Biodirected Isolation from *Forsskalea tenacissima*

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Summary: The methanolic extract of *Forsskalea tenacissima* was subjected to vacuum liquid chromatography (VLC). The ethyl acetate fraction was found to be most promising against *Candida albicans, Trichophyton mentagrophytes, Allescheria boydii, Microsporum canis, Aspergillus niger, Drechslera rostrata, Nigrospora oryzae, Stachybotrys atra, Curvularia lunata, Trichophyton semii* and *Trichophyton schoenleinii.* Phytochemical studies resulted in the isolation of six known compounds, for the first time from this plant.

Introduction

Forsskalea tenacissima belongs to family Urticaceae [1]. There are 45 genera and 600 species in this family. Forsskalea is a small genus of six species distributed from Canary Isles and southeastward Spain to Pakistan and India [2]. The plant is found in dry and hilly areas. In Baluchistan province it is used to cure cough, headache and also fed to goats [3]. *Forsskalea angustifolia* is another member of this genus but not found in Pakistan, it has been reported as diuretic, calculolytic and antiflu [4]. The plants, of this family, are reported to be used in the treatment of prostatic hyperplasia [5]. Literature survey revealed that no phytochemical work has been done so far on this species.

Results and Discussion

The methanolic extract of *Forsskalea tenacissima* was subjected to antibacterial and antifungal activities. The crude extract showed good antifungal activity against *Trichophyton mentagrophytes, Microsporum canis, Aspergillus niger, Dreschslera rostrata, Nigrospora oryzae, Stachborrys atra, Curvularia lunata, Trichophyton semii* and *Trichophyton schoenleinii.* The crude extract was then subjected to vacuum liquid chromatography (VLC), which resulted into nine fractions (F1—F9). The fractions were tested for antifungal activity. The ethyl acetate fraction (F5) showed most promising activity and was further subjected to phytochemical screening for the isolation of bioactive secondary metabolites responsible for the antifungal activity. Six known compounds were isolated and their structures were elucidated by spectroscopic techniques.

The electron impact mass spectrum (EI-MS) of Friedelin (**1**) showed a molecular ion $[M]^+$ at *m/z* 426.1. The high resolution electron impact mass spectrum (HR-EI-MS) of this compound resulted in the exact value of *m/z* 426.38644 which corresponded to the molecular formula $C_{30}H_{50}O$ (calcd. 426.38614). The IR spectrum revealed the presence of carbonyl group (1715) cm^{-1}. The ^1H -NMR spectrum revealed the presence of seven singlets each for three protons at δ 0.72, 0.85, 0.87, 0.95, 1.00, 1.18 and 1.25. These values were assigned to tertiary protons at H-27, H-23, H-29, H-24, H-25, H-26 and H-28 respectively. The spectrum also exhibits a doublet of three protons at δ 0.85 with a coupling constant of 6.5 Hz. The signal was attributed to the secondary methyl proton at H-23. The single secondary methyl group indicate the compound belong to friedelin subclass of pentacyclic triterpene. ^{13}C-NMR displayed signals for all thirty carbons. The broad band and DEPT experiments of friedelin (**1**) showed the presence of eight methyl, eleven methylene, four methine and seven quaternary carbon atoms. The signal at δ 213.2 in broad band indicated the presence of carbonyl group at C-3 position. The methine signals at δ 59.5, 53.1, 42.8 and 58.2 were assigned to C-10, C-8, C-18 and C-4 respectively.

