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Original Article

Isolation and antimutagenic activity of some flavanone compounds from *Kaempferia rotunda*

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ABSTRACT

Background/Aims: *Kaempferia rotunda* (Zingiberaceae), known as kunci pepet or kunir putih in Indonesia, has been traditionally used in abdominal pain, sputum laxative, wounds and diarrhea colic disorder. This study was conducted to isolate and to investigate antimutagenic activity of some flavanones from *K. rotunda*.

Methods: The milled dried rhizoma of *K. rotunda* (3 kg) was extracted exhaustively with methanol. The methanol extract was partitionated three times by *n*-hexane, chloroform, and ethyl acetate respectively. Each fraction was fractionated by vacuum liquid chromatography (VLC) and purified by column chromatography gravitation. Identification structures of all pure compounds were elucidated based on spectroscopic methods (UV, IR, and NMR) and compared to the spectroscopic previously reported data. Antimutagenic activity test was observed *in vivo* based on the number of micronucleated polychromatic cell erythrocytes (MNPCE) from male Balb-c mice (8–12 week) induced by cyclophosphamide.

Results: From the dried and milled rhizoma of *K. rotunda*, three known flavanones, namely 5-hydroxy-7-methoxyflavanone (1), 7-hydroxy-5-methoxyflavanone (2), and 5,7-dihydroxyflavanone (3) were isolated. The methanol extract and isolated flavanones from K. rotunda showed significant antimutagenic effect compared to control group.

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1. Introduction

Cancer becomes a significant health problem in the world. According to WHO in 2000–2010 cancer ranks as the second cause of death worldwide after heart disease and in the 2030 is expected to increase and become the first ranks.[1] Cancer is the uncontrolled growth of cells, followed by cell invasion into the surrounding tissue and spread to other body parts. The main characteristic of cancer is a continuous cell proliferation, causing an imbalance between life and death cells.[2] Cancer is a multi-factorial, multi-stage and multimechanistic complex process with multiple risk factors that involve interplay between genetic and environmental components.[3] One of the factors that cause cancer is the mutation in a DNA gene. When a mutation is happen in the DNA, then the cancer may be very difficult to cure. The mutations in somatic cells are not only involved in the carcinogenesis process but also play a role in the pathogenesis of other chronic degenerative diseases, such

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as atherosclerosis and heart diseases, which are the leading causes of death in the human population.[4] The mechanism of the mutation could be spontaneously and by the induction of some factors such as radiation, chemicals, and viruses. Mutagen is a substance that causes mutations, whereas compounds that can inhibit the mutation called antimutagenic.[5]

There is considerable evidence that the effects of mutagenic and carcinogenic agents can be altered by many dietary constituents or natural bioactive materials in many plant species. Investigations of antimutagenic potentials of herbal used in traditional medicine are generating great interest with the growing evidence of their safe consumption. Some herbs that have been studied as antimutagenic among others *Momordica charantia*,[5] ascorbic acid,[6] several compounds curcumin and its derivatives,[7] phenolic compounds such as ellagic acid,[8] and polyphenols in fruits, vegetables, and tea.[9]

Kaempferia genus is perennial member of the Zingiberaceae family and is cultivated in Indonesia and other parts of Southeast Asia. Number of studies has been conducted, providing information related to *Kaempferia* as chemopreventive agent. The methanol extract of *Kaempferia parviflora* showed a high cytotoxic activity against human cholangiocarcinoma (HuCCA-1 and RMCCA-1).[10] Some compounds such as panduratin A from *Kaempferia pandurata* showed high cytotoxic activity against human epidermis KB cancer cells,[11] showed high toxicity against human pancreatic cancer cells Panc-1.[12] The methanol extract of *K. parviflora* are also induced apoptosis of the cancer cells HL-60.[13] Previous studies showed that some compounds that have cytotoxic activity against cancer cells also showed antimutagenic properties.[4] This paper will report our investigation of some flavanones from *Kaempferia rotunda* and their antimutagenic activity.

