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A Coumarin from *Ageratum* Leaves (*Ageratum conyzoides* L.)

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Abstract: The aim of study was to isolate, characterize and elucidate of the antifungal compounds from acetone fraction of *Ageratum* leaves and to determine the antifungal activity of the isolated compounds. A coumarin compound has been isolated from acetone fraction of *Ageratum* leaves (*Ageratum conyzoides* L.) and chemical structure has been elucidated based on UV, IR, NMR and mass spectra. This compound showed an antifungal activity against the plant pathogenic fungi, *Aspergillus niger*. *A. niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes black mold on certain fruits and vegetables such as grape, onion and peanuts and is a common contaminant of food. The coumarin was tested for its antifungal activity against *A. niger* by disk diffusion method. The MIC values of coumarin was 62.5 μg mL^{-1}.

Key words: Coumarin, antifungal activity, acetone fraction, *Ageratum* leaves, *Aspergillus niger*

INTRODUCTION

Ageratum, is classified into two species, *Ageratum latifolium* and *A. conyzoides*. *A. conyzoides* is found in several countries in tropical and sub-tropical regions. Many of the secondary metabolites of this herb are biologically active (Ming, 1999).

Coumarin can be found in the plant of the family Umbelliferae, Leguminosae, Asteraceae, Rueae, Saxifragaceae and Thymelaeaceae. Coumarin compounds have been known as the pharmaceutical promiscuity for the extensive bioactivities. For example, as anticoagulant, estrogenic, anti-dermal photosensitizing activity, antibiotics, anthelmintics, sedatives and hypnotics, analgesics and hypothermal activity (Ming, 1999).

Antifungal activity of an Ag (I)-coumarin complex against pathogenic yeast *Candida albicans* have been observed and the result showed that coumarin complex lowered the ergosterol content of the fungal cells and increased the transmembrane leakage of amino acids (Thati *et al*., 2007). Iqbal *et al*. (2004) has found precocene II, a chromene compound isolated from the shoots of *A. conyzoides* L. This compound was active against some plant pathogenic fungi.

A. niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes black mold on certain fruits and vegetables such as grape, onion and peanuts and is a common contaminant of food. Infection of onion seedlings by *A. niger* can become systemic. The black conidia can observed between the scales of the bulb of onion (Samson *et al*., 2001). Some strain of *A. niger* have been reported to produce potent mycotoxins (Abarca *et al*., 1994). Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in *Aspergillus* spores.

The objectives of this research was to isolate, characterize and elucidate the antifungal compounds from acetone fraction of *Ageratum* leaves and to identify the antifungal activity of the isolated compounds.

MATERIALS AND METHODS

Aspergillus niger, *Candida albicans*, *Microsporum gypseum* and *Trichophyton mentagrophytes* were obtained from the School of Pharmacy ITB, Bandung, Indonesia, in December 2005. UV spectra were measured by Spectrophotometer UV-Vis Beckman DU6501, IR spectra were determined by Spectrophotometer JASCO FT/IR using KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded by a JNM ECA-500, JEOL, operating at 500 MHZ (^{1}H and ^{13}C), in $CDCl_3$ solution with TMS as an internal reference. Mass spectra were measured by Agilen GC type 6890 MS type 7973. Vacuum Liquid Chromatography (VLC) was carried out using Merck silica gel 60 GF_{254} and for TLC analysis were used precoated silica gel plates (Merck Kiesel-gel 60 GF_{254}, 0.25 mm).

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Plant material: *Ageratum conyzoides* fresh leaves were collected from a herbal medicinal garden in Lembang, East of Java, Indonesia in October-December 2005. The plant was determined by Herbarium Bandungense, Department Biology, ITB, Indonesia.

Extraction and isolation: The sun-dried and pulverised leaves (2.5 kg) were extracted with 95% ethanol at room temperature. The ethanol extract was concentrated in vacuum-rotary evaporator and fractionated with 80% acetone. The acetone fraction then was partitioned by liquid-liquid extraction with dichloromethane and continued by EtOAc and yielded the dichloromethane and ethyl acetate sub-fractions. The dichloromethane subfraction (FAM) was partitioned by Vacuum Liquid Chromatography (VLC) using n-hexane: chloroform eluent composition (4:6; 3:7 until 0:10). By this process, it has resulted several sub-subfractions (FAM1, FAM2, FAM3 and FAM4) and by recrystalisation from sub-subfraction 1 (FAM1) yielded the isolated compound (FAM1.1).

Characterization and structure elucidation: The isolated compound was characterized by measuring the melting range, UV and IR spectra. The structure of isolated compound was determined by ^{1}H NMR and ^{13}C NMR and mass spectra.

