New Thiophene and Flavonoid from *Tagetes minuta* Leaves Growing in Saudi Arabia

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**Abstract:** Phytochemical investigation of the methanolic extract of *Tagetes minuta* L. (Asteraceae) leaves resulted in the isolation and identification of two new compounds: 5-methyl-2,2',5',2'',5'',2''',5''',2''''-quinquethiophene (1) and quercetagetin-6-O-(6-O-caffeoyl-β-D-glucopyranoside) (9), in addition to seven known compounds: quercetin-3,6-dimethyl ether (2), quercetin-3-methyl ether (3), quercetin (4), axillarin-7-O-β-D-glucopyranoside (5), quercetagetin-3,7-dimethoxy-6-O-β-D-glucopyranoside (6), quercetagetin-7-methoxy-6-O-β-D-glucopyranoside (7), and quercetagetin-6-O-β-D-glucopyranoside (8). The compounds were identified by UV, IR, 1D, 2D NMR, and HRESIMS spectral data. They showed significant antioxidant activity, comparable with that of propyl gallate. Compounds 8 and 3 showed weak to moderate antileishmanial and antimalarial activities, with IC₅₀ values of 31.0 μg/mL and 4.37 μg/mL, respectively.

**Keywords:** *Tagetes minuta*; Asteraceae; thiophene; quercetagetin; antioxidant; antimicrobial; antileishmanial; antimalarial
1. Introduction

The genus Tagetes (Asteraceae) is mainly native to the central and southern part of America. It consists of approximately 30 species [1]. Members of the genus Tagetes have a long history of human use as beverages, condiments, ornamentals, and medicinal decoctions. Tagetes minuta L. has been used as anthelmintic, diuretic, antispasmodic, and for treatment of stomach and intestinal diseases [2]. Tagetes oil is used as a flavor component in food products including cola and alcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings, condiments, and relishes [3]. The oil has antibacterial [4], larvicidal [5], and insecticidal [6] activities. Previous phytochemical studies of T. minuta L. led to the isolation of terpenes [2,7], flavonoids [8], thiophenes, and aromatic compounds [9]. This article reports the isolation and characterization of two new compounds: 5-methyl-2,2',5',2'',5'',2''',5''',2''''-quinquethiophene (1) and quercetagetin-6-O-(6-O-caffeoyl-β-D-glucopyranoside) (9), together with seven known flavonoids (Figure 1).

Figure 1. Chemical structures of the isolated compounds 1–9.

2. Results and Discussion

Compound 1 was isolated as brown needles. HRESIMS gave an [M+H]^+ at m/z 427.6611 and 428.6613 [M+2H]^+, which is consistent with the molecular formula C_{21}H_{14}S_{5}, implying fifteen degrees of unsaturation. The UV absorption maxima at 387 and 334 nm indicated the presence of quinquethiophene moiety [10,11]. The ^1H-NMR spectrum showed eleven protons signals at δH 6.64–7.21 with coupling constants 5.5–3.5 Hz characteristic for 5-substituted quinquethiophenes [11]. Additionally, the proton signal at δH 2.42 (3H, s) indicated the presence of a methyl group (Table 1). The ^13C-NMR spectrum exhibited twenty one carbon resonances. The multiplicities of the carbons in 1 were confirmed with DEPT and HSQC experiments, which showed one methyl, eleven methines, and nine quaternary carbons. ^1H-^1H COSY provided five spin systems for five thiophene rings (Figure 2). The HMBC spectrum exhibited cross peaks from methyl protons at C-5 to C-4 and C-5. In HMBC spectrum, cross-peaks from H-3 to C-2', H-3' to C-2, H-4' to C-2'', H-3'' to C-5', H-4'' to C-2'''', H-3''' to C-5'', H-4''' to C-2''''', and H-3''''' to C-5''''confirmed the connectivity of thiophene rings [11].
Accordingly, 1 was 5-methyl-2', 2',5', 2''',5''', 2'''',5'''', 2''''-quinquethiophene. Compound 1 was isolated for the first time from natural origin.