2. Material and method

2.1. Apparatus

UV and IR spectra were measured with Varian Cary 100 Conc and Shimadzu 8300 FTIR, respectively. ^{1}H and ^{13}C NMR spectra were recorded with Jeol JNM A-5000 spectrometers, operating at 500.0 MHz (^{1}H) and 125.0 MHz (^{13}C) using residual and deuterated solvent peaks as internal standards. Evaporator Buchi Rotavapor R-114, vacuum liquid chromatography (VLC) was carried out using Si-gel Merck 60 GF254 (230–400 mesh), column chromatography using Si-gel Merck 60 (200–400 mesh), and TLC analysis on precoated Si gel plates Merck Kieselgel 60 F254 0.25 mm, 20 × 20 cm, water bath, shaker bath, microscup, camera, counter, deskglasser, eppendorf, object glass, and analytical balance.

2.2. Chemicals

Sodium carboxyl methyl cellulose (Na-CMC), cyclophosphamide monohydrate, physiological salin, xilol, Giemsa stain, were obtained from E. Merck in pure analytical grade. Several solvent such as chloroform, hexane, ethyl acetate, acetone, ethanol, and methanol.

2.3. Plant materials

Samples of the rizhoma of *K. rotunda* were collected in December 2010 from the Merapi Farma, Yogyakarta, Indonesia. The plant was identified by the staff at the Faculty Biology, Gadjah Mada University, Indonesia and a voucher specimen (KR-01–2012) was deposited at the Organic Laboratory, Yogyakarta State University, Indonesia.

2.4. Animal test

The experiment were carried out on adult male Balb-c mice (8–12 week) obtained from LPPT, Gadjah Mada University, Indonesia. All mice, 2–3 month old, weighed between 22, 5 and 27 g and were kept under constant environmental conditions with a 12: 12 light–dark cycle, at 23–25 °C room temperature. The animals were fed standard granulated chow (pelet 789) and had access to drinking water *ad libitum*. Animal experiments were done in accordance with Institutional Protocols of animal care. The mice were divided into ten groups consisting of six animal each. Group one served as normal control, group two was treated with cyclophosphamide doses at 50 mg/kg BW each day. Group three and four were treated with sample methanol extract *K. rotunda* doses at 300 and 600 mg/kg BW each day per *oral*, whereas group five until ten each group were treated with flavanone compounds (A, B, C) dose at 30 and 60 mg/kg BW daily per oral. Group three until ten at 30 min after treated with compounds followed cyclophosphamide doses at 50 mg/kg BW daily by *intravenal*. After 30 h treatment bone marrow from all the mice was collected respectively.

2.5. Antimutagenic assay

The antimutagenic assay of this experiment was determined by bone marrow micronucleus assay.[14] After 30 h the mice were anesthetized and the bone marrow was aspirated from femur and tibia into one ml of 1% physiological salin. The cell suspension was centrifuge (1000 rpm for 5 min) and the smears were prepared from the pellet on chemically cleaned glass slides and washed with ethanol absolute at 10 min, dried, and stained with Giemsa stain. To detect possible micronucleus, the portion of micronucleated polychromatic erythrocytes (MNPCE) in 1000 erythrocytes/mice was calculated by using a light microscope (1000× magnification). The frequency of MNPCE in individual mice was used as the experimental unit, with standard deviation based on difference among mice within the same group. The data from the micronucleus assay were statistically analyzed using Student's *t*-test, comparing the treated groups with control and significance level considered was $p < 0.05$. The percentage of antimutagenic activity calculated by the following formula:

$$\%\text{Activity} = \frac{(\text{mean CP} - (\text{mean S} + \text{mean N}))}{(\text{mean CP} - \text{mean N})} \times 100\%$$

Where CP = positive control group treated with cyclophosphamide; N = negative control group; S = group treated with methanol extracts or flavanones (A, B, or C)

