# Chemical Investigation and Antimicrobial Activity of Medicinal Plant *Toddalia asiatica* Lam

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***Abstract:*** *Medicinal plants have become important sources of natural products, which have been used in the development of therapeutic agents. Four new coumarins (**1**-**4**) have been isolated together with five known metabolites (**5**-**9**) from the medicinal plant* T. asiatica. *The structures of **1**-**9** were assigned based on their spectroscopic data. Compounds (**1**-**9**) inhibited the growth of the Gram-negative bacteria* E. coli *and Gram-positive bacteria* S. aureus *at a concentration of 25 and 50 µg/disc. Compounds (**2**-**4**) inhibited the phytopathogenic fungus* C. cucurbitarum *at 50 µg/disc.*

***Keywords:*** *natural products;* Toddalia asiatica*; antimicrobial activity; NMR*

## ■ INTRODUCTION

*Toddalia asiatica* Lam is a well-recognized medicinal plant in India, China, East Africa, Europe, and Okinawa [1-4]. Three main parts of this plant; fruits, roots, and leaves have been used to cure the same diseases like malaria, coughs, influenza, lung disease, rheumatisms, bronchial pains, stomachache, snake bites [1,5]. From the fact above, it is noted that many people already have traditional medical treatments using *T. asiatica*.

Previous phytochemical studies reported the isolation from *T. asiatica* that contain the following compounds like coumarins, few polyphenolic, triterpenoids, flavonoids, lignans, and alkaloids [6-10]. Crude extracts and isolated compounds from this plant are reported to have pharmacological activities such as antioxidant, cytotoxic [11-14], anti-leukemic [15], antiparasitic [16-17] antidiabetic [18], analgesic [19], phosphodiesterase-4 (PDE 4) inhibitory [20], and antibacterial activities [21-22].

During our screening for new bioactive metabolites from terrestrial and marine natural resources [23-25], we found that the acetone extract of the stems of *T. asiatica* collected in Okinawa, Japan inhibited the growth of microorganisms. The aim of this study was to investigate chemical constituents of the traditional medicinal plant *T. asiatica* from Okinawan Island.

This part describes the isolation, structure elucidation, and bioactivities of the four new coumarins class **1**-**4** together with five known compounds, toddalo lactone (**5**), toddaculin (**6**), toddanol (**7**), dihydrochelery thrine (**8**), and 6-Acetonyldihydrochelerythrine (**9**) from the medicinal plant *T. asiatica* [4,6,15,26].

## ■ EXPERIMENTAL SECTION

### Materials

The materials used were the steams of *T. asiatica* (collected from Okinawa Island Japan), and Merck silica gel 60 (particle size 0.063–0.200 mm, 70–230 mesh. 250 mm). Analytical TLC was performed using Kieselgel 60 F254 DC-fertigplatten (Merck). Microbial strains used were *E. coli*, *S. aureus*, *A. niger*, *Cladosporium* sp., and *C. cucurbitarum* (supplied by Natural Product Laboratory, Faculty of Science, University of the Ryukyus).

### Instrumentation

The $^{1}H$ (500 MHz) and $^{13}C$ (125 MHz) NMR spectra were measured on a JEOL α-500 spectrometer, HPLC was performed on a HITACHI L-6000 pump

equipped with a water RI detector (R401), using Nacalai tesque COSMOSIL packed column (5C18, 10 × 250 mm and 55 L, 10 × 250 mm), Merck Hibar pre-packed column (RT 250-10 RP-18, 7 μm). Chemical shifts were referenced to residual solvent signals ($CDCl_3$; $\delta_H$ 7.26, $\delta_C$ 77.0).

### Procedure

#### *Collection, extraction, and isolation*

The stems of *T. asiatica* were collected from Mibaru, Okinawa Island, in July 2008. The specimen was deposited at the University of the Ryukyus. The stems of *T. asiatica* (10 kg) was soaked in acetone and methanol three times at room temperature and filtered. The acetone and methanol extracts were combined and partitioned between water and ethyl acetate to yield a brown organic fraction.

