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Al-Majmaie, S, Nahar, L, Sharples, GP, Wadi, K and Sarker, SD (2019) Isolation and antimicrobial activity of rutin and its derivatives from Ruta chalepensis (Rutaceae) growing in Iraq. Records of Natural Products, 13 (1). pp. 64-70. ISSN 1307-6167

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Isolation and Antimicrobial Activity of Rutin and its Derivatives from *Ruta chalepensis* (Rutaceae) Growing in Iraq

Shaymaa Al-Majmaie¹, Lutfun Nahar¹, George P. Sharples¹, Khazal Wadi² and Satyajit D. Sarker*

¹Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Faculty of Science, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom
²Department of Biology, College of Science, University of Diyala, Baquba, Iraq

*Corresponding author: Email: S.Sarker@ljmu.ac.uk

(Received Month Day, 2018; Revised Month Day, 2018; Accepted Month Day, 2018)

Abstract: Rutin (1), rutin 3'-methyl ether (2), and a new flavonol glycoside, 6-hydroxy-rutin 3',7-dimethyl ether (3), were isolated from the methanol extract of the fruits of *Ruta chalepensis*, collected from Diyala, Iraq. Their structures were elucidated by spectroscopic analyses, including 1D-, 2D-NMR and HRESIMS. Antimicrobial activity of compounds 1-3 was tested against four Gram +ve and Gram –ve bacterial strains, and the only fungal strain, *Candida albicans*, using the 96-well based resazurin microtitre assay.

Keywords: *Ruta chalepensis*; Rutaceae; resazurin assay; antimicrobial activity; rutin; 6-hydroxy-rutin 3',7-dimethyl ether. © 201X ACG Publications. All rights reserved.

1. Introduction

*Ruta chalepensis* L. (family: Rutaceae), commonly known as “fringed rue”, is a perennial herb (ca. 80 cm tall), native to Eurasia and North Africa and introduced elsewhere [1-4]. Different parts of this plant have been used for centuries in traditional medicine to treat convulsions, dropsy, fever, mental disorders, menstrual problems, neuralgia, rheumatism, and other bleeding and nervous disorders [2, 4-6]. Whilst previous phytochemical studies on this plant revealed the presence of several alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, tannins and terpenoids [3, 4, 7-10], pharmacological evaluations established its analgesic, anthelmintic, anti-acetylcholinesterase, anticancer, anti-inflammatory, antimicrobial, antioxidant and antiparasitic properties [1, 5-8, 11-15]. We now report on the isolation, characterization and antimicrobial activity of three flavonol glycosides (1-3) (Figure 1) including a new one, 6-hydroxy-rutin 3',7-dimethyl ether (3) from the fruits of *R. chalepensis*, collected in Iraq.

2. Materials and Methods

2.1. General

Chromatographic solvents were purchased from Fisher Scientific, UK, and used without further purification. The NMR spectroscopic analyses were performed on a Bruker AMX600 NMR spectrometer (600 MHz for ¹H, and 150 MHz for ¹³C). MS analyses were performed on a Xevo G2-S ASAP or LTQ Orbitrap XL 1 spectrometers. UV spectra were recorded on Analytik Jena Specord 210 spectrophotometer. The SPE fractions were analysed on a Dionex Ultimate 3000 UHPLC coupled with a photodiode array (PDA) detector, using a Phenomenex Gemini-NX 5 U C₁₈ column (150 × 4.6 mm, 5 μm, Phenomenex, USA) and gradient solvent systems comprising MeOH (solvent B) and water
(solvent A) (both contained 0.1% TFA, flow rate: 1 mL/min) were employed to optimize separation of compounds for preparative HPLC. An Agilent 1260 Infinity series preparative HPLC coupled with a PDA detector was used to isolate compounds on an ACE preparative column (150 × 21.2 mm, 5 μm, Hichrom Ltd, UK; MeOH-water linear gradient, flow rate: 10 mL/min). The column temperature was set at 25 °C. The chromatogram was monitored at variable UV–vis wavelengths (215, 254, 280 and 320 nm).

2.2 Plant Materials
The fruits of _R. chalepensis_ L. were collected from Diyala, Central Iraq (N 33.79684 E 44.623337) in September 2015, air-dried at room temperature, and ground using a coffee grinder. A voucher specimen (33396) for this collection has been retained at the National Herbarium of Iraq.

