Isolation of Bioactive Compounds from *Aspergillus terreus*

Muhammad I. Choudhary, Syed G. Musharraf, Talat Mukhmoor, Farzana Shaheen, Shamsher Ali, and Atta-ur-Rahman

H. E. J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi-75270, Pakistan

Reprint requests to Prof. Dr. M. I. Choudhary. Fax: (92-21) 9243190-9243191. E-mail: hej@cyber.net.pk

Z. Naturforsch. **59b,** 324 – 328 (2004); received October 1, 2003

A new metabolite, 6-(4'-hydroxy-2'-methyl phenoxy)-(–)-(3*R*)-mellein (**1**) was isolated from the ethyl acetate extract of *Aspergillus terreus* culture medium along with three known isocoumarin derivatives, (–)-(3*R*)-6-methoxymellein (**2**), (–)-(3*R*)-6,7-dimethoxymellein (Kigelin) (**3**) and (3*R*, 4*R*)-6,7-dimethoxy-4-hydroxymellein (**4**). Metabolites **1** and **4** showed significant activity against human pathogenic dermatophytes, *Microsporum canis* and *Trichophyton longifusus*. Metabolite **1** also exhibited potent antioxidant activity. The structures of metabolites were characterized on the basis of spectroscopic techniques. ^{13}C NMR data of metabolites **2 – 4** are also being reported for the first time.

Key words: Aspergillus terreus, 6-(4'-Hydroxy-2'-methyl phenoxy)-(–)-(3*R*)-mellein, Antifungal Activity, Antioxidant

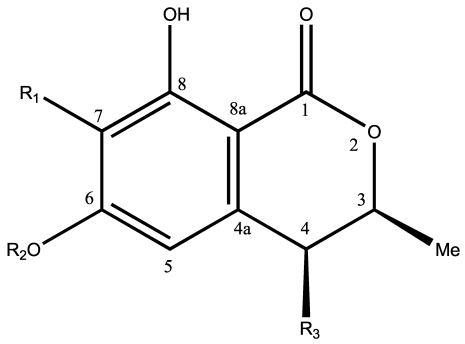
Introduction

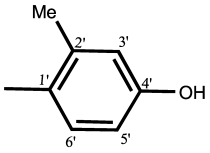
In the past few decades, systematic work on microbial metabolites has led to the isolation of thousands of natural products, most of them were found to have antibiotic and antitumor properties. This clearly indicates that the microorganisms are indeed a rich source of chemically unique and biologically potent substances [1, 2]. In continuation of our work on natural substances of terrestrial and marine origins [3, 4], we examined the chemistry of *Aspergillus terreus*, isolated from marine sediments collected from the Mubarak village beach, near Karachi city, which led to the isolation of a new bioactive metabolite **1** along with three known isocoumarin derivatives, (–)-(3*R*)-6-methoxymellein (**2**), (–)-(3*R*)-6,7-dimethoxymellein (Kigelin) (**3**) and (3*R*, 4*R*)-6,7-dimethoxy-4-hydroxymellein (**4**). Metabolite **2** was initially isolated from carrots [5] which had developed bitter taste during storage, subsequently this was isolated from different fungal cultures [6, 7]. Metabolite **3** and **4** were earlier isolated from *Kigelia pinnata* [8] and *Aspergillus terreus* [9], respectively. However metabolites **2** and **3** have been isolated for the first time from this species of *Aspergillus*. Metabolite **1** has exhibited good level of antioxidant activity in DPPH radical scavenging and xanthine oxidase inhibitory assays.

Antioxidants are the compounds which can protect the biological system from damaging oxidative reactions through scavenging reactive oxygen and nitrogen species within the body or by inhibiting the oxidative enzymes, hence hindering the process of oxidation [10, 11].

