PART-V
Experimental
PART-V: EXPERIMENTAL

General: Melting points were determined by the use of Kofler type electrical melting point apparatus and are uncorrected. All the analytical samples were tested for homogeneity on TLC plates in different solvent systems. TLC plates were prepared in glass plates using a slurry of Silica gel G (Merck, India) in EtOAc/ethanol and the spots of plates were visualized by either exposing the plates in iodine chamber or spraying with 10% H$_2$SO$_4$ in ethanol followed by heating at 110$^\circ$C. Silica gel (mesh 60-120, Merck, India), Diaion HP-20 (Mitsubishi Chemicals, Japan) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography (CC). CC and TLC were performed at room temperature (20-30$^\circ$C). The optical rotations were measured on a Jasco Dip-370 digital polarimeter. UV-vis spectra were recorded on Perkin Elmer Lambda 25 spectrophotometer and were expressed in $\lambda_{\text{max}}$ solvent nm (log$\varepsilon$). IR spectra in KBr disc were recorded on a Shimadzu 8100 FT-IR spectrophotometer and were expressed in $\nu_{\text{max}}$ cm$^{-1}$. $^1$H, $^{13}$C and 2D-NMR spectra were recorded on a Varian XL- 400 and 600 MHz NMR / Brucker Advance II 600 NMR spectrometer. Chemical shifts were expressed in $\delta$ (ppm) with tetramethylsilane (TMS) as an internal standard, and the coupling constants were expressed in hertz (Hz). EI-MS, HR-EI-MS were recorded on a Jeol JMS 700 mass spectrometer and FAB-MS and HR-FAB-MS were recorded on a Jeol JMS-HX 110 mass spectrometer. In MS the mass ion peaks were given in $m/z$ values with their relative abundances in % with respects to the base peak in a spectrum. NMR-DEPT experiments were carried out with flip an angle $\theta$ of 45$^0$, 90$^0$ and 135$^0$. GC/MS/MS analysis was carried out on a Hewlett-Packard (Palo Alto, CA) 5890 GC interfaced with a Finnigan MAT TSQ 700 triple-quadrupole mass spectrometer using a 30 m × 0.25 mm DB-5 column and GC injection port temperature, 250$^\circ$C. The helium carrier gas flow rate was 2.0 mL / min. The temperature of the transfer line was 250$^\circ$C. The initial oven temperature was 100$^\circ$C and was held for 1 min. The oven was then heated to 270$^\circ$C at a rate of 10$^\circ$C/min and was held at that temperature for 10 min. 1$\mu$L sample was injected in the splitless mode. Cholesterol was used as internal standard for
comparison of sterols. After a GC injection, the SRM scanning mode of mass spectrometer was set to detect cholesterol of mass m/z 386.

**Experimental**

**Extraction and isolation of phytochemicals**

1. **From *Vitex peduncularis***

Fresh leaves of *Vitex peduncularis* were collected from Dharmanagar, North Tripura in July 2012 and identified by Prof. B. K. Datta, Plant Taxonomist, Department of Botany, Tripura University. A voucher specimen (TU/H/1510) was deposited in the herbarium laboratory of B. K. D.

Air-dried leaves (2 kg) of *Vitex peduncularis* were extracted with MeOH (6L×2, 6 d each time) at room temperature. A semi-solid residue (160 g) was obtained after removal of solvent in a rotavapour. The major part (150 g) of the residue was suspended in H$_2$O (80 mL) and extracted successively with petroleum ether, CHCl$_3$, EtOAc and n-BuOH (each 3×150 mL).

The petroleum ether extract (15 g) was subjected to column chromatography (CC) over silica gel. Elution of the column with 5% EtOAc in CHCl$_3$ gave a colourless solid, which on repeated CC afforded 3α-friedelanol (1, 5 mg) and 3β-friedelanol (2, 42 mg). The CHCl$_3$ extract (35 g) was subjected to silica gel CC. Elution of the column with 15% EtOAc in CHCl$_3$ afforded vitezeticin (VPL-5) (3, 40 mg). Elution of the column with 20% EtOAc in CHCl$_3$ gave 4',5-dihydroxy-7-methoxyflavone (genkwanin) (4, 55 mg). While elution of the column with 25% EtOAc in CHCl$_3$ gave a solid residue of two compounds. The residue on repeated silica gel CC afforded 4'-acetoxy-5-hydroxy-6,7-dimethoxyflavonone (5, 10 mg) and 4',5-dihydroxy-6,7-dimethoxyflavone (cissimaritin) (6, 65 mg). Elution of the column with 50% EtOAc in CHCl$_3$ afforded a residue of two compounds. The residue on repeated silica gel CC afforded 2α-hydroxyursolic acid (corosolic acid) (7, 50 mg) from 50% EtOAc in CHCl$_3$ eluate and tormentic acid (8, 25 mg) from 60% EtOAc in CHCl$_3$ eluate. The EtOAc extract (40 g) was subjected to CC on Diaion HP-20. The elution of the column with 75% MeOH in H$_2$O afforded a residue, which on CC over silica
gel gave vitexin (9, 60 mg) from elution with EtOAc-MeOH-H$_2$O, 60:35:5 eluate and isovitexin (10, 15 mg) from elution with EtOAc-MeOH-H$_2$O, 60:40:10.

**Flow sheet 1: Isolation of compounds from *V. peduncularis***

The structures of the isolated compounds are provided in Fig. 1.

**3α-friedelinol (1)**
Colourless crystals, mp 280°C (lit. 278-280°C) [1]; [α]$_D^{25}$ +18.2°C (c = 0.1, CHCl$_3$); $^1$H-NMR (600 MHz, CDCl$_3$): δ 3.46 (1H, m, H-3), 0.78 (3H, d, J=6.8 Hz, H$_3$-23), 0.93 (3H, s, H$_3$-24), 0.85 (3H, s, H$_3$-25), 0.96 (3H, s, H$_3$-26), 0.98 (3H, s, H$_3$-27), 1.19 (3H, s, H$_3$-28), 0.91 (3H, s, H$_3$-29), 1.00 (3H, s, H$_3$-30) [2].

