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Isolation of Two New Mallotoates with Antifungal and Radical Scavenging Activities from *Mallotus phillipensis* muell

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Two new chalcone derivatives mallotoate A and mallotoate B were isolated from ethyl acetate fraction of *Mallotus philippensis* muell. Structure elucidation and the assignment of the isolates were achieved with the help of extensive 1D and 2D NMR studies. These compounds were identified using chemical and spectral data, as mallotoate A and mallotoate B, respectively. Both the compounds (mallotoate A and mallotoate B) showed significant fungicidal activity against *Cladosporium cladosporioides* in TLC bio-autography method. Using the same method, both mallotoate A and mallotoate B were tested for their antioxidant activities in DPPH radical scavenging activity in which, mallotoate B showed maximum and competitive activity (91.43 ± 0.82 %) against control drugs.

Keywords: ***Mallotus philippensis,*** **Mallotoate A, Mallotoate B, Antifungal activity, Antioxidant activity.**

INTRODUCTION

The present research work is based on the search for bioactive constituents of the plant *Mallotus philippensis* muell of the family Euphorbiaceae. *M. philippensis* muell is a woody plant, having wide geographical range, extending form E. Asia, Philippines, E. Malaysia and North-America to Northern Indo-Pak, China, Nepal and Japan. Its local name is Kamala. This plant exhibits antibacterial and antifungal activities. The bark of this plant shows substantial activity against Gram-positive and Gram-negative bacteria. The bark juice is taken for diarrhea and dysentery[1]. The fruit and roots are also a strong laxative. Rottlerine (chalcone), which is obtained from this species, is used to treat vomiting and is anthelmintic. Fruit is anthelmintic, vulnerary, detergent, maturant and carminative[2]. A compound isolated from the leaves of this plant strongly inhibited mouse skin tumor promotion[3]. The oil obtained from most parts of this plant is known as "Kamala oil" and was used as a constituent for tung oil[4]. In this paper, we report the isolation and structure elucidation of two new chalcone derivatives mallotoate A and B with antifungal activity against *Cladosperium cladosporiodes* and profound antioxidant activities in DPPH assay.

EXPERIMENTAL

Melting points were determined by Gallenkamp apparatus and are uncorrected. UV spectra were recorded on Waters 2998 diode array detector. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 500 MHz spectrometer in MeOD solutions. Tetramethylsilane was used as an internal standard. The samples were analyzed by LCMS using a Waters HPLC system (Waters 2545 Binary Gradient Module and Waters SFO System Fluidics Organizer). Chromatography (flow rate 1 mL min^{-1}) was achieved using Phenomenex LUNA column (5 µ, C_{18}, 100 Å, 4.6 × 250 mm). For preparation chromatography with flow rate 4 mL min^{-1} was achieved using Phenomenex LUNA column, 5 µ, C_{18}(2), 100 Å, 10.0 × 250 mm.

The plant *Mallotus philippensis* was collected from village Kuwari, District Mansehra, Khyber Pakhtunkhwa, Pakistan. Its botanical identification was done by Dr. Manzoor Ahmad, Chairman Department of Botany, Govt. College Abbottabad, Pakistan. Their botanical identities were determined and authenticated. Samples were deposited in the Botany Department, Govt. College Abbottabad, Pakistan. A voucher specimen has been deposited in the herbarium (Accession No. C-0027).

Extraction and isolation: The whole plant was shed dried for 2 months. The shed dried powdered material of whole plant body of *Mallotus philippensis* (2.5 Kg) was extracted three times in methanol. The resulting extract was evaporated with the help of rotary evaporator to obtain greenish gummy crude (81.6 g). This methanolic crude was then suspended in methanol-distilled water and successively partitioned with *n*-hexane (F1,

14.2 g), chloroform (F2, 14.8 g), ethyl acetate (F3, 18.8 g), *n*-butanol (F4, 14.1 g) and water soluble fraction (F5, 15.7 g). The ethyl acetate fraction (F3) was subjected to antifungal activity test on TLC bioautography method against *Cladosporium cladosperioides*. A portion of ethyl acetate fraction (10 g) was chromatographed over a column of silica gel (Merck Art 7734) with *n*-hexane-EtOAc to give 15 major fractions (Fr.1-Fr.15). Only two fractions Fr.3 and Fr.4 showed antifungal activity. Each fraction was further purified by a combination of chromatography over column of silica gel (eluent: with *n*-hexane-EtOAc) Sephadex LH20 (eluent: MeOH), RP-18 silica gel (eluant: CH_3CN-H_2O 15:25) and reversed phase HPLC (solvent system 20-15 % H_2O-MeOH, with flow rate 4 mL min^{-1} was achieved using Phenomenex LUNA column, 5 μ, C_{18}(2),100 Å, 10.0 × 250 mm) to give mallotoate A (47mg) from fr.3 and mallotoate B (65 mg) from Fr.4