Table 1. NMR spectral data for compound 1 (CDCl₃, 500 and 125 MHz).

| No. | δ_H [mult., J (Hz)] | δ_C (mult.) | HMBC |
|-----|----------------------|-------------|------|
| 2   | -                    | 133.5 (C)   | -    |
| 3   | 6.93 d (3.5)         | 125.9 (CH)  | 2, 4, 5 |
| 4   | 6.64 brs             | 124.0 (CH)  | 2, 3, 5 |
| 5   | -                    | 137.3 (C)   | -    |
| 2'  | -                    | 134.2 (C)   | -    |
| 3'  | 6.97 d (3.5)         | 121.2 (CH)  | 2    |
| 4'  | 6.96 d (3.5)         | 122.3 (CH)  | 2', 3', 2'' |
| 5'  | -                    | 128.9 (C)   | -    |
| 2'' | -                    | 134.7 (C)   | -    |
| 3'' | 6.98 d (3.5)         | 121.5 (CH)  | 4'', 2'' |
| 4'' | 7.02 d (3.5)         | 122.3 (CH)  | 2''  |
| 5'' | -                    | 126.1 (C)   | -    |
| 2'''| -                    | 135.1 (C)   | -    |
| 3'''| 6.99 d (3.5)         | 125.8 (CH)  | 4'', 5'', 2'' |
| 4'''| 7.12 d (3.5)         | 121.6 (CH)  | 2'', 2''', 3'', 5'' |
| 5'''| -                    | 132.8 (C)   | -    |
| 2''''| -                   | 135.3 (C)   | -    |
| 3''''| 7.04 brs            | 122.5 (CH)  | 4'', 5'' |
| 4''''| 7.14 brd (3.5)       | 121.7 (CH)  | 2'', 3'' |
| 5''''| 7.21 brd (5.5)       | 123.0 (CH)  | 3''', 4''' |
| 5-CH₃| 2.42 s              | 15.4 (CH₃)  | 4, 5 |

Figure 2. 1H-1H COSY and HMBC correlations of 1 and 9.

Compound 9 was isolated as a brown residue. It gave positive tests for flavonoids [12]. The HRESIMS spectrum showed a pseudo-molecular ion peak at m/z 643.1223, consistent with a molecular formula C₃₀H₂₆O₁₆. The UV spectrum of 9 showed absorption bands at 275 and 365 nm suggesting its flavonol nature [12]. IR spectrum showed absorption bands at 3,460 (OH), 2,976 (aromatic C-H), 1,668 (ester C=O), 1,608 (α,β-unsaturated C=O), and 1,058 (C-O) cm⁻¹. Analysis of
the NMR spectra of 9 showed the presence of quercetagetin, \textit{trans}-caffeoyl, and glucopyranosyl moieties and confirmed by significant fragment ion peaks at \textit{m}/\textit{z} 480.0826 \([\text{M+H-caffeoyl}]^+\) and \textit{m}/\textit{z} 318.0299 \([\text{M+H-(caffeoyl+hexose)}]^+\). The \(1^H\)-NMR spectrum revealed the presence of six singlets signals at \(\delta_H \text{ 6.59 (H-8), 8.50 (3-OH), 9.35 (3'-OH), 9.35 (4'-OH), 10.89 (7-OH), and 12.24 (5-OH).} \)