---

Natural product Chemistry 385
3β-friedelinol (2)

Colourless crystals, mp 168<sup>0</sup>C (dec)

<sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) ν: 1.88 (1H, dq, J=13.0, 3.0 Hz, H-2), 1.54 (1H, H-2), 3.73 (1H, q-like, J=2.0 Hz, H-3), 1.72 (1H, dt, J=12.0, 3.0 Hz, H-6), 0.98 (1H, H-6), 0.89 (3H, d, J=7.0 Hz, H-23), 0.91 (3H, s, H-24), 0.84 (3H, s, H-25), 0.96 (3H, s, H-26), 0.99 (3H, s, H-27), 1.18 (3H, s, H-28), 0.93 (3H, s, H-29), 0.98 (3H, s, H-30); <sup>13</sup>C-NMR (150 MHz, CDCl<sub>3</sub>) δ: 36.1 (CH<sub>2</sub>, C-1), 72.8 (CH, C-3), 49.2 (CH, C-4), 37.9 (C, C-5), 41.7 (CH<sub>2</sub>, C-6), 17.6 (CH<sub>2</sub>, C-7), 53.2 (CH, C-8), 37.1 (C, C-9), 61.4 (CH, C-10), 35.6 (CH<sub>2</sub>, C-11), 30.0 (CH<sub>2</sub>, C-12), 38.4 (C, C-13), 39.7 (C, C-14), 32.4 (CH<sub>2</sub>, C-15), 36.1 (CH<sub>2</sub>, C-16), 30.7 (C, C-17), 42.8 (CH, C-18), 35.4 (CH<sub>2</sub>, C-19), 28.2 (C, C-20), 32.8 (CH<sub>2</sub>, C-21), 39.3 (CH<sub>2</sub>, C-22), 11.6 (CH<sub>3</sub>, C-23), 16.4 (CH<sub>3</sub>, C-24), 18.3 (CH<sub>3</sub>, C-25), 20.3 (CH<sub>3</sub>, C-26), 18.7 (CH<sub>3</sub>, C-27), 32.1 (CH<sub>3</sub>, C-28), 35.1 (CH<sub>3</sub>, C-29), 31.8 (CH<sub>3</sub>, C-30); FAB-MS m/z (rel. int. %): 429 [M+H]<sup>+</sup> (100).

The spectral data of the compound were very similar to the literature data [2].

Vitecetin (3)

Yellow cubes, Mp 202<sup>0</sup>C; IR ν<sub>max</sub> (KBr) cm<sup>-1</sup>: 3472 (OH), 2872 (OCH<sub>3</sub>), 1643 (α,β-unsaturated C=O); UV λ<sub>max</sub> (MeOH) nm: 252, 263 sh, 360; λ<sub>max</sub> (NaOMe) nm: 268, dec; λ<sub>max</sub> (AlCl<sub>3</sub>) nm: 272, 310, 428; <sup>1</sup>H- and <sup>13</sup>C-NMR (600 MHz, DMSO-d<sub>6</sub>): Table 1.3 in part-I; FAB-MS (positive mode) m/z (%): 361 [(C<sub>18</sub>H<sub>16</sub>O<sub>8</sub>, M) +H]<sup>+</sup> (100), 360 [M]<sup>+</sup> (38), 345 (16), 317 (6), 299 (2), 167 (13).

Genkwanin (4', 5-Dihydroxy-7-methoxyflavone) (4)

Light yellow needles; M.p. 290<sup>0</sup>C (lit. 293–294<sup>0</sup>C) [3]; <sup>1</sup>H-NMR and <sup>13</sup>C-NMR data were similar to reported data [3]; FAB-MS m/z (rel. int. %): 285 [M+H]<sup>+</sup> (100), 257 [MH-CO]<sup>+</sup> (4), 167 (10), 121 (14).

4'-Acetoxy-5-hydroxy-6, 7-dimethoxyflavone (5)

Light yellow amorphous solid; UV λ<sub>max</sub> (MeOH) nm: 272 and 310; (+NaOMe): 263, 286 sh and 301; IR ν<sub>max</sub> (KBr) cm<sup>-1</sup>: 3467, 1755, 1643, 1358, 1499, 1254,
1086, 469; $^1$H and $^{13}$C-NMR (600 MHz, DMSO-$d_6$): Table 1.6 in part-1; FAB-MS $m/z$ (rel. int. %): 357 [M+H]$^+$ (6), 329 (3), 315 [M-42]$^+$ (100), 197 (6), 163 (12), 121 (8), 42 (30).

Cirsimaritin (4', 5-Dihydroxy-6, 7-dimethoxyflavone) (6)

Light yellow needles; M.p. 263°C (lit. 262–263°C) [4]; UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 236 (4.56), 274 (4.26), 336 (4.46); $^1$H-NMR (600 MHz, DMSO-$d_6$): 6.84 (1H, s, H-3), 6.92 (1H, s, H-8), 7.96 (2H, d, $J$=8.4 Hz, H-2', 6'), 6.93 (2H, d, $J$=8.4 Hz, H-3', 5'), 3.73 (3H, s, MeO-7), 3.98 (3H, s, MeO-6), 10.40 (1H, br s, HO-4'), 12.95 (1H, s, HO-5) [4]; $^{13}$C-NMR (150 MHz, DMSO-$d_6$) $\delta$: 164.1 (C, C-2), 102.7 (CH, C-3), 182.3 (C, C-4), 152.7 (C, C-5), 131.9 (C, C-6), 158.6 (C, C-7), 116.0 (CH, C-3', 5'), 161.3 (C, C-4'), 60.1 (CH$_3$, MeO-6), 56.5 (CH$_3$, MeO-7) [4]; FAB-MS $m/z$ (rel. int. %): 315 [M+H]$^+$ (100), 287 [MH-CO]$^+$ (9), 197 (6), 167 (8), 121 (15).

Corosolic acid (2α-hydroxyursolic acid) (7)

White amorphous powder, mp. 258-260°C (dec); $^1$H and $^{13}$C-NMR (600 MHz, DMSO-$d_6$): Table 1.8 in part-1; FAB-MS $m/z$ (rel. int. %): 495 [M+Na]$^+$ (67), 480 (41), 473 (18), 284 (36), 249 (36), 224 (18), 202 (23) and 146 (100) [5,6].

