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Full Length Research Paper

Antioxidant and antibacterial constituents from *Morus nigra*

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The objective of this study was to isolate and characterize the constituents of the local *Morus nigra* L. (Black mulberry), to compare its constituents with other studied Mulberries and to evaluate its anti-oxidative and anti-bacterial activities. The isolated compounds were identified by comparison of spectral data (UV, IR, MS and NMR) with literature values. The stem bark and wood of *M. nigra* yielded a stilbenoid oxyresveratrol 1, a 2- arylbenzofuran moracin M2, four isoprenylated flavonoids; cyclomorusin 3, morusin 4, kuwanon C5 and a derivative of kuwanon C6, two tritepenes; betulinic acid 7, -amyrin acetate 8 and a steroidal saponin -sitosterol-3-O- -D- glucoside 9. The phenolic isolates showed moderate DPPH radical scavenging activity (EC_{50} = 23-135 µgml^{-1}) compared to ascorbic acid (EC_{50} = 41 µgml^{-1}) after 30 min. Compounds 1, 2 and 4 to 6 showed activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococus flavus*, *Streptococcus faecalis*, *Salmonella abony*, *Pseudomonas aeruginosa*.

Key words: *Morus nigra*, antioxidant, antibacterial, oxyresveratrol, isoprenylated flavonoids.

INTRODUCTION

Morus nigra L. (Moraceae) belongs to the genus *Morus* which is widely distributed in Asia, Europe, North and South America and Africa. Mulberry (genus *Morus*) is an economically important plant used for sericulture, as a feed for the domesticated silkworm, *Bombyx mori* (Awasthi et al., 2004), and has a long history of medicinal use in Chinese medicine as a herbal medicine called “Sang Bai-Pi” (Nomura, 1988) . The root bark, twigs and fruits which contain phenolic compounds are used as refreshing substances, are prescribed to treat cough, asthma, other chest complaints and rheumatism (Nomura, 1988). The decoction of the leaves possesses blood purifying properties, reduces fever and is diuretic (Kumar and Gupta, 1996). The bark of *M. nigra* was reputed to be used to expel tape worm and its extracts have been reported to have antibacterial and fungicidal activity. The chemical constituents of White Mulbery have been well studied by Taro Nomura (Nomura, 1988) who has reported phenolic glycosides, prenylated flavonoids, Diels-Alder type adducts, *N*-containing sugars, terpenoids, coumarins and 2-arylbenzofurans. The medicinal importance of the genus *Morus* prompted the phytochemical investigations of this plant.

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EXPERIMENTAL

Plant material

The dry stem wood of black mulberry (*M. nigra*) was collected from Gaborone, Botswana in June 2006. The stem bark was harvested from Selibe-Phikwe, in the Central District of Botswana in July 2006. The plant species was verified by Mr Andrew Muzila of the Herbarium, Department of Biological Sciences, University of Botswana. A voucher specimen (OM112) has been deposited in the University of Botswana Herbarium.

General methods

Merck (Darmstadt, Germany) silica gel 60 (size 0.040 to 0.063 mm) was used for column chromatography. Thin layer chromatography (TLC) was carried out on 0.25 mm layer of Merck silica gel 60 F_{254} pre-coated on aluminium sheets. Merck silica gel 60 $HF_{254 + 366\ nm}$ coated on 20 x 20 cm glass plates (0.5 mm thickness) were used for preparative TLC (PTLC). The UV light ($_{max}$ 254 and 366 nm) and 1% vanillin-sulphuric acid spray were used for visualization.

The ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR, DEPT-135 (distortionless enhancement by polarization transfer), COSY

(Correlation spectroscopy), HMQC (heteronuclear multiple quantum correlation) HSQC (heteronuclear single quantum correlation), HMBC (heteronuclear multiple bond correlation) and TOCSY (total correlation spectroscopy), were acquired on on Bruker Avance DPX 300, and DRX 600 MHz. HR-MS were obtained on a GCT Premier Mass Spectrometer (Waters) in EI Mode. UV-Vis spectra were recorded on Shimadzu (UV-2101 PC) UV- Vis spectrometer (Kyoto, Japan). IR spectra were measured on a Shimadzu Hyper FT-IR 8700 (Kyoto, Japan). Melting point was recorded on Stuart Scientific melting point apparatus SMP1 (UK). Specific rotation $[\ ]_D$ was determined through Autopol IV (Rudolph Research Analytical) Automatic Polarimeter at = 589 nm.