The ethyl acetate extract (159 g) was suspended in methanol and water (1:1) and then successively extracted with hexane, chloroform, and 1-BuOH. The hexane extract showed strong inhibition against bacteria *E. coli* and *S. aureus* at 100 μg/disc. Hexane extract (36 g) was washed with hexane then purified by PTLC to give compound **5** (13 mg/0.0001% dry weight), **1** (2 mg/0.00002% dry weight), and **2** (2.0 mg/0.00002% dry weight).

The hexane fraction (26 g) was subjected to column chromatography on silica gel using hexane-EtOAc-MeOH solvent system to give 15 fractions. Fraction 12 (850 mg) was washed with hexane then purified by recrystallization to give compound **9** (2 mg/0.00013% dry weight). Fraction 3 was subjected to further purification by PTLC using EtOAc: hexane (1:2) to afford compound **6** (2 mg/0.00002% dry weight). Compounds **8** (2 mg/0.00002% dry weight), **9** (1.7 mg/0.000017% dry weight) and **2** (1.5 mg/0.00015% dry weight) were isolated from fraction 12-3-2 (50 mg) by HPLC using EtOAc:hexane (1:2). Fraction 14 (133.8 mg) was subjected to open column chromatography on silica gel using hexane-EtOAc-MeOH solvent system to give 10 fractions. Fraction 14-2 (18 mg) yielded compound **3** (2.2 mg/0.00022% dry weight) by HPLC on $S_i$-60 using EtOAc:hexane (1:2). Finally, compounds **7** (2.2 mg/0.000022% dry weight) and **4** (2.2 mg/0.000022%) were isolated from fraction 14-5 (90 mg) by HPLC using EtOAc:hexane (1:2).

#### *Microbial test cultures and growth conditions*

Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* were used for antibacterial tests and fungi *A. niger*, *Cladosporium* sp., and *C. cucurbitarum* were used for antifungal tests. Bacterial strains were maintained on bacteria medium agar (meat extract 0.05 g, peptone 0.1 g, NaCl 0.05 g, and agar 3 g in 100 mL $dH_2O$) petri dishes at 4 °C, while fungi were maintained on fungi medium agar (malt extract 1.48 g, glucose 1.4 g, peptone 0.08 g, and agar 3 g in 100 mL $dH_2O$). Antimicrobial in-vitro assays were done using the *disc* diffusion method. The fresh cultures were obtained by growing the test strains overnight at 37 °C for bacteria, while fungi were grown at 28 °C for 48 h.

#### *Antimicrobial activity assay*

The crude extracts and pure compounds were tested for antimicrobial activity against bacteria; *E. coli*, *S. aureus,* and phytopathogenic fungi; *A. niger*, *C. cucurbitarum*, and *Cladosporium* sp. Single colonies of the microorganisms used in the bioassay were sub-cultured in 5 mL of bacteria and fungus liquid medium and incubated for 24 h. Aliquots of the test solution were applied to sterile paper discs (8 mm diameter) using a final disc loading concentration of 25, 50, and 100 μg/disc for the crude extracts and 25 and 50 μg/disc for the pure compounds. The plates were incubated at 37 °C for 24 h, and antimicrobial activities were determined by measuring the diameter of the inhibitory zones in millimeters.

## ■ RESULTS AND DISCUSSION

The stems of *T. asiatica* were collected from Mibaru, Okinawa Island, in July 2008. The sample (10 kg wet weight) was extracted with methanol and acetone. The combined extracts were partitioned between water and EtOAc. The EtOAc extract was suspended in MeOH and water (1:1) and then successively extracted with hexane, chloroform, and 1-BuOH. Separation of the hexane extract by a series of chromatographic processes, including silica gel CC, PTLC, and HPLC, led to the isolation of four new compounds **1**-**4** together with five known compounds **5**-**9**.