Tormentic acid (8)

White powder, mp 262-264°C (dec); $^1$H-NMR (600 MHz, C$_5$D$_5$N): $\delta$ 1.02 (3H, s), 1.09 (3H, s), 1.13 (3H, s), 1.29 (3H, s), 1.45 (3H, s), 1.73 (3H, s), 1.12 (3H, d, $J$=6.6 Hz, H$_3$-30), 3.06 (1H, br s, H-18), 3.40 (1H, d, $J$=9.6 Hz, H-3), 4.12 (1H, m, H-2), 5.60 (1H, m, H-12) [5]; $^{13}$C-NMR (150 MHz, C$_5$D$_5$N): $\delta$ 48.0 (C-1), 68.8 (C-2), 83.8 (C-3), 40.0 (C-4), 56.2 (C-5), 19.0 (C-6), 33.6 (C-7), 40.4 (C-8), 48.5 (C-9), 38.5 (C-10), 24.2 (C-11), 127.9 (C-12), 140.0 (C-13), 42.3 (C-14), 29.3 (C-15), 26.4 (C-16), 48.0 (C-17), 54.8 (C-18), 72.9 (C-19), 42.5 (C-20), 26.9 (C-21), 38.7 (C-22), 29.3 (C-23), 17.0 (C-24), 17.3 (C-25), 17.7 (C-26), 26.9 (C-21), 38.7 (C-22), 29.3 (C-23), 17.0 (C-24), 17.3 (C-25), 17.7 (C-26),
24.7 (C-27), 180.9 (C-28), 27.3 (C-29), 16.9 (C-30) [65]; FAB-MS m/z (%): 511 [M+Na]+ (100).

**Vitexin (9)**
Yellow powder, mp 263-265°C; 1H and 13C-NMR (600 MHz, DMSO-d6): Table 1.8 in part-I; FAB-MS m/z (rel. int. %): 433 [M+H]+ (100), 391 (14), 315 (100), 269 (73) and 118 (76) [7].

**Isovitexin (10)**
Yellow powder; UV (MeOH): λmax 272 and 329 nm; IR (KBr): 3320, 1654, 1610, 1586, 1515, 1082 cm⁻¹; 1H-NMR (600 MHz, DMSO-d6): δ 6.73 (1H, s, H-3), 6.52 (1H, s, H-8), 7.92 (2H, d, J=8.4 Hz, H-2’,6’), 6.92 (2II, d, J=8.4 Hz, H-3’,5’), 4.62 (1H, d, J=10.0 Hz, H-2’); 13C-NMR (150 MHz, DMSO-d6): δ 163.4 (C-2), 103.0 (C-3), 182.1 (C-4), 161.2 (C-5), 108.9 (C-6), 163.2 (C-7), 93.9 (C-8), 156.2 (C-9), 103.4 (C-10), 121.1 (C-1’), 128.4 (C-2’,6’), 116.0 (C-3’,5’), 160.5 (C-4’), 73.1 (C-1’’), 70.6 (C-2’’), 78.9 (C-3’’), 70.4 (C-4’’), 81.4 (C-5’’), 61.3 (C-6’’) [8]; FAB-MS m/z (%): 433 [M+H]+ (100).

**Alkaline hydrolysis of compound 5:**
Compound 5 (3.5 mg) was heated with 0.5 N methanolic NaOH at 35–40°C with stirring for 2 hr. The reaction mixture was neutralized with Amberlite IRA-120 (H⁺ form) and the residue was removed by filtration. The filtrate was concentrated and subjected to silica gel CC to get compound 6 (1 mg), C₁₇H₁₄O₆ (M⁺ 314).

**Evaluation of antileishmanial activity of vitecetin (VPL-5, 3):**
1. Culture of parasite, *Leishmania donovani*
*Leishmania donovani* strain AG-83 (MHOM/IN/1983/AG83) promastigotes were cultured at 25°C in M-199 medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) (GibcoBRL, Grand Island, N.Y., USA), 20 mM HEPES, pH-7.4, 4 mM NaHCO₃, 100 IU/ml of Penicillin and 100 µg/ml of Streptomycin (Sigma-Aldrich, USA). The experiments were performed...
with stationary phase promastigotes, as stationary phase promastigotes of *L. donovani* were more infective in comparison to log phase promastigote [9].

2. **Culture of macrophage, THP-1 cells**

THP-1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 IU/ml of Penicillin and 100 µg/ml of Streptomycin. THP-1 cells were differentiated by incubation with 20ng/ml Phorbol 12-myristate 13 acetate (PMA; Sigma-Aldrich, USA) in culture medium at 37°C and 5% CO₂ for 72 h. Differentiated THP-1 cells were washed three times with fresh medium without PMA prior to infection. THP-1 macrophage cells were cultured on glass coverslips (18 mm²; 5 X 10⁵ macrophages/cover slip) in 0.5 ml of RPMI-1640 supplemented with 10% FCS and left to adhere overnight before in vitro infection studies.

3. **Preparation of stock solutions of vitezetin (3) and standard drug, SAG**

Vitezetin isolated from *Vitex peduncularis* was tested *in vitro* for its antileishmanial activity in MTT [3 (4,5-dimethyl thiazol 2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, USA) assay.

Sodium antimony gluconate or Sodium stibogluconate or pentostam (SAG) (Albert David Ltd, Kolkata, India) was used as a conventional antileishmanial drug for positive control.

Both VPL-5 and Sodium antimonygluconate were dissolved in sterile dimethyl sulfoxide (Sigma-Aldrich, USA) and stored in -80°C until used. From the stock solution of both the compounds serial dilution were made with phosphate buffered saline (PBS). The compounds were added at two fold dilutions up to 10 points in complete medium starting from 200 µM conc.

4. **Antiproliferative activity of 3 against *L. donovani* promastigotes in MTT assay**

The effect of VPL-5 on the viability of *Leishmania donovani* promastigotes was assessed by monitoring MTT metabolism after a 96 h culture period in presence of specified concentration of test compounds [10]. Parasites in stationary culture stage were seeded at 1 X 10⁶/100 µl Medium 199 per well in 96-well flat bottom microtitre plates (Nunc). The test compounds VPL-5 and SAG were added at ten
concentrations in two fold serial dilutions starting from 200 µM and the plates were then incubated at 25°C for 92 h, prior to MTT (20 µl per well of a 5 mg/ml PBS stock) addition and then for further 4–5 h. After incubation 100 µl of SDS-HCl (10% SDS in 0.01 N HCl) in each well was added to dissolve the MTT formazan produced. The relative amount of formazan produced by viable cells per well was measured photometrically at 570 nm using a plate reader (Biotek Synergy H1 Hybrid Reader). The IC$_{50}$ (measure of the concentration of drug needed to inhibit promastigote growth by 50%) and IC$_{90}$ (measure of the concentration of drug needed to inhibit promastigote growth by 90%) values of test compound and standard drug was determined from the graph representing different concentrations of the compounds plotted against percentage of promastigote growth.

