>
<td>56.41</td>
<td>5.67</td>
</tr>
<tr>
<td>48</td>
<td>66.26</td>
<td>66.08</td>
<td>58.59</td>
<td>63.64</td>
<td>4.38</td>
</tr>
<tr>
<td>72</td>
<td>77.24</td>
<td>78.26</td>
<td>68.79</td>
<td>74.76</td>
<td>5.19</td>
</tr>
<tr>
<td>96</td>
<td>85.63</td>
<td>87.05</td>
<td>78.43</td>
<td>83.71</td>
<td>4.62</td>
</tr>
<tr>
<td>120</td>
<td>91.80</td>
<td>93.47</td>
<td>85.85</td>
<td>90.37</td>
<td>4.00</td>
</tr>
<tr>
<td>144</td>
<td>96.13</td>
<td>98.07</td>
<td>91.09</td>
<td>95.10</td>
<td>3.60</td>
</tr>
<tr>
<td>168</td>
<td>99.31</td>
<td>101.33</td>
<td>95.26</td>
<td>98.63</td>
<td>3.09</td>
</tr>
</tbody>
</table>

Table 28 Raw data of percentage asiaticoside release from CD25 dressing

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1.5</td>
<td>14.46</td>
<td>12.92</td>
<td>13.37</td>
<td>13.58</td>
<td>0.79</td>
</tr>
<tr>
<td>3</td>
<td>17.12</td>
<td>16.36</td>
<td>16.15</td>
<td>16.54</td>
<td>0.51</td>
</tr>
<tr>
<td>6</td>
<td>22.16</td>
<td>21.32</td>
<td>21.90</td>
<td>21.79</td>
<td>0.43</td>
</tr>
<tr>
<td>12</td>
<td>28.04</td>
<td>27.00</td>
<td>27.10</td>
<td>27.38</td>
<td>0.58</td>
</tr>
<tr>
<td>24</td>
<td>35.83</td>
<td>35.11</td>
<td>36.16</td>
<td>35.70</td>
<td>0.53</td>
</tr>
<tr>
<td>36</td>
<td>43.41</td>
<td>42.16</td>
<td>45.55</td>
<td>43.71</td>
<td>1.71</td>
</tr>
<tr>
<td>48</td>
<td>51.62</td>
<td>49.20</td>
<td>52.45</td>
<td>51.09</td>
<td>1.69</td>
</tr>
<tr>
<td>72</td>
<td>61.79</td>
<td>59.85</td>
<td>63.44</td>
<td>61.69</td>
<td>1.80</td>
</tr>
<tr>
<td>96</td>
<td>71.32</td>
<td>68.33</td>
<td>73.09</td>
<td>70.91</td>
<td>2.40</td>
</tr>
<tr>
<td>120</td>
<td>78.19</td>
<td>74.53</td>
<td>79.73</td>
<td>77.48</td>
<td>2.67</td>
</tr>
<tr>
<td>144</td>
<td>83.41</td>
<td>79.63</td>
<td>84.99</td>
<td>82.68</td>
<td>2.76</td>
</tr>
<tr>
<td>168</td>
<td>86.43</td>
<td>84.25</td>
<td>90.35</td>
<td>87.01</td>
<td>3.09</td>
</tr>
</tbody>
</table>
**Table 29** Raw data of percentage asiaticoside release from CD50 dressing

