จุฬาลงกรณ์มหาวิทยาลัย

# ทุนวิจัย
# กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

**คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย**

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1. ชื่อโครงการ การพัฒนาเมมเบรนจากไบโอพอลิเมอร์เพื่อใช้ในการถนอมอาหาร

Development of biopolymer membrane for food preservation

2. ได้รับทุนวิจัย ปี...2553.....

3. ชื่อผู้วิจัยและผู้วิจัยร่วมทุกคน.....


ชื่อหัวหน้าโครงการ (ไทย) รศ. ดร.เหมือนเดือน พิศาลพงศ์

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4. บทนำ

เนื่องจากประเทศไทยมีศักยภาพในการเป็นประเทศผู้ผลิตอาหารและผู้ส่งออกอาหารที่สำคัญของโลก มีการใช้และส่งออกผลิตภัณฑ์ฟิล์มยืดหุ้มห่ออาหารเพิ่มทุกปี ตลอดจนมีการนำเข้าประเทศในปริมาณมาก และมีแนวโน้มที่เพิ่มสูงขึ้นอย่างต่อเนื่อง ยิ่งกว่านั้นพบว่ามีการนำฟิล์มยืดที่ไม่ใช่ฟิล์มยืดหุ้มห่ออาหารไปใช้หุ้มห่ออาหารซึ่งอาจส่งผลให้เกิดอันตรายต่อผู้บริโภค และมีผลเสียต่ออุตสาหกรรมการส่งออกผลิตภัณฑ์อาหารของประเทศ นอกจากนี้การใช้ฟิล์มจากสารสังเคราะห์ทางเคมีส่วนใหญ่ยังย่อยสลายยาก ก่อให้เกิดปัญหาสิ่งแวดล้อมเป็นพิษอันเนื่องจากปริมาณขยะจากฟิล์มที่ย่อยสลายไม่ได้เป็นปริมาณมากมายในแต่ละปี ในงานวิจัยนี้จึงมีจุดมุ่งหมายที่จะพัฒนาฟิล์มหุ้มห่ออาหารจากไบโอพอลิเมอร์ที่ผลิตได้โดยใช้กระบวนการทางชีวภาพซึ่งมีสมบัติที่สำคัญคือ

สามารถย่อยสลายได้เองตามธรรมชาติเมื่อถูกนำใช้งานแล้ว ตลอดจนพัฒนาให้มีความแข็งแรงเชิงกล ช่วยยืดอายุการเก็บรักษาอาหารและมีความปลอดภัยสูงในการนำไปใช้ห่อหุ้มอาหาร นอกจากนี้คณะผู้วิจัยยังมีความสนใจที่จะพัฒนาสมบัติของฟิล์มให้สามารถช่วยลดปริมาณ การเจริญเติบโตของจุลินทรีย์ปนเปื้อน ที่อาจปนอยู่บริเวณผิวของอาหารซึ่งพบว่าเป็นปัญหาที่ก่อให้เกิดการเน่าเสียของอาหารมากที่สุด โดยองค์ประกอบของแผ่นฟิลม์หุ้มห่ออาหารที่พัฒนาขึ้นนี้จะที่มีสารช่วยลดหรือยับยั้งการเจริญเติบโตของจุลินทรีย์ที่พบปนเปื้อนในอาหาร โดยสารที่ใช้จะเป็นกลุ่มสารสกัดจากพืชสมุนไพรหรือสารสกัดจากผลไม้บางชนิดที่เป็นที่ยอมรับในความปลอดภัยมานานแล้ว

5.วิธีดำเนินการวิจัย

5.1 การขึ้นรูปฟิล์ม

เตรียมฟิล์มจากวัสดุพอลิเมอร์ธรรมชาติโดยอาทิเช่น เซลลูโลส แป้ง เจลาตินโดยทำการทดสอบทั้งในรูปแบบสารเดียว และสารผสมในสัดส่วนต่างๆ โดยเริ่มจากเตรียมในรูปแบบสารละลายเข้มข้น จากนั้นนำไปรีดบนแผ่นเรียบ และทำการระเหยตัวทำละลายจนแห้ง จากนั้นจึงเก็บไว้ภายในถุงพลาสติกใสที่อุณหภูมิห้องก่อนนำไปใช้ต่อไป

5.2 การทดสอบสมบัติทางกายภาพและชีวภาพของฟิล์ม

ทำการทดสอบสมบัติทางกายภาพและชีวภาพของฟิล์มที่ได้พัฒนาขึ้น อาทิเช่น

- ลักษณะผิว ภายนอก โดยใช้เครื่อง scanning electron microscope (SEM)
- สมบัติในการดูดซับน้ำ
- สมบัติการละลายน้ำ
- สมบัติการแพร่ผ่านของไอน้ำ ออกซิเจน
- ลักษณะความพรุน ขนาดรูพรุน โดยใช้เครื่อง BET
- สมบัติทางกล ความแข็งแรงของฟิลม์ (tensile strength, Young's modulus, %Elongation )
- สมบัติในการย่อยสลายทางชีวภาพ

5.3 คัดเลือก หรือ นำพืชสมุนไพรที่สนใจมาทำการสกัดสารสำคัญที่สภาวะที่เหมาะสม จากนั้นกรอง โดยใช้กระดาษกรองเบอร์ 4 ด้วยเครื่อง Vacuum pump นำสารสกัดที่ได้ระเหยแห้งด้วยเครื่อง Rotary evaporator เติมสารละลาย Dimethyl sulfoxide (DMSO) 50 มิลลิลิตร เก็บไว้ที่อุณหภูมิ 4 องศาเซลเซียส เพื่อรอนำไปใช้งาน

5.4 ศึกษาเบื้องต้นเพื่อคัดเลือกสารที่มีประสิทธิภาพในการยับยั้งการเจริญของจุลินทรีย์ที่ก่อให้เกิดการเน่าเสีย โดยนำสารสกัดแต่ละชนิดมาทดสอบกับกลุ่มจุลินทรีย์ที่ก่อให้เกิดปัญหากับอาหารแต่ละชนิด ได้แก่ แบคทีเรียแกรมบวก *Staphylococcus aureus* แบคทีเรียแกรมลบ *Echerichia coli* และยีสต์ *Sacchoromyces cerevisiae* รวมทั้งรา *Aspergislus niger*

5.5 การศึกษาสมบัติการยับยั้งจุลินทรีย์ ของแผ่นฟิล์มที่มีการเติมสารสกัดที่มีผลยับยั้งจุลลินทรีย์

**6. ผลการวิจัย**

**6.1 การพัฒนาฟิลม์จากแบคทีเรียเซลลูโลสและเจลาติน**

นำแผ่นฟิล์มแบคทีเรียเซลลูโลสที่จากเชื้อแบคทีเรีย *Acetobacter Xylinum* มาปรับปรุงคุณภาพโดยทำให้มีองค์ประกอบของเจลาตินอยู่ในแผ่นฟิล์ม ด้วยกระบวนการแช่แผ่นฟิล์มแบคทีเรียเซลลูโลสเปล่าในสารละลายเจลาตินที่มีความเข้มข้น 15 และ 30% โดยน้ำหนัก แล้วจึงทำการเชื่อมพันธะ (cross link) ด้วยกรดแทนนิค ซึ่งเป็นสารสกัดจากเปลือกไม้ พบว่าแผ่นฟิล์มจะมีจำนวนเม็ดของเจลตินแทรกตามเส้นใยเซลลูโลส เพิ่มขึ้นแปรผันตามความเข้มข้นของสารละลายที่ใช้ จากการทดสอบด้วยอินฟราเรดทางโครงสร้างโมเลกุลพบว่า เซลลูโลสและเจลาตินมีปฏิสัมพันธ์ระหว่างกันแต่เป็นการปฏิสัมพันธ์แบบที่ไม่แข็งแรงและไม่เกิดพันธะเคมีต่อกัน จากการวิเคราะห์สมบัติทางกล การดูดซับน้ำ ความเป็นผลึก การผ่านของไอน้ำและออกซิเจน มีค่าใกล้เคียงกับฟิล์มแบคทีเรียเซลลูโลสที่ไม่ถูกปรับปรุง อย่างไรก็ตามแผ่นฟิล์มแบบที่ดัดแปลงมีสมบัติที่เปลี่ยนแปลงในส่วนมีความโปร่งแสงเพิ่มขึ้น และมีสมบัติในการต่อต้านแบคทีเรีย *Staphylococcus aureus* ซึ่งเป็นผลจากการเติมกรดเทนนิค แต่ไม่มีผลยับยั้ง*E.coli* หรือ *A.niger*

**6.2 การศึกษาการเติมสารสกัดจากเปลือกมังคุดในแผ่นฟิล์ม**

ศึกษาพัฒนาการขึ้นรูปแผ่นฟิลม์ไบโอพอลิเมอร์ที่มีการเติมสารสกัดจากสมุนไพรจากสารสกัดเปลือกมังคุดที่มีรายงานก่อนหน้านี้ถึงผลต่อการยับยั้งจุลินทรีย์บางชนิด โดยได้ทำการเตรียมฟิลม์จากวัสดุพอลิเมอร์ธรรมชาติคือแบคทีเรียเซลลูโลส ทำการโหลดสารสกัดจากเปลือกมังคุดทั้งแบบสกัดด้วยน้ำและสกัดโดยเอทานอลลงไป และเก็บในลักษณะฟิลม์แห้งซึ่งจะมีองค์ประกอบของสารสำคัญของสารสกัดสมุนไพรที่แตกต่างกัน จากการทดสอบสมบัติของแผ่นฟิลม์แห้งพบว่ามีสมบัติทางกายภาพและชีวภาพของไบโอฟิลม์ที่เปลี่ยนแปลงไป โดยพบว่าการโหลดสารสมุนไพรสกัดจากเปลือกมังคุดเขาไปในแผ่นฟิลม์มีผลต่อความแข็งแรงเชิงกลของแผ่นฟิลม์ ความสามารถในการดูดซับน้ำ และอัตราการแพร่ผ่านไอน้ำ ขึ้นกับปริมาณการโหลดและชนิดของสารสกัด (ในที่นี้คือ น้ำ หรือ เอทานอล) จากการศึกษาเบื้องต้นถึงประสิทธิภาพในการยับยั้งการเจริญของจุลินทรีย์ที่ก่อให้เกิดการเน่าเสีย โดยทดสอบกับกลุ่มจุลินทรีย์ที่ก่อให้เกิดปัญหากับอาหารแต่ละชนิด

ได้แก่ แบคทีเรียแกรมบวก *Staphylococcus aureus* แบคทีเรีย แกรมลบ *Echerichia coli* รวมทั้งรา *Aspergislus niger* พบว่าแผ่นฟิลม์ทีทำการโหลดสารสกัดสมุนไพรเปลือกมังคุดแสดงสมบัติการยับยั้งจุลินทรีย์หลายๆชนิด เช่น S*taphylococcus aureus, Staphylococcus epidermidis, Asperfillus niger* และ *Escherichia coli* ทั้งนี้ขึ้นกับปริมาณการโหลดและชนิดของสารสกัดโดยมีรายละเอียดตามบทความในหัวข้อที่8.2 และ 8.3

**6.3. ศึกษาผลของการใช้สารยับยั้งจุลินทรีย์ที่สกัดจากพืชสมุนไพรอื่นๆ**

ศึกษาประสิทธิภาพของสารสกัดจากสมุนไพรแห้ง กระชาย พลู ตำลึง เบญกานีและข่าต่อการยับยั้งเชื้อจุลินทรีย์ โดยใช้สารสมุนไพร จากการสกัดด้วยเอทานอล ที่ความเข้มข้น 1:1 (w/v) ที่ผ่านการกรอง อบแห้งและเตรียมในรูปแบบสารละลายใน DMSO

6.3.1 สารสกัดจากกระชายมีผลการยับยั้งการเจริญของเชื้อทั้ง 3 ชนิดได้แตกต่างกัน คือ สามารถยับยั้งเชื้อ *B. subtilis* ได้ดีที่สุด รองลงมา คือ เชื้อ *E. coli* และเชื้อ *S. aureus* ตามลำดับ เนื่องจากสารสกัดจากเหง้ากระชายมีฤทธิ์ในการยับยั้งการเจริญเติบโตของแบคทีเรียหลายชนิด และเป็นแหล่งของฟลาโวนอยด์ (flavonoid) หลายชนิดที่มีสมบัติทั้งทางเภสัชวิทยาและทางการแพทย์ เช่น ฟลาวาโนน (flavanone) ชาโคน (chalcone) และฟลาโวน (flavone) เป็นต้น นอกจากนี้ยังมีงานวิจัยที่พบว่า มี สาร 1,8-cineol มีคุณสมบัติต้านเชื้อ *B. subtilis* นอกจากนี้ pinostrobin ยังมีฤทธิ์ทำลายเชื้อ *E. coli*

6.3.2 สารสกัดจากเบญกานีมีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อทั้ง 3 ชนิด ได้แตกต่างกัน คือ สามารถยับยั้งเชื้อ *E. coli* ได้ดีที่สุด รองลงมา คือ เชื้อ *B. subtilis* และเชื้อ *S. aureus* ตามลำดับ โดยมีรายงานถึง ความมีฤทธิ์ของผลเบญจกานี ในการยับยั้งจุลินทรีย์ในทางเภสัชกรรมก่อนหน้านี้ รวมทั้งได้มีรายงานการวิจัยเกี่ยวกับผลในการยับยั้ง *E.coli* (Singh และคณะ 2005 , Basri และ Fan (2005) , Voravuthikunchai และ Suwalak (2008), Voravuthikunchai และ Suwalak (2008) Oussalah และคณะ (2006))