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Stigmast-4-ene-3-one (**2**) was isolated from the ethyl acetate (F_5) fraction. The mass spectrum showed molecular ion peak in HR-MS at *m/z* 412.3613 (calcd 412.3713 for $C_{29}H_{48}0$). The IR spectrum revealed an absorption band for ketonic group at 1680 cm^{-1}. The diagnostic fragments in the mass spectrum at *m/z* 397, 367, 272 were characteristic of sterol [6]. The ^{1}H-NMR spectrum exhibits the presence of six methyl at 0.69 (3H, s, H-18), 1.16 (1H, s, H-19), 0.90 (1H, d, J = 6.4 Hz, H-21), 0.82 (3H, d, J = 6.6 Hz, H-26), 0.79 (3H, d, J = 6.6 Hz, H-27) and 0.83 (3H, t, J = 6.6 Hz, H-29). The resonance of olefinic proton was observed at δ 5.72 (d, j= 1.41 Hz.) The broad band spectra of compound 2 revealed the presence of 29 carbon. While the DEPT experiments showed six methyl, eleven methylene, eight methine and four quaternary carbon atoms. On the basis of spectral data and the data reported in literature, the compound **2** was identified as stigmast-4-ene-3-one.

Other compounds such as β-amyrin (**3**), lupeol (**4**), β-sitosterol (**5**) and stigmasterol (**6**) were also isolated from the ethyl acetate fraction (F5) and their structures were confirmed on the basis of spectral data reported in the literature and TLC comparison with authentic compounds.

Experimental

General

Column chromatography was carried out using silica gel 70-230 mesh. Aluminium sheets precoated with silica gel 60 F_{254} (20 x 20 cm, 0.2 mm thick; E-Merck) were used for TLC to check the purity of compounds and were visualized under UV light (254 and 366 nm) followed by ceric sulphate and vanillin as spraying reagents. Optical rotation was measured on a Jasco DIP-360 digital polarimeter. The UV spectrum was recorded on a Hitachi UV-3200 spectrometer (λ_{max} in nm). IR spectra were recorded on Shimadzu RR-460 spectrophotometer (ν in cm^{-1}). EI-MS and HR-EI-MS were recorded on Jeol JMS-HX 110 spectrometer with data system. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Avance 500 MHz, 400 MHz and 300 MHz instruments, using TMS as internal standard. The chemical shift values were reported in ppm (*δ*) units and the scalar coupling constants (*J*) are in Hz.

Plant Material

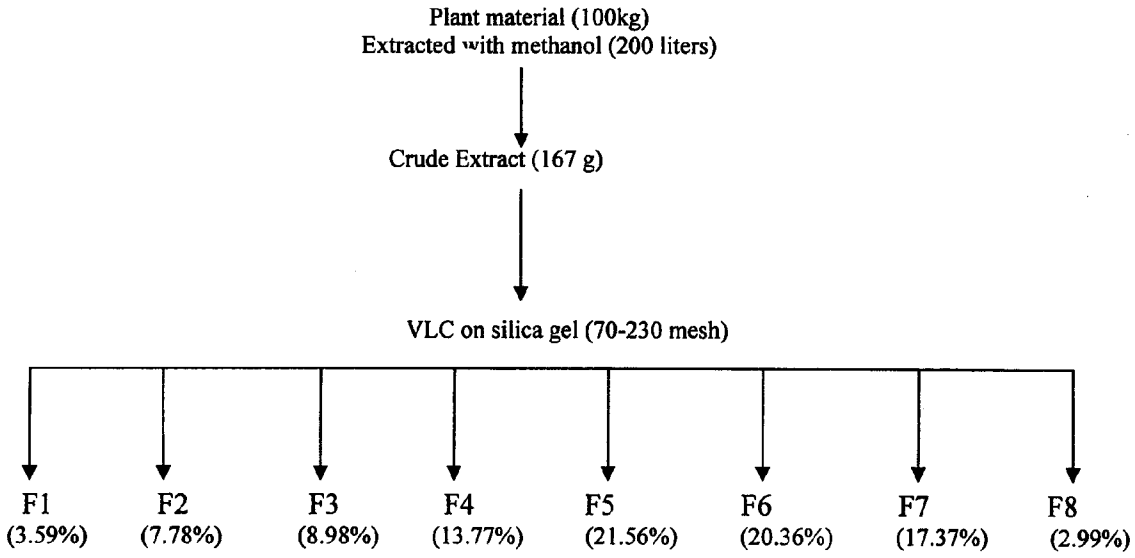
The whole plant of *Forsskalea tenacissima* was collected from Mohmand Agency (Tangi Area, of North West Frontier Province (NWFP) of Pakistan in the month of October. The plant was identified by the plant Taxonomist, Dr. Abdur Rasheed, Department of Botany, University of Peshawar, Pakistan. A plant (specimen # DB 1023) has been deposited in the Herbarium, Department of Botany, University of Peshawar, Pakistan.

