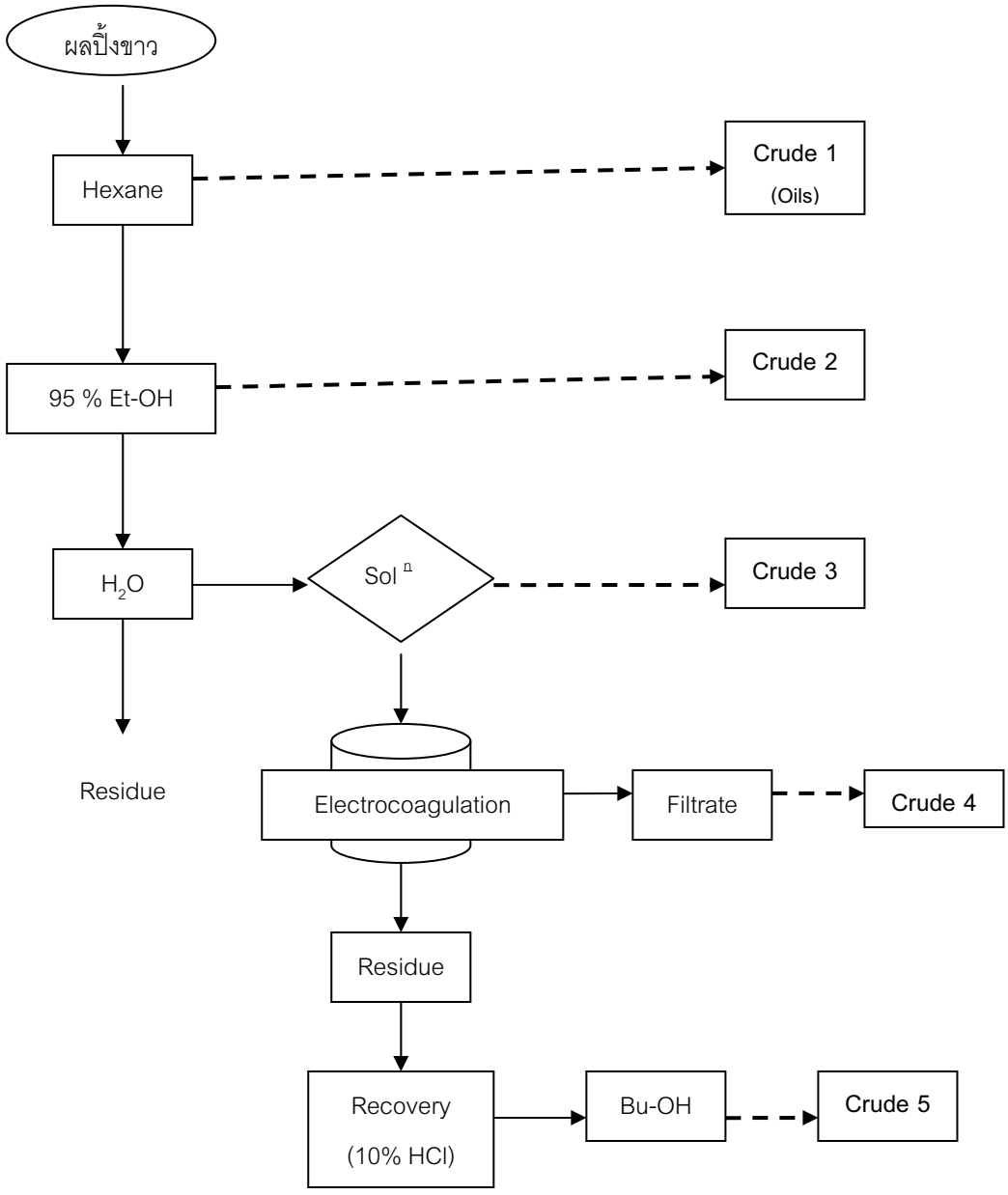
1. ปิ้งขาว (*Clerodendrum glandulosum*)

การทดลองที่ 1 การเตรียมสารสกัดหยาบและสารสกัดจากการตกตะกอนด้วยไฟฟ้าจากผลปิ้งขาว (วิธีที่ 1)

วิธีทดลอง

1. ปั่นผลปิ้งขาวแห้งให้ละเอียดพอประมาณ ปริมาณ 150 กรัม
2. นำไปสกัดด้วยเฮ็กเซน เพื่อสกัดสารพวกน้ำมันซึ่งมีมากให้ผลและเมล็ดของพืชทั่วไปออกด้วยชุดสกัดSoxhlet เป็นเวลา 2 ชั่วโมง นำสารละลายที่ได้ไประเหยออก ได้น้ำมันสีเหลืองหนืด 24.97 กรัม (Crude 1)
3. นำกากที่สกัดไขมันออกแล้วมาสกัดต่อด้วยเอธานอล 95% นาน 3 ชั่วโมง
4. ระเหยสารละลายสกัดด้วยเอธานอล 95% จะได้ของหนืดสีน้ำตาลเข้ม 12.57 กรัม (Crude 2)
5. นำกากที่เหลือจากการสกัดด้วยเอธานอล 95% มาสกัดต่อด้วยน้ำ 500 มล. นาน 3 ชั่วโมง
6. แบ่งสารละลายที่สกัดด้วยน้ำเป็นสองส่วน ส่วนแรก 200 มล. นำไประเหยน้ำออกจนหมด ได้ Crude 3 มีลักษณะเป็นของแข็ง สีน้ำตาลเข้ม19.45 กรัม ส่วนที่สอง 300 มล. นำไปผ่านการตกตะกอนด้วยไฟฟ้า
7. การตกตะกอนด้วยไฟฟ้า
    7.1 นำสารสกัดด้วยน้ำ 300 มล.ใส่ในบิกเกอร์ขนาด 600 มล. เติมอิเล็กโทรไลท์เสริมคือโซเดียมคลอไรด์ 0.5 กรัม
    7.2 จุ่มแผ่นอะลูมิเนียม ขนาด 8.5 x 14 ซม. เป็นอิเล็กโทรด แล้วผ่านกระแสไฟฟ้า (กระแสตรง) ประมาณ 0.3-0.4 แอ็มแปร์ ความต่างศักย์ 22 - 23 โวลต์ ด้วย DC power supply และคนด้วย magnetic stirrer ตลอดเวลา เป็นเวลา 1 ชั่วโมง
    7.3 เมื่อการตกตะกอนด้วยไฟฟ้าเสร็จ นำส่วนผสมทั้งหมดมากรอง เพื่อแยกสารละลายออก จากตะกอนล้างตะกอนด้วยน้ำกลั่น ทิ้งส่วนตะกอนให้แห้งที่อุณหภูมิห้อง 1 คืน
    7.4 นำส่วนสารละลายไประเหยจนแห้ง จะได้ของแข็งสีน้ำตาลอ่อน 5.38 กรัม (Crude 4)

7.5 นำส่วนตะกอนที่แห้งแล้วไปละลายด้วยกรดเกลือ 10% จนละลายหมด แล้วสกัดด้วย 1-บิวทานอล แยกสารละลายชั้น 1-บิวทานอล ไประเหยให้แห้ง ได้ของแข็งสีน้ำตาลเข้ม 4.66 กรัม (Crude 5) (ดูแผนภาพที่ 3 และตารางที่ 14)



แผนภาพที่ 3 การสกัดสารสกัดหยาบ (Crude 1-5)

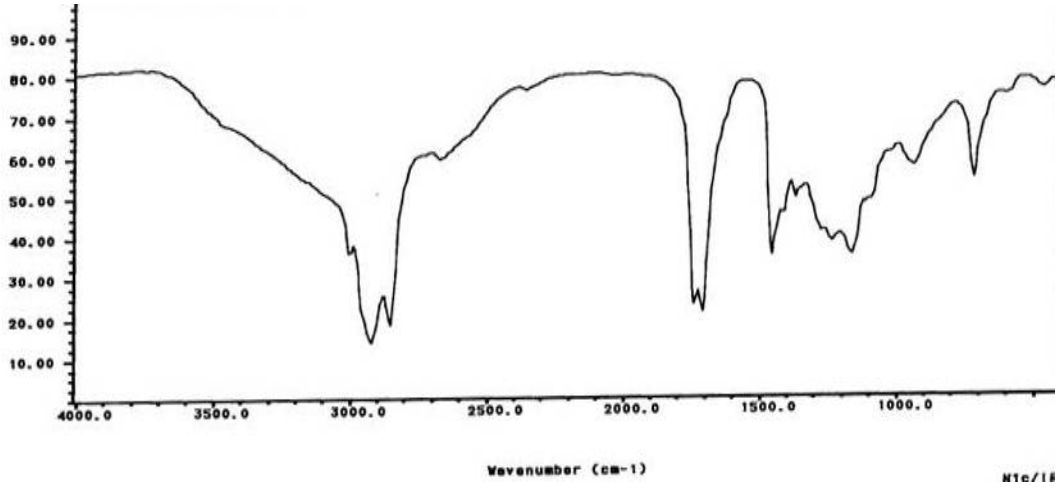
ตารางที่ 14 ผลการการสกัดสารสกัดหยาบ (Crude Extraction)

ตัวอย่าง	ลักษณะ	น้ำหนัก (กรัม)	% yield
ผลปิ้งขาว	ตัวอย่างแห้ง	150	-
Crude 1(oil)	น้ำมันหนืดสีเหลือง	24.97	16.65
Crude 2	ของหนืด สีน้ำตาลเข้ม	12.57	8.38
Crude 3	ของแข็ง สีน้ำตาลเข้ม	19.45	12.97
Crude 4	ของแข็ง สีน้ำตาลอ่อน	5.38	3.59
Crude 5	ของแข็ง สีน้ำตาลเข้ม	4.66	3.11

การทดลองที่ 2 การศึกษา Crude 1 (Oil)

1. **การวิเคราะห์ด้วย** IR Spectrum

จากอินฟราเรดสเปคตรัมของน้ำมัน (Crude 1) (รูปที่ 37) แสดงว่าประกอบด้วยกรดไขมันอิสระ (free fatty acids) ในสัดส่วนที่สูงมาก



รูปที่ 37 อินฟราเรดสเปคตรัมของน้ำมัน (Crude 1)

- นำน้ำมันผลปิ้งขาว 3.0 กรัม ละลายในเมธานอล 60 มล.แล้วเติม Amberlite IR-120(H+) 9 กรัม ในขวดก้นกลมขนาด 150 มล.

- ให้ความร้อนและทำการ reflux โดยมี magnetic stirrer คนตลอดเวลา นาน 6 ชั่วโมง

- กรองแยก Amberlite IR-120(H+) ออกจากส่วนของเหลว

- แยกสารละลายชั้นเมธานอลและชั้นน้ำมันด้วยกรวยแยก

- นำสารละลายชั้นเมธานอลไประเหยตัวทำละลายออกได้ crude methyl ester 1.03 กรัม

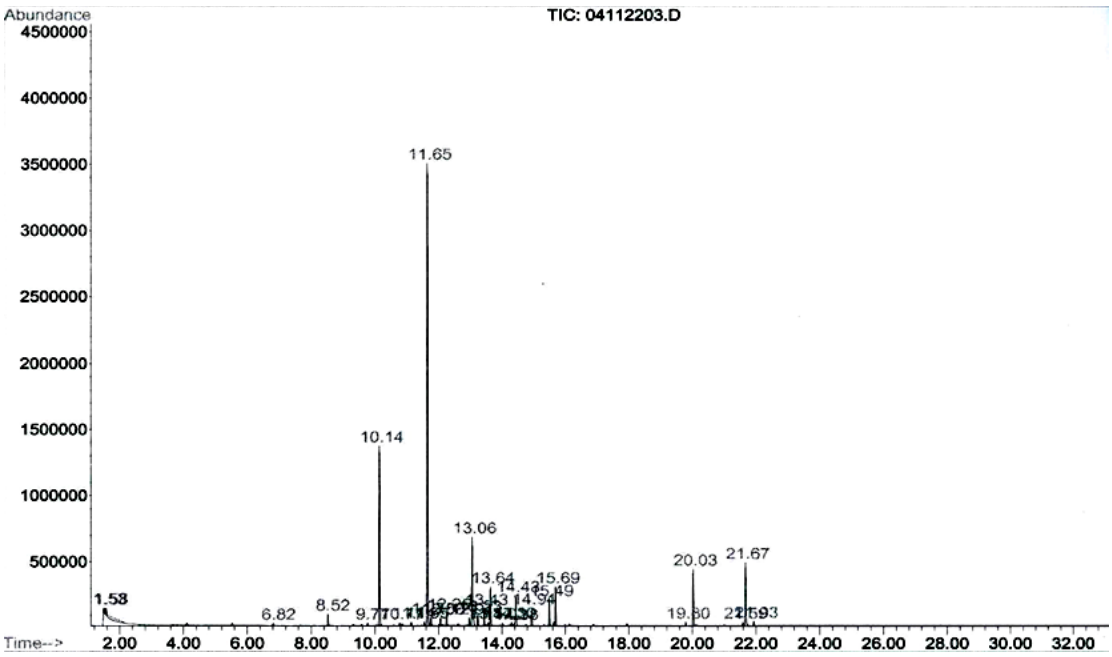
- นำ crude methyl ester ไปละลายด้วย ether และดูดความชื้นด้วย anhydrous $NaSO_4$

- วิเคราะห์ crude methyl ester ด้วย GC-MS ซึ่งมีรายละเอียดเครื่องมือและสภาวะดังตารางที่ 15

- ผลการวิเคราะห์ พบองค์ประกอบหลัก 9 ชนิด ดังรูปที่ 38 และตารางที่ 16

ตารางที่ 15 รายละเอียดเครื่องมือและสภาวะการวิเคราะห์ crude methyl esters โดย GC-MS

เครื่องมือ	**รายละเอียด/สภาวะในการวิเคราะห์**	
GC	รุ่น/บริษัท	GC 6890 / Agilent Technology
	Inlet	อุณหภูมิ 240°C
	SPME	100 μm, PDMS, 50°C 2 min.
	Oven	40°C (2 min.)→ 10°C/ min. →240°C (12 min.)
	Carrier gas	Helium 1.0 ml/min.
	Column	AT-1MS (30 m x 0.25 mm ID, 0.25 μm film thickness)
MS	รุ่น/บริษัท	MSD 5973 / Hewlett Packard
	EI	70 eV
	MS Quadrupole	150°C
	MS Source	230°C



รูปที่ 38 โครมาโทแกรมของ crude methyl ester

ตารางที่ 16 องค์ประกอบของ crude methyl ester จากน้ำมันผลปิ้งขาว

อันดับ	RT	% Peak Area	methyl ester ของ	ชื่ออื่นๆ ของกรดไขมัน
1	11.65	38.80	Nonanoic acid	Pelargonic acid
2	10.14	14.33	Octanoic acid	Caprylic acid
3	13.06	8.04	Decanoic acid	Capric acid
4	21.67	5.60	9-Octadecenoic acid	Red oil / Metaupon
5	20.03	4.66	Hexadecanoic acid	Cetylic acid
6	15.69	3.16	Nonanedioic acid	Azelaic acid
7	13.63	3.16	2-Decenoic acid	-
8	14.43	3.02	Octanedioic acid	Cork acid / Suberic acid
9	13.43	1.36	Nonanoic acid, 4-oxo-	-

การทดลองที่ 3 การศึกษาฤทธิ์ต้านออกซิเดชันของสารสกัดหยาบ (Crude 2 – 5)

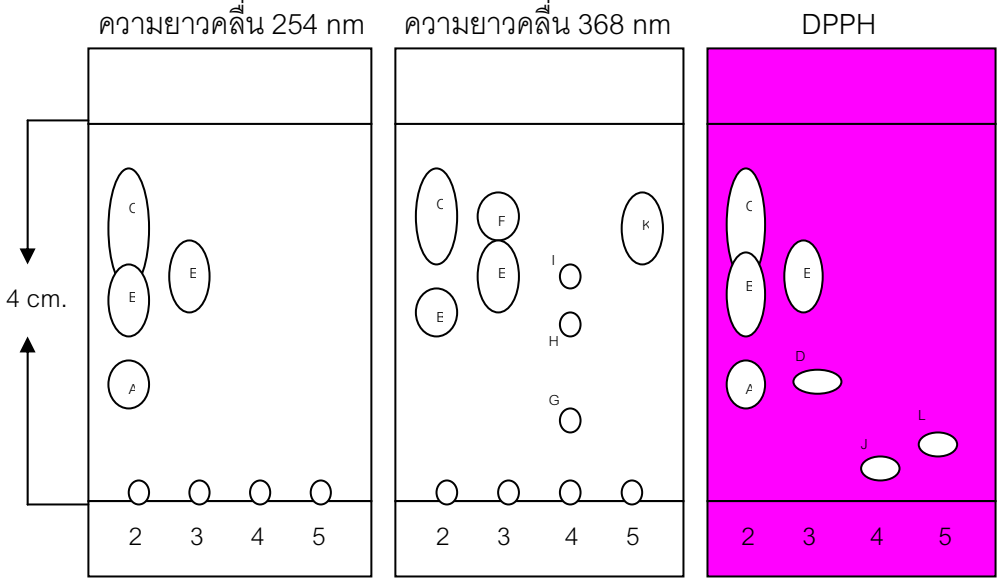
1. การตรวจสอบฤทธิ์ต้านออกซิเดชันด้วยวิธี TLC Screening

วิธีทดลอง

ใช้ DPPH (2,2-diphenyl-1-picrylhydrazyl) เป็นรีเอเจนต์ทดสอบ โดยพ่นสารละลายของสารดังกล่าวเข้มข้น 0.2 mM ในเมธานอล ลงบน TLC โครมาโตแกรมของสารสกัดหยาบ (Crude 2-5) สังเกตฤทธิ์การต้านอ็อกซิเดชันจากการฟอกจางสีของสารละลาย DPPH

ผลการทดลอง

ผลการตรวจสอบฤทธิ์ต้านอ็อกซิเดชัน (antioxidant activity) ของ Crude 2-5 โดยวิธี TLC screening แสดงดังรูปที่ 39 และตารางที่ 17



รูปที่ 39 ผลการทดสอบฤทธิ์ต้านอ็อกซิเดชันด้วย TLC

ตารางที่ 17 ผลการทดสอบการตรวจสอบฤทธิ์ต้านอ็อกซิเดชันด้วย TLC

Crude	สารที่พบ	ค่า R_f	254 nm	368 nm	DPPH
2	A	0.35	/		/
	B	0.57	/	/	/
	C	0.75	/	/	/
3	D	0.35			/
	E	0.57	/	/	/
	F	0.73		/	
4	G	0.21		/	
	H	0.5		/	
	I	0.61		/	
	J	0.09			/
5	K	0.75		/	
	L	0.1			/

2. **การศึกษาฤทธิ์ต้านออกซิเดชันเชิงปริมาณวิเคราะห์ด้วยวิธี** spectrophotometric assay

วิธีทดลอง[14]

1. เตรียมสารละลายสารสกัดตัวอย่างในเมธานอลที่มีความเข้มข้นต่างกัน 6 ชุด ปริมาตร 1 mL ให้ความเข้มข้นอยู่ในช่วง 1000-10 ppm
2. เตรียมสารละลาย control (DPPH 0.2 mM 2.0 mL + methanol 1.0 mL) นำไปหาค่าการดูดกลืนแสงสูงสุด (λmax) ในช่วงความยาวคลื่น 400 – 600 nm เพื่อหาความยาวคลื่นที่จะใช้วัดค่าการดูดกลืนแสง(Absorbance)
3. เติมสารละลาย DPPH 0.2 mM 2 mL ลงในสารละลายสารสกัดตัวอย่างแต่ละความเข้มข้นที่เตรียมไว้ในข้อ 1 เขย่าให้เข้ากันและตั้งทิ้งไว้ในที่มืด 30 นาที
4. นำไปวัดค่าการดูดกลืนแสงที่ความยาวคลื่นที่ได้จากข้อ3 (λmax = 517 nm)

5. ทำการทดลองโดยใช้สารสกัดเดิมซ้ำ 3 ครั้ง แล้วหาค่าเฉลี่ยของค่าการดูดกลืนแสงที่ได้แต่ละชุดความเข้มข้น
6. คำนวณค่า % radical scavenging จากสมการ

$$\% \text{ radical scavenging} = [1-(A_{sample}/A_{control})] \times 100$$

เมื่อ A_{sample} = ค่าการดูดกลืนแสงของสารละลายตัวอย่างกับสารละลาย DPPH

$A_{control}$ = ค่าการดูดกลืนแสงของสารละลาย control

7. นำค่าความเข้มข้นของสารละลายตัวอย่างและ % radical scavenging ไปสร้างกราฟเพื่อหาค่า IC_{50} โดยใช้โปรแกรม EXCEL เพื่อหาสมการ logarithmic ของเส้นกราฟระหว่างความเข้มข้นของสารละลายตัวอย่างและ % radical scavenging แล้วคำนวณค่า IC_{50} จากสมการดังกล่าว

ผลการทดลอง

ได้ผลดังตารางที่ 19 และสรุปผลดังตารางที่ 18

ตารางที่ 18 สรุปผลการศึกษาฤทธิ์ต้านอ็อกซิเดชันของ Crude 2-5

Crude	IC_{50}(ppm)
2	33.07
3	2.65
4	2,352.32
5	1.69
BHA	3.46

ตารางที่ 19 ผลการการศึกษาฤทธิ์ต้านอ็อกซิเดชันทางปริมาณวิเคราะห์ด้วยวิธี spectrophotometric assay

sample	Conc. (ppm)	UV absorbance				% radical scavenging	IC_{50} (ppm)
		1	2	3	average		
Crude 2	Control 2	0.675	0.678	0.670	0.674	-	33.07
	31.25	0.457	0.436	0.462	0.452	33.02	
	62.5	0.257	0.271	0.290	0.273	59.56	
	125	0.052	0.065	0.060	0.059	91.25	
	250	0.048	0.049	0.049	0.049	92.78	
	500	0.052	0.056	0.055	0.054	91.94	
	1000	0.069	0.07	0.072	0.070	89.57	
Crude 3	Control 3	0.66	0.664	0.655	0.660	-	2.65
	31.25	0.410	0.309	0.340	0.353	46.49	
	62.5	0.105	0.075	0.09	0.09	86.36	
	125	0.054	0.056	0.06	0.057	91.41	
	250	0.069	0.066	0.068	0.068	89.74	
	500	0.081	0.089	0.082	0.084	87.27	
	1000	0.12	0.125	0.122	0.122	81.45	
Crude 4	Control 4	0.622	0.623	0.624	0.623	-	2,352.32
	31.25	0.392	0.411	0.407	0.403	1.66	
	62.5	0.375	0.39	0.394	0.386	6.206	
	125	0.442	0.456	0.461	0.453	15.41	
	250	0.508	0.573	0.50	0.527	27.29	
	500	0.595	0.58	0.578	0.584	37.99	
	1000	0.624	0.592	0.622	0.612	35.26	
Crude 5	Control 5	0.663	0.66	0.662	0.662	-	1.69
	31.25	0.378	0.377	0.415	0.39	41.06	
	62.5	0.171	0.172	0.203	0.182	72.49	
	125	0.075	0.076	0.072	0.074	88.77	
	250	0.101	0.10	0.098	0.099	84.94	
	500	0.154	0.155	0.153	0.154	76.72	
	1000	0.260	0.255	0.259	0.258	61.01	

ตารางที่ 19 (ต่อ) ผลการการศึกษาฤทธิ์ต้านอ็อกซิเดชันทางปริมาณวิเคราะห์ด้วยวิธี spectrophotometric assay

sample	Conc. (ppm)	UV absorbance				% radical scavenging	IC_{50} (ppm)
		1	2	3	average		
BHA	Control	1.696	1.632	1.663	1.664	-	3.47
	31.25	0.639	0.623	0.641	0.634	61.87	
	62.5	0.341	0.335	0.339	0.338	79.66	
	125	0.121	0.118	0.125	0.121	92.70	
	250	0.064	0.059	0.066	0.063	96.21	
	500	0.054	0.051	0.055	0.053	96.79	
	1000	0.045	0.046	0.049	0.047	97.19	

การทดลองที่ 4 การศึกษาฤทธิ์ทางชีวภาพ

ได้ศึกษาฤทธิ์ทางชีวภาพของสารสกัดหยาบ (Crude 2-5) ของผลปิ้งขาวกับแบคทีเรียก่อโรค 5 ชนิดคือ *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi* และ *Shigella flexneri* ด้วยวิธี dish diffusion โดยการเลี้ยงเชื้อแบคทีเรียดังกล่าวในจานเพาะเชื้อ ใช้อาหารสำหรับเพาะเลี้ยงเชื้อแบคทีเรียชนิด Nutrient agar(NA) จากนั้นนำแผ่น Sterile paper disc ชุบสารละลายของสารสกัดหยาบที่ต้องการทดสอบ ความเข้มข้น 5, 2.5 และ1.25 mg/ml วางบนผิวหน้าของอาหารเพาะเลี้ยงเชื้อ แล้วนำไปบ่มที่อุณหภูมิ 37 ° C นาน 24 ชั่วโมง หลังจากนั้นจึงสังเกตผลจากขนาดวงใสของการยับยั้งที่เกิดขึ้นรอบแผ่น Sterile paper disc พบว่าสารสกัดจากผลปิ้งขาวทั้งหมดไม่แสดงผลยับยั้งการเติบโตของแบคทีเรียก่อโรคทั้ง 5 ชนิด

การทดลองที่ 5 การเตรียมสารสกัดหยาบและสารสกัดจากการตกตะกอนด้วยไฟฟ้าจากผลปิ้งขาว (วิธีที่ 2)

วิธีทดลอง

นำผลปิ้งขาวแห้งบดละเอียดปริมาณ 150 กรัม มาสกัดด้วยตัวทำละลายเฮกเซนปริมาตร 300 มล. โดยใช้เครื่องสกัดแบบ Soxhlet ทำการสกัดเป็นเวลา 2 ชั่วโมง จากนั้นนำสารละลายสกัดที่ได้ไประเหยตัวทำละลายออกด้วยเครื่องหมุนระเหยความดันต่ำแล้วทำให้สารสกัดแห้งปราศ จากความชื้นด้วยเครื่องดูดอากาศเป็นเวลา 1 ชั่วโมง จะได้น้ำมันสีเหลืองหนืด 25.15 กรัม นำกากที่เหลือจากการสกัดน้ำมันไปผึ่งให้แห้งที่อุณหภูมิห้อง 1 คืน

