Isolation and Structural Determination of New Sphingolipids and Pharmacological Activity of Africanene and Other Metabolites from *Sinularia leptoclados*

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Two new sphingolipids, (2*S*,3*S*,4*R*)-1,3,4-trihydroxy-2-[((*R*)-2′-hydroxytetradecanoyl) amino] tricosane (4) and (2*S*,3*S*,4*R*)-1,3,4-triacetoxy-2-[((*R*)-2′-acetoxyoctadecanoyl) amino]octadecane (5) along with africanene (1, reasonably good yield), 23-demethylgargosterol (2) and batylalcohol (3) have been isolated from the soft coral *Sinularia leptoclados*. Preliminary studies for pharmacological activity (blind screening and toxicity studies) of africanene were conducted. Africanene exhibited *in vitro* and *in vivo* cytotoxicity, dose dependent hypotensive activity as well as antiinflammatory activity. The pharmacological and toxicity studies on africanene are being reported for the first time and findings strongly encourage further investigation. Compounds 1, 4 and 5 were studied for the antibacterial, antifungal and antiviral activity while compounds 4 and 5 were also studied for the short term *in vitro* cytotoxic activity.

Key words soft coral; *Sinularia leptoclados*; sphingolipid; africanene; pharmacological study; toxicity

Soft corals are reported[1—3] to contain secondary metabolites which are shown to possess diverse pharmacological activities and also are good tools in analysis of various bioactive components. Africanene and batylalcohol which possess spermatostatic[4] and wound healing activities,[5] respectively, are the commonly reported compounds of soft corals. As part of continuing our studies on bioactive metabolites from marine organisms,[6] a soft coral *Sinularia leptoclados* was collected from the coast of Mandapam of Gulf of Mannar Island. In the present study the yield of africanene obtained was very high compared to other reports.[4,7—9] We report for the first time the toxicity and cytotoxic, hypotensive and antiinflammatory activity profiles of africanene (**1**) along with antibacterial, antifungal and antiviral properties of **1** and sphingolipids (**4**, **5**). Compounds **4** and **5** were also studied for their short term *in vitro* cytotoxic activity.

Results and Discussion

Chemistry Compound **4**: The IR spectrum (KBr) exhibited absorption bands of hydroxyl and amide NH (3500—3200 cm^{-1}) and amide carbonyl (1680 cm^{-1}). The tetraacetyl derivative, **4a** obtained from **4** with pyridine–Ac_2O, still showed a sharp IR absorption band at 3335 cm^{-1} indicating the presence of amide NH. ^{13}C-NMR distortionless enhancement by polarisation transfer (DEPT) spectrum of **4** (see Table 1 for NMR data) showed the signals at δ 175.68 of a carbonyl carbon and δ 51.95 of a methine carbon, both connected to amide nitrogen, indicating that **4** was a sphingolipid.[10,11] In addition, the spectrum exhibited signals at δ 86.18, 74.70, 72.59 of oxygenated methine carbons and δ 71.58 of an oxygenated methylene carbon. The identity of **4** as a sphingolipid was also confirmed from its ^{1}H-NMR spectrum by the presence of a characteristic[10,12,13] amide NH doublet at δ 8.52 (d, 1H, J=7.2 Hz; exchangeable with D_2O). The absence of any ^{13}C-NMR signal at δ 36—37 (lit.[13] δ 37.2) and ^{1}H-NMR signal at δ 2.22—2.36 (lit.[13,14] δ 2.22—2.28) as triplet and the presence of methylene signals at δ 35.59 (lit.[12,13,15,16] $\delta\cong$35.7) and δ 2.01 (m, 2H) in the ^{13}C-NMR and ^{1}H-NMR spectra, respectively, suggested that the methylene of fatty acyl chain is attached to an amide carbonyl only through an oxygenated methine carbon. Further, the appearance of resonance at δ 34.34 (lit.[12,13,15,16] $\delta\cong$34.4) clearly indicates the attachment of the methylene carbon of the fatty alkyl chain of sphingosine base to the oxygenated methine carbon. In the ^{1}H-NMR spectrum, the signal appeared at δ 4.80 (m, 1H) was assigned to nonoxygenated methine proton which is connected to amide NH. The signals displayed at δ 4.54 (m, 1H) and 3.92 (m, 1H) were assigned to oxygenated methylene protons. The oxygenated methine protons resonated at δ 4.66 (dd, 1H, J=7.5, 4.4 Hz), 4.33 (m, 1H) and 4.14 (dt, 1H, J= 8.8, 4.4 Hz). Both the carbon and proton spectra also exhibited the signals of methylene and terminal methyl groups of the fatty acyl/alkyl chains present in sphingolipids.

The length of the fatty acyl chain was determined on the basis of mass fragmentation pattern referring to earlier reports.[13,14,16] The FAB-MS showed the molecular ion at *m/z* 613 (M^+) in addition to the ion *m/z* 611 (M^+-2H). The prominent fragment ion at *m/z* 384 $[611-227]^+$ formed by the elimination of fatty acyl chain from the base moiety suggested that the molecular weight of the fatty acid is 228 and that the base is sphingosine. This was supported by the presence of the highest EI-MS peak at *m/z* 386. FAB-MS also showed fragment ions at *m/z* 595 (M^+-H_2O), 593 (M^+-H_2O, 2H) and prominent peaks at *m/z* 581 (M^+-CH_2OH, H) and 563 (M^+-CH_2OH, H_2O, H). The fragment ion at *m/z* 369 ($M^+-C_{14}H_{30}NO_2$) corresponds to the loss of amide NH along with the fatty acyl moiety. Indeed, the highest peak at *m/z* 386, corresponding to the sphingosine base, was observed from the EI-MS of acid hydrolysed (10% HCl in MeOH) product of **4**.