6.3.3 สารสกัดจากตำลึง มีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อทั้ง 3 ชนิดได้แตกต่างกัน คือ สามารถยับยั้งเชื้อ *E.coli* ได้ดีที่สุด รองลงมา คือ เชื้อ *S. aureus* แต่ไม่มีผลยับยั้งเชื้อ *B. subtilis*

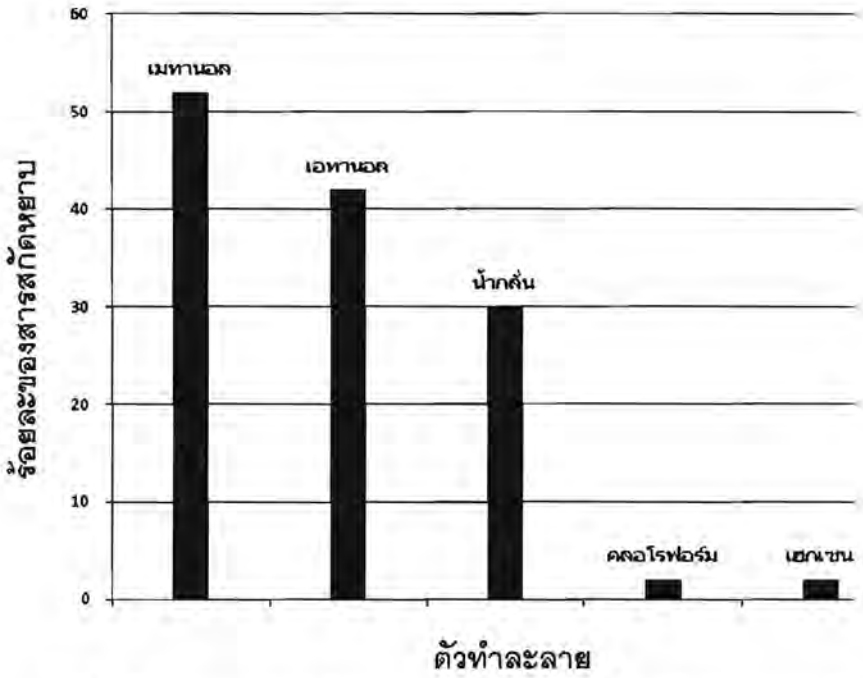
6.3.4 สารสกัดจากพลูมีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อทั้ง 3 ชนิดได้ในระดับต่ำ แม้ว่าเคยมีรายงานว่าองค์ประกอบน้ำมันหอมระเหย chavicol, chavibitol, และ β-situsterol ในใบพลูมีฤทธิ์ฆ่าแบคทีเรียได้ก็ตาม

6.3.5 สารสกัดจากข่ามีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อทั้ง 3 ชนิดได้ในระดับต่ำเมือเทียบกับสารสกัดอื่นๆ


รูปที่ 1 ผลการยับยั้งของสารสมุนไพรชนิดต่างๆ ที่สกัดด้วยเอทานอล ที่ความเข้มข้น 1:1 (w/v) ต่อเชื้อ*B.subtillis*


รูปที่ 2 ผลการยับยั้งของสารสมุนไพรชนิดต่างๆ ที่สกัดด้วยเอทานอล ที่ความเข้มข้น 1:1 (w/v) ต่อเชื้อ *E.coli*

เมื่อเปรียบเทียบผลสารจากการสกัดกระชาย พลู ตำลึง เบญกานีและข่า แห้งด้วยตัวทำละลายเอทานอล พบว่าสารสกัดที่มีผลยับยั้งจุลลินทรีย์สูงสุดคือสารสกัดเบญกานี โดยสารสกัดที่ได้มีลักษณะเป็นผลึกสีน้ำตาล ขาว เมื่อเปรียบเทียบผลการสกัดเบญกานี ด้วยตัวทำละลายชนิดต่างๆ ได้แก่ เมธานอล เอทานอล น้ำ คลอโรฟอร์ม และเฮกเซน พบว่าได้ค่าร้อยละของสารสกัดหยาบสูงสุดด้วยเมธานอล โดยร้อยละของสารสกัดเบญกานีด้วยเมทานอลสามารถสกัดได้ถึงครึ่งหนึ่งของน้ำหนักสมุนไพรเริ่มต้น รองมาคือเอทานอล น้ำกลั่น คลอโรฟอร์มและเฮกเซน ตามลำดับดังแสดงใน รูปที่ 3




**รูปที่ 3** ร้อยละของสารสกัดหยาบเบญกานีที่สกัดได้จากตัวทำละลายชนิดต่างๆ

โดยผลทดสอบฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์ด้วยวิธี Disc diffusion method ผลการยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์ของสารสกัดเบญกานีจากตัวทำละลายทั้ง 5 ชนิด ได้ผลดังแสดงในตารางที่ 1

**ตารางที่ 1** แสดงฤทธิ์ยับยั้งเชื้อจุลินทรีย์ของสารสกัดเบญกานีจากตัวทำละลายชนิดต่างๆ

| ตัวทำละลาย | ขนาดเส้นผ่านศูนย์กลางวงใสเฉลี่ย (มิลลิเมตร) ± S.D.[a] | | |
|---|---|---|---|
| | ***E. coli*** **ATCC 25922** | ***S. aerus*** **ATCC 25923** | ***B. subtilis*** **ATCC 6633** |
| เมทานอล | 20.3 ± 0.8 | 22.0 ± 0.7 | 25.3 ± 0.4 |
| เอทานอล | 19.0 ± 0.7 | 20.0± 0.7 | 24.0 ± 0.7 |
| น้ำกลั่น | 15.7 ± 0.4 | 18.0 ± 0.7 | 21.0 ± 0.7 |
| คลอโรฟอร์ม | 10.3 ± 0.9 | 12.7 ± 0.4 | 13.0 ± 0.7 |
| เฮกเซน | 10.0 ± 0.7 | 11.3 ± 0.8 | 12.0 ± 0.7 |
| Tetracycline | 19.0±0.6 | 28.9±0.4 | 20.5±0.6 |
| Kanamycin | 18.6±0.8 | 18.7±0.5 | 26.3±0.5 |

[a] หมายถึง ค่าเบี่ยงเบนมาตรฐานของขนาดเส้นผ่านศูนย์กลางวงใส

สารสกัดเบญกานีจากตัวทำละลายทั้ง 5 ชนิด สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียทั้ง 3 ชนิดได้ โดยสารสกัดเบญกานีจากเมทานอล เอทานอล และน้ำกลั่น ให้ผลการยับยั้งสูงกว่าสารสกัดจากเฮก เซนและคลอโรฟอร์ม โดยมีรายละเอียดตามบทความวิจัย 8.1 (ในเอกสารแนบ)

7. สรุปและวิจารณ์ผลงานวิจัย

ได้ข้อมูลจากการศึกษากระบวนการขึ้นรูปแผ่นฟิล์มแบคทีเรียเซลลูโลสและเจลาติน พบว่าจากการปรับปรุงสมบัติของแผ่นฟิล์มจากแบคทีเรียเซลลูโลสและเจลาติน ได้ฟิลม์ที่สามารถรับประทานได้ ที่มีความโปร่งแสง และฟิล์มมีสมบัติหลักคือเป็นแผ่นช่วยควบคุมความชื้น หรือลดการแพร่ผ่านของออกซิเจน คาร์บอนไดออกไซด์ ไอน้ำ หรือสารระเหยได้ นอกจากนี้ยังมีสมบัติช่วยยับยั้งการเจริญของเชื้อ *B. subtilis* ซึ่งเป็นจุลินทรีย์ที่อาจปนเปื้อนอยู่ในอาหาร และสามารถเพิ่มคุณสมบัติการยับยั้งจุลินทรีย์ของแผ่นฟิล์มโดยการเติมสารสกัดสมุนไพร โดยเมื่อเติมสารสกัดจากเปลือกมังคุดลงในแผ่นฟิลม์ พบว่าการเติมสารสกัดจากเปลือกมังคุดด้วยน้ำสามารถช่วยยับยั้งการเจริญของ *E.coli, S.aureus, S. epidermidis* และ *P.acnes* แต่ไม่ยับยั้งการเจริญของเชื้อรา *A.niger* ในขณะที่ การเติมสารสกัดจากเปลือกมังคุดด้วยเอทานอลสามารถช่วยยับยั้งการเจริญของ *S.aureus, S. epidermidis P.acnes* และ *A.niger* แต่ไม่ยับยั้งการเจริญของ *E.coli* และจากการศึกษาการสกัดสารสมุนไพรอื่นๆพบว่าสารสกัดจากเบญกานีและกระชายมีผลการยับยั้งจุลินทรีย์ที่ดีเช่นกัน โดยข้อมูลเหล่านี้จะเป็นประโยชน์ต่อการพัฒนาแผ่นฟิล์มจากไบโอพอลิเมอร์ที่เพื่อใช้ในการถนอมอาหาร ต่อไป โดยผลงานวิจัยส่วนหนึ่งได้รับการเผยแพร่ในรูปแบบ บทความวิจัยในวารสารระดับนานาชาติ 1 บทความ, บทความวิจัยในวารสารระดับชาติ 1 บทความ และบทความวิจัยในการนำเสนอผลงานวิชาการ(proceeding) ระดับนานาชาติ (2 เรื่อง)

8. ผลงาน (output)

8.1 บทความวิจัยในวารสารระดับนานาชาติ

8.1.1 C. Satirapathkul and T. Leela, Growth inhibition of pathogenic bacteria by extract of quercus infectoria galls, International Journal on Bioscience, Biochemistry and Bioinformatics, 2011, (n press)

8.2 บทความวิจัยในวารสารระดับชาติ

8.2.1 N. Nunkaew and M.Phisalaphong, Characteristics of bacterial cellulose film synthesized by *Acetobacter xylinum* containing extract from rind of *Garcinia mangostana*, วารสารมหาวิทยาลัยศรีนครินทรวิโรฒ(สาขาวิทยาศาสตร์และเทคโนโลยี), พ.ศ. 2554, ปีที่3 (ฉบับพิเศษที่ 1)

8.3 บทความวิจัยในการนำเสนอผลงานวิชาการ(proceeding) ระดับนานาชาติ จำนวน....2...เรื่อง ดังนี้

8.2.1. N. Nunkaew and M.Phisalaphong, Characteristics of bacterial cellulose film synthesized by Acetobacter xylinum containing extract from rind of Garcinia mangostana, The 3rd Pure and Applied Chemistry International Conference 2011 (PACCON 2011), Jan 5-7, 2011, Bangkok, Thailand.

8.2.2. N. Nunkaew and M. Phisalaphong, Antimicrobial activities of bacterial cellulose modified by supplementation of water and ethanolic extracts of fruit rind of Garcinia mangostana, The 3rd International Conference on Biochemistry and Molecular Biology 2011 (BMB2011), April 6-8, 2011, Chiang Mai, Thailand.

ลงชื่อผู้วิจัยหลัก.....

(รศ. ดร เหมือนเดือน พิศาลพงศ์)

ภาควิชา... วิศวกรรมเคมี....

วันที่...27...เดือน...ก.ค....พ.ศ...2554..

1. ชื่อผู้วิจัย (ทุกคน)... 1.1...รศ. ดร.เหมือนเดือน พิศาลพงศ์ (หัวหน้าโครงการ)

1.2 ดร. ชุติมณฑน์ สถิรพิพัฒนกุล (ผู้ร่วมโครงการ)

2. โครงการวิจัย(ภาษาไทย)...การพัฒนาเมมเบรนจากไบโอพอลิเมอร์เพื่อใช้ในการถนอมอาหาร

(ภาษาอังกฤษ)... Development of biopolymer membrane for food preservation...

3. ได้รับทุนวิจัยปี...2553.....

จำนวนเงิน...300,000 บาท (งวดที่ 1 =170,000 บาท, งวดที่ 2 =70,000 บาท)...

4. รายละเอียดค่าใช้จ่ายทั้งหมด

4.1 <u>หมวดค่าจ้างชั่วคราว</u>

(ชี้แจงรายละเอียดจำนวนบุคลากร / ระยะเวลา) 2 คน/ 12 เดือน

รวมเงิน ...100,000.00.....

4.2 <u>หมวดค่าใช้สอย</u> .....

ค่าจ้างเหมาบริการ (วิเคราะห์ตัวอย่าง)

รวมเงิน ...67,735.00.....

4.3 <u>หมวดค่าวัสดุภัณฑ์</u>

4.3.1 วัสดุวิทยาศาสตร์ ...70,944.67...บาท

4.3.2 วัสดุสำนักงาน .....-.....บาท

4.3.3 อื่นๆ .....-.....บาท

4.4 <u>ค่าถ่ายเอกสาร</u>

(ไม่เกินโครงการละ 1,000 บาท ตลอดโครงการยกเว้นโครงการวิจัยเอกสาร)

รวมเงิน .....963.00.....บาท

4.5 เบ็ดเตล็ด

(ไม่เกินโครงการละ 500 บาท ตลอดโครงการ) .....495.00.....บาท

4.6 รวมค่าใช้จ่ายทั้งหมดเป็นเงิน 240,137.67...บาท

(...สองแสนสี่หมื่นหนึ่งร้อยสามสิบเจ็ดบาทหกสิบเจ็ดสตางค์) ตัวอักษร

ลงชื่อผู้วิจัยหลัก. .....

(รศ. ดร.เหมือนเดือน พิศาลพงศ์)

วันที่...27.. เดือน.....ก.ค.....พ.ศ...2554.