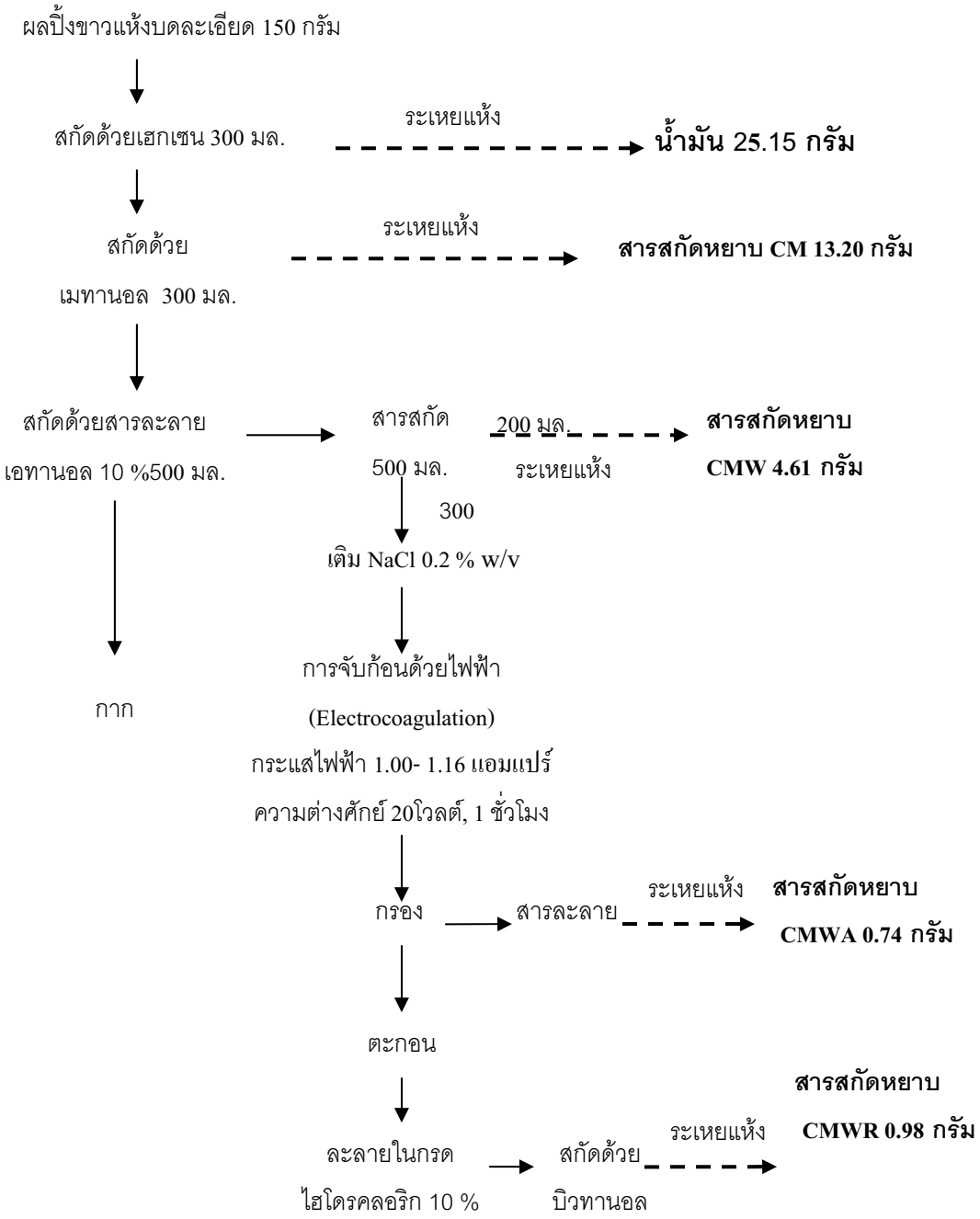
นำกากผลปิ้งขาวที่ผ่านการสกัดน้ำมันออกแล้วผึ่งไว้ 1 คืนมาสกัดด้วยเมทานอลปริมาณ 300 มล. โดยใช้เครื่องสกัดแบบ Soxhlet นาน 3 ชั่วโมง แล้วนำสารละลายสกัดที่ได้ไประเหยด้วยเครื่องหมุนระเหยความดันต่ำและทำให้แห้งสนิทด้วยเครื่องดูดอากาศนาน 1 ชั่วโมง จะได้สารสกัดหยาบเป็นของแข็งสีเขียวเข้มที่มีของหนืดสีน้ำตาลปนอยู่ 13.20 กรัม (CM) นำกากที่เหลือไปผึ่งให้แห้งที่อุณหภูมิห้อง 1 คืน

นำกากที่เหลือจากการสกัดด้วยเมทานอล มาสกัดด้วยสารละลายเอทานอลเจือจาง 10 % 500 มล. โดยใช้เครื่องสกัดแบบซ็อกซ์เล็ทนาน 3 ชั่วโมง นำสารละลายสกัดที่ได้แบ่งเป็นสองส่วน (ส่วนที่ 1 ปริมาตร 200 มล.และส่วนที่2 ปริมาตร 300 มล.) นำสารละลายสกัดส่วนที่ 1 ไประเหยแห้งด้วยเครื่องหมุนระเหยความดันต่ำและทำให้ปราศจากความชื้นด้วยเครื่องดูดอากาศนาน 1 ชั่วโมง ได้สารสกัดหยาบมีลักษณะเป็นของแข็ง สีน้ำตาลเข้ม4.61 กรัม (CMW) สำหรับสารละลายสกัดส่วนที่ 2 ปริมาตร 300 มล. นำไปผ่านการจับก้อนด้วยไฟฟ้า

การจับก้อนด้วยไฟฟ้า

นำสารละลายสกัดด้วยสารละลายเอทานอลเจือจาง 10 % ส่วนที่ 2 ปริมาตร 300 มล.ใส่ในบิกเกอร์ขนาด 600 มล. เติมอิเล็กโทรไลท์เสริมคือโซเดียมคลอไรด์(NaCl) 0.6 กรัม(0.2 % w/v) แล้วประกอบชุดอุปกรณ์การจับก้อนด้วยไฟฟ้า โดยใช้แผ่นอะลูมิเนียมขนาด 7.0 x 15 ซม. 2 แผ่น เป็นอิเล็กโทรด ห่างกัน 3.5 ซม. ผ่านกระแสไฟฟ้ากระแสตรงขนาด 1.65- 1.75 แอมแปร์ ความต่างศักย์ 20 โวลต์ และคนตลอดเวลาด้วยเครื่องกวนแม่เหล็กเป็นเวลานาน 1 ชั่วโมง จากนั้นนำส่วนผสมทั้งหมดมากรองแยกสารละลายออกจากตะกอน ล้างตะกอนด้วยน้ำกลั่นแล้วนำไปผึ่งให้แห้งที่อุณหภูมิห้อง 1 คืน นำส่วนสารละลายไประเหยจนแห้งด้วยเครื่องหมุนระเหยความดันต่ำและดูดความชื้นให้แห้งสนิทด้วยเครื่องดูดอากาศ จะได้สารสกัดหยาบของแข็งสีน้ำตาลอ่อน 0.74 กรัม (CMWA)

นำส่วนตะกอนจากการจับก้อนด้วยไฟฟ้าไปละลายด้วยสารละลายกรดไฮโดรคลอริกเจือจาง 10% จนละลายหมด (300 มล.) แล้วนำสารละลายกรดที่ได้ไปสกัดด้วยบิวทานอล ด้วยกรวยแยกครั้งละ 100 มล. 2 ครั้ง แยกสารละลายชั้นบิวทานอลไประเหยตัวทำละลายออกด้วยเครื่องหมุนระเหยความดันต่ำแล้วทำให้ปราศจากตัวทำละลายด้วยเครื่องดูดอากาศ ได้สารสกัดหยาบเป็นของแข็งสีน้ำตาลเข้ม 0.98 กรัม (CMWR) ผังการสกัดทั้งหมดแสดงในแผนภาพที่ 4



แผนภาพที่ 4 ผังการสกัดสารสกัดหยาบวิธีที่ 2

การทดลองที่ 6 การแยกและตรวจสอบโครงสร้างทางเคมีของสารสีน้ำเงิน (blue pigment) จากสารสกัดหยาบเมทานอล (CM)

วิธีทดลอง

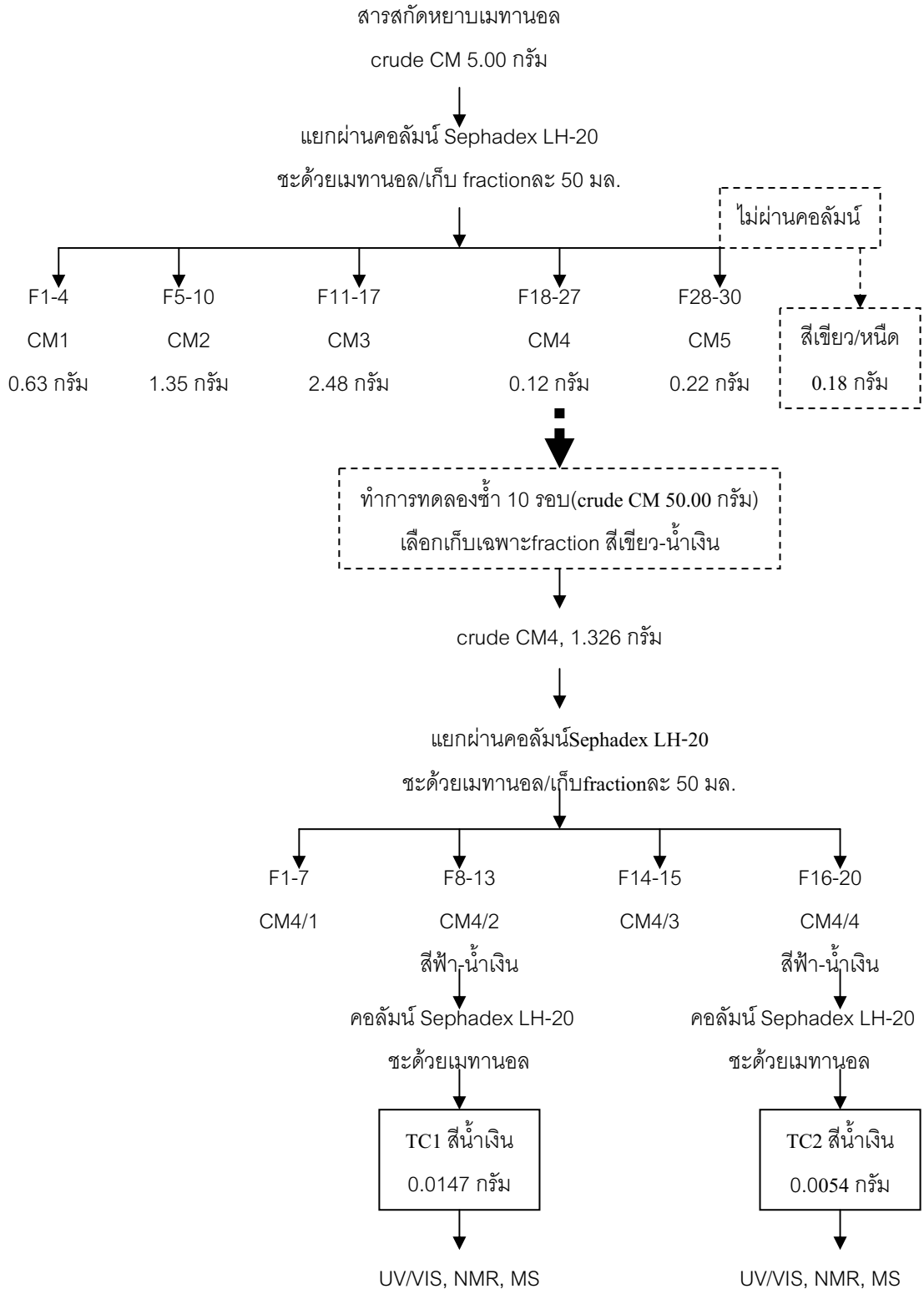
นำสารสกัดหยาบที่สกัดด้วยเมทานอล (CM) 5.00 กรัม (จากการทดลองที่ 5) ไปแยกด้วยโครมาโทกราฟีคอลัมน์โดยใช้ Sephadex LH-20 ชะด้วยเมทานอล เก็บ fractionละ 50 มล. จำนวน 30 fraction แล้วรวม fraction 1-4, 5-10, 11-17, 18-27และ28-30 นำไประเหยตัวทำละลายออกจนแห้ง จะได้ crude CM1(ของเหลวหนืดสีน้ำตาล,0.63กรัม) crude CM2 (ของเหลวหนืดสีเหลืองเข้ม,1.35กรัม) crude CM3 (ของเหลวหนืดสีน้ำตาล,2.48กรัม) crude CM4 (ของแข็งสีเขียว-น้ำเงิน,0.12กรัม) และcrude CM5 (ของเหลวหนืดสีเหลือง,0.22กรัม) ตามลำดับ และยังมีสารที่ไม่ผ่านคอลัมน์และไม่ละลายในเมทานอลค้างอยู่บนคอลัมน์ เป็นของเหลวคล้ายน้ำมันสีเขียวใส (0.18กรัม)

ทำการทดลองซ้ำตั้งแต่ต้นโดยใช้วิธีการเดิม แยกสารสกัดหยาบ CM ครั้งละ 5.00 กรัมโดยเลือกเก็บเฉพาะfraction ที่มีสีเขียวน้ำเงิน ทำการทดลองทั้งหมด 10 รอบ (ใช้สารสกัดหยาบ CM 50.00 กรัม) ได้ crude CM4 ของแข็งสีเขียวน้ำเงินรวม 1.326 กรัม

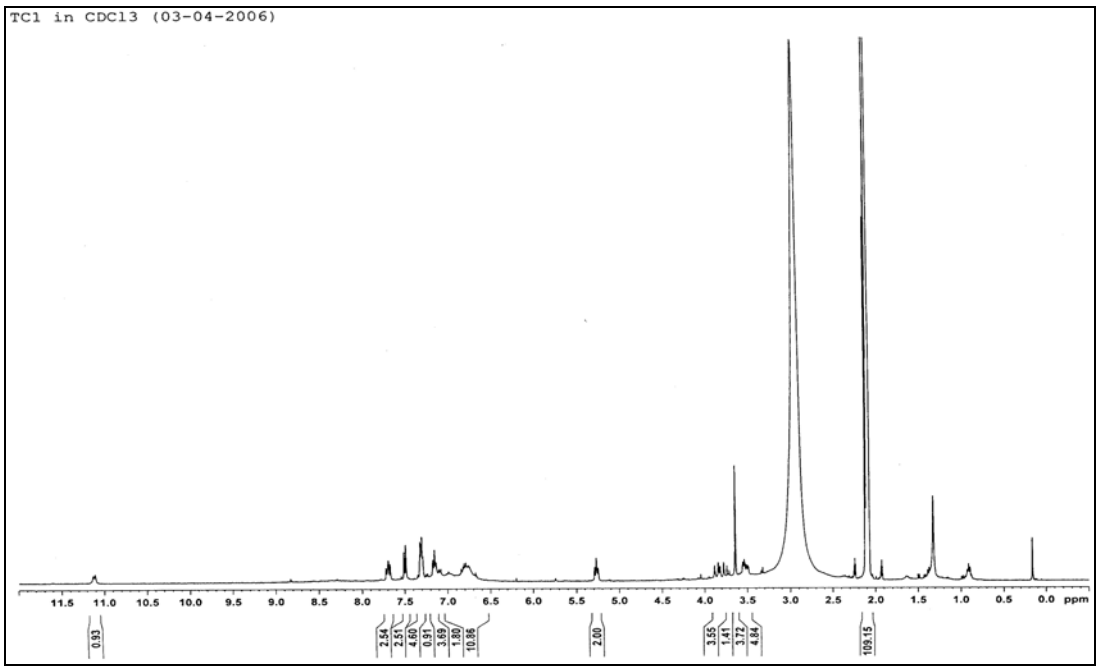
นำ crude CM4 ไปแยกด้วยคอลัมน์ Sephadex LH-20 ชะด้วยเมทานอลเจือจาง 80 % เก็บ fractionละ 50 มล.จำนวน 20 fraction แล้วรวมสารละลาย fractionที่ 1-7, 8-13, 14-45 และ 16-20 นำไประเหยจนแห้ง จะได้crude CM4/1(ของแข็งสีเขียว) crude CM4/2 (ของแข็งสีน้ำเงิน) crude CM4/3 (ของเหลวหนืดสีเหลือง) และ crude CM4/4 (ของแข็งสีน้ำเงิน) ตามลำดับ

นำ crude CM4/2 และ CM4/4 ซึ่งมีสีน้ำเงินไปแยกผ่านคอลัมน์ Sephadex LH-20 ชะด้วยเมทานอลเพื่อแยกเอาเฉพาะสารที่มีความบริสุทธิ์มากขึ้นจนได้สารTC1 (0.0147กรัม) และ TC2 (0.0054กรัม) ตามลำดับ (แผนภาพที่ 5)

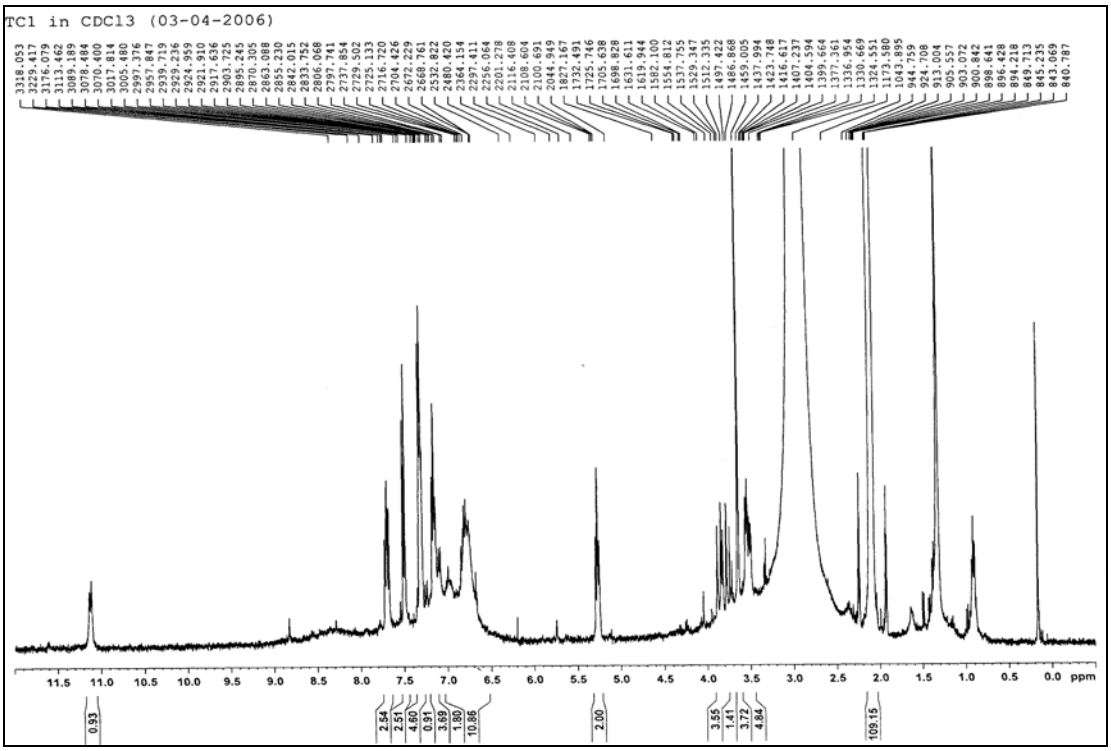
นำสารสีน้ำเงินที่แยกได้ (TC1 และ TC2) ไปวัด NMR สเปคตรา และ MS ดังรูปที่ 41-52



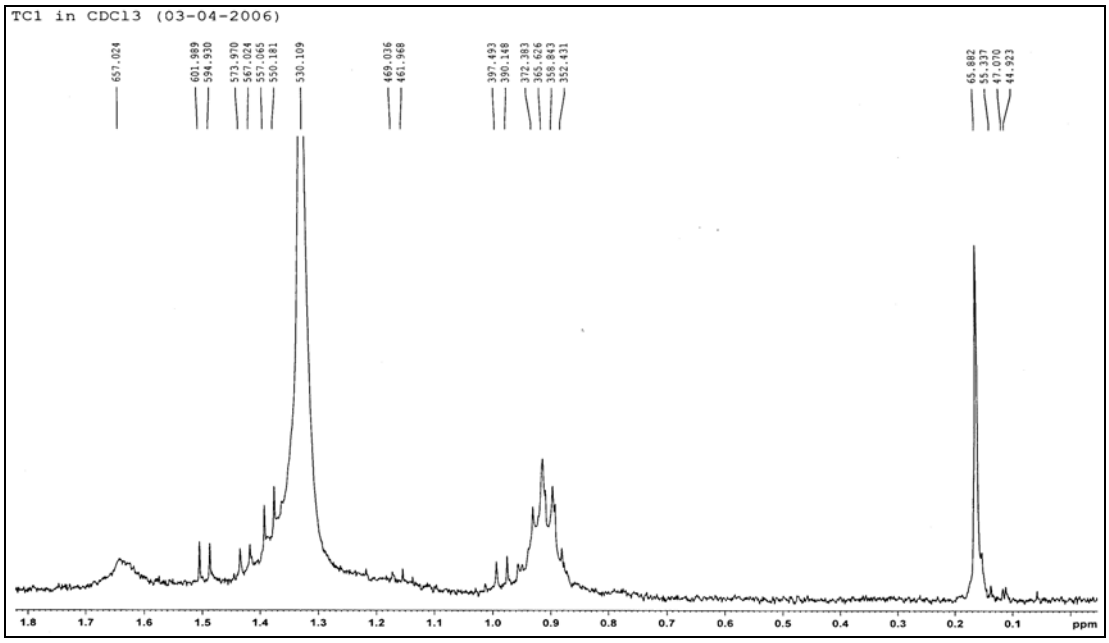
แผนภาพที่ 5 ผังการแยกและวิเคราะห์โครงสร้างทางเคมีของสารสีน้ำเงินจากสารสกัดหยาบเมทานอล (CM)



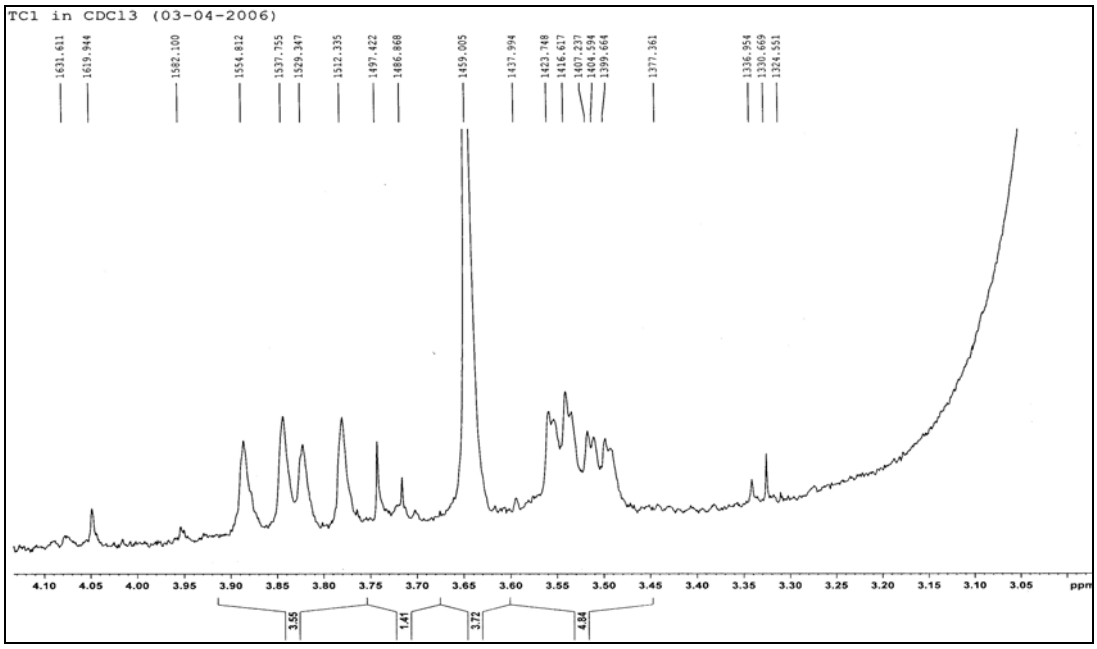
รูปที่ 40 สเปกตรัม ^{1}H-NMR ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



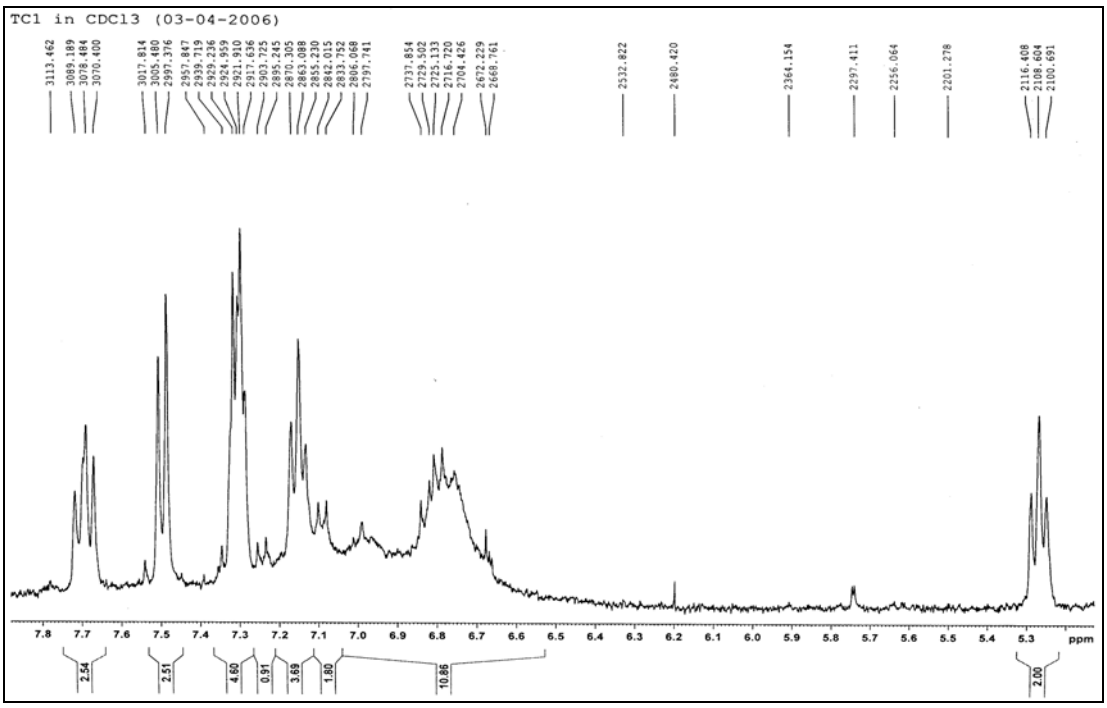
รูปที่ 41 สเปกตรัม ^{1}H-NMR ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



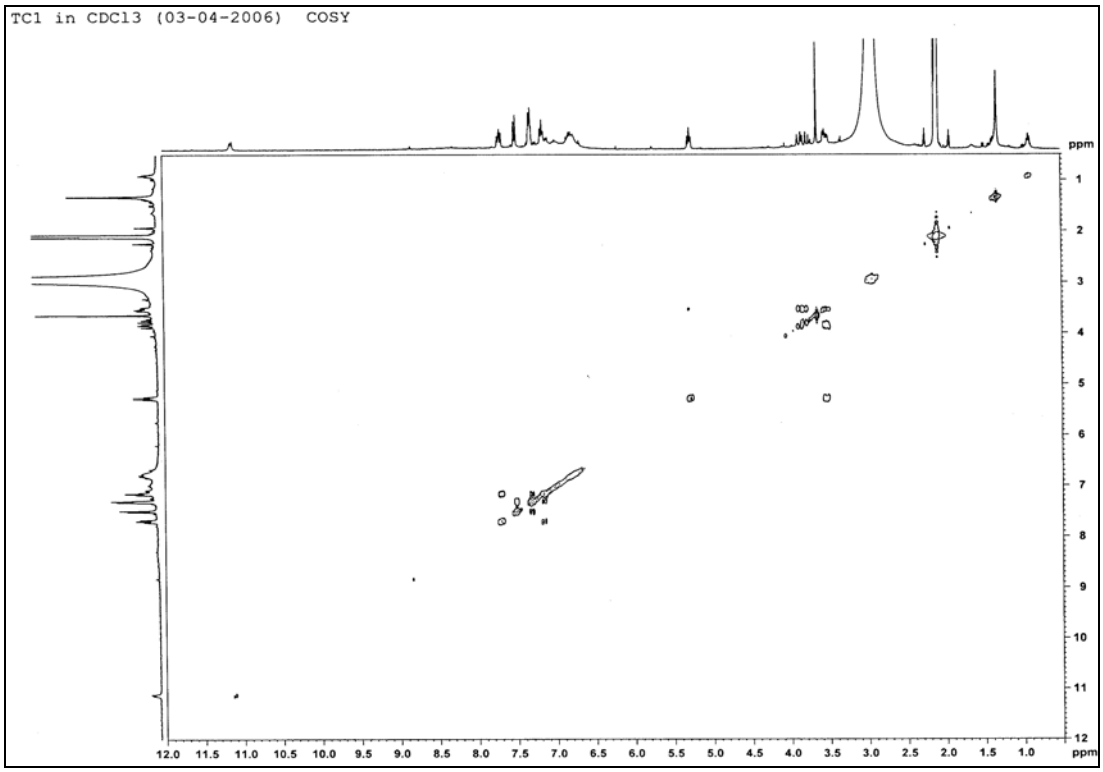
รูปที่ 42 สเปกตรัม ^{1}H-NMR ในช่วง 0.00-1.80 ppm ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