2.6. *Isolation and identification structure*

The milled dried rhizoma of *K. rotunda* (3 kg) was extracted exhaustively with methanol. The methanol extract on removal of the solvent under reduced pressure gave a brown residue (230 g). A portion (200 g) of the total methanol extract was partitionated three times by n-hexane, chloroform, and ethyl acetate respectively. Each fractions was evaporated to dryness under vacuum to yield brown residue n-hexane fraction (40 g), chloroform fraction (120 g), and ethyl acetate fractions (31 g). A portion (25 g) ethyl acetate fraction was fractionated by vacuum liquid chromatography (VLC) (silica gel GF 60 Merck 250 g; ϕ: 10 cm, t = 10 cm), using n-hexane, n-hexane-ethyl acetate (9:1; 8:2; 6:4; 5:5; 4:6; and 6:4), ethyl acetate, acetone, and methanol of increasing polarity as eluents to give twenty fractions. These fractions were combined based on the same TLC profiles and evaporated to give three major fractions A (1–5) (3.5 g), B (6–15) (10 g), and C (16–20) (6.6 g). Fraction A was recrystallized using methanol to give colorless crystal of 5-hydroxy-7-methoxyflavanone (1) (1.5 g). Fraction B was purified by column chromatography gravitation using Si-gel Merck 60 (200–400 mesh), (ϕ: 1.5 cm, t = 15 cm) eluted with hexane-ethyl acetate (6:4) as solvent to give 48 fraction. Fraction (13–21) were combined and evaporated to give a pale yellow crystal of 7-hydroxy-5-methoxyflavanone (2) (1.2 g). Fraction (30–45) yielded as yellow needle shaped crystals of 5,7-dihydroxyflavanone (3) (1.8 g).

Residue of n-hexane fraction (40 g), was recrystallized using methanol to give colorless crystal of 5-hydroxy-7-methoxyflavanone (1) (4, 5 g). Chloroform fraction (60 g) was fractionated by VLC (silica gel GF 60 Merck 250 g; ϕ: 10 cm, t = 10 cm), using n-hexane, n-hexane-ethyl acetate (9:1; 8:2; 6:4; 5:5; 4:6; and 6:4), ethyl acetate, acetone, and methanol of increasing polarity as eluents to give twenty fractions. Fraction (4–10) yielded colorless crystal of 5-hydroxy-7-methoxyflavanone (1) (3.0 g), whereas fraction (15–18) yielded as yellow needle shaped crystals of 5,7-dihydroxyflavanone (3) (2.5 g). The structures of these compounds (1–3) were established on the basis of their spectral data, including UV, IR and NMR one and two dimension HMQC and HMBC. A complete spectral data are included in the supplementary information (Fig. 3–14).

5-Hydroxy-7-methoxyflavanone (1) was obtained as a colorless crystal. The UV (in methanol solvent) λ_{max}: 213; 287 nm. The IR (KBr pellet) υ_{max}: 3444; 1645; 1621; 1581; 1381; and 1158 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) : δ 3.08 (1H, dd, 2.8; 12.0); 2, 84 (1H, d, 2.8); 3.81 (3H, s); 5.43 (1H, d, 12.0); 6.04 (1H, br s); 6.06 (1H, br s); 7.42 (2H, br s); 7.43 (3H, br s); and 12.03 (1H, s, OH) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 43.5; 55.85; 77.45; 94.43; 95.3; 103.3; 126.3 (3C); 129.0 (2C); 138.54; 163.14; 164.3; 168.3; 195.93 ppm. The result of this analysis compound 1 using NMR one and two dimension spectrophotometer listed in Table 1.

7-Hydroxy-5-methoxyflavanone (2) was obtained as a pale yellow crystal. The UV (in methanol solvent) λ_{max} : 227; 288 nm. The IR (KBr pellet) υ_{max}: 3450; 1645; 1621; 1581; 1381; and 1158 cm^{-1}. ^1H NMR (500 MHz, Acetone-d_6): δ 2.67 (1H,d, br s); 2.97 (1H, d, 12.6); 3.79 (3H, s); 5.48 (1H, d, 12.6); 6.09 (1H, br s); 6, 15 (1H, d, 2.5); 7.54 (2H, d, 8.6); 7.43 (2H, t, 8.6; 8.0); 7.37 (1H, t, 8.0; 8.0); 9.42 (1H, br s, OH). ^{13}C NMR (125 MHz, Acetone-d_6): δ 46.48; 56.14; 79.80; 94.23; 96.65; 106.18; 127.23 (2C); 129.22; 129.47 (2C); 140.67; 163.79; 165.06; 165.60; 187.84 ppm. The result of this analysis compound 2 using NMR one and two dimension spectrophotometer listed in Table 2.

