Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(4):41-49

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and Elucidation of Triterpenoid and Flavonoid Pure Compounds in *Morus alba* Stem Bark

D Rosa[1*], N Yuliana[2], YH Choi[3] and R Verpoorte[3]

[1]*Department of Biology, Universitas Pelita Harapan, Jl. M.H. Thamrin Boulevard, Tangerang, Indonesia*
[2]*Departement of Food Technology, Institut Pertanian Bogor, Jl. Raya Darmaga, Bogor, Indonesia*
[3]*Department of Biology, Section Pharmacognosy, Universiteit Leiden, Einsteinweg 55, The Netherlands*

ABSTRACT

Two pure compounds, morusin (a flavonoid, known for its bioactivity as tumor inhibitor, anti-bacterial, and anti-inflammatory agents) and betulinic acid (a triterpenoid, known for its anti-tumor activity), are isolated and elucidated from extract of Morus alba stem bark. Column chromatography and thin-layer chromatography techniques are used for the isolation, and nuclear magnetic resonance (NMR) analysis, both 1D and 2D, is used to elucidate the structure of the pure compounds.

Keywords: Flavonoid; Triterpenoid; *Morus alba*; Isolation procedure; Spectroscopic analysis

---

INTRODUCTION

Morus alba Linn or white mulberry is very famous especially in Far East Asia. This plant is native and cultivated in China, used as food source for *silk worm* (*Bombyx mori*) [1]. The plant itself is 1.8 meter tall, a medium sized, monoecious, deciduous tree with bark of large stems, brown, and rough. *M. alba* fruits are quite different than other Morus species. *M. alba* berries are white to pinkish while the others are red or black. The fruits are green when unripe and white when ripe.

Chinese Pharmacopoeia notes four parts of *M. alba* which are usually used as medicine. Those are Sangye or Folium Mori, Sangbaipi or Cortex Mori, Sangzhi or Ramulus Mori, and Sangren or Fructus Mori. Sangye or Folium Mori is the dry leaves of *M. alba* and is usually used as an antiphlogistic. Sangbaipi or Cortex Mori is the dry root bark of *M. alba* and can be used as an anti-inflammatory and a diuretic agent. Sangzhi or Ramulus Mori is the dry young branches of *M. alba* collected in the late of spring and early summer and is used for treatment of arthritis and rheumatism. Sangren or Fructus Mori is the ripe aggregate fruit of *M. alba* and is used as a tonic and sedative [2]. It is also noted that *M. alba* cortex is a constituent of herbal mixture called Jiang Qi Ding Chuan San which has bronchodilatory effect in asthmatics [1].

Despite the widespread use of *M. alba*, the first isolation of compounds from this plant was done only as late as 1960s [3]. Four flavone derivatives were isolated from the stem and root bark of *M. alba* such as mulberrin, cyclomulberrin, mulberrochromene, cyclomulberrochromene [2,3].

In this research pure compounds are isolated from *M. alba* stem bark using column chromatography techniques. The selection of the fraction to be further investigated is based on thin-layer chromatography analysis. It turns out that two pure compounds can be isolated in this way. One is betulinic acids which belongs to triterpenoid group, and the other is morusin which belongs to flavonoid groups.

MATERIALS AND METHODS

Plant Material

M. alba dried stem bark material was obtained from South Korea. The plant materials were identified by Y.H. Choi from Division of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University, Leiden, the Netherlands. A voucher specimen (ORST-Fcog-NL-230506) was deposited in the Division of Pharmacognosy, Institute of Biology, Leiden University, Leiden.

Chemical and Reagents

Methanol, chloroform, ethanol, sulfuric acid, glacial acetic acid, dimethylsulfoxide (DMSO) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Anisaldehyde was obtained from Acros Organis (Geel, Belgium) and *n*-buthanol was obtained from JT Baker BV (Deventer, The Netherlands). MeOH-d_4 and DMSO-d_6 NMR solvents was from Eurisotop (Yvette, France). Tris buffer was purchased from Gibco BRL (New York, NY, USA). All solvents and reagents were analytical grade.