5. Microscopic study on effect of vitecetin (3) against multiplication of intracellular L. donovani amastigotes
For assessing the activity of vitecetin against the intracellular L. donovani amastigote stage, THP-1 macrophages were cultured on glass coverslips (18 mm$^2$; 5 X 105 macrophages/cover slip), in 0.5 ml of RPMI-1640 supplemented with 10% fetal calf serum (FCS), were infected with L. donovani promastigotes at a 1:10 macrophage-to-parasite ratio for 4 h, after which the unbound parasites were washed off. The infected macrophages were incubated with test compounds at ten concentrations starting from 200 µM for 48 h at 37°C and 5% CO$_2$. The cells were washed with sterile PBS, fixed with chilled methanol and stained with Giemsa for the determination of Intracellular parasitic numbers [9]. The number of intracellular amastigotes were determined under optical microscopy and represented as number of amastigotes per 100 macrophages. IC$_{50}$ of antileishmanial activity was calculated using Microsoft Excel by analysis of the concentration response curve [11].

6. Cytotoxicity assay of vitecetin (3) and SAG against THP-1 cells by MTT method
Monolayers of THP-1 macrophages (1x 10$^5$cells/100 µl) in 96-well tissue culture plate (Tarson) were cultured in RPMI-1640 supplemented with 10% FCS and
incubated with test compounds at ten concentrations starting from 200 µM. After 48 hours of incubation the medium was replaced with fresh RPMI (w/o phenol red) containing 1mg/ml of MTT. Cells were incubated at 37°C for 3 h, the untransformed MTT was removed and 50 ml of 0.04 M HCl-isopropanolic solution was added to each well. After 15 minutes incubation at room temperature, absorbance was measured using an automatic plate reader (Biotek Synergy H1 Hybrid Reader), at a reference wavelength of 690 nm and test wavelength of 650 nm [12]. Fifty percent cytotoxic concentration (CC$_{50}$) values of test compounds were estimated. The selectivity index (SI) for vitecetin and SAG was calculated as ratio between, cytotoxicity (CC$_{50}$) and activity (IC$_{50}$) against Leishmania amastigotes [13].

7. **Nitrite generation assay of VPL-5 treated Leishmania parasites infected in THP-1 macrophage cells.**

Nitrite accumulation in culture was measured colorimetrically by the Griess reaction as described by Ding et al. 1988 [14]. Briefly, in a 24 well tissue culture plate (Tarson, India) THP-1 macrophages (1x10^6 cells/mL medium) infected with *Leishmania* parasite (1:10 ratio) were incubated with VPL-5 and sodium stibogluconate at IC$_{50}$ concentration for 48 h before nitrite assay. Cell-free supernatants were collected from different experimental sets after 48 h of treatment. 100 µl of supernatant were incubated with an equal volume of Griess reagent (1% sulphanilamide in 2·5% H$_3$PO$_4$ and 0·1% naphthyl-ethylene-diamine-dihydrochloride in distilled water; both solutions mixed in a ratio of 1:1 at room temperature) for 10 min in a 96-well microtitre plate. The absorbance at 550 nm was then determined by a microtitre plate reader (Biotek Synergy H1 Hybrid Reader). The standard curve for nitrite was prepared by using 10–100 µM sodium nitrite in distilled water. Data were expressed as micromoles of nitrite.

8. **Isolation of RNA and Real-time polymerase chain reaction (RT-PCR) for iNOS2 expression**

For studying the iNOS2 expression THP-1 macrophages (3X10^6 cells/mL medium) infected with *Leishmania* parasite (1:10 ratio) were incubated with vitecetin and sodium stibogluconate at IC$_{50}$ concentration for 5 h. Total RNA was
Experimental

extracted from 3X10^6 THP-1 cells of different experimental sets using TRITM Reagent (Sigma-Aldrich, USA) according to manufacturer's protocol. Isolated total RNA was then reverse transcribed using Revert AidTM, M-MULV Reverse Transcriptase (Fermentus, USA). The resulting cDNA was then used for Real Time PCR for iNOS2 using SYBR green and ABI 7500 real time PCR system (Applied Biosystems, UK). Glyceraldehyde-3-phosphate dehydrogenase was used as reference. The forward and reverse primer sequences used were as follows: For iNOS2 Forward: 5'-CCTGAGCTCTTCAAT-3', Reverse: 5'-AGGATTTGTAGCGCTGGA-3'; and for GAPDH Forward: 5'-TGTACCCACCAACTGCTTAGC-3', Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'. The reaction conditions consisted of the following steps: initial activation step (5 min at 95°C) and cycling step (denaturation for 30 s at 94°C; annealing for 30 s at 58°C and then extension for 1 min at 72°C X 40 cycles), after which the melt curve analysis were performed. Detection of the dequenched probe, calculation of threshold cycles (Ct values), and further analysis of these data were performed by the Sequence Detector Software (Version 1.4; Applied Biosystems). Relative changes in iNOS2 mRNA expression were compared to unstimulated control, normalized GAPDH and were quantified by the 2^-ddCT method [15]. Thus, all the values for experimental samples were expressed as fold differences between the sample mRNA and the calibrator (GAPDH) mRNA.

9. Statistical analysis

The experiments were performed at least three times and the data were represented as mean ± standard deviation (SD). A paired two-tailed Student's t test was employed to assess the significance of the differences between the mean values of control and experimental groups. P value of less than 0.05 was considered significant and less than 0.001 was considered highly significant.
2. From *Meyna spinosa*

The fresh leaves of *M. spinosa* Roxb. were collected from Dharmanagar, North Tripura in June 2011. The plant was identified by Prof. B. K. Datta, Plant Taxonomist, Department of Botany, Tripura University. A voucher specimen (TU/H/1535) has been deposited in the herbarium laboratory of B. K.D.