2.3 Extraction and Isolation
Ground air-dried fruits (103 g) were Soxhlet-extracted, successively, with _n_-hexane, dichloromethane (DCM) and methanol (MeOH) (0.9 L, 10 cycles each). All extracts were evaporated to dryness using a rotary evaporator at 45 °C to obtain 4.6 g, 4.2 g and 6.0 g of _n_-hexane, DCM and MeOH extracts, respectively. A portion (2 g) of the active MeOH extract was subjected to solid-phase extraction (SPE) on a Strata (20 g, Phenomenex) C18 reversed-phase cartridge eluted with water-MeOH mixture of decreasing polarity, water:MeOH 80:20, 50:50, 20:80 and 0:100 (250 mL each), to obtain four SPE fractions I-IV, respectively. All SPE fractions were dried using a rotary evaporator followed by freeze-drying and stored in sealed vials in a fridge for further work. Reversed-phase preparative HPLC purification of the SPE fraction II (linear gradient: solvent B in A, 30-100% in 30 min) afforded compounds 1 (3.5 mg, _t_ _R_ = 15.9 min), 2 (2.1 mg, _t_ _R_ = 17.1 min) and 3 (3.2 mg, _t_ _R_ = 18.0 min), respectively.

2.3.1. Rutin (1)
Yellow amorphous solid; UV _λ_ _max_ (MeOH) nm: 257, 267 (sh), 300 (sh) and 359 nm; 1H NMR (CD3OD, 600 MHz), see Table 1; 13C NMR (CD3OD, 150 MHz), see Table 2; HRESIMS (negative ion mode) _m/_z 609.1456 [M-H]−, (calcd. for C27H25O16, _m/_z 609.1456) [16].

2.3.2. Rutin 3'-methyl ether (2)
Brown amorphous solid; UV _λ_ _max_ (MeOH) nm: 254, 264 (sh), 304 (sh) and 354 nm; 1H NMR (CD3OD, 600 MHz), see Table 1; 13C NMR (CD3OD, 150 MHz), see Table 2; HRESIMS (positive ion mode) _m/_z 625.1761[M+H]+ (calcd. for C28H33O16, _m/_z 625.1769) [16-18].

2.3.3. 6-Hydroxy-rutin 3',7-dimethyl ether (3)
Brown amorphous solid; UV _λ_ _max_ (MeOH) nm: 252, 260 (sh), 306 (sh) and 360 nm; 1H NMR (CD3OD, 600 MHz), see Table 1; 13C NMR (CD3OD, 150 MHz), see Table 2; HRESIMS (positive ion mode) _m/_z 655.1870 [M+H]+ (calcd. for C20H13O17, _m/_z 655.1874).

| Compounds              | R  | R' | R'' |
|------------------------|----|----|-----|
| Rutin (1)              | H  | H  | H   |
| Rutin 3'-methyl ether (2) | Me | H  | H   |
| 6-Hydroxy-rutin 3',7-dimethyl ether (3) | Me | Me | OH  |

**Figure 1.** Structures of flavonol glycosides 1-3
2.4. Antimicrobial Assay (Resazurin Assay)

All three compounds (1-3) were screened for their potential antimicrobial activity against four bacterial strains, e.g., Gram +ve: *Micrococcus luteus* (NCTC 7508) and *Staphylococcus aureus* (NCTC 12981) and Gram –ve: *Escherichia coli* (NCTC12241) and *Pseudomonas aeruginosa* (NCTC 12903), and a fungal strain, *Candida albicans* (ATCC 90028) using the resazurin 96-well microtiter plate-based in vitro antimicrobial assay [19]. All bacterial strains were cultured on nutrient agar (Oxoid) and incubated for 24 h at 37°C to determine the MIC. Ciprofloxacin was used as a positive control for bacterial strains, and nystatin for *C. albicans*. Resazurin solution, prepared by dissolving 1 mg of resazurin in 5 mL of sterile distilled water, was used in this assay as an indicator of cell growth, and the method as was described by Sarker et al. [19]. This experiment was performed under aseptic conditions using a sterile 96 well plate. Compound solution (100 μL) was added into the first row of the plate and 50 μL of normal saline was added to all other wells. Serial dilutions were achieved using a multichannel pipette. To each well, 30 μL of nutrient broth and 10 μL of resazurin indicator solution were added. Finally, 10 μL of bacterial suspension (5×10⁵ cfu/mL) was added to each well. To prevent bacterial dehydration, each plate was wrapped loosely with cling film. Every plate contained a positive control. Any colour changes (or no changes in positive control), blue to pink, were noted. The lowest concentration was taken in the colour change that occurred as an MIC (minimum inhibitory concentration) value. The mean of three values was calculated.

