

Two novel antifungals, acornine 1 and acornine 2, from the bark of mangrove plant *Aegiceras corniculatum* (Linn.) Blanco from Sundarban Estuary

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ABSTRACT

Background: Microbes have been implicated in a wide variety of human diseases many of which are of life-threatening nature. New antimicrobials are urgently needed not only for combating these organisms but also to counter the menace of the harmful microbes developing resistance against drugs at alarming rates. Mangrove plants are rich sources of secondary metabolites having many beneficial biological activities including antimicrobial ones. True to this fact, this report describes identification, isolation and partial characterization of two novel antifungal compounds from *Aegiceras corniculatum*, a mangrove plant from Indian Sundarban estuary. **Materials and Methods:** Two compounds, named as Acornine 1 and Acornine 2, having antifungal activities were isolated from the bark of *A. corniculatum*, a mangrove plant, by using standard techniques. The compounds were characterized using routine microbiological and physicochemical methods. **Results:** Partial structural characterization of the two compounds indicated they are oleanane triterpenoids with linked sugar moieties. While both the compounds exhibited growth inhibition in tested Gram positive bacteria, Acornine 2 in particular demonstrated strong antifungal activities against several pathogenic fungi tested. Results also indicated that various environmental factors may govern the secondary metabolite profiles of the same mangrove plants growing in different geographical areas. **Conclusion:** Tissue extracts of *Aegiceras corniculatum*, a mangrove plant from Indian Sundarban estuary, exhibited the presence of remarkable antifungal activities. The isolated compounds responsible for such activities, named as Acornine 1 and Acornine 2, appear to have potential in food processing and health care industry. They need to be studied further.

Key words: Acornine 1, Acornine 2, *Aegiceras corniculatum*, Antifungal, Mangrove, Oleanane triterpenoid glycosides

INTRODUCTION

The Sundarban, covering about one million hectare in the delta of the rivers Ganges, Brahmaputra and Meghna, is shared between India and Bangladesh. It is also the world's largest coastal wetland forest with a rich diversity of mangrove plants.^[1] In this area the mangroves exist under very hostile and inhospitable conditions as the plants which grow there have to encounter constantly changing water pH, salinity and wind velocity, higher temperature and extreme humidity, tidal extremes, muddy anaerobic soil etc., No terrestrial plant can survive well under these adverse conditions.^[2] To exist in such hostile

living conditions with different biotic and abiotic stress factors the mangrove plants are known to synthesize a variety of phytochemicals that are known to have medicinal values.^[3] The local population in and around Sundarban have been using the tissue extracts of various mangrove plants for ages as cures for various diseases. The black mangrove *Aegiceras corniculatum* (Linn.) Blanco, which has many ethnopharmaceutical uses by the local people such as in the treatments of rheumatism, arthritis, inflammation, asthma, diabetes etc.^[4,5] has been selected for the present study. It belongs to the myrsinaceae family and is distributed in coastal and estuarine areas of India, including the Sundarban estuary. Probably because of the widespread ethnomedicinal uses, this plant has attracted interests of researchers from diverse areas. Roome *et al.*,^[6] and Agoramoorthy *et al.*,^[7] have studied the antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective actions of *A. corniculatum* stem extracts.

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Some researchers have analyzed the antimicrobial activity of *A. corniculatum*.^[8-11] Giron *et al.*,^[12] isolated two benzoquinone compounds, 2,5-dihydroxy-3tridecyl-1,4-benzoquinone and 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone from *A. corniculatum* which showed fungal inhibition. Wahidullah *et al.*,^[13] isolated and patented corniculatonin, an antifungal compound, from this plant. In this study we report the isolation and characterizations of two new antifungal compounds named as Acornine 1 and Acornine 2 from *Aegiceras corniculatum*. The results of these studies indicate that they may have potential uses in the human health care and food processing industries.