Isolation

The whole plant material (100 Kg) was dried under shade for two weeks, chopped into small pieces and finally pulverized into fine powder. The powdered plant material was soaked in methanol (2 x 100 liters) for ten days. The extract was filtered and dried at low temperature under reduced pressure in rotary evaporator (167 g, 0.167 % w/w). The vacuum liquid chromatography (VLC) of the crude extract was performed on silica gel (3 Kg, 70-230 mesh) and eluted with n-hexane, n-hexane-ethyl acetate, and methanol, which resulted into nine fractions (F1-F9).

The ethyl acetate fraction (F_5 36 g) was subjected to column chromatography on silica gel (70-230) using n-hexane-ethyl acetate as mobile phase. The aliquot obtained from n-hexane-ethyl acetate (50:50) was purified by recrystallization in chloroform to afforded needle like crystals of friedelin (**1**). It gave single spot on TLC using ceric sulphate as locating reagent.

Further elution with increasing polarity of n-hexane-ethyl acetate (40:60) resulted into the isolation of mixture of Friedelin (**1**) and Stigmat-4-ene-3-one (**2**) separated by repeated column chromatography. Compound 2 gave a faint spot on TLC using ceric sulphate as locating reagent. Elution of the same column with ethyl acetate and then with ethyl acetate-methanol (96:4) resulted into the isolation of β-amyrin (**3**) and lupeol (**4**). Gradual increase in polarity of ethyl acetate–methanol (95:5) resulted into isolation of mixture of β-sitosterol (**5**) and stigmasterol (**6**). Resolution of the mixture of compounds was achieved by column chromatography using the same mobile phase.



ISOLATION SCHEME

Friedelin **(1)**

Compound **1** (25 mg), was obtained as colourless needle like crystals, m.p 245-247°C; IR ($CHCl_3$) ν_{max} 2943, 2867, 1680, 1455, 1389, 1361, 1192, 1139, 110 cm^{-1};

^{1}H-NMR ($CDCl_3$, 400 MHz): δ_H 0.72 (3H, s, CH_3-27), 0.85 (3H, d, CH_3-23). 0.87 (3H, s, CH_3-29), 0.95 (3H, s, CH_3-24), 1.0, 1.18, 1.25 (9H, s, CH_3-25, CH_3-26, CH_3-28), 2.25 (1H, q; J = 7Hz, H-4), 2.42 (1H, dd, J = 5Hz, H-2), 2.38 (1H, dd, J = 5.2 Hz, H-2) ^{13}C-NMR (125 MHz, $CDCl_3$) δ 22.20 (C-1), 41.30 (C-2), 213.20 (C-3), 58.20 (C-4), 42.10 (C-5), 41.50 (C-6), 18.20 (C-7), 53.10 (C-8), 37.40 (C-9), 59.50 (C-10), 35.60 (C-11), 39.86 (C-12), 42.62 (C-13), 56.11 (C-14), 24.40 (C-15), 33.16 (C-16), 56.25 (C-17), 12.16 (C-18), 17.60 (C-19), 36.33 (C-20), 18.91 (C-21), 34.12 (C-22), 26.35 (C-23), 46.08 (C-24), 28.40 (C-25), 20.01 (C-26), 19.25 (C-27), 23.30 (C-28), 12.18 (C-29);

EI-MS *m/z* (Int. rel. %). 426.1 $[M]^+$ (19), 302 (23), 273(34), 246 (26), 218 (25), 205 (33), 191 (21), 179 (37), 163 (28), 137 (39), 123 (73), 109 (85), 95 (96), 69 (100), 55 (34). The physical and spectral data showed complete agreement with the literature [7-10].