5,7-Dihydroxyflavanone (3) was obtained as a yellow needle shaped crystals. UV (in methanol solvent) λ_{max} : 210; 288 nm. The IR (KBr pellet) υ_{max}: 3444; 1631; 1583; 1488; 1302; 1168; 1089 cm^{-1}. ^1H NMR (500 MHz, Acetone-d_6) : δ 2.82 (1H, dd, 2.9; 12.6); 3.18 (1H, dd, 2.9; 12.6); 5.56 (1H, dd, 2.9; 12.6); 5.98 (1H, d, 2.5); 6.01 (1H, br s); 7.42 (1H, t, 8.0; 8.0); 7.45 (2H, t, 8.0; 8.0); 7.56 (2H, d, 8.0); 9.63 (1H, br s, OH); 12.16 (1H, br s, OH) ppm. ^{13}C NMR (125 MHz, Acetone-d_6): δ 43.63; 79.47; 95.91; 96.96; 103.29; 127.32 (2C); 129.45 (2C); 129.51; 140.06; 164.19; 165.33; 167.38; 196.85 ppm. The result of this analysis compound 3 using NMR one and two dimension spectrophotometer listed in Table 3.

Table 1 – ^1H NMR, ^{13}C NMR, and HMBC data of compound 1 ($CDCl_3$).

No	δ C ppm	Δ H ($\sum$ H; m; J Hz)	HMBC (H→C)
1	–		
2	77.45	5.43 (1H, d, 12.0)	C4; C1′; C3
3	43.50	3.08 (1H, dd, 2.8; 12.0); 2.84 (1H, d, 2.8)	C4; C2
4	195.93	–	
5	164.30	–	C5; C7
6	95.30	6.04 (1H, br s)	C8; C5
7	168.30	–	
8	94.43	6.06 (1H, br s)	C10; C5
9	163.14	–	
10	103.30	–	
1′	138.54	–	
2′	126.30	7.43 (1H, br s)	C1′; C2
3′	129.00	7.42 (1H, br s)	
4′	126.30	7.43 (1H, br s)	
5′	129.00	7.42 (1H, br s)	
6′	126.30	7.43 (1H, br s)	
5-OH	–	12.03 (1H, s)	C6; C10; C5
7-OCH_3	55.85	3.81 (3H, s)	

Table 2 – ^1H NMR, ^{13}C NMR, and HMBC data of compound 2 (Acetone-d_6).

No	δ C ppm	Δ H ($\sum$ H; m; J Hz)	HMBC
1			
2	79.80	5.48 (1H, d, 12.6)	C1′; C3; C4
3	46.48	2.67 (1H, br s); 2.97 (1H, d, 12.6)	C4; C2
4	187.84	–	–
5	163.73		
6	96.65	6, 15 (1H, d, 2.5)	C8; C5
7	165.60	–	
8	94.23	6.09 (1H, br s)	C10; C9
9	165.06	–	
10	106.18	–	–
1′	140.67	–	
2′	127.23	7.54 (1H, d, 8.6)	C1′; C2
3′	129.47	7.43 (1H, t, 8.6; 8.0)	C1′; C2′
4′	129.22	7.37 (1H,t, 8.0; 8.0)	C2′
5′	129.47	7.43 (1H, t, 8.6; 8.0)	C1′; C2′
6′	127.43	7.54 (1H, d, 8.6)	C1′; C2
7-OH	–	9.42 (br s)	C8: C6
5-OCH_3	56.14	3.79 (s)	

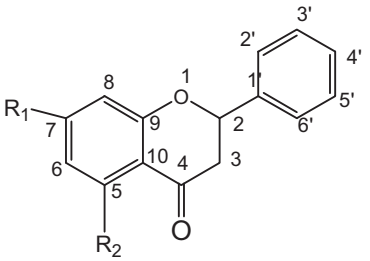
Table 3 – ^{1}H NMR, ^{13}C NMR, and HMBC data of compound 3 (Acetone-d_6).