Compound **1** was isolated as a yellow crystalline solid, and the molecular formula $C_{16}H_{20}O_6$ was elucidated on the basis of NMR spectral data (Table 1 and 2). NMR spectra were similar to those of the known aculeatin [4]. $^1$H and $^{13}$C-NMR spectra of compound **1** showed 16 carbon resonances including signals for two methoxy groups [$\delta_H$ 2.92 (3H, s), δc 56.0 ($CH_3$); $\delta_H$ 3.83 (3H, s), δc 63.1 ($CH_3$)], two methyls [$\delta_H$ 1.23 (3H, s), δc 18.9 (CH3); $\delta_H$ 1.38 (3H, s), δc 23.5 ($CH_3$)], one epoxide $\delta_H$ 2.92 (H, s), δc 56.0 (CH), δc 63.6], one $sp^3$ methylene [$\delta_H$ 2.84 (2H, dd, *J* = 5.8, 9.7 Hz), δc 24.7 ($CH_2$), three $sp^2$ methines [$\delta_H$ 6.22 (1H, d, *J* = 9.5 Hz), δc 112.4 (CH); $\delta_H$ 7.85 (1H, d, *J* = 9.5 Hz), δc 138.9 (CH); [$\delta_H$ 6.61 (1H, s), δc 95.4 (CH)], six quaternary carbons [δc 107.2 (C), 161.1 (C), 156.0 (C), 116.7 (C), 155.1 (C), 161.7 (C)]. The $^1$H and $^{13}$C-NMR correlations were demonstrated by the HMQC. The extensive 1D and 2D NMR analysis (Fig. 2) coupled with a comparison of spectral data with those of aculeatine allowed us to define the structure of **1**. Compounds **1** and **5** were positional isomers for a methoxyl group.

Compound **2** was isolated as a yellow crystalline solid, and the molecular formula $C_{16}H_{18}O_4$ was elucidated based on NMR spectral data (Table 1 and 2). $^1$H and $^{13}$C-NMR spectra of **2** were similar to those toddaculin (**6**). $^1$H and $^{13}$C-NMR spectra of compound **2** showed the presence of two methoxy groups [$\delta_H$ 3.83 (3H, s), δc 63.1 ($CH_3$); $\delta_H$ 2.92 (3H, s), δc 56.0 ($CH_3$)], two methyl [$\delta_H$ 1.68 (3H, s), δc 17.8 (CH3); $\delta_H$ 1.74 (3H, s), δc 22.8 ($CH_3$)], one $sp^3$ methylenes [$\delta_H$ 3.31 (2H, d, *J* = 6.8 Hz), δc 25.7 ($CH_2$)], four $sp^2$methines [$\delta_H$ 6.18 (1H, d, *J* = 9.5 Hz), δc 112.2 (CH); $\delta_H$ 7.82 (1H, d, *J* = 9.7 Hz), δc 139.0 (CH); $\delta_H$ 6.58 (1H, s), δc 95.3 (CH); $\delta_H$ 5.10 (1H, m),