Stagmast-4-ene-3-one **(2)**

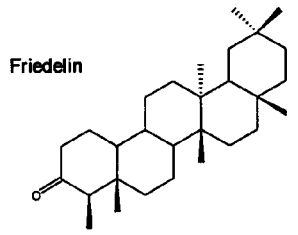
Compound **2** (15 mg), was isolated as white amorphous solid; IR ($CHCl_3$) ν_{max}. cm^{-1} 2938, 2365, 1680, 1455, 1373, 1240.

^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 5.70 (1H, d, J = 1.41 Hz, H-4), 0.69 (3H, s, H-18), 1.16 (1H, s, H-19), 0.90 (1H, d, J = 6.4 Hz, H-21), 0.82 (3H, d, J = 6.6 Hz, H-26), 0.79 (3H, d, J = 6.6 Hz, H-27), 0.83 (3H, t, J = 6.6 Hz, H-29). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 35.92 (C-1), 34.19 (C-2), 199.80 (C-3), 123.96 (C-4), 171.84 (C-5), 32.63 (C-6), 29.90 (C-7), 35.86 (C-8), 54.05 (C-9), 39.05 (C-10), 21.25 (C-11), 39.86 (C-12), 42.62 (C-13), 56.11 (C-14), 24.40 (C-15), 33.16 (C-16), 56.25 (C-17), 12.16 (C-18), 17.60 (C-19), 36.33 (C-20), 18.91 (C-21), 34.12 (C-22), 26.35 (C-23), 46.08 (C-24), 28.40 (C-25), 20.01 (C-26), 19.25 (C-27), 23.30 (C-28), 12.18 (C-29).

EI-MS showed $[M]^+$ *m/z* 412, 397 (M-Me), 367 (M-C_3H_7-2H), 272 (M-$C_{19}H_{28}O$). The physical and spectral data were in close agreement with the reported data [11].

β-Amyrin **(3)**

Compound **3** (35 mg), was isolated as white crystalline solid, m.p. 160 °C; IR ν_{max} (KBr) cm^{-1}: 3397, 2932, 1645, 1465, 1379, 1029, 900;



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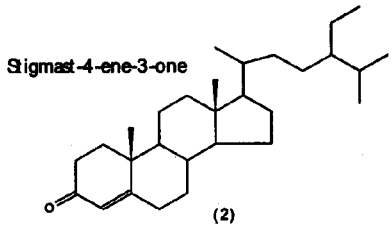

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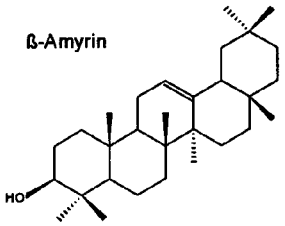

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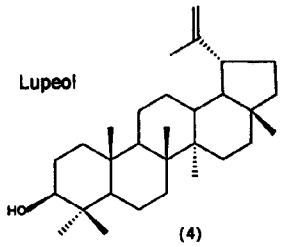

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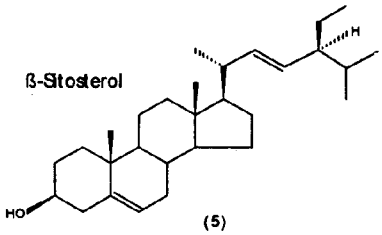

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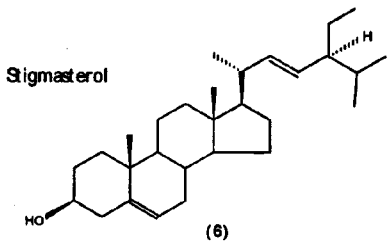

(6)