No	δ C ppm	Δ H (∑ H; m; J Hz)	HMBC
1	–		
2	79.47	5.56 (1H, dd, 2.9; 12.6)	C4; C1'; C3
3	43.63	2.82 (1H, dd, 2.9; 12.6); 3.18 (1H, dd, 2.9; 12.6)	C4; C2
4	196.85	–	
5	165.33	–	
6	96.60	5.98 (1H, d, 8.0)	C4
7	164.19	–	
8	95.91	6.01 (1H, br s)	C10; C5
9	167.38	–	
10	103.29	–	
1′	140.06	–	
2′	127.32	7.56 (1H, d, 8.0)	C1′; C2′
3′	129.45	7.45 (1H, t, 8.0; 8.0)	C2′; C1
4′	129.51	7.42 (1H, t, 8.0; 8.0)	C3′; C2′
5′	129.45	7.45 (1H, t, 8.0; 8.0)	C2′; C1′
6′	127.32	7.56 (1H, d, 8.0)	C1′; C2′
5-OH		12.16 (1H, br s)	C10; C6
7-OH		9.63 (1H, br s)	

3. Result and discussion

Characterization of isolated three flavanones by using NMR are listed in Tables 1–3. Structure of the isolated compounds at Fig. 1.

5-Hydroxy-7-methoxyflavanone (1) was obtained as a colorless crystal. Its UV (in methanol solvent) spectrum showed absorption maximum at 213; 287 nm suggesting the phenol ring. The IR (KBr pellet) spectrum exhibited hydroxyl group (3440 cm^{-1}), C=O carbonyl (1645 cm^{-1}), C=C aromatic (1621; 1581 cm^{-1}), and C–O–C bond (1158 cm^{-1}), these spectral characteristic absorptions supporting (1) to be a phenolic compound. ^{13}C NMR spectra showed one signals for carbon carbonyl at δ 195.93, three signal oxyaryl carbon at δ 168.3 (C-7), 164.3 (C-5), and 163.14 (C-9) ppm, nine aromatic carbon at δ 95.30 (C-6), 94.43 (C-8), 103.3 (C-10), 138.54 (C-1′), 126.30 (C-2′, C-4′ & C6′), 129.00 (C-3 & C-5) ppm. Additionally, the ^{13}C NMR also exhibited one oxyalkyl carbon at δ 77.45 (C-2) ppm, aliphatic carbon at δ 43.50 (C-3) ppm, and one methoxyl carbon at δ 55.85 (OCH_3). The ^{1}H NMR spectrum of (1) in $CDCl_3$ exhibited signals for a monosubstituted phenyl ring δ 7.43 (3H, br s, H-2′, H-6′, and H-4′) & 7.42 (2H, br s, H3′ & H5′) ppm. The ^{1}H NMR spectrum also showed one of meta-coupled aromatic protons signals at δ 6.04 (1H, br s, H-6) and 6.06 (1 H, br s, H-8) ppm, oxyalkyl proton at δ 5.43 (1H, d, 12.0 Hz, H-2), and aliphatic proton at δ 3.08 (1H, dd, 2.8; 12.0 Hz, H-3a) and δ 2.84 (1H, d, 2.8 Hz, H-3b). Additionally, the ^{1}H NMR spectrum exhibited signals for methoxyl group at δ 3.81 (3H, s), and hydroxyl proton at δ 12.03 (1 H, s). The connection between protons and their corresponding carbons was established by HMQC. Further support for the structure (1) was obtained from HMBC measurement (Table 1). These results suggested that compound (1) was a flavanone with substituted methoxyl and hydroxyl group. Further evidence for the structure assigned to compound (1) came from comparison of their spectral data with those reported in the literature. Therefore, it may be conclude that compound (1) is pinostrobin that isolated from *K. pandurata*.[15]



(1) $R_1 = OCH_3$; $R_2 = OH$
(2) $R_1 = OH$; $R_2 = OCH3$
(3) $R_1 = OH$; $R_2 = OH$

Fig. 1 – Structure of the isolated flavanones from *K. rotunda*.