Results and Discussion

The ethyl acetate extract of *Aspergillus terreus* was subjected to repeated column chromatography and preparative thin layer chromatography on silica gel to afford a new isocoumarin **1** and known isocoumarin derivatives **2 – 4**. The isocoumarins isolated can be regarded as closely related derivatives of 6-hydroxymellein differing only in the substitution pattern and showing characteristic similarities in the IR, UV and NMR spectra [12]. The ^{1}H NMR spectrum of compound **1** was very similar to the 6-hydroxymellein with additional aromatic proton signals at δ 6.52 (d, $J_{\text{meta}} = 2.6$ Hz), 6.42 (dd, $J_{\text{ortho}} = 8.5$ Hz, $J_{\text{meta}} = 2.8$ Hz) and 6.55 (br d, $J_{\text{ortho}} = 8.5$ Hz) which were assigned to H-3', H-5' and H-6', respectively, indicating the presence of a tri-substituted benzene ring as a substituent. An additional 3H singlet at δ 2.11 was assigned to C-2' methyl. The presence of two phenolic OH were inferred from the appearance of downfield signals in ^{1}H NMR spectrum (pyridine-d_5) at





1)- $R_1 = R_3 = H$, $R_2 =$

2)- $R_1 = R_3 = H$, $R_2 = Me$

3)- $R_1 = OMe$, $R_2 = Me$, $R_3 = H$

4)- $R_1 = OMe$, $R_2 = Me$, $R_3 = OH$

δ 10.15 (C-4'), while the chelated phenolic hydroxyl proton appeared at δ 11.5 (C-8). Presence of phenolic groups was further indicated by the appearance of purple color with $FeCl_3$ spray. The trisubstituted benzene substituent was ether linked with mellein, which was further supported by HREI MS which showed the cleavage of ether resulting in two major fragments, *m/z* 194.0545 ($C_{10}H_{10}O_4$, 194.0579) [M^+-C_7H_6O] as a base peak, and at *m/z* 124.0484 ($C_7H_8O_2$, 124.0524) [M^+- $C_{10}H_8O_3$]. A characteristic fragment of 3,4-dihydroisocumarin skeleton appeared at *m/z* 150.0318 ($C_9H_{10}O_2$, 150.0317), due to ring contraction *via* loss of a CO_2 unit from the base peak [13].

Using the ^{13}C NMR chemical shift values of 6-hydroxymellein as a reference [12], it was deduced that the compound **1** has an additional aromatic ring ether linked with the mellein at C-6. In the HMQC spectrum the protons which resonated at δ 6.52 (H-3'), 6.42 (H-5'), 6.55 (H-6'), 6.18 (H-5/H-7), 2.84 and 2.76 (2H-4), 4.61 (H-3), 1.41 (Me-3) and 2.11 (Me-2') were found to be coupled with the carbon atoms resonated at δ 118.4 (C-3'), 113.9 (C-5'), 116.4 (C-6'), 107.8 (C-5), 102.2 (C-7), 35.5 (C-4), 77.2 (C-3), 20.8 (Me-3) and 16.4 (Me-2'), respectively. The COSY-45° spectrum of compound **1** exhibited interactions between H-3'/H-5', CH_3-2' and H-5'/H-3', H-6' further supported the presence at a CH_3 at C-2'. Furthermore it showed the presence of a spin system comprising on three sets of protons H-4, H-3 and 3-CH_3. Similarly C-4 protons showed homoallylic coupling with H-5 (δ 6.18). In the HMBC spectrum of **1**, long-range couplings of H-3' with C-4' (δ 151.0), C-1' (δ 140.3) and CH_3-2' (δ 16.4) were observed. Similarly Me-2' (δ 2.11) showed couplings with C-3' (δ 118.4) and C-1', suggesting that the C-2' methyl and C-4' phenolic substituents were present on aromatic ring B.

Table 1. *In vitro* free radical and XO inhibitory activities of compounds **1 – 4.**.

	$IC_{50}(\mu M)^*$	
Compounds	DPPH Scavenging assay	XO Inhibition assay
1	159 ± 0.098	243 ± 0.544
4	–	707 ± 0.470
PG**	30 ± 0.27	628 ± 5.0
BHA**	44 ± 2.00	591 ± 8.0

* IC_{50} values are the mean ± standard mean (SEM) error of three assays; ** standard compounds; compounds **2** and **3** were not active.

Homo-decoupling of C-9 methyl signal resulted in the simplification of C-3 methine proton splitting pattern (dd, $J_{3a,4a} = 11.1$ Hz, $J_{3a,4e} = 3.1$ Hz) which was found to be coupled with C-4-H_a (δ 2.76, $J_{4a,e} = 16.2$ Hz, $J_{4a,3a} = 10.7$ Hz) and C-4-H_e (δ 2.84, $J_{4e,a} = 16.3$ Hz, $J_{4e,3a} = 3.3$ Hz). Similarly the sign of optical rotation of metabolite **1** was also found to be same as reported for (–)-3*R*-mellein [14]. The structure of compound **1** was concluded to be 6-(4'-hydroxy-2'-methyl phenoxy)-(–)-(3*R*)-mellein (**1**). In addition to the new metabolite **1**, the structures of known metabolites **2 – 4** were identified through comparison of their spectroscopic data with the literature values [6 – 9].