Antifungal activity test: The ethanol extract (2% w/v) was tested for screening antifungal activity against *A. niger*, *C. albicans*, *M. gypseum* and *T. mentagrophytes*. The acetone fraction (1% w/v), the dichloromethane and ethyl acetate sub-fractions (0.5%), FAM1, FAM2, FAM3 and FAM4 sub-sub-fractions (0.1% w/v) and FAM1.1 isolate (0.01% w/v) were tested for antifungal activity against *A. niger*. Antifungal activity test was done by disk diffusion method using Potato Dextrose Agar (PDA) medium, with incubation temperature at 22-25°C, for 24-48 h. Each solvents were used as control.

MIC determination MIC of the FAM1.1 was determined by using a serial concentrations (1000, 500, 250, 125, 62.5, 31.25 μg mL^{-1}) in dichloromethane.

Each experiment, both antifungal activity test and MIC determination, had four replicates (n = 4).

RESULTS AND DISCUSSION

The extraction process with 95% ethanol yielded 875 g concentrated extract, while fractionation yielded 302 g acetone fraction. The dichloromethane and EtOAc sub-fractions yields were 45.5 and 4.0 g, respectively. Partition process by VLC using n-hexane: chloroform eluent, has resulted 4 sub-subfraction (FAM1, FAM2, FAM3 and FAM4) and from FAM1 (4.4 g) yielded 150 mg FAM1.1 isolated compound.

Table 1: ^{1}H NMR and ^{13}C NMR spectra of the FAM1.1 isolate compound

No.	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)	HMBC
2	-	160.84	3, 4
3	6.42-6.44 (1H, d, J = 10)	116.65	5
4	7.72-7.74 (1H, d, J = 10)	143.51	5, 7
4a	-	118.82	3, 4, 6, 8
5	7.49-7.51 (1H, d, J = 10)	127.90	4, 6, 7, 8
6	7.27-7.30 (1H, t)	124.45	7, 8
7	7.54-7.55 (1H, t)	131.85	5, 6, 8
8	7.32-7.34 (1H, d, J = 10)	116.86	7
8a	-	154.01	4, 6, 8

Table 2: Antifungal screening activity of crude ethanol extract

Material tested	Inhibitory diameter (mm) (mean±SD)			
(conc. % w/v)	*C. albicans*	*A. niger*	*M. gypseum*	*T. mentagrophyta*
EtOH extract (2)	10.40±0.10	16.50±0.10	16.05±0.21	14.40±0.37
EtOH (control)	-	-	-	-

The characteristic of isolated compound was a white needle crystallin, with melting range 78.5-79.5°C. The UV spectra showed absorption bands at 273 and 311 nm which have been attributed to the benzene and pyrone rings, respectively. The IR spectra (KBr) showed C-H stretching at pyrone and benzene (δ 3054.69 and 3112.55 cm^{-1}), C = O stretching at pyrone (δ 1708.62 cm^{-1}) and C = C stretching (δ 1604.48 cm^{-1}). ^{1}H NMR ($CDCl_3$) (Table 1) exhibited 6 signals, consisting of 4 doublet signals at δ_H 6.42-6.44 ppm (1H, d, J = 10, H-3), 7.32-7.34 ppm (1 H, d, J = 10, H-8), 7.49-7.51 ppm (1 H, d, J = 10, H-5), 7.72-7.74 ppm (1H, d, J = 10, H-4) and 2 triplet signals at δ_H 7.27-7.30 ppm (1H, t, H-6), 7.54-7.55 ppm (1H, t, H-7). ^{13}C NMR ($CDCl_3$) exhibited 9 signals of 9 sp^2 carbon atom consisting of 3 signals of quarternary carbon at δ_C 118.83 (C-4a), 154.02 (C-8a) and 160.84 (C-2) and 6 signals of methine carbon at δ_C 116.65 (C-3), 116.86 (C-8), 124.46 (C-6), 127.90 (C-5), 131.85 (C-7) and 143.51 (C-4). This structure has been supported by HMBC spectrum which showed correlation between carbon signal of C-2 (carbonyl) with H-3 and H-4; C-3 with proton signal H-5; C-4 with H-5 and H-7; C-4a with H-3, H-4, H-6 and H-8; C-5 with H-4, H-6, H-7 and H-8; C-6 with H-7 and H-8; C-7 with H-5, H-6 and H-8; C-8 with H-7; C-8a with H-4, H-6 and H-8.

