

Metabolit Utama Metilripariokhromen-A, Senyawa Bioaktif dalam Daun *Eupatorium Riparium* Reg. dalam Urin Tikus Jantan

Major Metabolites of Methylripariochromene-A, a Bioactive Substance in The Leaves of *Eupatorium Riparium* Reg. in Male Rats Urine

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ABSTRAK

Metilripariokhromene-A (MRC) (1) adalah senyawa bioaktif yang diisolasi dari daun *Eupatorium riparium* Reg. hasil koleksi Taman Nasional G. Merapi. MRC diisolasi dari ekstrak kloroform daun dengan metoda *bioassay (BST) guided isolation*. MRC mempunyai efek diuretik, menurunkan tekanan darah, dan mengeluarkan mineral dari tubuh. Efek ini juga ditunjukkan oleh furosemide yang secara klinis telah digunakan sebagai diuretik. MRC merupakan senyawa khromen yang mempunyai 2 gugus -OCH₃ pada cincin aromatikanya, secara *in vivo* kemungkinan mengalami demetilasi memberikan gugus fenolik (-OH) yang bersifat antiseptik. Penelitian ini dimaksudkan untuk mengetahui efek diuretik dibandingkan dengan furosemide, mengisolasi dan mengidentifikasi metabolit utama yang terdapat dalam urin, dan kemungkinan MRC dapat digunakan sebagai obat anti-infeksi pada saluran kemih. Ekstrak n-hexana (81% MRC) (1,25 g/Kg BW) secara per oral diberikan kepada tikus jantan (\pm 200 g), dan furosemide juga diberikan sebagai kontrol positif untuk diuretik. Urin selama 48 jam dikumpulkan, dipartisi dengan EtOAc selama 15 menit sehingga diperoleh fraksi EtOAc. Fraksi EtOAc difraksinasi, dan metabolit utama yang terdapat dalam fr. 4-5, dan fr. 6-7 dipisahkan dengan KLT preparatif. Identifikasi struktur kimia metabolit utama dilakukan berdasarkan atas data ¹H- dan ¹³C-NMR. Efek diuretik dari ekstrak n-heksana (1,25 g / kg bb) sebanding dengan furosemid (8,83 mL/ tikus vs 8,70 mL/ tikus selama 24 jam). Data ¹H- dan ¹³C-NMR dari fr 4-5 menunjukkan bahwa tidak ada perubahan besar dalam penampilan spektrum ¹H- dan ¹³C-NMR dibandingkan dengan MRC, kecuali bahwa gugus 1 -OCH₃ didemetilasi menjadi demetil MRC (2) . Berdasarkan eksperimen HMBC dan HMQC, -OCH₃ di C-8 adalah yang demetilasi. Data ¹H-NMR dari fr. 6-7 mirip dengan fr. 4-5, demetilasi satu-OCH₃ grup pada C-8 tetapi hubungan eter dalam sistem ring furan rusak untuk memberikan 2 struktur tentatif (3 dan 4).

Kata kunci: Metilripariokhromene-A, urin tikus jantan, metabolit utama

ABSTRACT

Methylripariochromene-A (MRC) (1) is the principal bioactive compound isolated from *Eupatorium riparium* Reg. collected from the Mt. Merapi National Park. MRC was isolated from the chloroform extract of the leaves by using bioassay (BST) guided isolation method. MRC causes diuretic, reduces the blood pressure and excretes mineral from the body. This activity is comparable to a clinically used diuretic drug, furosemide. MRC is a