7-Hydroxy-5-methoxyflavanone (2) was obtained as a pale yellow crystal. The UV (in methanol solvent) λ_{max} : 227; 288 nm. The IR (KBr pellet) spectrum exhibited hydroxyl group (3450 cm^{-1}), C=O carbonyl (1645 cm^{-1}), C=C aromatic (1621; 1581 cm^{-1}), and C–O–C bond (1158 cm^{-1}). The ^{1}H and ^{13}C NMR spectrum showed the data that is very similar to compound 1. The ^{1}H NMR spectrum of (2) in Acetone-d_6 exhibited proton signals for a monosubstituted phenyl ring at δ 7.54 (2H, d, J = 8.6 Hz, H-2′, H-6′), 7.43 (2H, t, J = 8.6; 8.0 Hz, H3′ & H5′), and 7.37 (1H, t, J = 8.0) ppm. The ^{1}H NMR spectrum also showed one of meta-coupled aromatic protons signals at δ 6.15 (1H, br s, H-6) and 6.09 (1H, br s, H-8) ppm, oxyalkyl proton at δ 5.48 (1H, d, 12.6 Hz, H-2) ppm, aliphatic proton at δ 2.97 (1H, d, 12.6 Hz, H-3a) and δ 2.67 (1H, d, 2.8 Hz, H-3b) ppm, methoxyl group at δ 3.79 (3H, s) ppm, and hydroxyl proton at δ 9.42 (br s) ppm. ^{13}C NMR spectra showed sixteen carbon consisting of carbonyl carbon at δ 187.84, three signal oxyaryl carbon at δ 163.73 (C-5), 165.60 (C-7), and 165.06 (C-9) ppm, nine aromatic carbon at δ 96.65 (C-6), 94.23 (C-8), 106.18 (C-10), 140.67 (C-1′), 127.23 (C-2′, C6′), 129.47 (C-3, C-5), 129.47 (C-4′), one oxyalkyl carbon at δ 79.80 (C-2), alkyl carbon at δ 46.48 (C-3), and one methoxyl carbon at δ 56.14 (OCH_3) ppm. The HMQC spectrum supported complete assignment of all proton-bearing carbon signals of compound 2 (Table 2). Further support for the structure 2 was obtained from HMBC measurement. Compound 2 is flavanone with methoxyl group at C-5 and hydroxyl group at C-7. It is based from the HMBC spectrum showed a correlation between proton hydroxyl with C8 and C6, and there is no correlation between hydroxyl with C10, such as those in compound 1. Therefore, compound 2 is 7-Hydroxy-5-methoxyflavanone. These compound have the similar NMR data with alpinetin that isolated from *K. pandurata*.[15]

5,7-Dihydroxyflavanone (3) was obtained as a yellow needle shaped crystals. UV (in methanol solvent)λ_{max} : 210; 288 nm. The IR (KBr pellet) spectrum exhibited hydroxyl group (3444 cm^{-1}). Carbonyl group (1631 cm^{-1}), C=C aromatic (1583; 1488 cm^{-1}), and C–O–C group (1302 cm^{-1}). ^{1}H NMR (500 MHz, Acetone-d_6) showed aliphatic proton at δ 2.82 (1H, dd, J = 2.9; 12.6 Hz, H-3a); 3.18 (1H, dd, J = 2.9; 12.6 Hz, H-3b), oxyalkyl proton at δ 5.56 (1H, dd, J = 2.9; 12.6 Hz), one benzene ring with meta-coupled aromatic protons signals at δ 5.98 (1H, d, J = 2.5 Hz) and 6.01 (1H, br s); and five proton signals for a monosubstituted phenyl ring at δ 7.42 (1H, t, J = 8.0; 8.0 Hz, H-4′), 7.45 (2H, t, J = 8.0; 8.0 Hz, H 3′& 5′); and 7.56 (2H, d, J = 8.0 Hz,

Table 4 – Percentage antimutagenic activity of these groups.