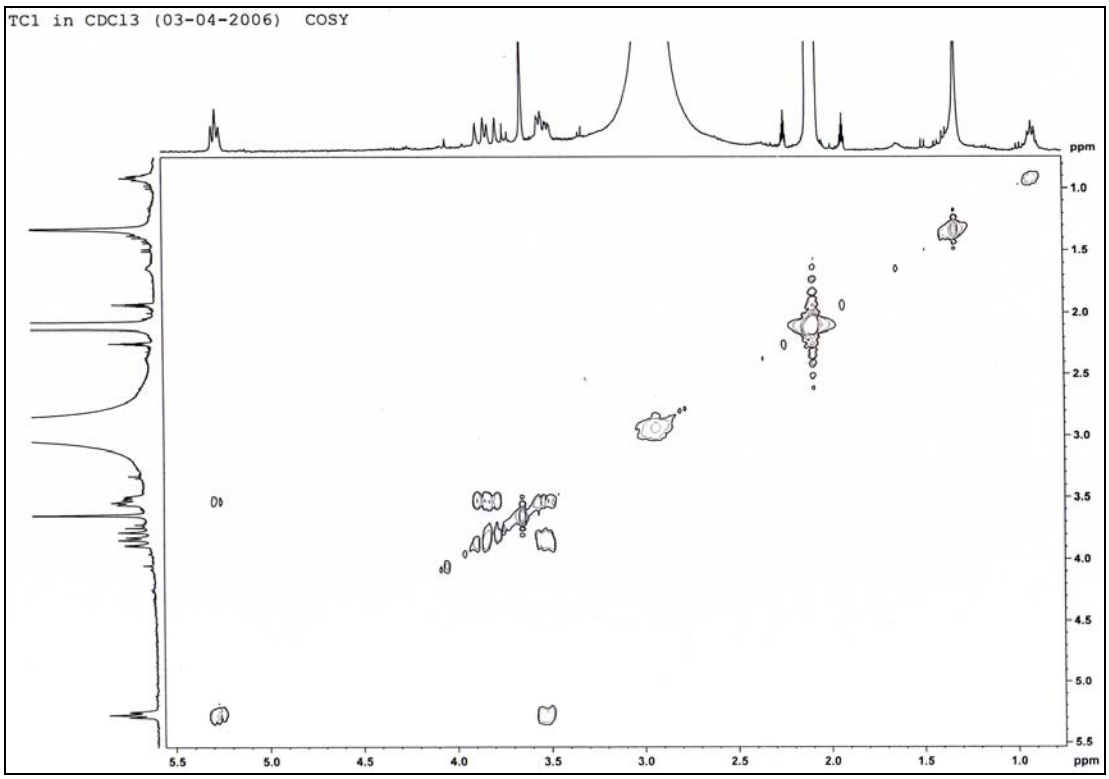
รูปที่ 43 สเปกตรัม ^{1}H-NMR ในช่วง 3.05-4.10 ppm ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



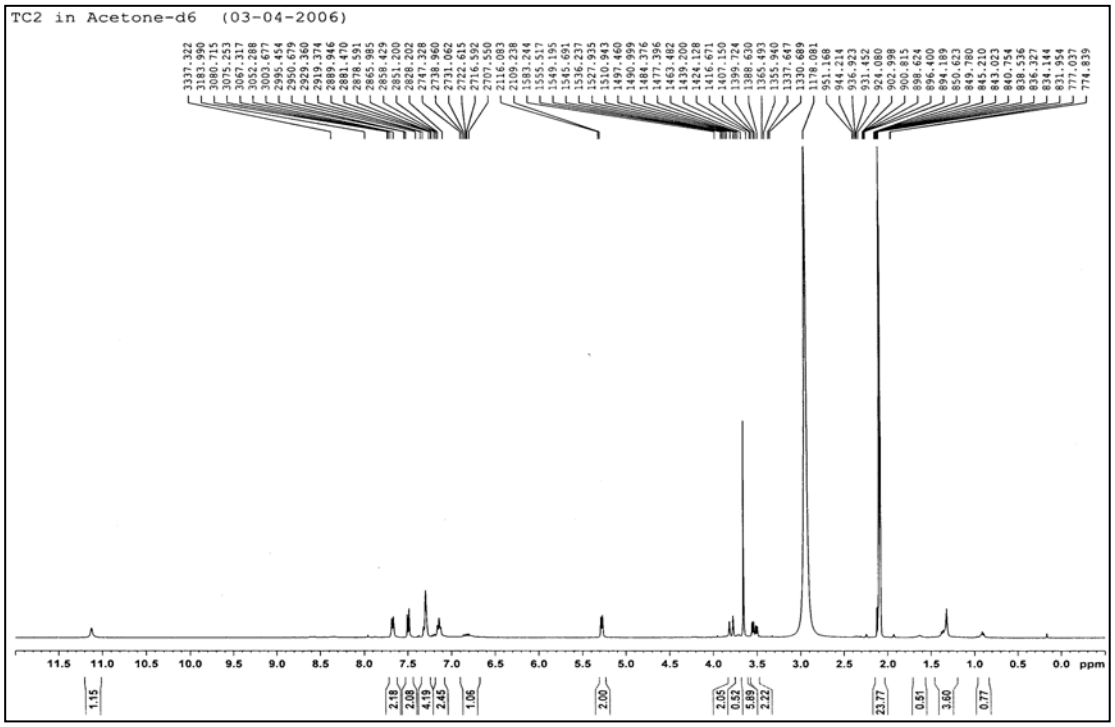
รูปที่ 44 สเปกตรัม ^{1}H-NMR ในช่วง 5.20-7.80 ppm ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



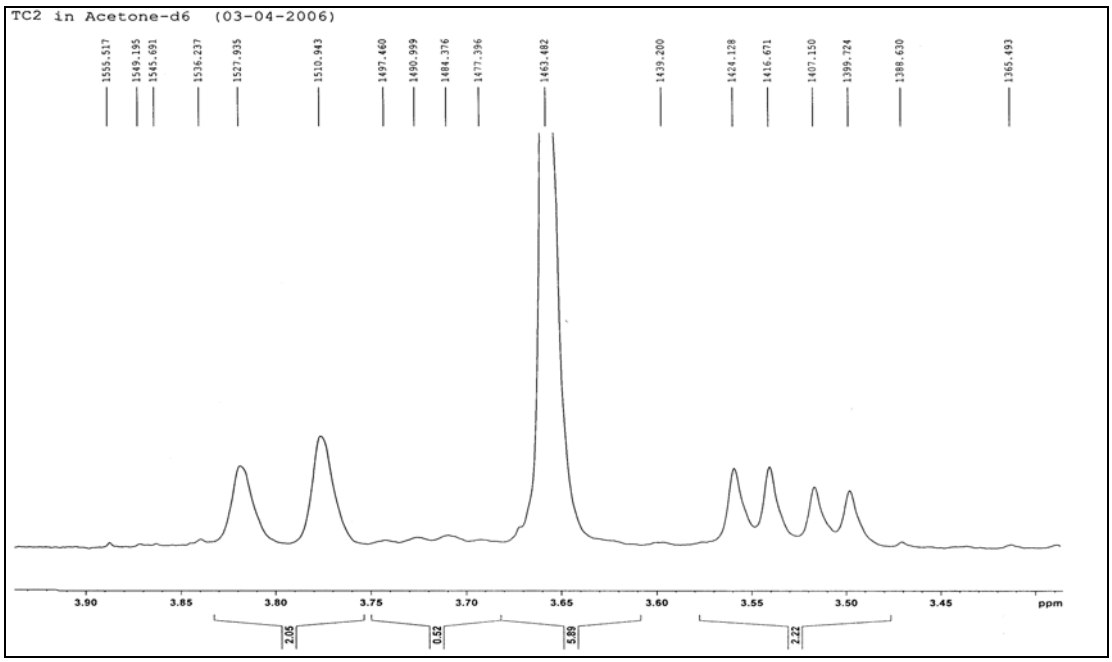
รูปที่ 45 สเปกตรัม COSY ของสารสีน้ำเงิน TC1 (ใน Acetone-D6)



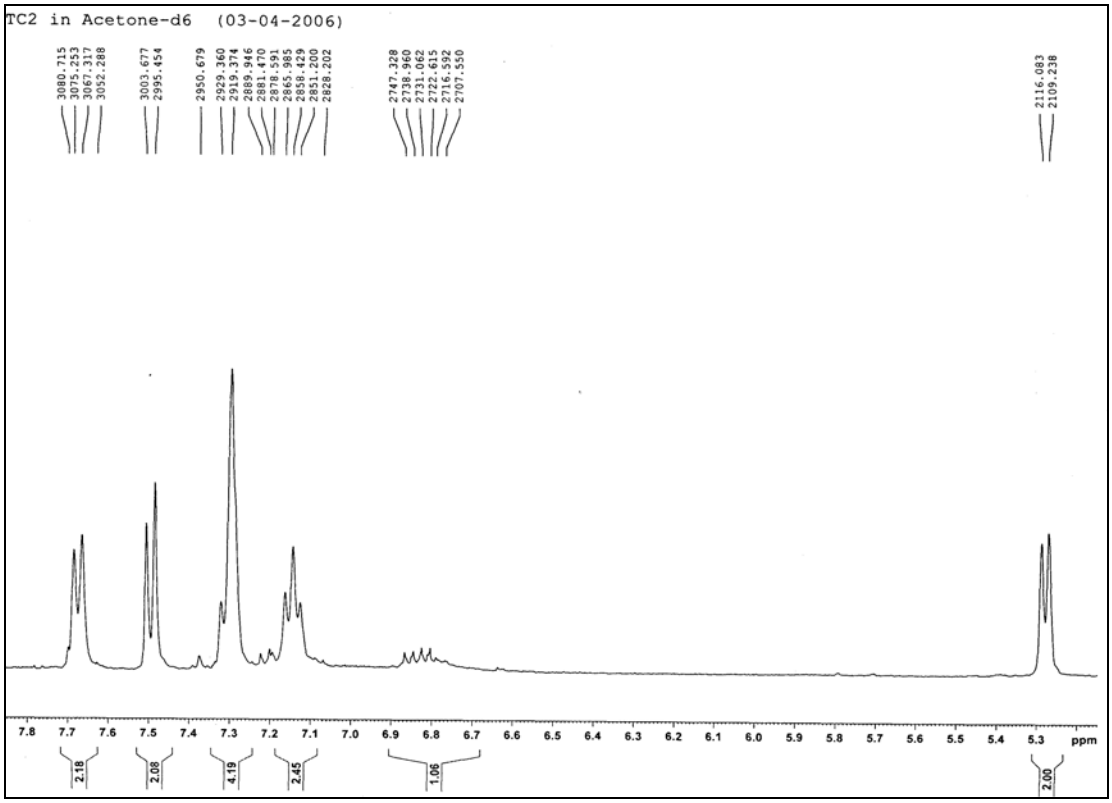
รูปที่ 46 สเปกตรัม COSY ของสารสีน้ำเงิน TC1 (ใน Acetone-D6) (รูปขยาย)



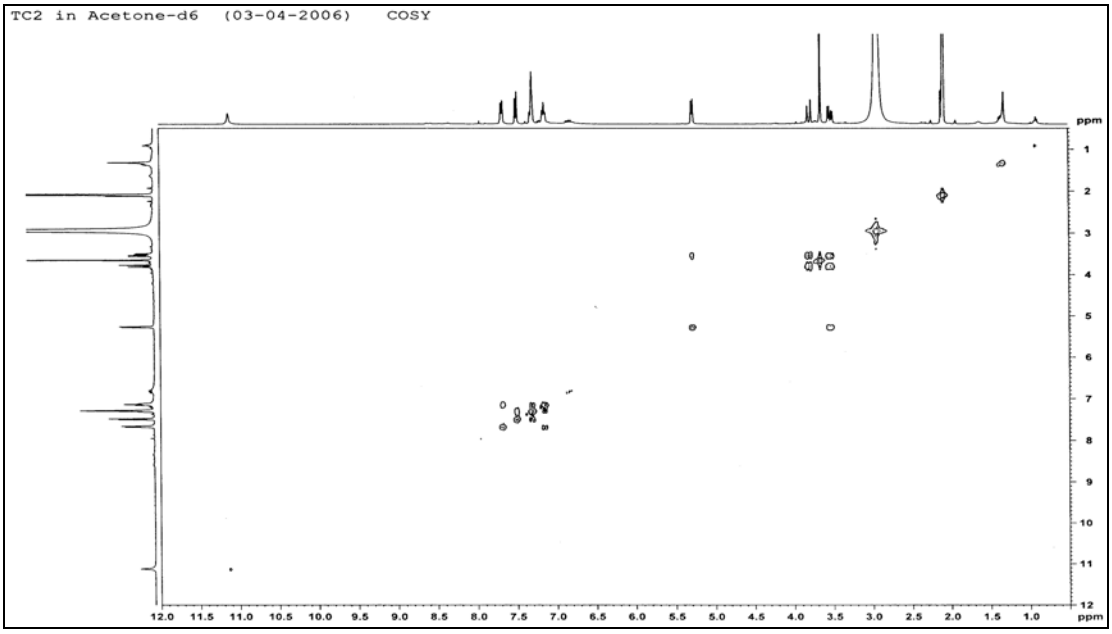
รูปที่ 47 สเปกตรัม ^{1}H-NMR ของสารสีน้ำเงิน TC2 (ใน Acetone-D6)



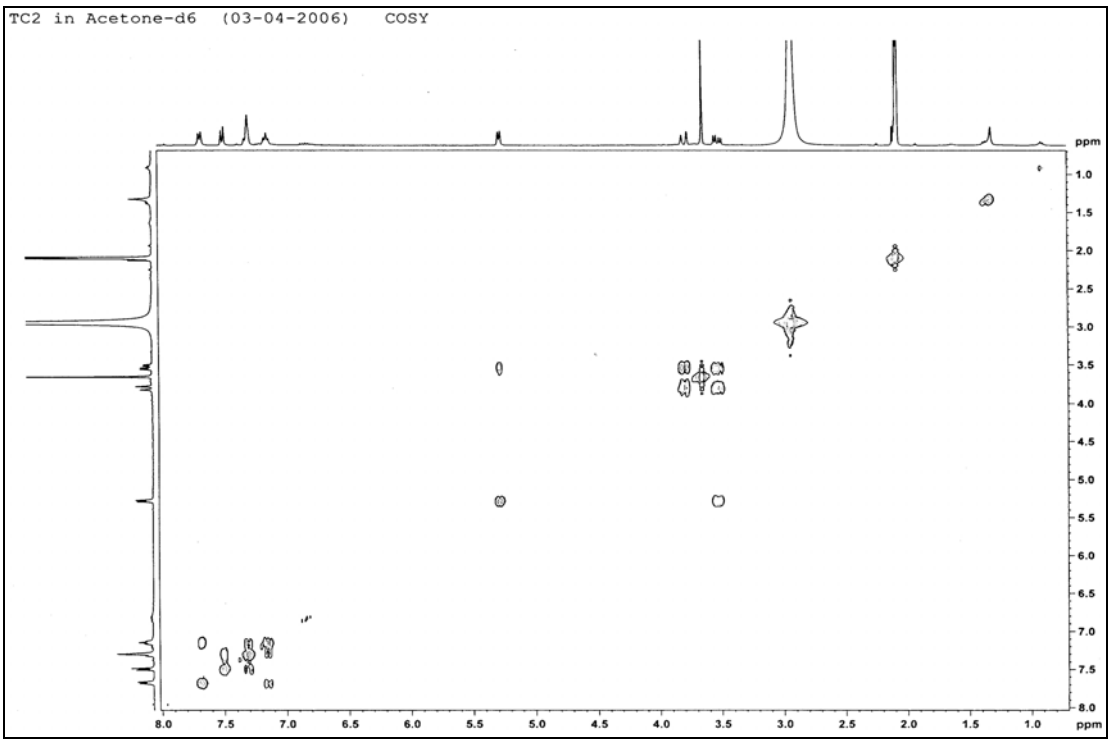
รูปที่ 48 สเปกตรัม ^{1}H-NMR ในช่วง 3.45-3.90 ppm ของสารสีน้ำเงิน TC2 (ใน Acetone-D6)



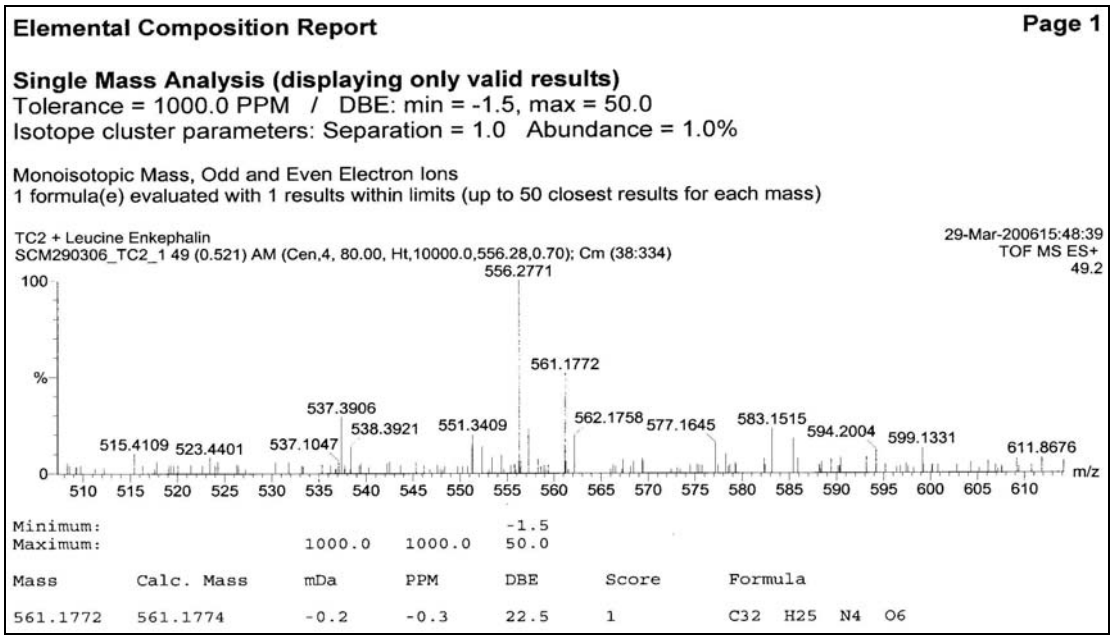
รูปที่ 49 สเปกตรัม ^{1}H-NMR ในช่วง 5.20-7.80 ppm ของสารสีน้ำเงิน TC2 (ใน Acetone-D6)



รูปที่ 50 สเปกตรัม COSY ของสารสีน้ำเงิน TC2 (ใน Acetone-D6)



รูปที่ 51 สเปกตรัม COSY ของสารสีน้ำเงิน TC2 (ใน Acetone-D6) (รูปขยาย)

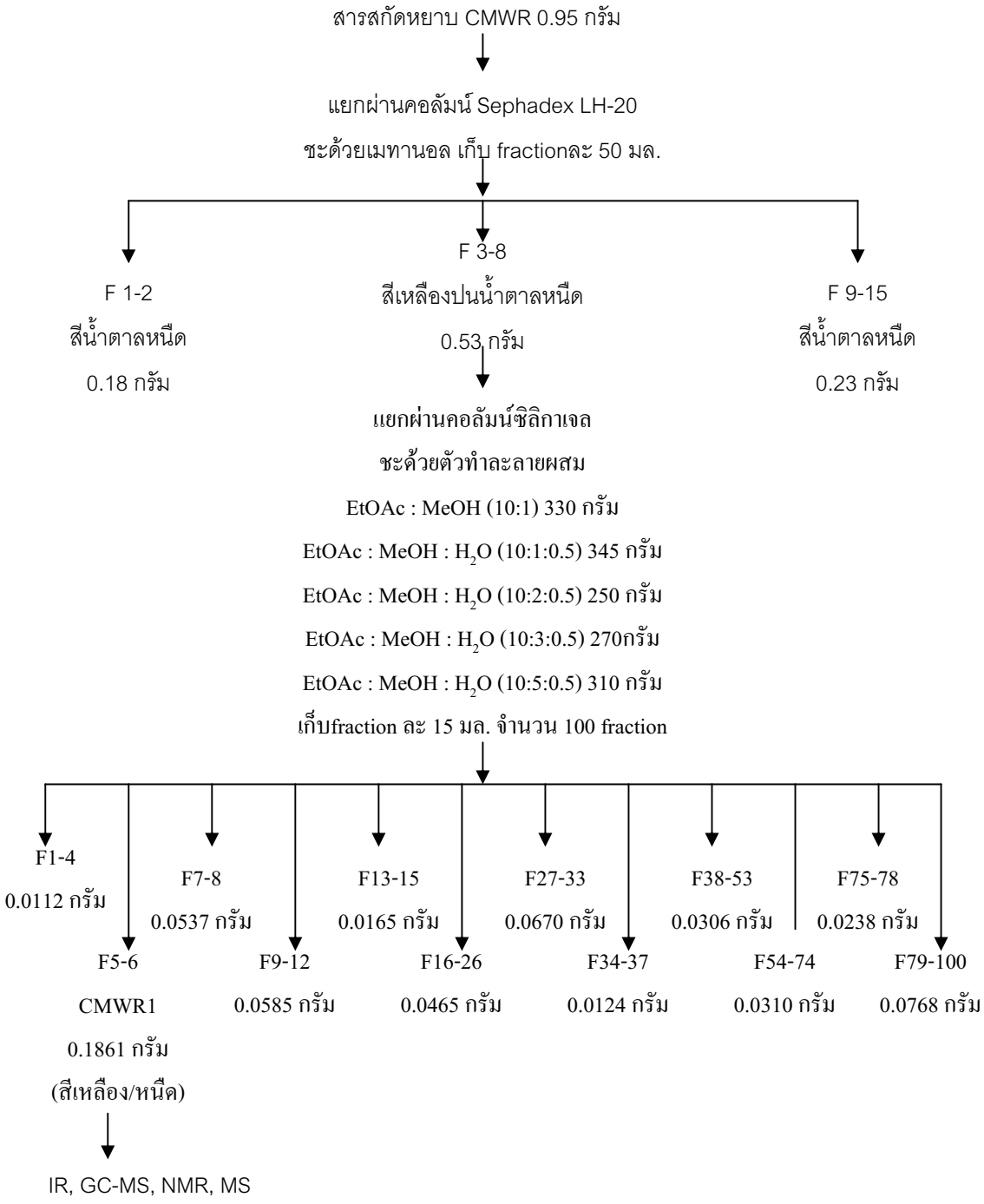


รูปที่ 52 ผลการวิเคราะห์มวลโมเลกุลสาร TC2 ด้วยแมสสเปกตรัม

การทดลองที่ 7 การแยกและตรวจสอบโครงสร้างทางเคมีของสารบริสุทธิ์จากสารสกัดหยาบจากการจับก้อนด้วยไฟฟ้า (CMWR)

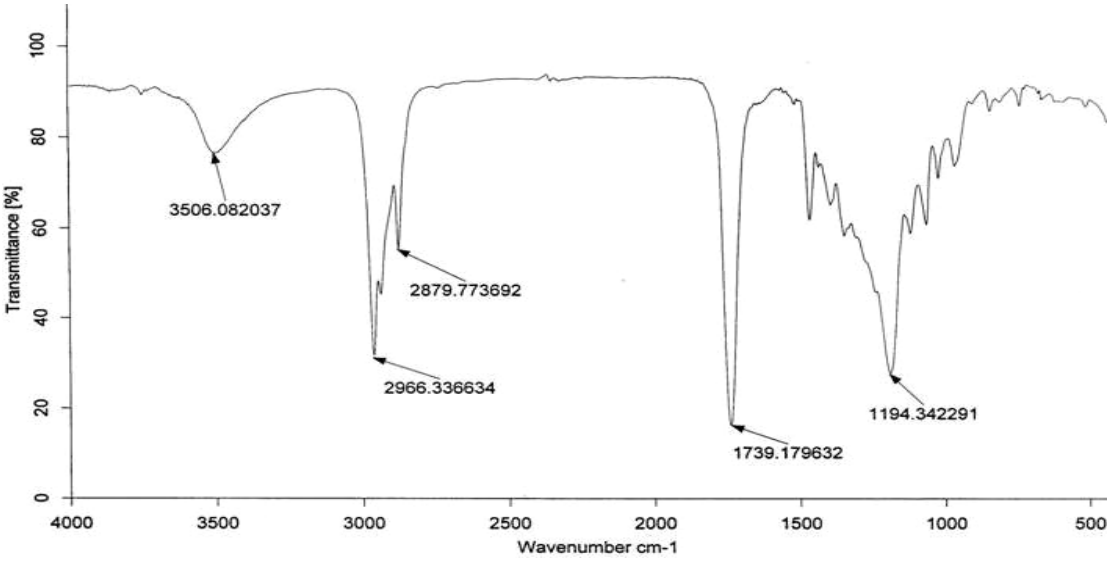
วิธีทดลอง

นำสารสกัดหยาบคืนสภาพของตะกอนจากการจับก้อนด้วยไฟฟ้า (CMWR) 0.95 กรัม (จากการทดลองที่ 5) ไปแยกด้วยโครมาโทรกราฟีคอลัมน์ Sephadex LH-20 ชะด้วยเมทานอล เก็บ fraction ละ 50 มล.จำนวน 15 fractions แล้วรวม fractions 1-2, 3-8 และ 9-15 นำไประเหยตัวทำละลายออกจนแห้ง นำcrude จาก fractions 3-8 (ของเหลวหนืดสีเหลืองปนน้ำตาล) 0.53 กรัมไปแยกผ่านคอลัมน์ซิลิกาเจล ชะด้วยตัวทำละลายผสม เอทิลอะซิเตตและเมทานอล(อัตราส่วน 10: 1, 330 มล.) และเอทิลอะซิเตต เมทานอลและน้ำ(อัตราส่วน 10: 1:0.5, 345 มล. อัตราส่วน 10: 2:0.5, 250 มล. อัตราส่วน 10: 3:0.5, 270 มล. และอัตราส่วน 10: 5:0.5, 310 มล. ตามลำดับ) เก็บ fraction ละ 15 มล. จำนวน 100 fractions จากนั้นรวม fractions 5-6 ซึ่งให้ผลทดสอบเป็นบวกกับสารละลายดราเจนดอร์ฟ (ให้จุดสีส้ม) ไประเหยตัวทำละลายออกจนแห้งได้สาร CMWR1 (0.1861 กรัม) (แผนภาพที่ 6)



แผนภาพที่ 6 ผังการแยกและตรวจสอบโครงสร้างทางเคมีของสารบริสุทธิ์จากสารสกัดหยาบจากการจับก้อนด้วยไฟฟ้า (CMWR)

