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A bioactive chromene from *Eupatorium toppingianum*

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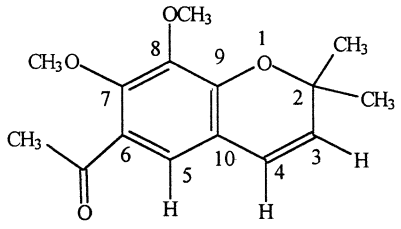
A chromene isolated from *Eupatorium toppingianum* has been identified as 6-acetyl-7,8-dimethoxy-2,2-dimethylchromene. Its structure was elucidated by extensive 1D and 2D NMR techniques, IR, UV and MS. Bioassay of the chromene indicated that it has high cytotoxic, antimutagenic and antimicrobial properties.

Keywords: *Eupatorium toppingianum*, midasol, Compositae, chromene, cytotoxic, antimutagen, antimicrobial

THIS STUDY WAS CONDUCTED TO ISOLATE ANY BIOACTIVE CONSTITUents that may be present in the weed, *Eupatorium toppingianum or* midasol (Family: Compositae) which is found only in the highlands of the Philippines. Congeners of this weed have known medicinal properties, although the plant itself has not been studied and has no medicinal applications. The aerial parts of its congener *E. ataechadosman* possess antiseptic, tonachis and haemagogic properties [1]. The leaves of *E. japonicum* are used as diuretics, while *E. triplinerve* is used to cure fever, colds and diarrhea [2]. A number of chemical studies have been conducted on the genus *Eupatorium* which report details of the isolation of chromenes [3-8], terpenes [9], flavones [10], alkaloids [11], and flavonol glycosides [12]. An antifungal chromene from *E. riparium* [5] and antitumor sesquiterpenes from *E. leukolepis* [9] have also been reported. We now report the isolation, identification and preliminary bioassay results of 6-acetyl-7, 8-dimethoxy-2,2-dimethylchromene (**1**) from *E. toppingianum*.



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Results and Discussion

The chloroform extract of the air-dried leaves of *Eupatorium toppingianum* afforded **1**. Its structure was elucidated by NMR, IR, UV spectroscopy and mass spectrometry as follows.

The low resolution mass spectrum of **1** afforded a molecular ion peak m/z 262 which corresponds to a molecular formula of $C_{15}H_{18}O_4$. Its ^1H NMR spectrum showed a six proton methyl singlet at d 1.41, an acetyl group at δ 2.50 (3H, s), two methoxy groups at δ 3.80 (3H, s) and δ 3.89 (3H, s), two olefinic H's at δ 5.52 (1H, d, J = 9.9 Hz) and δ 6.25 (1H, d, J = 9.9 Hz) and an aromatic H at δ 7.15 (s). The COSY spectrum of **1** showed only one isolated 2-spin system: -CH=CH-. The ^{13}C and DEPT spectra revealed fifteen carbon atoms with the following functionalities: the carbonyl of a conjugated ketone at 197.4 ppm (IR nmax 1677 cm^{-1}); olefinic methine C's at 129.5, 122.0 and 121.3 ppm; non-protonated olefinic C's at 154.3, 150.6, 141.0, 124.6 and 117.5 ppm; an oxygenated quaternary C at 77.4 ppm (IR ν_{max} 1200 cm^{-1}); two oxygenated methyl C's at 61.1 and 60.5 ppm (IR ν_{max} 1050, 1110 cm^{-1}); two methyl C's at 28.0 (2CH3) and one at 30.0 ppm (IR ν_{max} 2979, 2940 cm^{-1}). The carbonyl and four olefins accounted for five double bond equivalents. From the molecular formula of **1**, the remaining hydrogen deficiency could be attributed to a bicyclic system. The UV spectrum gave absorption at 254.4 nm which is as expected for an acyl derivative [8]. This was supported by the resonance for an aromatic H at δ 7.15 (s) and the IR absorptions at ν_{max} 3039, 1604, 769, 610 cm^{-1}.

The ^{13}C and ^1H assignments for **1** were verified by a short range heteronuclear 2D experiment (Bruker XHCORRD program) (Table 1) and connectivity was verified by the inverse long-range heteronuclear experiment HMBC optimized for J = 8 Hz (Table 2). All long-range correlations observed were consistent with the proposed structure of **1**.

The structure of **1** was confirmed by NOESY which indicated correlation through space of the ^1H nuclei in the

molecule (Figure 1). This was supported by the nuclear Overhauser effect difference spectra obtained by irradiation of the acetyl H's and H-5. Irradiation of the acetyl H at d 2.50 enhanced the intensity of the absorption at d 7.15 (H-5), while irradiation of the H at d 7.15 enhanced the intensity of the absorption at d 6.25 (H-4).

A formula index search indicated that **1** has been isolated from *Eupatorium riparium* [7].