The proton assignments made were confirmed further by

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Table 1. ^{1}H, ^{13}C DEPT and ^{1}H–^{1}H COSY NMR Spectral Data of **4** and **5**

H or C	δ ^{1}H, ppm (multi., J in Hz)		δ ^{13}C, ppm (multi.) (100 MHz, $CDCl_3$)		^{1}H–^{1}H COSY[a]	
	4 (400 MHz, d_5-pyridine)	**5** (400 MHz, $CDCl_3$)	**4**	**5**	**4** (200 MHz, d_5-pyridine)	**5** (200 MHz, $CDCl_3$)
1_a	4.54 (m)	4.35 (dd , J=4.0, 10.6)	71.58 (t)	62.37 (t)	1_a-H, 1_b-H; 1_a-H, 2-H	1_a-H, 1_b-H; 1_a-H, 2-H
1_b	3.92 (m)	4.03 (dd, J=3.1, 10.6)	—	—	1_b-H, 2-H	1_b-H, 2-H
2	4.80 (m)	4.46 (m)	51.95 (d)	47.80 (d)	2-H, NH; 2-H, 3-H	2-H, NH; 2-H, 3-H
3	4.33 (m)	5.09 (dd, J=3.0, 9.3)[e]	72.59 (d)	72.68 (d)	3-H, 4H	3-H, 4H
4	4.14 (dt, J=4.4, 8.8)	4.93 (br dt, J=9.8, 2.8)[e]	74.70 (d)	72.21 (d)	4-H, 5-H_2	4-H, 5-H_2
5	1.71 (m)	1.66 (m)	34.34 (t)[c]	31.76 (t)	5-H_2, 6-H_2	5-H_2—17-H_2
6	1.46 (m)[b]	1.25 (br s)	32.15 (t)[d]	29.69—24.87 (t)	6-H_2, 7-H_2	—
7—16	1.25 (br s)	1.25 (br s)	29.99—25.88 (t)	29.69—24.87 (t)	7-H_2—22-H_2	—
17	1.25 (br s)	1.25 (br s)	29.99—25.88 (t)	22.68 (t)	—	17-H_2, 18-H_3
18	1.25 (br s)	0.88 (t, J=6.8)	29.99—25.88 (t)	14.11 (q)	—	—
19—21	1.25 (br s)	—	29.99—25.88 (t)	—	—	—
22	1.25 (br s)	—	22.95 (t)	—	22-H_2, 23-H_3	—
23	0.86 (t, J=6.0)	—	14.29 (q)	—	—	—
1′	—	—	175.68 (s)	171.29 (s)	—	—
2′	4.66 (dd, J=4.4, 7.5)	5.09 (t, J=8.0)[e]	86.18 (d)	73.98 (d)	2′-H, 3′-H_2	2′-H, 3′-H_2
3′	2.01 (m)	1.83 (m)	35.59 (t)[c]	31.91 (t)	3′-H_2, 4′-H_2	3′-H_2, 4′-H_2
4′	1.71 (m)	1.66 (m)	32.90 (t)[d]	31.76 (t)	4′-H_2—13′-H_2	4′-H_2—17′-H_2
5′	1.58 (m)[b]	1.25 (br s)	29.99 (t)	29.69—24.87 (t)	—	—
6′—12′	1.25 (br s)	1.25 (br s)	29.99—25.88 (t)	29.69—24.87 (t)	—	—
13′	1.25 (br s)	1.25 (br s)	22.95 (t)	29.69—24.87 (t)	13′-H_2, 14′-H_3	—
14′	0.86 (t, J=6.0)	1.25 (br s)	14.29 (q)	29.69—24.87 (t)	—	—
15′—16′	—	1.25 (br s)	—	29.69—24.87 (t)	—	—
17′	—	1.25 (br s)	—	22.68 (t)	—	17′-H_2, 18′-H_3
18′	—	0.88 (t, J=6.8)	—	14.11 (q)	—	—
NH	8.52 (d, J=7.2)	6.63 (d, J=8.9)	—	—	2-H, NH	2-H, NH
Acetate carbonyls (×4)	—	—	—	169.96 (s), 170.02 (s), 169.98 (s), 170.91 (s)	—	—
Acetate methyls (×4)	—	2.09 (s), 2.06 (s), 2.03 (s), 2.19 (s)	—	20.67 (q), 20.74 (q), 20.86 (q), 21.01 (q)	—	—

Proton assignments based on the ^{1}H–^{1}H COSY. *a*) Type of spin interaction observed is vicinal coupling among all the protons and the coupling between 1_a-H, 1_b-H is geminal coupling. *b—d*) Assignments having same superscript are interchangeable; other assignments having nearly identical values may be interchangeable. *e*) Signals are overlapped (signals taken from the δ scale expansion spectra).

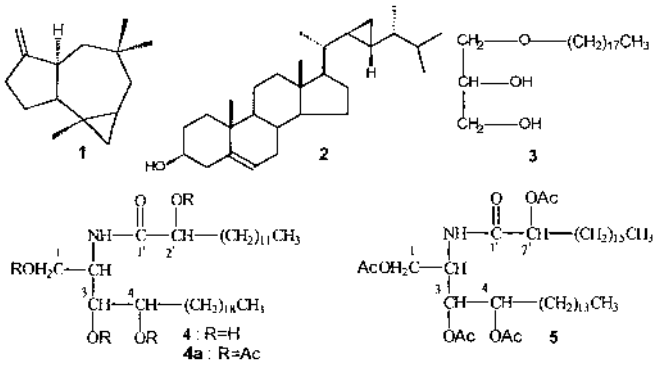
the analysis of ^{1}H–^{1}H correlation spectroscopy (COSY). The relative stereochemistry of **4** at C-2, C-3, C-4 and C-2′ was predicted to be the same as that of natural cerebrosides agelasphin-7a, 9a, 9b, 11 and 13 (isolated from the sponge *Agelas mauritianus*[12]) and of the synthetic ceramide (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-1,3,4-heptadecanetriol[12] and its related analogues.[10,12,17] ^{1}H-NMR spectral data of **4** [d_5-pyridine; 1_a-H at δ 4.54 (m), 1_b-H at δ 3.92 (m), 2-H at δ 4.80 (m), 3-H at δ 4.33 (m), 4-H at δ 4.14 (dt, J=4.4, 8.8 Hz) and 2′-H at δ 4.66 (dd, J=4.4, 7.5 Hz)] is more or less in agreement with those of natural agelasphin-9a [d_5-pyridine; 1_a-H at δ 4.58 (m), 1_b-H at δ 4.32 (m), 2-H at δ 5.2 (m), 3-H at δ 4.38 (m), 4-H at δ 4.26 (m), 2′-H at δ 4.63 (m)] and of synthetic ceramide [1_a-H at δ 4.47 (m), 1_b-H at δ 4.43 (m), 2-H at δ 5.07 (m), 3-H at δ 4.38 (m), 4-H at δ 4.28 (m), 2′-H at δ 5.5 (dd, J=5.3, 7.3 Hz)]. The coupling constants $J_{1,2}$, $J_{2,3}$, $J_{2,NH}$, $J_{3,4}$ and $J_{2',3'}$ observed for the tetraacetyl derivative of **4** (4, 3.1, 3.0, 8.5, 3.5, 7.3 Hz, respectively) were almost in agreement with those of (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-3,4-di-*O*-benzoyl-1-*O*-triphenylmethyl-1,3,4-heptadecanetriol[12] (3.7, 2.9, 2.4, 9.2, 2.9, 7.3 Hz, respectively) and (2*S*,3*S*,4*R*)-2-acetamino-1,3,4-triacetoxyheptadecane[12] (4.3, 3.1, 3.1, 9.2, 3.1 Hz, respectively). Methanolysis of **4** followed by separation of the ester and the long chain base and acetylation of the base gave methyl 2-hydroxytetradecanoate (**4b**) and 2-acetamino-1,3,4-triacetoxytricosane (**4c**). The specific rotation of **4b**, $[\alpha]_D^{28}$ −2.8° (c=0.1, $CHCl_3$) is very close to that of the methyl esters of 2-(*R*)-hydroxy fatty acids reported earlier [lit.[10,12,17] $[\alpha]_D^{23}$ −2.4° (c=3.0, $CHCl_3$), $[\alpha]_D^{23}$ −2.2° (c=0.5, $CHCl_3$), $[\alpha]_D^{25}$ −2.2° (c=2.0, $CHCl_3$)]. Moreover, the specific rotations of **4**, $[\alpha]_D^{28}$ +7.0° (c=0.1, $CHCl_3$) and **4c**, $[\alpha]_D^{28}$ +6.8° (c=0.1, $CHCl_3$) are also almost in agreement with that of synthetic ceramide (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-1,3,4-heptadecanetriol, $[\alpha]_D^{23}$ +6.0° (c=0.1, $CHCl_3$). So, the stereochemistry of **4** at C-2, C-2′, C-3 and C-4 could be 2*S*,2′*R*,3*S* and 4*R*. Based on the spectral data, **4** was characterized as (2*S*,3*S*,4*R*)-1,3,4-trihydroxy-2-[((*R*)-2′-hydroxytetradecanoyl) amino] tricosane.