Mallotoate A (1): Colourless amorphous solid. $[\alpha]^{22}_D$ ± 0° (c = 0.5, MeOH), IR (KBr, ν_{max}, cm^{-1}): 3451-3310 (OH) and 1610-1500 (aromatic). UV (MeOH) λ_{max} nm (log ε): 380 (4.34), 259 (4.31) and 206 (4.63). ^1H NMR, ^{13}C NMR and HMBC correlation (Table-1). HREIMS: *m/z* = 518.2757 (calcd. 518.2700 for $C_{32}H_{38}O_6$ $[M]^+$).

Mallotoate B (2): Colourless amorphous solid. $[\alpha]^{22}_D$ ± 0° (c = 0.5, MeOH), IR (KBr, ν_{max}, cm^{-1}): 3451-3310 (OH) and 1610-1500 (aromatic). UV (MeOH) λ_{max} nm (log ε): 380 (4.34), 270 (4.39), 206 (4.63). ^1H NMR, ^{13}C NMR and HMBC correlation (Table-1). HREIMS: *m/z* = 450.5251(calcd. *m/z* 450.5200 for $C_{27}H_{30}O_6$ $[M]^+$).

Antifungal bioassay: The EtOAc extract, mallotoate A and mallotoate B were tested for antifungal activity by direct bioautography on aluminium-backed TLC sheets[5] against the wheat pathogenic fungus, *Cladosporium cladosporioides* was assessed on glass-backed TLC plates in agar overlay assay[6]. After elution, the chromatograms were thoroughly dried to remove any solvent residues before being sprayed with suspension of the fungus. Nystatine (Sigma) and Amphotericin B (Sigma) were used as controls.

Test against *Cladosporium cladosporioides* strains: Inoculated sabouraud maltose liquid medium with *Cladosporium cladosporioides* was sprayed on TLC. Clear inhibition zones were observed against a blue-reddish background after 48 h incubation at room temperature in humid atmosphere. Conidial suspension of *Cladosporium cladosporioides* was supplemented with a solution of thiazolium (0.25 % MTT) before being sprayed on the TLC. The activity of the extract and compound appeared as clear inhibition zones against reddish background 48 h post-incubation.

Radical scavenging assays with DPPH: The TLC-DPPH assay was performed as described in literature[7]. Briefly, after developing and drying, aluminium-backed TLC sheets were

TABLE-1
^1H AND ^{13}C NMR DATA OF MALLOTOATE A AND MALLOTOATE B ($CDCl_3$, δ IN ppm, *J* IN Hz) AND IMPORTANT HMBC CORRELATIONS

Position	Mallotoate A			Mallotoate B		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	-	127.2		-	127.3	
2	7.02	113.3		7.01	113.4	
3	-	144.2		-	144.3	
4	-	146.5			146.4	
5	6.70 d (7.1)	114.5		6.71 d (7.1)	114.9	
6	7.0 brd	122.4		7.0 brd	122.8	
7	3.85 s	56.1		3.85 s	56.1	
8	3.85 s	56.1		3.85 s	56.1	
α	7.90 d (15.6)	124.3		7.91 d (15.6)	124.9	
β	7.60 d (15.6)	142.0		7.62 d (15.6)	142.2	
C=O	-	192.0		-	192.8	
2'	-	81.4	C(1''), C (2'')	-	81.4	
3'	5.40 d (9.8)	122.5		5.41 d (9.8)	122.5	
4'	6.60 d (9.8)	116.2		6.62 d (9.8)	116.2	
4a'	-	103.0		-	103.7	
5'	-	154.5	C(4'), C(1')	-	154.6	C(4'), C(1')
6'	-	105.2		-	105.2	
7'	-	164.6	C(7'), C(6'), C(8')	-	164.9	C(7'),(6'),C(8')
8'	-	106.3		-	105.0	
8a'	-	154.0		-	154.0	
1''	1.69-1.99 m	42.4		3.50 d (7.1)	21.7	
2''	2.02-2.29m	22.2		5.32 t (7.1)	122.2	
3''	5.19 t (6.6)	125.0		-	137.7	
4''	-	133.9		1.82 s	25.2	C(3''), C(2'')
5''	1.39 s	26.6	C(4''), C(3'')	1.85 s	16.3	C(3''), C2'')
6''	1.50 s	18.7	C(4''), C(3'')			
1'''	3.48 d (7.1)	21.7				
2'''	5.30 t (7.1)	122.9				
3'''	-	137.7				
4'''	1.80 s	25.8				
5'''	1.85 s	16.8				
5' –OH	6.53 s			6.53 s		
7' –OH	6.53 s			6.53 s		