chromene type of compound, having 2 -OCH₃ groups on the aromatic ring that are possibly demethylated *in vivo* to give phenolic compounds well known for having antiseptic properties. This study was aimed to evaluate diuretic properties compared to furosemide, isolate and identify MRC major metabolites present in urine. If it is true, then MRC probably can be used also as anti-infectious diseases in the tractus urinary. n-Hexane extract (81% MRC) of *E. riparium* (1.25 g/kg bw) was applied p.o on male rats (+ 200g) and furosemide was used as a positive control of diuretic activity. Urine was collected during 48 hours, partitioned with EtOAc for 15 minutes to give EtOAc fraction at the upper layer. The EtOAc fraction was then checked by tlc with MRC as the reference standard. The major metabolites were isolated by fractionating the EtOAc fraction followed by purification using preparative tlc (fr. 4-5 and 6-7). The major metabolites obtained were identified based on their ¹H- and ¹³C-NMR data. Diuretic effect of n-hexane extract (1.25 g/kg bw) was comparable to furosemide (8.83 mL/rat vs 8.70 mL/rat for 24 hours). The ¹H- and ¹³C-NMR data of fr 4-5 indicated that there was not major changing in the ¹H- and ¹³C-NMR spectra appearance compared to that of MRC, except that 1 -OCH₃ group was demethylated became demethyl MRC (2). Based on HMBC and HMQC experiments, -OCH₃ at C-8 was the one demethylated. The ¹H-NMR data of fr. 6-7 was similar to fr. 4-5, demethylation of one -OCH₃ group on C-8 but the ether linkage in the furan ring system was broken to give 2 tentative structures (3 and 4).

Keyword: Methylripariochromene-A, urine male rats, major metabolites

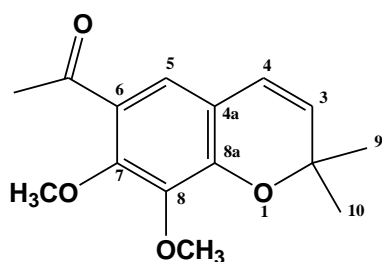
INTRODUCTION

Searching of bioactive compounds has been done in the rain forest of central Kalimantan, Mt. Merapi National Park, Ternate-Halmahera and Batam islands. The bioactive compounds search in Ternate-Halmahera and Batam islands was emphasized on the presence of Calanolide-A in *Calophyllum* sp. in which calanolide-A was reported present in huge amount at 38% of latex. Calanolide-A is a coumarin type of compound having anti HIV virus by inhibiting m-RNA transcriptase enzyme (Subagus *et al*, 2006). While the search in central Kalimantan and Mt. Merapi National Park was based on the guidance of a local medicine man (Subagus *et al*, 2004, 2005). There were 130 medicinal plants were collected from central Kalimantan, and only 30 medicinal plants species from Mt. Merapi National Park. Those medicinal plants were dried, pulverized, extracted, and screened by BST (*Brine Shrimps Lethality test*) at 1000, 500, 100, 10 µg/mL. Twenty medicinal plants collected from central Kalimantan were categorized as potentially

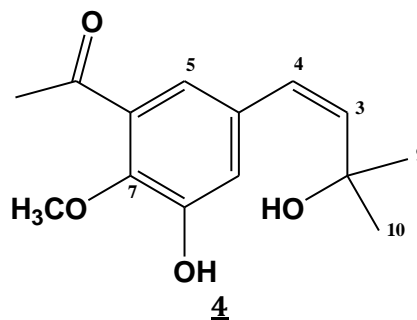
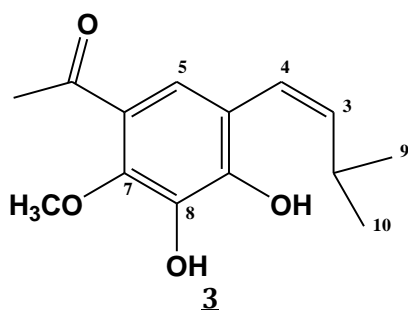
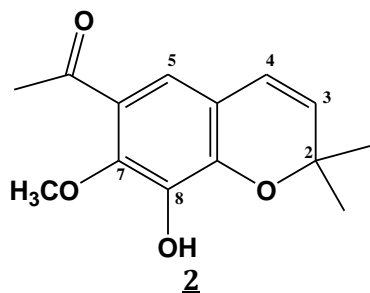
high to be studied further and three species were categorized as potentially very-high to be developed further (Subagus *et al*, 2006). In contrast to the above result, there were only two species out of 30 species collected from Mt. Merapi National Park performed potentially high to be studied further. These were methanol extract of *Ficus septica* (# 04-TN 10-13) and chloroform extract of *Eupatorium riparium* Reg. (# 04-TN 10-06). The chloroform extract of *E. riparium* was selected for the study as this extract was more potential (lower LC₅₀ value) than that of *F. septica* (Nanang *et al*, 2005).