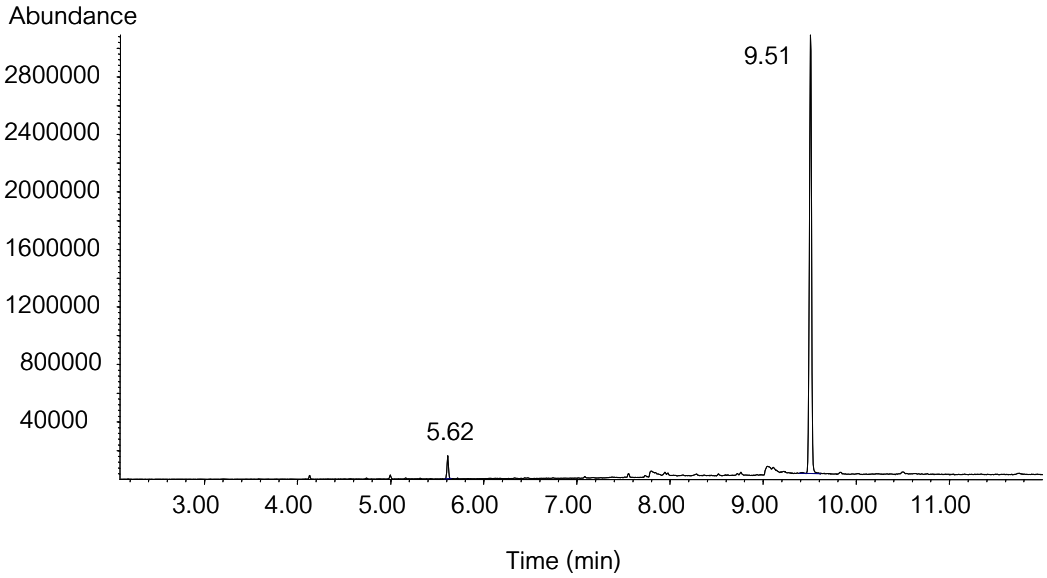
นำสาร CMWR1 ที่แยกได้ไปวิเคราะห์ด้วย IR (รูปที่ 53) และ GC-MS โดยมีรายละเอียดเครื่องมือและสภาวะการวิเคราะห์ดังแสดงในตาราง 20 และโครมาโทแกรมและสเปคตรัมดังแสดงในรูปที่ 54-55 ซึ่งพบว่าเหมือนกับสเป็คตรัมของ butyl citrate



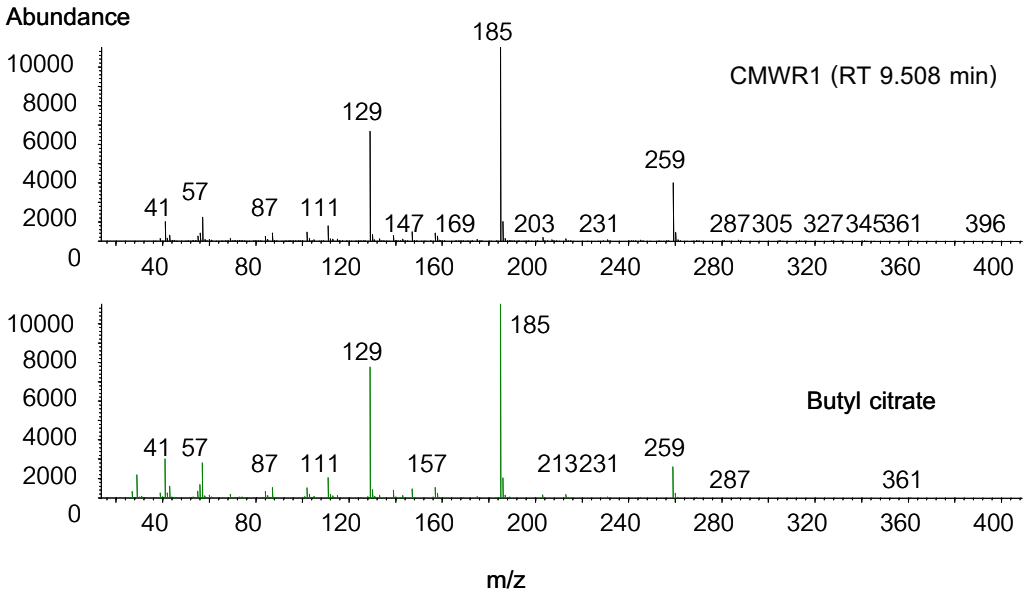
รูปที่ 53 อินฟราเรดสเปกตรัม ของสาร CMWR1

ตาราง 20 รายละเอียดเครื่องมือและสภาวะการวิเคราะห์ CMWR1 ด้วย GC-MS

เครื่องมือ	**รายละเอียด/สภาวะในการวิเคราะห์**	
GC	รุ่น/บริษัท	GC 6890 / Agilent Technology
	ส่วนฉีดสาร (Inlet)	อุณหภูมิ 250°C
	อุณหภูมิตู้อบ (Oven)	เริ่มต้น120°C (คงที่ 30 วินาที) เพิ่มอุณหภูมิ 15°C/นาทีจนถึง 240°C (คงที่4 นาที)
	แก๊สพา (Carrier gas)	ฮีเลียม(He) อัตราการไหล 1.0 มล./นาที
	คอลัมน์ (Column)	คอลัมน์ HP-5 (5% phenyl methyl sioxane) ความยาว 30 เมตร เส้นผ่าศูนย์กลาง 0.25 มิลลิเมตร ความหนาของลิควิดเฟส 0.25 ไมโครเมตร
MS	รุ่น/บริษัท	MSD 5973 / Hewlett Packard
	พลังงานอิเล็คตรอน(EI)	70 eV
	อุณหภูมิ MS Quadrupole	150°C
	อุณหภูมิ MS Source	230°C



รูปที่ 54 GC ของสาร CMWR1



รูปที่ 55 แมสสเปกตรัมของสาร CMWR1 เทียบกับ Butyl citrate จากฐานข้อมูล WILEY275

เอกสารอ้างอิง

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Output ที่ได้จากโครงการ

ก. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับชาติและนานาชาติ

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ข. จำนวนนักวิทยาศาสตร์รุ่นใหม่

ได้นักวิทยาศาสตร์รุ่นใหม่ที่มีประสบการณ์ในงานวิจัยเกี่ยวกับการสกัดสารโดยวิธีจับก้อนด้วยไฟฟ้าจำนวน 5 คน ได้แก่

1. นางสาวกัลยา จำปาทอง (นักศึกษาปริญญาเอก)
2. นางสาวนีรนุช ไชยรังสี (นักศึกษาปริญญาเอก)
3. นายวรณ์ ดอนชัย (นักศึกษาปริญญาโท)
4. นางสาวณัฐพร พุทธวงศ์ (นักศึกษาปริญญาโท)
5. นางสาวประไพ ประดับคำ (นักศึกษาปริญญาโท)

ค. องค์ความรู้ใหม่

ได้องค์ความรู้ใหม่เกี่ยวกับการประยุกต์การจับก้อนด้วยไฟฟ้าในการสกัดแยกองค์ประกอบทางเคมีจากพืช ซึ่งสามารถนำไปใช้ประโยชน์ในทางปฏิบัติได้ เช่น

- สามารถนำไปสกัดสารสำคัญ (asiaticoside) จากใบบัวบกในระดับอุตสาหกรรม ซึ่งกำลังดำเนินการอยู่ ณ นิคมอุสาหกรรมลำพูนในขณะนี้ (โดย ดร.พิเชษฐ์ วิริยะจิตรา จากบริษัท Asian Life)
- สามารถนำไปสกัดสารสำคัญ (plumbagin) จากรากเจตมูลเพลิงแดงในระดับอุตสาหกรรมเพื่อจำหน่ายในราคาที่ต่ำกว่าที่ขายในต่างประเทศ (ดำเนินการโดย ดร.วีรชัย พุทธวงศ์ จากมหาวิทยาลัยแม่โจ้)

ภาคผนวก

(Reprints และ manuscript ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการ
ระดับชาติและนานาชาติ)

Chapter

ELECTROCOAGULATION IN AQUEOUS ALCOHOLIC SOLUTIONS

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ABSTRACT

As a model study of electrolytic decolourisation of aqueous alcoholic extracts of natural products, electrocoagulation of a green dye (chlorophyllin) and a yellow dye (crocin) was performed in aqueous alcoholic solutions, using aluminium and iron plates as electrodes and sodium chloride as supporting electrolyte. The aqueous alcoholic solutions studied contained 25, 50, 75, and 85% (v/v) ethanol. Effective decolourisation was observed in every experiment performed.

To illustrate the potential application of the above electrochemical technique to the isolation of natural products, extraction of a known plant (*Centella asiatica*) and an unstudied one (*Lithocarpus elegans*) was carried out with the inclusion of an electrocoagulation step in the isolation process for each plant.

INTRODUCTION

The first step in studying a majority of natural products from plants generally consists of extraction of substances of interest, which are normally secondary metabolites, from the crude

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extract. A major problem in this step is to eliminate the unwanted so-called organic "matrix" materials, notably various plants pigments, tannins, and biopolymers (eg. carbohydrates, proteins). In a laboratory scale or even larger, this step is almost always carried out by a combination of solvent extraction and fractionation, and various chromatographic techniques. This usually involves using toxic organic solvents, and expensive specific adsorbents and a large volume of organic eluents, again consisting of mixtures of organic solvents. Also, both the adsorbents and the solvents after being used normally cannot be recycled or recovered, and consequently become foul and hazardous chemical wastes. Thus, if an alternative technique can be used to wholly, or even partly, replace the solvent fractionation and chromatographic separation, this may contribute toward lowering the expenditure of extraction of natural compounds from natural sources. Certainly, it will help reduce the amount of adsorbents and solvents used and subsequently cast away as contaminating wastes.

Electrocoagulation is originally an electrochemical technique by which a variety of unwanted dissolved particles and suspended matter, both organic and inorganic, can be effectively removed from an aqueous solution by electrolysis [1-18]. Our preliminary work [19-24] on electrocoagulation, which is an extension of the work on extraction of stevioside by Miwa *et al.*[25], shows that this technique, normally applied to the treatment of various aqueous organic and inorganic wastes, or as a method in clarifying potable water in some areas [26, 27], may be incorporated as a step in isolating certain kinds of organic compounds, including a few natural products, resulting in the reduction of the usage of organic solvents and chromatographic adsorbents.

Electrocoagulation seems to be a suitable method for clarifying the aqueous solution of a plant extract, rendering it free from the above interfering organic "matrix" materials, and thus leaving the compounds of interest more readily processable. By this method we have isolated caffeine from tea [28]; stevioside, a sweet glycoside from stevia (*Stevia rebaudiana)* [20]; glycyrrhizin, a sweet glycoside from *Glycyrrhiza radix* (licorice) [19]; D-pinitol, a cyclic polyol, from *Cassia siamea* [21]; and mukurrozioside, a saponin, from *Sapindus rarak* [24].

However, all of the work above dealed with aqueous extracts, and, to our knowledge, there has been no other investigations on electrocoagulation that is performed in organic solvents or aqueous organic solvents, the systems that are undoubtedly more useful in isolating natural products in general. Thus, in the present work we report the results of our preliminary study on electrolytic removal of colouring matters in aqueous alcoholic solutions using model compounds, i.e. chlorophyllin (a representative green dye derived from chlorophyll) and crocin (a representative yellow dye which is a glycoside of the carotenoid crocetin). After that, to illustrate the potential application of the above electrochemical technique to the isolation of natural products, extraction of a known plant (*Centella asiatica*) and an unstudied one (*Lithocarpus elegans*), which were carried out employing electrocoagulation as a step in the isolation process for each plant, is presented.

ELECTROCOAGULATION EXPERIMENTS

Materials and Methods

Chemicals

Absolute ethanol and sodium chloride (98.0%) were purchased from E. Merck (Darmstadt, Germany). Chlorophyllin (sodium-copper salt) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Crocin was purchased from Fluka Chemie AG (Buchs, Switzerland). Acetone used was of a commercial grade.

Equipment

Direct current was sustained by a GW Instek DC power supply; visible absorbance was measured on a Perkin-Elmer Lambda 25 and a Genesys10 spectrophotometer.

Electrolytic Decolourisation and Colour Measurement

Aluminium and iron plates (15.0×3.0 cm) were washed prior to use with acetone to remove surface grease. A pair of aluminium or iron plates was immersed 1.5 cm apart and 7.0 cm deep into 200 ml of various aqueous alcoholic solutions (0, 25, 50, 75, 85 %) of 0.025 % w/v chlorophyllin or crocin in a beaker (diameter: 5.7 cm, height: 10.8 cm). These solutions were agitated throughout the experiment with a magnetic stirrer (250 r.p.m.). Sodium chloride (0.2 g) was added as supporting electrolyte. Direct current (0.3A, 10-30V) was then passed through the solution *via* the two electrodes. At every 15-minute interval during a 2-hour period of electrolysis, a 4-cm^3 aliquot sample of the solution was drawn and centrifuged for 10 minutes, and the absorbance of the supernatant solution was measured at an appropriate wavelength of the absorption maximum for each dye as follows: chlorophyllin: 626 nm (green range) and 406 nm (yellow range); crocin: 440 nm (yellow range). The measured absorbance was then converted to the residual weight percentage of chlorophyllin or crocin by a calibration curve obtained by a plot between the absorbance *versus* the concentration for each compound.

Results and Discussion

The mechanism of coagulation by electrolysis has been elaborated by Mollah *et al.* [29] in their review of electrocoagulation and its application for waste water treatment. When aluminium is used as a sacrificial electrode, the coagulating agent is usually aluminium hydroxide, its polymer $Al_n(OH)_{3n}$, or various forms of gelatinous charged hydroxo cationic Al complexes, which can effectively remove pollutants by adsorption to produce charge neutralization and by enmeshment in a precipitate. Al^{3+} produced *in situ* may also react directly with acidic species such as carboxylic acids and phenolic compounds present in the solution. When iron is used as the sacrificial electrode, Fe^{2+}, Fe^{3+}, $Fe(OH)_2$, $Fe(OH)_3$, and $Fe(OH)_n$ are formed. The $Fe(OH)_n$, present as a gelatious suspension, can remove pollutants either by complexation or by electrostatic attraction, followed by coagulation. Other species

which may be formed, depending on the pH of the solution, include $Fe(OH)^{2+}$, $Fe(OH)_2^{+}$, $Fe(OH)_6^{-}$, and $Fe(OH)_4^{-}$. It is, therefore, quite apparent that electrocoagulation of both cationic and anionic species is possible by using iron as the sacrificial electrode.

In the first part of this experiment, the efficiency of chlorophyllin precipitation by each type of electrode was investigated. The plots of the residual weight percentage of chlorophyllin *versus* electrolysis time for various aqueous alcoholic solutions are shown in Figures 1A and 1B. In Figure 1A, with aluminium as electrodes, the results show that cholorophyllin was effectively removed at every concentration of ethanol used although the result of 50 % ethanol giving the lowest removal efficiency is rather surprising. In the case of iron being used as electrodes, the efficiency of chlorophyllin removal in all aqueous alcoholic solutions (0, 25, 50, 75, 85 %) was high (Figure 1B).

After electrolysis for 2 hours, some of the chlorophyllin solutions were colourless, while some were of a pale yellow. The 0 and 25 % ethanolic solutions and 0-50 % ethanolic solutions were colourless when Al and Fe were used as electrodes, respectively. On the other hand, the solutions of 50-85 % ethanol concentrations (Al as electrodes) and 75 and 85 % ethanol concentrations (Fe as electrodes) were a clear pale yellow. Accordingly, the absorbance of these solutions were measured again at the wavelength of the absorption maximum for the yellow range (406 nm). The results are shown in Figures 2A and 2B. It is clear from the figures that the decolourisation of chlorophyllin in the yellow range was of the same efficiency as that in the green range.

Figures 3A and 3B show the plots of the residual weight percentage of crocin *versus* electrolysis time for various alcoholic solutions when Al and Fe were used as electrodes, respectively. As shown in Figure 3A, the coagulation of crocin seems to fluctuate somewhat when alumimiun was used as electrodes. The results reveal that crocin was effectively removed in 75 and 85 % ethanol. In the case of 0, 25, and 50 % ethanol, the removal efficiency decreased when the concentration of ethanol in the mixture was increased. Using iron as electrodes, on the other hand, the efficiency of coagulation was high in all alcoholic solutions.

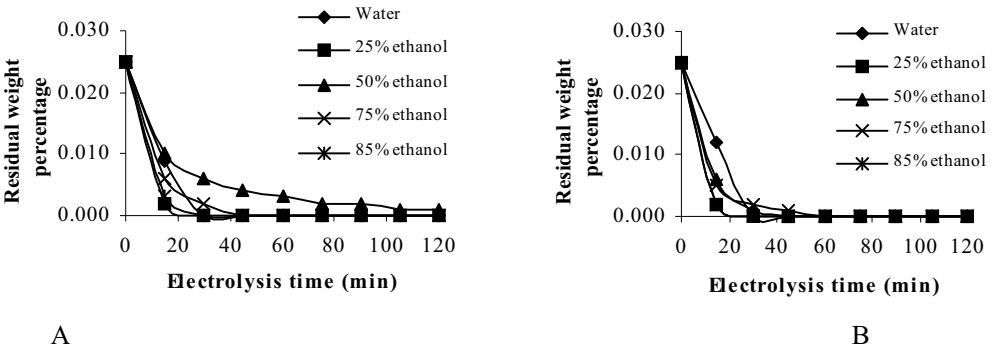


Figure 1. Plots of residual weight percentage and electrolysis time for each ethanol concentration of chlorophyllin solution at 626 nm; A: with aluminium as electrodes, B: with iron as electrodes; ◆, 0% ethanol (water); ■, 25 % ethanol; ▲, 50 % ethanol; ×, 75 % ethanol; ✻, 85 % ethanol.

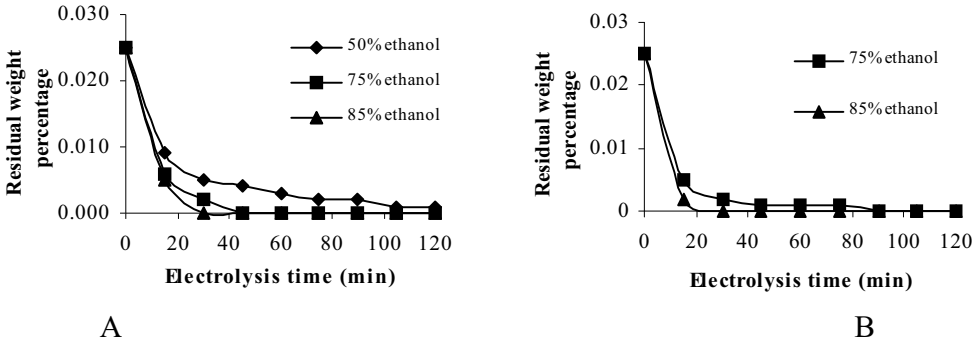


Figure 2. Plots of residual weight percentage and electrolysis time for each ethanol concentration of chlorophyllin solution at 406 nm; A: with aluminium as electrodes, B: with iron as electrodes; ▲, 50%ethanol; ■, 75%ethanol; ▲ , 85%ethanol.

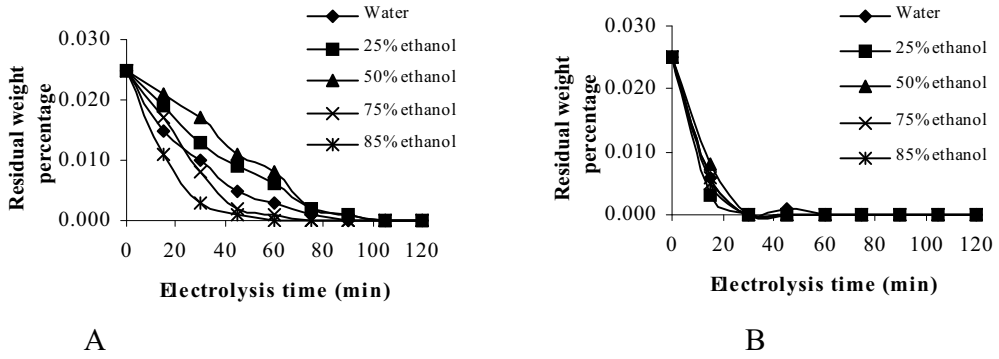


Figure 3. Plots of residual weight percentage and electrolysis time for each ethanol concentration of crocin solution at 440 nm; A: with aluminium as electrodes, B: with iron as electrodes ; ◆, 0 % ethanol (water); ■, 25 % ethanol; ▲, 50 % ethanol; ×, 75 % ethanol; ✻, 85 % ethanol.

Thus, in concluding, electrocoagulation in aqueous alcoholic solutions has been demonstrated to be practical, at least with model compounds used in this experiment, in which a green dye and a yellow dye were efficiently coagulated from the aqueous alcoholic solutions of various concentrations. Since these two dyes are representative to some extent of those colouring matters normally found in the majority of plant extracts, it is evident that electrocoagulation may be of use in removing these usually undesirable contaminants from the aqueous alcoholic plant extracts in practice.

APPLICATIONS TO NATURAL PRODUCTS ISOLATION

Centella Asiatica

Discussion

Centella asiatica, or Asiatic Pennywort, is a herb found in the marshy areas throughout India and Ceylon. It is also found in subtropical Africa, South America, southern parts of America and also widespread in Southeast Asia. In Thailand, it is called "Bua Bok", literally "land lotus", because of its miniature lotus-like leaf. The plant is greatly valued for its time-honoured use as an efficient wound healing promotant, among many other activities. This has been demonstrated to be due mainly to the presence of asiaticoside in the leaves [30-34]. Asiaticoside, or *O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(2α,3β,4α)-2,3,23-trihydroxyurs-12-en-28-oate, is a triterpenoid trisaccharide glycoside [30].

Isolation of asiaticoside from the leaves of *C. asiatica* was normally carried out using a series of solvent extraction and fractionation. For example, Pasich *et al.* [35] defatted the plant material first with benzene, then extracted the defatted material with ethanol. The concentrated ethanolic extract was further extracted with acetone, and the acetone-soluble fraction was repeatedly extracted with ether. The ether-soluble fraction was treated with a cation exchange resin. The resulting supernatant solution was decanted, treated with absolute ethanol in excess, and the raw asiaticoside fraction setting out was recrystallised. Luo and Chin [36] used 95% ethanol as extracting solvent, decolourised the solution with active carbon, treated the concentrated alcoholic solution with petroleum ether, diethyl ether, and ethyl acetate, and then extracted with water-saturated butanol to give a fraction containing asiaticoside, which was crystallised. Kim *et al.* [37] extracted the plant material with an aqueous alcoholic solution, which was then treated with a halogenated solvent. The aqueous layer was extracted with a higher alcohol, whose layer was washed several times with an aqueous alkaline solution, then with water, concentrated in vacuo, and the resulting residue crystallised.

In this study, electrocoagulation with iron as electrodes and sodium chloride as supporting electrolyte, was performed on an aqueous ethanolic extract of *C. asiatica* to eliminate plant pigments, fatty material, and other suspended particles. The resulting decolourised solution was passed through a column of a mixed-bed ion exchange resin to trap the added electrolyte, as well as further purify the extract. The resulting demineralised solution, after concentration, was eluted through a silica gel column with aqueous alcohol to afford a major component. This was easily recrystallised as a pure white solid, which was obtained in 0.036 % (from fresh leaves). Characterisation of this solid as asiaticoside was carried out by a spectroscopic study. The main advantage of this isolation technique of asiaticoside was a minimum use of different types of organic solvents, viz. only alcohol (ethanol and methanol) was used throughout the process.

Experimental

Fresh leaves of *C. asiatica* (670 g) were extracted with 25 % (v/v) ethanol (1,000 ml) by heating at reflux for 3 hours. After one coarse filtration, 800 ml of a dark green ethanolic

aqueous extract was obtained. Two iron plates (dimension 30 x 10 x 0.05 cm) were used as electrodes for electrocoagulation of the extract. These were vertically dipped 3 cm apart and 9 cm deep into a magnetically-stirred aqueous ethanolic solution in a round glass jar. Sodium chloride (2 g) was added, and direct current (0.5 A, 22 V) from a DC power supplier was then passed through the solution *via* the two electrodes for 2 hours. The resulting mixture was filtered and the filtered solution obtained was subjected to a second electrolysis for 30 minutes under the same condition. After filtration, a clear pale yellow solution (700 ml) was obtained. This doubly-electrolysed solution was passed through a column of a mix-bed ion-exchange resin (Amberlite MB-1, 200 ml), and the resulting clear, colourless aqueous solution (conductivity < 50 μS $cm^{-}1$) was collected. Evaporation gave a liquid residue (3.89 g), which showed an intense spot and some light spots on a RP18 TLC plate (70 % MeOH as eluent, and visualised by spraying with 30 % H_2SO_4 and heating). The residue was chromatographed over a RP18 silica gel column (70 % MeOH as eluent) to collect the major component, which easily crystallised in methanol-water as a white amorphous solid (0.24 g, 0.036 % from fresh leaves), m.p. 229-231^{o} (literature value for asiaticoside = 230-232^{o}) [38]. Characterisation of asiaticoside was carried out by NMR, and mass spectrometry. (See below)

Spectroscopic Study

All ^{1}H NMR spectra (1D and 2D) were measured on a Varian Mercury 500 MHz spectrometer with CD_3OD as solvent; ^{13}C NMR spectra and DEPT were run on a Varian Mercury 300 MHz spectrometer with CD_3OD as solvent; mass spectrum was recorded on a VG Quattro LC-MS.

ES-MS (Positive ion) *m/z* (% relative intensity) 959 $[M]^{+}$ (29), 797 $[M\text{-}Rha]^{+}$ (9), 635 (15), 499 (20), 381 (30), 359 (100), 214 (87), 157 (40), 71 (45); ^{1}H NMR (500 MHz, CD_3OD) δ 0.90 (3H, *d*, *J*=6.6 Hz, Me-29), 0.97 (3H, *d*, *J*=6.4 Hz, Me-30), 0.71, 0.84, 1.05, 1.13 (each 3H, *s*, Me-24, Me-26, Me-25, Me-27), 1.27 (3H, *d*, *J*=6.1 Hz, Me of Rha), 1.38 (2H, *m*, H-19), 2.24 (1H, *d*, *J*=10.8 Hz, H-18), 3.34 (1H, *d*, *J*=11.7 Hz, H-3), 3.46 (2H, *d*, *J*=10.8 Hz, H-23), 3.67 (1H, *ddd*, *J*=10, 9.5, 4 Hz, H-2), 4.37 (1H, *d*, *J*=7.3 Hz, H-1 of Glc), 4.84 (1H, *br s*, H-1 of Rha), 5.24 (1H, *br t*, *J*=3.4, H-12), 5.29 (1H, *d*, *J*=8.1 Hz, H-1 of Glc); ^{13}C NMR (75 MHz, CD_3OD) δ 47.3 (C-1), 69.4 (C-2), 76.9 (C-3), 42.9 (C-4), 47.4 (C-5), 17.8 (C-6), 32.4 (C-7), 39.7 (C-8), 48.3 (C-9), 37.8 (C-10), 23.3 (C-11), 125.7 (C-12), 138.1 (C-13), 42.2 (C-14), 28.0 (C-15), 24.0 (C-16), 48.5 (C-17), 52.9 (C-18), 39.0 (C-19), 39.2 (C-20), 30.5 (C-21), 36.4 (C-22), 65.0 (C-23), 12.8 (C-24), 16.6 (C-25), 16.7 (C-26), 22.8 (C-27), 176.7 (C-28), 16.4 (C-29), 20.4 (C-30).

The crystallised solid was analysed for $C_{48}H_{78}O_{19}$ by its ^{13}C NMR data as well as from the ES (positive ion mode)-MS data at *m/z* 959 [M]+ and 797 [M-Rha]+. The ^{1}H and ^{13}C-NMR spectra display resonances due to the six methyl signals (four singlets, a broad singlet, and a doublet) indicating the presence of a Δ12-ursene skeleton. The sugar part of the 1H NMR spectrum (Figure 4) showed, beside other signals, the three doublets for the three anomeric protons (δ 5.29 *d*, *J*=7.9 Hz; 4.84, *d*, *J*=1.5 Hz; 4.37, *d*, *J*=7.9 Hz) indicating that it contains three monosaccharides with many overlapping multiplets, which present an unambiguous assignment even by performing an ordinary COSY experiment However, the 2D TOCSY experiment allows an easy identification of the three sugar spin systems. The

sequence of three sugar chains was deduced unequivocally from the HMBC and TOCSY information, as illustrated in Figures 5, 6, and 7.