sprayed with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution (1 mg/mL in MeOH). Active compounds appeared as yellow spots against purple background and were selected for quantitative estimation of radical scavenging activity (RSA) according to the standard method[8]. 2.5 mL of 0.04 % DPPH radical solution (MeOH) was added to each sample solution (100 μL) ranging from 192.5 to 6 μg/mL. The mixtures were vortex-mixed and kept under darkroom conditions for 0.5 h. The optical density (OD) was measured at 517 nm. Methanol was used as baseline control. Ascorbic acid and Gallic acid were used as positive controls. The antioxidant activity was carried out in triplicate and the reading was averaged. The scavenging activity was measured as a decrease in absorbance of the samples *versus* DPPH standard solution. The DPPH radical concentration was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \frac{(A_o - A_s)}{A_o} \times 100$$

where A_o is the absorbance of the control reaction and A_s is the absorbance of the samples.

Statistical analysis: The data were presented as mean ± standard deviation (SD) for the three determinations. The data of DPPH assays were analyzed for statistical significance using analysis of variance (one-way ANOVA) with SPSS 12 software.

RESULTS AND DISCUSSION

Structure elucidation: The EtOAc-soluble sub-fraction of the methanolic extract of *M. philippensis* was subjected to a series of chromatographic techniques to obtained mallotoate A and mallotoate B.

Mallotoate A was obtained as a colourless amorphous solid, which gave a positive ferric chloride test for phenolic moiety. The HREIMS gave the molecular ion peak at *m/z* 518.2757, corresponding to molecular formula $C_{32}H_{38}O_6$ which was also confirmed by positive-mode of FABMS ion peak at *m/z* 519 $[M+H]^+$. The IR spectrum showed bands for hydroxyl (3451-3310 cm^{-1}) and aromatic (1610-1500 cm^{-1}) groups. The UV spectrum showed maxima at 380, 259 and 206 nm, respectively.

Support for chalcone structure for mallotoate A was provided by the signals observed in its ^1H NMR (Table-1). Two *trans*-olefinicprotons are readily identified at δ 7.62 and δ 7.90 (*J* 15.6 Hz) and attributed to the β and α-positions, respectively. The aromatic protons show the ABX pattern typical of a 3,4-substituted A ring. The signals for the isoprenyl group two vinyl methyl signals, two benzylic proton signals, (δ, 3.48), a triplet proton are also clear. Furthermore, a 4-methyl-pent-3-enyl side chain is characterized by two methyl resonance, one methine proton and two methylene protons. Beside that two methoxyl group were observed as singlets at δ 3.85 (6H).

The ^{13}C NMR spectra of mallotoate A showed 32 signals comprising seven methyl, three methylene, nine methine and thirteen quaternary carbon atoms, in line with the proposed structure. The oxygenated aromatic carbon observed at δ 164.6, 154.5, 146.5 and 144.2, respectively. The benzylic carbon resonates at δ 21.7 and two methine carbons appeared at δ 125.0 and 122.9, respectively. The olefinic β and α-positions carbon resonated at δ 142 and 124.3 and methoxy carbon gave a signal at δ 56.1. Four methyl groups carbon appeared at δ 26.6, 25.8, 16.8 and 18.7. The important carbonyl carbon gave a single at δ 192.0.