No	Groups treatment	Mean of MNPCE ± SD	% Antimutagenic activity
1	Negative control (blanco) (Na-CMC 1%)	0.0 ± 0.0	–
2	Positive control (cyclophosphamide dose 50 mg/kg BW)	5.75 ± 3.4	–
3	Methanol extract dose 300 mg/kg bw followed cyclophosphamide 50 mg/kg BW	2.25 ± 1.7	55.0
4	Methanol extract dose 600 mg/kg BW followed cyclophosphamide 50 mg/kg BW	1.0 ± 1.4	80.0
5	5-hydroxy-7-methoxyflavanone (A1) dose 30 mg/kg BW followed cyclophosphamide 50 mg/kg BW	2.5 ± 3.0	56.5
6	5- hydroxy-7-methoxyflavanone (A2) dose 60 mg/kg BW followed cyclophosphamide 50 mg/kg BW	0.2 ± 0.44	96.5
7	7-hydroxy-5-methoxyflavanone (B1) dose 30 mg/kg BW, followed cyclophosphamide 50 mg/kg BW	0.2 ± 0.44	96.5
8	7-hydroxy-5-methoxyflavanone (B2) dose 60 mg/kg BW, followed cyclophosphamide 50 mg/kg BW	0.0 ± 0.0	100
9	5,7-dihydroxyflavanone (C1) dose 30 mg/kg BW followed cyclophosphamide 50 mg/kg BW	0.4 ± 0.89	93.0
10	5,7-dihydroxyflavanone (C2) dose 60 mg/kg BW followed cyclophosphamide 50 mg/kg BW	0.0 ± 0.0	100

H-2′ & H-6′); and two proton signal from hydroxyl group at δ 9.63 (1H, br s, OH); 12.16 (1H, br s, OH) ppm. ^{13}C NMR (125 MHz, Acetone-d_6) showed sixteen carbon consisting of aliphatic carbon at δ 43.63; oxyalkyl carbon at δ 79.47 ppm, aromatic carbon at δ 95.91; 96.96; 103.29; 127.32 (2C); 129.45 (2C); 129.51; and 140.06 ppm, oxyaryl carbon at δ 164.19; 165.33; and 167.38 ppm, and carbon carbonyl at δ 196.85 ppm. The connection between protons and their corresponding carbons was established by HMQC. Further support for the structure (3) was obtained from HMBC measurement (Table 3). These results suggested that compound (3) was a flavanone with substituted two hydroxyl group. Therefore, compound 3 is 5,7-dihydroxyflavanone. These compound have the similar NMR data with pinocembrin that isolated from *K. pandurata*.[15]

The antimutagenic assay of this experiment was determined by bone marrow micronucleus assay.[14] The bone marrow micronucleus test is one of the most suitable antimutagenic assay by *in vivo*. The antimutagenic activity of methanol extract and isolated flavanone compounds was evaluated by measuring their inhibitory effect on cyclophosphamide induced mutagenesis. The oral administration of 30 and 60 mg/kg BW of flavanone prior to cyclophosphamide exposure reduced the frequency of MNPCE in all groups studied. The results as shown in Table 4, demonstrated that the flavanone isolated from *K. rotunda* at a dose of 30 mg/kg BW, which is 5-hydroxy-7-methoxyflavanone (A1), 5,7-dihydroxyflavanone (C1), 7-hydroxy-5-methoxyflavanone (B1), have antimutagenic activity percentage of 56.5%; 93.0% and 96.5% respectively. Meanwhile, at a dose of 60 mg/kg BW, three compounds showed a very high activity. Methanol extract of *K. rotunda* which is a crude extract contain a mixture of some flavanone compounds. It showed significant activity but lower than pure compounds. The polychromatic erythrocytes can be showed at Fig. 2.

