Antimicrobial Flavonoid from *Hibiscus rosa-sinensis* Linn.

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The air-dried flowers of *Hibiscus rosa-sinensis* Linn., commonly known as gumamela afforded 5,7,4'-trihydroxyflavanone (**1**), also known as naringenin, and a mixture of hydrocarbons and squalene. The structure of **1** was elucidated by extensive 1D and 2D NMR analyses. Antimicrobial tests on **1** indicated low activity against the bacterium, *S. aureus* and fungi, *C. albicans* and *T. mentagrophytes.* Compound **1** did not exhibit cytotoxicity against a human lung non-small cell adenocarcinoma (A549) cell line and a normal Chinese hamster ovarian (AA8) cell line.

Keywords: *Hibiscus rosa-sinensis,* Malvaceae, 5,7,4'-trihydroxyflavanone, naringenin, antimicrobial

1. INTRODUCTION

Hibiscus rosa-sinensis, locally known as gumamela is commonly grown as an ornamental plant with a wide range of medicinal applications. The red flower is used against paralysis and to regulate menstruation, a purgative, a dysmenorrheal, and sometimes it causes abortion. An infusion of the flowers is used as an expectorant in bronchitis and gonorrhea and as a refrigerant drink in fevers and a demulcent in coughs. Dark red petals in the form of mucilaginous infusion are employed to treat ardor-urinae, strangury, cystitis, and other irritable conditions of the genitor-urinary tract. Paste from the flower buds are applied as a poultice to boils, cancerous swellings and mumps (Quisumbing, 1978). Previous studies on the plant reported the isolation of β-rosasterol oxide which is an antifertility agent (Zhou, Jiang, Zhao, & Li, 1998), β-rosasterol (Yu, Hu, Sha, Zheng, & Zhou, 1991), campesterol, cholesterol, ergosterol, and stigmasterol (Chauhan, 1984), β-sitosterol and taraxeryl acetate (Agarwal & Rastogi, 1971), quercetin-3,7-diglucoside, cyanidin-3,5-diglucoside(I), cyanidin-3-sophoroside-5-glucoside, kaempferol-3-xylosylglucoside, gossypetin-8-glucoside, and gossypetin-7-glucoside (Subramanian & Nair, 1972).

We report herein the isolation, structure elucidation, and antimicrobial and cytotoxicity assays of 5,7,4'-trihydroxyflavanone (**1**), also known as naringenin, from the flowers of *H. rosa-sinensis*. A mixture of hydrocarbons and squalene was also obtained. To the best of our knowledge, this is the first report on the isolation of **1** from *Hibiscus rosa-sinensis.* To facilitate the identification of **1**, extensive 1D and 2D NMR spectra were obtained to elucidate its structure.

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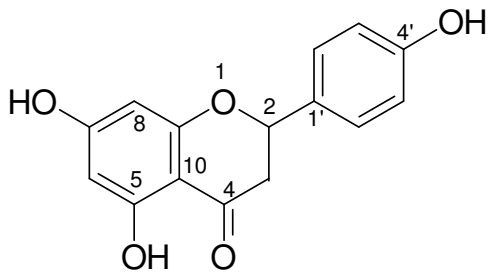

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2. MATERIALS AND METHODS

2.1. General Experimental Procedures

NMR spectra were recorded on a Bruker Avance 400 in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C. Column chromatography was performed with silica gel 60 (70-230 mesh), while the TLC was performed with plastic backed plates coated with silica gel F_{254}. The plates were visualized with vanillin-H_2SO_4 and warming.

2.2. Sample Collection

H. rosa-sinensis flowers (2 kg) were obtained from Fairview, Quezon City in September 2004. It was identified as *Hibiscus rosa-sinensis* at the Philippine National Museum.

2.3. Isolation

The air-dried flowers (582 g) of *H. rosa-sinensis* were ground in an osterizer, soaked in dichloromethane for three days, then filtered. The filtrate was concentrated under vacuum to afford a crude extract (9 g) which was chromatographed in increasing proportions of acetone in dichloromethane at 10% increment. The 60% to 70% acetone in dichloromethane fractions were combined and rechromatographed (3x) in dichloromethane:diethyl ether:acetonitrile (9:0.5:0.5) to afford **1** (5 mg, yellowish solid). The dichloromethane fraction was rexhromatographed in petroleum ether to afford a mixture of hydrocarbons and squalene (10 mg, colorless oil).

2.3.1. Antimicrobial Tests

The microorganisms used in these tests were obtained from the University of the Philippines Culture Collection (UPCC). These are *Aspergillus niger* UPCC 4219, *Candida albicans* UPCC 2168, *Bacillus subtilis* UPCC 1295, *Pseudomonas aeruginosa* UPCC 1244, *Escherichia coli* UPCC 1195, *Staphylococcus aureus* UPCC 1143, and *Trichophyton mentagrophyte* UPCC 4193. The test compound was dissolved in 95% ethanol. The antimicrobial assay procedure reported in the literature (Guevara & Recio, 1985) was employed. The activity index was computed by subtracting the diameter of the well from the diameter of the clearing zone divided by the diameter of the well.