Column and Thin Layer Chromatography

Column chromatography was performed with silica gel 60 (230-400) mesh from Merck (Darmstadt, Germany), in 1.8 × 30 cm column from Sigma Aldrich Chemie BV (Zwijndrecht, The Netherlands), SPE C-18 column from Macherey-Nagel GmbH & Co. (Duren, Germany), and Sephadex LH-20 gel from Sigma Aldrich Chemie BV (Zwijndrecht, The Netherlands) in a 2.75 × 140 cm column. Preparative and analytical TLC were performed using alumunium TLC plates of 20 × 20 cm silica gel 60 F 254 from Merck (Darmstadt, Germany). High Performance Liquid Chromatography was performed using Luna pentafluorophenyl propyl (PFP) from Phenomenex with 5 µm particles, 100 Å pore size, 250 × 10 mm column. Spectra were recorded by PL-ELS 2100 ice. The solvent system was water – methanol with a gradual change in proportion to have a better separation according to the polarity of the compound:

a. from time 0 – 30 minutes : methanol : water = 90 : 10
b. from time 30 – 51 minutes: methanol : water = 100 : 0
c. from time 51 – 60 minutes : methanol : water = 90 : 10

The flow rate used was 1 ml/minute and the injection volume was 100 µL.

Plant Material Extraction

The powdered dried plant material (100 g) was extracted 3 times with 800 mL of 80% MeOH then sonicated for one hour at room temperature. After the filtration, centrifugation, and evaporation, MeOH extracts (7.58 g) was added 100 mL H_2O and partitioned 3 times by 100 mL of $CHCl_3$. The partitioning was done using liquid-liquid partition method, which means each time the mixture of extract, water, and $CHCl_3$ was mixed together, and then left to settle. The $CHCl_3$ and water separated, and the $CHCl_3$ was then taken for further isolation. The amount of $CHCl_3$ fraction was 724 mg. The reason of choosing fraction $CHCl_3$ for further process is as follows. Most of the pure compounds with pharmaceutical properties in *Morus alba* are compounds of middle polarity [4-6]. These middle polar compounds should be included in fraction $CHCl_3$.

Compound Isolation

The $CHCl_3$ fraction was separated with a silica gel column using sequentially changed solvent mixture of $CHCl_3$:MeOH, first 100% : 0%, then 50% : 50%, and at last 0% : 100%. Every 10 ml of eluate was analyzed using Thin Layer Chromatography (TLC) technique, and those with similar TLC patterns were put together. In this way four major fractions were obtained, whose amount were 109.5 mg, 107.5 mg, 449 mg, and 46.1 mg. Since the amount of fraction 3 was the largest, it was reasonable to assume that the major compounds would be in this fraction. The third fraction was therefore selected to be separated further with C-18 Solid Phase Extraction (SPE) column using gradient solvent of H_2O:MeOH (1:0 → 0:1). Again every 10 ml of eluate was analyzed using TLC method and then grouped together based on the TLC patterns. The result of this process is compound **1** (0.2 mg), which came out as a crystalline substance, and four fractions: 3A 12.6 mg, 3B 5.7 mg, 3C 81.5 mg, and 3D 161.8 mg. These fractions were analyzed further using TLC technique. The fraction that showed good separation and had thick band gave the highest chance of finding pure compound in it. Based on the TLC pattern, fraction 3C and 3D were chosen to be further separated. Fraction 3C was further separated with preparative HPLC using MeOH:H_2O (90:10) as solvent system, yielded 8 fractions: 3C1 6.4 mg, 3C2 8.5 mg, 3C3 6.1 mg, 3C4 5.1 mg, 3C5 5.0 mg, 3C6 7.7 mg, 3C7 6.7 mg, and 3C8 7.0 mg. Unfortunately, NMR spectra of these 8 fractions only showed minor compounds which were not promising to be further isolated.

Fractions 3D was put into Sephadex LH-20 column chromatography with 500 mL of MeOH and a flow rate of 2 mL/minute for further separation, resulting in eleven fractions: 3D1 23.0 mg, 3D2 24.7 mg, 3D3 15.9 mg, 3D4 1.38 mg, 3D5 8.02 mg, 3D6 3.83 mg, 3D7 3.57 mg, 3D8 25.6 mg, 3D9 2.6 mg, 3D10 0.9 mg, and 3D11 0.5 mg. Since fraction 3DE5 showed major compound (thick band) on TLC plate, this fraction was subjected to TLC preparative separation using mobile solvent system $CHCl_3$/MeOH (10:1), yielded compound **2** (2.02 mg). Fractionation and isolation were monitored by TLC with visualization under UV (254 and 365 nm) using anisaldehyde-H_2SO_4 spray reagent followed by heating. The structure were assigned on the basis of ^{1}H, J resolved, COSY, HMBC NMR, and APCI-MS spectra. The obtained data were compared with previous reports.

NMR Measurement

Each pure compound was dissolved in MeOD or DMSO-d_6 for 1D and 2D NMR. NMR spectra were recorded on 600 MHz Bruker DMX 600 Spectrometer. 1D (^{1}H) and 2D (J resolved, COSY, HMBC) NMR experiments were performed at 25^{o}C. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are reported in Hz.