Compound **5**: Spectral data was recorded for the tetraacetate **5**, which was obtained following acetylation and purification of the crude solid isolated (refer to Experimental and also to the literature[11]) by chromatography of the ethyl acetate soluble fraction of the methanolic extract of *S. leptoclados*. The IR spectrum (KBr) of **5** exhibited strong absorption bands at 3344 (amide NH), 1744 (acetate carbonyls), 1664

(amide carbonyl), and 2912 and 2864 cm^{-1} (long aliphatic chain). The ^{13}C-NMR DEPT spectrum (see Table 1 for NMR data) showed the signals of four acetyl methyls at δ 21.01 (q), 20.86 (q), 20.74 (q), 20.67 (q) and the corresponding carbonyl carbons at δ 170.91 (s), 170.02 (s), 169.98 (s) and 169.96 indicating that **5** is a tetraacetyl derivative. This was supported by the presence of signals of four acetoxy methyls in the ^{1}H-NMR spectrum also. The characteristic signals appearing due to an amide carbonyl at δ 171.29, a methine carbon linked to amide nitrogen at δ 47.80 in ^{13}C-NMR DEPT spectrum and an amide NH doublet at δ 6.63 (d, 1H, J=8.9 Hz, exchangeable with D_2O) in ^{1}H-NMR spectrum, also suggest **5** to be a sphingolipid.[10—13] The spectrum also showed four oxygenated carbon signals at δ 73.98 (d), 72.68 (d), 72.21 (d) and 62.37 (t). The absence of any signal at δ 2.22—2.36 (lit.[13,14] δ 2.22—2.28) as triplet in the proton spectrum, δ 36—37 (lit.[13] δ 37.12) in the carbon spectrum and the presence of high frequency acetoxy methyl signal at δ 2.19 (s, 3H; lit.[11] δ 2.19, tetraacetyl ptiloceramide), methylene signals at δ 1.83 (m, 2H; lit.[11] δ 1.82) and δ 31.91 (t; lit.[11,14] δ 31.9) in the proton and carbon spectrum, respectively, inferred that the carbonyl function is not directly bridged to the methylenes of the fatty acyl chain but only through a –CH(OAc)–. The signals at δ 5.09 (dd, 1H, J=3, 9.3 Hz and t, 1H, J=8.0 Hz; signals overlapped[18]) were assigned for two acetoxy bearing methine protons, of which one connected to the amide carbonyl of the fatty acyl chain and the other connected to the nonoxygenated methine proton of the sphingosine base. The presence of a 2-*N*-acyl-1,3,4-triacetoxy chain[10—14] in **5** was inferred from the ^{1}H-NMR spectral resonances at δ 4.35 (dd, 1H, J=4.0, 10.6 Hz, 1_a-H), 4.03 (dd, 1H, J=3.1, 10.6 Hz, 1_b-H), 4.46 (m, 1H, 2-H), 5.09 (dd, 1H, J=3, 9.3 Hz, 3-H), 4.93 (br dt, 1H, J=9.8, 2.8 Hz, 4-H) and 6.63 (d, 1H, J=8.9 Hz, NH). The carbon and proton spectra also displayed the signals of methylene and terminal methyl groups of the fatty acyl/alkyl chains present in sphingolipids.

The length of the alkyl chain of the fatty acyl moiety and of the sphingosine base was established by FAB-MS fragmentation pattern referring to the earlier reports.[13,14,16] The FAB-MS of the compound showed the molecular ion at *m/z* 767 (M^+) in addition to the ion *m/z* 768 (MH^+). The FAB-MS also showed the base peak at *m/z* 708 (MH^+-AcOH) in addition to the ions of *m/z* 648 ($MH^+-2AcOH$), 588 ($MH^+-3AcOH$). The prominent ion peak at *m/z* 264 [$M^+-COCH(OAc)\cdot(CH_2)_{15}CH_3$, 3OAc,H], *i.e.* ($767^+-503$) formed by elimination of a fatty acyl group from the base moiety, established that the molecular weight of the fatty acid is 325 and that the base is sphingosine. The same was supported by the presence of the highest EI-MS peak at *m/z* 316, corresponding to sphingosine base, obtained from the alkaline hydrolysed (10% KOH in MeOH) product of **5**.