สารบัญภาพ

เอกสาร 8, 1, 1

# Growth Inhibition of Pathogenic Bacteria By Extract of *Quercus Infectoria* Galls

C. Satirapathkul and T. Leela, *APCBEES Member*

***Abstract*— Extract of *Quercus infectoria* galls was evaluated for its antimicrobial activity against a wide variety of pathogenic bacteria such as Escheria coli ATCC 25922, Staphylococus aurus ATCC 25923 and Bacillus subtilis ATCC 6633. The antimicrobial activities of Q. infectoria extracts prepared from different solvents of varying polarity were examined and their efficacies were then compared by the disc diffusion method. Crude extracts of the solvents exhibited the anitmicrobial effect to a different extent as seen in the varying diameters of the zones of inhibition. The antimicrobial activity of the methanol extract was found to be superior to all other extracts. Ethanol and aqueous extracts showed a strong though lower antimicrobial effect against all the tested organisms while chloroform and hexane extracts of Q. infactoria were found to be least active. Compared to the commercial antibiotics, all the extracts exhibited a good antimicrobial activity. All the gram-positive bacteria and gram-negative bacteria tested were susceptible to all aqueous and solvent extracts of Q. infectoria galls. The methanol extracts at different concentrations were then incubated with the three bacteria strains, and the minimum inhibitory concentrations (MICs) of each bacterial strain were determined. The MIC values of methanol extracts for E. coli ATCC 25922, S. aureus ATCC 25923 and B. subtilis ATCC 6633 are 2.500, 1.25 and 0.625 mg ml$^{-1}$ respectively. Microscopic observation under scanning electron microscopy (SEM) revealed a sharp decline in bacterial population density for all three bacterial strains. The bacterial morphology of all the strains became more irregular in shape differing from their respective originally homogeneous forms.***

*Index Terms*— **antimicrobial activity, Quercus infectoria galls, pathogenic bacteria, the extracts**

## I. Introduction

Recently, there has been an increase in the number of reported poisoning outbreaks caused by food-borne pathogenic bacteria. The evolution of bacterial resistance to currently available antibiotics has necessitated the research for more novel antimicrobial compounds. Many local plants are cheap, readily available and widely used in traditional folk medicine since they produce a diverse range of bioactive compounds. In several occasions, the use of plant extracts is more preferable over the use of human-synthesized medicine. Unlike human-synthesized medicine which normally consists of only a single bioactive compound, the extracts from plants may contain more than one bioactive ingredients which synergistically work against a particular disease. In addition, being from nature is normally perceived as safer and therefore, more acceptable by humans.

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Antimicrobial, antifungal and antiviral activities are among the medicinally useful properties for which many plant extracts are employed. Particularly, the antimicrobial activity of such extracts has formed the basis of various applications, including raw and processed food preservation (antimicrobial cans or food preservatives), textile industries (antimicrobial dyes or antimicrobial cloths), pharmaceuticals (natural antibiotics), and alternative medicine (substitutes for human-synthesized drugs). Therefore, plants with possible antimicrobial activity should be tested against an appropriate microbial model to validate their effectiveness and to ascertain the parameters at which they best work.

*Quercus infectoria* Olivier (Fagaceae) is a small shrub mainly present in Greece, Asia Minor, Syria and Iran. It is locally cultivated for its valuable medicinal properties. The medicinal properties of the plant have been a subject of numerous investigations. In traditional folk medicine, the galls are extracted with hot water for use as a gargle to relieve inflamed tonsils or directly applied onto the inflamed skin to reduce swelling. Ethyl acetate extract of the galls has been reported to be very effective in killing mosquito larvae and is currently a promising candidate for the development of environmentally-friendly larvicide [1]. In addition, they have also been known to produce many bioactive compounds [2,3] with antibacterial [4], antifungal [5], antidiabetic [6], local anaesthetic [7], antiviral [8], and anti-inflammatory [9] activities. Moreover, they contains the mixture of tannin (60-70%) with antitumor activity [10], polyphenols with antivenom activity [11], gallalic acid, methyl gallate and ellagic acid with antioxidant activity [12,13]. Tannins are generally defined as water–soluble polyphenolic compounds ranging in molecular weight from 500 to 3000 Daltons and having the ability to precipitate proteins [14].

Due to its myriad of useful medicinal properties mentioned above, there is a further incentive to find out more about the potential of this plant as an antimicrobial agent. Hence, this study was designed to assess the effectiveness of different solvent extracts of *Q. infectoria* galls toward the growth inhibition of gram-positive and gram-negative bacteria. We also investigated the effect of the extracts on the morphological changes of the test pathogenic bacteria under scanning electron microscope.

## II. Materials and Methods

### A. Test microorganisms

Tested pathogenic bacteria comprised *Escherichia coli ATCC 25922*, *Bacillus subtilis ATCC 6633*, and *Staphylococcus aureus ATCC 25923*. The bacteria were maintained by subculturing periodically on nutrient agar and were preserved at 4 °C prior to use.

*B. Culture media*

The nutrient agar was prepared by dissolving 5 g peptone, 1.5 g beef extract, 1.5 g yeast extract, 5 g NaCl and 20 g agar in 1000 ml distilled water, boiling the mixture and adjusting its pH value to between 6.4–6.8. The nutrient mixture was then sterilized by autoclaving at 15 psi pressure (121 °C) for 20 min. Nutrient agar was prepared by pouring the nutrient mixture to the same thickness on sterilized petri plates. The test bacteria were then grown overnight at 37 °C, 120 rpm in 10 ml nutrient broth. This broth was used for seeding the bacteria onto the agar plates.

*C. Plant materials*

The galls of *Q. infectoria* were obtained commercially from a Thai traditional drug store. All samples were washed with distilled water, cut into small pieces and dried at 60°C overnight. They were crushed with a mechanical mortar into fine powder before the extraction (Fig 1).

*D. Extraction conditions*

Extracts of dried plant materials were prepared by using solvents of varying polarity. The dried plant materials of 5 g each were extracted by maceration in different solvents (25 ml) for 5 days at room temperature in a dark place. The solvents used were methanol, ethanol, hexane, chloroform, and distilled water. Following the solvent maceration, the extract was filtered through Whatman filter paper IV. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely dry. The weight of the solid residue was recorded and taken as yield of crude extracts. The extracts were stored at -20 °C and freshly dissolved in 10 % dimethyl sulfoxide (DMSO, Merck, Germany) before use. The corresponding concentration was expressed in term of mg of extract per ml of solvent (mg $ml^{-1}$).

*E. Antimicrobial assay*

Antimicrobial activity was tested by the disc diffusion method. Small discs of filter paper (diameter 6.0 mm) were impregnated with 50 µl of different extracts and placed on top of the seeded media of the three bacterial strains. The antibacterial assay plates were incubated at 37 °C for 24 h and the diameters of the zones of clearing were noted. In addition, the discs of the standard antibiotics; tetracycline (10 µg per disc) and kanamycin (10 µg per disc) were also experimented separately which would serve as positive antibacterial controls. For this study, the diameter of the zone of inhibition around each disc (disc diameter included) was taken as a measure of the antibacterial activity. The diameters of the zones of inhibition by the samples were then compared with the diameters of the zones of inhibition produced by the standard antibiotic discs. Each experiment was carried out in triplicate and the mean diameter of the inhibition zones was recorded.

*F. Minimum inhibitory concentration (MIC)*

MICs of the extracts were determined by the broth dilution method using serial dilution of the plant extracts as described previously by *Evans et al.* [15]. Briefly, the test bacteria were prepared in nutrient broth and incubated at 37 °C for 24 h. After that, the cultures' concentrations were adjusted with sterilized saline to bring the optical density at 660 nm to 0.04. Serial two-fold dilutions of the extracts were prepared in nutrient broth with concentrations ranging from 5.0000 to 0.0049 mg/ml. The 1 ml of each serially diluted extract was separately added to the tubes containing an equal volume of the inoculum (1 ml). All the tubes (total volume of 2 ml) were then incubated at 37 °C for 24 h.

The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity. Solvent blanks and positive controls were also included. All tests were performed in triplicate.

*G. Morphological observations under scanning electron microscope (SEM)*

Cells of each strain at a logarithmic phase in nutrient agar were treated with the extracts for 12 h. The bacterial cells treated with 10 % DMSO were used as control. The cells were collected by centrifugation and washed with the sodium phosphate buffer. Following that, the samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4°C overnight, and postfixed in 1% osmium tetroxide in the phosphate buffer for 1 h at room temperature before processing for the observations under scanning electron microscope.

## III. Result and Discussion

*A. Yield of extracts*

The solvents with an increasing order of polarity were used for the extraction of *Q. infectoria* galls. The percentage yields of the extracts for *Q. infectoria* were: 52% (methanol), 42% (ethanol), 30% (water), 2% (hexane), and 2% (chloroform) as shown in Fig. 2. Methanol extracted the most materials from the plant followed by ethanol and water. The extracts of chloroform and hexane yielded the lowest amount.

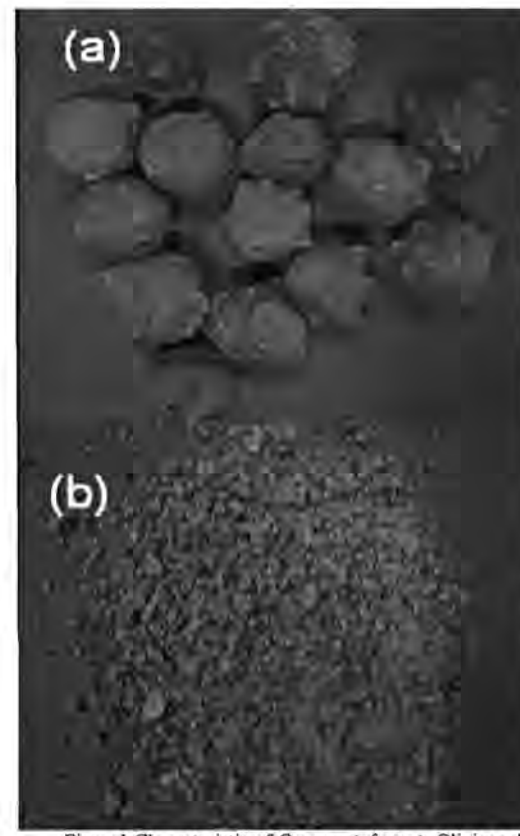


Figure1 Characteristic of *Quercus infectoria* Olivier galls: dried samples (a) and powder (b)

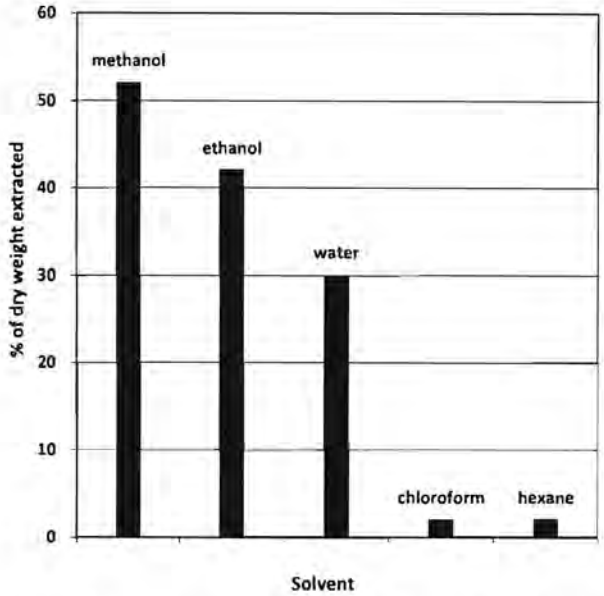


Figure 2. Percentage of 5 g dried material extracted after extractions with 25 ml of methanol, ethanol, water, chloroform and hexane.

### B. *Antimicrobial assay*

The antimicrobial activity of five solvent extracts of *Q. infectoria* galls was studied by the disc diffusion method and the results are shown (Table 1, Fig. 3-5). All the solvent extracts showed a significant inhibitory activity against all pathogenic bacterial strains. Methanol, ethanol and aqueous extracts exhibited a higher antibacterial activity than chloroform and hexane extracts did and produced inhibition zones ranging from 19.0 to 25.3 mm in diameter at the concentration of 5 mg ml $^{-1}$. Methanol extract displayed an excellent activity against gram-positive *B. subtilis ATCC 6633* (25.3 mm), *S. aureus ATCC 25923* (22.0 mm) and gram-negative *E. coli ATCC 25922* (20.3 mm). Ethanol extracts showed a strong though a little bit lower activity against gram-positive *B. subtilis ATCC 6633* (24.0 mm), *S. aureus ATCC 25923* (20.0 mm) and gram-negative *E. coli ATCC 25922* (19.0 mm). Similarly, aqueous extracts also showed a strong activity against gram-positive *B. subtilis ATCC 6633* (21.0 mm), *S. aureus ATCC 25923* (18.0 mm) and gram-negative *E. coli ATCC 25922* (15.7 mm). However, chloroform and hexane extracts only displayed a mild to moderate activity against all of the tested bacteria. Chloroform extracts were found to be active against gram-positive *B. subtilis ATCC 6633* (13.0 mm), *S. aureus ATCC 25923* (12.7 mm) and gram-negative *E. coli ATCC 25922* (10.3 mm). Finally, hexane extracts were found to be active against gram positive *B. subtilis ATCC 6633* (12.0 mm), *S. aureus ATCC 25923* (11.3 mm) and gram-negative *E. coli ATCC 25922* (10.0 mm). The results were compared with those of tetracycline, and kanamycin as standard antibiotics. On overall consideration, the antimicrobial activities of the alcoholic extracts were higher as compared to those of other less polar extracts. This may imply that the bioactive molecules responsible for the antimicrobial action should be more hydrophilic in nature. In addition, a higher antibacterial activity was also observed against gram-positive bacteria.