The fragmentation pattern of mass spectrum of the isolated compound (Fig. 1) showed strong molecular ion (M^+, m/e 146). The later ion is formed directly from the molecular ion by loss of carbon monoxide (CO), from the pyrone ring, resulting the benzofuran ion (m/e 118). The benzofuran ion is decomposed further by consecutive loss of CO (m/e 90) and a hydrogen atom (m/e 89) (Murray *et al*., 1982). The chemical structure of the isolated compound was coumarin compound (Fig. 2).

In vitro antifungal screening (Table 2) showed that the crude ethanol extract had activity against *C. albicans*, *A. niger*, *M. gypseum* and *T. mentagrophytes*. This extract showed strong activity especially against *A. niger*. In

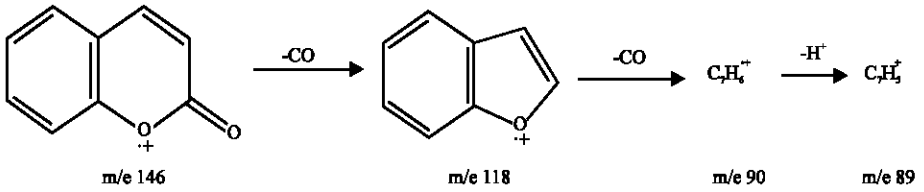

Fig. 1: Fragmentation of coumarin (Murray *et al*., 1982)

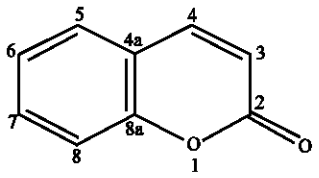

Fig. 2: Chemical structure of coumarin

this study, the crude ethanol extract, had less activity against the bacterial tested, i.e., *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. This agreed with the result reported by Durodola (1997) and Almagboul *et al*. (1985), that methanolic extract of stem and root had no inhibitory effect to all the bacteria isolates used (*S. aureus*, *Y. enterolitica*, *Salmonella gallinarum* and *E. coli*). The active component of this plant may be due to its high non polar compounds (Okwoni *et al*., 2007), so the use of methanol and ethanol (70-80%) extracts showed less activity against bacterial tested. The dichloromethane sub-fraction inhibited *A. niger* growth stronger than acetone fraction, but decreasing activity was showed by ethyl acetate sub-fraction (Table 3). Further assay show that FAM1 has strongest activity and did not show a significant difference with the isolate compound activity (Table 4). All control did not show a significant effect, especially against *Aspergillus niger*.

To predict the character of the isolated compound, as a fungicide or fungistatic, we compared antifungal activity between the isolated compound and antifungal (fungistatic) standard medicine (miconazole nitrate BPFI) against *A. niger*. The isolate (100 μg mL^{-1}) showed a clear inhibition zone up to 72 h incubation time, while miconazole nitrate was only 24 h, at the same concentration. This isolated compound was suggested as a fungicide agent against *A. niger*. MIC value of this isolate was 62.5 μg mL^{-1}.

In this study we found that the active compound of the acetone fraction of *A. conyzoides* leaves was coumarin. This compound was suggested as a fungicide, especially against *A. niger*. The fungicidal action of coumarin was proposed by lowered the ergosterol content of the fungal cells and increased the transmembrane

Table 3: Antifungal activity of fraction and sub-fraction against *A. niger*

Material tested (conc. in % w/v)	Inhibitory diameter (mm) (Mean±SD)
Acetone fraction (1)	17.67±0.29
Dichloromethane sub-fr. (0.5)	24.30±0.30
EtOAc sub-fr. (0.5)	9.30±0.24
Acetone	-
Dichloromethane	-
Ethyl acetate	-

Table 4: Antifungal activity of sub-subfraction and isolate compound against *A. niger*

Material tested (conc. % w/v)	Inhibitory diameter (mm) (Mean±SD)
FAM1 (0.1)	23.43±0.49
FAM2 (0.1)	9.83±0.35
FAM3 (0.1)	8.27±0.25
FAM4 (0.1)	-
FAM1.1 (0.01)	23.58±0.33
Dichloromethane	-

leakage of amino acids (Thati *et al*., 2007). However, the fungicide mechanism of isolated compound on *A. niger* has not been known yet.

CONCLUSION

A coumarin compound has been isolated from acetone fraction of *Ageratum conyzoides* leaves (*Ageratum conyzoides* L.) and its chemical structure has been elucidated based on UV, IR, NMR and mass spectra data. This compound showed an antifungal activity against pathogenic fungi, *Aspergillus niger*. This study recommended the potential use of dichloromethane sub-fraction and coumarin, as fungicidal agents, on pathogenic fungi, especially *A. niger*.

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