The protection mechanism against mutagenic agent has not been known yet, however, it may scavenge free radicals or inhibiting DNA strand breaks or may enhance DNA repair mechanism. Micronucleus is one indicator of DNA mutation. Micronucleus is the result of a broken chromosome, and then appears as a small nucleus in the cell. The micronucleus derived from acentric fragments or lagging chromosome which only appear in the anaphase stage of the mitosis process. So that the micronucleus are easily observed in the cells that constantly divide, such as cells in the bone marrow. The number of micronucleated polychromatic erythrocytes (MNPCE) shows the level of genetic damage in the erythropoiesis process.

Alkylation on agent such as cyclophosphamide has cytotoxic effects through the transfer of alkyl cluster to various elements of the cell. Alkylation of DNA in the nucleus is a major mechanism leads to the cell death. Cyclophosphamide is converted by cytochrome P450

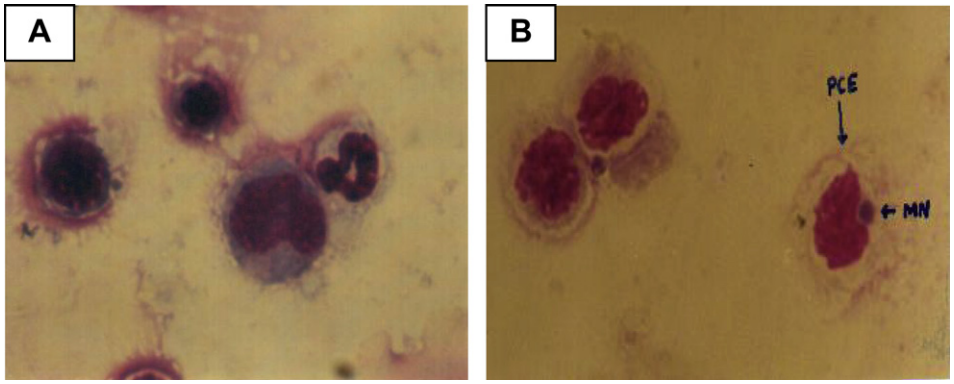


Fig. 2 – The normal polychromatic erythrocytes (A) and MNPCE (B).

isoenzymes into 4-hydroxycyclophosphamide in the liver. 4-hydroxycyclophosphamide is oxidized into aldophosphamide as the active metabolites. Furthermore, aldophosphamide will break down into phosphamide mustard and acrolein, which is highly toxic. This mechanism of toxicity happens in the bone marrow.

Based on the antimutagenic activity and molecular structure relationship, known that the hydroxyl group at position C-7 can increase antimutagenic activity. This is related to the nature of the two compounds flavanones polarity which is having hydroxyl group at position C-7. Compound 5-hydroxy-7-methoxyflavanone or pinostrobin do not have a hydroxyl group at position C-7, but has a hydroxyl group at position C-5. Hydroxyl group at position C-5 is not in the free state, because it can form hydrogen bonds with the carbonyl group (C3). Edenharder R, *et al*[16] reported that the correlation of antimutagenicity of natural compounds of plant origin was equivalent with polarity of flavonols.

Although the biochemical mechanisms underlying flavanone compounds activities are not yet clear, our results demonstrated that *K. rotunda* has a preventive effect against chromosome fragmentation *in vivo*, probably due to its free radical scavenging capability. Our observations are consistent with those in the other studies. Moreover, study showed that phenolic compounds have bioactivity as antioxidant, antimutagenic, and chemopreventive. Therefore, our results confirm and extend our knowledge on the ability of methanol extract and isolated flavanone compounds from *K. rotunda* to protect DNA, showing that both prevent chromosome damage after cyclophosphamide exposure in mice.

4. Conclusion

From the dried and milled rhizoma of *K. rotunda*, three known flavanones, namely 5-hydroxy-7-methoxyflavanone (1), 7-hydroxy-5-methoxyflavanone (2), and 5,7-dihydroxyflavanone (3) were isolated. The methanol extract and isolated flavanones from *K. rotunda* showed significant antimutagenic effect compared to control group.

Conflicts of interest

All authors have none to declare.

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