TABLE 1. ANTIBACTERIAL ACTIVITIES OF DIFFERENT EXTRACTS OF Q. INFECTORIA GALLS

| Solvent | Diameter of inhibition zone (mm) ± S.D.[a] | | |
|---|---|---|---|
| | *E. coli ATCC 25922* | *S. aerus ATCC 25923* | *B. subtilis ATCC 6633* |
| Methanol | 20.3 ± 0.8 | 22.0 ± 0.7 | 25.3 ± 0.4 |
| Ethanol | 19.0 ± 0.7 | 20.0± 0.7 | 24.0 ± 0.7 |
| Distilled water | 15.7 ± 0.4 | 18.0 ± 0.7 | 21.0 ± 0.7 |
| Chloroform | 10.3 ± 0.9 | 12.7 ± 0.4 | 13.0 ± 0.7 |
| Hexane | 10.0 ± 0.7 | 11.3 ± 0.8 | 12.0 ± 0.7 |
| Tetracycline | 19.0±0.6 | 28.9±0.4 | 20.5±0.6 |
| Kanamycin | 18.6±0.8 | 18.7±0.5 | 26.3±0.5 |

[a] Mean value of three determinations, each from a different plate


Figure 3 Antimicrobial activity of the extracts on *E. coli ATCC 25922*: Disc diffusion test for the effect of *Q. infectoria* against *E .coli ATCC 25922* grown on nutrient agar medium.1. ethanol; 2. methanol; 3.chloroform; 4. hexane; 5.water.


Figure 4 Antimicrobial activity of the extracts on *S. aureus ATCC 25923*: Disc diffusion test for the effect of *Q. infectoria* against *S. aureus ATCC 25923* grown on nutrient agar medium. 1. ethanol; 2. methanol; 3.chloroform; 4.hexane; 5.water.

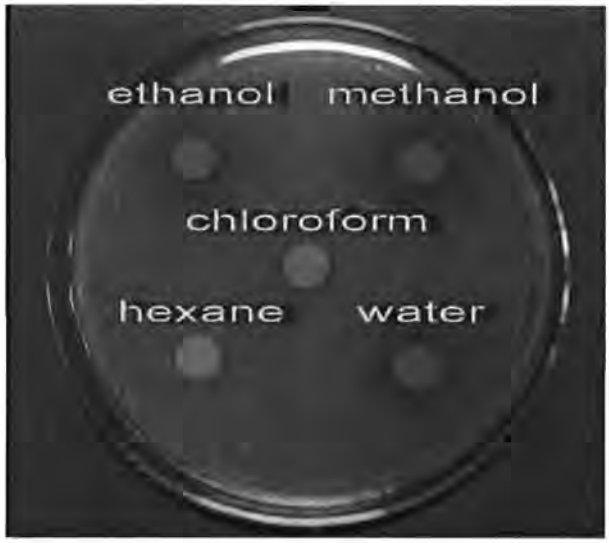


Figure 5 Antimicrobial activity of the extracts on *B. subtilis ATCC 6633*: Disc diffusion test for the effect of *Q. infectoria* against *B. subtilis ATCC 6633* grown on nutrient agar medium. 1. ethanol; 2. methanol; 3.chloroform; 4.hexane; 5.water.

The results from this study demonstrate that the gram-positive bacteria like *S. aureus ATCC 25923* and *B. subtilis ATCC 6633* are more susceptible to the extracts than gram-negative bacteria such as *E. coli ATCC 25922* were. The reason behind the more resistant behavior of such gram-negative bacteria could be due to the lipopolysaccharide (LPS) layer in their outer membrane which acts as an extra barrier against the entry of the bioactive molecules. In addition, there is also a report from Ikigai H et al. that the more resistant behavior of gram-negative bacteria is actually partly pertaining to the highly negative charges on the LPS layer even though its exact mechanism is not yet understood [16]. High amounts of tannin present in the galls of *Q. infectoria* may suggest that tannin is the active compound responsible for the antibacterial activity in this study [17,18]. When considering the nature of tannin, it is a phenolic compound which is soluble in water and alcohol so it is hydrophilic in nature which is in correspondence to what we have predicted earlier about the nature of the bioactive molecules in the *Q. infectoria* extracts [16, 18]. Hence, it may be concluded that the active compounds in the extracts are likely to be hydrophilic tannins and that gram-positive bacteria are more resistant to such compounds due to the LPS layer which forms an extra protective coating.

### *C. Minimum Inhibition concentration (MIC)*

Table 2 indicates the minimum concentrations of the methanol extracts required to completely inhibit the growth of the three bacterial pathogens. The relative growth of each microorganism after 24 h of incubation in the presence of different concentrations of *Q. infectoria* extracts was compared to the control. Methanol extract of *Q. infectoria* suppresses the growth of *E. coli ATCC 25922* at the MIC of 2.500 mg $ml^{-1}$while the growth of *S. aerus ATCC 25923* is affected at the MIC of 1.250 mg $ml^{-1}$. The most susceptible bacterial strain in the study is *B. subtilis ATCC 6633* which requires only the MIC of 0.625 mg $ml^{-1}$ to inhibit their growth.

TABLE 2. MICS OF DIFFERENT EXTRACTS OF Q. INFECTORIA GALLS

| Solvent extract | MICs of solvent extracts (mg/ml)[a] | | | | |
|---|---|---|---|---|---|
| | *E. coli* ATCC 25922 | *S. aerus ATCC 25923* | *B. subtilis ATCC 6633* | *Positive Control* | *Negative Control* |
| 5.0000 | - | - | - | + | - |
| 2.5000 | - | - | - | + | - |
| 1.2500 | + | - | - | + | - |
| 0.6250 | + | + | - | + | - |
| 0.3125 | + | + | + | + | - |
| 0.1563 | + | + | + | + | - |
| 0.0781 | + | + | + | + | - |
| 0.0391 | + | + | + | + | - |
| 0.0195 | + | + | + | + | - |
| 0.0098 | + | + | + | + | - |
| 0.0049 | + | + | + | + | - |

[a] - Absence of growth, Positive Control: Bacterial suspension and saline, + Presence of growth, Negative Control: Extracts and broth

Figure 6 Scanning electron microscope images of *E. coli ATCC 25922* with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically rod-shaped and the cell surfaces were relatively smooth (a). The cells treated with the extract at 5 mg $ml^{-1}$ (b).

*D. Scanning electron microscope observation at 24 h*

The effects of the methanol extracts of *Q. infectoria* galls on the surface morphology of gram-positive and gram-negative bacteria during its logarithmic phase of growth were shown in Fig. 6-8. The spectrum of antimicrobial activities on the surface morphology of individual cells and bacterial populations was visualized.

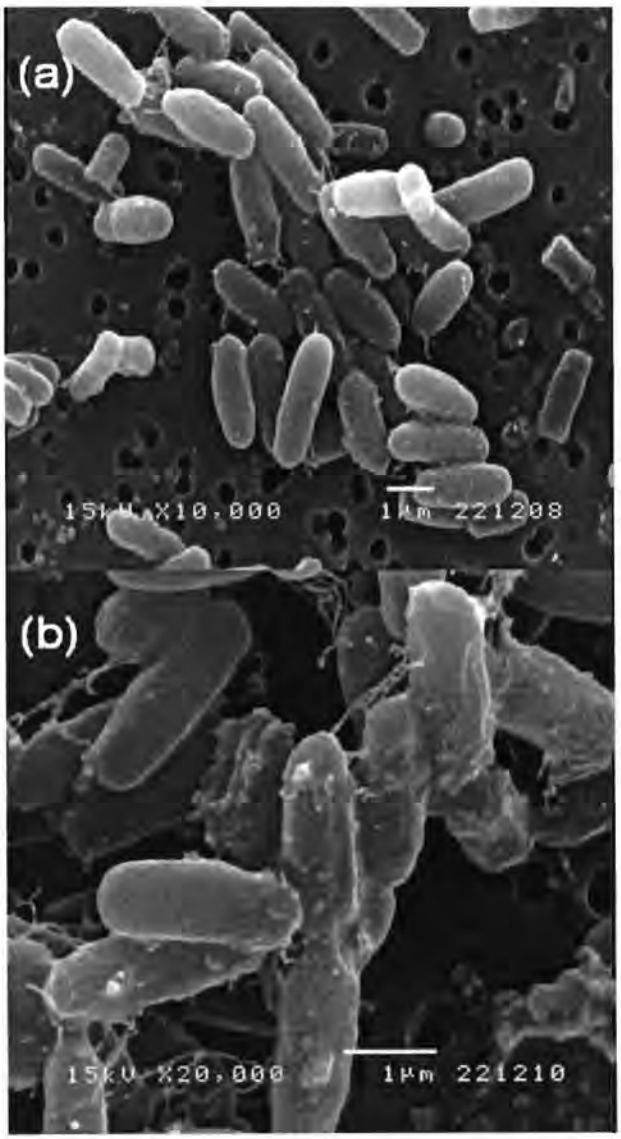


Figure 7 Scanning electron microscope images of *S. aureus ATCC 25923* with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically spherical in shape (a). The cells treated with the extract at 5 mg $ml^{-1}$ (b).

The effects of the methanol extract on the surface morphology of *E. coli ATCC 25922* were generally similar. Untreated organisms (Fig. 6a) appeared rod-shaped. Exposure to the extract resulted in only occasional morphologic defects characterized by tubular outpouchings from the cell wall (Fig. 6b).

The effects of the methanol extract on the surface morphology of *S. aureus ATCC 25923* during its logarithmic phase of growth were similar under the experimental conditions utilized. Untreated staphylococci (Fig. 7a) appeared to be smooth and spherical in grapelike clusters. Exposure to the extract resulted in the appearance of small bleb-like structures on the surface of some cells; irregular spherical structures lying free or appearing to extrude from cells were also observed (Fig. 7b).

The effects of the methanol extract on the surface morphology of *B. subtilis ATCC 6633* were also relatively similar. Untreated organism (Fig. 8a) appeared rod-shaped. Exposure of this organism to the extract resulted in morphological abnormality as seen in the formation of spherical globules and collapse in structures of the treated cells (Fig. 8b).

For all strains of bacteria tested, treatments with methanol extract of *Q. infectoria* clearly result in a sharp decline in bacterial population density observed under scanning electron micrographs. Morphology of the bacteria was also altered becoming more irregular and inhomogeneous in shape. Small degraded pieces of cellular debrises were also observed in all micrographs following the treatment with the *Q. infectoria* extract.

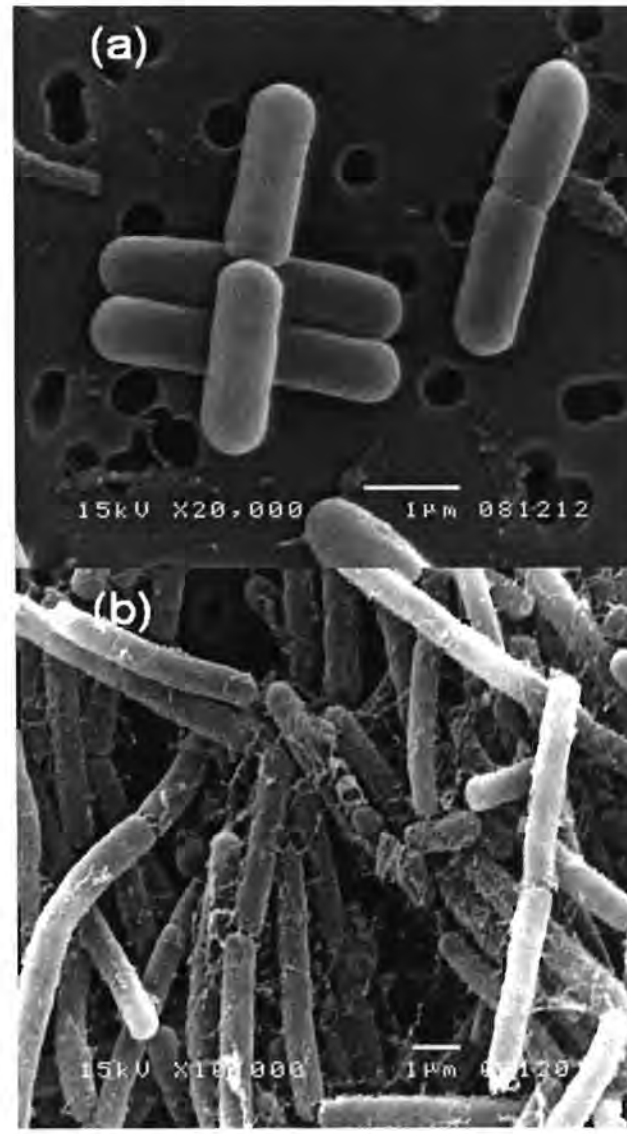


Figure 8 Scanning electron microscope images of *B. subtilis ATCC 6633* with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically spherical in shape (a). The cells treated with the extract at 5 mg $ml^{-1}$ (b).

## IV. CONCLUSION

The results obtained from this study reveal that *Quercus infectoria* galls have an antimicrobial activity against gram-positive and gram-negative bacteria. In this study, it was observed that the potency of this medicinal plant was enhanced by the type of solvents used with methanol being the most effective solvent. This study corresponds with the results of other researchers who have observed that the alcoholic extracts of *Q. infectoria* were found to be more active against all bacteria ever studied till present. Hence, the active materials, most likely to be tannins, in *Q. infectoria* were probably be better dissolved in alcohol and water rather than in hexane or chloroform.