In view of the biological activity of some compounds isolated from the genus *Eupatorium*, **1** was tested for its cytotoxic, antimutagenicity and antimicrobial potentials.

A brine shrimp bioassay was conducted on **1** to determine its cytotoxicity. Results of the test are summarized in Table 3. As shown in the table, the LC_{50} of **1** is 7.7 µg/mL. According to the National Cancer Institute of the US, only extracts of $LC_{50} \leq 30$ µg/mL are considered bioactive, hence most of the anticancer compounds have LC_{50} within this range [13].

Table 1. One-Bond ^{13}C-^{1}H Correlation Spectral Data of **1**

Carbon	ppm	^{1}H-^{13}C COSY, d
C-2	77.4	
C-3	129.5	5.52 (d,9.9Hz)
C-4	121.3	6.25 (d,9.9Hz)
C-5	122.0	7.15 (s)
C-6	124.6	
C-7	154.3	
C-8	141.0	
C-9	150.6	
C-10	117.5	
2 CH_3	28.0	1.41 (s)
OCH_3	60.5	3.80 (s)
OCH_3	61.1	3.89 (s)
$CH_3C{=}O$	30.0	2.50 (s)
C=O	197.4	

Table 2. Long-Range 1H-13C Correlation Spectral Data of **1**.

Carbon	^{1}H-^{13}C COSY, d	long-range het.corr. expt.
H-3	5.52 (d,9.9Hz)	77.4, 117.5
H-4	6.25 (d,9.9Hz)	77.4, 150.6
H-5	7.15 (s)	150.6, 154.3
2 CH3	1.41 (s)	77.4, 129.5
OCH3	3.80 (s)	141.0
OCH3	3.89 (s)	154.3
CH3C=O	2.50 (s)	197.4

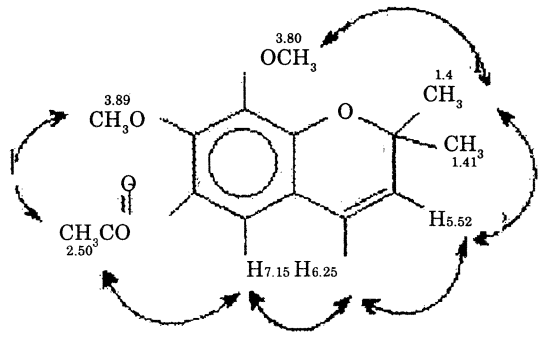

Figure 1. Correlation of ^{1}H NMR Nuclei from NOESY

Table 3. Brine Shrimp Bioassay Data of **1**

Concentration (µg/mL)	% Death of Nauplii[a] Isolate B
10	9.25
100	100
1,000	100
*LC_{50}	7.7 µg/mL

[a] determined from 12 replicates, methanol for control which has zero % death nauplii

* calculated using probit analysis

The LD_{50} of **1** was found to be 31 mg/kg mouse. This experiment was conducted using mice (Swiss strain) administered orally with four dosages of **1** (10, 40, 150, 600 mg/kg).

The antimutagenicity of **1** was evaluated by the Micronucleus test. Results of the study (Table 4) indicated a significant reduction of micronucleated polychromatic erythrocytes (11.4%, 35.8% and 79.8% for doses of 0.025, 0.075 and 0.20 mg/kg, respectively) induced by mitomycin C. Statistical analysis using the T-test showed that there is a significant decrease in MPCE at $\alpha = 0.01$. This implies that **1** is a potent antimutagen. Because of a strong correlation between antimutagen and antitumor, **1** may be a potential antitumor compound. The antimutagenicity test results support the results obtained from the cytotoxicity test.

The antimicrobial activity test results are shown in Table 5. Compound **1** showed complete inhibition against the growth of common pathogenic bacteria, such as *Salmonella typhi*, *Staphylococus aureus*, *Vibrio cholera* and *Shigella dysenteriae* at 2.0 mg/mL. It also showed promising antibacterial potential at the same concentration in *Bacillus subtilis* and *Echerichia coli*. This implies that **1** can prevent the proliferation of the tissues of any infections caused by these pathogens. Compound **1** also has antifungal activity since it inhibited the growth of *Aspergillus niger*, a fungi that attacks fruit-bearing crops causing browning of the leaves and rinds of fruits [14]. This suggests that **1** can be used to protect the crops against infection from these fungi.