Air-dried leaves (2 kg) of *M. spinosa* were extracted with methanol by maceration at room temperature (6 L×2, 6 d each time) and the methanolic extract was concentrated *in vacuo* using a rotavapour to a residue (550 g). This crude residue was suspended in water (80 mL) and partitioned against petroleum ether, chloroform, ethyl acetate and *n*-butanol successively. The residue from petroleum ether extract (20 g) on silica gel CC afforded compounds myricyl pentadecanoate (11, 30 mg), myrricyl alcohol (12, 42 mg) and β-sitosterol (13, 60 mg) on elution with different ratios of petroleum ether-ethyl acetate mixture. Elution of the column with petroleum ether-ethylacetate (9:1) afforded a solid

---

**Fig. 5.1.** Structures of compounds 1-10 isolated from *V. peduncularis*
residue which on repeated CC on silica gel using petroleum-ethylacetate (15:1, 12:1, 10:1) mixture gave 11, 12 and 13. The chloroform extract (80 g) on silica gel CC gave compounds 2α,3α,19α,24,28-pentahydroxyurs-12-ene (14, 45 mg), meyanthic acid (15, 20 mg), 19α-hydroxyasiatic acid (16, 50 mg), oleanolic acid (17, 100mg) and β-sitosterol glucoside (18, 60 mg). Elution of the column with CHCl₃-EtOAc (9:1) mixture afforded oleanolic acid (17) and CHCl₃-EtOAc (6:1) afforded 2α,3α,19α,24,28-pentahydroxyurs-12-ene (14), while CHCl₃-EtOAc (4:1) afforded a mixture of two compounds, which on repeated CC on silica gel using CHCl₃-EtOAc (5:1) and (4:1) mixture gave meyanthic acid (15) and 19α-hydroxyasiatic acid (16). CHCl₃-EtOAc (1:1) eluate gave a residue which on repeated CC using CHCl₃-EtOAc (3:1, 2:1 and 1:1) mixture on silica gel afforded β-sitosterol glucoside (18). Ethyl acetate fraction on CC over silica gel gave oleanolic acid (17, 100 mg) and 19α-hydroxyasiatic acid (16, 60 mg) from CHCl₃-EtOAc (4:1) and (3:1) eluates.
The structures of the isolated compounds are provided in Fig. 4.2.

**Myricyl pentadecanoate (11)**
Amorphous powder; mp 76–78°C; FAB-MS \( m/z \) (rel. int.): 663 [M+H]⁺ (33), 635 (2), 437 (3), 243 (4), 225 (5), 111 (12), 97 (21), 83 (41), 69 (6), 57 (100); IR (KBr)νmax cm⁻¹: 1752, 1416, 1075; \(^1\)H and \(^{13}\)C-NMR (600 MHz, CDCl₃): Table 2.12 in part-II.

**Myricyl alcohol (12)**
White powder, mp 87°C (lit. mp 88°C [6]); FAB-MS (\( m/z \)): 461 [M (C₃₀H₆₂O) + Na]⁺ (100%).
**β-Stosterol (13)**
Colourless needle like crystals, mp 138°C; EI-MS m/z (%): 414 [M] (100), 399 (30), 396 (40), 381 (25), 329 (50), 303 (50), 273 (40), 255 (50), 231 (50), 229 (25) and 213 (78) [17].

**2a,3a,19α,24,28-Pentahydroxyurs-12-ene (14)**
Colorless amorphous powder; \([\alpha]_D^{24} + 21.2 (c 0.03, \text{MeOH})\); HR-FAB-MS m/z: 491.3733 \([\text{M+H}^+]\); IR (KBr)\(\nu_{\text{max}}\) cm\(^{-1}\): 3436, 1636, 1565, 1468, 1418, 1380, 1178, 1102, 800, 779; \(^1\)H and \(^{13}\)C-NMR data (C\(_5\)D\(_5\)N): Table 2.2 in part-II.

**Meyanthic acid (15)**
Colorless powder; mp 286–288\(^0\)C (dec); \([\alpha]_D^{25} + 10.6 (c 0.2, \text{MeOH})\); FAB-MS m/z (%): 569 [M+Na]\(^+\) (100), 547 [M+H]\(^+\) (21), 532 (23), 282 (10), 264 (8), 246 (9), 222 (7); \(^1\)H and \(^{13}\)C-NMR data (C\(_5\)D\(_5\)N): Table 2.3 in part-II.

**19α-Hydroxyasiatic acid (16)**
Colorless powder; mp 276–78\(^0\)C; FAB-MS m/z (rel. int.): 5027 [M+Na]\(^+\) (100), 512 (33), 246 (9), 240 (11); \(^1\)H-NMR (600 MHz, C\(_5\)D\(_5\)N): \(\delta\) 4.27 (ddd, \(J=10.0, 9.0, 4.2\) Hz, H-2), 4.25 (d, \(J=10.0,\) Hz, H-3), 5.60 (t-like, H-12), 3.06 (s, H-18), 3.74 (d, \(J=10.2\) Hz, H-23), 4.21 (d, \(J=10.2\) Hz, H-23), 1.0 (s, H-24), 1.15 (s, H-25), 1.11 (s, H-26), 1.41 (s, H-27), 1.67 (s, H-29), 1.12 (d, \(J=6.6\) Hz, H-30). \(^{13}\)C-NMR (150 MHz, C\(_5\)D\(_5\)N): similar to reported data [18].

**Oleanolic acid (17)**
White amorphous solid, mp 275-277\(^0\)C; IR (KBr) \(\nu_{\text{max}}\) cm\(^{-1}\): 3430, 1690, 1608, 1580; \(^1\)H-NMR (600 MHz, CDCl\(_3\)): \(\delta\) 3.24 (1H, dd, \(J=10.5\) and 4.8 Hz, H-3), 5.24 (1H, t, \(J=3.6\) Hz, H-12), 2.82 (1H, dd, \(J=13.0, 4.5\) Hz, H-18), 0.97 (3H, s, H-23), 0.78 (3H, s, H-24), 0.84 (3H, s, H-25), 0.76 (3H, s, H-26), 1.25 (3H, s, H-27), 0.87 (3H, s, H-29), 0.92 (3H, s, H-30); \(^{13}\)C-NMR (150 MHz, CDCl\(_3\)+MeOH): \(\delta\) 38.6 (C-1), 26.7 (C-2), 78.7 (C-3), 39.2 (C-4), 55.6 (C-5), 18.3 (C-6), 32.7 (C-7), 39.6 (C-8), 48.2 (C-9), 37.0 (C-10), 22.8 (C-11), 122.4 (C-12),
Experimental

144.5 (C-13), 42.0 (C-14), 27.7 (C-15), 22.8 (C-16), 46.8 (C-17), 41.5 (C-18), 46.1 (C-19), 30.4 (C-20), 33.7 (C-21), 32.3 (C-22), 28.7 (C-23), 14.7 (C-24), 15.1 (C-25), 16.5 (C-26), 25.2 (C-27), 180.6 (C-28), 32.8 (C-29), 23.4 (C-30) [19,20].