In addition, it was also found that all extracts from the galls inhibited the growth of gram-positive bacteria more effectively than they did on the gram-negative bacteria since the gram-negative bacteria possess an extra protective layer of LPS on the cell membrane which is highly negatively charged.

This study also demonstrates visually the spectrum of the effects of the methanol extract on the surface morphology of specific bacteria making them more irregular and inhomogeneous in shape. The more thorough investigation under scanning electron microscope may help to correlate these various morphologic forms with biochemical alternations occurring within the cell wall.


## ACKNOWLEDGMENT

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## REFERENCES


[1] M.Digraki, M.H. Alma, A. Ilcim , and S. Sen, "Antibacterial and antifungal effects of various commercial plant extracts," *Pharm Biol.*, vol.37, 1993, pp. 216-20.

[2] M.Ikram, F. Nowshad, "Constituents of Quercus infectoria", *Planta Med.*, vol.31, 1977,pp. 286-7.

[3] E.C. Bate-Smith and T. Swain, "Flavonoid compounds, In comparative Biochemistry, H.S. Mason and M. A. Florkin eds, Academic Press, New York, 1962,pp. 755-809.

[4] S. Fatima, A.H.AFarooqi, R. Kumar,T.R.S. Kumar,S.PS. Khanuja, "Antibacterial activity possessed by medicinal plants used in tooth powders," *J. Med Aromatic Plant Sci., vol.22*,2001, pp.187-9.

[5] A. Redwane,H.B. Lazrek, S. Bouallam, M. Markouk, H. Amarouch,and M. Jana, "Larvicidal activity of extracts from Quercus lusitania var. infectoria galls (Oliv.)," *J. Ethnopharmacol*, vol. 79, pp. 261-3, 2002.

[6] J.K.Hwang,T.W. Kong, TW,N.I. Baek,and Y.R.Pyun, "α-Glycosidase Inhibitory Activity of hexagalloylglucose from the galls of Quercus infectoria," *Planta Med*, vol. 66,2000, pp. 73-4.

[7] M.R.Dar,S, Ikram, M, Fakouhi T, "Pharmacology of Quercus infectoria", *J. Pharm Sci*, vol.65, 1976, pp.1791-4.

[8] G. Hussein, H. Miyashiro, N. Nakamura, M.Hattori,N. Kakiuchi, and K. Shimotohno, "Inhibitory effects of Sudanese medicinal plant extracts on hepatitis C virus protease," *Phytoiher Res.*, vol.14, 2000,pp.510-6.

[9] G. Kaur, H. Hamid, A. Ali, MS Alam, and M. Athar, "Antiinflammatory evaluation of alcoholic extract of galls of Quercus infectoria," *J.Ethnopharmacol.* vol.90, 2004,pp 285-92.

[10] H.U Gai, E.M. Perchellet, X.M. Bottaro and JP Perchellet, "Antitumor promoting effects of gallotannins extracted from various sources in mouse skin in vivo", *Anticancer Res.*, vol.13,1993,pp.915-922.

[11] P. Pithayanukul, P. Ruenraroengsak, R. Bavovada, N. Pakkmanee, R. Suttisri, and S. Saen-oon, "Inhibition of Naja kaouthia venom activities by plant phenols" *J. Ethnopharmacol.*, vol.97,2005, pp. 527-533.

[12] H. Hamid, G. Kaur , S.T.Abdullah, M. Ali, M. Athar and M. S. Alam, Two new compounds from the galls of *Quercus infectoria* with nitric oxide and superoxide inhibiting ability", *Phar.Biol.vol.*43, 2005, pp.317–323.

[13] A.V. Krishnaraju, C.V. Rao,D. Sundararaju,and G.V. Subbaraju, "Antioxidant constituents from stem galls of *Quercusinfectoria* Olivier", *Asian J. Chem., vol.* 17, 2005,pp. 1833–1838.

[14] M. Vaara, "The outer membrane as the penetration barrier against mupirocin in gram-negative enteric bacteria," *J. Antimicrob. Chemother*, vol. 29, 1992,pp. 221–222.

[15] .W.C. Evans, Pharmacopoeial and related drugs of biological origin. In: Trease and Evan's Pharmacognosy. London: WB Saunders Co Ltd.; 1996

[16] H. Ikigai, T. Nakae, Y. Hara, and T. Shimamura, "Bactericidal catechins damage the lipid bilayer" Biochim. Biophys. Acta, vol. 1147,1993,pp. 132–136.

[17] C.K. Leach, "The phenolic contents of some British cynipid galls", *Cecidology*, vol. 1, 1986,pp.10-12.

[18] B.D. Fredalina, " The potential of aqueous and acetone extracts of gall of Quercus infectoria as antimicrobial agents," *India J. Pharmacol.*, vol. 37, 2005, pp. 26-39.

# CHARACTERISTICS OF BACTERIAL CELLULOSE FILM SYNTHESIZED BY ACETOBACTER XYLINUM CONTAINING EXTRACT FROM RIND OF GARCINIA MANGOSTANA

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## Abstract

A bacterial cellulose (BC) film was synthesized by *Acetobacter xylinum* in static cultivation. Afterwards, physical and biological properties of the BC films were modified by supplementation of crude extract from fruit rind of mangosteen (*Garcinia mangostana*), using extract concentrations of 2.5-10.0%wt for the BC film in the wet form (never dried film) immersion. From FT-IR analysis, the interaction between BC functional groups and mangosteen extract compounds, which absorbed into the BC film, was indicated. The tensile strength and Young's modulus of the BC film containing mangosteen extract were decreased by increasing extract concentration in the solution, whereas the elongation at break was increased. In addition, antimicrobial tests showed that the growths of *Escherichia coli* and *Staphylococcus aureus* were inhibited by the BC film containing extract from rind of *G. mangostana*.

**Keywords**: Bactorial cellulose film, Autobacter xylinum, Garcinia mangostana

## Introduction

Cellulose is a biopolymer consisting of β-(1,4) glucose in its structure. Cellulose from plants is usually found in a mixture with hemicellulose, lignin, pectin and biogenetic products. Cellulose can also be generated by some microbial cells such as Acetobacter, Agrobacteria, Rhizobia, and Sarcina strains. However, only Acetobacter species produce enough amount of cellulose for commercial interest. The major advantages of BC, which differ from planted cellulose, are its free of other bio-products, excellent mechanical strength, fine fiber network structure, large specific surface, high water absorption capacity, and high crystallinity. BC has been used for several applications such as food supplement, modified paper pulp, membranes, high performance speaker diaphragms, paint thickeners, biomedical applications (artificial skin, artificial bone, tissue engineering and others) [1-2].

Mangosteen (*Garcinia mangostana* Linn.) is a tropical fruit tree in plant family "Guttiferae". It is planted widely in area of Asia such as India, Srilanka, Myanmar, Malaysia, Indonesia, Philippines and especially Thailand. The fruit rind of *G. mangostana* has been used as traditional medicines of these countries, for treatments for abdominal pain, diarrhoea, dysentery, leucorrhoea, gonorrhoea, skin infection, wounds, etc. Besides, the extract from fruit rind of *G. mangostana* has been documented for inflammatory inhibition, antitumor ability, antioxidant activity, and antibacterial activity [3]. It was reported that the extract from rind of *Garcinia mangostana* has many complex phenolic compounds such as xanthones, flavonoids, tannins, and other bioactive substances [4].

Because of many advantages of BC on wound healing, for instance, wound exudates control, ability to retain moisture, biocompatibility, and so on, it is one of biomaterials which has been interested for using as a wound dressing. Previously, in order to generate antimicrobial ability of this biocompatible and nontoxic material, the addition of antimicrobial chemical agents into the films has been applied. However, many of these agents were often toxic to human body and its degradation in the environment was not easy [5-6].

In this research, with the aim to create antimicrobial ability of BC film, the modification of the film by supplementation of crude extract from fruit rind of *G. mangostana* was studied. Mangosteen extract was impregnated into the BC film by means of immersion of film samples in mangosteen extract solution.

## Materials and Methods

**Microbial strains:** The *A. xylinum* strain was isolated from nata de coco. The stock culture was kindly supplied by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand.

**Culture media and method:** The culture medium for the inoculums was coconut-water consisting of 5.0% sucrose, 0.5% ammonium sulfate and 1.0% of 30%v/v acetic acid. The medium was sterilized at 110°C for 5 min. Precultures was prepared by transferring 15 mL of the stock culture to 300 mL medium in a 500 mL Erlenmeyer flask and incubated statically at 30°C for 7 days. After that, the 5%v/v preculture broth was added to the main culture medium. The activated main medium 75 mL was inoculated in a in a 14.5cm diameter Preti-dish and kept at 30°C for 7-10 days. The bacterial cellulose pellicles were purified by washing with deionized (DI) water, treated with 1%w/v sodium hydroxide at room temperature for 24 hours to remove bacterial cells, neutralized by 1%v/v acetic acid for 1 hour and then rinsed with DI water until pH became neutral. Afterward, the films were immersed into 20%v/v ethanol for 10 minutes before were air dried at room temperature for 3-5 days and stored in plastic film.

**Impregnating BC films with mangosteen extract:** The purified BC films in wet state were

immersed in a solution of mangosteen extract (purchased from Thiptipa Co., Ltd., Pathumthani, Thailand) with concentrations of 2.5, 5.0 and 10.0%wt at room temperature for 2 days. Afterward, the films were air dried at room temperature for 3-5 days and stored in plastic film.

In this study, BCM and BC film refers to BC film with and without the mangosteen extract impregnation, respectively.

**Fourier Transform Infrared Spectroscopy analysis**: FTIR spectroscopy was used primarily to identify the chemical structure of the sample. FTIR spectra of the films were recorded with Perkin Elmer (Spectrum One, Massachusetts, USA) in the region of 4000–450 $cm^{-1}$.

**Mechanical property test**: All BC and BCM films in dry form were tested for tensile strength, Young's modulus and elongation at break. From the test conditions followed ASTM D 882, the film samples were cut into strip-shaped specimens 10 mm width and 10 cm length. All tensile elastic properties were the average values determined from at least five specimens and measured by Hounsfield Universal Testing Machine (H 10KM, Redhill, England).

**Antibacterial test**: The antibacterial test of BC and BCM films against *Escherichia coli* (*E. coli*) and Staphylococcus aureus (*S. aureus*) bacteria was determined. The film samples were cut into round-shaped sample of 3.8 cm diameter according to the method described by AATCC TM 39-1989 (Assessment on Textile Materials: Mildew and Rot Resistance of Textile). Before the antibacterial assay, all BC and BCM films were sterilized by UV irradiation for 20 min in each side. The incubation was 24 hours at 37°C.

## Results

BC was synthesized by *Acetobacter xylinum* in form of a pellicle which floated on culture medium in static condition. To create antimicrobial activity of the BC film, the crude extract from fruit rind of *G. mangostana* was added into the BC film. All films were dried by air drying process and kept in dry place at room temperature. The physical and biological properties such as mechanical properties and antimicrobial ability of the BCM films were then investigated.

**FTIR analysis**: Fourier transform infrared (FTIR) spectroscopy of BC and BCM films was examined for the detection of functional groups or chemical bonds that exist in BC and mangosteen extract compounds. The FTIR spectra of all samples were detected at wavenumbers ranging from 4000 to 450 $cm^{-1}$ as demonstrated in Figure 1.

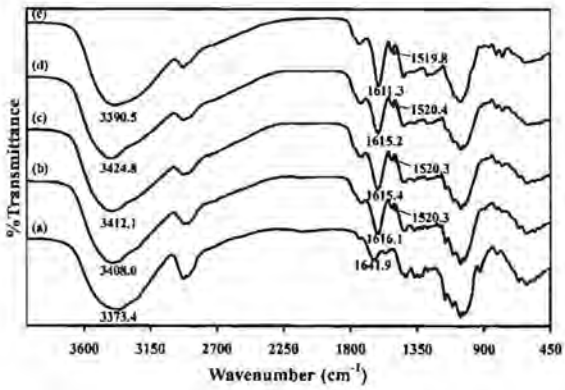


**Figure 1** The FTIR spectra in wave number ranging from 4000 to 450 $cm^{-1}$ of (a) BC, (b–d) BCM films and (e) mangosteen extract powder. The supplementation of mangosteen extract (%wt) in BCM were: (b) 2.5%, (c) 5.0% and (d) 10.0%.

The BC film showed a band at 3373.4 $cm^{-1}$ and 1641.9 $cm^{-1}$ which were attributed to hydroxyl group (-OH) and glucose carbonyl group (C=O) of cellulose. Meanwhile, the characteristic absorptions of mangosteen extract compounds were the bands at 1611.3 and 1519.8 $cm^{-1}$, which were represented to double bond (C=C) in aromatic ring and O-C-O of ether. The peaks attributed to O-H and C=O stretching vibrations of BC film were apparently shifted and there were some new peaks on the BCM films around 1520.3-1520.4 $cm^{-1}$ and 1615.2-1616.1 $cm^{-1}$. These changes indicated interactions between the functional groups of BC film and mangosteen extract compounds.

**Mechanical property:** Figure 2 shows the effects of mangosteen extract addition on tensile strength of the films. The tensile strength of the BC film was 32.04 MPa. After the films were immersed into mangosteen extract solution at concentration of 2.5%wt, the tensile strength of the BCM-2.5%wt was increased to 52.69 MPa.