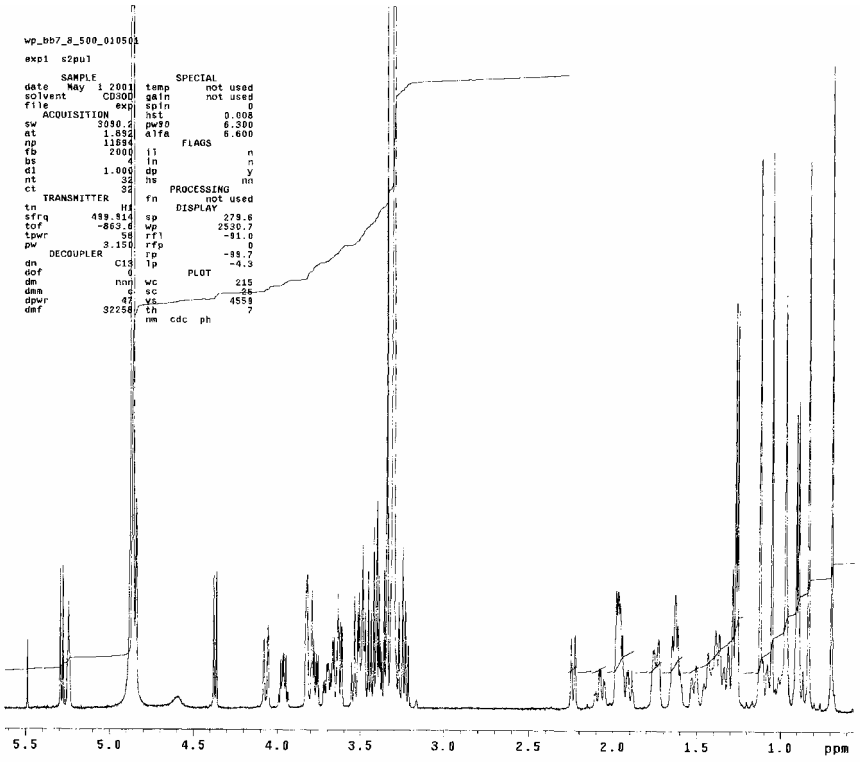
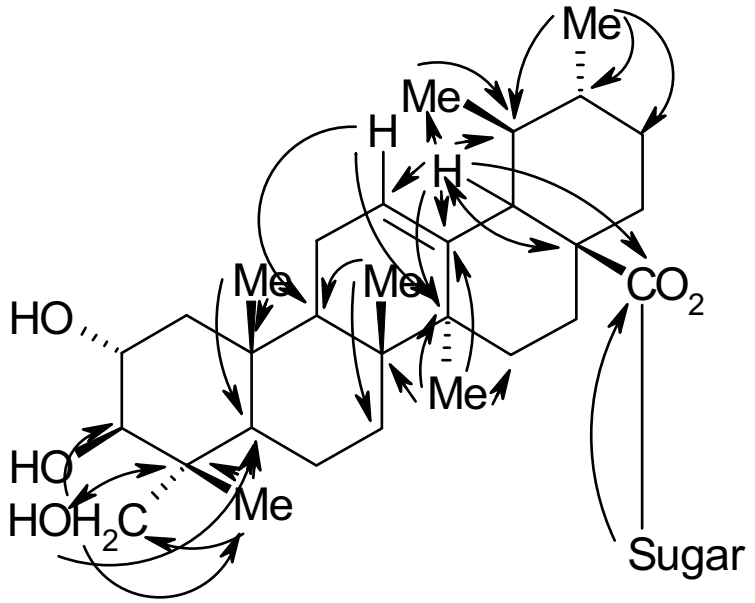
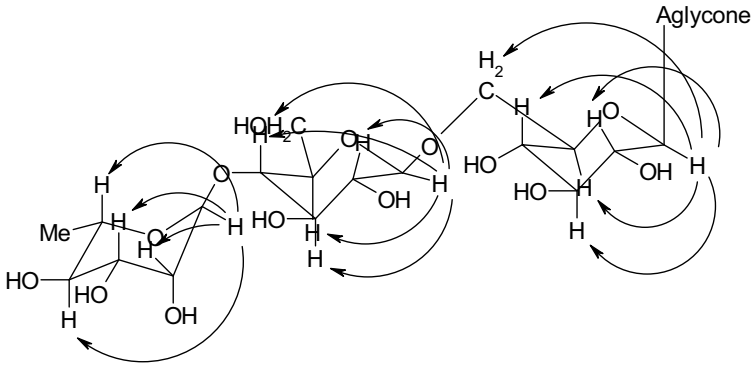


Figure 4. 500 MHz ^{1}H-NMR spectrum (methanol-d_4) of the crystallised solid.



HMBC



TOCSY

Figure 5. The sequence and linkage position of monosaccharide and triterpenoid established by HMBC and TOCSY experiments.

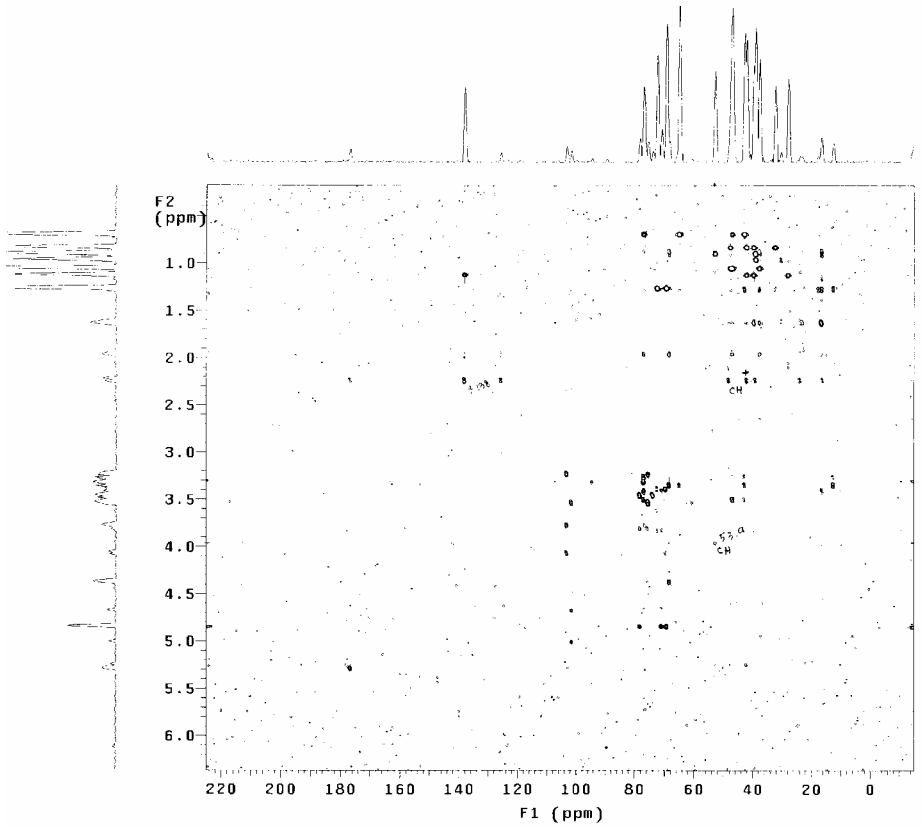


Figure 6. 500 MHz HMBC NMR spectrum for the sequence and linkage position of monosaccharide and triterpenoid (methanol-d_4) of the crystallised solid.

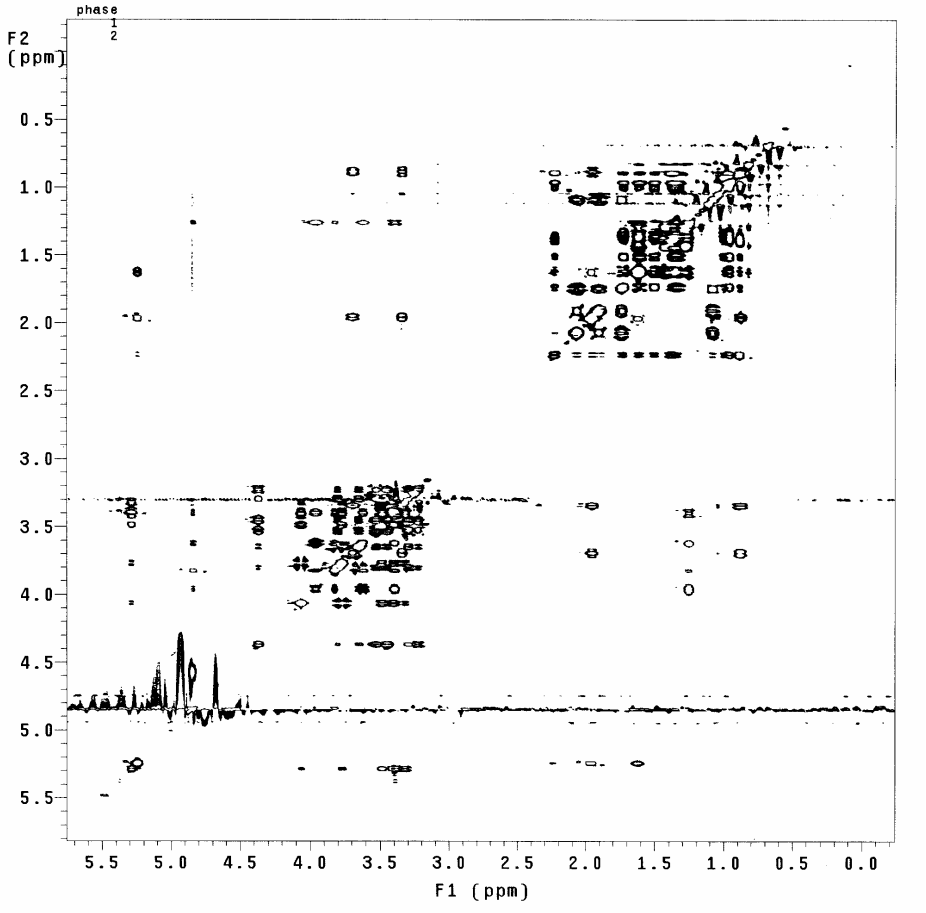


Figure 7. 500 MHz TOCSY NMR spectrum for the sequence and linkage position of monosaccharide and triterpenoid (methanol-d_4) of the crystallised solid.

The glycoside was acetylated with acetic anhydride-pyridine and then flash-chromatographed to give the acetate form, which showed the ^{1}H spectrum of acetate grouping indicating 12 hydroxyl groups in the structure. The ^{13}C NMR spectrum showed 48 signals, of which 30 were assigned to the triterpenoid moiety, and 18 to the saccharide portion units. From the HMBC spectrum, the long-range correlation of H-1 (δ 5.29) of Glc' and H-18 (δ 2.23) of the aglycone with C-28 (δ 176.7) established that the sugar part was attached to C-17 of the aglycone.

Lithocarpus Elegans

Discussion

Lithocarpus elegans is a forest evergreen tree known in Thailand as “Gor Mone” with no information on its chemical constituents so far available in the literature. The phytochemical

investigation of other members of the genus *Lithocarpus* (family: Fagaceae) reveals the presence of triterpenoids, steroids, and flavonoids [39-45]. *L. elegans* was selected for our study in the present work for the pure reason of its accidental availability to us when we came across it being left knocked down in the forest by someone. Its trunk when cut showed a bright red colour of the bark, which was then collected for this study.

Since the chemical constituents of the plant were unknown, we separately extracted the collected bark both by a standard method and that which incorporated an electrocoagulation step, in order to test the effect the electrocoagulation might have on the extraction process. In the former method, the bark was extracted with aqueous ethanol to give a crude extract, which was solvent fractionated into 2 fractions, viz. the water-methanol and the dichloromethane-water fractions. The first fraction was then further solvent fractionated and chromatographed to give 3 known flavonoid compounds: catechin, epicatechin, and proanthocyanidin B. The latter fraction was chromatographed to give 3 known triterpenoid compounds: lupeol, betulin, and betulinic acid. (See Figure 8.)

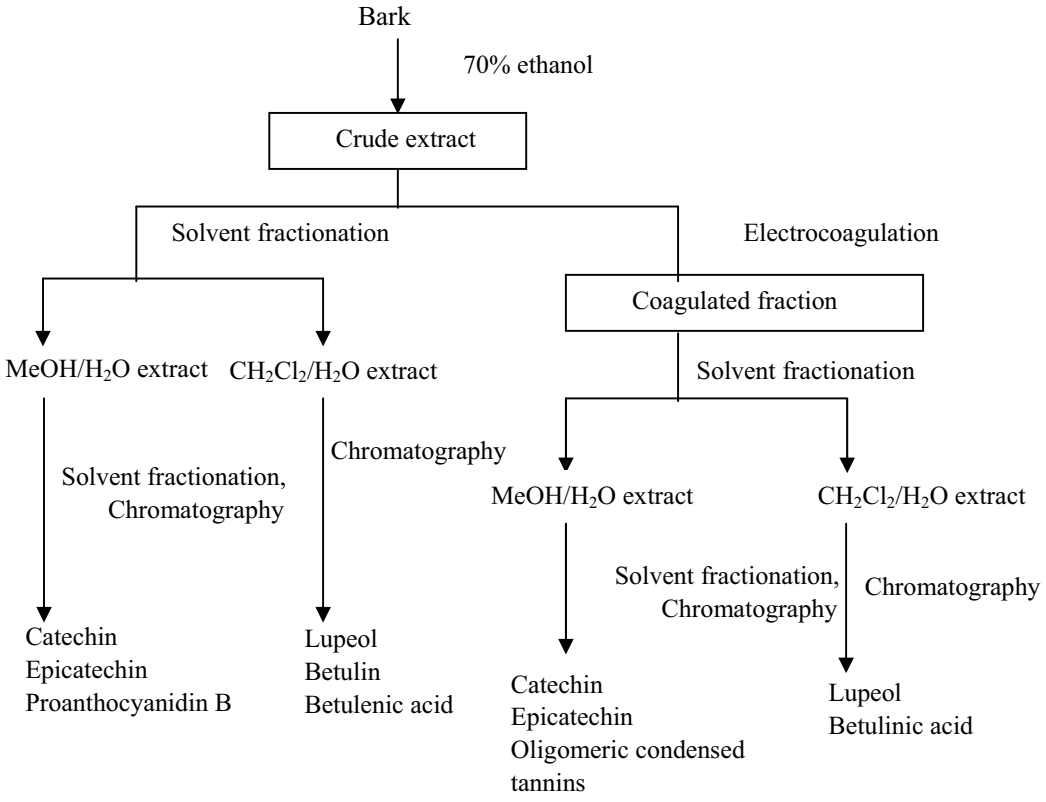


Figure 8. Extraction scheme for the bark of *L. elegans*

In the procedure in which an electrocoagulation step was included (Figure 8), the original crude extract was electrocoagulated in 25 % ethanol and a coagulated fraction was obtained, which was then treated similarly by solvent fractionation to give the water-methanol and the dichloromethane-water fractions. The former fraction, after identical treatment as above, also gave more or less the same set of flavonoid compounds. The latter fraction after the same

treatment as above, however, gave somewhat different results, i.e. only 2 triterpenoid compounds were present: lupeol and betulinic acid, while betulin was absent. Many other minor compounds present in the dichloromethane-water fraction of the original crude extract were also missing, thus giving a much cleaner appearance of the TLC chromatogram (Figure 9). These missing components were presumably left uncoagulated in the electrolysed solution, which, unfortunatedly, we had no time to examine further.

Thus, from the above results, it seems that, firstly, the electrocoagulation step, normally performed with aqueous extracts of natural products, can also be carried out in an aqueous alcoholic solution, a more useful system. Secondly, the electrocoagulation condition used in this experiment does not affect the integrity of the isolated components present in the extract of natural products. And thirdly, the electrocoagulation step can be of use in fractionating those components into 2 main fractions, viz. the coagulated and the uncoagulated parts. Other things being equal, large molecules tend to be preferentially coagulated, as well as compounds with certain functional groups, e.g. carboxyl group and 1,2-disubstituted phenolic groups [46-47]. In the above experiment, however, why lupeol, a hydroxytriterpene, was coagulated, but betulin, a similar triterpene with 2 hydroxyl groups, was not coagulated is still not readily explainable.

Experimental

Plant Material

The stem bark of *Lithocarpus elegans* was collected from Doi Suthep-Pui National Park, east side of Doi Suthep at Pah Lad Temple, Chiang Mai , Thailand, on 6 September 2000.The plant material was identified by J. Maxwell from the Department of Biology at Chiang Mai University. A voucher specimen was deposited at the Department of Biology Herbarium, Chiang Mai University with the Accession Nos. S-01 and S-12.

Instrumentation

Melting points were recorded either on a Fisher-Johns melting point apparatus or a Gallenkamp melting point apparatus. Electrospray (ES) mass spectra were obtained on a Finigan Mat 900 XL double focussing magnetic sector spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) experiments, including COSY, HMQC, and HMBC techniques were carried on a Bruker DRX –500 (500 MHz). Chemical shifts (δ) were recorded in part per million (ppm). Reverse phase high performance liquid chromatography (RP HPLC) was connected to a Waters 501 pump or an ICI Instruments 1500 pump connected with the Diode array detection. TLC was carried out on aluminium plates coated with Merck silica gel 60 F_{254}. NPCC (normal phase flash column chromatography) was carried out using silica gel S (0.032 – 0.063 mm) under a pressure of compressed air.

Extraction and Analysis of the Crude Bark Extract

The dried stem bark of *L. elegans* (1.4 kg) was extracted with 70% ethanol (4 litres) at room temperature for 2 days. The red extract was filtered and the remaining bark reextracted.

This procedure was repeated until a pale red solution was afforded. The combined solution was evaporated and dried under vacuum to afford the crude ethanolic extract (170 g) as a deep red solid substance. A portion (5.58g) of this ethanolic extract was dissolved in methanol:H_2O (1:1, 80 ml) and partitioned with CH_2Cl_2 (30 ml) to give, after evaporation, the CH_2Cl_2/H_2O fraction (1.58 g) and H_2O/MeOH fraction (4.6 g). The CH_2Cl_2/H_2O fraction (LC29-DCM) was then chromatographed over a silica gel column eluted with the gradient hexane, CH_2Cl_2, EtOAc, and MeOH, respectively. The resulting eluate was monitored by TLC (developed with acetone:hexane = 1:5) to yield 8 fractions, i.e. fractions 25-1 to 25-8. Fraction 25-5 was recrystallised from MeOH to yield lupeol (15 mg). Fraction 25-8 was then separated by reverse phase HPLC, which was carried out with C-18 Phenomenex R 10x250 mm column, with the diode array detector at 202 nm. The elution conditions were as follows: flow rate 2.0 mlmin-1, temperature 28^o, gradient solvent system A (100 % H_2O) and B (100% MeOH). The samples were eluted according to the following binary gradient: 70 % B reaching 80 % B at 20min, 85 % at 30 min and 90 % B at 50 min. Betulin (4 mg) and betulinic acid (3 mg) were obtained at retention times 41.6 and 39.9 min., respectively. Characterisation of the isolated lupeol, betulin, and betulinic acid was done by comparison of their respective spectra (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) with those reported in the literature [41, 48, 49-52].

The H_2O/MeOH fraction was partitioned with 1-butanol (2x30 ml) to gain, after evaporation, the butanol-soluble extract (3.59 g), which was subjected to gel filtration chromatography using a column prepacked with methacrylate copolymer matrix: Fractogel TSK HW-40 (particle diameter 25-40 μm, MerckR) of length 38 cm, with i.d. 2 cm. The column was connected with a peristaltic pump and eluted with gradient polarity eluents (70 % aqueous MeOH, 80 % aqueous MeOH, MeOH:H_2O:acetone = 7:2:1, 6:2:2, 5:2:3, 3:2:5). The filtrate passing through the column was monitored with TLC (developed with 1-butanol:CH_3COOH:H_2O = 6:1:2, and visualised under short UV wavelength at 254 nm, together with spraying with 2 % $FeCl_3$ alcoholic colouring reagent) to yield 8 fractions, viz. LC29t1-LC29t8. The LC29t1 (50 mg) was further separated by reversed phase HPLC, which was carried out with C-18 Phenomenex, spherex column, 10x250 mm, with a UV detector at 280 nm. The elution condition was as follows: flow-rate 1.0 ml min-1, temperature 28^o, gradient solvent system A (0.1 % CH_3COOH in water) and B (100 % MeOH). The system of binary gradient eluent was started with 5 % B in order to reach 50 % B at 20 min, 80 % B at 25 min and 100 % B at 30 min, to afford catechin (12 mg) and epicatechin (18 mg) at retention times 10.20 and 11.80 min., respectively. The LC29t2 fraction (30 mg) was investigated by NMR and MS spectrometry, which showed that it was a mixture containing proanthocyanidin B as a major constituent The other remaining fractions, according to their NMR spectral data, contained oligomeric condensed tannins as the main components, which were not purified further. Characterisation of the isolated catechin, epicatechin, and proanthocyanidin B was done by comparison of their respective spectra (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) with those reported in the literature [53-55].

Electrolysis and Recovery of Electrocoagulated Compounds

A portion of the original crude *L. elagans* ethanolic extract (1.67 g) was dissolved in 25 % ethanol in water (1 litre). The resulting solution was placed in a glass jar (diameter 11 cm, height 23 cm). Two aluminium plates (dimension 30x10x0.05 cm) were used as electrodes.

These were dipped vertically 3 cm apart and 9 cm deep into the magnetically-stirred solution. Sodium chloride (2 g) was added as supporting electrolyte. Direct current (2.0A, 16V) from a DC power supplier was then passed through the solution *via* the two electrodes for 2 hours. The resulting mixture was filtered and the coagulum was collected. The coagulated substances were recovered [46] by dissolving the coagulum in 10% HCl (100 ml). The obtained acidic solution was extracted with a small quantity of 1-butanol. Evaporation of the alcohol then afforded the recovery sample (0.98 g).

Fractionation and Analysis of Electrocoagulated Sample

The recovery sample from above (0.30 g) was dissolved in 50 % aqueous methanol and partitioned with CH_2Cl_2 to give, after evaporation, CH_2Cl_2/H_2O fraction (LC33-DCM, 0.09 gm) and H_2O/MeOH fraction (0.25 g). Comparison of the CH_2Cl_2/H_2O fraction with the previous corresponding fraction obtained without electrocoagulation treatment (fraction LC29-DCM) by TLC (developed with acetone:hexane = 1:5, sprayed with phosphomolybdic acid reagent and heated) gave the result as shown in Figure 9. Then the CH_2Cl_2/H_2O fraction was chromatographed over a silica gel column eluted with hexane, CH_2Cl_2, EtOAc, and MeOH, respectively to yield 5 fractions, i.e. fractions LC33-1 to LC33-5. Lupeol (10 mg) was recovered from fraction LC33-2. Fraction LC33-5 (30 mg) was then subjected to reverse-phase HPLC using the same system as in the isolation of the components from fraction 25-8 to yield betulinic acid (3 mg).

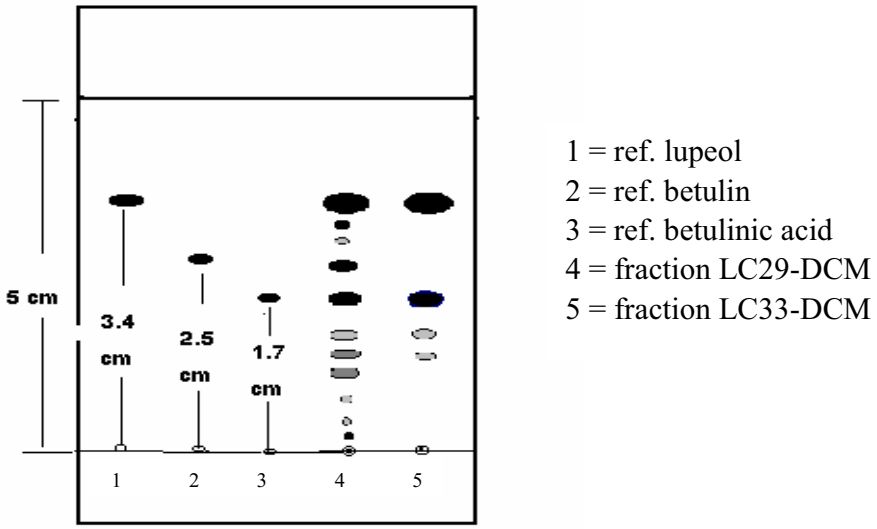


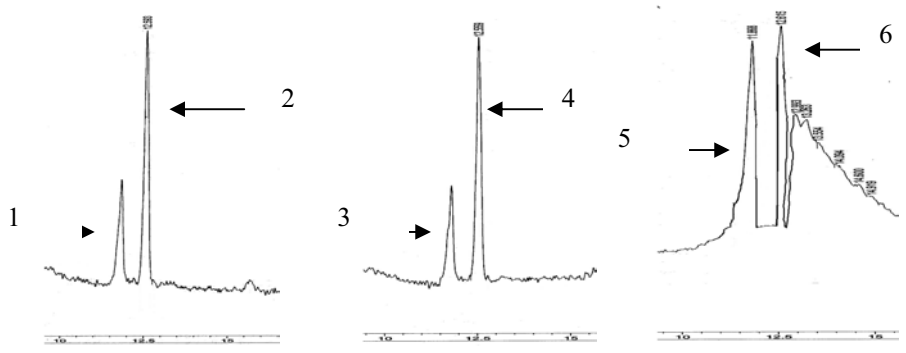
Figure 9. TLC of uncoagulated CH_2Cl_2/H_2O fraction (LC29-DCM) compared with coagulated CH_2Cl_2/H_2O fraction (LC33-DCM)

The H_2O/MeOH fraction (0.25 g) was repartitioned with 1-butanol to gain the butanol-soluble extract (0.15 g). It was then fractionated by a gel filtration chromatography column prepacked with methacrylate copolymer matrix, Fractogel TSK HW-40, and eluted with the

same gradient polarity system used to isolate the untreated sample to yield 6 main fractions, i.e. LC34t1 to LC34t6. Each fraction was investigated by ^{1}H NMR and MS (EIMS, negative ion mode). The ^{1}H NMR spectrum of LC34t2 (10 mg) was almost identical to that of LC29t1 containing a mixture of monomeric condensed tannins, viz. catechin and epicatechin. Moreover the mass spectrum of this recovery fraction has a prominent peak of $[M-H]^-$ at *m/z* 290 suggesting the molecular peak of monomeric condensed tannins, catechin and epicatechin. However, since the obtained sample size of this fraction (LC34t2, 10 mg) was rather small, it was not further separated by HPLC like fraction LC29t1, but analysed instead by HPLC/MS under the following conditions. The MS apparatus coupled to the chromatographic system was a Hewlett Packard simple quadrupole mass spectrometer with a mass range of 400 mass units, equipped with an electrospray ion source. The mass spectrometer was operated in the negative ion mode. Ion spray voltage was set at -4000 V and orifice voltage at –50 V with nitrogen flow rate 13 L/min. HPLC was carried out on narrow-bore reverse phase column (Water μ-bondapak, C-18, porasil semi-preparative column, 3.6x300 mm). The sample was injected with a rotary valve fitted with a 20 μL sample loop. Elution was achieved with solvent A (0.01 M aqueous phosphoric acid) and B (methanol). The sample was eluted according to the following binary gradient: 5 % B reaching 50 % at 10 min, 70 % at 15 min, 80 % at 20 min and 100 % at 25 min. The flow rate was 0.5 ml min-1. DAD instrument was operated at UV wavelength 280 nm. The temperature was constant at 29^{o}.

The result of the above operation showed that fraction LC34t2 gave 2 sharp signals at retention times 11.8 and 12.6 min., each showing the molecular ion peak in the mass spectrum at *m/z* 289 (ESI, negative ion mode), and a very broad signal immediately after. Fraction LC29t1 under the same condition gave 2 sharp signals at retention times 11.8 and 12.7 min. Those of authentic catechin and epicatechin were 11.8 and 12.7 min. respectively (Figure 10).

