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In vitro Antibacterial Activity of Quercetin Containing Extract from Hibiscus Sabdariffa L. Calyxes

Do Thi Hai Anh[1], Le Huy Hoang[1,2],
Kitsamone Shihavong[1], Nguyen Thai Uy[1], Nguyen Quang Huy[1,*]

[1]*Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam*
[2]*Institute of New Technology, Academy of Military Science and Technology, 17 Hoang Sam, Cau Giay, Hanoi, Vietnam*

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Abstract: *Hibiscus sabdariffa* L. has been used traditionally as food and in herbal medicine because its calyxes are rich of flavonoids source especially quercetin and anthocyanin. In addition, its antibacterial activity is implied to be helpful for human health even at the low concentration. In this study, we investigated the *in vitro* antibacterial activity and quantified the free quercetin in hibiscus extract using sensitive and reliable methods such as agar disk diffusion, HPTLC and LC-MS/MS. The results indicated that crude methanol extract of hibiscus calyxes had strong antibacterial activity. The total phenolic and flavonoid contents of hibiscus calyxes were 25.07 mg GAE/g DW and 29.96 mg QE/g DW, respectively. From methanol hibiscus extract, quercetin was determined by using HPTLC and LC-MS/MS methods. There was presence of quercetin aglycone in one fraction (which showed highest antibacterial activity) at the concentration of 11.3 ng/ml. This study provided useful information in using reliable and sensitive methods for screening and determining antibacterial activity of free quercetin at the low concentration based on any plant raw material.

Keywords: Quercetin, *Hibiscus sabdariffa* L., antibacterial, HPTLC.

1. Introduction

Flavonoids are polyphenol compounds occurring in fruit and vegetables. Flavonoids are believed to be responsible for the wide spectrum of pharmacological activities seen in many plants [1]. Moreover, flavonoids have remarkable health promoting effects, such as anti-inflammatory [2], anti-microbial [3], and antioxidant [4] activity in which flavonoid quercetin is mostly interested

Quercetin is flavonoid that has been extensively studied over many years. Quercetin occurs naturally in plants as conjugated glycosides, with the most common glycosides being quercetin-3,4-O-diglucoside, quercetin-4-O-monoglucoside and quercetin 3-monoglucoside. Quercetin has been detected in many fruits and vegetables in varied concentration. For instance, the flesh of onions contains mostly quercetin glucoside, the skin

*Corresponding author. Tel.: 84-904263388
Email: nguyenquanghuy@vnu.edu.vn

and outer layers of an onion have much more quercetin aglycone [5]. Quercetin is one of the most studied plants flavonoids and has been reported to have antibacterial effects. For example, Quercetin had inhibitory effects on *Streptococcus mutans* with minimum inhibitory concentration (MIC) of 2 mg/ml, *Streptococcus sobrinus* with MIC of 1 mg/ml and *Prevotella intermedia* with MIC 4 mg/ml [6]. Many reports showed that quercetin at low concentration have capacity to interact with enzymes *in vitro* and also showed bioactive properties such as antibacterial activity. At the concentration of 0.001 ppm to 100 ppm Quercetin-3-glucuronic can inhibit angiotensin converting enzyme (ACE) [7]. However, there are limited reports illustrating clearly the methods to investigate the relation between bioactive properties and quercetin at the low concentration.

Hibiscus sabdariffa L., commonly named as "roselle" is rich of flavonoid source, especially flavonols [8]. According to Lorrainer *et al*., the two most commonly found flavonoid aglycones in Hibiscus were the flavonol quercetin and the anthocyanin cyanidin [9]. The aim of this study was to use simple and sensitive methods for screening bioactive constituents/components such as quercetin aglycone and investigating the antibacterial activity of hibiscus calyxes extract.

2. Material and methods

2.1. Material

Tested microorganisms were provided by the VNU-Institute of Microbiology and Biotechnology, including *Escherichia coli (*ATCC 25922), *Staphylococcus aureus (*ATCC 25923), *Bacillus subtilis (*ATCC 23857) and *Bacillus cereus (*ATCC 14579).

Reagents and solvents were purchased from well-known companies such as Folin-Ciocalteu (SigmaAldrich), Gallic acid (BDH chemical Ltd, England), Quercetin (National institute of drug quality control), 2,2-diphenyl-1-picrylhydrazyl (SigmaAldrich, USA). Others solvents and reagents were of analytical grade.

