ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):36 - 41

eISSN-0128-1119

Secondary metabolites from the leave extracts of *Morus alba* L.

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Abstract: *Morus alba* L., a commercially important plant has medicinal value and grows naturally in Bangladesh. The leaves of *M. alba* were extracted with aqueous 80% ethanol and was evaporated into dryness. The dry mass was suspended in water and was successively partitioned with dichloromethane, ethyl acetate, and n-butanol. By repeated normal phase silica gel column chromatography of dichloromethane and ethyl acetate extracts afforded four secondary metabolites namely, octadecanol, 4-hydroxy octadec-6,9-dienoic acid, *β*-sitosterol, and stigmasterol. The structures of all metabolites were confirmed by ^{1}H- and ^{13}C-NMR spectroscopic studies. Analysis of fatty acid compositions of an oily fraction obtained from a column of DCM extract by gas chromatography equipped with a flame ionization detector (GC-FID) revealed that palmitic acid was predominant acid in the oil.

Keyword: Morus alba, fatty acids, Bangladesh, flame ionization detector

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INTRODUCTION

Morus alba L., commonly known as white Mulberry belongs to the family Moraceae (Jeszka-Skowron et al., 2014). The genus Morus comprises approximately nineteen members and more than 150 species. *M. alba* is a dominant species among them and is primarily distributed in the temperate and tropical regions including South Europe, North America, East and Southeast Asia, and Southeastern Australia, and also some parts of Africa (The Plant List, 2017, Srivastava et al., 2006 and Encyclopedia of Life, 2017). The plant is cultivated in the northern zone of Bangladesh, particularly in Rajshahi for the production of silk (Rahman & Khanom, 2013). The plant contained various secondary metabolites including steroids, flavonoids, amino acids, vitamins, triterpenes and other trace elements (Deshmukh et al., 1993). *M. alba* is reported to have antioxidant, anti-hyperglycemic, antibacterial, antihypertensive, anti-hyperlipidemic activities, and neuroprotective properties (Nomura et al., 1980). The white mulberry leaves, an important food for silkworm, are used to treat hypertension, arthritis, and the fruit is a diuretic and tonic agent. The root bark of the plant is considered as an important medicine to treat cough, inflammation, diabetes, cancer, hepatitis

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and heart diseases (Vo VC. Dictionary of Vietnamese Medicinal plants, 1999). Here, isolation and structure elucidation of four secondary metabolites is reported for the first time for the variety from Bangladesh.

MATERIALS AND METHODS

Instruments: Shimadzu Prestige-21 (KBr pellet) was used to record IR spectra. NMR spectra of pure compounds were recorded by using a High-resolution ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) (Bruker, Switzerland) spectrometer. The spectra were recorded using $CDCl_3$. Shimadzu GC 2025 Gas Chromatograph with FID detector (Shimadzu, Japan) was used for fatty acid analysis.

Collection of plants: The matured leaves of *M. alba* were collected from Madhupur, Tangail, Bangladesh and identified by the Taxonomist, Department of Botany, University of Dhaka. The leaves were cleaned to remove mud and dust particles immediately and were air-dried at room temperature and then in the oven below 40 °C within seven days. Dried leaves were ground into powder.

Extraction: Dried powdered leaves (1700 g) were extracted with aqueous 80% ethanol (6 L x 3 times) for 2 days. The combined extracts were filtered. The filtrate was evaporated below 40° C with rotary evaporator, and 167.2 g crude ethanol extract was obtained. Ethanol extract (60.0 g) was partitioned with dichloromethane, ethyl acetate and butanol by suspending the extract in water. Each of the extracts was evaporated and thus dichloromethane (36.10 g), ethyl acetate (1.70 g) and butanol extracts (0.8 g) were obtained.