The remaining fractions from the gel filtration column (LC34t3-LC34t6) were examined by ^{1}H and ^{13}C NMR, which indicated the presence of oligomeric condensed tannins as the major component.



(a) (b) (c)

1 = catechin 11.8 min 3 = catechin 11.8 min 5 = catechin 11.8 min
2 = epicatechin 12.7 min 4 = epicatechin 12.7 min 6 = epicatechin 12.6 min
a = standard mixture of catechin and epicatechin
b = fraction LC29t1 c = fraction LC34t2

Spectroscopic Data

Lupeol. M.p. 212°C (lit. 215-216°C) [48]; EIMS *m/z* (% relative intensity) 426 (41), 411 (13), 315 (12), 272 (6), 257 (13), 218 (39), 207 (74), 189 (75), 147 (33), 135 (84), 121 (64), 109 (89), 95 (100), 81 (100), 69 (90), 55 (84), 41 (72); HR-EIMS: 426. 6067 [M+]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.67 (1H, *d*, *J* = 9.0 Hz, H-5), 0.75 (3H, *s*, H-24), 0.78 (3H, *s*, H-28), 0.84 (3H, *s*, H-25), 0.93 (3H, *s*, H-27), 0.95 (3H, *s*, H-23), 1.02 (3H, *s*, H-26), 1.65 (3H, *s*, H-30), 1.22 (1H, *m*, H-22a), 1.42 (1H, *m*, H-22b), 1.33 (1H, *m*, H-21a), 1.90 (1H, *m*, H-21b), 2.38 (1H, *m*, H-19), 3.18 (1H, *dd*, *J* = 11.2, 4 Hz, H-3), 4.55 (1H, *dd*, *J* = 2.5, 1.5 Hz, H-29a), 4.67 (1H, *d*, *J* = 2.5 Hz, H-29b);.^{13}C NMR (125 MHz, $CDCl_3$) δ 38.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.8 (C-8), 50.4 (C-9), 37.1 (C-10), 20.9 (C-11), 25.1 (C-12), 38.0 (C-13), 42.8 (C-14), 27.4 (C-15), 35.5 (C-16), 43.0 (C-17), 48.2 (C-18), 47.9 (C-19), 150.9 (C-20), 29.8 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30).

Betulin. M.p. 237°C (lit. 236-238°C) [50]; ESI +ve *m/z* (% relative intensity) 443 (100), 412 (26), 384 (38), 361 (15), 344 (12), 325 (22), 318 (29), 288(5), 257 (8), 248 (30), 207 (55), 189 (100), 175 (32), 135 (62), 121 (50), 107 (52), 95 (67), 81 (70), 69 (63), 55 (62), 41 (58); LR-EIMS: 443 [M+]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.67 (1H, *d*, *J* = 8.0 Hz, H-5), 0.76 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.89 (1H, *d*, *J* = 2 Hz, H-1b), 0.97 (3H, *s*, H-23), 0.99 (3H, *s*, H-27), 1.02 (3H, *s*, H-26), 1.18 (1H, *d*, *J* = 4.0 Hz, H-11b), 1.21 (1H, *d*, *J* = 3.5 Hz, H-16b), 1.68 (3H, *s*, H-30), 2.38 (1H, *m*, H-19), 3.18 (1H, *dd*, *J* = 11.4, 5 Hz, H-3), 3.34 (1H, *d*, *J* = 10.6Hz, H-28b), 3.80 (1H, *d*, *J* = 10.6 Hz, H-28a), 4.58 (1H, *d*, *J* = 1.4, H-29a), 4.69 (1H, *d*, *J* = 1.4 Hz, H-29b); ^{13}C NMR (125 MHz, $CDCl_3$) δ 38.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.7 (C-4), 55.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.9 (C-8), 50.4 (C-9), 37.2 (C-10), 20.8 (C-11), 25.2 (C-12), 37.3 (C-13), 42.7 (C-14), 27.0 (C-15), 29.2 (C-16), 47.7 (C-17), 48.7(C-18), 47.8 (C-19), 150.6 (C-20), 29.7 (C-21), 34.0 (C-22), 27.9 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.7 (C-27), 60.5 (C-28), 109.7 (C-29), 20.8 (C-30)

Betulinic acid. M.p. 280 °C (lit. 279-283 °C) [52]; ESI +ve *m/z* (% relative intensity) 457 (20), 412 (23), 316 (3), 302 (4), 248(30), 218 (39), 208 (74), 190(75), 147 (33), 135 (84), 121 (64), 109 (89), 95 (70), 81 (100), 69 (90); HR-EIMS: 455[M^-]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.68 (1H, *d*, *J* = 9.0 Hz, H-5), 0.75 (3H, *s*, H-24), 0.82 (3H, *s*, H-25), 0.97 (3H, *s*, H-23), 0.94 (3H, *s*, H-26), 0.98 (3H, *s*, H-27), 1.69 (3H, *s*, H-30), 1.96 (1H, *m*, H-21b), 1.39 (1H, *m*, H-21a), 2.28 (1H, *m*, H-21b), 2.99 (1H, *ddd*, *J* = 11, 11, 6 Hz, H-19), 3.18 (1H, *dd*, *J* = 11.3, 5 Hz, H-3), 4.60 (1H, *d*, *J* = 1.5 Hz, H-29a), 4.74 (1H, *d*, *J* = 1.5 Hz, H-29b); ^{13}C NMR (125 MHz, CDCl3) δ 38.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.3 (C-7), 40.7 (C-8), 50.5 (C-9), 37.2 (C-10), 20.8 (C-11), 25.5 (C-12), 38.4 (C-13), 42.4 (C-14), 30.5 (C-15), 32.1 (C-16), 56.3 (C-17), 46.8 (C-18), 49.2 (C-19), 150.3 (C-20), 29.7 (C-21), 37.0 (C-22), 27.9 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.7 (C-27), 180.5 (C-28), 109.6 (C-29), 19.4 (C-30).

Catechin. IR (KBr) υ max 3350, 1440, 1600, 1160, 780 cm^{-1}; HR-EIMS: 289 [M-]; ^{1}H NMR (500 MHz, acetone-d_6: D_2O = 1: 1) δ (signal for H-2 is obscured by solvent peak), 4.02 (1H, *m*, H-3), 2.40 (1H, *dd*, *J* = 16.2, 7.9 Hz, H-4ax), 2.71 (1H, *dd*, 16.2, 5.4 Hz, H-4eq), 5.91 (1H, *d*, *J* = 2.3 Hz, H-6), 5.81 (1H, *d*, *J* = 2.3 Hz, H-8), 6.77 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.74

(1H, *d*, 8.1 Hz, H-5'), 6.67(1H, *dd*, *J* = 8.2, 2.0 Hz, H-6'); ^{13}C NMR (125 MHz, acetone-d_6: D_2O = 1: 1 v/v) δ 81.4 (C-2), 67.1 (C-3), 27.3 (C-4), 156.0 (C-5), 96.2 (C-6), 156.1 (C-7), 95.3 (C-8), 155.7 (C-9), 100.6 (C-10), 130.9 (C-1'), 115.1 (C-2'), 144.8 (C-3'), 144.9 (C-4'), 116.3 (C-5'), 120.0 (C-6').

Epicatechin. IR (KBr) υ max 3400, 1460, 1617, 1140, 750 cm^{-1}; HR-EIMS: 289 [M-]; ^{1}H NMR (500 MHz, acetone-d_6: D_2O = 1: 1) δ 4.79 (1H, *s*, H-2), 4.18 (1H, *br*. H-3), 2.76 (1H, *dd*, *J* = 16.8, 4.44 Hz, H-4ax), 2.59 (1H, *dd*, 16.6, 2.8 Hz, H-4eq), 5.91 (1H, *d*, *J* = 2.3 Hz, H-6), 5.89 (1H, *d*, *J* = 2.3 Hz, H-8), 6.89 (1H, *d*, *s*, H-2'), 6.76 (1H, *s*, H-5'), 6.66 (1H, *s*, H-6'); ^{13}C NMR (125 MHz, acetone-d_6: D_2O = 1: 1) δ 78.5 (C-2), 66.1 (C-3), 27.9 (C-4), 156.4 (C-5), 95.9 (C-6), 156.1 (C-7), 95.3 (C-8), 155.9 (C-9), 99.6 (C-10), 131.2 (C-1'), 115.7 (C-2'), 144.3 (C-3'), 144.4 (C-4'), 114.8 (C-5'), 119.0 (C-6').

Proanthocyanidin B. HR-EIMS: 577 [M-]; ^{1}H NMR (500 MHz, acetone-d_6: D_2O = 1: 1) δ 8.25 (*s*, ArOH), 5.9-6.1 (*m*, ArH of ring A, D), 6.6-7.2 (*m*, ArH of ring B, E), 4.04 (*m*, OH), 4.8 (*s*, H-2 of ring C), 4.35 (*s*, H-3 of ring C), 4.1 (*s*, H-4 of ring C), 5.05 (*m*, H-2 of ring F), 5.2 (*m*, H-3 of ring F), 2.8 (*m*, H-4 of ring F); ^{13}C NMR (125 MHz, acetone-d_6: D_2O = 1: 1) δ upper: 83.2 (C-2), 73.5 (C-3), 37.9 (C-4), 96.4 (C-6), 96.6 (C-8), 131.4 (C-1'), 115.4 (C-2'), 144.8 (C-3'), 144.9 (C-4'), 116.5 (C-5'), 119.8 (C-6'), lower: 81.4 (C-2), 68.2 (C-3), 97.2 (C-6), 108.6 (C-8), 132.1 (C-1'), 116.2 (C-2'), 145.0 (C-3'), 145.4 (C-4'), 116.6 (C-5'), 120.8 (C-6').

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Communication

Dechlorophyllation by Electrocoagulation

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Abstract: Electrocoagulation was used for dechlorophyllation of alcoholic extracts from five plants. The results showed that for every plant extract studied, electrocoagulation was more efficient than the classical solvent extraction method in removing plant pigments, while not affecting the important secondary metabolites in those extracts.

Keywords: Dechlorophyllation, electrolytic decolourisation, electrocoagulation

Introduction

In the process of isolation of natural products from plants, the so-called "organic matrix" consisting of such substances as pigments, tannins, carbohydrates, etc. normally must be removed from the crude extract so the desired secondary metabolites may be obtained in a purified form. This problem is more pronounced when the extracted plant part is the leaf, which always contains plant pigments, notably the chlorophylls and the carotenoids, apart from other normally undesirable components, especially the tannins. Conventional methods for removing these substances are solvent extraction and chromatography. However, typically one or more toxic organic solvents must be used in large amounts in both methods, after which these solvents eventually end up as a problematic volatile chemical waste. Also, in chromatography, expensive and usually unrecoverable adsorbents must be used, and even then, the efficiency of pigment removal is usually far from satisfactory. In this report, we present an alternative method of plant pigment removal, in which usage of a large amount of extracting solvents can be dispensed with. The pioneer in this field is Miwa [1], who in 1978 used the process of electrocoagulation to effect dechlorophyllation of an aqueous extract of stevia in order

to obtain the sweet glycosidic components from stevia leaves. However, no further work in which electrocoagulation has been similarly applied has appeared in the literature since then. Instead, electrocoagulation (EC), an electrochemical technique by which a variety of unwanted dissolved particles and suspended matter, both organic and inorganic, can be effectively removed from an aqueous solution by electrolysis, has been mainly used to treat various aqueous organic and inorganic wastes [2-19], or sometimes as a method in clarifying potable water [20-21]. As an extension of the work by Miwa who used only aqueous plant extracts, we report here the dechlorophyllation of alcoholic plant extracts by electrocoagulation. To our knowledge, apart from the work of our group [22-25], there have been no other investigations on electrocoagulation that are performed in solutions containing an organic solvent, the system that is undoubtedly more useful in isolating natural products in general.

Results and Discussion

As shown in Table 1, absorbances at 665-666 nm (corresponding to the green pigments) and at 408-410 nm (corresponding to the yellow pigments) for Solutions 1 (from the EC method) are all lower than the corresponding values for Solutions 2 (from the solvent extraction method). This is also evident visually from Figure 1, thus indicating that dechlorophyllation by EC is more efficient than the conventional solvent fractionation method for all of the plants studied.

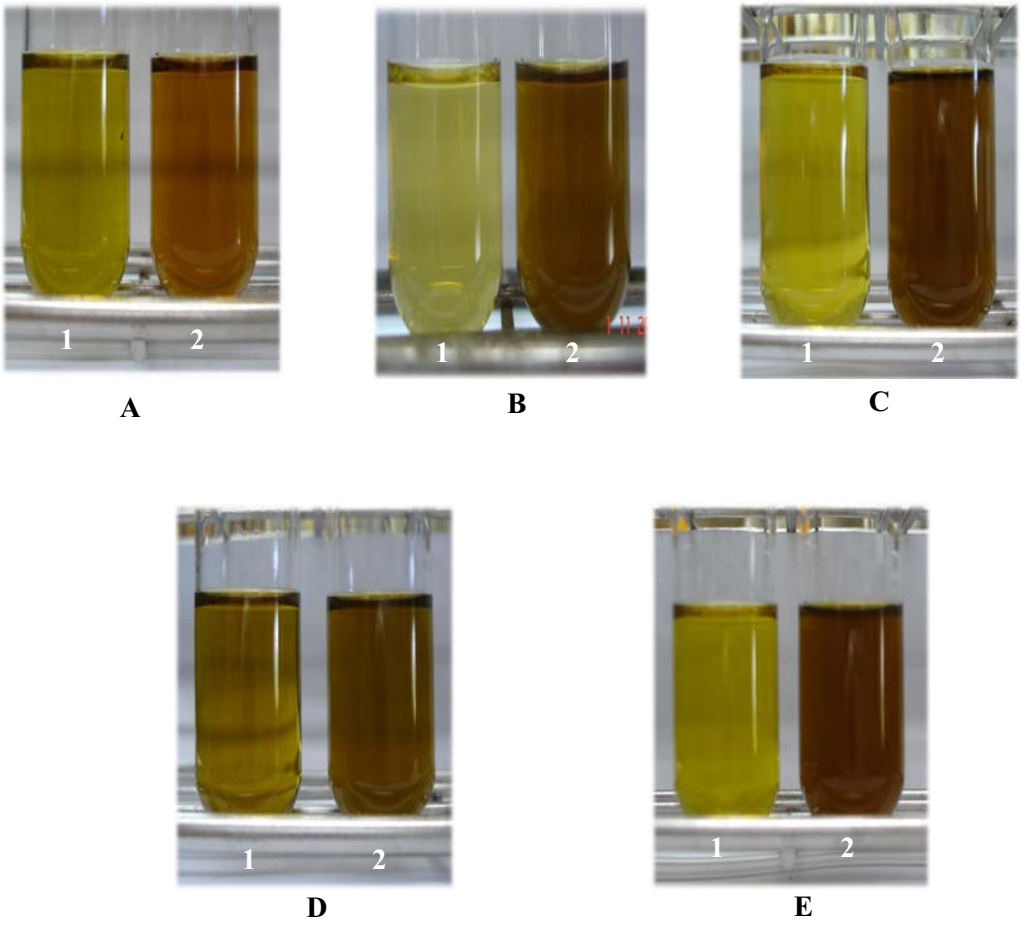
Table 1. Absorbances of plant extracts obtained after dechlorophyllation by EC (Solution 1) and by solvent extraction (Solution 2).

Plant	Absorbance			
	Solution 1		Solution 2	
	665 – 666 nm	408 – 410 nm	665 – 666 nm	408 – 410 nm
Solanum laciniatum	0.35	5.40	1.18	9.03
Andrographis paniculata	0.30	1.85	0.70	5.70
Stevia rebaudiana	0.27	-*	1.50	-*
Centella asiatica	0.65	3.50	1.60	6.50
Cassia siamea	0.30	-*	1.75	-*

* unmeasurable due to interfering peaks.

To ensure that the natural products of interest are not affected by the process of electrocoagulation, we also carried out their isolation from some of the plants. Thus, for stevia leaves, after dechlorophyllation of the alcoholic solution by EC, the sweet glycosidic substances were isolated from the alcoholic extract in the usual manner [26], resulting in a yield of 8-10% for the required glycosides, a normal yield for these compounds obtained by other classical methods in which chemical adsorbents were used for decolourisation.

Figure 1. Plant extracts after dechlorophyllation by (1) electrocoagulation and (2) solvent extraction: A) *Solanum laciniatum*; B) *Andrographis paniculata*; C) *Stevia rebaudiana*; D) *Centella asiatica*; E) *Cassia siamea*.



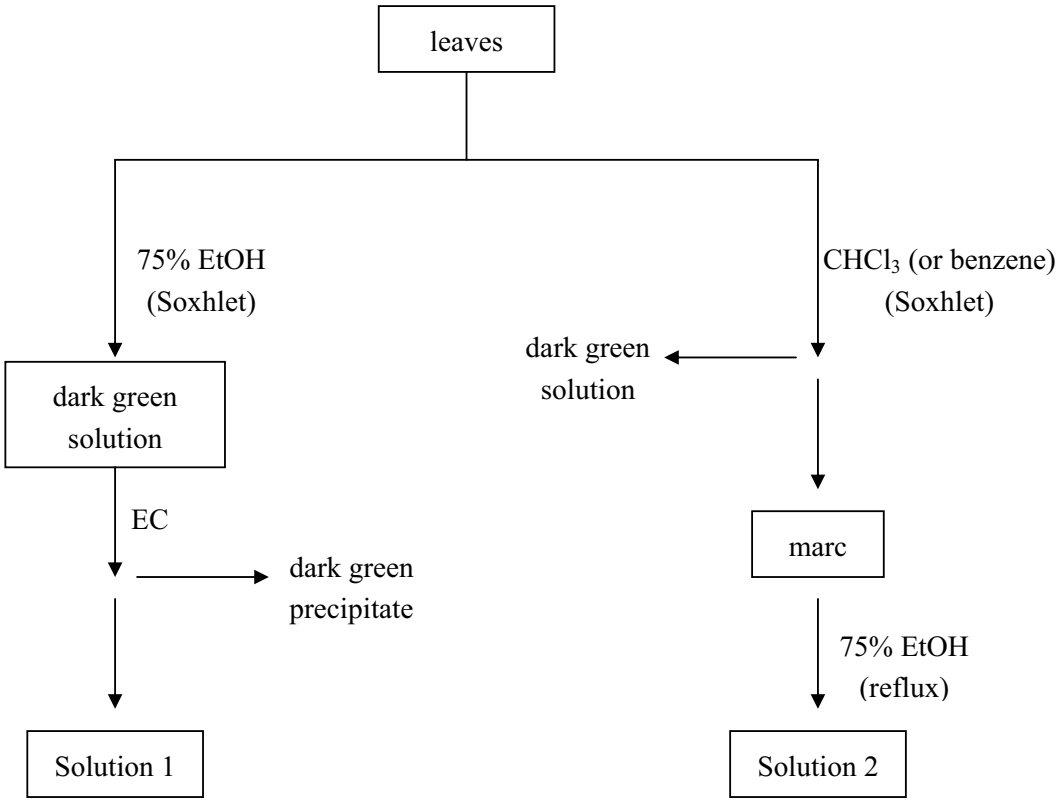
For *S. laciniatum*, solasodine, a steroidal alkaloid used as a starting material for steroid synthesis, was obtained in 1% yield by the EC method. The compound was also isolated from the same leaf samples in only about 0.5% yield by a similar method [27,28] in which chloroform was used conventionally as a dechlorophyllating solvent. For *A. paniculata*, the bioactive lactone content in the crude extract, obtained by simple alcoholic extraction, was about 8% [29,30]. However, when the 75% ethanolic solution, obtained from extracting the leaves from the same source under the same conditions, was subjected to the usual process of electrocoagulation followed by desalting with an ion exchange resin, the resulting crude extract was much more enriched with the lactones (about 38%, as determined by the same method [30]). For *C. asiatica*, our method consisted of extracting the dry leaves with absolute ethanol, diluting the ethanolic solution with water before dechlorophyllating by electrolysis for 2 hours using aluminium electrodes, filtering the resulting electrolysed mixture, evaporating the filtered solution to dryness, and extracting the residue with a little ethanol to afford a crude extract containing 4% (by HPLC) of asiaticoside after evaporation of the ethanol. A classical solvent extraction method [31], in our hands, afforded only 3% (by HPLC) of the same medicinally important glycoside in the crude extract (the asiaticoside content in the dry leaves is about 0.1% [32]).

As for *C. siamea*, we isolated pure D-pinitol, a cyclic polyol, from the dry leaves for the first time by a similar EC method a few years ago [22], although without proper monitoring of the extent of dechlorophyllation as in this report.

Conclusions

Electrocoagulation seems be a method of choice for dechlorophyllation of some plant extracts, rendering them relatively freer from the interfering plant pigments, and thus leaving the compounds of interest more readily processable. This is in addition to the fact that it is a technique which is more environmentally friendly than the conventional usage of various toxic organic solvents.

Scheme 1.



Experimental

The ground dry leaves of five well known plant species, viz. *Stevia rebaudiana* (stevia), *Cassia siamea*, *Solanum laciniatum*, *Andrographis paniculata*, and *Centella asiatica* (10 g each), which were collected locally, were separately extracted with 75% ethanol (200 mL) in a Soxhlet extractor until colourless. The resulting dark green solution was then subject to electrocoagulation for 2.5 hours.

The conditions for electrocoagulation were as follows: two clean (acetone-washed) aluminium plates (iron plates in case of *S. laciniatum*), each of 3x15 cm dimensions were used as electrodes. These were spaced 1.5 cm apart and dipped 7 cm into the magnetically-stirred solution containing

0.1% (w/v) NaCl as supporting electrolyte. Direct current (0.9 A, 16.9-31.6 V) from a power supply was then passed *via* the two electrodes through the solution, which was placed in a jacketed 250-mL beaker for occasional cooling during electrolysis. After 2.5 hours of electrolysis, the mixture in the beaker was filtered to afford a decolourised solution (Solution 1, 150 mL), which was taken for absorbance measurement to determine the extent of decolourisation at 665-666 nm (corresponding to the presence of the green pigments) and at 408-410 nm (corresponding to the presence of the yellow pigments).

As a comparison, dechlorophyllation by solvent extraction was performed by extracting the same amount of plant material (10 g) with an organic solvent (chloroform or benzene) using a Soxhlet extractor until colourless. After drying 75% ethanol (150 mL) was then added to the marc and the mixture was refluxed for 2 hours, then filtered and the solution obtained (Solution 2, 150 mL) was taken for absorbance measurement as above (See Scheme 1).

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Full Paper

Solvent Effects in Electrocoagulation of Selected Plant Pigments and Tannin

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Abstract: Electrocoagulation of a plant extract and certain substances representative of selected classes of plant pigments, viz. chlorophyll, a carotenoid, a phenolic substance and a tannin, was performed in ethanol containing varying amounts of water (15-75%). The results showed that the extent and efficiency of coagulation of these substances tends to vary in a manner directly related to the water content of the solvent, although the tannin and the phenolic substance were less sensitive to the solvent composition and are equally well coagulated in all solvent systems studied. The findings can be applied to the removal of these substances from aqueous alcoholic plant extracts using the electrocoagulation technique.

Keywords: Electrocoagulation, electrolytic decolourisation, plant pigments, phenolic compounds, tannins

Introduction

Electrocoagulation has been a useful alternative technique for clarifying and decolourising certain solutions containing unwanted dissolved substances or suspended matter. However, those solutions were almost always aqueous in nature, for example, potable water [1-2], food wastewater [3], tar-sand and oil-shale wastewater [4], phosphate-containing sewage [5-6], industrial wastes containing cyanide and heavy metals [7-9], and dye-containing textile wastewater [10-11]. Electrocoagulation has also

been used as a purification step in the isolation of a few natural products from crude plant extracts [12-17]. Again, the solvent used in those isolations was also purely aqueous. Trials of natural product isolation using electrocoagulation in alcoholic solutions as a part of the process have been reported in the literature, for example, for the isolation of asiaticoside [18] and the extraction of phenolic compounds from the bark of *Lithocarpus elegans* [19], but a systematic study of electrocoagulation in alcoholic solutions has, to our knowledge, not been undertaken. In view of the fact that a system containing an organic solvent is undoubtedly more useful in isolating natural products in general, we would like to report our study on the effects of solvent in the electrocoagulation of some selected organic substances and plant extracts. For a start, we chose as our model study the system in which ethanol with varying amounts of water is used as solvent.

Results and Discussion

Our previous work carried out with water and alcohol soluble natural dyes (chlorophyllin and crocin) [20] showed that although decreases in the water content of the solvent (ethanol) has some effect on the coagulation efficiency for the dyes, coagulation was more or less complete after 2 hours, especially when iron is used for the electrodes (Figures 1-2).

Figure 1. Plots of residual weight percentage and electrolysis time for each ethanol concentration of chlorophyllin solution at 626 nm; A: with aluminium as electrodes, B: with iron as electrodes.

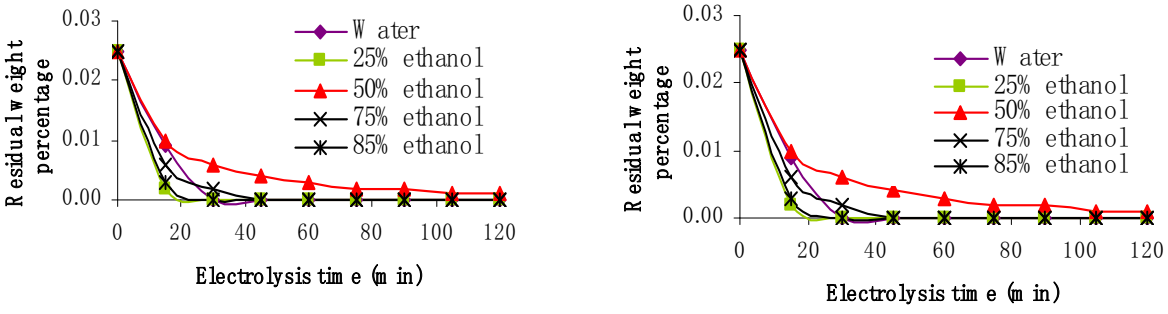
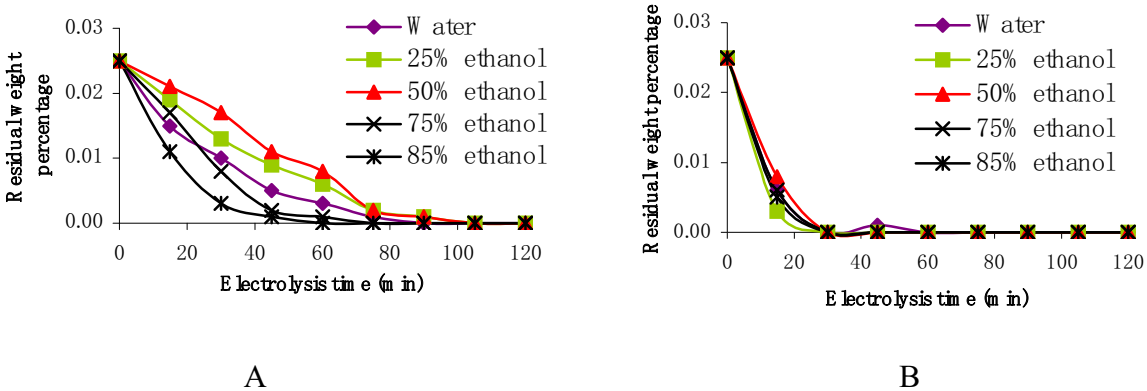
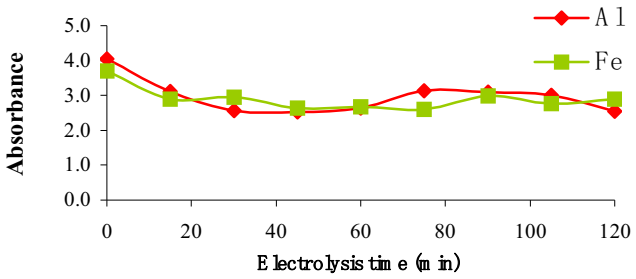


Figure 2. Plots of residual weight percentage and electrolysis time for each ethanol concentration of crocin solution at 440 nm; A: with aluminium as electrodes, B: with iron as electrodes.