APCI Mass Spectrometry

Spectra were recorded on Agilent 1100 MSD single quadrapole mass spectrometer using probe positive-ion and Phenomenex RP 18 (4.6 × 150 mm, 5 micron) column. The mass scan range was 100-700 m/z. The solvent system was MeOH + 0.1% HCOOH : H_2O + 0.1% HCOOH with flow rate of 0.5 mL/minute and injection volume of 5 µL/minute.

RESULTS AND DISCUSSION

Fractionation of *Morus. alba* stem bark MeOH 80% extract resulted in 2 pure compounds, compound **1** and compound **2**. The structures of these compounds were elucidated using LC MS and NMR spectra as explained below. Compound **1** was isolated from the solid phase extraction (SPE) as transparent crystal in one of 37 fractions before pooled into four fractions (3A-3D). Compound **2** was obtained as yellow powder from TLC preparative separation from fraction 3DE5.

Structure Elucidation

The structures of compounds isolated from *Morus alba* stem bark were determined by analysis of APCI/MS, 1D and 2D NMR (J resolved, COSY, and HMBC) spectroscopy data, and comparison with literatures.

Structure of compound 1-Betulinic Acid:

The first compound was isolated as transparent cyrstal which showed blue fluorescence under UV light of 366 nm wave length. The spectrum from negative APCI-MS exhibited a molecular ion peak at *m/z* 455.6 ([M-H]$^-$), indicating a molecular weight of 456.6. The molecular formula was established as $C_{30}H_{48}O_5$ indicating 7 degrees of in saturation, with hypothesized structure depicted in Figure 1.

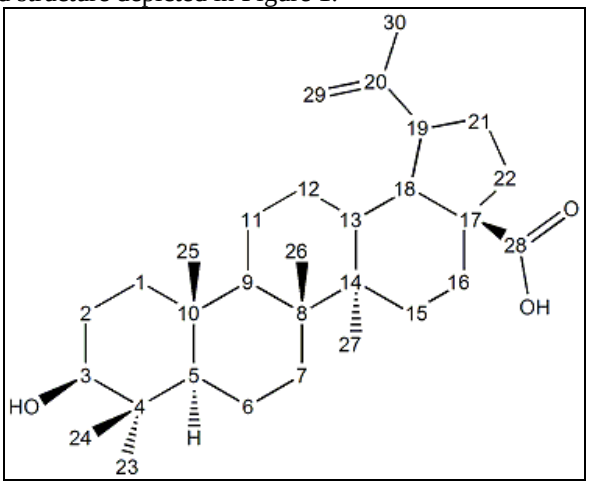

Figure 1: Structure of compound 1 (betulinic acid)

The ^{1}H NMR spectrum of compound **1** (Figure 2) displayed signals for typical triterpenoid methyl groups at δ 0.75 (3H, H_3-24), 0.86 (3H, H_3-25), 0.95 (3H, H_3-23), 0.97 (3H, H_3-26), 1.00 (3H, H_3-27) and 1.69 (3H, H_3-30).

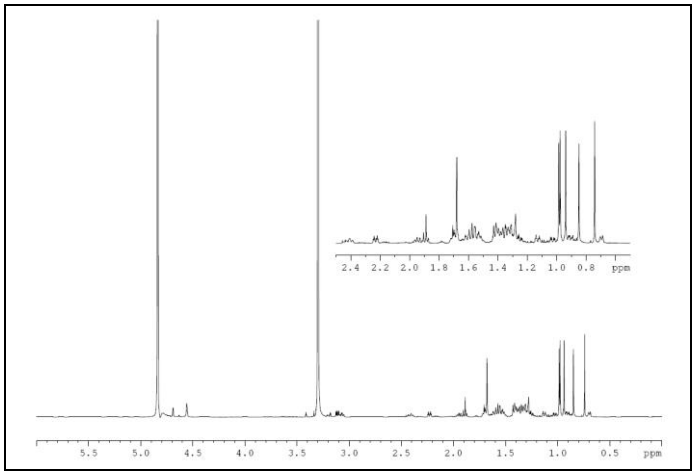

Figure 2: ^{1}H NMR spectra of compound 1 (betulinic acid) in MeOH-*d_4*

The presence of a vinyl methyl proton signal at δ 1.69 and two broad vinyl proton signals at δ 4.58 (1H, H-29a) and 4.70 (1H, H-29b) were characteristic of an isopropenyl group of lupene triterpenes [7]. The ^{1}H NMR spectrum also showed a double doublet signal at δ 3.12 (1H, H-3, J = 11 Hz, 5 Hz) due to a hydroxymethylene group. The splitting pattern of this proton was confirmed by J resolved spectra (Figure 3).