^{1}H-NMR (300 MHz, $CDCl_3$): δ_H 5.11 (1H, m, H-12), 3.19 (1H, dd, $J_{ax,ax}$=10.0 Hz, $J_{ax,eq}$ = 4.5 Hz, H-3), 1.08 (3H, s, Me-27), 1.02 (3H, s, Me-26), 1.01 (3H, s, Me-23), 0.96 (3H, s, Me-25), 0.91 (3H, d, J=6.6 Hz, Me-30), 0.81 (6H, s, Me-24, Me-28) and 0.80 (3H, d, J=6.8 Hz, Me-29). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 38.14 (C-1), 27.51 (C-2), 79.12 (C-3), 38.88 (C-4), 55.27 (C-5), 18.44 (C-6), 33.03 (C-7), 38.82 (C-8), 47.81 (C-9), 37.00 (C-10), 23.48 (C-11), 121.82 (C-12), 145.28(C-13), 42.18 (C-14), 26.12 (C-15), 27.37 (C-16), 32.04 (C-17), 47.22 (C-18), 46.93 (C-19), 31.34 (C-20), 34.83 (C-21), 37.26 (C-22), 28.22 (C-23), 15.47 (C-24), 15.72 (C-25), 16.97 (C-26), 25.56 (C-27), 28.44 (C-28), 33.44 (C-29), 23.48 (C-30); EI-MS: *m/z* 426 (M^+, 0.6 %), 411 (0.1 %), 218 (100%), 272 (0.1 %), 189 (30 %), 135 (34 %), 95 (48 %). The physical and spectral data of compound 3 matched the data reported in literature [12-15].

Lupeol (4)

Lupeol 4 (20 mg), was obtained as white crystals, m.p. 210-212°C; $[\alpha]_D$ + 30.4 (C, 0.58 in $CHCl_3$); IR ν_{max}: 3610, 3070, 3015, 1640, 1520, 1380, 1217, 1020, 887 cm^{-1};

^{1}H -NMR ($CDCl_3$ 300 MHz):: δ_H 0.75, 0.78, 0.81, 0.92, 0.94, 1.02 (Me-28, Me-23, Me-24, Me-25, Me-26, Me-27), 1.67 (3H, br d, J=0.5 Hz, Me-30), 3.18 (1H, dd, J=9.6, 6.2 Hz, H-3), 4.56 (1H, d, J=0.4 Hz, H-29), 4.67 (1H, dq, J=0.4, 0.5 Hz, H-29); ^{13}C-NMR: δ: 38.0 (C-1), 27.4 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 55.3 (C-5), 18.3 (C-5), 18.3 (C-6), 34.2 (C-7), 40.1 (C-8), 50.4 (C-9), 37.7 (C-10), 20.9 (C-11), 25.1 (C-12), 38.0 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 42.8 (C-17), 48.2 (C-17), 48.2 (C-18), 48.0 (C-19), 150.9 (C-20), 28.5 (C-21), 40.0

(C-22), 28.1 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.6 (C-27), 18.0 (C-28), 109.5 (C-29), 19.4 (C-30).

EI-MS *m/z* (rel. int. %): 426 $[M]^+$ (20), 411 $[M^+-CH_3]$ (3), 408 $[M^+-H_2O]$ (3), 218 (5), 207 (6), 189 (58), 163 (80), 135 (57), 107 (68), 105 (55), 79 (54), 41 (100). The physical and spectral data coincided with the reported data [16, 17].

β-sitosterol **(5)**

β-sitosterol **5** (700 mg) was obtained as white solid, m.p. 134.4°C; IR (CHCl3) ν_{max}: cm^{-1} 3450 (hydroxy group), 3050, 1650 and 815 (trisubstituted double bond).