All the proton assignments made were confirmed further by the analysis of ^{1}H–^{1}H COSY. The coupling between 2′-H at δ 5.09 and 3′-H_2 at δ 1.83 indicated that the methylene protons of the fatty acyl chain are not directly linked to the amide carbonyl but only through a –CH(OAc)–. The absolute configuration of **5** at C-2, C-3, C-4 and C-2′ was predicted to be the same as that of the reported compounds[12] (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-3,4-di-*O*-benzoyl-1-*O*-triphenylmethyl-1,3,4-heptadecanetriol and (2*S*,3*S*,4*R*)-2-acetamino-1,3,4-triacetoxyheptadecane. The ^{1}H-NMR chemical shifts of 1-H_2, 2-H, 3-H and 4-H for **5** [$CDCl_3$; 1_a-H at δ 4.35 (dd, J=4.0, 10.6 Hz), 1_b-H at δ 4.03 (dd, J=3.1, 10.6 Hz), 2-H at δ 4.46 (m), 3-H at δ 5.09 (dd, J=3.0, 9.3 Hz), 4-H at δ 4.93 (br dt, J=2.8, 9.8 Hz)] are more or less in agreement with those of (2*S*,3*S*,4*R*)-2-acetamino-1,3,4-triacetoxyheptadecane [lit.[12] $CDCl_3$; 1_a-H at δ 4.29 (dd, J=11.6, 4.3 Hz), 1_b-H at δ 4.00 (dd, J=11.6, 3.1 Hz), 2-H at δ 4.47 (m), 3-H at 5.10 (dd, J=8.5, 3.1 Hz), 4-H at δ 4.93 (dt, J=9.8, 3.1 Hz)]. Further, the coupling constants $J_{1,2}$, $J_{2,3}$, $J_{2,NH}$, $J_{3,4}$ and $J_{2',3'}$ for **5** (4, 3.1, 3.0, 8.9, 2.8, 8.0 Hz, respectively) are very close to those of (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-3,4-di-*O*-benzoyl-1-*O*-triphenylmethyl-1,3,4-heptadecanetriol (3.7, 2.9, 2.4, 9.2, 2.9, 7.3 Hz, respectively) and (2*S*,3*S*,4*R*)-2-acetamino-1,3,4-triacetoxyheptadecane (4.3, 3.1, 3.1, 9.2, 3.1 Hz, respectively). The coupling constants observed for **5** are also well in agreement with those of several synthetic analogues reported by Natori *et al.*[12] Methanolysis of **5** followed by separation of the ester and the long chain base and acetylation of the base gave methyl 2-hydroxyoctadecanoate (**5a**) and 2-acetamino-1,3,4-triacetoxyoctadecane (**5b**). The specific rotation of **5a**, $[\alpha]_D^{28}$ −3.0° (c=0.1, $CHCl_3$) is very close to that of the methyl esters of 2-(*R*)-hydroxy fatty acids reported earlier [lit.[10,12,17] $[\alpha]_D^{23}$ −2.4° (c=3.0, $CHCl_3$), $[\alpha]_D^{23}$ −2.2° (c=0.5, $CHCl_3$), $[\alpha]_D^{25}$ −2.2° (c=2.0, $CHCl_3$)]. Moreover, the specific rotations of **5**, $[\alpha]_D^{28}$ +8.0° (c=0.1, $CHCl_3$) and **5b**, $[\alpha]_D^{28}$ +7.1° (c=0.1, $CHCl_3$) are also almost in agreement with that of synthetic ceramide (2*S*,3*S*,4*R*)-*N*-[(*R*)-2′-acetoxytetracosanoyl]-2-amino-1,3,4-heptadecanetriol,[12] $[\alpha]_D^{23}$ +6.0° (c=0.1, $CHCl_3$). The results indicated the absolute configuration of **5** to be 2*S*, 2′*R*, 3*S* and 4*R*. Based on the spectral data, **5** was characterized as (2*S*,3*S*,4*R*)-1,3,4-triacetoxy-2-[((*R*)-2′-acetoxyoctadecanoyl) amino] octadecane.



Biological Compounds **4** and **5** from the coral *Sinularia leptoclados* exhibited mild antibacterial activity against the gram negative bacteria tested at a concentration of MIC 200 μg/ml, and no activity was found against the gram positive bacteria tested. None of the other compounds studied possessed antibacterial or antifungal activity against the organisms tested. No notable antiviral activity was observed at 2 mg/ml concentration for **1**, **4** or **5**. None of these compounds produced any cytopathic effects by Herpes simplex virus-1 (HSV-1) and none was found to be cytotoxic in Vero cell line culture at a concentration of 2 mg/ml.

In performing preliminary tests for pharmacological activity, the behavioural (awareness, mood, motor activity, *etc.*),