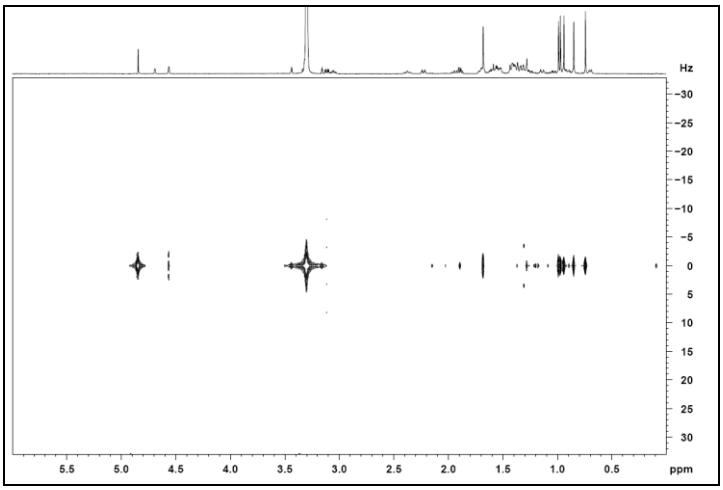

Figure 3: J resolved spectra of compound 1 (betulinic acid) in MeOH-*d_4*

The heteronuclear multiple bond connectivity (HMBC) as depicted in Figure 4 showed correlations of H-18 (δ 1.95) to carboxylic carbon, indicating the carboxyl group (δ 180) was adjacent to C-17. The long-range correlation of two methyl groups (H_3-23 and H_3-24) to carbon signal at δ 78 ppm confirmed the position of hydroxyl group at position 3. This is confirmed by COSY spectra (Figure 5) that showed correlation between H-2 (δ 1.55, m) with H-3 (δ 3.12). On the other hand, the HMBC correlations between vinyl protons and a carbon signal at δ 48 (C-19), indicated that an isopropenyl group was attached to C-19.

This isopropenyl group conformation was also confirmed by COSY that displayed the correlation between H-29a (δ 4.58) and H-29b (δ 4.70) and also there is relationship between both H-29a and H-29b with H_3-30 (δ 1.69). Other COSY and HMBC correlations can be seen in Figure 6.