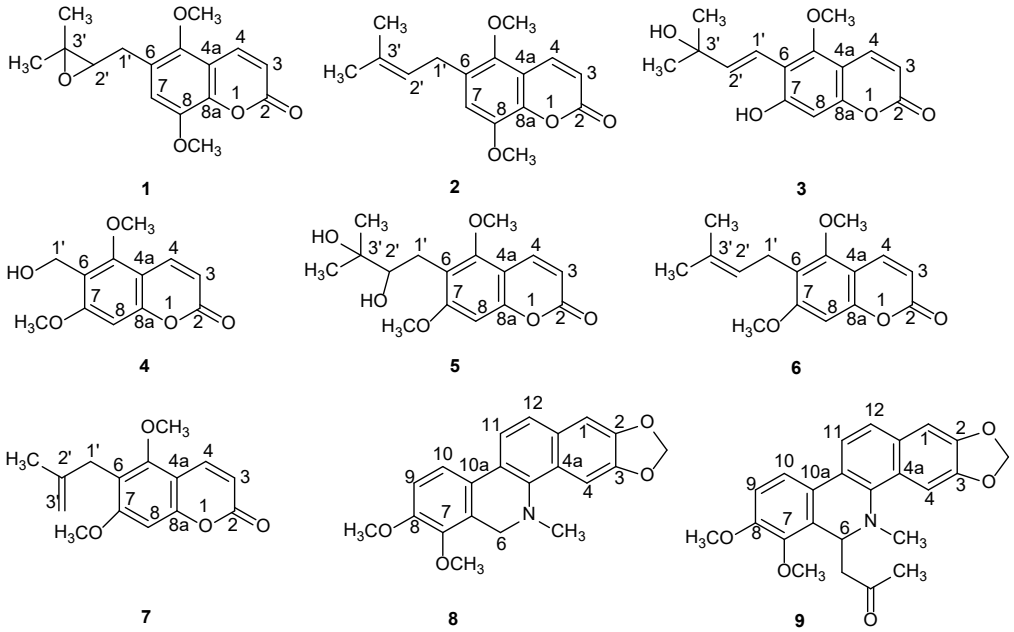


**Fig 1.** Structures of compounds **1**-**9** isolated from medicinal plant *T. asiatica*

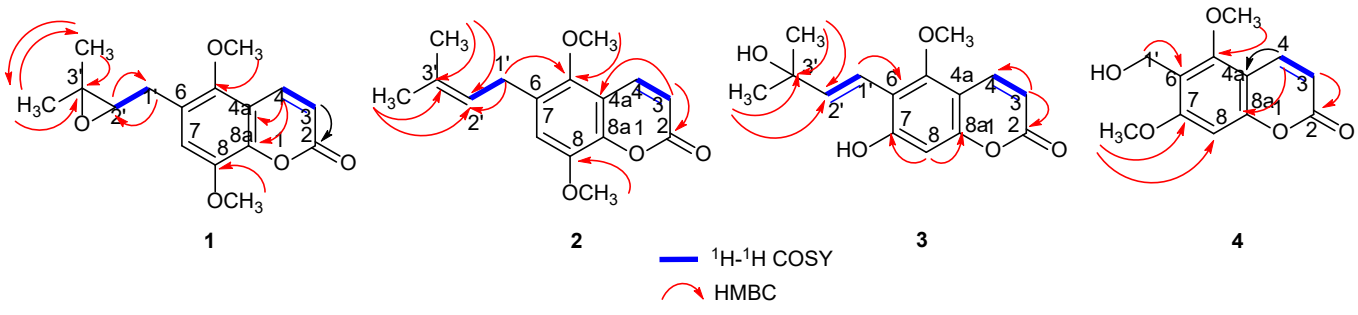


**Fig 2.** $^1$H–$^1$H COSY and key HMBC correlations for **1**-**4**

**Table 1.** $^{13}$C-NMR data for compounds **1**-**4**

| Position | $\delta_C$ (mult.)[a] | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| 1 | | | | |
| 2 | 161.7 (s) | 161.6 (s) | 163.0 (s) | 160.9 (s) |
| 3 | 112.4 (d) | 112.2 (d) | 113.2 (d) | 112.2 (d) |
| 4 | 138.9 (d) | 139.0 (d) | 130.8 (d) | 138.8 (d) |
| 4a | 107.2 (s) | 107.1 (s) | 111.4 (s) | 104.2 (s) |
| 5 | 161.1 (s) | 161.2 (s) | 161.2 (s) | 156.1 (s) |
| 6 | 156.0 (s) | 155.2 (s) | 145.8 (s) | 148.7 (s) |
| 7 | 95.4 (d) | 95.3 (d) | 143.7 (s) | 138.8 (s) |
| 8 | 116.7 (s) | 120.2 (s) | 108.5 (d) | 91.3 (d) |
| 8a | 155.1 (s) | 155.1 (s) | 143.7 (s) | 152.3 (s) |
| 5-$OCH_3$ | 63.1 (q) | 63.1 (q) | 56.5 (q) | 56.5 (q) |
| 8-$OCH_3$ | 56.0 (q) | 56.0 (q) | | 56.5 (q) |
| 1' | 24.7 (t) | 25.7 (t) | 115.2 (d) | 61.6 (t) |
| 2' | 56.0 (d) | 122.1 (d) | 130.8 (d) | |
| 3' | 63.6 (s) | 132.0 (s) | 78.0 (s) | |
| $CH_3$ | 18.9 (q) | 17.8 (q) | 28.0 (q) | |
| $CH_3$ | 23.5 (q) | 22.6 (q) | 28.0 (q) | |