The ethyl acetate extract of *Aspergillus terreus* showed good antifungal activity, while compounds **1**, **3** and **4** showed significant activity against human pathogenic fungi (dermatophytes), *Microsporum canis* and *Trichophyton longifusus* (Table 1). In addition to antifungal activity, metabolite **1** showed radical scavenging potential against DPPH radicals with IC_{50} value of 159 μM. The same compound also inhibited xanthine oxidase (XO) with IC_{50} value of 243 μM was found to be a better inhibitor of XO than 3-*t*-butyl-4-hydroxyanisole (BHA) and propyl gallate (PG) (Table 2).

Experimental Section

General

The melting points were measured on a Buchi 535 melting point apparatus. Optical rotations were measured in MeOH on a Jasco DIP-360 digital polarimeter. IR spectra were

Name of fungus	% Inhibition Ethyl acetate extract	Compound **1**	Compound **3**	Compound **4**	Conc. of standard drugs (μg/ml)
T. longifusus	33	55	45	70	Mic (70)
A. flavus	45	0	20	0	Amp (20)
M. canis	65	70	50	50	Mic (98.4)
F. solani	35	30	0	20	Mic (73.2)
C. glaberata	21	0	0	0	Mic (110.8)
C. albicans	14	0	0	0	Mic (110.8)

Table 4. antifungal activity of crude and pure compounds **1**, **3** and **4**.

Mic. = miconazole, Amp. = amphotericin B, % inhibition of std. Drug = 100; concentration of crude = 400 μg/ml; concentration of sample = 200 μg/ml.

recorded in $CHCl_3$ on a FT IR-8900 spectrophotometer. UV Spectra were recorded in MeOH on a Hitachi U-3200 spectrophotometer. The ^{1}H NMR were recorded in CD_3OD and pyridine-d_5 on Bruker Avance-500 NMR at 500 MHz, while ^{13}C NMR spectra were recorded in CD_3OD on the same instrument at 125 MHz. Chemical shifts δ in ppm. rel. to $SiMe_4$ as internal standard, coupling constants J in Hz. The EI and HREI MS were measured on Jeol JMS-600H mass spectrometer. Column chromatography was performed on silica gel (E. Merck, 70 – 230 mesh). TLC separation were performed on precoated plates (Silica gel 60, PF_{254}, 0.2 mm, E. Merck).

Collection and identification

The *Aspergillus terrus* was separated using serial dilution methods from marine sediment collected from the Mubarak village beach, near Karachi city during mid April 2001, and identified by Dr. Saleem Shahzad (Mycologist), Department of Botany, University of Karachi. A subculture has been deposited at the Mycological Collection of Botany Department, University of Karachi and was assigned the accession number BDKU 1164.

Culturing of Aspergillus terrus

The growth medium for *Aspergillus terrus* (BDKU 1164) was prepared by mixing 2 kg of glucose and fresh potato extract (2 l) in 38 l distilled water. The fresh potato extract was prepared by cutting 2 kg potatoes into small cubes and boiled with 2 l of distilled water, for 30 min. The medium thus obtained was distributed among 50 flasks of 1 l capacity (800 ml in each) and autoclaved at 121 °C for 30 min. Sample of five days fresh mycelium of the fungus grown on potato dextrose agar (PDA) medium with 1.5% in a petri dish at 28 °C were inoculated into 1 l flask containing medium (800 ml), for 2 days to get seed broth which was inoculated aseptically into a 49 media flasks and placed on shaker at 200 rpm (29 °C) for 30 days.

Extraction and purification

The culture media and mycelium were separated by filtration. The mycelium was washed with EtOAc (6 l) and the filtrate was extracted with EtOAc (3×10 l). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure to afford a brown oily gummy residue (16.7 gm). The EtOAc extract was subjected to column chromatography (CC) on silica gel (70 – 230-mesh size) to yield a new isocoumarin derivative **1**, along with three known compounds **2 – 4**. The column was initially eluted with gradient polarities of n-hexane and EtOAc, which afforded three fractions (F-1 to F-3). F-1 (20% EtOAc: hexane, 2.1 g), when subjected to column chromatography, afforded compound **2** (15.4 mg) (12% EtOAc: hexane). Fraction F-2 (40% EtOAc: hexane, 1.32 g) after column chromatography afforded compounds **3** (100.2 mg) (22% EtOAc: hexane) and **1** (19.3 mg) (31% EtOAc: hexane). Fraction F-3 (60% EtOAc: hexane, 4.2 g) on column chromatography afforded compound **4** (17.5 mg) (57% EtOAc: hexane).