Also, it showed three coupled protons at \(\delta_H \text{ 6.88 (1H, d, \(J = 7.0 \text{ Hz, H-5'}\)), 7.57 (1H, brd, \(J = 7.0 \text{ Hz, H-6'}\), and 7.74 (1H, brs, H-2') for a \textit{tri}-substituted B-ring (Table 2). Furthermore, an anomic proton signal at \(\delta_H \text{ 5.02 (1H, d, \(J = 6.5 \text{ Hz, H-1''}\)) indicated \(\beta\)-configuration of the glycosidic linkage [13]. In addition, signals at \(\delta_H \text{ 6.96 (1H, d, \(J = 1.5 \text{ Hz, H-2''}\)), 6.93 (1H, dd, \(J = 6.8, 1.5 \text{ Hz, H-6''}\) ), and 6.77 (1H, d, \(J = 6.8 \text{ Hz, H-5''}\)} for a \textit{tri}-substituted phenyl ring (ABX pattern) and two \textit{trans}-coupled olefinic protons at \(\delta_H \text{ 7.43 (1H, d, \(J = 16.0 \text{ Hz, H-7''}\}) and 6.23 (1H, d, \(J = 16.0 \text{ Hz, H-8''}\)} indicating the presence of \textit{trans} caffeyol moiety (Table 2) [14–16] in which confirmed by \(13^C\)-NMR signals at \(\delta_C \text{ 113.4 (C-8''), 115.3 (C-5''), 115.7 (C-2''), 120.5 (C-6''), 145.3 (C-4''), 145.5 (C-7''), 148.3 (C-3''), and 166.5 (C-9'').} \)

\[\begin{array}{c|c|c|c}
\text{No.} & \delta_H [\text{mult., } J (\text{Hz})] & \delta_C (\text{mult.}) & \text{HMBC} \\
\hline
2 & - & 148.1 (C) & - \\
3 & - & 135.6 (C) & - \\
4 & - & 176.1 (C) & - \\
5 & - & 151.5 (C) & - \\
6 & - & 129.6 (C) & - \\
7 & - & 151.4 (C) & \\
8 & 6.59 s & 93.5 (CH) & 6, 7, 10 \\
9 & - & 147.7 (C) & - \\
10 & - & 105.1 (C) & - \\
1' & - & 122.0 (C) & - \\
2' & 7.74 brs & 115.5 (CH) & 2, 4', 6' \\
3' & - & 145.0 (C) & - \\
4' & - & 147.5 (C) & - \\
5' & 6.88 d (7.0) & 115.3 (CH) & 3', 6' \\
6' & 7.57 brd (7.0) & 119.9 (CH) & 2, 1', 4' \\
1'' & 5.02 d (6.5) & 100.9 (CH) & 6 \\
2'' & 3.77 dd (7.0, 9.0) & 73.1 (CH) & - \\
3'' & 3.86 m & 75.7 (CH) & - \\
4'' & 3.23 dd (9.0, 9.5) & 69.6 (CH) & - \\
5'' & 4.35 m & 77.2 (CH) & - \\
6'' & 4.41 dd (2.8, 12.0) & 64.6 (CH2) & 9'' \\
1''' & - & 125.2 (C) & - \\
2''' & 6.96 d (1.5) & 115.7 (CH) & 6'', 7'' \\
3''' & - & 148.3 (C) & - \\
4''' & - & 145.3 (C) & - \\
5''' & 6.77 d (6.8) & 115.3 (CH) & 3'' \\
6''' & 6.93 dd (1.5, 6.8) & 120.5 (CH) & 4'', 5'', 7'', 8'' \\
\end{array}\]
Table 2. Cont.

| No.  | $\delta_H$ [mult., $J$ (Hz)] | $\delta_C$ (mult.) | HMBC     |
|------|-----------------------------|--------------------|----------|
| 7'   | 7.43 d (16.0)               | 145.5 (CH)         | 9''      |
| 8'   | 6.23 d (16.0)               | 113.4 (CH)         | 1'', 9'' |
| 9''  | -                           | 166.5 (C)          | -        |
| 5-OH | 12.24 s                     | -                  | -        |
| 7-OH | 10.89 s                     | -                  | -        |
| 3'-OH| 9.35 s                      | -                  | -        |
| 4'-OH| 9.35 s                      | -                  | -        |
| 3-OH | 8.50 s                      | -                  | -        |