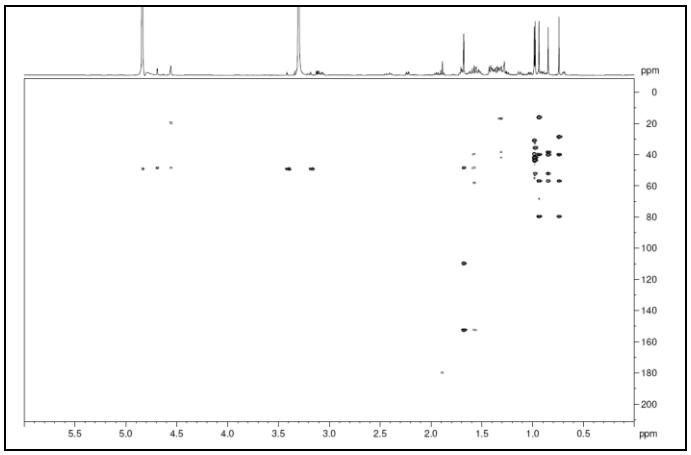

Figure 4: HMBC spectra of compound 1 (betulinic acid) in MeOH-d_4

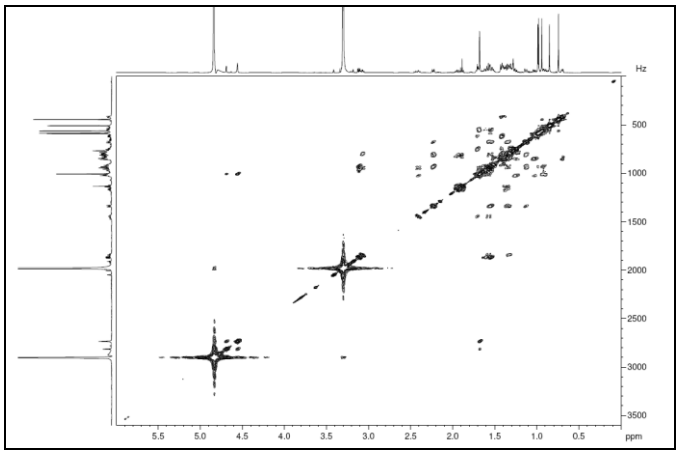

Figure 5: COSY spectra of compound 1 (betulinic acid) in MeOH-d_4

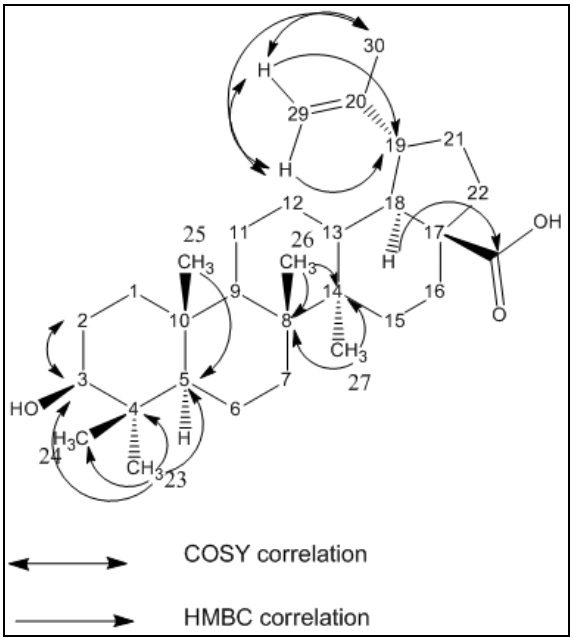

Figure 6: COSY and HMBC spectra of compound 1 (betulinic acid) in MeOH-d_4

The analysis of mass and NMR spectra led to conclusion that compound **1** was betulinic acid (3β-3-hydorxy-lup-20 (29)-en-28-oic acid). This is confirmed by comparing spectroscopy data to reference compound analysis and spectra reported for betulinic acid in the literatures [7-10]. This triterpenoid was also isolated from stem of *Morus alba* in previous research [3].

Structure of compound 2-Morusin:

The second compound was isolated as yellow powder which gave dark blue fluorescence under UV light of 254 nm wave length. The spectrum from positive APCI-MS exhibited a molecular ion peak at m/z 421.3 ($[M-H]^+$), indicating a molecular weight of 420.3, in agreement with the molecular formula $C_{25}H_{24}O_6$. The hypothesized structure for this compound is shown in Figure 7.

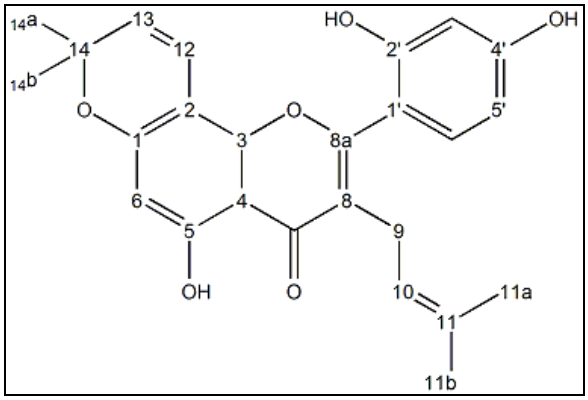

Figure 7: Structure of compound 2 (morusin)

^{1}H NMR spectra of compound **2** (Figure 8) showed a typical moiety of prenylflavonoid structure. Previous researches showed that indeed prenylflavonoids were commonly found in Moraceae family, including *M. alba* [11,12]. These prenylated flavonoids are characterized by a flavonoid structure bearing one or more isoprenoid side chain(s) at positions 6 or 8.

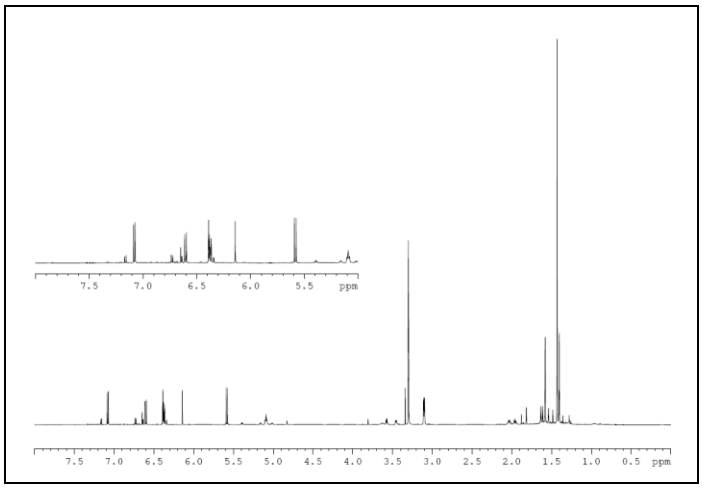

Figure 8: ^{1}H NMR of compound 2 (morusin) in MeOH-d_4

The presence of a prenylated group was described by COSY correlations (Figure 9) between both two singlets at δ 1.40 (3H, H_3-11a) and δ 1.58 (3H, H_3-11b) with both a broad doublet at δ 3.10 (2H, H_2-9, J=10Hz) and a broad triplet at δ 5.09 (1H, H-10, J=10Hz).