Biological Activity

DPPH radical scavenging assay

Different concentrations ranging from 1,000 to 10 μM of compounds in DMSO were allowed to react with DPPH radicals at 37 °C for 30 min, while the concentration of DPPH was 300 μM in EtOH. The ratio of sample solution, to DPPH was 5:95. The reaction was carried out in 96-well microtitre plate reader (Spectramax 340, Molecular Devices, USA). Finally the absorbance was measured at 515 nm and percent radical scavenging activity of samples was determined with comparison of DMSO treating control group. The IC_{50} values were calculated by using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst, U.S.A.*). 3-*t*-Butyl-4-hydroxyanisole (BHA) and propyl gallate (PG) were used as positive control [15, 16].

Xanthine oxidase inhibition activity

The xanthine oxidase (XO) inhibition activity was assayed in phosphate buffer (0.1 M, pH 7.5) 250 μl, XO (0.003 unit/well) 20 μl and test sample in 10 μl DMSO was diluted to the desired range of concentrations, were mixed in 96-well microplate and pre-incubated for 10 min. at r. t. The reaction was initiated

by adding 20 μl of 0.1 mM of xanthine as substrate. The uric acid formation was measured spectrophotometrically at 295 nm by using microtiter plate reader.

Antifungal activity

Ethyl acetate extract of *Aspergillus terreus* culture and pure compounds **1, 3** and **4** were tested for antifungal activity against six pathogenic fungi, including three human pathogens (*Trichophyton longifusus*, *Microsporum canis* and *Candida albicans*), a plant pathogen (*Fusarium solani*) and two animal pathogens (*Candida glabrata* and *Aspergillus flavus*) by agar tube dilution methods [17, 18]. Amphotericin B and miconazole were used as standard drugs. Stock solutions of crude sample (24 mg) and pure compounds (12 mg) were prepared in sterile DMSO (1 ml). Test compounds (66.6 μl) were added from the stock soln. to non-solidified Sabouraud agar media (50). The final concentrations of crude and pure compounds were 400 μg/ml and 200 μg/ml, respectively. Tubes were allowed to solidify at room temperature in slanting position and inoculated with 4 mm diameter of inocula derived from seven-days-old fungal cultures. For nonmycelial fungi, an agar surface streak was employed. These tubes were incubated at 27 – 29 °C for 7 – 10 days, while for mycelial fungi tubes were incubated for 3 days and the growth in the compound-containing media was measured by the linear growth (mm) and growth inhibition were calculated with reference to the respective control.

6-(4'-Hydroxy-2'-methyl phenoxy)-(–)-(3R)-mellein (**1**): Light brown crystalline solid (19.3 mg). – M.P. 186 – 187 °C. – $[\alpha]_D^{25}$ −18.2° (c = 0.2, MeOH). – UV/vis (MeOH): λ_{max} nm (lg ε) = 300 (4.2), 268 (4.4), 216 (4.6). – IR ($CHCl_3$): ν_{max} = 3206, 3077, 1631, 1587, 1476, 1383, 1255 cm^{-1}. – ^{1}H NMR (CD_3OD, 500 MHz): (Table 3). – ^{13}C NMR (CD_3OD, 125 MHz): (Table 3). – MS (EI, 70 eV): *m/z* (%) = 300 (4) [M^+], 272 (14), 256 (31), 244 (11), 194 (100), 166 (8), 150 (81), 138 (21), 124 (92), 119 (17), 93 (28), 65 (34). – MS (HREI): *m/z* = 300.1749 ($C_{19}H_{24}O_3$, calcd. 300.1725).