2.4.1. Determination of bacteriostatic or bactericidal property

To determine whether the compounds were bacteriostatic or bactericidal, under aseptic condition, 100 μL of culture from the rows of MIC in the microtiter plates was transferred to an empty petri dish, 20 mL of nutrient agar was poured on to the dish, and incubated at 37°C for 24 h. Any microbial regrowth indicated bacteriostatic, and no growth indicated bactericidal activity of the test sample.

3. Results and Discussion

3.1. Extraction, Isolation and Structure Elucidation of Rutin Derivatives (1-3)

Soxhlet extraction of the dried and ground fruits of *Ruta chalepensis* afforded 4.6, 4.2 and 6.0 g of n-hexane, DCM and MeOH extracts, respectively. SPE of the MeOH extract, the most active extract of the three, produced 0.45, 0.49, 0.50 and 0.40 g of SPE fractions, eluted with 20, 50, 80 and 100% MeOH in water, indicating an excellent recovery of the sample from the SPE cartridge. Antimicrobial assay-guided and preparative HPLC-assisted purification of the most active SPE fraction II afforded compounds 1-3. The UV-Vis spectral analyses of the compounds 1-3 indicated that they were flavonoid glycosides, possibly with rutin-type skeleton [18]. The HRESIMS analyses of these compounds (1-3) provided their molecular formula, 

| Compound | Molecular Formula |
|----------|------------------|
| 1        | C_{27}H_{30}O_{16} |
| 2        | C_{26}H_{28}O_{16} |
| 3        | C_{26}H_{24}O_{17} |

respectively, suggesting compound 1 could be rutin. It also added further evidence to the assumption made from the UV-Vis data that compounds 1-3 could be rutin derivatives. In fact, according to the HRESIMS data, compound 2 had just 14 amu unit more than that of rutin (1) suggesting this compound could be a methyl ether of rutin (1). Similarly, the molecular mass of compound 3 was 44 amu unit higher than that of rutin (1), indicating this compound could be a dimethyl ether analogue of 1 with an additional hydroxyl group. The 

\[ \text{H and }^{13} \text{C NMR data (Tables 1 and 2) of compounds 1 and 2 were in good agreement with respective published data for rutin (1) and rutin 3'-methyl ether (2).} \]

The attachment of the rutinosyl moiety at C-3 in compounds 1 and 2, and the methyl ether formation at C-3' in compound 2 were confirmed from extensive HSQC and HMBC experiments.

The \[ \text{H NMR spectrum (Table 1) of compound 3 was quite similar to that of compound 2, with the exception that there was a downfield 6H singlet at } \delta 3.97, \text{ and instead of the usual shielded aromatic meta-coupled proton signals for H-6 and H-8 in flavonol skeleton, there was only a 1H singlet at } \delta 6.53, \text{ suggesting that one of those protons was substituted by an extra functional group, most likely a hydroxyl group based on the HRESIMS data. The }^{13} \text{C NMR spectrum of 3 (Table 2) showed the presence of an additional oxygenated aromatic quaternary signal at } \delta 145.3, \text{ which could be assigned to C-6 or C-8.} \]

Whilst a COSY experiment of compound 3 established all major \[ \text{H-1H scalar couplings. }^{1} \text{H-}^{13} \text{C direct (J) couplings were obtained from an HSQC experiment confirming the} \]
assignment of all protonated $^{13}$C signals to their respective $^1$H signals. An HMBC experiment displayed all major $^{1}$H-$^{13}$C long-range ($^2$J and $^3$J) correlations (Figure 2). The rhamnose anomeric proton (H-$^{13}$C) showed a $^3$J correlation to C-$^6$ of the glucose unit, and the glucose anomeric proton (H-$^{1}$) displayed a $^2$J correlation to C-$3$ of the flavonol aglycones, confirming the formation of the disaccharide, rutinose, and its connection to C-$3$ (as in compounds 1 and 2). Similarly, $^3$J correlations from the methoxyl signal at δ3.97 (6H) (Table 1) to C-$7$ and C-$3'$ (Table 2) established methyl ether formation at C-$7$ and C-$3'$ (Figure 2). The $^{13}$C NMR (Table 2) signal at 89.64 confirmed that the hydroxylation was indeed on C-$6$ in compound 3, and this fact was further corroborated from the $^{1}$H-$^{13}$C HMBC long-range correlations from H-$8$ signal to C-$6$ and C-$10$ (Figure 2). Thus, the compound 3 was identified as 6-hydroxy-rutin 3',7-dimethyl ether (3), which to the best of our knowledge, is a new natural product. This is also the first report on any assay-guided phytochemical analysis of the fruits of Iraqi R. chalepensis.