By bioassay (BST) guided isolation, a bioactive compound was isolated and identified as methylripariochromene-A (MRC) on the basis of its spectroscopic data. MRC displayed LC₅₀ at 4.23 µg/mL (1.6 x 10⁻⁵ M) (Nanang *et al*, 2007). This value indicated that MRC is valuable enough for further biological study. Indeed this compound was not a new compound and had been isolated in 1969 (Devon and Scott, 1972), but MRC was reported active as hypotensive due to diuretic, vasodilating,

and decrease cardiac output actions (Matsubara, 1999). As MRC is methyl ether of a phenolic type of compound, it was suspected that MRC would be demethylated in the *in vivo* to give a free -OH of phenolic compound that mostly possesses antibacterial activity that would be excreted via urinary tractus. In the future, we expect that MRC displayed hypotensive activity and antiinfective in the urinary tractus as well. So the main aim of this study is to isolate and identify the major metabolites of MRC present in rats urine treated with MRC orally. Instead of pure MRC, standardized extract of *E. riparium* containing 81% of MRC was applied orally to rats due to limited amount of pure MRC.



Methyripariokhromene-A (MRC) (**1**)



METHODOLOGY

Materials, n-hexane extract of *E. riparium* (collected from Mt. Merapi National Park, consist of 81% MRC.), Male Rats Wistar strain (± 200 g), Furosemide (0.6 mg/rat, positive control).

Method, MRC treatment. N-hexane extract (80% MRC) (**A**) per orally (1.25 g/Kg bw) was applied to male Wistar-strain rats. The extract was suspended in 0.5% tween 80 (in water), Urine collection. The urine (**B**) collected for 24 hours, Metabolites extraction. The urine was partitioned (15 minutes) in a separatory funnel with EtOAc (50 mL, 2 x). The EtOAc layers were separated, combined and evaporated to dryness (**C**). The water layers were combined, evaporated (water-bath) to dry. The residue obtained was triturated with MeOH to give MeOH soluble (**D**) and insoluble fractions (**E**). MRC (**St**) was used as a standard. EtOAc fraction (**F**) of *E. riparium* water extract.

Analysis, Urine volume due to n-hexane extract treatment, compared to control and the structures of the major metabolites were determined by NMR spectroscopy.

RESULTS AND DISCUSSION

The MRC applied per orally to the rats was replaced by a standardized n-hexane extract of *E. riparium* leaves contained 81% of MRC. This step was undertaken as a large

Table I. Urine collection data

No.	Dates of urine Collection (2008)	Number of rats	Total urine (mL)	Urine (mL)/rat	Number of dead Rat
1.	30 th Jan	4	42	10.5	-
2.	31 st Jan	6	53	8.8	1
3.	1 st Feb	4	33	8.3	3
4.	4 th Feb	5	45	8.1	-
5.	5 th Feb	4	33	8.3	-
6.	6 th Feb	5	50	10.0	1
7.	7 th Feb	5	47	9.4	1
8.	8 th Feb	4	38	9.5	-
9.	11 th Feb	4	31	7.8	1
10.	12 th Feb	6	56	9.3	-
11.	13 th Feb	5	44	8.8	1
12.	15 th Feb	2	15	7.5	1
Total :		54	487	9.02	9 (16.6%)

amount of isolated MRC was required for this *in vivo* study (1.25 g/mL Kg bw). The urines were collected after 24 hours, measured the urine volume and the rats behavior was observed during 24 hours, and no specific behavior was observed during 24 hours; however, 9 rats were found dead (16.6%) (Table I). Our suspicion about the rats dead was due to dehydration, the water in the cage was practically untouched. Other possibility was that the rats were intoxicated by MRC [LC_{50} (BST), 1.6×10^{-5} M], in that case lower dose applied should be altered. The diuretic effect of MRC is higher to the clinically used medicine furosemide (0.6 mg/rat) (9.02 vs 8.7 mL/rat) (Ernst *et al.*, 1995), and effect of both treatments (furosemide and MRC) are twice higher than that of negative control (3.7 mL/rat) (Table II). The collected urine were combined and partitioned further in order to separate major metabolites according to their polarity (Scheme 1).