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.5</td>
<td>9.81</td>
<td>9.63</td>
<td>9.04</td>
<td>9.49</td>
<td>0.40</td>
</tr>
<tr>
<td>1</td>
<td>10.79</td>
<td>10.59</td>
<td>9.95</td>
<td>10.44</td>
<td>0.44</td>
</tr>
<tr>
<td>4</td>
<td>15.20</td>
<td>11.55</td>
<td>10.85</td>
<td>12.54</td>
<td>2.34</td>
</tr>
<tr>
<td>8</td>
<td>18.90</td>
<td>15.98</td>
<td>15.04</td>
<td>16.64</td>
<td>2.02</td>
</tr>
<tr>
<td>12</td>
<td>22.60</td>
<td>18.93</td>
<td>18.04</td>
<td>19.85</td>
<td>2.42</td>
</tr>
<tr>
<td>16</td>
<td>25.73</td>
<td>21.62</td>
<td>20.75</td>
<td>22.70</td>
<td>2.66</td>
</tr>
<tr>
<td>24</td>
<td>30.53</td>
<td>26.34</td>
<td>24.31</td>
<td>27.06</td>
<td>3.17</td>
</tr>
<tr>
<td>36</td>
<td>34.78</td>
<td>31.51</td>
<td>31.28</td>
<td>32.52</td>
<td>1.96</td>
</tr>
<tr>
<td>48</td>
<td>43.29</td>
<td>37.95</td>
<td>42.41</td>
<td>41.22</td>
<td>2.86</td>
</tr>
<tr>
<td>72</td>
<td>55.31</td>
<td>49.63</td>
<td>54.96</td>
<td>53.30</td>
<td>3.18</td>
</tr>
<tr>
<td>96</td>
<td>64.96</td>
<td>62.07</td>
<td>65.34</td>
<td>64.12</td>
<td>1.78</td>
</tr>
<tr>
<td>120</td>
<td>72.67</td>
<td>76.52</td>
<td>71.17</td>
<td>73.45</td>
<td>2.76</td>
</tr>
<tr>
<td>144</td>
<td>79.95</td>
<td>80.55</td>
<td>81.64</td>
<td>80.71</td>
<td>0.86</td>
</tr>
<tr>
<td>168</td>
<td>89.24</td>
<td>88.99</td>
<td>88.53</td>
<td>88.92</td>
<td>0.36</td>
</tr>
</tbody>
</table>
Appendix II

Powder x-ray diffraction
Appendix II

Powder x-ray diffraction

Table 30 20 degree and d-spacing of the PXRD peak of raw materials, chitosan sponge and chitosan dressings (related to the x-ray diffractogram in the section 3.1.3 and 4.1.3 of the chapter 4)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak No.</th>
<th>2θ (°)</th>
<th>d-spacing (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>1</td>
<td>10.47</td>
<td>8.44</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.68</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20.02</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22.51</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29.18</td>
<td>3.06</td>
</tr>
<tr>
<td>Alst</td>
<td>1</td>
<td>6.67</td>
<td>13.23</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11.38</td>
<td>7.77</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19.55</td>
<td>4.54</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22.87</td>
<td>3.88</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>1</td>
<td>4.38</td>
<td>20.16</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.60</td>
<td>13.38</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11.04</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21.39</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>23.69</td>
<td>3.75</td>
</tr>
<tr>
<td>Asiaticoside</td>
<td>1</td>
<td>4.19</td>
<td>21.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.28</td>
<td>16.73</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12.60</td>
<td>7.02</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.33</td>
<td>6.63</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>13.91</td>
<td>6.36</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>15.31</td>
<td>5.78</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>15.83</td>
<td>5.59</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16.71</td>
<td>5.30</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>19.76</td>
<td>4.49</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21.40</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>22.97</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>24.55</td>
<td>3.62</td>
</tr>
<tr>
<td>Asiatic acid</td>
<td>1</td>
<td>7.56</td>
<td>11.68</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.89</td>
<td>5.95</td>
</tr>
<tr>
<td>CL</td>
<td>1</td>
<td>18.62</td>
<td>4.76</td>
</tr>
<tr>
<td>CLA25</td>
<td>1</td>
<td>20.95</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.70</td>
<td>3.75</td>
</tr>
<tr>
<td>CL-DHT</td>
<td>1</td>
<td>20.96</td>
<td>4.23</td>
</tr>
<tr>
<td>CLA25-DHT</td>
<td>1</td>
<td>21.30</td>
<td>4.17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.37</td>
<td>3.80</td>
</tr>
<tr>
<td>CD0</td>
<td>1</td>
<td>20.01</td>
<td>4.53</td>
</tr>
<tr>
<td>CD05</td>
<td>1</td>
<td>21.39</td>
<td>4.15</td>
</tr>
<tr>
<td>CD25</td>
<td>1</td>
<td>20.99</td>
<td>4.23</td>
</tr>
<tr>
<td>CD5</td>
<td>1</td>
<td>20.70</td>
<td>4.29</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22.43</td>
<td>3.96</td>
</tr>
<tr>
<td>CD0-DHT</td>
<td>1</td>
<td>19.55</td>
<td>4.53</td>
</tr>
<tr>
<td>CD05-DHT</td>
<td>1</td>
<td>19.90</td>
<td>4.46</td>
</tr>
<tr>
<td>CD25-DHT</td>
<td>1</td>
<td>21.43</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.71</td>
<td>3.75</td>
</tr>
<tr>
<td>CD5-DHT</td>
<td>1</td>
<td>21.61</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22.48</td>
<td>3.89</td>
</tr>
</tbody>
</table>
Appendix III