Extraction and Isolation

Stem bark

The dried and powdered stem bark (3.1 kg) was soaked in acetone (7 L) for three days followed by ethanol (5 L) for three days. The combined extract was evaporated using rotary evaporator to yield 136.7 g black jelly. The crude extract was suspended in water, and liquid-liquid partitioning was performed successively with *n*-hexane and ethyl acetate to yield *n*-hexane (10.3 g), ethyl acetate (91.6 g) and residual water (23.8 g) extracts. The EtOAc extract (91.6 g) was fractionated over silica gel column eluted successively with $CHCl_3$/MeOH mixtures of increasing polarity to afford eleven fractions, which were mixed based on similarity of fraction TLC profiles [T1, T2, T3, T4]. Fraction T1 yielded white crystals of compound 8 (1.4 g). Fraction T2 (730 mg) was fractionated into sub-fractions [T2a (30 mg), T2b (40 mg), T2c (20 mg) and T2d (100 mg)] using silica gel column using step wise gradients of *n*-hexane, *n*-hexane/acetone (8:2 and 6:4) and acetone. The sub- fractions were subjected to preparative TLC using the solvent system toluene- EtOAc (6:1) to afford compound 3 (10 mg) and betulinic acid (7; 10 mg). Fraction T3 (2.0 g) was fractionated over a silica gel column eluted with *n*-hexane/acetone (9:1 to 1:1) to afford three sub-fractions T3a (10 mg), T3b (40 mg) and T3c (480 mg). T3c was subjected to PTLC (TEA 6:1:1) and yielded 4 (90 mg), 5 (10 mg) and 6 (40 mg).

Stem wood

The dry powdered stem wood (5.3 kg) was soaked in methanol (5 L, three days) which upon solvent evaporation yielded 19.3 g of black residue. The residue (18 g) was chromatographed on a silica gel column, eluted successively with $CHCl_3$/MeOH (9:1 to 3:7) and sixty five fractions were collected. These were combined based on similarity of fraction TLC profiles [W1-7]. Fraction W1 (9.8 g) was re-chromatographed ($CHCl_3$/acetone 9:1to 1:1) to give four sub-fractions W1a (9 mg), W1b (49 mg), W1c (420 mg), and W1d (3.3 g). Sub-fraction W1c was repeatedly subjected to Sephadex LH-20 ($CHCl_3$/MeOH (1:1)) to give 1 (210 mg) . Part of fraction W2 (160 mg) was fractionated on a silica gel column and eluted with $CHCl_3$/MeOH (6:2) to give eight fractions, which were mixed [W2a (100 mg), W2b (10 mg), W2c (20 mg) and W2d (30 mg]. A PTLC of sub-fraction W2a was run using the solvent system toluene/EtOAc (6:1) developed three times to give Moracin M (2; 10 mg). Fraction W3 (350 mg) yielded white crystals (9; 200 mg), which were recrystallized in methanol. The other fractions when worked on yielded compounds already isolated.

DPPH radical scavenging assays

The antioxidant potential of *M. nigra* extracts and compounds was measured using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) obtained from Aldrich (Munich, Germany) and following the method described by Yeboah and Majinda (2009). Briefly, methanolic samples (2 ml) of various concentrations (0.005, 0.05, 0.1, 0.2 and 0.5) $mgml^{-1}$ were prepared and 2 ml of 2 % DPPH was added. Ascorbic acid was used as a standard and all measurements were done in triplicates. The absorbance of the mixture was measured at 517 nm at time intervals of 0.5, 1, 3 and 6 h.

Antibacterial screening

The antibacterial activity of control drug tetracycline (Aldrich, Germany), extracts and compounds (1 to 6) was studied using serial micro-dilution method (Danielle, 2006). The test microorganisms were supplied by the Microbiology unit of the Department of Basic Sciences, Botswana College of Agriculture. The test microorganisms were *Staphylococcus aureus* (NCTC 4163), *Bacillus subtilis* (NCTC 10073), *Micrococus flavus* (NCTC 2665), *Streptococcus faecalis* (NCTC 775), *Salmonella abony* (NCIMB 6017), *Pseudomonas aeruginosa* (NCIMB 10421). The antibacterial activity was expressed as minimum inhibitory concentration (MIC) . The assay was also used to establish whether the crude extract or pure compounds were bacteriostatic (MBSC) or bactericidal (MBCC).