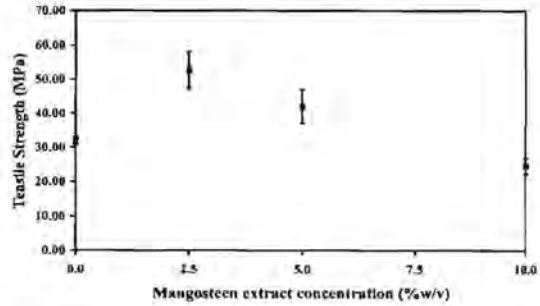


**Figure 2** Tensile strength of BC and BCM films as a function of mangosteen extractsupplementation (%wt).

Therefore, the binding between the extract compounds and the film, to some extent, could enhance the tensile strength of the films. However, with the increase of the extract concentration to 5.0 and 10.0%wt, the tensile strengths of the BCM films were decreased to 41.99 and 24.52 MPa, respectively. Due to the presence of high content of mangosteen extract compounds inserting BC fibrils, the films became more amorphous. Amorphous films are usually less rigid, weaker and more easily deformed. Therefore, the BCM films at high content of the extract compounds became less stable and less durable than the unmodified one.

Figure 3 demonstrates the effects of the extract compounds on percentage of elongation at break of the films.

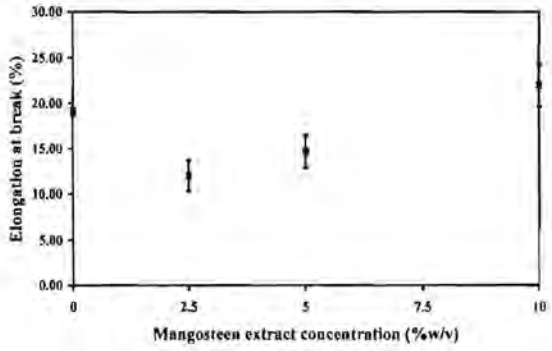


**Figure 3** Elongation at break of BC and BCM films as a function of mangosteen extract supplementation (%wt).

The elongation at break of the films of BC and BCM-2.5, 5.0 and 10.0%wt film were 19.06, 12.00, 14.67, and 21.93%, respectively. The tendency of the percentage of elongation at break of the BCM films was opposite to its tensile strength. The BCM films of higher content of the extract compounds have higher elongation at break than lower ones. When the content of extract compounds increased, the amorphous fraction of the films increased allowing the increase of percentage of elongation.

Young's modulus of BCM films as a function of mangosteen extract content is presented in Figure 4.

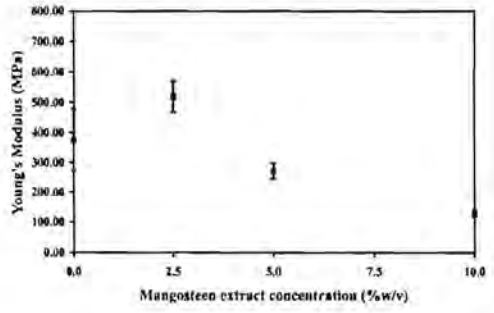


**Figure 4** Young's modulus of BC and BCM films as a function of mangosteen extract supplementation (%wt).

By addition of mangosteen extract with concentrations of 2.5, 5.0, and 10.0%wt, the modulus of elasticity of the BCM films were 519.88, 270.57 and 129.64 MPa, respectively, whereas that of the BC film was 375.82 MPa. The reduction of elastic modulus should be arised from the increase of amorphous structure in the BCM films. The flexibility and endurance of the BCM films were less than the BC film, since the unmodified BC film has more organized fiber-network structure.

**Antibacterial ability**: *E. coli* and *S. aureus*, representative gram negative and gram positive bacteria, were used for bacteria testing to examine the antibacterial properties of the BC and BCM films. The results of antibacterial testing of the films are shown in table 1 and figure 5.

**Table 1** The antibacterial activity of BC and BCM films against *E. coli* and *S. aureus* at the end of the incubation for 24 hours.

| Microbial strains | Sample | Result |
|---|---|---|
| *E. coli* | BC | Inhibition of growth under the sample.No clear zone. |
| | BCM 5.0 %wt | Inhibition of growth 6.0mm in width of clear zone. |
| | BCM 10.0 %wt | Inhibition of growth 6.67±0.06mm in width of clear zone. |
| *S. aureus* | BC | Inhibition of growth under the sample.No clear zone. |
| | BCM 5.0 %wt | Inhibition of growth 4.0±0.05mm in width of clear zone. |
| | BCM 10.0 %wt | Inhibition of growth 5.17±0.08mm in width of clear zone. |

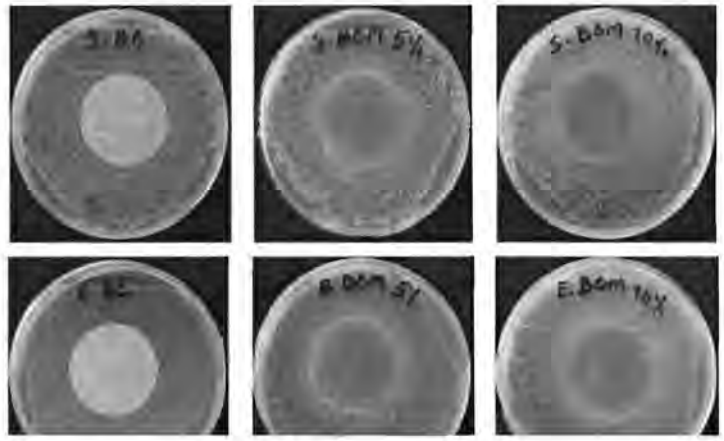

**Figure 5** The results from antibacterial testing of BC, BCM-5.0%wt and BCM-10.0%wt (from left to right) on *S. aureus* (upper row) and *E. coli* (lower row) for 24 hours, incubated at 37°C.

The result indicated that antibacterial ability of the BCM films were better than that of the BC film. The BCM films showed the inhibition of growths of *E. coli* and *S. aureus* under and around the film samples, which could be observed by clear zones. This showed potent inhibitory activity of mangosteen extract against the gram negative and gram positive bacteria. However, the degree of antibacterial activities does not depend linearly on the mangosteen extract concentration in the BCM films. Therefore, the release rate of the active components should be an important factor for the antimicrobial control.

## Conclusions and Discussion

The modification of BC films by mangosteen extract supplementation was used to improve antibacterial ability of the films. The FT-IR results indicated interactions between the functional groups of BC film and mangosteen extract compounds. The tensile strength and Young's modulus of the BCM films were

decreased with the increase of concentration of the extract compounds, whereas the percentages of elongation at break were increased. The changes of mechanical properties were owing to the interactions between mangosteen extract compounds and the functional groups of BC, as well as the increase of amorphous fraction and toughness of the BCM films at high content of the extract compounds. The BCM films showed antibacterial activity towards *E. coli* and *S. aureus* under and around the film samples. The antibacterial ability was controlled by the release of the active components from mangosteen extract.

## Acknowledgments

The authors would like to acknowledge the financial support from Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

## References

[1] S. Keshk; & K. Sameshima. (2006). *Enzyme. Microb. Tech.* 40: 4-8.

[2] J. Y. Jung; J. K. Park; & H. N. Chang. (2005). *Enzyme. Microb. Tech.* 37: 347-354.

[3] L. Yu; et al. (2007). *Food. Chem.* 104: 176-181.

[4] W. Phothitirat; et al. (2009). *Fitoterapia.* 80: 442-447.

[5] T. Maneerung; S. Tokura; & R. Rujiravanit. (2008). *Carbohyd. Polym.* 72: 43-51.

[6] A. Hou; M. Zhou; & X. Wang. (2009). *Carbohyd. Polym.* 75: 328-332.

# Characteristics of bacterial cellulose film synthesized by Acetobacter xylinum containing extract from rind of Garcinia mangostana

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**Abstract: A bacterial cellulose (BC) film was synthesized by *Acetobacter xylinum* in static cultivation. Afterwards, physical and biological properties of the BC films were modified by supplementation of crude extract from fruit rind of mangosteen (*Garcinia mangostana*), using extract concentrations of 2.5-10.0%wt for the BC film in the wet form (never dried film) immersion. From FT-IR analysis, the interaction between BC functional groups and mangosteen extract compounds, which absorbed into the BC film, was indicated. The tensile strength and Young's modulus of the BC film containing mangosteen extract were decreased by increasing extract concentration in the solution, whereas the elongation at break was increased. In addition, antimicrobial tests showed that the growths of *Escherichia coli* and *Staphylococcus aureus* were inhibited by the BC film containing extract from rind of *G. mangostana*.**

## Introduction

Cellulose is a biopolymer consisting of β-(1,4) glucose in its structure. Cellulose from plants is usually found in a mixture with hemicellulose, lignin, pectin and biogenetic products. Cellulose can also be generated by some microbial cells such as Acetobacter, Agrobacteria, Rhizobia, and Sarcina strains. However, only Acetobacter species produce enough amount of cellulose for commercial interest. The major advantages of BC, which differ from planted cellulose, are its free of other bio-products, excellent mechanical strength, fine fiber network structure, large specific surface, high water absorption capacity, and high crystallinity. BC has been used for several applications such as food supplement, modified paper pulp, membranes, high performance speaker diaphragms, paint thickeners, biomedical applications (artificial skin, artificial bone, tissue engineering and others) [1,2].

Mangosteen (*Garcinia mangostana* Linn.) is a tropical fruit tree in plant family "Guttiferae". It is planted widely in area of Asia such as India, Srilanka, Myanmar, Malaysia, Indonesia, Philippines and especially Thailand. The fruit rind of *G. mangostana* has been used as traditional medicines of these countries, for treatments for abdominal pain, diarrhoea, dysentery, leucorrhoea, gonorrhoea, skin infection, wounds, etc. Besides, the extract from fruit rind of *G. mangostana* has been documented for inflammatory inhibition, antitumor ability, antioxidant activity, and antibacterial activity [3]. It was reported that the extract from rind of *Garcinia mangostana* has many complex phenolic compounds such as xanthones, flavonoids, tannins, and other bioactive substances [4].

Because of many advantages of BC on wound healing, for instance, wound exudates control, ability to retain moisture, biocompatibility, and so on, it is one of biomaterials which has been interested for using as a wound dressing. Previously, in order to generate antimicrobial ability of this biocompatible and nontoxic material, the addition of antimicrobial chemical agents into the films has been applied. However, many of these agents were often toxic to human body and its degradation in the environment was not easy [5,6].

In this research, with the aim to create antimicrobial ability of BC film, the modification of the film by supplementation of crude extract from fruit rind of *G. mangostana* was studied. Mangosteen extract was impregnated into the BC film by means of immersion of film samples in mangosteen extract solution.

## Materials and Methods

*Microbial strains:* The *A. xylinum* strain was isolated from nata de coco. The stock culture was kindly supplied by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand.

*Culture media and method:* The culture medium for the inoculums was coconut-water consisting of 5.0% sucrose, 0.5% ammonium sulfate and 1.0% of 30%v/v acetic acid. The medium was sterilized at 110 °C for 5 min. Precultures was prepared by transferring 15 mL of the stock culture to 300 mL medium in a 500 mL Erlenmeyer flask and incubated statically at 30 °C for 7 days. After that, the 5%v/v preculture broth was added to the main culture medium. The activated main medium 75 mL was inoculated in a in a 14.5 cm diameter Preti-dish and kept at 30 °C for 7–10 days. The bacterial cellulose pellicles were purified by washing with deionized (DI) water, treated with 1%w/v sodium hydroxide at room temperature for 24 hours to remove bacterial cells, neutralized by 1%v/v acetic acid for 1 hour and then rinsed with DI water until pH became neutral. Afterward, the films were

immersed into 20%v/v ethanol for 10 minutes before were air dried at room temperature for 3-5 days and stored in plastic film.

*Impregnating BC films with mangosteen extract:* The purified BC films in wet state were immersed in a solution of mangosteen extract (purchased from Thiptipa Co., Ltd., Pathumthani, Thailand) with concentrations of 2.5, 5.0 and 10.0%wt at room temperature for 2 days. Afterward, the films were air dried at room temperature for 3-5 days and stored in plastic film.

In this study, BCM and BC film refers to BC film with and without the mangosteen extract impregnation, respectively.

*Fourier Transform Infrared Spectroscopy analysis:* FTIR spectroscopy was used primarily to identify the chemical structure of the sample. FTIR spectra of the films were recorded with Perkin Elmer (Spectrum One, Massachusetts, USA) in the region of 4000–450 $cm^{-1}$.

*Mechanical property test:* All BC and BCM films in dry form were tested for tensile strength, Young's modulus and elongation at break. From the test conditions followed ASTM D 882, the film samples were cut into strip-shaped specimens 10 mm width and 10 cm length. All tensile elastic properties were the average values determined from at least five specimens and measured by Hounsfield Universal Testing Machine (H 10 KM, Redhill, England).

*Antibacterial test:* The antibacterial test of BC and BCM films against *Escherichia coli* (*E. coli*) and Staphylococcus aureus (*S. aureus*) bacteria was determined. The film samples were cut into round-shaped sample of 3.8 cm diameter according to the method described by AATCC TM 39-1989 (Assessment on Textile Materials: Mildew and Rot Resistance of Textile). Before the antibacterial assay, all BC and BCM films were sterilized by UV irradiation for 20 min in each side. The incubation was 24 hours at 37 °C.