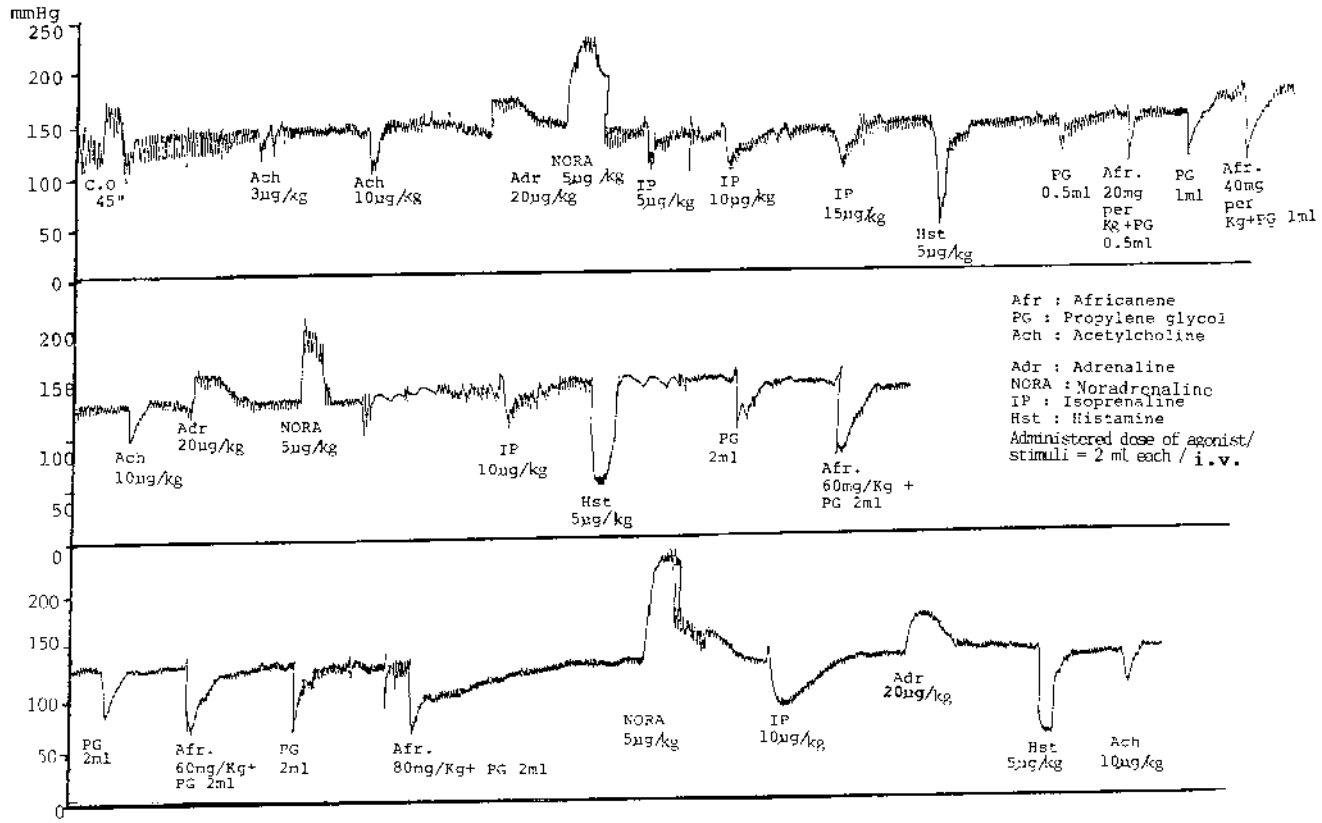


Fig. 1. Effect of Africanene on Blood Pressure of the Dog (Route: i.v.; Dog Weight: 8 kg)

autonomic (urination, salivation, heart rate, *etc*.) and neurological responses (motor incoordination, muscle tone, posture, reflexes, *etc*.) were observed in male Swiss albino mice. The oral administration of africanene at the dose of 300 mg/kg body weight at the 3rd h produced mild central nervous system (CNS) depressant activity which was observed from the changes in behavioural responses (awareness, mood, motor activity). The changes in neurological responses (muscle tone, ipsilateral flexor reflexes) noticed at the above dose level at the 3rd h suggested mild muscle relaxant activity for africanene. In another study, it was also noticed that the normal animal rested on the rotarod apparatus (rotating at 15 rpm) for an average period of 300 s and then tried to jump from the hot plate of an analgesia apparatus chamber (55 ± 0.5 °C) within 4—5 s. The animals that received the test dose of 300 mg/kg body weight, were found to fall from the rotarod at the 3rd h within 180 s and did not jump off the hot plate of the analgesia chamber within the stipulated period, in contrast to normal. The mild response of the animals in these tests may be due to the muscle relaxant and CNS depressant activity. A higher dose of 600 mg/kg body weight of africanene at the 1st h produced CNS depressant and muscle relaxant activities which were also confirmed by the rotarod and hot plate analgesia experiments. However, this dose was also found to be toxic and the pharmacological effects produced at this dose level may be due to the toxicity of africanene. Lacrimal secretions were observed from the 3rd h onwards at this dose. As a compound producing CNS depressant activity may also possess narcotic analgesic, anticonvulsant, hypnotic or angiolytic activities, africanene which showed CNS depressant activity is worth studying for these activities, too. Though no visible toxic symptoms were observed during the first 4 h of study in mice on oral administration of africanene at doses 300 and 600 mg/kg body weight, death was observed within 24—36 h and 5—12 h, respectively, which may be due to respiratory distress and circulatory failure. The LD_{50} (oral) of africanene was found to be approximately 300 mg/kg body weight.

The effect of africanene on isolated frog rectus abdominis muscle was studied for contractile or relaxation response of the tissue. At a level of 100 μg/ml in propylene glycol, africanene did not produce any response on the muscle tissue. Higher concentrations were not attempted since the solubility of the compound is a limiting factor.

Africanene was screened for its cardiovascular activity in anaesthetised dogs. It showed dose dependent hypotension in the range of 20—80 mg/kg body weight/i.v. and produced transient to profound fall of blood pressure at doses of 60 and 80 mg/kg body weight. Africanene did not alter the responses of carotid occlusion (CO), noradrenaline, adrenaline, acetylcholine, histamine or isoprenaline, indicating that it may not possess antimonoaminooxidase, sympatholytic, ganglion blocking or ataractic activities, anticholinesterase or muscarinic activity and histaminergic or its blocking activity, respectively. It is also possible that africanene may act directly on the vascular system as vasodilator or may affect the cardiac function. Further experiments are to be conducted to elucidate its mechanism of hypotension. The cardiovascular activity of africanene is shown in Fig. 1.

In acute inflammatory (carrageenin induced rat oedema) studies africanene at the dose of 10 mg/kg body weight orally produced promising reduction ($p < 0.119 \times 10^{-6}$, com-

Table 2. Short Term *in Vitro* Cytotoxicity Assay of **1**

Test material	Concentration (μg/ml)	DLAT cells		EAC cells	
		% Dead cells	Mean % of dead cells	% Dead cells	Mean % of dead cells
Control	0	9, 5	7	10, 6	8
Africanene	0.5	11, 13	12	8, 12	10
	1	25, 31	28	21, 25	23
	2	35, 37	36	35, 39	37
	5	46, 42	44	43, 53	48
	10	100, 100	100	88, 96	92
	20	100, 100	100	100, 100	100
	50	100, 100	100	100, 100	100
	100	100, 100	100	100, 100	100

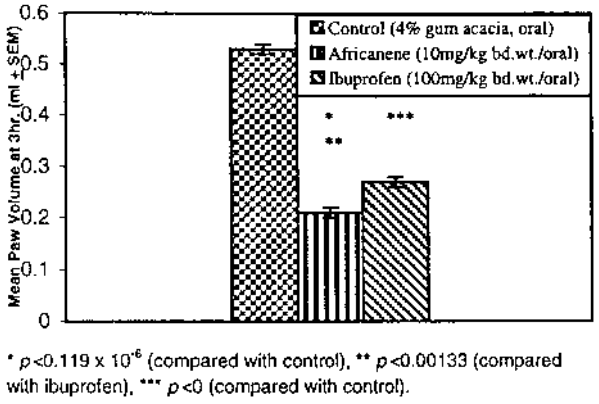


Fig. 2. Antiinflammatory Activity of Africanene on Rats (Carrageenin Induced Paw Oedema Model)

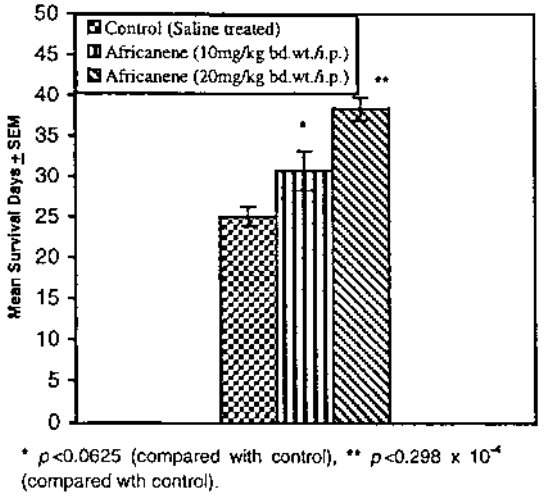


Fig. 3. Effect of Africanene on Reduction of Ehrlich Ascites Tumour in Mice (*in Vivo* Antitumour Assay)

pared to control and $p<0.00133$ compared to ibuprofen) in paw volume at the 3rd h (0.21 ± 0.01 ml, 60.37% oedema inhibition), while ibuprofen showed only 49.05% reduction in oedema at a higher dose of 100 mg/kg body weight/oral. The results of the activity are shown in Fig. 2.