The $^{13}$C-NMR spectrum displayed fifteen carbon signals were attributed to quercetagetin skeleton [16,17] and six carbons for glucose. The multiplicity of each carbon was determined by HSQC experiment. The glucose moiety was located at C-6 based on the HMBC cross peak of H-1'' at $\delta_H$ 5.02 (1H, d, $J$ = 6.5 Hz) to C-6 ($\delta_C$ 129.6) and further confirmed by its reaction with diagnostic shift reagents. In the HMBC spectrum, the methylene protons at $\delta_H$ 4.41 (H-6"B) and 4.30 (H-6"A) correlated with the caffeoyl carbonyl group at $\delta_C$ 166.5 suggesting the connectivity of caffeoyl moiety at C-6" and confirmed by the downfield shift of C-6" ($\delta_C$ 64.6). Acid hydrolysis of 9 afforded quercetagetin, caffeic acid, and $\beta$-D-glucose. They were identified by co-chromatography with authentic samples using (S5) [14]. Accordingly, 9 was identified as quercetagetin-6-O-(6-O-caffeoyl-$\beta$-D-glucopyranoside).

The other compounds were identified as quercetin-3,6-dimethyl ether (2) [18], quercetin-3-methyl ether (3) [18], quercetin (4) [18], axillarin-7-O-$\beta$-D-glucopyranoside (5) [19], quercetagetin-3,7-dimethoxy-6-O-$\beta$-D-glucopyranoside (6) [20], quercetagetin-7-methoxy-6-O-$\beta$-D-glucopyranoside (7) [20], and quercetagetin-6-O-$\beta$-D-glucopyranoside (8) [20] by comparison of their physical and spectral data with those in the literature. The antioxidant activity of the isolated compounds 2-9 was determined by using a DPPH free radical scavenging system. The antioxidant percentage activity ranged from 91.6 to 68.3% (Table 3). The antioxidant effect of these compounds was related to the number of free phenolic hydroxyl groups in the 3,4-dihydroxy form in their structures, which explains the close similarity of their antioxidant activity. Absence or blocking of the hydroxyl groups by a methyl or glucose moiety leads to a decrease of the antioxidant activity [21].

Table 3. Antioxidant activity of the isolated compounds.

| Comp. | % Activity |
|-------|------------|
| 2     | 81.1       |
| 3     | 82.4       |
| 4     | 91.6       |
| 5     | 68.3       |
| 6     | 69.1       |
| 7     | 71.3       |
| 8     | 83.0       |
| 9     | 89.1       |

Compounds 1–9 were evaluated for their antimicrobial, antimalarial and antileishmanial activities. None of the isolated compounds 1–9 showed any antimicrobial activity. Compound 8 showed weak
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antileishmanial activity with an IC$_{50}$ 31.0 μg/mL. Compound 3 showed moderate antimalarial activity against chloroquine sensitive (D6) clones of *P. falciparum* with an IC$_{50}$ of 4.37 μg/mL.

3. Experimental

3.1. General Procedures

Melting points were measured on an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering Ltd, Essex, England). Optical rotation was measured with a Perkin-Elmer 241 automatic polarimeter (Perkin-Elmer Inc, Massachusetts, MA, USA). HRESIMS was recorded on a LTQ Orbitrap (ThermoFinnigan, Bremen, Germany) mass spectrometer. Low resolution mass spectra were determined using a Finnigan MAT TSQ-7000 mass spectrometer. UV spectra were recorded on a Shimadzu 1601 UV/VIS spectrophotometer (Kyoto, Japan). The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer. 1D and 2D NMR spectra were recorded on a Bruker Avance DRX 500 instrument (Bruker BioSpin, Massachusetts, MA, USA). Column chromatography separations were performed on silica gel 60 (0.04–0.063 mm), RP$_{18}$ (0.04–0.063 mm Merck, Darmstadt, Germany), and Sephadex LH-20 (0.25–0.1 mm, Merck, Darmstadt, Germany). TLC was performed on pre-coated plates with silica gel 60 F$_{254}$ (0.2 mm, Merck). The solvent systems used for TLC analyses were *n*-hexane/EtOAc (97:3, S1), CHCl$_3$/MeOH (95:5, S2), CHCl$_3$/MeOH (90:10, S3), CHCl$_3$/MeOH (85:15, S4), and *n*-BuOH/HOAc/H$_2$O (4:1:5, S5).