| Position (H) | 1 | 2 | 3 |
|-------------|---|---|---|
| Aglycone   |   |   |   |
| 6          | 6.23 (1H, d, J = 2.1) | 6.23 (1H, d, J = 2.0) | - |
| 8          | 6.42 (1H, d, J = 2.1) | 6.44 (1H, d, J = 2.0) | 6.53 (1H, s) |
| 2'         | 7.69 (1H, d, J = 2.0) | 7.96 (1H, d, J = 2.0) | 8.00 (1H, d, J = 2.0) |
| 5'         | 6.89 (1H, d, J = 8.4) | 6.94 (1H, d, J = 8.4) | 6.94 (1H, d, J = 8.4) |
| 6'         | 7.65 (1H, dd, J = 2.0, 8.4) | 7.65 (1H, dd, J = 2.0, 8.4) | 7.80 (1H, dd, J = 2.0, 8.4) |
| 7'-OMe     | - | - | 3.97 (3H, s) |
| 3'-OMe     | - | - | 3.97 (3H, s) |
| Glucose moiety |   |   |   |
| 1'         | 5.12 (1H, d, J = 7.7) | 5.25 (1H, d, J = 7.6) | 5.25 (1H, d, J = 7.4) |
| 2'         | 3.49* | 3.49* | 3.49* |
| 3'         | 3.31* | 3.32* | 3.31* |
| 4'         | 3.29* | 3.30* | 3.29* |
| 5'         | 3.43* | 3.46* | 3.43* |
| 6'         | 3.41* | 3.44* | 3.44* |
| 3.81 (1H, m) | 3.84 (1H, m) | 3.84 (1H, m) |
| Rhamnose moiety |   |   |   |
| 1''        | 4.54 (1H, d, J = 1.5) | 4.55 (1H, d, J = 1.5) | 4.54 (1H, d, J = 1.4) |
| 2''        | 3.65* | 3.63* | 3.60* |
| 3''        | 3.55* | 3.56* | 3.54* |
| 4''        | 3.30* | 3.28* | 3.28* |
| 5''        | 3.46* | 3.43* | 3.43* |
| 6''        | 1.14 (3H, d, J = 6.2) | 1.11 (3H, d, J = 6.2) | 1.11 (3H, d, J = 6.3) |

*Overlapped peaks – confirmed from COSY. HSQC and HMBC experiments

3.2 Antimicrobial activity

Potential antimicrobial properties of rutin (1) and its two derivatives (2 and 3) were assessed against two Gram +ve, two Gram –ve bacterial species, and a fungal strain (Table 3). All compounds (1-3) showed various degrees of activities against all tested organisms in the resazurin microtitre assay [19]. Among three compounds, compound 3 was the most potent one, and the order of antimicrobial potency among these compounds was 3>2>1, which indicated that the addition of methyl ether functionality on rutin (1) enhanced the potency; more the number of methoxyl groups, the better was the activity. In fact, the cell membrane is considered as a direct target of antibacterial action of flavonoids, suggesting that membrane interaction could be an important mechanism of antibacterial activity of flavonoids, and increased lipophilicity in flavonoids might lead to enhanced membrane interaction [20]. All three compounds were particularly active against the fungal strain Candida albicans (MICs = 96-205 µM). Ruta chalepensis has long been used in Iraqi traditional medicine to
treat various diseases including microbial infections [3,4]. Antimicrobial assay-guided isolation of rutin (1) and its derivatives (2 and 3) as active components and subsequent assessment of their antimicrobial properties have provided some scientific evidence for the traditional use of R. chalepensis to treat infections.