Africanene and compounds **4** and **5** were tested for the short term *in vitro* cytotoxic activity against the Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites tumour (DLAT) cells. Africanene at 10 μg/ml concentration caused the death of the EAC and DLAT cells to 100% and 92%, respectively. **4** and **5** did not show any notable *in vitro* cytotoxicity. In an *in vivo* cytotoxic assay, determination of the inhibition of the Ehrlich ascites tumours was made in africanene administered mice. The life span of the mice increased by 22.68% and 53.32% at the concentrations of 10 and 20 mg/kg body weight, respectively, with intraperitoneal administration of africanene. As per National Cancer Institute protocol, a compound that produces increase in life span (ILS) of greater than 20% is worthy of further study. The results of the *in vitro* and *in vivo* cytotoxicity assay of africanene are presented in Table 2 and Fig. 3, respectively.

Experimental

All the melting points were determined on an Automelpo HMK dreader hot plate and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on 400 MHz (Bruker FT WM-400/Unity Varian Vista), 200 MHz (Gemini) or 90 MHz (JEOL JNM FX-90Q/Perkin Elmer R32) spectrometers with tetramethylsilane as internal standard. EI-MS and FAB-MS were taken on JEOL D-300 and JEOL SX-102 spectrometers, respectively. Elemental analysis was carried out on a Carlo Erba 1108 analyzer. Optical rotations were measured on a Rudolf Autopol III polarimeter. The IR spectra were taken on a Perkin-Elmer (FT-IR) 1800. Antiinflammtory studies were done at the Pharmacological Division of the Indian Institute of Chemical Technology, Hyderabad by the author.[19] Cytotoxicity studies were carried out at the Amala Cancer Research Centre, Amala Nagar, Trichur, Kerala by the author. Antiviral studies were performed through the courtesy of Prof. S. P. Tyagarajan, Dr. A. L. Murugappa Postgraduate Institute of Basic Medical Sciences, Madras. The rest of the biological and pharmacological studies were conducted by the author in the Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam.

Extraction and Isolation The organism was collected during December 1992 from the coasts of Mandapam (9°18′N, 79°08′E) of the Gulf of Mannar Islands at a depth of 10—15 metres. Specimen was identified by Dr. Phil Alderslade, Curator of Coelenterates, Northern Territory Museum of Arts and Sciences, Australia and was found to be *Sinularia leptoclados* var. *Gonadates*. The organism was extracted with methanol six times at room temperature at 48 h intervals. The combined alcoholic extracts were concentrated under reduced pressure. The concentrated extract was fractionated with ethyl acetate and the ethyl acetate soluble fraction was concentrated under vacuum to yield a crude dark brown coloured gummy residue (30 g). Weight of the air dried organism after extraction was 1.5 kg. The ethyl acetate soluble fraction of the methanolic extract was chromatographed over silica gel using eluants with increasing polarity starting from hexane through ethyl acetate to methanol. Fractions were collected and monitored through silica gel TLC and a total of seven fractions (I to VII) were collected. Fraction I (*n*-hexane) on further column chromatography over silica gel using hexane yielded colourless liquid (**1**, 3.1 g). Fraction II (3—5% EtOAc–hexane) did not show distinct spots on TLC and no further investigation was carried out. Fraction III (10% EtOAc–hexane) on further column chromatography (2% EtOAc–hexane) over silica gel yielded colourless crystalline solid (**2**, 11 mg, positive to Liebermann and Burchard test). Fraction IV (15—20% EtOAc–hexane) on repeated crystallization from methanol gave colourless flakes (**3**, 200 mg). Fraction V (40% EtOAc–hexane) was further purified by column chromatography (20% EtOAc–hexane) over silica gel; it

yielded colourless amorphous solid (**4**, 21 mg). Fraction VI (60—80% EtOAc–hexane) on chromatography (70% EtOAc–hexane) over silica gel yielded a crude solid which was contaminated with inseparable colouring matter. Hence, to facilitate the separation of pure compound from the colouring matter and for spectral data measurements, the crude solid was acetylated with C_5H_5N and Ac_2O. The acetylated mixture on chromatography (10% EtOAc–hexane) over silica gel gave colourless solid (**5**, 20 mg). In fact, the crude solid did not show any acetyl protons in its PMR spectrum. However, the PMR spectrum of **5**[20] showed acetyl protons which were introduced in the process of separation from the crude solid. Fraction VII (EtOAc/methanol) was obtained as intractable gum and no investigations were made.

$\Delta^{9(15)}$-Africanene (**1**): Colourless liquid, $[\alpha]_D^{28}$ +82° (c=0.23, $CHCl_3$). Survey of the literature revealed that its spectral data is in agreement with those reported in the literature.[4,7—9]

Demethylgargosterol (**2**): Colourless crystals, mp 162—164 °C, $[\alpha]_D^{28}$ +34.86° (c=0.1, $CHCl_3$). It was identified by comparison with the spectral data of the literature.[21—23]

Batylalcohol (**3**): Colourless flakes, mp 69—71 °C, $[\alpha]_D^{28}$ +2.58° (c=1.0, $CHCl_3$). Its identification was confirmed by comparison of the spectral data with those reported in the literature.[24]

(2*S*,3*S*,4*R*)-1,3,4-Trihydroxy-2-[((*R*)-2′-hydroxytetradecanoyl) amino] tricosane (**4**): Colourless amorphous powder, mp 105—107 °C $[\alpha]_D^{28}$ +7.0° (c=0.1, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1} 3500—3200, 2912, 2856, 1680, 1552, 1504, 1272, 1056, 736. FAB-MS *m*/*z* (%): 613 (M^+, 9), 611 (13), 595 (8), 593 (8), 581 (68), 563 (9), 384 (10), 369 (8), 354 (9), 340 (14), 326 (9), 300 (13), 298 (13), 280 (10), 278 (9), 257 (8), 239 (7), 226 (8), 202 (8), 178 (10), 165 (12), 113 (41), 99 (47), 85 (54), 71 (70), 57 (81), 43 (54), 29 (60). *Anal*. Calcd for $C_{37}H_{75}NO_5$: C, 72.44%; H, 12.24%; N, 2.28%. Found: C, 72.45%; H, 12.22%; N, 2.27%. The ^{1}H-NMR, ^{13}C-NMR DEPT and ^{1}H–^{1}H COSY spectral data are shown in Table 1.