3.2. Plant Material

The leaves of *Tagetes minuta* L. (Asteraceae) were collected in June 2012 from Al-Baha, Saudi Arabia. The plant was identified by Dr. A. A. Fayed, Prof. of Plant Taxonomy, Faculty of Science, Assiut University, Egypt. A voucher specimen (TM-1-2012) was deposited at the herbarium of the research center for medicinal, aromatic and poisonous plants, King Saud University.

3.3. Extraction and Isolation

The air-dried powdered leaves (1.1 kg) were extracted with MeOH (4 × 5 L, 24 h each) at room temperature. The combined extracts were concentrated under reduced pressure to afford a dark green residue (30.8 g) which was suspended in distilled water (250 mL) then partitioned successively between *n*-hexane (3 × 500 mL), EtOAc (3 × 500 mL), and *n*-BuOH (3 × 500 mL). Each fraction was concentrated to give *n*-hexane (4.2 g), EtOAc (3.1 g), *n*-BuOH (2.6 g), and aqueous (17.8 g) fractions. The *n*-hexane fraction (4.2 g) was subjected to vacuum liquid chromatography (VLC) using a *n*-hexane-EtOAc gradient to afford four subfractions: H-1 to H-4. Subfraction H-1 (0.52 g) was chromatographed over a silica gel column (100 g × 50 × 2 cm) using *n*-hexane/EtOAc (99:1 to 90:10) to give 1 (17 mg, brown needles). The EtOAc fraction (3.1 g) was subjected to VLC using a CHCl$_3$-MeOH gradient, to afford four subfractions: E-1 to E-4. Subfraction E-1 (0.69 g) was chromatographed over a silica gel column (100 g × 50 × 2 cm) using CHCl$_3$-MeOH gradients to give 2 (12 mg, yellow needles) and 3 (17 mg, yellow needles). Subfraction E-2 (0.90 g) was similarly like subfraction E-1 to give 4 (9 mg, yellow needles). Silica gel column chromatography of subfraction E-3 (0.51 g) (150 g × 50 × 3 cm) using CHCl$_3$-MeOH gradients yielded 5 (11 mg, yellow residue) and 6 (7 mg,
yellow residue). Subfraction E-4 (0.81 g) was chromatographed over a Sephadex LH-20 column (100 g × 50 × 3 cm) using MeOH as an eluent to give two subfractions: E-4A (295 mg) and E-4B (430 mg). Subfraction E-4B was subjected to RP$_{18}$ column chromatography (100 g × 50 × 2 cm) using a MeOH-H$_2$O gradient to afford 7 (16 mg, yellow residue). The n-BuOH fraction (2.6 g) was subjected to Sephadex LH-20 column chromatography (100 g × 50 × 3 cm) using MeOH as an eluent to give three subfractions: B-1 (611 mg), B-2 (355 mg), and B-3 (760 mg). Separately, subfractions B-2 and B-3, each one was chromatographed over a RP$_{18}$ column (40 g × 25 × 1 cm) using a MeOH-H$_2$O gradient to give 8 (13 mg, yellow residue) and 9 (11 mg, brown residue). The other subfractions were retained for further investigation.