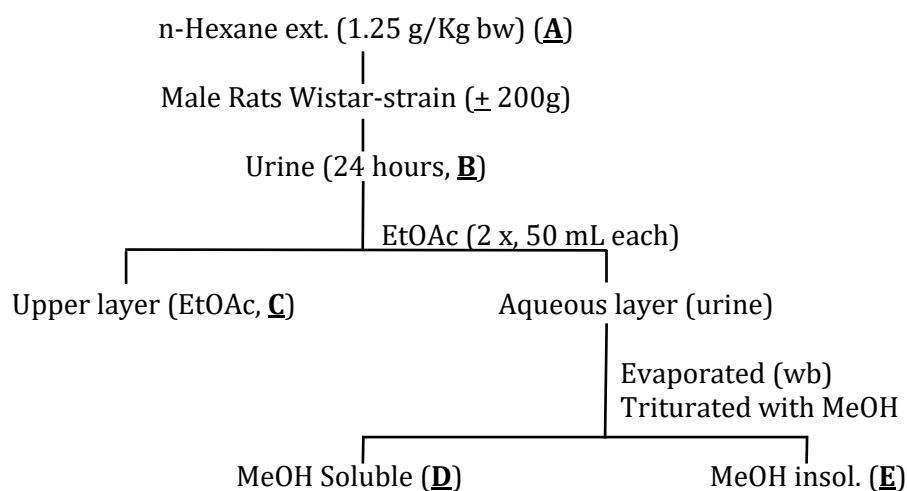
Fractions **A**, **C**, **D**, **E** were subjected on TLC (Thin Layer Chromatography) using selected stationary and mobile phases similar to MRC system. MRC of n-hexane extract was observed at similar rf. value of MRC standard (**St.**), and there was MRC-like

spot present on **C** except that their rf. values were a bit lower than that of **St.** While no specific spot was observed on both **D** and **E**. It meant that MRC major metabolites extraction from the rats urine has completed. In addition, fraction **F** was prepared in order to see whether MRC present in the water extract or not. This data is very important to be understood as local people use the water extract of the leaves to cure their illness. By a simple detection using TLC picture, the MRC presence in the water extract was detected although MRC known to be fat soluble substance. However, the MRC content in the water extract should be determined. The number of rats dead (16.6%) indicated that the dose of MRC consumed possibly too high [1.013 g/Kg bw (81% of 1.25 g/Kg bw)], although *in vivo* assay of cranberry proanthocyanidins uses 1.5 g/Kg bw (Amy, 2007). In the case of MRC, the dose applied in rats should be lower than 1.013 g/Kg bw in order to have effective dose.

As the **C** is still showing a complex mixture, the **C** was then fractionated by VLC (Vacuum Liquid Chromatography) using silica gel to give 10 fractions. Based on the similar appearance to MRC in the TLC

Table II. Diuretic effect of n-hexane extract of *E. riparium*

Treatment	Urine volume (mL, after 24 hours)
Control (-) (no treatment)	3.4, 4.2, 3.6 Average : 3.7 mL
Control (+) (Furosemide) (0.6 mg/rat)	7.6, 8.8, 9.6 Average : 8.7 mL
MRC (1.5 g/Kg BW)	487 mL of 54 rats Average : 9.02 mL



pictures, fractions 4 + 5 and 6 + 7 were combined separately. Those two combined fractions (4 + 5) and (6+7) were subjected into preparative TLC for major metabolites purification.