**β-Sitosterol glucoside**

White amorphous powder; mp 278-280°C; IR (KBr) v\(_{\text{max}}\) cm\(^{-1}\): 3392, 2958, 2870, 1650, 1445, 1378, 1028 and 972; \(^1\)H-NMR (600 MHz, C\(_5\)D\(_5\)N): δ 0.68 (3H, s, H\(_3\)-18), 0.96 (3H, s, H\(_3\)-19), 0.86 (3H, d, J=6.6 Hz, H\(_3\)-26), 0.90 (3H, d, J=6.6 Hz, H\(_3\)-27), 1.01 (3H, d, J=5.8 Hz, H\(_3\)-21), 5.37 (1H, br s, H-6), 5.07 (1H, d, J=7.2 Hz, H-1’’), 4.08 (1H, dd, J=7.5,7.8 Hz, H-2’’), 4.31 (1H, dd, J=8.8, 7.8 Hz, H-3’’), 4.28 (1H, dd, J=8.7, 8.1 Hz, H-4’’), 3.94 (1H, m, H-5’’), 4.59 (1H, br d, J=11.4 Hz, H-6’’), 4.45 (1H, dd, J=11.7, 4.8 Hz, H-6’’) [21]; FAB-MS m/z (%): 599 [M+Na]\(^+\) (29), 413 [M-glycosyl]\(^+\) (43), 397 (100), 329 (71), 273 (70), 255 (36) and 219 (82).

**Saponification of compound 11**

Compound 11 (6 mg) was refluxed with 1N methanolic KOH (10 mL) under N\(_2\) atmosphere for 2 hr. After reflux, the mixture was concentrated, diluted with water and extracted with CHCl\(_3\). The CHCl\(_3\) extract was concentrated and column chromatographed over silica gel to get myricyl alcohol (1.5 mg), CH\(_3\)(CH\(_2\))\(_{29}\)-OH, mp 89\(^\circ\)C (lit. 88\(^\circ\)C [22]) and pentadecanoic acid (1.3 mg), mp 53\(^\circ\)C (lit. 53–54\(^\circ\)C [22]).
3. From *Cornus mascula* (syn. *C. mas*)

The red cherry fruits of *Cornus mascula* were collected in the flowering-fruit making season, September, 2013 from virgin forests of Northern Greece. The plant was identified by Dr. Th. Constantinidis, Faculty of Biology, Department of Ecology & Systematics, National and Kapodistrian University of Athens, Greece and the voucher specimen (No. AMK/01/14) has been deposited in Herbarium Laboratory of the University.

Air-dried semi-powdered fruits (750 g) of *C. mascula* were extracted with methanol by maceration at room temperature (2.5 L× 2, 6 d each time) and the methanolic extract was concentrated in vacuo using a rotavapor to a residue (200 g). The crude residue was suspended in water (35 mL) and partitioned against dichloromethane, ethyl acetate and *n*-butanol, successively. The residue from
dichloromethane extract (22 g) on silica gel column chromatography (CC) afforded 4-acetoxy-5,2',4',6',β-pentahydroxy-3-methoxychalcone (19, 16 mg) from elution with 5% EtOAc in hexane and ursolic acid (20, 4 mg) from elution with 40% EtOAc in hexane. The EtOAc extract (36 g) on silica gel CC afforded naringenin-7-O-methyl ether (21, 12 mg), 7,3′-dihydroxy-5,4′-dimethoxyflavanone (22, 8 mg), 2R,3R-tans-aromadendrin (23, 7 mg), myricetin (24, 5 mg), ursolic acid (20, 26 mg) and gallic acid (25, 32 mg) eluted with a solvent gradient of EtOAc-CHCl₃ (20:80, 25:75, 30:70, 35:65, 40:60 and 90:10, respectively). The n-BuOH extract (7 g) was subjected to CC on diaion HP-20 and eluted with a solvent gradient of H₂O-MeOH (1:0, 3:1, 1:1, 1:3, 0:1). The residue obtained from H₂O-MeOH (1:1) eluate was CC over silica gel to afford quercetin-3-O-rutinoside (26, 22 mg) from elution with MeOH-CHCl₃ (30:70). The aqueous part remaining after extraction with organic solvents was concentrated to a residue (16 g). A part of the residue (10 g) was acetylated with Ac₂O and pyridine at room temperature for 24 h. The acetylated mixture on usual work up and silica gel CC afforded D-glucose tetraacetate (27, 60 mg).
Flow sheet 3: Isolation of compounds from *Cornus mascula*

**C. mascula**

MeOH, percolation, rt.

MeOH extract (160 g) → Merck

Fractionated

CH$_2$Cl$_2$ sol. fr. → CC over silica gel

- 4-acetoxy-5,2',4',6',β-pentahydroxy-3-methoxychalcone (19)
- ursolic acid (20)

EtOAc sol. fr. → n-BuOH sol. fr.

- quercetin-3-O-rutinoside (26)
- D-glucose isolated as tetraacetate (27)

1. CC over Diaion HP-20
2. CC over silica gel

Aqueous fr.

CC over silica gel

- naringenin-7-O-methyl ether (21)
- 2R,3R-trans-aromadendrin (23)
- ursolic acid (20)
- 7,3'-dihydroxy-5,4'-dimethoxyflavanone (22)
- myricetin (24)
- gallic acid (25)

---

4-Acetoxy-5,2',4',6',β-pentahydroxy-3-methoxychalcone (19)