2.3.2. Cytotoxicity Tests

Compound **1** was tested for cytotoxic activity against a human lung non-small cell adenocarcinoma (A549) cell line and a normal Chinese hamster ovarian (AA8) cell line at the Institute of Biology, University of the Philippines, Diliman, Quezon City. Doxorubicin was used as the positive control, while DMSO was used as the negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed (Freshney, 1994).

3. RESULTS AND DISCUSSION

The dichloromethane extract of the air-dried flowers of *H. rosa-sinensis* afforded **1** and a mixture of hydrocarbons and squalene by silica gel chromatography. The structure of **1** was elucidated by extensive 1D and 2D NMR spectroscopy as follows.

The ^1H NMR spectrum of **1** (Table 1) indicated resonances for geminal protons at δ 2.73 (dd, J = 4, 12 Hz) and 3.18 (dd, J = 12,16 Hz) which are coupled to the carbinyl proton at δ 5.45 (dd, J = 4, 16 Hz); ortho aromatic protons at δ 6.90 (2H, d, J = 8 Hz) and 7.40

(2H, d, J = 8 Hz); meta aromatic protons at δ 5.95 (d, J = 2 Hz) and 5.96 (d, J = 2 Hz); aromatic hydroxyl protons at δ 9.68, 8.51, and δ 12.18.

Table 1.
100 MHz ^{13}C and 400 MHz ^{1}H NMR spectral data of 1 in $(CD_3)_2CO$

Position	1, δ_C	1, δ_H
2	78.80	5.45 (dd, J= 4,16 Hz)
3	42.35	2.73 (dd, J= 4,12 Hz) 3.18 (dd, J= 12,16 Hz)
4	196.11	--
5	164.16	--
6	95.67	5.95 (d, J= 4 Hz)
7	166.19	--
8	94.70	5.96 (d, J= 4 Hz)
9	163.24	--
10	102.08	--
1'	129.66	--
2', 6'	127.88	7.40 (d, J= 8 Hz)
3', 5'	115.04	6.90 (d, J= 8 Hz)
4'	157.56	--
5-OH		12.18 s
7-OH		9.68 s
4′-OH		8.51 s

The ^{13}C and DEPT NMR spectral data of **1** (Table 1) showed 15 carbon resonances with the following functionalities: a methylene carbon at δ 42.35, a carbinyl carbon at δ 78.80, twelve aromatic carbons at δ 94.70, 95.67, 102.08, 115.04 (2C), 127.88 (2C), 129.66, 157.56, 163.24, 164.16, 166.19, and a carbonyl carbon at δ 196.11. The deshielded aromatic carbons at δ157.56-166.19 are oxygenated. These spectral data indicated a flavanol with three hydroxyl groups.

The ^{1}H and ^{13}C assignments of **1** (Table 1) were verified by HSQC and connectivity was verified by HMBC (Fig. 1). Based on deshielded resonance, the hydroxyl at δ 12.18 was attached to C-5 which was chelated to the carbonyl at C-4. Long-range correlations were observed between this proton and C-5, C-6, and C-10. The hydroxyl at δ 9.68 was attached to C-7 on the basis of long-range correlation between this proton and the carbons at C-6, C-7, and C-8. The third hydroxyl at δ 8.51 was attached to C-4' due to long-range correlation between this proton and the carbons at C-3' and C-4'. The carbinyl proton at δ 5.45 was placed at C-2 on the basis of its long-range correlation to C-3, C-1', and C-2'.

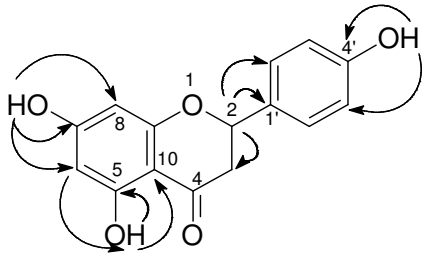

Figure 1. **Key ^{1}H-^{13}C HMBC long-range correlations for 1.**

Thus, **1** was identified as 5,7,4'-trihydroxyflavanone (naringenin) which was previously reported as a constituent of *Prunus cerasus* (Ibrahim, Galal, Ahmed, & Mossa, 2003), *Lysidice rhodostegia* Hance (Gao, Fu,

Fan, Yu, S.S., & Yu, DQ, 2005), *Citrus janos* (Heo et al., 2004) and grapefruit (Felgines et al., 2000). Naringenin is associated with healthful benefits, such as in osteoporosis, cancer and cardiovascular diseases (Galluzo, Ascenzi, Bulzomi, & Marino, 2008). Naringenin is reported to have antiproliferative effects in different cancer cell lines, such as breast (So, Guthri, Chambers, Moussa, & Carroll, 1996; Harmon & Patel, 2004) and colon (Frydoonfar, McGrath, & Spigelman, 2003). Several mechanisms proposed for naringenin-induced antiproliferative effects are antioxidant activities and kinase and glucose uptake inhibition (Harmon & Patel, 2004; Moon, Wang, & Morris, 2006) and the ability of naringenin to hamper cell proliferation by binding to estrogen receptor (Totta, Acconcia, Leone, Cardillo, & Marino, 2004; Virgili et al., 2004).