Acetylation of 4 To a solution of **4** (3 mg) in pyridine (1 ml), Ac_2O (0.5 ml) was added and the mixture was kept overnight at room temperature. The excess reagents were removed *in vacuo* and the residue was partitioned between water and ether. Evaporation of the solvent left a residue which on chromatography over a small column of silica gel yielded a tetraacetyl derivative (**4a**, 3 mg). **4a**: a white amorphous powder, mp 60—63 °C, $[\alpha]_D^{28}$ +9.0° (c=0.1, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1} 3335, 2910, 2850, 1740, 1675, 1270, 1050, 730. *Anal*. Calcd for $C_{45}H_{83}NO_9$: C, 69.14%; H,10.63%; N, 1.79%. Found: C, 69.05%; H, 10.73%; N, 1.78%. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 6.59 (d, J=8.5 Hz, 1H), 5.38 (dt, J=9.0, 3.0 Hz, 1H), 5.16 (t, J=7.3 Hz, 1H), 4.95 (dd, J=9.0, 3.5 Hz, 1H), 4.6 (m, 1H), 4.25 (dd, J=10.6, 4 Hz, 1H), 4.12 (dd, J=10.6, 3.1 Hz, 1H), 2.16 (s, 3H), 2.12 (s, 6H), 2.09 (s, 3H), 1.91 (m, 2H), 1.82 (m, 2H), 1.54 (m, 4H), 1.25 (br s, 50H), 0.88 (t, J=6.9 Hz, 6H).

Methanolysis of 4 and Acetylation of the Long Chain Base A 4 mg sample of **4** was treated with 10% HCl in MeOH (3 ml) for 15 h at 80 °C. The reaction mixture was extracted with *n*-hexane and the hexane layer was concentrated and chromatographed over silica gel (25% EtOAc–hexane) to give methyl 2-hydroxytetradecanoate (**4b**, 1.5 mg). **4b**: $[\alpha]_D^{28}$ −2.8° (c=0.1, $CHCl_3$), EI-MS *m*/*z* 258 (M^+). The aqueous methanolic layer was neutralized with Amberlite CG-400 and concentrated, and the residue was dissolved in water and extracted with EtOAc. The EtOAc phase afforded the crude long chain base, which was acetylated with acetic anhydride/pyridine (1 : 1) for 2 h at 80 °C. The reaction mixture was diluted with water and extracted with EtOAc. The residue of the ethyl acetate layer was chromatographed using silica gel (40% EtOAc–hexane) to give 2-acetamino-1,3,4-triacetoxytricosane (**4c**, 2 mg). **4c**: $[\alpha]_D^{28}$ +6.8° (c=0.1, $CHCl_3$); ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 5.95 (d, J=9.2 Hz, 1H), 5.10 (dd, J=8.3, 3.3 Hz, 1H), 4.89 (dt, J=9.75, 3.1 Hz, 1H), 4.45 (m, 1H), 4.28 (dd, J=11.0, 4.2 Hz, 1H), 4.00 (dd, J=11.6, 3.5 Hz, 1H), 2.08 (s, 3H), 2.05 (s, 6H), 2.02 (s, 3H), 1.12—1.70 (m, long chain –CH_2–), 0.88 (t, J=6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1,3,4-triacetoxy-2-[((*R*)-2′-acetoxyocatadecanoyl) amino] octadecane (**5**): Colourless amorphous powder, mp 54—57 °C, $[\alpha]_D^{28}$ +8° (c= 0.1, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1} 3344, 2912, 2864, 1744, 1664, 1552, 1488, 1376, 1248, 1046, 736. FAB-MS *m*/*z* (%): 768 (MH^+, 41), 767 (20), 708 (100), 666 (20), 664 (20), 648 (23), 606 (6), 604 (6), 588 (5), 264 (52), 252 (8), 250 (8), 113 (14), 99 (25), 85 (31), 71 (20), 57 (52), 43 (100), 29 (14). *Anal*. Calcd for $C_{44}H_{81}NO_9$: C, 68.84%; H, 10.56%; N, 1.83%. Found: C, 68.89%; H, 10.54%; N, 1.80%. The ^{1}H-NMR, ^{13}C-NMR DEPT and ^{1}H–^{1}H COSY spectral data are shown in Table 1.

Methanolysis of 5 and Acetylation of the Long Chain Base Compound **5** was treated in the same way as described for **4**, to yield methyl 2-hydroxyoctadecanoate (**5a**, 2 mg) and 2-acetamino-1,3,4-triacetoxyoctadecane (**5b**, 2 mg). **5a**: $[\alpha]_D^{28}$ −3.0° (c=0.1, $CHCl_3$), EI-MS *m*/*z* 314 (M^+). **5b**: $[\alpha]_D^{28}$ +7.1° (c=0.1, $CHCl_3$), ^{1}H-NMR spectral data of **5b** was identical with that of **4c** except for differences in aliphatic chain protons.

Acute Toxicity Studies and Preliminary Tests of Africanene for Pharmacological Activity (Blind Screening Studies)[25] Swiss albino male mice, weighing 20—22 g were obtained from the animal house, Ghosh Enterprise, Calcutta, India. They were housed in standard conditions and received standard diet and water *ad libitum*. The animals were divided into various groups of six animals each. The test compound was suspended in 5% gum acacia and the suspension was administered orally to each group of animals in doses of 100, 300, 600 and 1000 mg/kg body weight and the control group received only an equal volume of 5% gum acacia mucilage. The animals were observed for 4 h after administration of the test compound for any behavioural, autonomic or neurological responses following Irwin's method.[26] Animals were also observed for any toxic symptoms and mortality during the first 24 h post-treatment of the test compound.

Effect of Africanene on Isolated Frog Rectus Abdominis Muscle The effect of africanene, at a concentration of 100 and 500 μg/ml in propylene glycol, on isolated frog rectus abdominis muscle was studied[27] to see if there was any contractile response of the tissue using acetylcholine as reference.