The arrangement of the substituents and the placement of the pyran at the 4a/8a positions were established from the results of the hetero nuclear multiple bond connectivity (HMBC) experiments (Fig. 1). The chelated hydroxyl group in ring B showed the expected ^2J correlation between its proton and C-7' (164.6). The HMBC experiment showed a clear ^3J correlation between H-4' and C-5' (154.6) and between H-3' and C-4a' (103.7); while the chelated hydroxyl proton showed the expected ^3J correlation with C-6' (105.2) and C-8' (106.3). The isoprenoid chain showed ^2J and ^3J correlation between H-1", H-2" and C-2'(84.4).From these spectral data, the structure of **1** was determined to be (E)-1-(5,7-dihydroxy-2-methyl-6-(3-methyl-but-2-en-1-yl)-2-(4-Methyl-pent-3-en-1-yl)-2H-chromen-8-yl)-3-(3,4-dimethoxyphenyl)-prop-2-en-1-one and named mallotoate A (**1**).

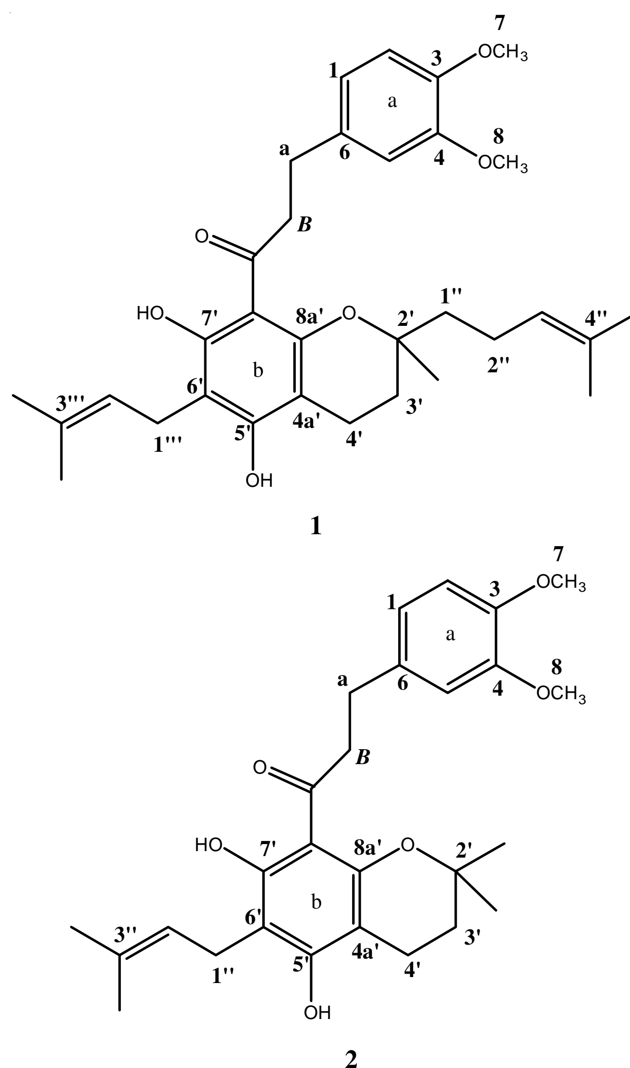

Fig. 1. Structures of and mallotoate A (**1**) and mallotoate B (**2**)

The ^1H NMR and ^{13}C NMR spectrum of mallotoate B was very similar to that of mallotoate A (Table-1). Compound mallotoate B were also isolated as colourless amorphous solid

had the molecular formula $C_{27}H_{30}O_6$ as found from its HR-EIMS (*m/z* 450.5251). The IR spectrum shows hydroxy group (3451-3310 cm^{-1}) absorption; showing twelve degree of unsaturation. The UV spectrum showed maxima at 380, 270, 206 nm. The ^1H NMR and ^{13}C NMR of spectrum of mallotoate B were also demonstrated a saturated chromeno chalcone. The presence of two *cis*-coupled olefinic protons, together with two methyl group resonances and a quaternary carbon at δ 81.4 in the ^{13}C NMR spectrum (Table-1), indicated a 2,2-dimethyl-pyran system. HMBC correlation is presented in Fig. 2. From these spectral data, the structure of mallotoate B was determined to be (E)-1-(5,7-dihydroxy-2,2-dimethyl-6-(3-methylbut-2-en-1-yl)-2H-chromen-8-yl)-3-(3,4-dimethoxy-phenyl)prop-2-en-1-one and named mallotoate B (**2**).