From fraction 4-5, a pure metabolite (TLC) (**2**) was isolated and the structure was identified by 1- and 2-D NMR (600 MHz) (Umar *et al*, 2006). According to the ¹³C-NMR data (Figure 2), compound **2** carbon signals were very similar to that of MRC (**1**), except that 1-OCH₃ was missing. Compound **1** has 2 -OCH₃ groups at C-7 and C-8, now the question arises of which -OCH₃ is cleaved to give **2**. The ¹H-NMR of **2** (Figure 3) was also similar to that of **1**, and there was no additional Ar-proton signal. It means that the -OCH₃ group cleaved does not produce an Ar-H, but -OH (phenolic) group formed instead, that is shown by an additional of broad signal at δ, 1.66 ppm in the ¹H-NMR spectra of **2** indicating the presence of -OH group. The structure of **2** (6- acetyl- 7- methoxy- 8- hydroxy -2,2

dimethyl- 2H- 1- benzopyran) was deduced using 2D NMR techniques (COSY, HetCor and HMBC). The ¹³C-NMR and ¹H-NMR (600 MHz, CDCl₃) data of **2** (Table III and IV) in comparison with **1** (100 MHz, CDCl₃).

Based on ¹H-NMR (CDCl₃) of fr. 6-7 (Figure 4), the isolated compound appeared to be a mixture of **3** and **4**. Similar to **2**, the ¹H NMR spectra showed that only 1 -OCH₃ signal was observed on **3** and **4**; however, the Ar-H (H-5) was shifted downfield to δ, 7.80 ppm and one of gem-dimethyl shifted downfield to δ 1.85 ppm. The structures of **3** and **4** were deduced tentatively, and further 2D-NMR data were needed to determine their definite structures.

CONCLUSION

One of the -OCH₃ group (C-8) of MRC was de-methylated, and the ether bond of the pyran ring system was cleaved to give metabolite **2** and two possible metabolite **3** and **4** due to cleavage at

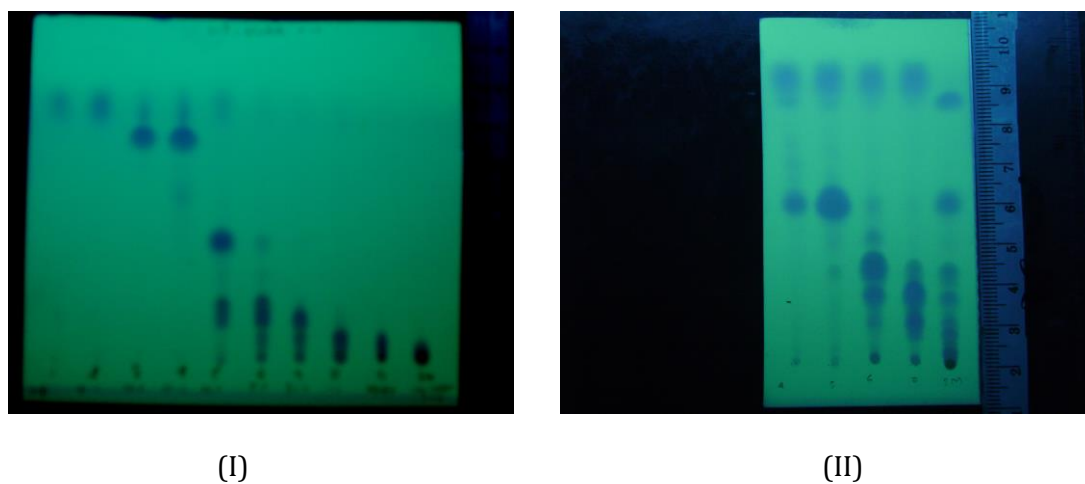


Figure 1. TLC pictures of fractions obtained from VLC of **C** (I) [n-hex – EtOAc (6:1 v/v)], and fr. 4-7 and ext. (II) [n-hex – EtOAc (4:1 v/v)]