[a] Data recorded at 125 MHz

**Table 2.** $^{1}$H-NMR data for compounds **1**-**4**

| Position | $\delta_H$ (mult., J/Hz)[a] | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| 1 | | | | |
| 2 | | | | |
| 3 | 6.22 (d, 9.5) | 6.18 (d, 9.7) | 6.23 (d, 9.5 ) | 6.13 (d, 9.7) |
| 4 | 7.85 (d, 9.5) | 7.82(d, 9.7) | 7.55 (d, 9.5) | 7.97 (d, 9.7) |
| 4a | | | | |
| 5 | | | | |
| 6 | | | | |
| 7 | 6.61 (s) | 6.58 (s) | | |
| 8 | | | 6.75 (s) | 6.32 (s) |
| 8a | | | | |
| 5-$OCH_3$ | 3.83 (s) | 3.83 (s) | 3.87 (s) | 3.87 (s) |
| 8-$OCH_3$ | 2.92 (s) | 2.92 (s) | | 3.85 (s) |
| 1' | 2.84 (dd, 5.8, 9.7) | 3.31 (d, 6.8) | 6.86 (d, 10.0) | 3.93 (t, 1.7) |
| 2' | 2.92 (s) | 5.10 (m) | 5.72 (d, 10.0) | |
| 3' | | | | |
| $CH_3$ | 1.23 (s) | 1.68 (s) | 1.54 (s) | |
| $CH_3$ | 1.38 (s) | 1.74 (s) | 1.53 (s) | |

[a] Data recorded at 500 MHz

δc 122.1 (CH)], seven quaternary carbons [δc 161.6 (C), 107.2 (C), δc 107.2 (C), 155.2 (C), 161.2 (C), 120.2 (C), 132.0 (C)]. $^{1}$H and $^{13}$C-NMR correlations were demonstrated by the HMQC. The structure of **2** was elucidated to be as depicted in the formula **2** by the extensive analysis of 1D and 2D NMR data (Fig. 2), and

by comparison of the NMR data with those of **6**. Compound **2** differed from **6** only in the position of a methoxyl group.

Compound **3** was isolated as a yellow crystalline solid, and the molecular formula $C_{15}H_{17}O_5$ was established by NMR spectra (Table 1 and 2). $^{13}$C-NMR spectra of compound **3** showed 15 carbons resonances and $^{1}$H and $^{13}$C-NMR data indicated the presence of a methoxy group [$\delta_H$ 3.87 (3H, s), δc 56.5 ($CH_3$], two methyls [$\delta_H$ 1.54 (3H, s), δc 28.0 ($CH_3$); $\delta_H$ 1.53 (3H, s), δc 28.0 ($CH_3$)], one oxygenated quaternary carbon [δc 78.0 (C)], five $sp^2$ methines [$\delta_H$ 6.23 (1H, d, *J* = 6.2 Hz), δc 113.2 (CH); $\delta_H$ 5.72 (1H, d, *J* = 10.0 Hz), δc 130.8 (CH); $\delta_H$ 6.78 (1H, s), δc 108.5 (CH); $\delta_H$ 6.86 (1H, d, *J* = 10.0 Hz), δc 115.2 (CH], six quaternary carbons [δc 163.0 (C), 111.4 (C), δc 161.2 (C), 145.8 (C), 143.7 (C)], and three oxygenated sp2 carbon [δc 143.7(C), 143.7 (C), 161.2 (C)]. $^{1}$H and $^{13}$C-NMR correlations were demonstrated by the HMQC experiment. The $^{1}$H-NMR spectrum of **3** was similar to that of toddalolactone (**5**). The main differences were the presence of two olefinic protons in **3** instead of a hydroxyl group in **5** and the lack of three protons for a methoxyl group in **3**. The extensive 1D and 2D NMR analysis and comparison of the spectral data with those of **5** led to the structure determination for **3**. The *Z* geometry of the $\Delta^{2'}$ double bond was established by the large coupling constant observed between H-1' and H-2' (*J* = 10.0 Hz) [27-28].