Table 2. $^{13}$C NMR data for compounds 1-3 (at 150 MHz in CD$_3$OD, $\delta$ in ppm)

| Position (C) | 1       | 2       | 3       |
|--------------|---------|---------|---------|
| Aglycone     |         |         |         |
| 2            | 159.5   | 159.0   | 159.3   |
| 3            | 135.7   | 135.6   | 135.3   |
| 4            | 179.5   | 179.5   | 180.0   |
| 5            | 163.2   | 163.2   | 153.2   |
| 6            | 100.0   | 100.1   | 127.8   |
| 7            | 166.2   | 166.2   | 155.5   |
| 8            | 95.0    | 95.0    | 96.4    |
| 9            | 158.5   | 158.7   | 145.3   |
| 10           | 105.7   | 105.9   | 106.0   |
| 1'           | 123.2   | 123.2   | 123.3   |
| 2'           | 117.8   | 114.7   | 114.8   |
| 3'           | 146.0   | 148.5   | 148.5   |
| 4'           | 150.4   | 151.0   | 151.0   |
| 5'           | 116.2   | 116.3   | 116.2   |
| 6'           | 123.7   | 124.2   | 124.7   |
| 7-OMe        | -       | -       | 57.2    |
| 3'-OMe       | -       | 56.9    | 56.9    |
| Glucose moiety |       |         |         |
| 1''          | 104.8   | 104.5   | 104.5   |
| 2''          | 75.9    | 76.1    | 76.1    |
| 3''          | 77.4    | 72.4    | 72.4    |
| 4''          | 71.5    | 77.5    | 77.5    |
| 5''          | 78.3    | 78.3    | 78.3    |
| 6''          | 68.7    | 68.1    | 68.7    |
| Rhamnose moiety |   |         |         |
| 1'''         | 102.5   | 102.7   | 102.7   |
| 2'''         | 72.2    | 72.2    | 72.2    |
| 3'''         | 72.4    | 71.8    | 71.8    |
| 4'''         | 74.1    | 74.0    | 74.0    |
| 5'''         | 69.8    | 69.9    | 69.9    |
| 6'''         | 18.0    | 18.0    | 18.0    |

Table 3. Antimicrobial activity of compounds 1-3

| Test organisms            | Minimum inhibitory concentration (MIC) in $\mu$M |
|---------------------------|-----------------------------------------------|
|                           | Positive controls                             | Ciprofloxacin | Nystatin | Compounds       |
|                           |                                               | 1            | 2        | 3              |
| Escherichia coli (NCTC 12241) | 1.473                                      | N/A          | 1638.9   | 1601.2 | 774.5         |
| Micrococcus luteus (NCTC 7508) | 2.947                                      | N/A          | 409.7    | 200.1 | 193.6         |
| Pseudomonas aeruginosa (NCTC 12903) | 7.36 x 10$^{-1}$                            | N/A          | 1638.9   | 800.6 | 387.3         |
| Staphylococcus aureus (NCTC 12981) | 2.947                                      | N/A          | 1638.9   | 1601.2 | 1459.1        |
| Candida albicans (ATCC 90028) | N/A                                         | 1.054        | 204.9    | 100.1 | 96.8          |
Figure 2. $^1$H-$^{13}$C HMBC selected long-range key correlations of compound 3

Rutin (1) is one of the most common flavonol glycosides, especially found in the plants from the family Rutaceae, and has been extensively studied to establish its various bioactivities including antibacterial and antifungal properties [16, 21-23]. Therefore, the antimicrobial activities of rutin (1) and its analogues (2 and 3), including a new one (3), as observed in the present study, are in agreement with previous reports [16, 21-23]. All these compounds (1-3) were found to be bacteriostatic [24,25] as there was microbial regrowth, when under aseptic condition, 100 μL of culture from the rows of MIC in the microtitre plates was transferred to an empty petri dish, 20 mL of nutrient agar was added, and incubated at 37°C for 24 h.

Acknowledgments
Mass spectrometry data were acquired at the EPSRC UK National Mass Spectrometry Facility at Swansea University. Shaymaa Al-Majmaie thanks the Iraqi Ministry of Higher Education and Scientific Research, and Department of Biosciences, College of Science, University of Diyala, Iraq, for a PhD Scholarship to carry out this study.

Supporting Information
Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

ORCID
Shaymaa Al-Majmaie: https://orcid.org/0000-0003-8680-8188
Lutfun Nahar: https://orcid.org/0000-0002-1157-2405
George P. Sharples: https://orcid.org/0000-0003-0492-0253
Khazal Wadi: https://orcid.org/0000-0003-136-0485
Satyajit Dey Sarker: https://orcid.org/0000-0003-4038-0514

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