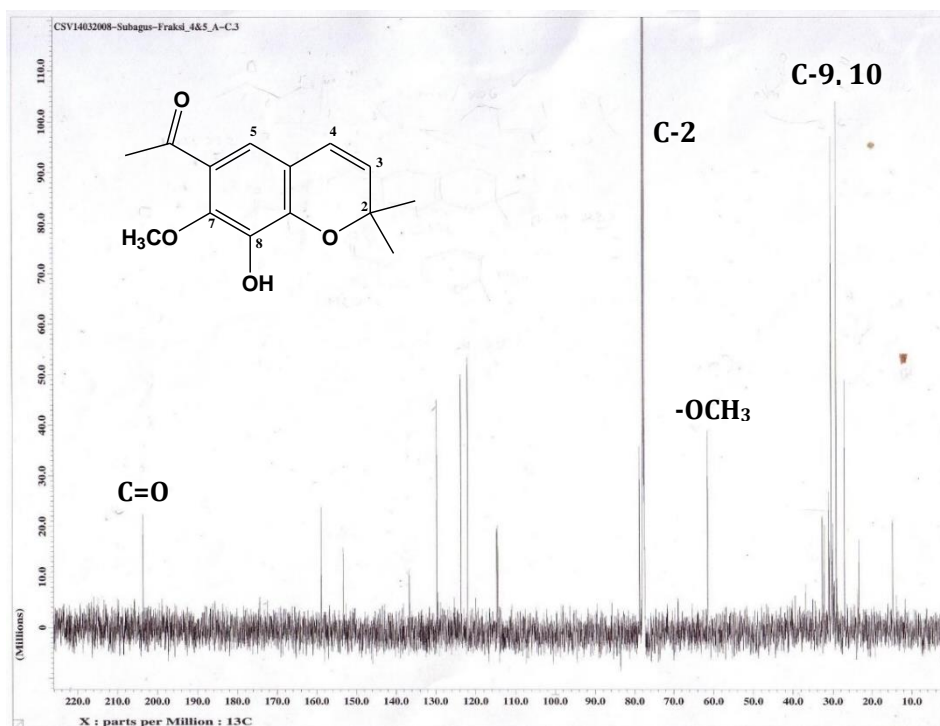


Figure 2. ^{13}C -NMR (600 MHz, CDCl_3) spectra of **2**

different bonds. Additional 2D-NMR data are needed to identify two other major metabolites (**3** and **4**). Antiinfective activity of the metabolites on gram (+) and (-)

bacteria and fungi should be performed, and it is important to evaluate the benefits of the double actions (diuretic and antimicrobial on urinary tract).