^1H -NMR ($CDCl_3$ 300 MHz): δ_H 5.23 (1H, m, H-6), 3.32 (1H, m, H-3), 1.01 (3H, s, Me-19), 0.92 (3H, d, J = 6.2 Hz, Me-21), 0.84 (3H, t, J = 7.0 Hz, Me-29), 0.83 (3H, d, J = 6.5 Hz, Me-26), 0.81 (3H, d, J = 6.5 Hz, Me-27) and 0.68 (3H, s, Me-18)^{13}C NMR ($CDCl_3$, 75 MHz), δ 37.31 (C-1), 31.81 (C-2), 71.90 (C-3), 42.40 (C-4), 140.90 (C-5), 121.87 (C-6), 32.07 (C-7), 32.0 (C-8), 50.81 (C-9), 36.61 (C-10), 21.12 (C-11), 40.00 (C-12), 42.61 (C-13), 56.78 (C-14), 24.32 (C-15), 28.24 (C-16), 56.20 (C-17), 11.90 (C-18), 19.44 (C-19), 36.26 (C-20), 19.10 (C-21), 34.00 (C-22), 129.31 (C-23), 45.80 (C-24), 26.21 (C-25), 18.80 (C-26), 19.80 (C-27), 23.10 (C-28), 11.92 (C-29).

EI-MS (rel. int. %) 414 $[M]^+$ (30), 399 (10), 396 (15), 381 (75), 329 (26), 303 (20), 275 (14), 273 (35), 255 (35). The physical and spectral data matched the data reported in literature [18].

Stigmasterol **(6)**

Compound **6** (30 mg), was obtained as crystalline solid, m.p. 169.5°C; IR ($CHCl_3$) ν_{max}: cm^{-1} 3440 (hydroxy group), 3025, 1650, 1410, 1250, 690 and 820 (olefinic region).

^1H-NMR ($CDCl_3$ 300 MHz): δ_H 5.33 (1H, m, H-6), 5.15 (2H, m, H-22, H-23), 3.21 (1H, m, H-3), 0.90 (3H, d, J = 6.5 Hz, Me-21), 0.84 (3H, t, J = 7 Hz, Me-29), 0.83 (3H, d, J = 6.6 Hz, Me-26), 0.81 (3H, d, J = 6.5 Hz, Me-27, 0.80 (3H, s, Me-19) and 0.65 (3H, s, Me-18). ^{13}C-NMR ($CDCl_3$, 75 MHz), δ 37.42 (C-1), 31.81 (C-2), 71.92 (C-3), 42.40 (C-4), 140.92 (C-5), 121.78 (C-6), 31.91 (C-7), 31.89 (C-8), 50.37 (C-9), 36.61 (C-10), 21.01 (C-11), 39.78 (C-12), 42.40 (C-13), 36.61 (C-14), 24.41 (C-15), 28.90 (C-16), 56.00 (C-17), 12.43 (C-18), 19.40 (C-19), 40.50 (C-20), 21.12 (C-21), 138.40 (C-22), 129.48 (C-23), 45.80 (C-24), 26.21 (C-25), 18.80 (C-26), 19.80 (C-27), 23.10 (C-28), 11.92 (C-29).

EI-MS (rel. int. %): 412 $[C_{29}H_{48}O, M^+]$ (15), 397 $[M-Me]^+$(10), 394 $[M-H_2O]^+$ (38), 379 $[M-Me-H_2O]^+$ (20), 369 $[M-C_3H_7]^+$(36), 351 $[M-C_3H_7-H_2O]^+$ (65), 327 $[M-H_2O-C_6H_7]^+$ (70), 301 $[M-H_2O-C_7H_9]^+$ (20), 300 $[M-C_8H_{16}]^+$ (59), 273 $[M-H_2O-C_7H_{13}]^+$ (30) and [M-side chain at C-17 2H]$^+$ (26). The physical and spectral data were in close agreement with the reported data [19].

The crude extract was chromatographed on silica gel (mesh size 70-230) eluted with 5 liters of mobile phase as F1 (n-hexane 100 %), F2 (n-hexane-ethyl acetate 75:25), F3 (n-hexane-ethyl acetate 50:50), F4 (Hexane-Ethyl acetate 25:75), F5 (Ethyl acetate 100 %), F6 (ethyl acetate-methanol 75:25), F7 (ethyl acetate-methanol 50:50), F8 (ethyl acetate-methanol 25:75) and F9 (methanol 100 %).

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