It is also confirmed by HMBC data (Figure 10) that showed relationship between both H_3-11a and H_3-11b to both carbon signals at δ 121.5 (C-10) and at δ 131.8 (C-11), indicating that both these methyl groups were connected to carbons which have double bonds. The position of this group was assigned by HMBC correlations between both H_2-9 and H-10 with C-8 at δ 120.5, showing that prenylated group was attached to C-8.

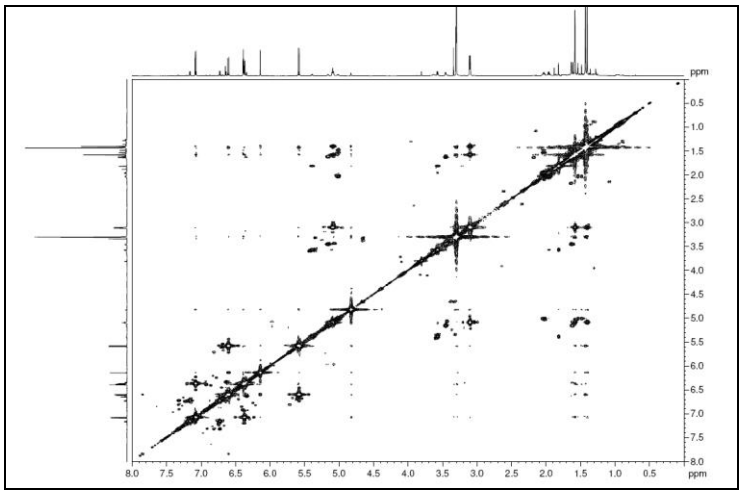

Figure 9: COSY of compound 2 (morusin) in MeOH-d_4

Meanwhile, the presence of a 2,2-dimethylchromene ring was displayed by the singlet signals at δ 1.42 (3H, H_3-14a and 3H, H_3-14b), doublets at δ 6.61 (1H, H-12, J=10Hz) and at δ 5.59 (H, H-13, J=10Hz). The correlation of both doublets H-12 and H-13 can be seen in COSY (Figure 9) with coupling constant 10 Hz, indicating that C-12 and C-13 were adjacent to each other and connected with double bound. HMBC spectroscopy (Figure 10) showed correlations between H-12, H-13, H_3-14a, and H_3-14b with carbon signal at δ 79, showing that the methyl groups were connected to the ring via this carbon (C-14).

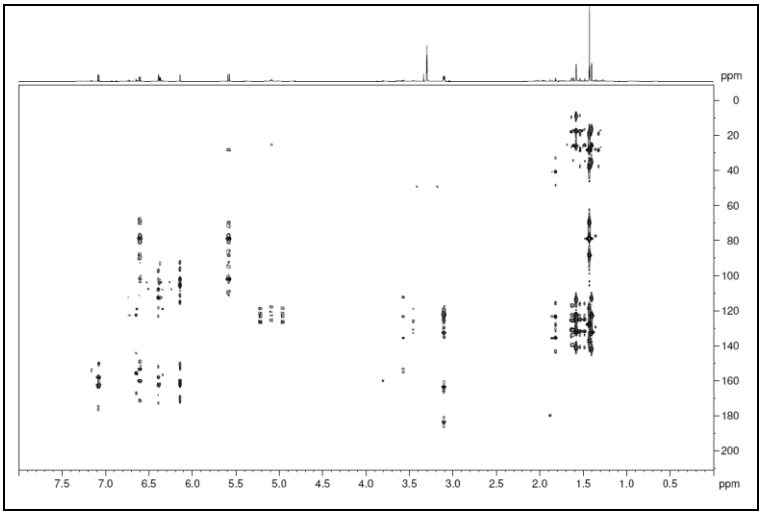

Figure 10: HMBC of compound 2 (morusin) in MeOH-d_4

The downfield chemical shift of C-14 indicated that this carbon was connected to oxygen atom. The connections of C-12 to other components of the ring was shown by HMBC correlations (Figure 10) between both doublet at δ 6.61 (H-12) and singlet at δ 6.14 (1H, H-6) to carbon signals at δ 160 (C-1) and δ 101.5 (C-2). By their chemical shifts, C-1 and C-2 could be deduced to be conjugated alkene, and therefore the C-6 should be a part of other ring (ring A) which was connected to 2,2-dimethylchromene ring at position C-1 and C-2.