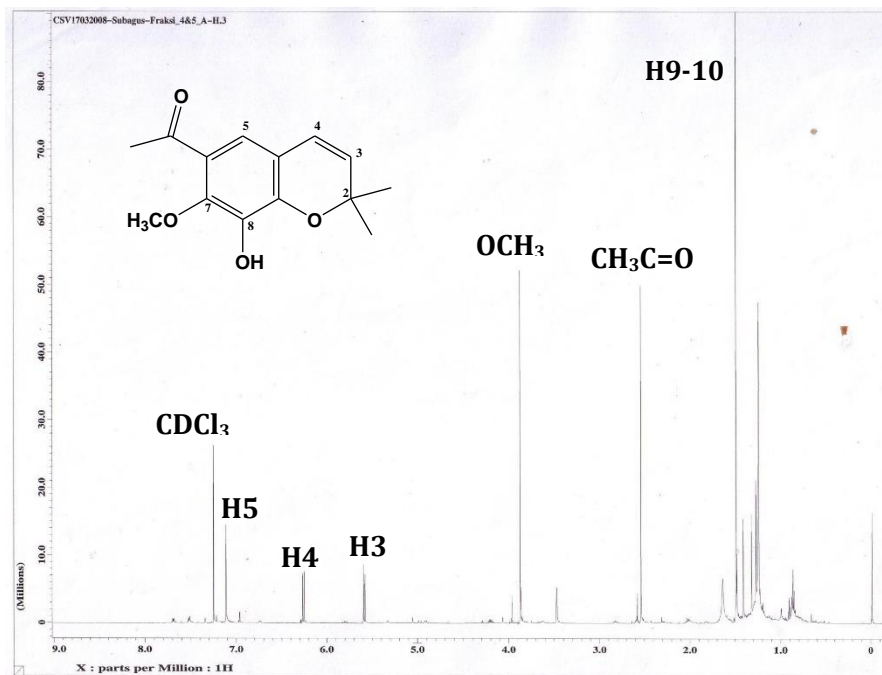


Figure 3. ¹H-NMR (600 MHz, CDCl₃) spectra of 2

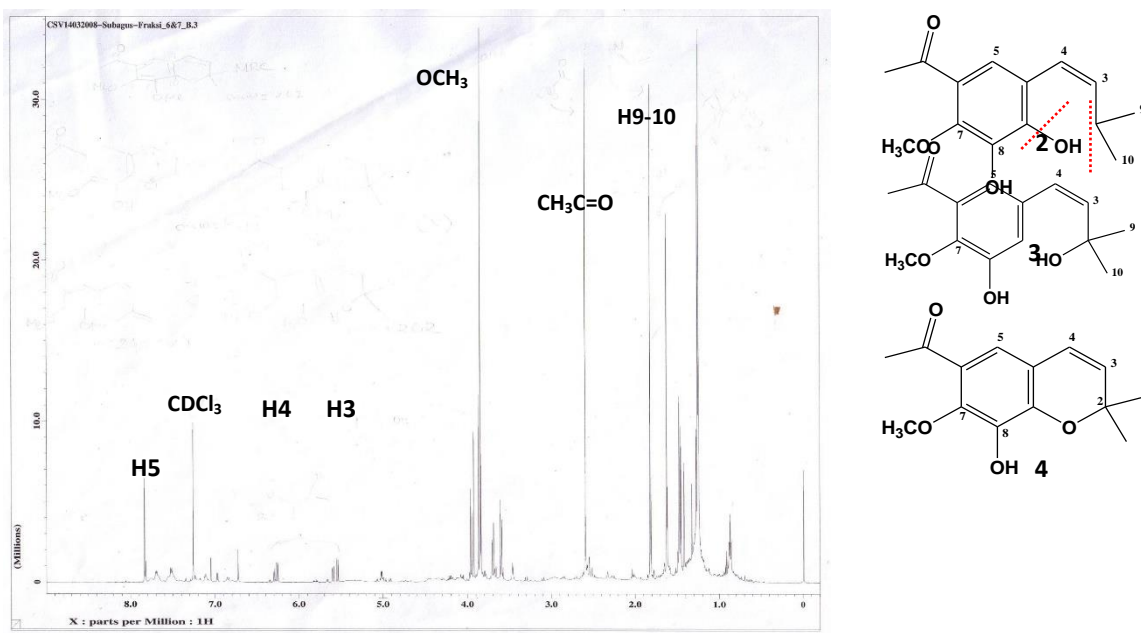


Figure 4. ¹H-NMR (600 MHz, CDCl₃) spectra of 3 and 4

Table III. ¹³C-NMR data of **1** and **2** (CDCl₃)

- C-	δ (ppm) (1) (100 MHz)	δ (ppm) (2) (600 MHz)
2	77.5	78.1
3	117.6	113.9
4	121.4	114.1
4a	122.1	121.4
5	129.6	129.1
6	124.6	123.1
7	154.3	158.2
8	141.12	136.0
8a	150.6	152.6
9	28.2	28.5
10	28.2	28.5
-C=O	197.4	202.9
-OMe	60.7	60.8
-OMe	61.3	-

Table IV. ¹H-NMR data of **1** and **2** (CDCl₃)

-H	δ (ppm) (1) (100 MHz)	δ (ppm) (2) (600 MHz)
H-3	5.60 (<i>d</i>)	5.60 (<i>d</i> , 9.7 Hz)
H-4	6.30 (<i>d</i>)	6.30 (<i>d</i> , 10.3 Hz)
H-5	7.22 (<i>s</i>)	7.11 (<i>s</i>)
H-9	1.49 (<i>s</i>)	1.49 (<i>s</i>)
H-10	1.49 (<i>s</i>)	1.49 (<i>s</i>)
CH ₃ -C=O	2.58 (<i>s</i>)	2.53 (<i>s</i>)
-OCH ₃	3.88 (<i>s</i>)	3.87 (<i>s</i>)
-OCH ₃	3.97 (<i>s</i>)	-

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