RESULTS AND DISCUSSION

The aerial parts of *M. nigra* were extracted using EtOAc and MeOH. The stem bark EtOAc extract yielded compounds 1 to 6 and 8, while the stem wood MeOH extract afforded compounds 1, 2, 8 and 9. The spectral data of compounds 1 (Takasugi et al., 1978), 2 (Basnet et al., 1993), 3 (Nomura et al., 1976), 4-5 (Nomura et al., 1978a,b), 6 (Nomura, 1988), 7-8 (Wu et al., 2003) and 9 (Basnet et al., 1993) were in agreement with those reported in Literature. Moracin M (2) (Zheng et al., 2010) and Morusin, (4) (De Souza et al., 2000) were previously reported from *M. nigra* root and leaves while other compounds have been reported from other Mulberries (*Morus alba*, *Morus insgnis* and *Morus macroura*) (Nomura, 1988; Zheng et al., 2010) except Black mulberry. The DPPH radical scavenging activity of compounds 1-6 was assessed using the free stable DPPH radical (Table 1). The crude extracts and isolated compounds all showed activity (Table 1). The higher free radical scavenging activity of the stem wood extract could be attributed to the presence of compound 1, which was the major constituent of the stem wood (Figure 1).

The extracts of *M. nigra* fruit were reported to have a protective action against peroxidative damage to biomembranes and biomolecules (Naderi et al., 2004), while the roots methanolic extract showed mushroom tyrosinase inhibitory activity (Zheng et al., 2010). The flavonoids, 3-5 are C-3 and C-8 isoprenylated, and have closely related structures. Compounds 4 and 5 have higher free radical scavenging activities compared to 3 and 6, which have the C-3 isoprenyl group cyclised. This could be attributed to the involvement of the C_2-C_3 double bond in the formation of partial aromaticity of the C_3-$C_{2'}$ pyrone rings. The higher activity of 4 over 5 was

Table 1. DPPH radical scavenging activity of extracts and isolates from *M. nigra*

Compounds	30 mins EC_{50}, μgml^{-1}	1 h EC_{50}, μgml^{-1}	3 h EC_{50}, μgml^{-1}	6 h EC_{50}, μgml^{-1}
SB EtOAc	73.10	59.60	38.1	37.00
SW MeOH	71.20	44.61	35.80	31.7
1	23.22	21.81	20.84	19.95
2	98.46	49.22	48.39	39.01
3	>250	>250	>250	216
4	100.31	85.24	70.6	68.29
5	122.14	105.08	80.38	72.99
6	135.75	121.37	101.04	94.20
Ascorbic acid	41.08	26.20	25.00	23.55

Each value is the mean ± standard deviation of a triplicate determinations, SD ± 0.04-, 0.08 μgmL^{-1}. SB = stem bark, SW = stem wood.

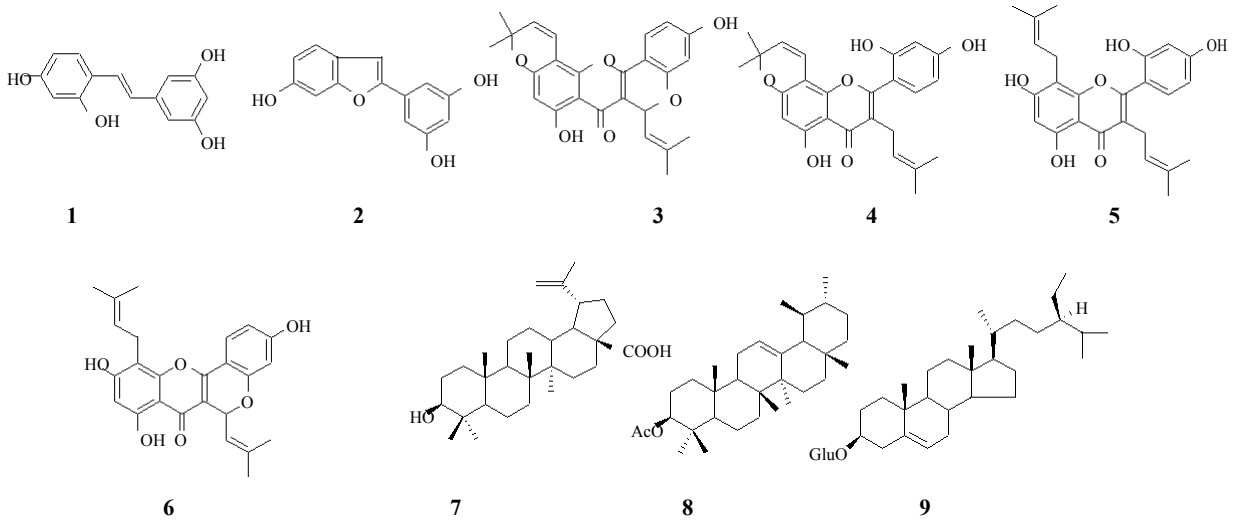

Figure 1. Compounds isolated from *M. nigra*.