2.2. Experimental methods

Ultrasound-assisted extraction

Hibiscus calyxes were purchased from a grocery store in Laos, and classified by Nguyen Anh Duc (Botany Department, Faculty of Biology, VNU University of Science) based on the visible physical characteristics of the plant (flower, leave and seed). The dried calyxes were grounded to powder and then stored at 4^{o}C until use. The hibiscus calyxes were extracted by different solvents which have different polarization including n-hexane, ethyl acetate, methanol and 80% ethanol. 5 g sample and 50 ml solvent were mixed well and then was treated with 37 kHz ultrasonic wave, power 140W for 1 hour. The extract was centrifuged at 2000 rpm in 5 minutes at room temperature. The residue was removed by using filter. The solvent was evaporated by a rotary evaporator at 40 ^{o}C. The extract was kept in a freezer at 4 ^{o}C for further studies.

Quantitative analysis of total polyphenol and flavonoid content

Methanol solution of the extract was used in the polyphenol analysis using Folin-Ciocalteu reagent to determine the total phenolic content [10]. The phenols values are expressed in terms of gallic acid equivalent. The linear equation was $y = 0.0109x + 0.036$ and $R^2 = 0.991$.

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method at λ_{max} = 415nm and the reagent was aluminum chloride [11]. The calibration curve was made by preparing quercetin solutions at different concentrations in methanol. The linear equation was $y = 0{,}0048x + 0{,}018$ and $R^2 = 0{,}994$.

Column chromatography

The usual adsorbents employed in column chromatography are silica, the solvents used to separate different compounds were the mixtures

of different solvents including (n-hexane: ethyl acetate: methanol) with different ratio: 50:50:0, 30:70:0, 0:90:10, 0:60:40, 0:70:30.

The identity of the fractions were identified by color. Then each fraction was screened for antibacterial activity. Free quercetin was detected in fraction which has remarkable antibacterial activity by using HPTLC and LC-MS/MS methods.

HPTLC method

Instrument: CAMAG Automatic TLC Sampler 4 (Camag, Switzerland) with win CATS software. Stationary phase: TLC plates silica gel 60 F_{254} pre coated layer (20 ×10 cm), thickness 0.2 mm, number of tracks: 16, band length: 8 mm. Mobile phase: Toluene: Ethyl acetate: Formic acid: Methanol (5.5:3:1:0.5); Standard: Quercetin. Sample: Fraction 3. Solubility: Methanol standard concentration: 150 µg/ml; Standard injection volumes (µl): 1, 2, 3, 4, 5. Sample concentration: 160 µg/ml. Sample application volumes (µl): 1, 2, 3, 4, 5. Development chamber: Twin trough chamber (20×10). Development mode: Ascending mode. Distance run: 75 mm. Scanning wavelength: 386 nm. Lamp: D2Slit dimensions 4.0×0.3mm, Micro measurement mode: absorbance.

LC-MS/MS method

Apparatus and chromatograph system: The mobile phase consists of Methanol and 0.1% (v/v) formic acid. The flow rate and infection volume were set at 300 µl/min and 30 µl, respectively. The optimum interface conditions were: interface temperature of 500 ^{o}C; delclustering potential of -60V; entrance potential of -12V; collision energy at -28V; and collision exit potential -16V.

Determining bioactive properties of extract by agar disk diffusion and DPPH methods

Agar disk diffusion method (Kirby-Bauer antibiotic testing) was used to identify the antimicrobial activity of different extracts. The concentration of tested microorganisms was determined by using the $OD_{620\ nm}$ value (the value between 0.08-0.1 is appropriate with 10^6 CFU/ml). 50 µl of each extracts were injected into the well and plates were firstly kept at room temperature for 2 hours to allow the diffusion of any produced antimicrobial. All plates were incubated at 37 ^{o}C. Each experiment was performed in triplicates. Antimicrobial activity was determined by measuring the diameter of antibacterial zone: Without activity when diameter of antibacterial zone <10 mm; Weak activity when diameter of antibacterial zone of 10-12 mm; Moderate activity when diameter of antibacterial zone of 13-15 mm and high activity when diameter of antibacterial zone > 15 mm. Positive control was Ampicillin with different concentrations for different tested microorganisms (1.25 µg/ml for *B. cereus, B. subtilis,* and *S. aureus,* 10 µg/ml for *E. coli*). Negative control is methanol [12].