Isolation of secondary metabolites: The DCM extract (36.1 g) was fractionated by silica gel column chromatography. A glass column was made with the slurry of silica gel 60 (0.063 - 0.200 mm) using n-hexane as column packing and equilibrating solvent. The DCM extract was applied to the column and was eluted with 0 to 100% DCM in hexane followed by 5 to 20% MeOH in DCM and finally eluted with 30% MeOH and seven fractions (T_1-T_7) were collected. One of the fractions eluted from 80% DCM in hexane was subjected to sub column chromatography in silica gel and six fractions (J_1-J_6) were obtained. One fraction obtained from 70% DCM in hexane and another one 80% DCM in hexane gave separately single spots on TLC and were purified by washing with *n*-hexane several times to get compound 1 (18 mg) and 2 (12 mg), respectively. The ethyl acetate extract (1.7 g) was further fractionated by silica gel column chromatography and 7 fractions were obtained. Two fractions eluted from 20% ethyl acetate in hexane became pure on the TLC spot after washing with n-hexane and collected as compound 3 (24 mg) and 4 (26 mg).

Compound 1: Gummy and oily (18mg), soluble in a mixture of hexane and DCM, FT-IR (KBr pellets): ν_{max} 3369, 2916, 1376 and 719 cm^{-1}. ^{1}H -NMR ($CDCl_3$): δ 3.56, 2.0, 1.22, and 0.85 ppm. ^{13}C-NMR ($CDCl_3$): δ 62.6, 29.0, 25.7, 22.6 and 13.9 ppm.

Compound 2: Gummy and oily (~ 12 mg), soluble in DCM, ^{1}H -NMR ($CDCl_3$): δ 5.48, 5.14, 5.14, 5.14, 4.12, 3.68, 2.09, 1.27 and 0.90 ppm. ^{13}C-NMR ($CDCl_3$): δ 173.2, 132.0, 130.0, 128.2, 127.2, 69.0, 63.1, 62.1, 32.8, 31.7, 29.6, 26.4, 26.3, 25.6, 23.4, 22.7, 15.9 and 14.0 ppm.

Compound 3: Solid (~ 24.7mg), soluble in EtOAc, IR (KBr pellet): ν_{max} 3421, 2932, 1459, 1379, 1174 and 1053 cm^{-1}. ^{1}H -NMR ($CDCl_3$): δ 5.36, 3.53, 2.25, 2.03, 1.43, 1.02, 0.95, 0.87, 0.85, 0.82 and 0.67 ppm. ^{13}C-NMR ($CDCl_3$): δ 140.8, 121.7, 71.8, 56.8, 56.1, 50.1, 45.9, 42.3, 42.2, 39.9, 37.4, 36.5,

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eISSN-0128-1119

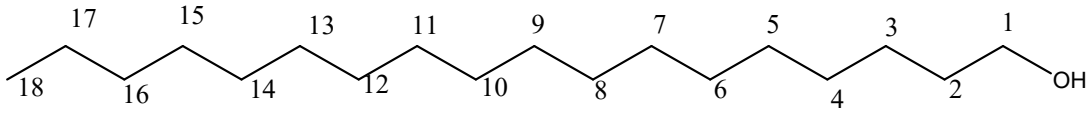
36.2, 33.9, 32.8, 32.6, 31.9, 29.7, 28.2, 26.2, 24.3, 23.1, 21.0, 19.8, 19.7, 19.0, 18.8, 14.1 and 11.9 ppm.

Compound 4: Solid (26.7mg), soluble in EtOAc. ^{1}H NMR ($CDCl_3$): δ 5.36, 5.30, 5.15, 3.53, 2.25, 2.03, 1.43, 1.02, 0.95, 0.87, 0.85, 0.82 and 0.67 ppm. ^{13}C-NMR ($CDCl_3$): δ 140.8, 140.0, 123.1, 121.7, 71.8, 56.8, 56.1, 50.1, 45.9, 42.3, 42.2, 39.9, 37.4, 36.5, 36.2, 32.8, 32.6, 31.9, 29.7, 28.7, 24.3, 23.1, 21.0, 19.8, 19.7, 19.0, 18.8, 14.1 and 11.9 ppm.