(–)-(3R)-6-Methoxymellein (**2**): Colorless crystalline solid (8.4 mg). – M.P. 72 – 73 °C (lit.,[8] 75 – 76 °C). – $[\alpha]_D^{25}$ −46.5 (c = 0.14, MeOH). – UV/vis (MeOH): λ_{max} nm (lg ε) = 300 (3.7), 267 (4.1), 222 (4.0). – IR ($CHCl_3$): ν_{max} = 2979, 2936, 1666, 1628, 1583, 1371, 1251, 1160, 1181 cm^{-1}. – ^{1}H NMR (CD_3OD, 500 MHz): δ = 6.31 (2H, s, H-5/H-7), 4.65 (IH, m, H-3α), 2.93 (1H, dd, J = 16.3 Hz, J = 3.3 Hz, H_a-4), 2.82 (1H, dd, J = 16.3 Hz, J = 4.2 Hz, H_b-4), 1.46 (3H, d, J = 6.3, 3-Meβ), 3.81 (3H, s, 6-OMe). – ^{13}C NMR (CD_3OD, 125 MHz): (Table 4). – MS (EI, 70 eV): *m/z* (%) = 208(100) $[M]^+$ 190 (52), 179 (46), 165 (57), 164 (96), 147 (29), 119 (36), 93 (27), 69 (46), 65 (37). – MS (HREI): *m/z* = 208.2763 ($C_{11}H_{12}O_4$, calcd. 208.2741).

(–)-(3R)-6,7-Dimethoxymellein (Kigelin) (**3**): Colorless crystalline solid (100.2 mg). – M.P. 143 – 144 °C (lit.,[8] 144 °C). – $[\alpha]_D^{25}$ −7.5 (c = 0.1, MeOH). – UV/vis (MeOH): λ_{max} nm (lg ε) = 389 (2.9), 273 (3.7), 220 (3.9). – IR ($CHCl_3$): ν_{max} = 2924, 2852, 1663, 1426,

Table 3. NMR data of compound **1**.

Position	δ_C	δ_H (J in Hz)	HMBC (H→C)	^{1}H-COSY
1	171.7, s	–	–	–
3	77.2, d	4.61, m	4, 3-Me	H_a-4, H_b-4, βMe-3
4	35.5, t	2.84, dd, (16.3, 3.3)	3, 5, 4a, 8a	H-3, H-5
		2.76, dd, (16.2, 10.7)	4a	H-3, H-5
4a	143.4, s	–	–	–
5	107.8, d	6.18, br s	6, 7, 8a	H_a-4, H_b-4
6	166.1, s	–	–	–
7	102.2, d	6.18, br s	5, 6, 8, 8a	–
8	165.5, s	–	–	–
8a	101.4, s	–	–	–
3-Me	20.8, q	1.41, d, (6.3)	3, 4	H-3
1'	149.3, s	–	–	–
2'	126.5, s	–	–	–
3'	118.4, d	6.52, d, (2.6)	1', 4', 2'-Me	H-5', Me-2'
4'	151.0, s	–	–	–
5'	113.9, d	6.42, dd, (8.5, 2.8)	1', 4'	H-3', H-5'
6'	116.4, d	6.55, br d, (8.5)	2'	H-5'
2'-Me	16.4, q	2.11, s	1', 3'	H-1', H-3'

Table 4. ^{13}C NMR data of compounds **2 – 4**.

Position	**2**	**3**	**4**
1	171.5, s	169.8, s	170.9, s
3	77.3, d	75.7, d	79.8, d
4	35.5, t	34.7, t	67.7, d
4a	143.2, s	135.4[a], s	139.3, s
5	106.9, d	102.0, d	104.3, d
6	167.5, s	158.4, s	160.2, s
7	100.4, s	135.3[a], s	134.2, s
8	165.6, s	156.2, s	156.7, s
8a	102.4, s	102.8, s	102.9, s
3-Me	20.8, q	20.7, q	16.3, q
6-OMe	56.1, q	56.1, q	56.8, q
7-OMe	–	60.7, q	60.9, q

[a] These values can be interchanged.

1269, 1121 cm^{-1}. – ^1H NMR (CD_3OD, 500 MHz): $\delta = 6.51$ (1H, s, H-5), 4.69 (IH, m, H-3α), 3.77 (3H, s, 6-OMe), 3.90 (3H, s, 7-OMe), 2.96 (1H, dd, $J =$ 16.2 Hz, $J = 3.4$ Hz, H_a-4), 2.87 (1H, dd, $J = 16.1$ Hz, $J = 11.1$ Hz, H_b-4), 1.46 (3H, d, $J = 6.3$, 3-Meβ). – ^{13}C NMR (CD_3OD, 125 MHz): (Table 4). – MS (EI, 70 eV): *m/z* (%) = 238 (100) $[M]^+$, 223 (25), 179 (46), 209 (16), 195 (34), 177 (40), 151 (37), 136 (15), 106 (19), 77 (27), 51 (62). – MS (HREI): *m/z* = 238.0884 ($C_{12}H_{14}O_5$, calcd. 238.0841).