In the J resolved (Figure 11) and COSY (Figure 9), the arrangement of the outer ring (ring B) can be seen. A double doublet signal at δ 6.39 (1H, H-5', J=2Hz, 8Hz) couples with doublet at δ 7.08 (1H, H-6', J=8Hz) and doublet at 6.36 (1H, H-3', J=2Hz). It means that ring B is substituted in the 2' and 4' position.

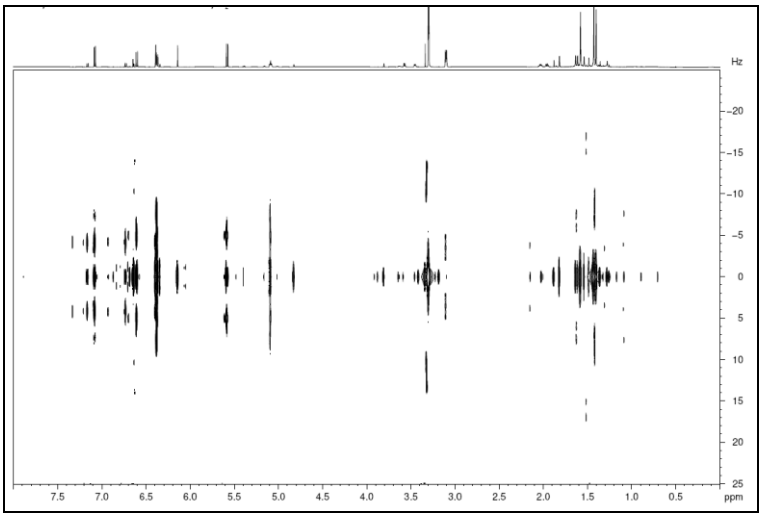

Figure 11: J Resolved of compound 2 (morusin) in MeOH-d_4

The assignments of these signals were confirmed by HMBC data (Figure 10). Ring B was connected to the rest of the structure at carbon at δ 162 (C-8a) since HMBC spectroscopy also showed that this carbon was related with H-6' and H_2-9. Other COSY and HMBC correlations are shown in Figure 12.

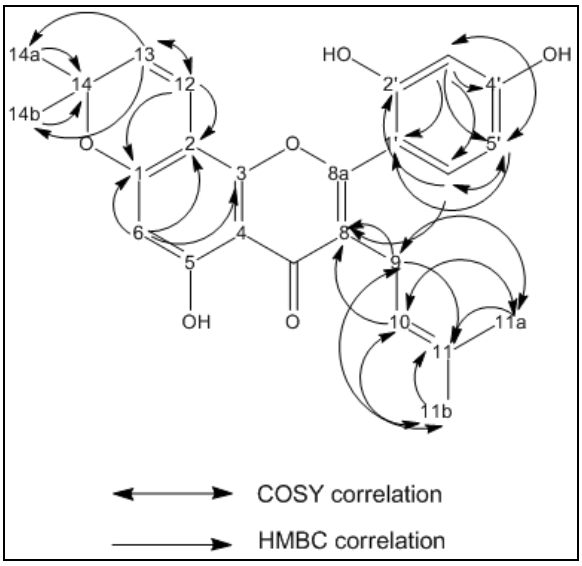

Figure 12: COSY and HMBC spectra of compound 2 (morusin) in MeOH-d_4

Based on the above mentioned analyses it can be concluded that compound **2** isolated from *M. alba* stem bark was morusin (2-(2,4-dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-3-(3-methylbut-2-enyl)pyrano[2,3-h]chromen-4-one). This is also supported by comparison with previous researches [13-15] which provided ^{1}H- and ^{13}C-NMR spectrums of morusin.

CONCLUSION

In this research there are two pure compounds which are isolated from the *M. alba* stembark. The first compound is betulinic acid. Some studies about betulinic acid show that this compound has a lot of biological activities such as inhibition of human immunodeficiency virus (HIV) replication in H9 lymphocyte cells [16], inhibition of DNA polymerase β [17], and blockage of HIV type 1 entry into cells [18]. Moreover betulinic acid derivatives are also shown to inhibit HIV type 2 [19].