However, experiments carried out in this study with water-insoluble dyes (the chlorophylls and the carotenoids) seem to indicate that, contrary to the above result, these pigments seem to be more difficult to coagulate. For example, in Figure 3 it can be seen that the model pigment β-carotene is hardly affected and barely removed from the solution by the electrolytic decolourisation process applied, even when iron electrodes are used (due to the difficulties in solubilizing this pigment, electrocoagulation was only examined in a single mixed solvent system.)

Figure 3. Plots of absorbance at 460 nm and electrolysis time for β-carotene (0.01%) in 27% ethanol; ♦: with aluminium as electrodes, ■: with iron as electrodes.



Similarly, for solutions of extracted chlorophyll, the trend is that coagulation seems to be less efficient as the water content in the solvent decreases (Figures 4-5). This may not only be due to the fact that, as the water content in the solvent decreases the process of electrocoagulation itself is naturally retarded, but also to the fact that more pigment is dissolved in the solution as the water content in the solvent decreases and the alcohol content accordingly increases. This trend was repeated in nearly every case of green plant extracts we studied. Thus, for example, when the alcoholic extract of the leaves of *Solanum laciniatum*, which contain solasonine (an important starting compound for steroid synthesis), was subject to decolourisation by electrocoagulation, typical results as shown in Figure 6 (for the yellow pigments) and Figure 7 (for the green pigments) were obtained.

Figure 4. Plots of absorbance at 440 nm and electrolysis time for each ethanol concentration of extracted chlorophyll solution; A: with aluminium as electrodes, B: with iron as electrodes; ♦, 75% ethanol; ■, 85% ethanol

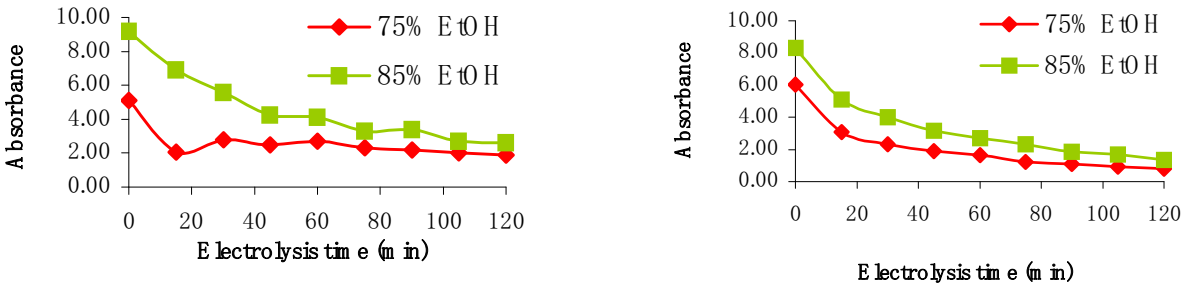


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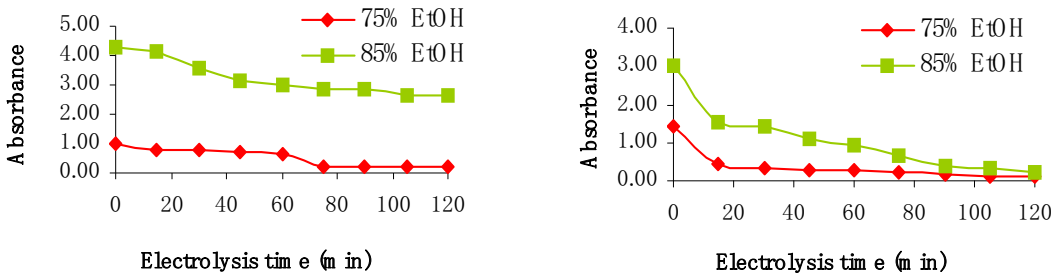
Figure 5. Plots of absorbance at 665 nm and electrolysis time for each ethanol concentration of extracted chlorophyll solution; A: with aluminium as electrodes, B: with iron as electrodes; ♦, 75% ethanol; ■, 80% ethanol



A B

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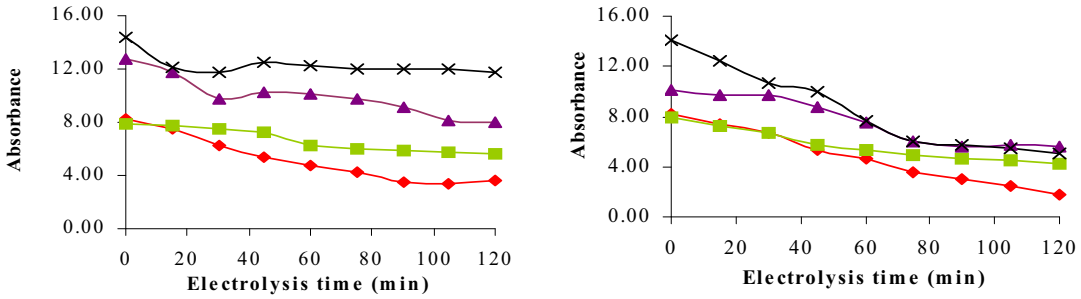
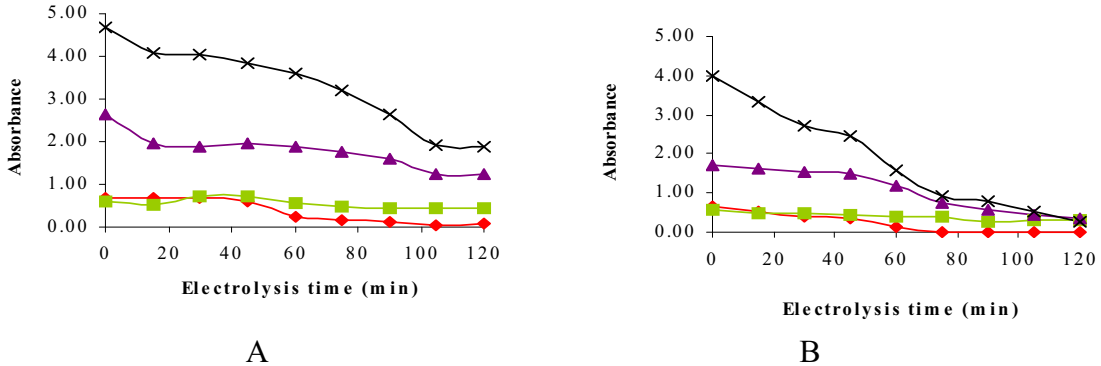
Importantly, however, it has been shown by our group that even with this general decreased efficacy of the pigment removal, electrocoagulation is still more efficient than the conventional method of extraction with organic solvents and does not affect the desired natural products, which were subsequently isolated [21].

Figure 6. Plots of absorbance and electrolysis time for each ethanol concentration of *Solanum laciniatum* extract at 408 nm; A: with aluminium as electrodes, B: with iron as electrodes; ♦ , 25% ethanol; ■, 50% ethanol; ▲, 75% ethanol; ×, 85% ethanol.



A B

Figure 7. Plots of absorbance and electrolysis time for each ethanol concentration of *Solanum laciniatum* extract at 666 nm; A: with aluminium as electrodes, B: with iron as electrodes; ♦, 25% ethanol; ■, 50% ethanol; ▲, 75% ethanol; ×, 85% ethanol



A B

Another important class of plant pigments is the phenolic substances, which include the tannins, the flavonoids, and the various quinone compounds. Most of these materials (except perhaps the tannins) are poorly soluble in water but more soluble in alcohol or other organic solvents. Many of them are valuable natural products, while others (especially the tannins) are regarded as little more than intractable mixtures with unfavourable biological activities and it is generally preferable to remove them along with the pigments.

In a 100% aqueous medium, it has been shown that tannins can be very efficiently coagulated and removed by electrolysis [22-23]. In this study, we tried to repeat the process in aqueous alcoholic solutions. The hypothesis is that tannins, owing to their polyphenolic nature, should still be easily coagulated by the phenolate salt forming mechanism in addition to the adsorption mechanism [23]. Our experiments showed this to be the case. Thus, for example, at a concentration of 0.1% tannin in up to 85% ethanol, a 250-mL solution was almost completely de-tannized within 15 minutes, using aluminium as electrodes and a current of 0.3 A (Figure 8). At a concentration of 1.0% tannin, the complete detannization time was increased to 80 minutes (Figure 9).

Figure 8. Plot of the residual weight percentage and electrolysis time for 0.1% w/v tannin at 275 nm ; ●, 25% ethanol , ▲, 50% ethanol, ■, 75% ethanol and ◆, 85% ethanolic solution.

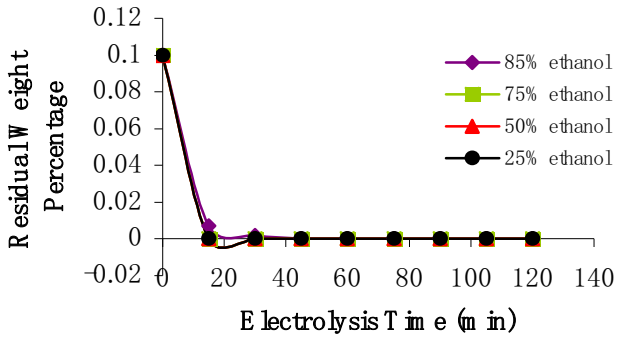
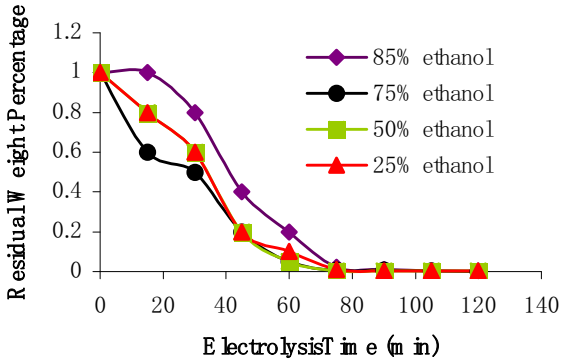
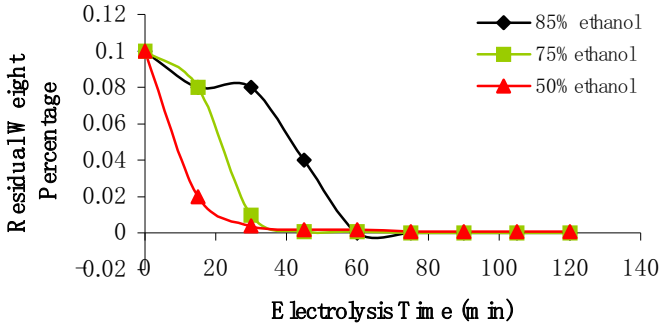


Figure 9. Plot of the residual weight percentage and electrolysis time for 1.0% w/v tannin at 275 nm; ▲, 25% ethanol, ■, 50% ethanol, ●, 75% ethanol, and ◆, 85% ethanolic solution.



Next, to demonstrate the general trend of the effect of water content in the solvent on the coagulation of phenolic compounds other than tannins, the result of electrocoagulation of a known flavonoid, morin, is presented here as a typical example (Figure 10). It can thus be seen that the effect of the decrease of water content in the solvent on the coagulation of this type of substances is similar to
that of the chlorophylls and the carotenoids, i.e., as the water content in the solvent decreases (or the alcohol content increases), coagulating efficacy also seems to decrease proportionally.

Figure 10. Plot of the residual weight percentage and electrolysis time for 0.1% w/v morin at 415, 420 nm ; ▲,50% ethanol, ■,75% ethanol and ◆85% ethanolic solution (a 0.1% solution of morin in 25% ethanol cannot be prepared.)



For most natural phenolic compounds, however, probably owing to the active polyhydroxy functions of the substances, coagulation which can occur both by reaction and by adsorption tends to be more complete than that of the chlorophylls and the carotenoids, so that after some time (60 minutes in this case), coagulation is virtually complete, even in solvents with a high percentage of alcohol.

Conclusions

It has been demonstrated in a systematic manner that in the electrocoagulation in aqueous alcoholic solutions of some important plant pigments, including tannins, the decrease in the percentage of water in the solvent has some negative effects on the degree and efficiency of their coagulation compared with that observed in 100% aqueous solution. However, the effect is small in the case of tannins and some other phenolic substances. For chlorophylls and carotenoids, this retarding effect is somewhat higher, probably due to only a single mode of coagulation being in operation (viz. adsorption mode). However, even with this unfavourable effect being present, electrocoagulation is still more efficient in removing these organic matrix substances than the conventional method of solvent extraction.

Experimental Section

General

All of the tested compounds used were of standard reagent grade, and were used as received. Tannin and β-carotene were purchased from Fluka Chemica AG (Buchs, Switzerland); morin

(3,5,7,2',4'-pentahydroxyflavone) was purchased from May & Baker Ltd (Dagenham, England). Crude chlorophyll was obtained from *Spinach oleracea.* Air-dried leaves of *Solanum laciniatum* were obtained from Dr. Jiradej Manosroi, Faculty of Pharmacy, Chiangmai University. Sodium chloride (99.9%, AR grade) was purchased from Ajax Chemical Co. (Sydney, Australia). Absolute ethanol was purchased from E. Merck (Darmstadt, Germany). Acetone and ethanol (95%) were of a commercial grade. Aluminium and iron plates were purchased locally. Direct current was sustained by a GW Instek DC power supply. Absorbance was measured on a Genesys 10 spectrophotometer.

Preparation of solutions for electrocoagulation

A solution of tannin (tannic acid) or morin (0.01, 0.1, or 1.0% w/v) was prepared in aqueous ethanol (25%, 50%, 75% or 85% v/v). Beta-carotene was dissolved in 27% ethanol to give a 0.01% w/v solution. Crude chlorophyll was extracted from *Spinach oleracea* with 75 or 85% ethanol at room temperature by grinding 40 g of the dry plant material with solvent (200 mL). The filtered solution was used directly in the electrocoagulation experiments. For *Solanum laciniatum*, the powdered air-dried leaves (20 g) were refluxed for 3 hours with each aqueous alcoholic solution (25%, 50%, 75%, 85%, 200 mL). After filtration, the deep green solutions obtained were used directly in the electrocoagulation experiments.

Electrocoagulation procedures

1) Morin and tannic acid

Two aluminum plates (dimensions 15 x 4 cm) were used as electrodes. These were spaced 3 cm apart and dipped 5.5 cm deep into a magnetically-stirred aqueous solution (250 mL) of the tested compound (0.01, 0.1 or 1.0% w/v solution) in a 400 mL beaker. Sodium chloride (0.5 g) was added as an electrolyte. Direct current (0.3 A) from the DC power supplier was then passed through the solution. Every 15 minutes during a 2 hour period of electrolysis, a 4 mL sample of the solution was withdrawn, centrifuged and taken for an absorbance measurement at an appropriate wavelength (275 nm for tannin and 415-420 nm for morin). The measured absorbance was then converted into the residual weight percentage of the compound by a calibration curve obtained from a plot of the absorbance versus the concentration for each compound.

2) Chlorophyll, β-carotene, and Solanum laciniatum

Aluminium or iron plates (15.0 × 3.0 cm.) were washed prior to use with acetone to remove surface grease. A pair of aluminium or iron plates 1.5 cm apart was immersed 7.0 cm deep into 200 mL of each solution in a jacketed 250-mL beaker for occasional cooling during electrolysis. The solution was agitated throughout the experiment with a magnetic stirrer (250 r.p.m.). Sodium chloride (0.2 g) was added as supporting electrolyte. Direct current (0.9 A, 16.9-31.6 V) was then passed through the solution *via* the two electrodes. At every 15-minute interval during a 2-hour period of electrolysis, a 4-mL aliquot of the solution was withdrawn and centrifuged for 10 minutes, and the absorbance of the

supernatant solution was measured at an appropriate wavelength of the absorption maximum for each plant solution (440 and 665 nm for chlorophyll, 460 nm for β-carotene and 408 and 666 nm for *Solanum laciniatum*).

Acknowledgements

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Full Paper

Electrocoagulation of Quinone Pigments

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Abstract: Some representative quinones, viz. one naphthoquinone (plumbagin) and five anthraquinones (alizarin, purpurin, chrysazin, emodin, and anthrarufin), were subjected to electrocoagulation. It was found that the rate and extent of coagulation of these compounds appears to correlate with the number and relative position of their phenolic substituent groups, and that all of the coagulated quinones could be recovered. Attempts were then made to electrochemically isolate three quinones, namely plumbagin, morindone and erythrolaccin, from natural sources.

Keywords: Electrocoagulation, quinones, naphthoquinones, anthraquinones, phenolic compounds, alizarin, purpurin, chrysazin, emodin, anthrarufin, plumbagin, morindone, erythrolaccin, seedlac, *Plumbago rosea*, *Morinda angustifolia*

Introduction

Quinones, notably naphthoquinones and anthraquinones, are among the most widely distributed natural products. The majority of them exist as coloured phenolic compounds, useful as dyes and pigments. Isolation of these substances from their natural sources normally requires the use of organic

solvents before the required compound can be obtained in a reasonably pure state, either to first extract them from the raw material and then to partition them between various solvent phases or as eluents for chromatographic separations. These organic solvents are usually not only costly but also a potential toxic burden to the environment, even if treated in the proper way.

With the advent of electrocoagulation, it has been known for some time that this process is capable of fractionating a number of organic substances in a rather efficient manner by electrochemically coagulating some of them, while leaving other components in solution. For example, the technique can be used to remove coloured matter from drinking water [1], textile wastewater [2,3] and from crude aqueous plant extracts [4-6]. It can coagulate starch and protein, while leaving sugars and polyols intact [7]. Electrocoagulation is one of the most effective methods for removing tannins from a liquid medium [8-10], while for other phenolic substances, their structure, including the number and the location of the hydroxyl groups, seems to dictate whether they will be coagulated or not [7,9]. Conversely, many glycosides [4,5,9a] and alkaloids [11] are unaffected by electrocoagulation and consequently these substances conveniently remain in the electrolysed solutions, relatively free from other impurities which are coagulated out.

Our group has recently demonstrated that compounds of interest may be recovered from the coagulated materials by following a novel specific procedure [7,9,12]. With this technique now available, and with the intention to contributing to "Green Chemistry", we hypothesized that like glycosides and alkaloids, the application of electrocoagulation to this important class of natural products, viz. quinones, might lead to a simple and environmentally friendly alternative method for their isolation, which is, to the best of our knowledge, an operation that has not been reported elsewhere. The results of the consequent investigation are reported in this paper, in which authentic samples of some typical quinones were electrocoagulated and recovered experimentally, and then attempts were made to isolate some of them electrochemically from their respective natural sources.

Results and Discussion

In the first part of this investigation, a number of pure samples of representative quinones, viz. alizarin, purpurin, chrysazin, emodin, anthrarufin and plumbagin (Figure 1) were separately electrocoagulated using direct current and aluminium plates as electrodes in an aqueous alcoholic solution containing sodium chloride as the supporting electrolyte. The absorbance of each electrolysed sample solution was measured at an appropriate wavelength at regular intervals during electrolysis and electrocoagulation curves were obtained for each quinone as a plot of percent residual weight of the sample or absorbance versus electrocoagulation time, as shown in Figures 2-3.

From the results above it can be seen that the quinones under study may be roughly divided into three categories. The two most rapidly and completely coagulated quinones were alizarin and purpurin, being completely coagulated within 15 minutes under the experimental conditions used. It can also be noted that these two compounds have adjacent phenolic hydroxyl groups (Figure 1). This catechol-like structure thus seems to be particularly prone to coagulation by a metal ion, probably by complexation, a fact consistent with similar cases encountered and reported earlier [9].

Figure 1. Structures of the quinones studied.

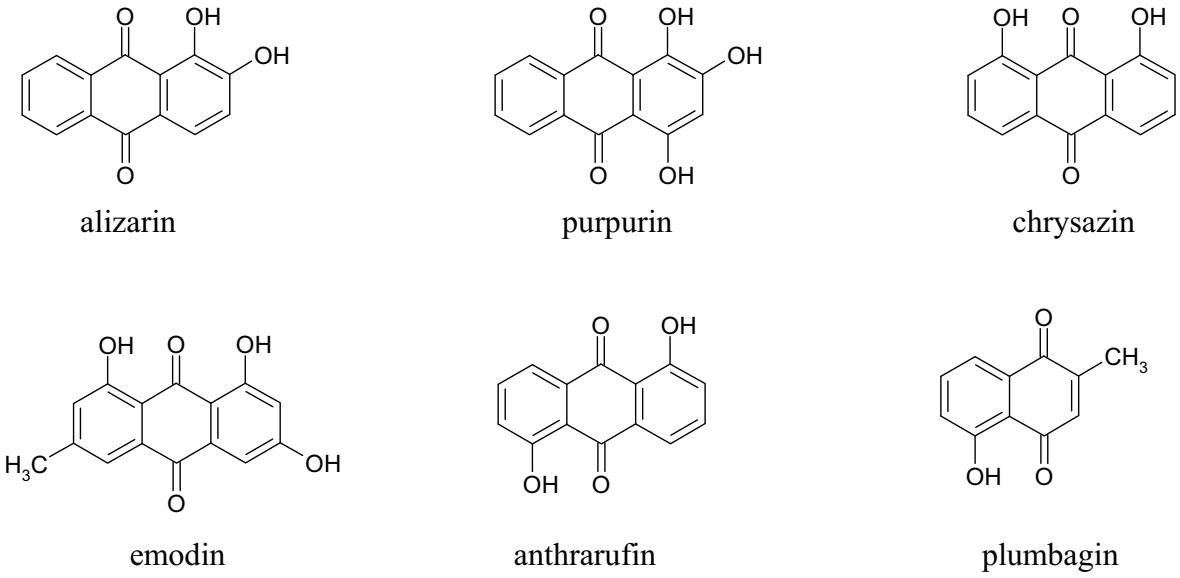
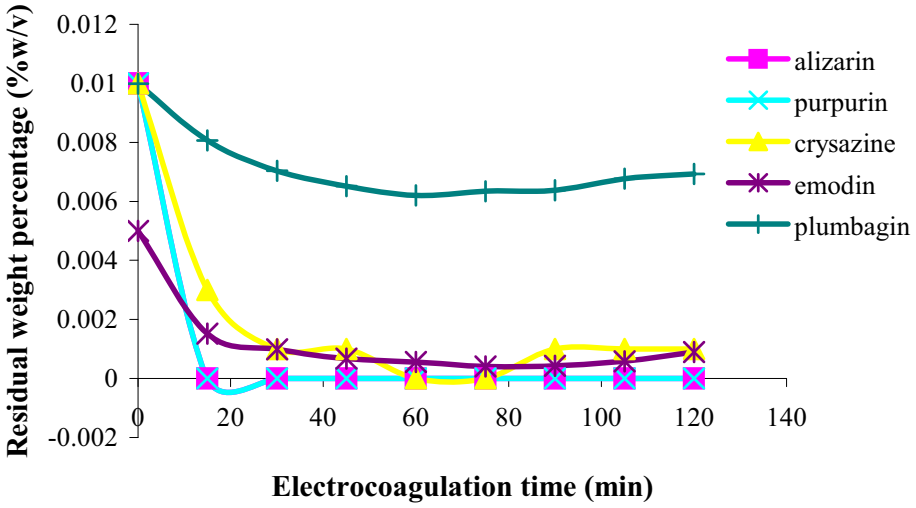


Figure 2. Plots of residual weight percentage vs. electrolysis time for quinones in 85% ethanol solution; ■ alizarin, x purpurin, ▲ chrysazine, * emodin, and + plumbagin.

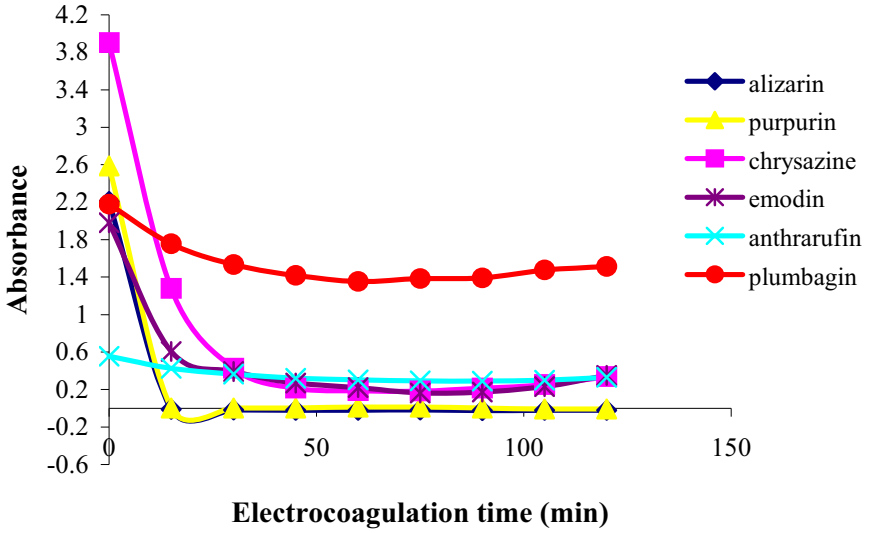


The second category of quinones comprised chrysazin and emodin, which were coagulated somewhat less completely and more slowly than the first two ones. In this case, it can also be seen from their structures that they have no adjacent hydroxyl groups, although they both possess two phenolic groups flanking a quinone carbonyl group (Figure 1). This may then be the next best functional group arrangement of a quinone for complexing with metal ions released by the electrolytic process, although exactly why this should be so remains to be explained. In any case, however, this is better than the arrangement seen in the third category, in which only *one* phenolic hydroxyl group flanks a carbonyl group, as seen in anthrarufin and plumbagin (Figure 1), which were barely coagulated and give nearly horizontal coagulation curves (Figures 2-3).

Next, all the coagulated quinones, i.e. alizarin, purpurin, chrysazin, and emodin, were recovered using the previously reported procedure [7,9,12], which essentially consists of dissociating the

aluminium complexes of each phenolic quinone with dilute mineral acid (HCl) and separating the thus freed quinones by extraction with a polar solvent (in this case 1-butanol). The integrity of each recovered quinone was confirmed by established chemical and physical methods. Having done this successfully, we then embarked on the isolation of some natural quinones, namely plumbagin, morindone, and erythrolaccin, from the roots of *Plumbago rosea, Morinda angustifolia* and from seedlac, respectively. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a well-known natural naphthoquinone [13], while morindone (1,2,5-trihydroxy-6-methylanthraquinone) is a less well-known isomer of emodin, useful as a red dye for cotton [14]. Erythrolaccin (1,3,5,6-tetrahydroxy-8-methyl-anthraquinone) is a yellow dyestuff present in seedlac obtained from crude lac, a resinous substance secreted by the scale insect *Laccifer lacca* [15a].

Figure 3. Plots of absorbance vs. electrolysis time for quinones in 85% ethanol solution; ◆ alizarin, ▲ purpurin, ■ chrysazine, * emodin, x anthrarufin, and ● plumbagin.



Plumbagin was extracted with 50% aqueous ethanol and the resulting extract was then electrolysed using conditions similar to those used in the experimental electrocoagulation of pure plumbagin. After filtering off the precipitate formed, the remaining electrolysed solution contained nearly pure plumbagin, plus a little of the salt added as supporting electrolyte. This can be eliminated by ion exchange, but we used distillation, whereby we obtained an aqueous alcoholic solution of purified plumbagin, which sublimed out from the distillation flask into the collection flask. Partitioning of this solution with a little dichloromethane afforded pure plumbagin in 0.49 g (0.54 % yield). By applying a reported isolation method involving organic solvent extraction and chromatographic purification [16], a slightly lower yield (0.47g, 0.51%) was obtained from the same root sample.