**Results and Discussion**

BC was synthesized by *Acetobacter xylinum* in form of a pellicle which floated on culture medium in static condition. To create antimicrobial activity of the BC film, the crude extract from fruit rind of *G. mangostana* was added into the BC film. All films were dried by air drying process and kept in dry place at room temperature. The physical and biological properties such as mechanical properties and antimicrobial ability of the BCM films were then investigated.

*FTIR analysis:* Fourier transform infrared (FTIR) spectroscopy of BC and BCM films was examined for the detection of functional groups or chemical bonds

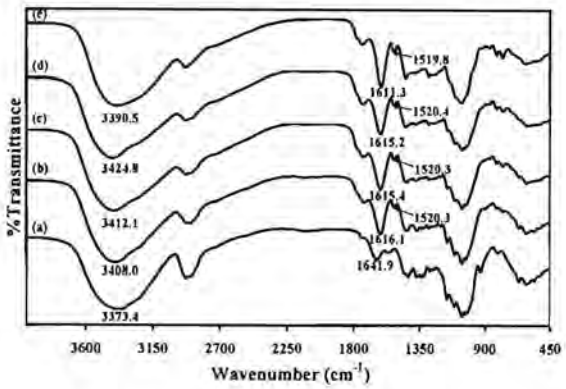


Figure 1. The FTIR spectra in wave number ranging from 4000 to 450 $cm^{-1}$ of (a) BC, (b–d) BCM films and (e) mangosteen extract powder. The supplementation of mangosteen extract (%wt) in BCM were: (b) 2.5%, (c) 5.0% and (d) 10.0%

that exist in BC and mangosteen extract compounds. The FTIR spectra of all samples were detected at wavenumbers ranging from 4000 to 450 $cm^{-1}$ as demonstrated in Figure 1. The BC film showed a band at 3373.4 $cm^{-1}$ and 1641.9 $cm^{-1}$ which were attributed to hydroxyl group (-OH) and glucose carbonyl group (C=O) of cellulose. Meanwhile, the characteristic absorptions of mangosteen extract compounds were the bands at 1611.3 and 1519.8 $cm^{-1}$, which were represented to double bond (C=C) in aromatic ring and O-C-O of ether. The peaks attributed to O-H and C=O stretching vibrations of BC film were apparently shifted and there were some new peaks on the BCM films around 1520.3-1520.4 $cm^{-1}$ and 1615.2-1616.1 $cm^{-1}$. These changes indicated interactions between the functional groups of BC film and mangosteen extract compounds.

*Mechanical property:* Figure 2. shows the effects of mangosteen extract addition on tensile strength of the films. The tensile strength of the BC film was 32.04 MPa. After the films were immersed into mangosteen extract solution at concentration of 2.5%wt, the tensile strength of the BCM-2.5%wt was increased to 52.69 MPa. Therefore, the binding between the extract compounds and the film, to some extent, could enhance the tensile strength of the films. However, with the increase of the extract concentration to 5.0 and 10.0%wt, the tensile strengths of the BCM films were decreased to 41.99 and 24.52 MPa, respectively. Due to the presence of high content of mangosteen extract compounds inserting BC fibrils, the films became more amorphous. Amorphous films are usually less rigid, weaker and more easily deformed. Therefore, the BCM films at high content of the extract compounds became less stable and less durable than the unmodified one.

Figure 3. demonstrates the effects of the extract compounds on percentage of elongation at break of the films. The elongation at break of the films of BC and BCM-2.5, 5.0 and 10.0%wt film were 19.06, 12.00,

14.67, and 21.93%, respectively. The tendency of the percentage of elongation at break of the BCM films was opposite to its tensile strength. The BCM films of higher content of the extract compounds have higher elongation at break than lower ones. When the content of extract compounds increased, the amorphous fraction of the films increased allowing the increase of percentage of elongation.

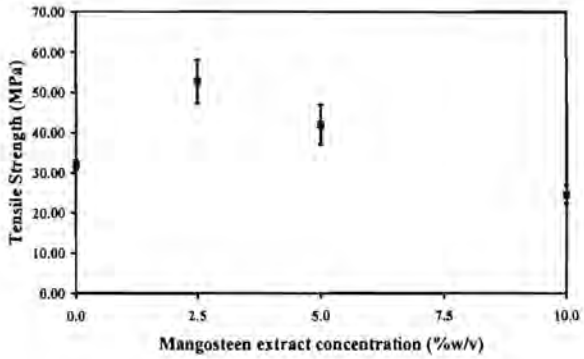


Figure 2. Tensile strength of BC and BCM films as a function of mangosteen extract supplementation (%wt)

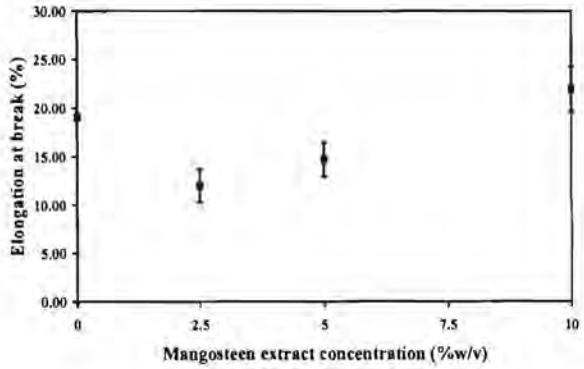


Figure 3. Elongation at break of BC and BCM films as a function of mangosteen extract supplementation (%wt)

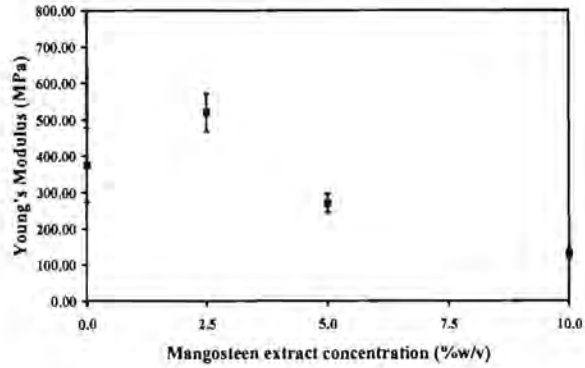


Figure 4. Young's modulus of BC and BCM films as a function of mangosteen extract supplementation (%wt)

Young's modulus of BCM films as a function of mangosteen extract content is presented in Figure 4. By addition of mangosteen extract with concentrations of 2.5, 5.0, and 10.0%wt, the modulus of elasticity of the BCM films were 519.88, 270.57 and 129.64 MPa, respectively, whereas that of the BC film was 375.82 MPa. The reduction of elastic modulus should be arised from the increase of amorphous structure in the BCM films. The flexibility and endurance of the BCM films were less than the BC film, since the unmodified BC film has more organized fiber-network structure.

*Antibacterial ability*: *E. coli* and *S. aureus*, representative gram negative and gram positive bacteria, were used for bacteria testing to examine the antibacterial properties of the BC and BCM films. The results of antibacterial testing of the films are shown in table 1. and figure 5.

The result indicated that antibacterial ability of the BCM films were better than that of the BC film. The BCM films showed the inhibition of growths of *E. coli* and *S. aureus* under and around the film samples, which could be observed by clear zones. This showed potent inhibitory activity of mangosteen extract against the gram negative and gram positive bacteria. However, the degree of antibacterial activities does not depend linearly on the mangosteen extract concentration in the BCM films. Therefore, the release rate of the active components should be an important factor for the antimicrobial control.

Table 1: The antibacterial activity of BC and BCM films against *E. coli* and *S. aureus* at the end of the incubation for 24 hours

| Microbial strains | Sample | Result |
|---|---|---|
| *E. coli* | BC | Inhibition of growth under the sample. No clear zone. |
| | BCM 5.0 %wt | Inhibition of growth 6.0 mm in width of clear zone. |
| | BCM 10.0 %wt | Inhibition of growth 6.67±0.06 mm in width of clear zone. |
| *S. aureus* | BC | Inhibition of growth under the sample. No clear zone. |
| | BCM 5.0 %wt | Inhibition of growth 4.0±0.05 mm in width of clear zone. |
| | BCM 10.0 %wt | Inhibition of growth 5.17±0.08 mm in width of clear zone. |

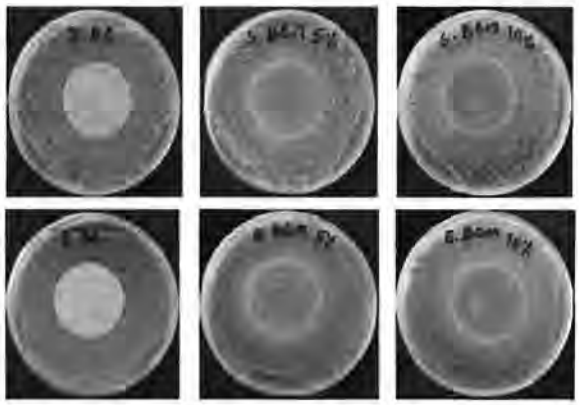

Figure 5. The results from antibacterial testing of BC, BCM-5.0%wt and BCM-10.0%wt (from left to right) on *S. aureus* (upper row) and *E. coli* (lower row) for 24 hours, incubated at 37 °C

## Conclusions

The modification of BC films by mangosteen extract supplementation was used to improve antibacterial ability of the films. The FT-IR results indicated interactions between the functional groups of BC film and mangosteen extract compounds. The tensile strength and Young's modulus of the BCM films were decreased with the increase of concentration of the extract compounds, whereas the percentages of elongation at break were increased. The changes of mechanical properties were owing to the interactions between mangosteen extract compounds and the functional groups of BC, as well as the increase of amorphous fraction and toughness of the BCM films at high content of the extract compounds. The BCM films showed antibacterial activity towards *E. coli* and *S. aureus* under and around the film samples. The antibacterial ability was controlled by the release of the active components from mangosteen extract.

## Acknowledgments

The authors would like to acknowledge the financial support from Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University.

## References

[1] S. Keshk and K. Sameshima, *Enzyme. Microb. Tech.* **40** (2006), pp. 4-8.
[2] J. Y. Jung, J. K. Park and H. N. Chang, *Enzyme. Microb. Tech.* **37** (2005), pp. 347-354.
[3] L. Yu, M. Zhao, B. Yang, Q. Zhao and Y. Jiang, *Food. Chem.* **104** (2007), pp. 176-181.
[4] W. Phothitirat, M. T.Chomnawang , R. Supabphol and W. Gritsanapan, *Fitoterapia.* **80** (2009), pp. 442-447.
[5] T. Maneerung, S. Tokura and R. Rujiravanit, *Carbohyd. Polym.* **72** (2008), pp. 43-51.
[6] A. Hou, M. Zhou and X. Wang, *Carbohyd. Polym.* **75** (2009), pp. 328-332.
[7] M. Phisalaphong and N. Jatupaiboon, *Carbohyd. Polym.* **74** (2008), pp. 482-488.
[8] O. Saibuatong and M. Phisalaphong, *Carbohyd. Polym.* **79** (2010), pp. 455-460.

# Antimicrobial Activities of Bacterial Cellulose Modified by Supplementation of Water and Ethanolic Extracts of Fruit Rind of Garcinia mangostana

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**Abstract** Modified bacterial cellulose (BC) films with water and ethanolic *Garcinia mangostana* extracts supplementation were studied to compare the inhibitory effects against 6 microbial strains: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Aspergillus niger* (*A. niger*), *Staphylococcus epidermidis* (*S. epidermidis*), *Propionibacterium acnes* (*P. acnes*), and *Candida albicans* (*C. albicans*). After immersion the BC films into distinct concentrations (2.5-10%w/v for the water extract and 0.25-1.00%v/v for the ethanolic extract), physical and biological properties of the BC films containing *G. mangostana* extract were investigated. From the analysis, the water absorption capacity (WAC) of films were higher with the addition of water *Garcinia mangostana* extracts. The antimicrobial tests showed that the growths of *S. aureus, S. epidermidis* and *P. acnes* were inhibited by the supplementation of water and ethanolic *G. mangostana* extracts except the growth of *E. coli* which was inhibited only by the supplementation of the water extract compounds. However, no inhibitions were observed in the cultures of *A. niger* and *C. albicans*.

**Keywords:** bacterial cellulose, film, *Garcinia mangostana*, mangosteen extract, antimicrobial ability

## Introduction

Bacterial cellulose (BC) which synthesized by some microbial strains (i.e. Acetobacter, Agrobacteria, Rhizobia, and Sarcina) has the same chemical composition as plant cellulose composing of β-(1,4) glucose in its structure, but free from hemicellulose, lignin, and pectin. Moreover, BC microfibrils have unique properties which distinct from plant cellulose such as high tensile strength, high water holding capacity, moldability, high crystallinity, large specific surface, and fine fiber network structure. Owing to its excellent mechanical properties, BC has been used for various applications especially biomedical applications (i.e. artificial skin, artificial bone, tissue engineering, and others) (1). However, BC has no antimicrobial activity in itself. Previously, there have been many attempts to generate antimicrobial activity of this biocompatible and nontoxic material by the supplementation of antimicrobial chemical agents, but these agents is not easy to decompose in the environment and have toxicity to human (2).

Mangosteen or *Garcinia mangostana* Linn. is an indigenous plant widely spread in South and Southeast Asian countries, for instance, India, Srilanka, Myanmar, Malaysia, Indonesia, Philippines and Thailand. It has been known as folk medicines in treatments of abdominal pain, diarrhea, dysentery, infected wounds, suppuration, chronic ulcer, leucorrhoea and gonorrhoea. Recent studies reported that various phytochemical substances exist in extract from the rind of mangosteen such as phenolic compounds particularly xanthones, anthocyanins, tannins, triterpenes, polysaccharides, vitamins B1, B2, C, and other bioactive substances (3). Furthermore, *G. mangostana* extract has been found that it has an anti-inflammatory inhibition, antitumor ability, antioxidant activity and antibacterial acivity (4).