Table 4. Micronucleus Test Results of **1**

Sample dosage (μg/kg mouse)	Average No. of MPCE/1000 PCE(per slide) + σ*	Percent reduction (%)
25	15.6 ± 0.53	11.4
75	11.3 ± 0.71	35.8
200	3.56 ± 0.88	79.8
(+) Control	17.6 ± 0.52	
(-) Control	2.6 ± 0.52	

* determined from 9 slides

Experimental

General

The identity of **1** was established by spectroscopic methods. Spectra were recorded with the use of Bruker AM 300 (300 MHz NMR), Nicolet FT-IR, Jeol D-100 mass spectrometer and Hitachi uv-vis spectrophotometer. The number of MPCE /1000 PCE were counted by Zeiss microscope. All NMR spectra were recorded in $CDCl_3$, ^1H nmr at 300 MHz and ^{13}C nmr at 75 MHz. Chloroform was the solvent for FT-IR spectral measurements and methanol for UV-VIS spectroscopy. Fractions were monitored by TLC and spots were visualized by spraying with vanillin/H_2SO_4, then warming.

Biological Material

The plant sample was collected from the Botanical Gardens, Baguio City in January 1994 and a voucher specimen is kept at the Chemistry Department of De La Salle University. The plant sample was identified at the National Museum by comparing it with the sample (accession no. PNH 104063) identified by Jacob as *Eupatorium toppingianum* and deposited at the National Museum on February 14, 1968.

Isolation

Five hundred grams of air-dried leaves were ground in an osterizer, soaked in $CHCl_3$ (2L), then filtered. The filtrate was concentrated *in vacuo* to afford the crude extract (42.5 g). The crude extract (10 g) was subjected to vacuum liquid chromatography using silica gel (60G, 70-230 mesh) as adsorbent. The gradient elution technique was employed with increasing proportions of EtOAc in

Table 5. Antimicrobial Bioassay Data of **1**

Microorganism	Zone of Inhibition (mm)*									
	After 24 hours					After 48 hours				
	0.5	0.7	1.0	1.4	2.0	0.5	0.7	1.0	1.4	2.0 (μg/mL)
S. typhi	10.0	11.5	13.5	18.7	20.0					
B. subtilis	9.1	12.0	13.1	16.8	18.1					
S. aureus	9.0	10.5	13.2	15.9	17.2					
E. coli	9.0	11.2	14.1	16.8	18.5					
V. cholera	9.5	12.1	14.2	16.1	19.0					
S. dysenteriae	9.0	10.2	13.1	15.4	17.8					
S. aeruginosa	-	-	-	-	T					
S. cerevisae						-	-	-	-	-
A. niger						9.8	13.8	19.2	22.1	23.0
C. albicans						-	-	-	T	T

* average diameter of 9 filter discs (8-mm diameter), chloroform was used as control which showed no significant inhibition zones.
T: Thinning
–: No zone of inhibition

petroleum ether (10% increment). Eleven (100 mL) fractions were collected. Fractions 5 to 6 were combined and rechromatograped by gravity column with 30% ethyl accetate in petroleum ether as eluent to afford **1** [Rf = 0.62 (30% EtOAc in petrol), 1.62 g].

Cytotoxicity Test

Artemia salina eggs (brine shrimp eggs) were used as test organism for cytotoxicity test [15]. These eggs were hatched in a rectangular hatchery tank. Ten shrimps were transferred to each vial, then seawater was added to make 5 mL. The discs containing **1** (10, 100, 1000 mg/ml) were placed in vials. Twelve replicates were prepared for each dose level. Survivors were counted after 24 hours with the aid of a magnifying glass and the % death for each dose and control were determined. The LC_{50} was determined using probit analysis [16].

Determination of Median Lethal Dose (LD_{50})

Increasing dosages of **1** (10, 40, 150 and 600 mg/kg mouse) were administered orally to five groups of mice (10 mice/group, one control negative group). Mortality rate was observed for seven days. LD_{50} was calculated using Litchfield and Wilcoxon's methods [18].

Micronucleus Test

Solutions of compound **1** with concentrations 0.025, 0.075 and 0.20 mg/kg (solvent DMSO) were prepared. These were orally and simultaneously administered with Mitomycin C (2.75 mg/kg mouse) to mice of the Swiss strain (source: DOST). For the control, only the mutagen Mitomycin C (positive control) and DMSO (7.5 mL/kg mouse, solvent control) were administered orally to mice of the Swiss strain. Three mice were tested for each concentration and control. The second administration was done after twenty-four hours. Six hours after the second administration, the mice were killed by dislocation of the neck. Blood from the bone marrow was smeared on slides (three per mouse). The slides were stained with May-Grunwald and Giemsa solutions [18]. The numbers of MPCE/1000 PCE were counted by the use of a high power microscope.

Antimicrobial Test

The test bacteria (clinical isolation) used were *Salmonella typhi, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Vibrio cholera, Shigella dysenteriae* and *Pseudomonas aeruginosa,* while the test fungi were *Candida albicans, Aspergillus niger* and *Saccharomyces cerevisiae*. Concentrations of 0.5, 0.7, 1.0, 1.4 and 2.0 µg/mL of **1** were used. The petri dishes were incubated at 37°C and evaluated for antimicrobial activity by measuring the diameter of the inhibition zones after 24 for bacteria and 48 hours for fungi.

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