(3R, 4R)-6,7-Dimethoxy-4-hydroxymellein (**4**): Colorless crystalline needles (17.5 mg). – M.P. 189–190 °C (lit.,[9] 190–192 °C). – $[\alpha]_D^{25}$ −4 ($c = 0.2$, MeOH). – UV/vis (MeOH): λ_{max} nm $(\lg \varepsilon) = 274$ (3.7), 219 (3.9). – IR ($CHCl_3$): $\nu_{max} = 3496$, 2929, 2851, 1661, 1368, 1277, 1106 cm^{-1}. – ^1H NMR (CD_3OD, 500 MHz): $\delta = 6.70$ (1H, s, H-5), 4.65 (IH, dd, $J = 2.1$ Hz, $J = 6.7$ Hz, H-3α), 4.50 (IH, d, $J = 1.9$ Hz, H-4α), 3.94 (3H, s, 6-OMe), 3.80 (3H, s, 7-OMe), 1.49 (3H, d, $J = 6.6$ Hz, 3-Meβ). – ^{13}C NMR (CD_3OD, 125 MHz): (Table 4). – MS (EI, 70 eV): *m/z* (%) = 254 (100) $[M]^+$, 221 (13), 207 (11), 182 (29), 167 (26), 136 (7), 111 (36), 93 (8), 55 (21). – MS (HREI): *m/z* = 254.6491 ($C_{12}H_{14}O_6$, calcd. 254.6477).

Acknowledgements

We are very thankful to Dr. Saleem Shahzad (Department of Botany, University of Karachi, Karachi-75270, Pakistan) for the identification of fungi. We are also thankful to Medics Laboratories, Karachi, Pakistan for providing financial assistance to S. G. M.

[1] W. Fenical, Chem. Rev. **93**, 1673 (1993).
[2] R. G. Kerr, S. S. Kerr, Exp. Opin. Ther. **9**, 1207 (1999).
[3] Atta-ur-Rahman, M. Sabir, A. M. Khan, S. Hayat, A. Nasreen, S. Malik, M. I. Choudhary, Nat. Prod. Lett. **13**, 255 (1999).
[4] Atta-ur-Rahman, M. I. Choudhary, S. Hayat, A. M. Khan, A. Ahmad, Chem. Pharm. Bull. **49**, 105 (2001).
[5] E. Sondheimer, J. Am. Chem. Soc. **79**, 5036 (1957).
[6] W. J. McGahren, L. A. Mitscher, J. Org. Chem. **33**, 1577 (1968).
[7] R. Aue, R. Mauli, H. P. Sigg, Experientia **22**, 575 (1966).
[8] T. R. Govindachari, S. J. Patankar, N. Viswanathan, Phytochemistry **10**, 1603 (1971).
[9] A. Kunizo, Y. Tadashi, I. Yooshitaka, Y. Yuzuru, Chem. Pharm. Bull. **31**, 925 (1983).
[10] S. R. J. Maxwell, Drugs **49**, 345 (1995).
[11] K. H. Cheeseman. Mol. Aspects Med. **14**, 191 (1993).
[12] J. K. Kendall, T. H. Fisher, H. P. Schultz, T. P. Schultz, J. Org. Chem. **54**, 4218 (1989).
[13] N. H. Rama, R. Iqbal, K. Zamani, Chem. Soc. Pak. **20**, 66 (1998).
[14] H. Arakawa, Bull. Chem. Soc. Jpn. **41**, 2541 (1968).
[15] R. C. Smith, J. C. Reeves, R. C. Dage, R. A. Schnettler, Biochem. Pharmacol. **36**, 1457 (1987).
[16] Y. Fujita, I. Uehara, Y. Morimoto, M. Nakashima, T. Hatano, Yakugaku Zasshi **108**, 129 (1998).
[17] H. Blank, G. Rewbell, Arch. Derm. **92**, 322 (1965).
[18] S. S. Shaukat, N. A. Khan, F. Ahmed, Pak. J. Bot. **12**, 97 (1980).