In the isolation of morindone, the situation was somewhat different, due its being coagulated by the electrochemical process. Thus, after filtering the electrolysed solution, the coagulum or precipitate formed was saved for recovery of the desired phenolic anthraquinone. When this was done, a moderately pure morindone was obtained (matching IR, but broad melting point).

In our attempt to isolate erythrolaccin from seedlac, an aqueous alcoholic solution of this substance was treated under similar electrocoagulation conditions and using the same subsequent recovery

operations as described above for the isolation of morindone. The recovered colouring matter obtained, however, was not identical to that which was isolated from seedlac by an established procedure for erythrolaccin isolation [15a], thus suggesting that the recovered pigment, though potentially useful in itself, might be an artefact. This might occur due to the fact that the coagulation time required in this case was rather long (1-2 hours), which according to our experience, can be somewhat detrimental in general to the coagulated compounds. Also, it has been mentioned that in a prolonged electrolysing operation of this type, certain compounds present in the solutions may undergo reactions (e.g. oxidation or reduction) before coagulating [10,17]. As to the reason why erythrolaccin was only slowly electrocoagulated when it possesses adjacent phenolic groups like alizarin or purpurin, this is not clear, although it was mentioned some time ago that erythrolaccin does not all occur in a free state in lac or seedlac but is partially bound with the resinous substance [15b]. On the other hand, however, it was noted that the electrolysed seedlac was very pale in colour. Normally, this material is decolourised using various forms of carbon but the results are usually far from satisfactory. We have found that electrocoagulation is far more efficient than carbon treatment in decolourising seedlac by coagulating out the yellow erythrolaccin. However, in our hand the yield of decolourised seedlac was still low, due to the fact that a good part of the resinous substances themselves tended to co-coagulate with the colouring matter.

Conclusions

It has been determined that a variety of structurally different quinones were susceptible to electrocoagulation in different degrees depending on the number and position of their phenolic hydroxyl groups. Those quinones that were well coagulated could be recovered by a simple procedure. This finding was applied to the isolation of some quinones from natural source by a process in which the use of toxic organic solvents is kept to a minimum.

Experimental Section

General

The tested compounds were used as received. Alizarin (1,2-dihydroxyanthraquinone), anthrarufin (1,5-dihydroxyanthraquinone) and purpurin (1,2,4-trihydroxyanthraquinone) were of standard grade and were purchased from Fluka Chemica AG (Buchs, Switzerland). Chrysazin (1,8-dihydroxy-anthraquinone, 90-95%) and emodin (1,3,8-trihydroxy-6-methylanthraquinone, 95%) were purchased from ACROS Organics (New Jersey, USA). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was of standard reagent grade and was purchased from Sigma (USA). Authentic morindone was a donated product, courtesy of Dr. Suree Phutrakul, Chemistry Department, Chiang Mai University. Sodium chloride (99.9%, AR grade) was purchased from Ajax Chemical Co. (Sydney, Australia). 1-Butanol (AR grade) was purchased from Fisher Scientific UK Limited (UK). Hydrochloric acid (37%) was purchased from Carlo Erba Reagent Co. (Ronando, MI, Italy), and 95% ethanol was of a commercial grade and purchased from a local brewery. Aluminium plate was purchased from a local store. Direct current was sustained by a GW Instek DC power supply. Absorbance was measured on a

Genesys 10 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 300 spectrometer. GC-MS and FT-IR were performed on Agilent 6890(GC)/HP 5975(MS) and Perkin Elmer 1725X instruments, respectively. The roots of *Plumbago rosea* were obtained from the botanical garden of the Faculty of Agricultural Production, Maejo University, Chiang Mai, Thailand. The roots of *Morinda angustifolia* were from Mae Hong Son Province, courtesy of Dr. Suree Phutrakul of the Chemistry Department, Chiang Mai University, Thailand. The seedlac was a local product obtained courtesy of Dr. Pichai Pirakitikulr of the Faculty of Pharmacy, Payap University, Chiang Mai, Thailand.

Preparation of sample solutions for electrocoagulation

0.01% (w/v) sample solutions of each of the four quinone compounds, viz. alizarin, chrysazin, purpurin, and plumbagin were prepared in aqueous (85% v/v) ethanol. In the case of anthrarufin, its concentration in this solvent system was somewhat less than 0.01%, due to incomplete dissolution, and for emodin its concentration in the same solvent was reduced to 0.005% due to poor solubility.

Electrocoagulation experiments

A pair of 15 x 4 cm aluminium plates spaced 3 cm apart was immersed 5.5 cm into each sample solution (250 mL) contained in a 400-mL beaker. The solution was agitated throughout the experiment with a magnetic stirrer. Sodium chloride (0.5 g or 0.2% w/v) was added as supporting electrolyte. Direct current (0.3 A, 24-31 V) was passed through the solution *via* the two electrodes. At 15-minute intervals over a 2-hour period of electrolysis, a 4-mL aliquot of the solution was withdrawn, centrifuged, and the absorbance of the supernatant solution was measured at an appropriate wavelength corresponding to the absorption maximum for each quinone solution as follows: alizarin 435 nm, anthrarufin 420 nm, chrysazin 430 nm, emodin 440 nm, purpurin 485 nm and plumbagin, 420 nm. The data thus obtained for each compound were used to construct the electrocoagulation curves as presented in Figures 2-3.

Compound recovery experiments

A sample solution (200 mL) of each of the four well-coagulated quinone compounds, viz. alizarin, chrysazin, purpurin and emodin, was placed in a 250-mL beaker. Two aluminium plates (15 x 4 cm) were used as electrodes. These were placed 3 cm apart and dipped 6.5 cm into the magnetically-stirred solution. Sodium chloride (0.4 g) was added as supporting electrolyte. Direct current (0.3 A, 22-24 V) was then passed through the solution *via* the two electrodes for an appropriate time as follows: 40 min for alizarin, 75 min for chrysazin and emodin, and 15 min for purpurin. The resulting mixture was filtered through a Buchner funnel. The precipitate was collected and stirred in a sufficient volume of 10% hydrochloric acid solution to completely dissolve it. The acidic solution obtained was extracted with 1-butanol (50 mL), the alcoholic solution was evaporated to dryness and the residual solid treated again with a small amount of 10% HCl, then filtered, washed with water and dried to afford the recovered compound. TLC, IR, m.p., and mixed m.p. were used to confirm the integrity of each

recovered quinone (except in the case of emodin, whose m.p. could not be determined due to insufficient sample). The percentages of recovery obtained were 55, 28, 56 and 64 % for alizarin, purpurin, chrysaszin, and emodin, respectively.

Isolation of plumbagin

Air-dried roots of *Plumbago rosea* (91.7 g) were immersed in 50% ethanol for 24 hours and then the mixture was refluxed for 2 hours. Filtration gave a dark solution, which was concentrated to a volume of 1 L, transferred to a beaker, sodium chloride (0.2%) was added as supporting electrolyte and the solution was electrocoagulated with a pair of 10 x 30 cm aluminium electrodes spaced 3 cm apart and dipped into the magnetically-stirred mixture. After 2 hours of electrolysis with direct current (2.0 A, 12 V), the mixture was filtered and the filtrate re-electrolysed under the same conditions for an additional 30 minutes, refiltered, and distilled on a rotary evaporator. The yellow distillate was extracted with a small volume of dichloromethane and the dichloromethane layer was concentrated to afford orange needles (0.49 g, 0.54%), m.p. 74-75 ^{o}C (lit. [13] 78-79 ^{o}C), with spectroscopic data (IR, NMR, MS) identical with those of authentic plumbagin.

Isolation of morindone

Dry roots of *Morinda angustifolia* (29 g) were extracted with methanol (500 mL) in a Soxhlet extractor for 8 hrs. The resulting solution, after diluting with a little water to an 85% alcohol concentration, was transferred to a beaker and electrocoagulated with a pair of 9 x 20 cm aluminium electrodes, spaced 1.5 cm apart and dipped 5.5 cm into the magnetically-stirred solution which contained 0.1% sodium chloride as supporting electrolyte. After 30 minutes of electrolysis with direct current (1.7 A, 30V) the mixture was filtered and the precipitate collected was dissolved in 10% HCl and the acidic solution extracted with 1-butanol. After evaporating off the alcohol, the extract yielded a coloured solid (2.72 g), a portion of which was recrystallised from acidified methanol to give a solid product (m.p. 274-280 ^{o}C) with an IR spectrum identical to that of authentic morindone (m.p. [14] 280-284 ^{o}C).

Example of attempted isolation of erythrolaccin

A 1% solution of seedlac in 85% ethanol (250 mL) contained in a 400-mL beaker was electrocoagulated with a pair of 15 x 4 cm aluminium electrodes, spaced 3 cm apart and dipped 5.5 cm into the magnetically-stirred solution containing 0.2% sodium chloride as supporting electrolyte. After 1 hour of electrolysis with direct current (0.3 A, 22-27 V), the mixture was filtered and the filtrate was evaporated. The resinous brown residue was dissolved in absolute ethanol and the remaining undissolved inorganic matter was then filtered off from the solution which, after evaporating the solvent, gave a pale-coloured resinous lac (0.62 g). The precipitate obtained from the electrocoagulation above was dissolved in 10% hydrochloric acid solution and the resulting acidic solution was extracted with 1-butanol. The alcoholic solution was evaporated and the solid residue was collected, treated with a little 10% HCl, filtered, and washed free of acid with water. The IR spectrum

of the resulting dried solid (0.37 g) was compared with that of a yellow pigment obtained from seedlac by the known method of erythrolaccin isolation [15a] and found to be non-identical.

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Contributed Paper

Application of Electrocoagulation to the Isolation of Alkaloids

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ABSTRACT

Electrocoagulation technique was applied to the isolation of seven known alkaloids. The result showed that they could be extracted successfully with a yield comparable to that obtained by conventional methods. However, with respect to the number and amount of organic solvents used in the isolation process, all isolations incorporating electrocoagulation as a fractionating step utilized a minimum number and amount of organic solvents, especially those that were notorious for harming the environment.

Keywords : Electrocoagulation, alkaloids, caffeine, capsaicin, dihydrocapsaicin, reserpine, ajmaline, arecoline, solasodine.

1. INTRODUCTION

Alkaloids comprise the largest single class of secondary plant substances with a wide spectrum of physiological activities, hence their wide use in medicine. Being chemically basic in nature, their isolation normally requires the use of acid-base partitioning, as well as a number of organic solvents, first to extract it out of the raw material, and then to fractionate it by chromatographic methods, before the required alkaloid in a reasonably pure state can be obtained. Those organic solvents are usually not only costly and toxic, but also eventually a burden to the environment even if treated in a proper way.

With the advent of electrocoagulation (EC), it has been known for some time that this process is capable of fractionating in a rather efficient manner a number of both organic and inorganic substances by electrochemically coagulating some of them, while leaving the other components free in the solution. The process comprises an electrolysis setup (Figure 1), normally with aluminium or iron plates being used as both electrodes to generate *in situ* such species as Al^{3+}, Fe^{2+}, Fe^{3+}, OH^-, $Al(OH)_3$, $Fe(OH)_3$, etc., which are capable of selectively coagulating some particles soluble or suspended in the electrolysed solution [1]. The advantage of fractionating by EC is that it is a process in which the use of organic solvents, especially those that are toxic, is dispensed with or kept to a minimum. In our experience in applying this technique to the isolation of natural organic substances, we were successful to some extent in isolating a number of such products as a few glycosides [2-5], a cyclic polyol [6], some triterpenoids [7,8], tannins [9,10], and some phenolic compounds including quinones [10-12]. However, to our

knowledge, no study has been carried out which applies EC to the isolation of alkaloids, another important class of natural products. We thus report here our work on this novel method of isolation of some alkaloids by EC.

2. MATERIALS AND METHODS

2.1 General

The authentic compounds were used as such. Nicotine and reserpine were of laboratory grade and were purchased from BDH (England). Piperine (purum grade) and capsaicin (HPLC grade, > 97.0%) were purchased from Fluka Chemica AG (Buchs, Switzerland). Ajmaline and arecoline were of laboratory grade and were purchased from Merck (Germany) and Sigma (USA), respectively.

All other chemicals and solvents were of commercial grade, laboratory grade, or analytical grade, and were used without further purification. Aluminium plate used for electrodes was purchased from a local store.

Direct current for electrolysis was sustained by a GPR-1810HD or GPS-3030D Model DC power supply from Good Will Instrument Co. Ltd. Absorbance was measured on a Genesys 10 spectrometer.

Melting points were measured on a Mel-Temp II melting point apparatus and were uncorrected. The infrared spectra were recorded as KBr discs or thin films on a Bruker FTIR Tenser27 instrument or a Nicolet FTIR 510 instrument. The UV-VIS spectra were measured on a Genesys 10 spectrometer. The ^{1}H NMR spectra were obtained in deuterochloroform on a Bruker DRX400 (400 MHz) spectrometer with tetramethyl-silane (TMS) as an internal standard. Thin layer chromatography (TLC) analyses were performed on silica gel plates (Merck 60 F_{254}, 0.2 mm thickness), and the components were detected by UV light (254, 365 nm). High performance liquid chromatography (HPLC) analyses were carried out with an Agilent Technologies HP1100 Quaternary instrument with a UV detector, an Eclipse XDB-C8 column (4.6x150 mm), and 0.1% trifluoroacetic acid in acetonitrile as eluent (gradient elution). Gas chromatographs were run on an Agilent Technologies 6890 instrument. Mass spectra were obtained on a Hewlett Packard 5973 spectrometer with an ionization potential of 70 eV.

Dry tea leaves, dry tobacco leaves, dry black pepper fruits, areca nuts, and the fruits of *Capsicum frutescens* were purchased from a local market. The roots of *Rauvolfia serpentina* were purchased from a local herbal drug store.

2.2 Electrocoagulation Experiments

Solutions of each of the five alkaloids (0.005-0.025% w/v), viz. caffeine, capsaicin, reserpine, ajmaline, and arecoline, were prepared in water or aqueous ethanol. A pair of 15 x 4 cm aluminium plates spaced 3 cm apart was immersed 6.5 cm into each alkaloid solution (200 ml) contained in a 250-ml beaker. The solution was agitated throughout the experiment with a magnetic stirrer. Sodium chloride (0.4 g or 0.2% w/v) was added as supporting electrolyte. Direct current (0.2-2.6 A, 19-31 V) was passed through the solution *via* the two electrodes. At 15-minute intervals over a 2-hour period of electrolysis, a 5-ml aliquot of the solution was withdrawn, centrifuged, and the absorbance of the supernatant solution was measured at an appropriate wavelength corresponding to the absorption maximum for each alkaloid solution as follows: caffeine 275 nm, capsaicin 230 nm, reserpine 270 nm, ajmaline 245-250 nm, and arecoline 205 nm.

2.3 Purification by Electrocoagulation (EC)

Previous procedures [3-6,11] for EC were generally followed. An aqueous, aqueous ethanolic, or aqueous methanolic extract of each studied plant part, usually obtained by a Soxhlet extraction or maceration, was rid of undesirable impurities, e.g. tannins, pigments,

by electrolysis. For this process, a pair of aluminium or iron plates of suitable dimension placed 1.5-3 cm apart was used as electrodes and sodium chloride (0.2%) was used as supporting electrolyte. Direct current at low voltage (up to 8 A and 31 V) from a power supplier was passed through the magnetically-stirred extract for 0.5-2.5 hours (Figure 1). The resulting mixture was filtered and the filtrate normally was rid of solvent by a rotary evaporator. The resulting residue which contained the added salt was extracted with a small amount of ethanol or acetone and the ethanolic or acetone solution obtained was evaporated to dryness to give a crude alkaloid, which was subsequently purified by crystallisation, or directly analysed by a suitable method.

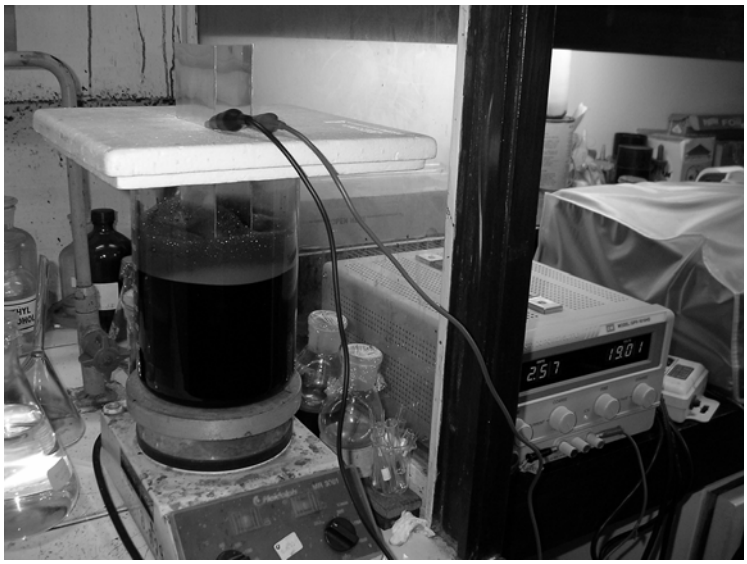

Figure 1. A typical electrocoagulation setup.

3. RESULTS AND DISCUSSION

When a pure alkaloid sample chosen for this study was subjected to EC under a normal condition, the result shows that the alkaloid is little affected by the process and remains in the electrolysed solution (Figure 2). This result is more or less the same as that obtained when EC is applied to glycosides [2-4,11]. Thus, it appears that, like glycoside, the amine function in the alkaloid seems to be unreactive to coagulation under the electrochemical condition used in our operation. EC in this case will then serve to clear up the undesirable impurities, e.g. tannins and pigments, by coagulating them out. What remains in the solution is therefore a purer alkaloid fraction, which can be easily separated. In most cases, this is done simply by evaporating out the solvent from the filtrate obtained after filtering out the coagulated impurities. The residual crude alkaloid, still containing the salt added as supporting electrolyte, is dissolved in a little nonaqueous solvent, usually ethanol or acetone, to rid it of salt in the final step.

By using this general method, we could extract crude caffeine from tea in a purer form (white powder, higher m.p.), although in a somewhat lower yield (0.4 vs. 0.7%), than that obtained by a classical laboratory procedure [13]. However, the latter method requires a large amount of chlorinated solvent

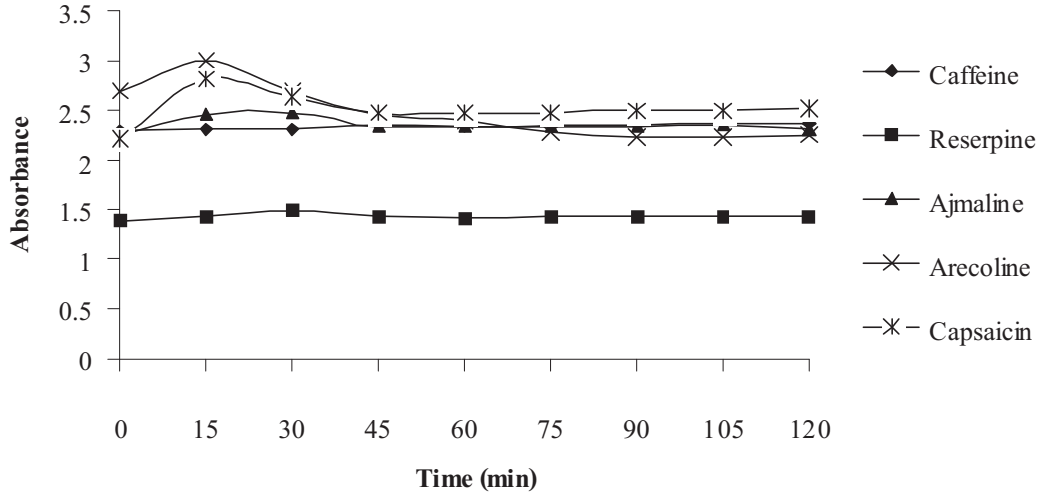

Figure 2. Plot of absorbance against electrocoagulation time for each alkaloid solution (Due to an increase in pH of the electrolysed solution, this might initially affect the absorbance of a strong base like arecoline slightly. Similar phenomenon may occur also to capsaicin, the only alkaloid which has a phenolic function).

(usually dichloromethane) to partition the caffeine out of the aqueous tea solution before a crude slightly green-coloured caffeine is gotten. Similarly, capsaicin and dihydrocapsaicin, the major pungent alkaloids from red chilli, were isolated electrolytically using only 75% ethanol as solvent. With a typical conventional method [14], four organic solvents, viz. acetone, toluene, benzene, and 50% ethanol, were utilized before the same alkaloids were obtained in comparable quantity (0.3%) and purity (as characterised by NMR and determined by GC, Figure 3).

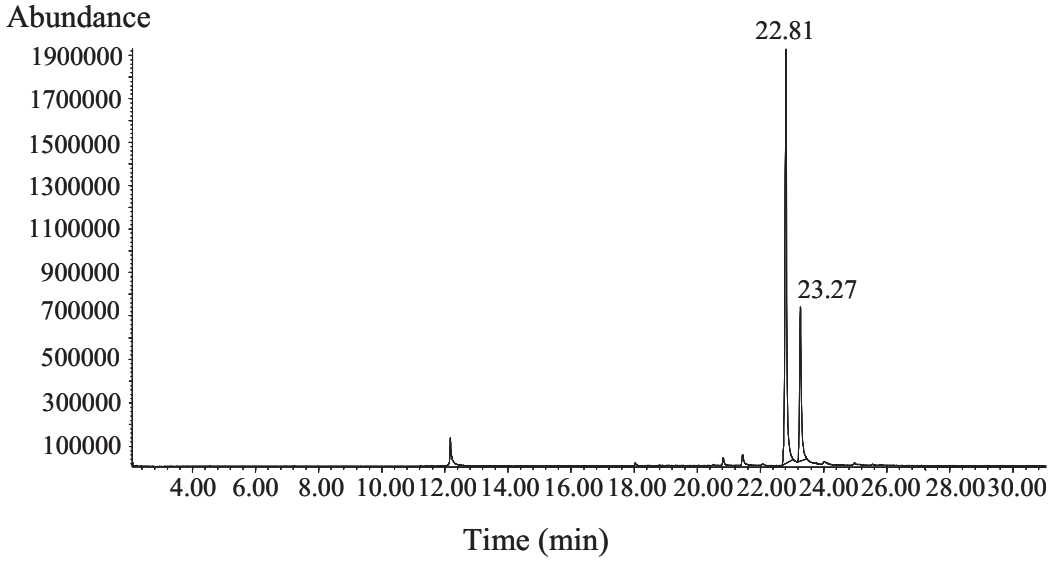

Figure 3. GC of red chilli crude extract obtained by EC method, showing capsaicin (retention time 22.81 min.) and dihydrocapsaicin (retention time 23.27 min.).

In order to isolate the medicinally important alkaloids (reserpine and ajmaline) from the root of *Rauvolfia serpentina*, it usually has to be soaked with dilute ammonia before being extracted with an organic solvent (e.g. ether) [15]. The extracted alkaloids were then partitioned with an acid solution, converted back into the free bases, and taken into another water-insoluble organic solvent (e.g. benzene) to give, after evaporation, the crude alkaloid extract. With our EC method, we directly extracted the root with ethanol, diluted the ethanolic extract with a small amount of water before subjecting it to EC for 2 hours. The resulting electrolysed mixture was filtered and the filtrate evaporated to dryness. Dissolving the resulting residue with a small amount of ethanol directly afforded, after evaporating off the ethanol, the crude extract containing the two alkaloids in about the same amounts (although in a more dilute form) as those in the above extract obtained conventionally (0.1% and 0.8% of the root for reserpine and almaline respectively, as determined by HPLC, Figure 4).

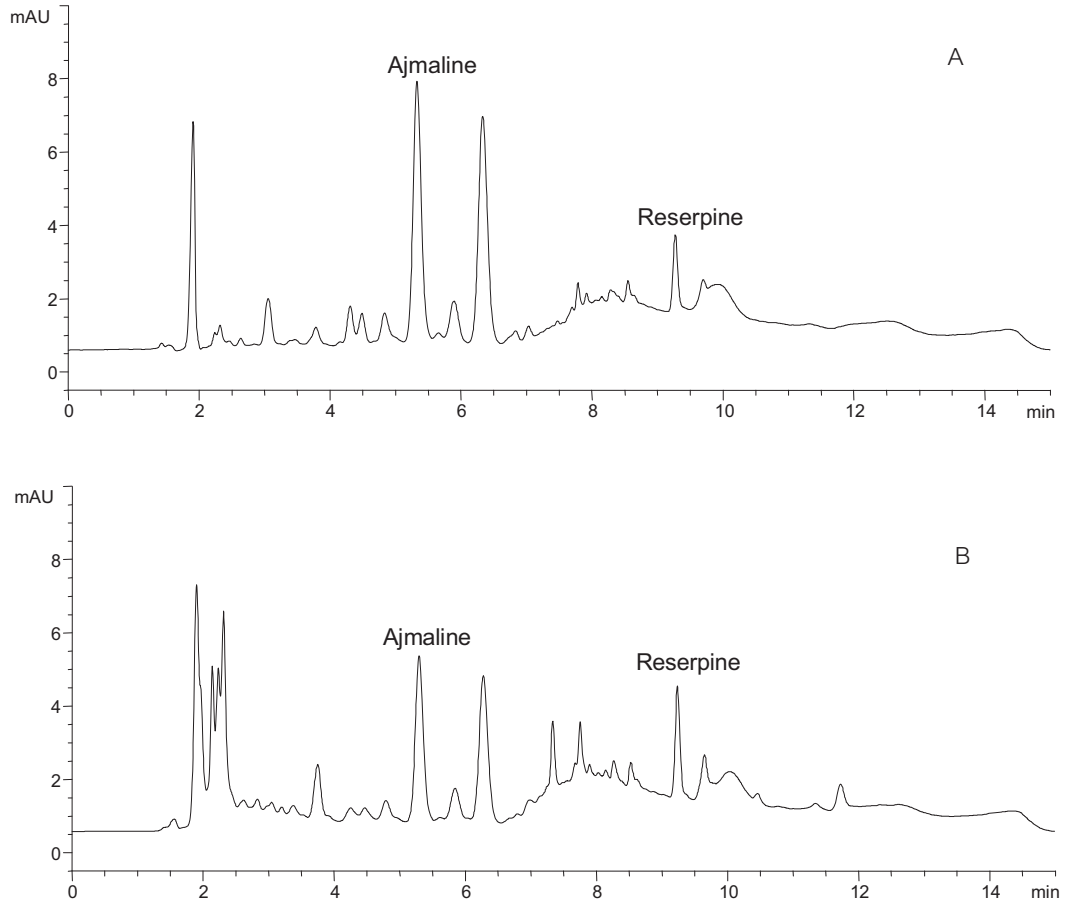

Figure 4. HPLC of crude extracts of *Rauvolfia serpentina* roots obtained by conventional method (A) and EC method (B) [Extra peaks in B might stem from alcohol, a solvent with a wider extracting range, being used as extracting solvent].

Similarly, arecoline was electrochemically extracted from arecanut in approximately the same percentage yield (0.1%) as by a normal method in the literature [16], but with less use of organic solvent (ether). Moreover, a red dye, which readily coagulated out, was obtained as a by-product. Finally, when solasodine, a steroidal alkaloid from the leaf of *Solanum laciniatum*, was extracted using EC as the dechlorophyllation step [5] in place of the conventional dechlorophyllation by organic solvent (e.g. chloroform) [17,18], we obtained the pure alkaloid (matching m.p., IR, NMR with authentic sample) in a much better yield (1% vs. 0.5%). This steroidal alkaloid is an important starting material for the synthesis of many steroids.

4. CONCLUSION

It has been demonstrated that EC can be successfully applied to the isolation of some alkaloids, with an important advantage over conventional isolation methods. This is the reduction in both the number and amount of toxic organic solvents involved in the isolation process. This method of isolation of alkaloids therefore seems to be a novel alternative method, which is also a more environmentally friendly one.

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