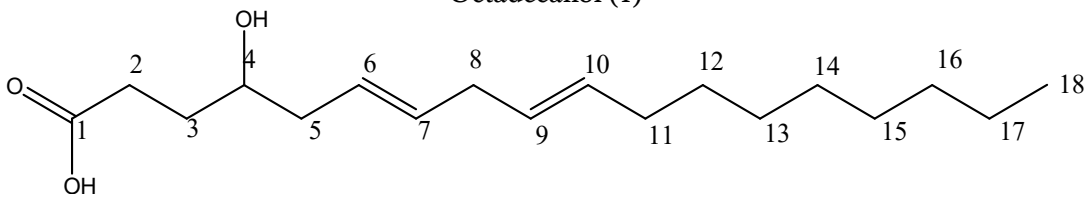
Analysis of fatty acid composition: Oil (100 mg) obtained from 40% DCM in hexane fraction was saponified with 1.0 ml of 0.5 M methanolic NaOH solution in a boiling water bath at about 60 ºC for 3 hours and evaporated to dryness. The dried material was dissolved in water (~ 1.0 ml) acidified with a few drops 2M HCl and partitioned with *n*-hexane. The hexane extract was evaporated to dryness under blowing nitrogen. Boron trifluoride-methanol (BF_3-MeOH, 1 ml) complex was added to the dried free fatty acids, ultrasonicated (vortexed 30 sec.), heated in a boiling water bath for 30 min and evaporated into dryness. *n*-hexane (~ 1 ml) was added to the dried methyl ester, filtered through Pasteur pipette containing cotton, transferred to a GC vial and analyzed by GC-FID. Separation was performed on the HP-5 column (30 m in length, 0.25 mm in diameter and film thickness 0.25 µm). The temperature program in the oven was as followed: 140 °C for 1 min (hold) gradually increased by 7 °C/min to 270 °C and again hold for 10 min. N_2 (g) was used as a carrier gas with a column flow rate of 2 mL/min. Air and nitrogen gases were used as fuel for FID. Injector temperature was 280 °C and the detector temperature 290 °C. Injection volume: 1 mL.

RESULTS AND DISCUSSION

Silica gel column chromatography of the DCM and ethyl acetate extracts of *M. alba* afforded compounds **1** and **2**, and compounds **3** and **4**, respectively (Figure 1).



Octadecanol (1)



4-Hydroxy octadec-6,9-dienoic acid (2)

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eISSN-0128-1119

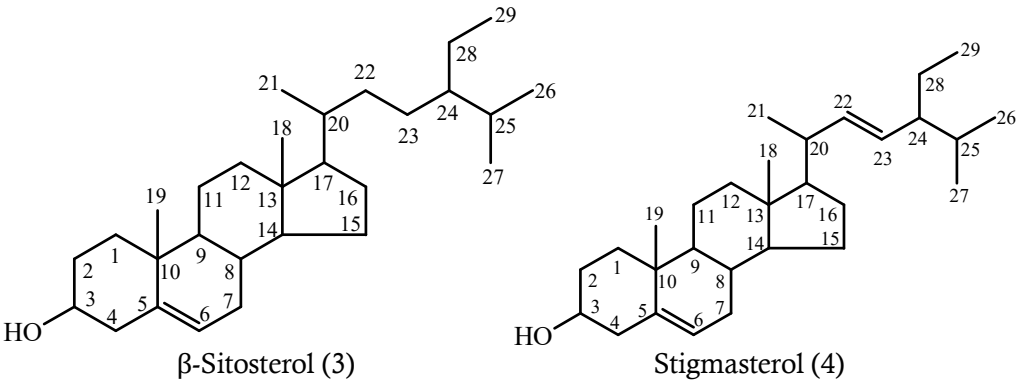

Figure 1: Structure of isolated compounds

The FT-IR spectrum of **1** showed absorption band at 3369, 2916, 1376, and 719 cm^{-1} indicating the presence of O-H, C-H stretching (sp^3 C-H; either $–CH_3$, or $-CH_2-$), C-O stretching, and $–CH_2-$ bending and rocking of a long aliphatic chain group, respectively. The ^1H NMR spectrum of **1** had signals at δ_H 0.85, 1.22, 2.00 and 3.56 ppm for CH_3, CH_2, CH, and CH_2OH, respectively. The ^{13}C NMR spectrum showed signals at δ_C 13.9, 22.6, 25.7, 29.0, and 62.6 ppm for CH_3, CH_2, CH_2, CH_2, and CH_2OH, respectively. All spectroscopic data confirmed that compound **1** is octadecanol**.**