Based on the above literature, naringenin is known to exhibit anti-breast cancer and anti-colon cancer activities. To test if **1** also exhibits anti-lung cancer potential, it was tested for cytotoxicity using a human lung non-small cell adenocarcinoma (A549) cell line at the Institute of Biology, University of the Philippines, Diliman, Quezon City. It was also tested for cytotoxicity against a normal Chinese hamster ovarian (AA8) cell line. Results of the study indicated that **1** had no linear interpolation with the A549 and AA8 cell lines, thus IC_{50} could not be computed. This implied that **1** did not exhibit cytotoxic effect against these cell lines.

As part of our continuing search for antimicrobial compounds from Philippine medicinal plants and since some flavonoids are known to possess antimicrobial properties (Ragasa, Cruz, Chiong, Tada, & Rideout, 1997; Ragasa, Pendon, Sangalang, & Rideout, 1999; Ragasa, Wong, & Rideout, 2007), **1** was tested for antimicrobial activity against seven microorganisms, namely: *B.subtilis*, *E.coli*, *P. aeruginosa*, *S.aureus*, *C. albicans*, *A. niger*, and *T. mentagrophytes*. Results of the study (Table 2) indicated that **1** exhibited a low activity index (AI) of 0.2 against the fungi: *C. albicans,* an oval budding yeast and *T. mentagrophytes*, a parasite, as compared to the standard antibiotic canesten that contains 1% clotrimazole with AI of 0.8 and 4.5, respectively. The compound also exhibited low activity (AI = 0.3) against the gram-positive bacteria *S. aureus*, as compared to the standard antibiotic chloramphenicol with an AI of 3.2. The results of the study implied that **1** can be used to prevent the proliferation of the infection on tissues caused by *C. albicans, T. mentagrophytes* and *S. aureus.* It was found inactive against *E. coli*, *P. aeruginosa*, *B. subtilis,* and *A. niger*.

Table 2.
Antimicrobial Test Results of 1

Organism	Sample (30 µg concentration)	Clearing zone (mm)	Activity Index (AI)
E. coli	**1**	-	0
	Chloramphenicol	23	2.8
P. aeruginosa	**1**	-	0
	Chloramphenicol	14	1.3
S. aureus	**1**	13	0.3
	Chloramphenicol	25	3.2
B. subtilis	**1**	thinning	0
	Chloramphenicol	20	2.3
C. albicans	**1**	12	0.2
	Canesten[a] (0.2g)	18	0.8
T. mentagrophytes	**1**	12	0.2
	Canesten[a] (0.2g)	55	4.5
A. niger	**1**	-	0
	Canesten[a] (0.2g)	23	1.3

[a]contains 1% clotrimazole

The mixture of hydrocarbon and squalene was deduced from the high intensity resonance at δ 1.26 for the methylene protons and the methyl triplet at δ 0.88 of the hydrocarbons and the resonances at δ 5.10-5.15 for olefinic, δ 1.60 and 1.68 for allylic methyl, and 1.96-2.07 for allylic methylene protons of squlene (Inte, Ragasa, & Rideout, 1998).

Squalene has cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties (Farvin et al., 2006). It significantly suppresses colonic ACF formation and crypt multiplicity. This strengthens the hypothesis that squalene possesses chemopreventive activity against colon carcinogenesis (Rao, Newmark, & Reddy, 1998). Squalene is a potential chemopreventive agent and an antioxidant (Smith, 2000; Owen et al., 2004), and it potentiates cytotoxicity of anticancer agents (Nakagawa et al., 1985). It may counteract the increase in body fat, blood pressure, and levels of plasma liptin, glucose, cholesterol, and triglycerides (Liu et al., 2009). Squalene also improves mitochondrial function during aging and minimizes age associated disorders (Buddhan, Sivakumar, Dhandapani, Ganesa, & Amandan, 2007).

ACKNOWLEDGEMENT

The authors are grateful to Dr. John A. Rideout for conducting the NMR analyses at the School of Chemical and Biomedical Sciences, Central Queensland University, Rockhampton, Queensland, Australia. The antimicrobial tests were conducted at the University of the Philippines – Natural Sciences Research Institute. The MTT assay was conducted at the Institute of Biology, University of the Philippines, Diliman, Quezon City.

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