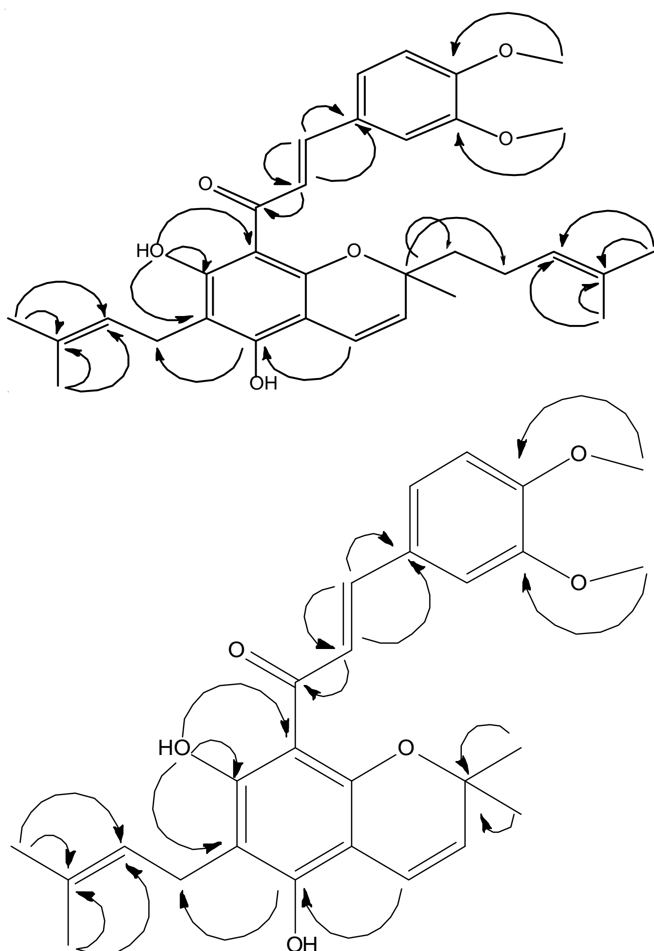

Fig. 2. Important HMBC correlation of mallotoate A and mallotoate B

Both compounds showed promising fungicidal activity at **1** (5 μg/spot) and **2** (2 μg/spot) against *Cladosporium cladosporioides* on TLC bio-autography method, wherein spore germinates as black zones and antifungal compounds appear as white zones[5]. Benlate was used as positive control.

Antioxidant properties of mallotoate A and mallotoate B were evaluated against the DPPH radical by TLC bio-autography method[7]. In this assay antioxidants react with the free radical DPPH and produce colourless 2,20-phenyl-1-picryl-hydrazine. α-Tocopherol was used as the positive control. Both mallotoate A and mallotoate B exhibited off-white spots in purple back-ground at the level of 1 μg/spot. In quantitative DPPH test, the IC_{50} values for mallotoate A and mallotoate B were obtained as 4.71 and 3.54 μg/mL, respectively. mallotoate B showed the highest radical scavenging activity (RSA) as 91.43 ± 0.82 % DPPH inhibition against the control drugs, gallic acid (IC_{50} = 3.09 μg/mL, 97.35 % ± 0.82) and ascorbic acid IC_{50} =3.14 μg/mL, 96.32 ± 0.57 respectively (Table-2).

TABLE-2
RADICAL SCAVENGING ACTIVITY AND IC_{50} VALUES OF MALLOTOATE A AND MALLOTOATE B FROM *M. philippensis*

S. No.	Test sample[a]	IC_{50} (μg/mL)	% DPPH inhibition ± SEM[c]
1	Mallotoate A	4.71	83.64 ± 1.51
2	Mallotoate B	3.42	91.43 ± 0.82
3	Gallic acid[b]	3.09	97.35 ± 0.27
4	Ascorbic acid[b]	3.14	96.32 ± 0.57

[a]Test samples were TLC DPPH scavengers at amount of 1 μg/spot.
[b]Positive control used in assays.
[c]Standard error of mean of three assays.

Conclusion

Both the new chalcone derivatives and mallotoate B were isolated from the ethyl acetate solvent fraction of *M. philippensis*, which showed fungicidal activity against *Cladosporium cladosporioides* and profound antioxidative activities in DPPH assay by TLC bio-autography method. They may be used against different human infirmities. This plant is used as one of the ingredients of conventional medicines in different parts of the world. More investigations are recommended to discover the potential medicinal value of this plant.

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