MATERIALS AND METHODS

General experimental procedures

For the purity analysis of two extracted bands of Acornine 1 and Acornine 2 from preparative Thin layer chromatography (TLC) plates, analytical HPLC experiment on a C-18 column was carried out with solvent A-water/ac and solvent B-methanol. Liebermann-Burchard test for triterpenoids was carried out by dissolving 0.001 gm of the sample in chloroform and treating the solution with one drop of concentrated H₂SO₄ and one drop of acetic anhydride when a pink coloration is developed. Optical rotations were measured on a Bellingham + Stanley Ltd. ADP-410 polarimeter at 20°C. The Fourier transform infrared (FTIR) spectra were recorded on a Varian FTIR 640 spectrometer with 2 mg of the sample milled in 50 mg of potassium bromide (KBr) and then compressed into a thin pellet which was analyzed. The FTIR spectra were recorded within the range of 400-4000 cm⁻¹. The DART-MS was recorded on a JEOL-AccuTOF JMS-T100LC mass spectrometer having a DART source. The samples were subjected as such in front of the DART source. Dry helium was used with 4.0 LPM flow rate for ionization at 350°C. The orifice 1 potential was set at 28V. Data acquisition was from m/z 50 to 1000 and print outs were average spectra of 6-8 scans. The ¹H NMR spectra (for both Acornine 1 and 2) and ¹³C NMR spectra for Acornine 1 were recorded on a Bruker spectrometer (300 MHz) with Methanol-d₄ (MeOD) as the solvent; the chemical shifts are reported in parts per million (d). ¹³C NMR analysis of Acornine 2 (in MeOD) was carried out in a Bruker Avance NMR equipment at 600 MHz with TCI cryoprobe and data analyzed using standard Bruker Pulse program.

Plant materials

The mangrove plants used in this study were collected from Sundarban estuary in West Bengal state, India. These plant materials were identified by Dr. K. Naskar and Prof. K. Bhattacharya. The dried specimens of these plants were deposited in the Herbarium of Botany

department (Voucher no. VB/CBT/AR/005), Visva-Bharati University, India. Fresh bark of *A. corniculatum* was washed thoroughly with tap water to get rid of all foreign deposits and air dried under shade. Any area of the plant tissue that could not be cleaned totally free of foreign deposits were discarded. Dried tissues were then crushed into fine powder with the help of a mechanical grinder and stored at room temperature until used.

Extraction and isolation of Acornine 1 and Acornine 2

Powdered bark (1.5 kg) of *A. corniculatum* was used for the extraction of Acornine 1 and Acornine 2. The bark powder was extracted sequentially with 2 L each of hexane, benzene and chloroform in a 3 L soxhlet apparatus for 40 h each. The temperature for extractions was 65°C for hexane, 80°C for benzene and 60°C for chloroform. After the final extraction with chloroform in the soxhlet, the residual material was collected from the soxhlet apparatus and extracted further with 2 L of methanol at room temperature for 10 days after which the suspension was filtered. The filtrate containing the active substances was concentrated by air drying to a volume of about 150 mL. For the next round of purification, preparative TLC method was used. 75 g silica Gel G (Merck), as adsorbent, was mixed with 150 mL of distilled water to make a thick slurry. The slurry was immediately poured into a spreader and plates for TLC were prepared by spreading the slurry on 56 × 16.7 cm size glass plates with the thickness of the layer fixed at 2 mm. Plates were allowed to air dry overnight and the adsorbent layer on glass plate was fixed by drying it further at 75°C for 1 h just before use. Using a micropipette, about 700 μL of bark methanol extract of *A. corniculatum* was applied gradually at the point of application on the preparative TLC plates. The plates were then air-dried again and run in BAW (n-butanol-acetic acid-water: 4: 1.15: 5) running solvent in a closed glass chamber already saturated with BAW. The positions of the two separated bands with R_f 0.36 (representing Acornine 1) and R_f 0.32 (representing Acornine 2) on the chromatograms were localized after spraying carefully only a part of the TLC bed on one side with H₂SO₄/C₂H₅OH solution and heating it at 75°C for 5 min. The desired two bands from unstained portions of the plates were then scraped off and the substances inside were eluted from the silica with methanol which was next evaporated to dryness to obtain the pure compound in a powdery form. The pure materials were stored at 4°C till use. This process of preparative TLC was repeated several times to accumulate enough of the two compounds for subsequent analyses.