3. Results and Discussion

3.1. Bioactive properties and quantitative phenolic compounds of hibiscus extract

The antimicrobial activity of the extract was investigated using the disk diffusion method. Methanol was used as control in order to analyze the effect of this solvent on microbial growth, while ampicillin was used as the referent sample. Based on the obtained results (table 1), it can be seen that hibiscus methanol extract at the concentration of 1 mg/ml had the remarkable antimicrobial activity on *S. aureus, B. cereus and B. subtilis* strains. All tested microorganism remained resistant to the effect of methanol solvent.

The total phenolic and flavonoid content of *Hibiscus sabdariffa* calyxes were 25.07 mg GAE/g DW and 29.96 mg QE/g DW, respectively. In 2014 research of Daniele *et al.* showed that on experimental conditions, total polyphenol content from Hibiscus ranged from 4.60 mg GAE/g DW to 50.12 mg GAE/g DW [13].

Table 1. Antibacterial activity of methanol hibiscus extract

Tested organisms	*Agar disk -diffusion method*			
	Hibiscus extract	*Ampicillin*	*Methanol*	*Quercetin*
E.coli	++	++	-	+
S.aureus	+++	++	-	+++
B.cereus	+++	++	-	+++
B.subtilis	+++	++	-	+++

Without activity (-), Weak activity (+), Moderate activity (++), High activity (+++)

The antibacterial activity of the Hibiscus extract may be attributed from its phytochemical compounds especially those of phenolic compounds such as quercetin. Therefore, we used column chromatography, HPTLC and LC-MS/MS, to detect and quantify quercetin in hibiscus extract.

3.2. Column chromatography result and quercetin detection by HPTLC method

After separating by column chromatography, 100 small fractions (5 ml/fraction) were collected. We separated them by their color and there were 5 fractions collected, namely F.1 (colorless), F2 (colorless), F.3 (orange – yellow), F.4 (pink), F.5 (purple). Then the solvent was removed by rotary evaporation.

The antibacterial activity of these fractions were determined by using agar disk – diffusion method. Quercetin-containing fractions were detected by using HPTLC methods.

Table 2. Antibacterial activity of different fractions of Hibiscus extract

Test samples	*Agar disk – diffusion method*				*The detection by HPTLC method*
	E.coli	S.aure	B.cere	B.subtil	*Quercetin*
F.1	-	-	-	-	*ND*
F.2	-	-	-	-	*ND*
F.3	+++	+++	++	+++	*D*
F.4	-	+	-	+	*ND*
F.5	-	++	-	+	*ND*
Ampicill	++	++	++	++	
Methano	-	-	-	-	

Non-detected (ND), detected (D),

Without activity (-), Weak activity (+), Moderate activity (++), High activity (+++)

From the Table 2, fraction 3 had the highest antimicrobial activity on all tested microorganisms. In contrast, fraction 1-2 did not show the antimicrobial activity. Both fraction number 4 and 5 could inhibit the growth of 2 strains *S. aureus* and *B. subtilis* and had weak activity with other bacteria strains.

Quercetin containing fractions were detected by using HPTLC method. Five different concentrations of quercetin (150-750 µg/ml) were prepared as standard. There was good correlation between peak area, height and the corresponding concentration of quercetin with line equation was y = -29.642 + 0.645x,

R^2= 0.9995. The standard quercetin has R_f value of 0.49. This result indicated that HPTLC is a reliable method to detect bioactive compounds such as quercetin. Only fraction 3 had compounds which had the same R_f as quercetin, however, 2 peaks were observed (Figure 1). Therefore, we used LC-MS/MS method to quantitative free quercetin in this fraction.