In this present work, the BC films were modified by supplementation of extracts of fruit rind of *G. mangostana* to enhance antimicrobial ability. Immersion of the BC film into mangosteen extract solution was

applied for the impregnation at various concentrations. Antimicrobial activities of the modified BC films by supplementation of the water extract (BCWM) and the modified BC films by supplementation of the ethanolic extract (BCEM) were examined to investigate the inhibitory effects of the impregnation of *G. mangostana* extracts.

## Materials & Methods

### *Microbial strains*

The stock culture of *Acetobater xylinum* AGR 60 was kindly supported by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand. This strain was isolated from nata de coco and kept at 4 °C before further use.

### *Culture media and method*

The culture medium for the inoculums was coconut-water consisting of 5.0% sucrose, 0.5% ammonium sulfate and 1.0% of 30%v/v acetic acid. The medium was sterilized at 110 °C for 5 min. Precultures was prepared by transferring 15 mL of the stock culture to 300 mL medium in a 500 mL Erlenmeyer flask and incubated statically at 30 °C for 7 days. After that, the 5%v/v preculture broth was added to the main culture medium. The activated main medium 75 mL was inoculated in a 14.5 cm diameter Preti-dish and kept at 30 °C for 7–10 days. The bacterial cellulose pellicles were purified by washing with deionized (DI) water, treated with 1%w/v sodium hydroxide at room temperature for 24 hours to remove bacterial cells, neutralized by 1%v/v acetic acid for 1 hour and then rinsed with DI water until pH became neutral. Afterward, the films were air dried at room temperature for 3-5 days and stored in plastic film.

### *BC films impregnation with water and ethanolic mangosteen extracts*

The purified BC films in wet state were immersed in a solution of water mangosteen extract (purchased from Thiptipa Co., Ltd., Pathumthani, Thailand) with concentrations of 2.5, 5.0, and 10.0%w/v or in an ethanolic extract solution of mangosteen (kindly supported by Thai-China Flavours and Fragrances Industry Co., Ltd., Phra Nakhon Si Ayutthaya, Thailand) with solution concentrations of 0.25, 0.50, and 1.00%v/v at room temperature for 2 days. The excess content of the extract compounds on BC film surface was removed by rinsing with 20%v/v ethanol solution and then the films were air dried at room temperature for 3-5 days and stored in plastic film.

### *Water Absorption Capacity (WAC)*

Water absorption capacity was determined by immersing the dried BC, BCWM, and BCEM films in distilled water at room temperature until equilibration. The films were then removed from the water and excess water at the surface of the film was blotted out with Kim wipes paper. The weights of the swollen films were measured and the procedure was repeated until there was no further weight change. The water content was calculated using the following formula:

$$WAC(\%) = \frac{W_h - W_d}{W_d} x100$$

Where $W_h$ and $W_d$ denoted the weight of hydrate and dry membrane, respectively.

### *Antimicrobial test*

The antimicrobial properties of BC, BCWM, and BCEM films were examined against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Aspergillus niger* (*A. niger*), *Staphylococcus epidermidis* (*S. epidermidis*), *Propionibacterium acnes* (*P. acnes*), and *Candida albicans* (*C. albicans*). The film samples were cut into round-shaped sample of 38 mm diameter according to the method described by AATCC TM 39-1989. The incubation was 24 hours at 37 °C under aerobic conditions for *E. coli*, *S. aureus*, *S. epidermidis*, *and C. albicans* while the inoculated plates of *P. acnes* was incubated anaerobically at 37 °C for 72 hours and the test of *A. niger* was performed in the AGAR plate for a week of inoculation at 30 °C. Before the antimicrobial assay, all BC, BCWM, and BCEM films were sterilized by UV irradiation for 20 min in each side.

## Results & Discussions

### *WAC analysis*

Wound exudates control and ability to retain moisture are essential properties for wound healing process. Therefore, the WAC analysis of BC, BCWM, and BCEM films was examined by immersing the dried films in distilled water at room temperature until equilibration. Figure 1 showed that the WAC of the BC film was 328.77%. After the BC films were immersed into water extract solution at concentrations varied from 2.5, 5.0 and 10.0%w/v, the WACs of BCWM films were increased related to the amount of the supplementation to 577.09, 651.14 and 756.88%, respectively, owing to the more hydrophilic property of the BCWM films.

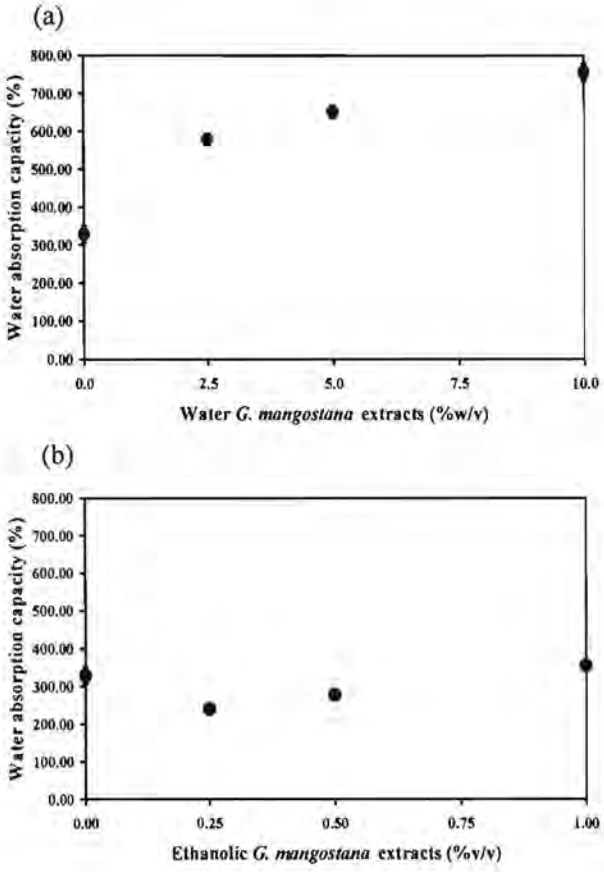


**Figure 1** The water absorption capacity of a) BCWM and b) BCEM films as a function of water and ethanolic extract concentration

On the other hand, the WACs of the BCEM films were slightly lower than that of the BC film. The WACs of BCEM films with the supplementation of 0.25, 0.50, and 1.00%v/v of ethanolic *G. mangostana* extracts were 239.50, 277.14, and 354.87%, respectively. Because of the hydrophobic property of ethanolic *G. mangostana* extract compounds, compared to the BC film, the WACs of the BCEM films tended to decrease. However, the impregnation of the films with the extract compounds at high content could have constructive interfere in chemical bonds of the films and might result in a looser film structure. This could be a reason for relatively higher WAC of the BCEM film with the supplementation of 1.00% ethanolic *G. mangostana* extract.

### *Antimicrobial ability*

On the examination of antimicrobial ability of the modified BC films, *E. coli* and *S. aureus* were used as the representative gram negative and gram positive bacteria, respectively. *S. epidermidis*, *P. acnes*, *C. albicans* and *A. niger* illustrated the pathogenic bacteria and fungus. From tables 1 and 2, the results indicated that the antimicrobial activities of the BCWM and BCEM films were better than those of the BC film. The BCWM films showed the inhibition of growths of *E. coli*, *S. aureus*, *S. epidermidis* and *P. acnes*, meanwhile the BCEM films expressed the inhibitory effects against *S. aureus*, *S. epidermidis* and *P. acnes*. However, both of BCWM and BCEM films did not show inhibition effect on the growth of *C. albicans* and *A. niger*. The differences of bactericidal inhibition against gram negative (*E. coli*) and gram positive (*S. aureus*) could be explained by their compositions and the arrangement of cell membrane. Peptidoglycan, an outer membrane of gram positive bacteria, is not a barrier to solutes. Conversely, the gram negative cell membrane does not permit lipophilic solutes into cytoplasm because of an outer phospholipidic layer. However, there are porins on the layer of the gram negative cell membrane causing the permeability of hydrophilic solutes (5). Therefore, the BCWM films showed the inhibitory effect on *E. coli*, but the BCEM films did not.

Mostly, the antimicrobial activities were increased with the concentration of the *G. mangostana* extracts but not as a linear relationship. However, for the test of antimicrobial activity of BCWM against *P. acnes*, the clear zone in a dose of 5 and

10%w/v is roughly the same size. The concentration of released extract compounds and the diffusion length should be the most important factors affecting on the inhibition of microbial growths (observed by clear zone).

Therefore, not only the initial concentration of impregnated *G. mangostana* extracts but also the rate of bioactive compounds release should be considered for the antimicrobial control of the modified BC films.

**Conclusions**

The WAC of BC film could be improved by the addition of water extract of *G. mangostana*. The antimicrobial activity of the BC film could be improved by the supplementation of water and ethanolic extracts of fruit rind of *G. mangostana*. The BCWM showed antimicrobial activities against *E. coli*, *S. aureus*, *S. epidermidis* and *P. acnes* and the BCEM expressed antimicrobial activities against *S. aureus*, *S. epidermidis* and *P. acnes*.

**Table 1** Antimicrobial activities of BC, BCWM, and BCEM films against *E. coli*, *S. aureus*, *A. niger*, *S. epidermidis*, *P. acnes*, and *C. albicans*

| Microorganisms | Sample | Clear zone (mm) | Microorganisms | Sample | Clear zone (mm) |
|---|---|---|---|---|---|
| *E. coli* | BC | 0 | *P. acnes* | BC | 0 |
| | BCWM-2.5%w/v | 7.50±1.32 | | BCWM-2.5%w/v | 5.50±0.87 |
| | BCWM-5.0%w/v | 8.33±0.29 | | BCWM-5.0%w/v | 6.00±0.00 |
| | BCWM-10.0%w/v | 9.33±1.44 | | BCWM-10.0%w/v | 6.00±0.00 |
| | BCEM-0.25%v/v | 0 | | BCEM-0.25%v/v | 4.83±1.89 |
| | BCEM-0.50%v/v | 0 | | BCEM-0.50%v/v | 5.00±1.52 |
| | BCEM-1.00%v/v | 0 | | BCEM-1.00%v/v | 7.42±1.28 |
| *S. aureus* | BC | 0 | *C. albicans* | BC | 0 |
| | BCWM-2.5%w/v | 5.00±1.00 | | BCWM-2.5%w/v | 0 |
| | BCWM-5.0%w/v | 5.50±0.50 | | BCWM-5.0%w/v | 0 |
| | BCWM-10.0%w/v | 6.50±0.87 | | BCWM-10.0%w/v | 0 |
| | BCEM-0.25%v/v | 2.00±0.87 | | BCEM-0.25%v/v | 0 |
| | BCEM-0.50%v/v | 5.33±0.58 | | BCEM-0.50%v/v | 0 |
| | BCEM-1.00%v/v | 6.83±1.44 | | BCEM-1.00%v/v | 0 |
| *S. epidermidis* | BC | 0 | | | |
| | BCWM-2.5%w/v | 1.33±0.58 | | | |
| | BCWM-5.0%w/v | 4.17±0.29 | | | |
| | BCWM-10.0%w/v | 5.00±0.50 | | | |
| | BCEM-0.25%v/v | 2.17±1.26 | | | |
| | BCEM-0.50%v/v | 2.33±0.29 | | | |
| | BCEM-1.00%v/v | 3.50±0.00 | | | |

**Table 2** Antifungal activity of BC, BCWM, and BCEM films against *A. niger*

| Microorganisms | Sample | Observed growth | |
|---|---|---|---|
| | | Grade | Clear zone (mm) |
| *A. niger* | BC | 5 | 0 |
| | BCWM-2.5%w/v | 3 | 0 |
| | BCWM-5.0%w/v | 4 | 0 |
| | BCWM-10.0%w/v | 5 | 0 |
| | BCEM-0.25%v/v | 2 | 0 |
| | BCEM-0.50%v/v | 2 | 0 |
| | BCEM-1.00%v/v | 2 | 0 |

*Grade was used as a measurement of fungal growth: 0 = none, 1 = only apparent under microscope, 2 = trace (<10%), 3 = light growth (10-30%), 4 = medium growth (30-60%) and 5 = heavy growth (> 60%)

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**References**

1. Jung JY, Park JK and Chang HN. Bacterial cellulose production by *Gluconacetobacter hansenii* in anagitated culture without living non-cellulose producing cells. Enzyme and Microbial Technology 2005; 37: 347-354.
2. Hou A, Zhou M, Wang X. Preparation and characterization of durable antibacterial cellulose biomaterials modified with triazine derivatives. Carbohydrate Polymers 2009; 75: 328-332.
3. Yu L, Zhao M, Yang B, Zhao Q, Jiang Y. Phenolics from hull of *Garcinia mangostana* fruit and their antioxidant activities. Food Chemistry 2007; 104: 176-181.
4. Kosem N, Han YH, Moongkarndi P. Antioxidant and cytoprotective activities of methanolic extract from *Garcinia mangostana* hulls. ScienceAsia 2007; 33: 283-292.
5. Palakawong C, Sophanodora P, Pisuchpen S, Phongpaichit S. Antioxidant and antimicrobial activities of crude extracts from mangosteen (*Garcinia mangostana* L.) parts and some essential oils. International Food Research Journal 2010; 17: 583-589.