Orange needles (hexane-EtOAc mixture), mp 200°C; UV (MeOH) $\lambda_{max}$ nm: 281 and 368; IR (KBr) $\nu_{max}$ cm$^{-1}$: 3436, 2855, 1675, 1608, 1570, 1483, 757; $^1$H- and $^{13}$C-NMR data (CDCl$_3$): Table 3.3 in part-III; ESI-TOF-MS m/z: 377 [M+H]$^+$, 335 [MH - 42]$^+$, 251, 209, 153; HR-ESI-TOF-MS m/z: 377.0870 [M+H]$^+$ (Calcd for C$_{18}$H$_{17}$O$_9$, 377.0873); ESI-TOF-MS m/z (%): 377 (46), 251 (21), 209 (29), 165 (15), 153 (100), 125 (37).
Ursolic acid (20)
Amphorus solid, mp 268-270°; ESI-TOF-MS m/z: 457 [M+H]+; IR (KBr) νₘₐₓ cm⁻¹: 3434, 2934, 1708, 1639, 1468, 1380 and 972; ¹H- NMR (600 MHz, CD₃OD): δH 0.78 (3H, s, H₃-25), 0.85 (3H, s, H₃-26), 0.88 (3H, d, J=6.6 Hz, H₃-29), 0.96 (3H, s, H₃-24), 0.97 (3H, d, J=6.6 Hz, H₃-30), 1.12 (3H, s, H₃-27), 1.29 (3H, s, H₃-23), 2.20 (1H, d, J=10.8 Hz, H-18), 3.15 (1H, dd, J=11.4, 4.8 Hz, H₃-29), 5.23 (1H, t, J=3.0 Hz, H-12) [23]; ¹³C- NMR (150 MHz, CD₃OD): δC 40.0 (C-1), 27.9 (C-2), 79.7 (C-3), 39.8 (C-4), 56.7 (C-5), 19.5 (C-6), 34.3 (C-7), 40.8 (C-8), 47.9 (C-9), 38.1 (C-10), 24.4 (C-11), 126.9 (C-12), 139.9 (C-13), 43.2 (C-14), 29.2 (C-15), 25.2 (C-16), 47.9 (C-17), 54.5 (C-18), 40.0 (C-19), 40.4 (C-20), 31.8 (C-21), 38.3 (C-22), 28.8 (C-23), 16.0 (C-24), 16.4 (C-25), 17.6 (C-26), 24.2 (C-27), 181.8 (C-28), 18.0 (C-29), 21.6 (C-30) [24]; ESI-TOF-MS m/z (%): 457 [M+H]+ (60), 441 [M-Me]+ (40), 438 (20), 248 (100), 203 (55), 207 (63) and 189 (38).

Naringenin-7-O-methylether (21)
Pale yellow amorphous solid; ¹H- NMR (600 MHz, CD₃OD): δH 5.34 (1H, dd, J=13.0, 3.0 Hz, H-2), 2.74 (1H, dd, J=17.0, 3.0 Hz, H-3), 3.16 (1H, dd, J=17.0, 12.8 Hz, H-3), 5.91 (1H, d, J=2.4 Hz, H-6), 6.07 (1H, d, J=2.4 Hz, H-8), 7.32 (2H, d, J=8.4 Hz, H-2’,6’), 6.81 (2H, d, J=8.4 Hz, H-3’,5’), 3.90 (3H, s, MeO-7) [25]; ¹³C- NMR (150 MHz, CD₃OD): δC 80.5 (C-2), 44.5 (C-3), 197.8 (C-4), 165.7 (C-5), 96.4 (C-6), 168.6 (C-7), 93.8 (C-8), 165.1 (C-9), 103.5 (C-10), 131.2 (C-1’), 129.2 (C-2’,6’), 116.6 (C-3’,5’), 159.8 (C-4’), 57.0 (MeO-7) [25,26].

(2S)-7,3'-Dihydroxy-5,4'-dimethoxyflavanone (22)
Bright yellow amorphous solid; UV (MeOH) λₘₐₓ nm: 283 and 325; IR (KBr) νₘₐₓ cm⁻¹: 3430, 1690, 1630, 1570, 1516, 1483, 1455, 1275; CD (MeOH, c 0.21): Δε₂₈₂ -0.20, Δε₃₂₄ +0.09; ¹H- and ¹³C-NMR data (DMSO-d₆): Table 3.2 in part-III; FAB-MS m/z (%): 317 (70), 315 (12), 289 (46), 193 (23), 167 (19), 150 (49), 135 (100).
2R,3R-trans-Aromadendrin (23)
Pale yellow powder; $^1$H- NMR (600 MHz, DMSO-$d_6$): $\delta_H$ 5.06 (1H, d, $J=12.0$ Hz, H-2), 4.60 (1H, dd, $J=12.0$, 6.0 Hz, H-3), 5.84 (1H, d, $J=2.4$ Hz, H-6), 5.90 (1H, d, $J=2.4$ Hz, H-8), 7.32 (2H, d, $J=8.4$ Hz, H-2',6'), 6.78 (2H, d, $J=8.4$ Hz, H-3',5'), 5.64 (HO-3), 11.44 (1H, s, HO-5), 10.80 (1H, br s, HO-7), 9.54 (1H, s, HO-4') [27]; $^{13}$C- NMR (150 MHz, DMSO-$d_6$): $\delta_C$ 82.8 (C-2), 71.4 (C-3), 198.2 (C-4), 163.3 (C-5), 96.0 (C-6), 166.7 (C-7), 95.1 (C-8), 162.6 (C-9), 100.6 (C-10), 127.5 (C-1'), 129.4 (C-2',6'), 114.9 (C-3',5'), 157.7 (C-4') [26].

Myricetin (24)
Yellow powder; $^1$H- NMR (600 MHz, CD$_3$OD): $\delta_H$ 6.20 (1H, d, $J=2.0$ Hz, H-6), 6.50 (1H, d, $J=2.0$ Hz, H-8), 6.98 (2H, br s, H-2',6') [26]; $^{13}$C- NMR (150 MHz, CD$_3$OD): $\delta_C$ 148.2 (C-2), 137.0 (C-3), 178.1 (C-4), 163.5 (C-5), 99.1 (C-6), 165.7 (C-7), 92.2 (C-8), 158.8 (C-9), 104.6 (C-10), 122.1 (C-1'), 109.2 (C-2',6'), 146.8 (C-3',5'), 136.5 (C-4') [26].

Gallic acid (25)
White powder; $^1$H- NMR (600 MHz, DMSO-$d_6$): $\delta_H$ 6.91 (2H, s, H-2,6), 9.19 (2H, s, HO-3,5), 8.24 (1H, s, HO-4), 12.23 (1H, br s, HOOC-1); $^{13}$C- NMR (150 MHz, DMSO-$d_6$): $\delta_C$ 120.4 (C-1), 108.7 (C-2,6), 145.2 (C-3,5), 138.0 (C-4), 167.5 (COOH) [28].