Betulinic acid has also cytotoxic effect. It can trigger the mitochondrial pathway of apoptosis in cancer cells. Apoptosis is an intrinsic program of cell death that is present in every cell and regulated by defined signaling

pathways [20]. Therefore, it is believed that betulinic acid is a candidate to be used against cancer, for example human melanoma [21], but also for a variety of other cancers [20]. This cytotoxic effect of betulinic acid is not observed for normal cells and tissues, underlining its activity as promising anti-cancer agent [20]. In fact, another study has confirmed that in combination with ionizing radiation, the effect of betulinic acid on growth inhibition is additive in colony forming assays for melanoma cells, making this compound an interesting candidate for anti-cancer agent, not only as a single agent, but also in combination with radiotherapy [22].

The second compound isolated in this research is morusin. This compound may be active as anti-tumour promoter, at least for tumour on mouse skin promoted by teleocidin [6]. It is also demonstrated to significantly inhibit the growth and clonogenicity of human colorectal cancer HT-29 cells by inducing apoptosis and suppressing nuclear translocation NF-κB in DNA binding activities in those cells [23]. Morusin is even patented as anti-bacterial agent, especially against oral pathogenic microorganisms (*Actinomyces viscosus*, *Actinobacillus actino-mycetemcomitans*, *Streptococcus mutans*, and *Porphyromonas gingivalis*) [24]. The same patent also mentions the anti-inflammatory use of morusin.

REFERENCES

[1] VR Kumar; S Chauhan. *J Med Plant Res*. **2008**, 2(10), 271-278.
[2] W Tang; G Eisenbrand. Chinese drugs of plant origin chemistry, pharmacology, and use in traditional and modern medicine, Springer-Verlag, Heidelberg. **1992**.
[3] VH Deshpande; PC Parthasaranthy; K Venkataraman. *Tetrahedron Lett.* **1968**, 14, 1715-1719.
[4] L Cui; M Na; H Oh; EY Bae; DG Jeong; SE Ryu; S Kim; BY Kim; WK Oh; JS Ahn. *Bioorg Med Chem Lett*. **2006**, 16(5), 1426-1429.
[5] JM Rollinger; R Spitaler; M Menz; K Marschall; R Zelger; EP Ellmerer; P Schneider; H Stuppner. *J Agr Food Chem*. **2006**, 54(22), 8432-8436.
[6] S Yoshizawa; M Suganuma; H Fujiki; T Fukai; T Nomura; T Sugimura. *Phytotherap Res*. **1989**, 3(5), 193-195.
[7] DK Kim; IY Nam; JW Kim; TY Shin; JP Lim. *Arch Pharm Res*. **2002**, 25(5), 617-620.
[8] MS Rahman. *Pharm J*. **2005**, 4(1).
[9] OB Flekhter; LR Nigmatullina; LA Baltina; LT Karachurina; FZ Galin; FS Zarudii; GA Tolstikov; EI Boreko; NI Pavlova; SN Nikolaeva; OV Savinova. *Pharm Chem J*. **2002**, 36(9), 484-487.
[10] M Sholichin. *Chem Pharm Bull*. **1980**, 28(3), 1006-1008.
[11] D Barron; RK Ibrahim. *Phythochem*. **1996**, 43(5), 921-982.
[12] MS Butt; A Nazir; MT Sultan; K Schroën. *Trends Food Sci Tech*. **2008**, 19(10), 505-512.
[13] T Nomura. *Chem Pharm Bull*. **1978**, 26(5), 1394-1402.
[14] T Nomura. *Chem Pharm Bull*. **1976**, 24(11), 2898-2900.
[15] T Nomura; T Fukai. *Heterocycles*. **1979**, 12(10), 1289-1295.
[16] M Evers. *J Med Chem*. **1996**, 39(5), 1056-1058.
[17] J Ma; SR Starck; SM Hecht. *J Nat Prod*. **1999**, 62, 1660-1663.
[18] JF Mayaux; A Bousseau; R Pauwels; T Huet; Y Henin; N Dereu; M Evers; F Soler; C Poujade; E De Clercq. *Proc Natl Acad Sci*. **1994**, 91, 3564-3568.
[19] Z Dang. *J Med Chem*. **2009**, 40(30).
[20] S Fulda. *J Mol Sci*. **2008**, 9, 1096-1107.
[21] P Chatterjee; SA Kouzi; JM Pezzuto; MT Hamann. *Appl Environ Microb*. **2000**, 66(9), 3850-3885.
[22] E Selzer; E Pimentel; V Wacheck; W Schlegel; H Pehamberger; B Jansen; R Kodym. *J Invest Dermatol*. **2000,** 114(5), 935-940.
[23] JC Lee; SJ Won; CL Chao; FL Wu; HS Liu; P Ling; CN Lin; CL Su. *Biochem Bioph Res Co*. **2008**, 372(1), 236-242.
[24] http://www.faqs.org/patents/app/20080286213