Chick chorio-allantoic membrane (CAM) assay
Certification of Approval for the Care and Use of Laboratory Animals at Silpakorn University

by Ethics Committee for the Use of Laboratory Animals
Faculty of Pharmacy, Silpakorn University

Title of Project  Chitosan-aluminium monostearate composite sponges for promoting angiogenesis in wound

Principal Investigator  Associate Professor Thawatchai Phaecharnud, Ph.D.

Protocol Number  003/2013

Name of Faculty  Faculty of Pharmacy, Silpakorn University.

The aforementioned protocol have been reviewed and approved by Ethics Committee for the Use of Laboratory Animals, Faculty of Pharmacy, Silpakorn University.

Date of Approval  26 September 2013

( Pornsak Sriomonsak, Ph.D. )
Chairman
Ethics Committee for the Use of Laboratory Animals
Faculty of Pharmacy, Silpakorn University

( Jurapat Nunthanid, Ph.D. )
Dean
Faculty of Pharmacy, Silpakorn University
ในรูปกระดาษอนุญาตให้สัมภาษณ์และใช้สิทธิ์ที่มีชีวิต
เพื่องานทางวิทยาศาสตร์ที่มหาวิทยาลัยศิลปากร
คณะเภสัชศาสตร์ มหาวิทยาลัยศิลปากร

ชื่อโครงการ
ระบบโครงสร้างความพร้อมรุ่น comparatively กิจกรรมและคุณสมบัติใหม่ในสารเคมี
สำหรับใช้ในการทดสอบการสำเร็จของการผลิตในแผ่น

ชื่อหัวหน้าโครงการ
เภสัชกร ระดับ licentiate ฝ่ายวิจัย แพทย์

เลขที่โครงการ/รหัส
101/4556

สังกัดหน่วยงาน/กรม
คณะกรรมการกองยาภูมิคุ้มค้ำ

การรับเรื่อง
ขอรับรองโครงการวิจัยดังกล่าวข้างต้น ได้ดำเนินการพิจารณาและรับรอง
จากคณะกรรมการจริยธรรมการวิจัยด้านการแพทย์ คณะเภสัชศาสตร์
มหาวิทยาลัยศิลปากร เมื่อวันที่ 6 กันยายน 2556

ลงนาม
(ลงชื่อ ระดับ licentiate ผู้นิติบัญญัติ ศรีวิชัยกลิ่น)
ประธานคณะกรรมการจริยธรรมการวิจัยด้านการแพทย์

ลงนาม
(ลงชื่อ глаชาติจิตร รก. นิติบัญญัติ นักการบริหาร)
คณะกรรมการกองยาภูมิคุ้มค้ำ มหาวิทยาลัยศิลปากร)
Biography

Name Kotchamon Yodkhum, Ms.
Date of Birth September 16, 1985
Place of Birth Suphan Buri, Thailand
Nationality/Religion Thai/Buddhism
E-mail address marskotchamon@gmail.com

Education
2009 - 2013 Doctor of Philosophy, Ph.D. in Pharmaceutical Technology
Silpakorn University, Thailand
2004 - 2008 Bachelor of Pharmacy
Silpakorn University, Thailand

Scholarship
2008 Industrial Research Projects for Undergraduate Students (IRPUS) 2008
2009 - 2013 Thailand Research Funds through the Golden Jubilee Ph.D. Program (Grant No. PHD/0074/2551)

Publications


Presentation (Poster)


Award