Quercetin-3-O-rutinoside (26)
Yellow amorphous solid; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3360, 1650, 1260; $^1$H- NMR (600 MHz, DMSO-$d_6$): $\delta_H$ 6.19 (1H, d, $J=1.8$ Hz, H-6), 6.38 (1H, d, $J=1.8$ Hz, H-8), 7.54 (1H, d, $J=1.8$ Hz, H-2'), 6.83 (1H, d, $J=8.4$ Hz, H-5'), 7.52 (1H, dd, $J=8.4$, 1.8 Hz, H-6'), 5.34 (1H, d, $J=7.2$ Hz, H-1''), 5.10 (1H, br d, $J=14.4$ Hz, H-6''), 4.54 (1H, br d, $J=9.0$ Hz, H-6''), 4.38 (1H, br d, H-1''), 0.98 (3H, d, $J=6.6$ Hz, H-6''), 3.05-3.71 (8H, m, H-2'',3'',4'',5'',2''',3''',4''',5''') [29]; $^{13}$C- NMR (150 MHz, DMSO-$d_6$): Table 4.3 in Part-IV.
D-Glucose (27)
Isolated as tetraacetate
Colourless needles; IR (KBr) νmax cm⁻¹: 3474, 1747, 1370, 1228, 1080, 1068, 1045, 914; ¹H- NMR (600 MHz, CDCl₃): δH 6.33 (1H, d, J=3.6 Hz, H-1), 5.10 (1H, dd, J=9.6, 3.6 Hz, H-2), 5.72 (1H, dd, J=9.6, 9.6 Hz, H-3), 5.24 (1H, dd, J=9.6, 9.6 Hz, H-4), 4.26-4.31 (2H, m, H-5,6), 4.11 (1H, dd, J=10.2, 2.4 Hz, H-6), 2.02, 2.04, 2.10, 2.12 (each 3H, s, Ac × 4); ¹³C- NMR (150 MHz, CDCl₃): δC 91.6 (C-1), 72.7 (C-2), 70.2 (C-3), 67.7 (C-4), 72.7 (C-5), 61.4 (C-6), 170.6, 169.4, 169.2, 168.9, 20.5, 20.6, 20.7, 20.8 (Ac × 4) [30].

![Diagram of compounds](image)

**Fig. 5.3.** Structures of isolated compounds 19-27 from *C. mascula.*
From Morinda citrifolia

Fresh fruits and leaves of Morinda citrifolia L. (Rubiaceae) were collected from Morinda, Inc. formerly known as Tahitian Noni International and Morinda Bioactives, American Fork, Provo, Utah, USA in August, 2011.

Air-dried fruits (1.5 kg) of M. citrifolia were extracted with MeOH at room temperature (2.0 L× 2, 6d each time) and the methanolic extract was concentrated to a semisolid residue (~250 g). The residue was suspended in water (100 mL) and extracted with dichloromethane (3 × 100 mL). The CH₂Cl₂ extract (35 g) was subjected to column chromatography over silica gel (120 g). Elution of the column with 5% EtOAc in hexane afforded scopoletin (28, 180 mg).

Air-dried powdered leaves (1.0 kg) of M. citrifolia were extracted with MeOH (2.0 L× 2, 6d each time) at room temperature. The methanolic extract was concentrated under reduced pressure to a residue (150 g). The residue was suspended in water (75 mL) and extracted successively with dichloromethane, ethyl acetate and n-butanol (3 × 100 mL, each solvent). The major butanol extract (30 g) was subjected to column chromatography over Diaion HP-20. The column eluted with H₂O, H₂O-MeOH (3:1, 1:1, 1:3) and MeOH. The residue from H₂O-MeOH (1:1, 1:3) eluates were identical in TLC. The residue were mixed together and subjected to silica gel CC. The elution of the column with 75% CHCl₃ in MeOH afforded quercetin-3-O-rutinoside (29, 70 g).
Flow sheet 4: Isolation of compounds from *Morinda citrifolia*

**Morinda citrifolia** fruits
- Extraction with MeOH, rt.
- MeOH extract
  - MeOH extract
    - Extracted with DCM
      - DCM extract
        - CC over silica gel
          - scopoletin (28)

**Morinda citrifolia** leaves
- Extraction with MeOH, rt.
- MeOH extract
  - Fractionation
    - DCM fr.
      - EtOAc fr.
    - n-BuOH fr.
      - 1. CC over Diaion HP-20
        - 2. CC over silica gel
          - quercetin-3-O-rutinoside (29)

**Scopoletin (28)**
Yellow needles, mp 208-210°C; C_{10}H_{8}O_{4} (M$^+$ 192); IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3341, 1708, 1618, 1607 and 1568; $^1$H- and $^{13}$C-NMR data (DMSO-$d_6$): Table 4.3 in part-IV; FAB-MS m/z (%): 193 [M+H$^+$] (100), 164 (71) and 136 (50), 107 (12) [31].

**Quercetin-3-O-rutinoside (29)**
Yellow amorphous solid; C_{27}H_{30}O_{16} (M$^+$ 610); IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3360, 1650, 1260; $^1$H- and $^{13}$C-NMR data (DMSO-$d_6$): Table 4.4 in part-IV; FAB-MS m/z (%): 633 [M+Na$^+$] (100), 611 [M+H$^+$] (15), 301 (12).

**Furin inhibition assay of scopoletin (28)**
In vitro assay of furin activity was performed by using three different concentrations of substrate Boc-RVRR-MCA namely 20, 40 and 60 μM. The tested compounds scopoletin named as MCD-1 was dissolved in DMSO and by
proper dilution with the buffer (consisted of 25 mM Mes, 25 mM Tris and 2.5 mM CaCl$_2$, pH 7.4), different concentrations (10-5 – 100 µM) of it were prepared. Furin (5 µL of 50 fold diluted from stock sample) was incubated with each solution of MCD-1 (5 µL) in presence of a fixed concentration of Boc-RVRR in DMSO (5 µL) in buffer (35 µL) in 96-well plates. In parallel, a control experiment was performed in identical condition without the MCD-1. The release of fluorescent AMC (7-amino-4-methylcoumarin) moiety was measured by stop-time method using the fluorometer instrument with excitation and emission wavelengths fixed at 370 and 460 nm, respectively [32]. A standard curve correlating fluorescence with the amount of free AMC was developed from this purpose. The inhibition constant $K_i$ values were derived from Cornish-Bowden plots. For measurement of IC$_{50}$ values, the data were plotted using Grafit 4 software. Each experiment was repeated twice, each time in duplicate and the result was averaged.

![Fig. 5.4. Structures of compounds isolated from M. citrifolia](image-url)
References