3.4. Spectral Data

5-Methyl-2,2',5',2'',5''',2''''-quinquethiophene (1). Brown needles (17 mg), m.p. 215–216 °C. R$_f$ 0.86, silica gel 60 F$_{254}$ (S1). UV (MeOH): $\lambda_{max}$ 334, 387 nm. IR (KBr): $\nu_{max}$ 2870, 1600 cm$^{-1}$. NMR data: see Table 1. HRESIMS: $m/z$ 427.6611 (calcd for C$_{21}$H$_{15}$S$_5$, [M+H]$^+$, 427.6609); 428.6613 (calcd for C$_{21}$H$_{16}$S$_5$, [M+2H]$^+$, 428.6609).

Quercetagetin-6-O-(6-O-caffeoyl-$\beta$-D-glucopyranoside) (9). Brown residue (11 mg), R$_f$ 0.76, silica gel 60 F$_{254}$ (S4). $\alpha$$_D$ $-$176 (0.5, MeOH). UV (MeOH): $\lambda_{max}$ 275, 365 nm; +NaOMe: 282, 405 nm; +AlCl$_3$: 295, 410 nm; +AlCl$_3$/HCl: 293 388 nm; +NaOAc: 296, 385 nm; +NaOAc/H$_3$BO$_3$: 280, 385 nm. IR (KBr): $\nu_{max}$ 3460, 2976, 1668, 1608, 1565, 1058 cm$^{-1}$. NMR data: see Table 2. HRESIMS: $m/z$ 643.1223 (calcd for C$_{30}$H$_{27}$O$_{16}$, [M+H]$^+$, 643.1221).

3.5. Acid Hydrolysis of 9

Compound 9 (3 mg) was refluxed in 10 mL of 1 N HCl for 4 h. The aglycone was extracted with CHCl$_3$. The sugar in the aqueous layer was identified by co-paper chromatography (PC) with authentic materials using solvent system (S5) and aniline phthalate spray as detection reagent [14].

3.6. Antimicrobial Assay

All the isolated compounds 2–9 were tested for antimicrobial activity against Candida albicans ATCC 90028, Candida glabrata ATCC90030, Candida krusei ATCC 6258, Asperigillus fumigates ATCC 90906, methicillin-resistant Staphylococcus aureus ATCC 33591, Cryptococcus neoformans ATCC 90113, Staphylococcus aureus ATCC 2921, Escherichia coli ATCC 35218, Pseudomonas aeruginosa ATCC 27853, and Mycobacterium intracellulare ATCC 23068 as described previously [22–24]. Ciprofloxacin and amphotericin B were used as positive standards.

3.7. Antimalarial Assay

The isolated compounds were tested on chloroquine sensitive (D6, Sierraleon) and resistant (W2, Indo-china) strains of Plasmodium falciparum using previously reported method [22,25]. Artemisinin and chloroquine were included in each assay as anti-malarial drug controls.
3.8. Antileishmanial Assay

The anti-leishmanial activity of the isolated metabolites was tested in vitro against a culture of *L. donovani* promastigotes as previously outlined [26]. Pentamidine and amphotericin B were used as positive standards.

3.9. Antioxidant Activity

The antioxidant activity of the isolated compounds 2–9 (20 μM) in DPPH solution (4 mg was dissolved in HPLC MeOH 50 mL to obtain a concentration 80 μg/mL) was determined as previously outlined [27–29].

4. Conclusions

In conclusion, in this study nine compounds were isolated and elucidated from *T. minuta* L. two of them (compounds 1 and 9) are new. The antioxidant, antimicrobial, antimalarial, and antileishmanial activities of the isolated compounds were evaluated. They showed antioxidant activity ranging from 91.6% to 68.3%. Compound 8 showed weak antileishmanial activity with an IC$_{50}$ 31.0 μg/mL, while compound 3 showed moderate antimalarial activity against chloroquine sensitive (D6) clones of *P. falciparum* with an IC$_{50}$ 4.37 μg/mL.

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Conflicts of Interest

The authors declare no conflict of interest

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Sample Availability: Samples of the isolated compounds are available from the authors.

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