Compound **4** was isolated as a yellow crystalline solid, and the molecular formula was $C_{12}H_{12}O_6$ deduced from NMR spectral data (Table 1 and 2). $^{13}$C-NMR spectra of compound **4** showed 12 carbon resonances. $^{1}$H and $^{13}$C-NMR data indicated the presence of two methoxy groups [$\delta_H$ 3.87 (3H, s), δc 56.5 ($CH_3$), $\delta_H$ 3.85 (3H, s), δc 56.5 ($CH_3$)], two methyls [$\delta_H$ 1.54 (3H, s), δc 28.0 ($CH_3$); $\delta_H$ 1.53 (3H, s), δc 28.0 ($CH_3$)], one hydroxyl group [$\delta_H$ 3.93 (1H, t, *J* = 1.7 Hz), δc 61.6 ($CH_2$), three $sp^2$ methines [$\delta_H$ 6.13 (1H, d, *J* = 6.2 Hz), δc 112.2 (CH); $\delta_H$ 7.97 (1H, d, *J* = 9.7 Hz), δc 138.8 (CH); $\delta_H$ 6.32 (1H, s), δc 91.3 (CH)], six quaternary carbons [δc 160.9 (C),

**Table 3.** Antibacterial and antifungal activity of crude extracts of *T. asiatica*

| Crude extract | *E. coli* | | | *S. aureus* | | | *A. niger* | | | *Cladosporium* sp. | | | *C. cucurbitarum* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 | 50 | 100 | 25 | 50 | 100 | 25 | 50 | 100 | 25 | 50 | 100 | 25 | 50 | 100 |
| $CHCl_3$ | + | + | ++ | + | + | ++ | - | - | - | - | - | - | - | - | - |
| Hexane | + | + | ++ | + | + | ++ | - | - | - | - | - | - | - | + | ++ |

Diameter of inhibition zone (mm); + (11–12.5), ++ (13–15.5), - no activity
Diameter of a paper disc; 8 mm
Concentration: (μg/disc)

**Table 4.** Antibacterial and antifungal activity of compounds (**1**-**9**) isolated from the stems of the plant *T. asiatica*

| Compounds | *E. coli* | | *S. aureus* | | *A. niger* | | *Cladosporium* sp. | | *C. cucurbitarum* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 25 | 50 | 25 | 50 | 25 | 50 | 25 | 50 | 25 | 50 |
| **1** | + | ++ | + | ++ | - | - | - | - | - | - |
| **2** | + | ++ | + | ++ | - | - | - | - | - | + |
| **3** | + | ++ | + | ++ | - | - | - | - | - | + |
| **4** | + | ++ | + | ++ | - | - | - | - | - | + |
| **5** | + | ++ | + | ++ | - | - | - | - | - | - |
| **6** | + | ++ | + | ++ | - | - | - | - | - | - |
| **7** | + | ++ | + | ++ | - | - | - | - | - | - |
| **8** | + | ++ | + | ++ | - | - | - | - | - | - |
| **9** | + | ++ | + | ++ | - | - | - | - | - | - |

Diameter of inhibition zone (mm); + (11–12.5), ++ (13–15.5), - no activity
Diameter of a paper disc; 8 mm
Concentration: (μg/disc)

104.2 (C), 156.1 (C), 138.8 (C), 152.3 (C)]. The $^{1}H$ and $^{13}C$-NMR correlations were demonstrated by the HMQC experiment. An extensive analysis of 2D NMR spectra (Fig. 2) led to the planar structure of **4**. HMBC correlations between $H_3$-5-$OCH_3$/C-5 and $H_3$-7-$OCH_3$/C-7 and C-8 confirmed the location of the methoxy groups.

### Antibacterial and Antifungal Assays

The antibacterial and antifungal assays were carried out at the concentration of 25 and 50 µg/disc for pure compounds and 25, 50, and 100 µg/disc for crude extracts. The results are tabulated in Tables **3** and 4.

The two extracts (chloroform and hexane) from *T. asiatica* showed activity against *E. coli* and *S. aureus* at 25, 50, and 100 µg/disc, only hexane extract showed activity against *C. cucurbitarum* at 100 µg/disc. Compounds **1**-**9** showed strong antibacterial activity at 25 and 50 µg/disc. Compounds **2**-**4** showed activity against the phytopathogenic fungi *C. cucurbitarum* at 50 µg/disc.

## ■ CONCLUSION

Chemical investigation of the stems of *T. asiatica* led to the isolation of four new metabolites **1**-**4**, along with five known metabolites **5**-**9**. Compounds **1**-**9** were active against the bacteria *E. coli* and *S. aureus* at 25 and 50 µg/disc. Compounds **2**-**4** showed activity against *C. cucurbitarum* at 50 µg/disc in disc diffusion antimicrobial assay.

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