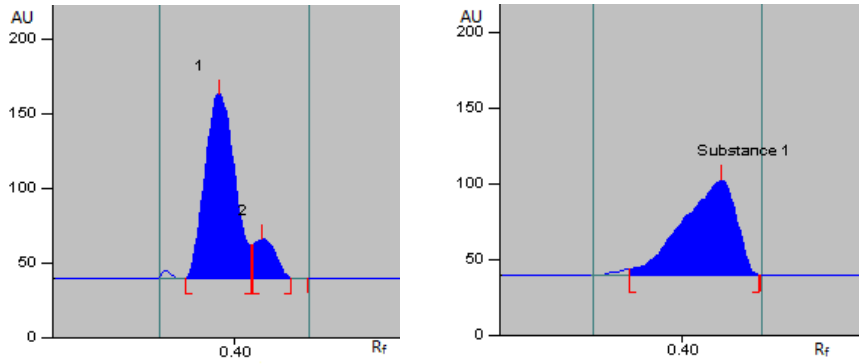

Figure 1. Chromatogram of fraction 3(left side) and standard quercetin (right side)

3.3. Quantitative analysis of quercetin by using LC-MS/MS methods

From quercetin detection result, quercetin was investigated by using LC-MS/MS method. The quantification of analysis was performed by negative ionization mode of LC-MS/MS for high sensitivity and selectivity of data. The selected reaction monitoring pair monitored the ion transition of Q1:Q3 m/z 301.1/150.9 for quercetin. Finally, the quercetin in fraction 3 was estimated at the concentration of 11.3 ng/ml.

4. Conclusion

The methanol extract of *Hibiscus sabdariffa* calyxes had strong antibacterial activity. The total phenolic and flavonoid content of calyxes were 25.07 mg GAE/g DW and 29.96 mg QE/g DW, respectively. By column chromatography, 5 fractions were separated, and fraction 3 had the highest antimicrobial activity on all tested microorganisms and contained free quercetin with concentration of 11.3 ng/ml.

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Nghiên cứu tác dụng kháng khuẩn *in vitro* của dịch chiết chứa Quercetin từ đài hoa bụp giấm (*Hibiscus sabdariffa* L.)

Đỗ Thị Hải Anh[1], Lê Huy Hoàng[1,2],
Kitsamone Shihavong[1], Nguyễn Thái Uy[1], Nguyễn Quang Huy[1]

[1]*Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt nam*
[2]*Viện Công nghệ mới,Viện Khoa học và Công nghệ Quân sự, 17 Hoàng Sâm, Cầu Giấy, Hà Nội*

Tóm tắt: *Hibiscus sabdariffa* L. (Bụp giấm) đã và đang được sử dụng rộng rãi trong đời sống thường ngày như một loại thực phẩm giàu dinh dưỡng, cũng như trong đông y vì là nguồn giàu flavonoid đặc biệt là quercetin và anthocyanin. Hơn nữa, hoạt tính kháng khuẩn của Bụp giấm được cho là hữu ích đối với sức khỏe con người kể cả ở nồng độ thấp. Nghiên cứu của chúng tôi được tiến hành nhằm đánh giá hoạt tính kháng khuẩn *in vitro* và định lượng hàm lượng quercetin tự do trong dịch chiết của đài hoa bụp giấm sử dụng các phương pháp nghiên cứu có độ nhạy và độ tin cậy cao như khuếch tán đĩa thạch, sắc ký lớp mỏng hiệu năng cao (HPTLC), sắc ký lỏng hai lần khối phổ (LC/MS-MS). Kết quả nghiên cứu cho thấy dịch chiết đài hoa bụp giấm trong methanol có hoạt tính kháng các vi sinh vật kiểm định tốt nhất. Hàm lượng phenolic và flavonoid tổng số có trong dịch chiết đạt tương ứng là 25,07 mg GAE/g DW và 29,96 mg QE/g DW. Hàm lượng quercetin được xác định từ dịch chiết bằng methanol sử dụng phương pháp HPTLC và LC/MS-MS. Kết quả cho thấy quercetin có mặt ở một phân đoạn (phân đoạn này có hoạt tính kháng khuẩn cao nhất) với hàm lượng đạt 11,3 ng/ml. Kết quả này đã bước đầu đóng góp dữ liệu vào việc chọn lọc các phương pháp có độ nhạy cao trong sàng lọc hoạt tính kháng khuẩn của quercetin tự do với hàm lượng thấp từ nguyên liệu thực vật.

Từ khóa: Quercetin, *Hibiscus sabdariffa* L, kháng khuẩn, HPTLC (sắc ký lớp mỏng hiệu năng cao).