attributed to the increased conjugation of 4 by cyclisation of C-8 prenyl group. The antibacterial activity of the extracts and compounds (1 to 6) was studied using the serial dilution method (Danielle, 2006) and tetracycline was used as a standard (Table 2). Compounds 1 and 2 showed bactericidal activity against *S. aureus* (MBCC = 125 and 62.5 μgml^{-1} resp.) and 2 also showed bactericidal activity against *S. faecalis* (MBCC = 500 μgml^{-1}). The structure activity relationship of 3 and 4 showed that the cyclization of prenyl unit at C- 3 in 3 reduced activity, whereas presence of free prenyl unit at C-3 in 4 enhances the activity. When comparing the activities of 4 and 6 it was observed that, when the prenyl unit at C -3 was cyclised (6) then to have better activity the C-8 attached prenyl unit should be open (6). Compound 3 has both prenyl units cyclised and 5 has both prenyl units open, these have lower activities than compounds 4 and 6 which have one of the prenyl units open. Though it is inconclusive, the results show that the electron donating group (-OH) at positions 7 and/or 2' was vital for increased activity as observed for 4 and 6. A more detailed study would have to be carried out to ascertain the role of hydroxyl groups, open and closed prenyl group in increased activity (Table 1).

The activities of 2, 4, and 5 were compared to previously reported activities (Sohn et al., 2004; Fukai et al., 2005). Fukai reported activities of 2 against methicillin-sensitive and methicillin-resistant *S. aureus* strains (MIC = 25 gml^{-1}) and *P. aeruginosa* (MIC > 100 gml^{-1}) (Fukai et al., 2005). Sohn reported MIC > 100 gml^{-1} against *E. coli* and *S. typhimurium* and activity specific towards *S. epidermis* and *S. aureus* (MIC < 25 gml^{-1}) for 4 (Sohn et al., 2004). Nomura reported that Kuwanon C (5) was inactive against *E. coli* and *P. aeruginosa* (100 gml^{-1}) (Nomura, 1988) while Sohn reported activities against *E. coli* and *S. typhimurium* (MIC < 25 gml^{-1}) (Sohn et al., 2004). The differences observed might be due to the methods used, level of

Table 2. Antibacterial activity of extracts and isolates from *M. nigra*

Samples	Microorganism and MIC in µgml^{-1}					
	SA	BS	MF	SF	SAB	PA
SB EtOAc	125	125	500	500	250	500
SW MeOH	62.5	62.5	250	250	125	500
1	125	250	250	125	250	250
2	62.5	31.25	125	62.5	62.5	125
3	125	500	NA	NA	NA	NA
4	7.81	3.91	62.5	31.25	7.81	62.5
5	62.5	125	500	31.25	125	250
6	15.63	15.63	125	62.5	62.5	125
tetracycline	1.95	1.95	3.91	3.91	1.95	15.63

SA = *Staphylococcus aureus*, BS = *Bacillus subtilis*, MF = *Micrococus flavus*, SF = *Streptococcus faecalis*, SAB = *Salmonella abony*, PA = *Pseudomonas aeruginosa. Experiment done in triplicate.* SB= stem bark, SW = stem wood. NA = no activity up to 500 gml^{-1}.

purity of compounds and bacterial strains used (Table 2).

Conclusion

The investigation of *M. nigra* bark and leaves yielded a stilbenoid, a 2-arylbenzofuran, four isoprenylated flavonoids, two tritepenes and a steroidal saponin. Moracin M (2) and Morusin, (4) were the only compounds to have been previously reported from *M. nigra* root and leaves, the other compounds have been reported from other Mulberries (*M. alba*, *M. insgnis* and *M. macroura*) except Black mulberry. The phenolic compounds showed moderate anti-oxidant and anti-bacterial properties. The results add to the use of phenolic compounds presence in Mulberries to partially explain their reported pharmacological activities which include use as refreshing substances and as antibacterial.

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