Effect of Africanene on Blood Pressure of Anaesthetised Dogs Investigation was carried out to study the effect of the africanene on normal blood pressure of dog[28,29] and its influence on the changes in blood pressure produced by a series of chemical and neural stimuli following a group of tests described by Nieschulz *et al*.[30] and elaborated by Smith.[31] In this experiment a) the effect of africanene (at doses 20, 40, 60 and 80 mg/kg body weight/i.v.) on blood pressure and b) its influence on the responses to the stimuli on the blood pressure of CO (for 45 s), adrenaline i.v. injection (10—30 μg/kg), noradrenaline i.v. injection (5—30 μg/kg), acetylcholine i.v. injection (5—20 μg/kg), histamine i.v. injection (5—20 μg/kg) and isoprenaline i.v. injection (5—30 μg/kg) were studied. Not less than three dogs were employed for each experiment to show that the effect produced by the test compound is reproducible. Africanene, in propylene glycol at doses of 20 to 80 mg/kg body weight, was administered through the femoral vein and responses were recorded.

Acute Inflammatory Studies Wistar albino rats (120—160 g) of either sex supplied by the National Institute of Nutrition, Hyderabad, India were used. They were housed in the animal house (25±2 °C, relative humidity of 60±5% and 12 h light–dark cycle) with access to standard food and water *ad libitum*. Employing the method of Winter *et al*.,[32] carrageenin induced rat paw oedema was compared at 0 and 3 h to that of control (4% gum acacia mucilage). Groups of six rats were injected with 0.1 ml of 1% carrageenin solution in the subplantar region of the right hind paw 30 min after oral administration of africanene (10 mg/kg body weight). The paw volume was measured plethysmographically at 3 h after carrageenin injection. Ibuprofen (100 mg/kg body weight/oral) was used as reference substance. The results were analysed using ANOVA followed by Dunnette's *t*-test.[33,34]

Cytotoxic Activity Testing Africanene (**1**) and compounds **4** and **5** were tested for the short term *in vitro* cytotoxic activity against EAC and DLAT cells which were originally procured from the National Cancer Research Institute, Bombay, India were maintained as ascites tumours in Swiss albino mice. Africanene was tested also for the *in vivo* antitumour activity against Ehrlich ascites tumour in these mice. Swiss albino mice (6—7 weeks old and weighing between 20—22 g) were supplied by the National Institute of Nutrition, Hyderabad, India. The animals were kept in an air-controlled room and fed normal mouse chow (Lipton, India) and water *ad libitum*.

Short Term *in Vitro* Cytotoxicity Assay The short term *in vitro* cytotoxic studies were done using EAC and/or DLAT cells.[35] Test compounds **1**, **4** and **5** at various concentrations (0.5—500 μg/ml) in dimethyl sulfoxide (DMSO) were added separately to phosphate-buffered saline (PBS, *ca*. 0.7—0.8 ml, pH 7.2). DLAT and EAC cells were grown in the peritoneal cavity of the Swiss albino mice. Cells were removed by aspirator and washed with PBS. Tumour cells (one million) were added and final volume was made to 1 ml with PBS and incubated for 3 h at 37 °C. Then, 0.1 ml of 0.1% of trypan blue was added and the number of live or dead cells was counted using a haemocytometer. The control experiment contained only an equal volume of DMSO (without test compound) as that of the test experiment in 1 ml PBS solution with one million tumour cells and the live cells were counted as in the test experiment. The experiments were performed in duplicate for each concentration of test compound as well as control and the mean percentage of dead cells was recorded.

Determination of the Inhibition of the Tumour Development (*in Vivo* Anti-tumour Assay) The *in vivo* antitumour activity of africanene was studied by noting the death pattern of animals due to tumour burden and the

percentage increase in life span was calculated following the method reported earlier.[36—38] Swiss albino mice (six per group) were injected intraperitoneally with Ehrlich ascites tumour cells (one million cells) on day 0, *i.e*. the day of transplantation, for tumour development. Visible tumours appeared within 10—15 d.

The test compound was suspended in normal saline at a concentration of 0.1 g/ml, and from this, appropriate doses were selected for the study. According to the treatment schedule the tumour transplanted animals were divided into three groups, A, B and C (six per group). The animals of groups A and B received the test compound intraperitoneally at a dose of 10 and 20 mg/kg body weight, respectively, for five alternate days. The treatment was started 24 h after the tumour transplantation. The mice in control group C received normal saline by the same route. The animals were fed normal colony diet (composition: cracked wheat 70%, cracked bengal gram 20%, yeast 4%, fish meal 5%, shark liver oil 0.25% and sesame oil 0.75%) and water was given *ad libitum*. ILS of animals was calculated according to the formula $[(T-C)/C\times100]$ where T is the average number of days the treated mice lived after the transplantation of tumour cells and C is the average number of days the untreated mice lived after the transplantation.

Antibacterial and Antifungal Activity Compounds **1**, **4** and **5** were studied for the antibacterial (against three gram positive bacteria, *viz. Bacillus pumilis*, *Bacillus subtilis* and *Staphylococcus aureus* and two gram negative bacteria, *viz. Escherichia coli* and *Pseudomonas aeruginosa*) and antifungal (against *Candida albicans*, *Aspergillus niger* and *Rhizopus nigricans*) activities. They were tested for determination of the inhibition zone by the cup-diffusion method.[39,40]

Antiviral Studies.[41] **Antihepatitis-B Virus Properties by Hepatitis-B Surface Antigen (HBsAg) Binding Studies** HBsAg binding studies were carried out as the first part of the antiviral studies for compounds **1**, **4** and **5**. Equal volumes of HBsAg positive plasma and the test compound (2 mg/ml) were added and incubated at 37 °C for a period of 5 d. The binding effect of these compounds which equates with *in vitro* inactivation of HBsAg was analyzed by ELISA procedure conducted every day.

Anti HSV Properties Using Vero Cell Line[41] The anti HSV studies were carried out for compounds **1**, **4** and **5**.

Preparation of the Compounds for Anti HSV Studies: The test compound was dissolved in DMSO and centrifuged at 2000 rpm for 10 min. The supernatant was removed and filtered in a membrane filter with porosity of 0.2 micron. The filtrate was used for the antiviral studies against HSV-1.

Study Protocol : Vero cell monolayers were used for the anti HSV-1 evaluatory studies. Four sets of six Vero cell culture tubes were used. Group A which receives 0.1 ml each of sterile DMSO acted as DMSO control. Group B tubes received 0.1 ml of each test compound, 2 mg/ml, and acted as compound control. Group C tubes were inoculated with 0.1 ml of AC strain of HSV-1 at a concentration of 10 pfu/ml (positive virus control) and group D received the same as group C. All the tubes were incubated at room temperature for 90 min. After incubation, group D tubes alone were inoculated with 0.1 ml of test compound at a concentration of 2 mg/ml (test group). Two ml of Eagle's maintenance minimal essential medium (MEM) was added to all the tubes and incubated at 37 °C for two weeks. They were observed every day for any evidence of cytopathic effect by HSV-1 and cytotoxicity by the compound itself.

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