The ^1H NMR spectrum of **2** gave signal at δ_H 5.48, 5.14, 5.14, 5.14, 4.12, 3.68, 2.09, 1.27, 0.90 ppm. Two signals at δ_H 5.48 and 5.14 ppm were due to the presence of two olefinic protons. Signals at δ_H 5.14 ppm were also for another two olefinic protons. A distorted triplet at δ_H 0.90 ppm is for one methyl group. A broad signal at δ_H 1.24 ppm is due to methylene proton. One doublet at δ_H 4.12 (2H) ppm is for one oxygenated methylene group. The ^{13}C NMR spectrum of **2** gave signal at δ_C 173.2, 132.0, 130.0, 128.2, 127.2, 69.0, 62.1, 29.6, 63.1, 26.3, 25.6, 23.4, 22.7, 14.0, 31.7, 32.8, 26.4 and 15.9 ppm. All these data confirmed that the structure of **2** is 4-hydroxy octadec-6,9-dienoic acid.

The FT-IR spectrum of **3** showed an absorption band at 3421 cm^{-1} assignable for the O-H group and 2932 cm^{-1} which was due to the presence of aliphatic C-H stretching. The two peaks at 1459 and 1379 cm^{-1} were indicative of - CH_2- and $–CH_3$ groups, respectively. The absorption band at 1174 cm^{-1} was due to C-O stretching and 1053 cm^{-1} was suggestive of >C=C-H. The ^1H-NMR spectrum of **3** had two sharp singlets (s) at δ_H 0.67 and 0.95 ppm typical for the presence of methyl protons. The spectrum had a multiplet at δ_H 3.53 ppm indicated the presence of oxymethine proton (H-3α). The distorted multiplet at δ_H 5.36 ppm was indicative of the presence of olefinic proton (H-6) at C-6. The three doublets at δ_H 0.87, 0.85 and 0.82 ppm were due to the presence of three methyl protons. The spectrum had a triplet at δ_H 0.87 ppm was due to the presence of the methyl group. The other signals between δ_H 1.43–2.25 ppm were due to the presence of different methylene ($-CH_2-$) and methine (>CH-) protons. The ^{13}C-NMR spectrum of **3** showed signals at δc 140.8 and 121.7 ppm which were due to two olefinic carbons and signals at δ_C 36.5 and 42.3 ppm were assignable for two quaternary carbons. The signals at δ_C 11.9, 14.1, 18.8, 19.0, 19.7 and 19.8 were due to the presence of six methyl carbons and the signals at δ_C 37.4, 31.6, 42.3, 31.9, 21.0, 39.9, 23.1, 28.2, 33.9, 26.2 and 24.3 ppm were due to the presence of eleven methylene carbons. The signals at δc 71.8, 32.8, 50.1, 56.8, 29.7, 56.1, 36.2 and 45.9 ppm were due to the presence of eight methine carbons. The signal at δ_C 71.8 ppm was due to oxymethine carbon. ^1H- and ^{13}C-NMR data of **3** was compared with reported NMR data and found identified with *β*-sitosterol (Greca et al., 1990, Shoeb et al., 2013).

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Compound **4** had similar spectral data of compound **3** with additional signals at δ_H 5.30 and 5.15 ppm and δ_C 140.0 and 123.1 ppm in the ^1H and ^{13}C NMR spectrum, respectively. The spectroscopic data of compound **4** was compared with the reported data of stigmasterol and found identical (Chaturvedula & Prakash, 2012). Thus, compound **4** was confirmed as stigmasterol.

Relative fatty acid composition of fractions: The fatty acids in the oil from DCM in hexane fractions were made into their methyl ester by saponification with methanolic NaOH followed by esterification with BF_3-MeOH complex, and analyzed by GC-FID. The relative percentage of methyl ester of fatty acids in oil was identified by comparing their retention time with that of methyl ester of standard fatty acids. It was found that myristic, palmitic, stearic, arachidic, and behenic acid were found in fractions and their relative percentages were 0.98, 37.66, 27.39, 20.92 and 13.05, respectively.

CONCLUSION

The leaves of *M. alba* L. afforded octadecanol, 4-hydroxy octadec-6,9-dienoic acid, β-sitosterol, and stigmasterol. Analysis of fatty acids compositions in oil by GC-FID revealed that palmitic acid was a predominante fatty acid.

ACKNOWLEDGMENT

Authors are grateful to International Science Program (ISP), Uppsala University, Sweden, University Grants Commission of Bangladesh (UGC), Ministry of Education, Government of the People's Republic of Bangladesh for financial supports.

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

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eISSN-0128-1119

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