Structure elucidation

Partial structure elucidation of Acornine 1 and Acornine 2 was achieved using standard spectroscopic techniques: FTIR, ¹H and ¹³C NMR, DART-MS (data not shown). The

backbone structures were confirmed by comparison of the spectroscopy data with that in the literature.^[13]

Test organisms and other microbiological methods

The samples were screened against non-pathogenic fungi *S. cerevisiae* and *T. chryseus* and Gram-positive bacteria *Bacillus subtilis* and *Bacillus coagulans* as model organisms for routine tests. For testing against pathogenic fungi the purified compounds were screened against *Candida albicans*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The composition and preparation semi-solid (YPD-agar or Sabouard dextrose agar) or liquid (YPD or Sabouard dextrose broth) media, maintenance, propagation and growth of microorganisms etc., all were as described.^[14] Stock cultures of the microorganisms were maintained at 4°C on slants of YPD-agar. For experimental purpose, fungi were grown in Sabouard dextrose broth or YPD-broth. Active and healthy cultures of microorganisms for experiments were prepared by transferring single colony from the stock culture to 1.5 mL of Sabouard dextrose broth or YPD-broth and incubated at 180 rpm for 24 h at 30°C. These healthy mini cultures were used routinely as inoculums, after diluting them suitably in 0.9% saline, for the disc diffusion assays (Bauer *et al.*,^[15] and determination of MIC.^[16] The basidiomycetes fungus *T. chryseus* was maintained and grown in the laboratory as previously described.^[17,18]

Antifungal activity assay

The antifungal activity of different samples were determined by disc diffusion method^[14] using YPD-agar and PD-agar plates. The assay plates were prepared by pouring 30 mL of sterile agar-media into sterile 90 mm petridish and allowed to solidify. The inoculums prepared as described above were then spread uniformly on agar plates using a sterile cotton swab dipped into the experimental microbial suspension. Then paper discs (made from Whatman No. 1 filter paper) of 6 mm diameter were placed on the agar surface and 8 µL extract of concentration 250 mg/mL dissolved in dimethyl sulphoxide (DMSO) was spotted on each disc (2 mg/disc). The plates were then incubated at 30°C up to 36 h. Diameter of the inhibition zone around each disc was measured in mm. 3 µL of antibiotics (10 mg/mL) such as commercial fluconazole, an antifungal, were used as standards and DMSO as a negative control to determine the sensitivity of the microorganisms tested.

Minimum inhibitory concentration (MIC) was determined using Inhibitory Concentration in Diffusion (ICD) method.^[16] In this method the discs of sterile Whatman no. 1 filter papers (6 mm diameter) were placed on the surface of agar plates containing the spread-out microbial culture with the help of forceps; they were then spotted with serially diluted concentrations of Acornine 1 and

Acornine 2. For making these dilutions a stock solution each of Acornine 1 and 2 was prepared and then serially diluted (1:1) in DMSO to obtain different concentrations. The lowest concentration of a particular extract that inhibited the growth of a test microorganism was noted as the MIC value of that extract for that strain. MIC tests for antifungal activities using pathogenic fungi were carried out by Genohelix Corporation, Bangalore, India using the disc diffusion assays.

Thin layer chromatography and Bioautography

Silica GF₂₅₄ plates (Merck), 20 × 20 cm² and 0.2 mm thick, were used for analytical TLC. Methanol extracts of bark at a concentration of 100 mg/mL (5 µL) were applied on TLC plates. The chromatograms (in triplicate) were run in BAW as running solvents (composition described earlier). At the end of the run, the TLC plates were either (i) sprayed with C₂H₅OH-H₂SO₄ solution 95:5, v/v) and heated at 100°C for 5 min for the purpose of visualizing separated phytochemical components or (ii) subjected to bioautography (detailed below) for the purpose of identifying components having antifungal activities.

Bioautography is a useful technique to determine bioactive compound with antimicrobial activity from plant extract on TLC plate. For the purpose of detection of antifungal components present in the methanolic bark extracts of *A. corniculatum*, bioautography technique of Nostro *et al.*,^[19] was used with some modifications, after first separating these phytochemical components on TLC plates as described above. At the end of TLC run, YPD-agar containing 1% inoculums of *S. cerevisiae* were poured on these TLC plates. After solidification of the YPD-agar medium on the TLC plates, they were incubated for 24 h at 30°C. Subsequently, the bioautogram was sprayed with a 1% aqueous solution of 3-(4-5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incubated at 30°C for 1-2 h. Zones in which fungal growth was inhibited (due to the presence of a phytochemical component having antifungal activities on the TLC plate) failed to take the stain and indicated the presence of active compounds in that area of TLC plate. TLC-Bioautography technique showed how many active compounds are present in the experimental sample.

RESULTS AND DISCUSSION

Earlier we have reported a detailed characterization of antimicrobial activities found in the different tissues of this plant elsewhere.^[11] Following this lead, we characterized the antifungal activities in the bark extracts of *A. corniculatum* further. Figure 1 shows that the bark extract (methanol) of *A. corniculatum*, run in a TLC plate with BAW as the running

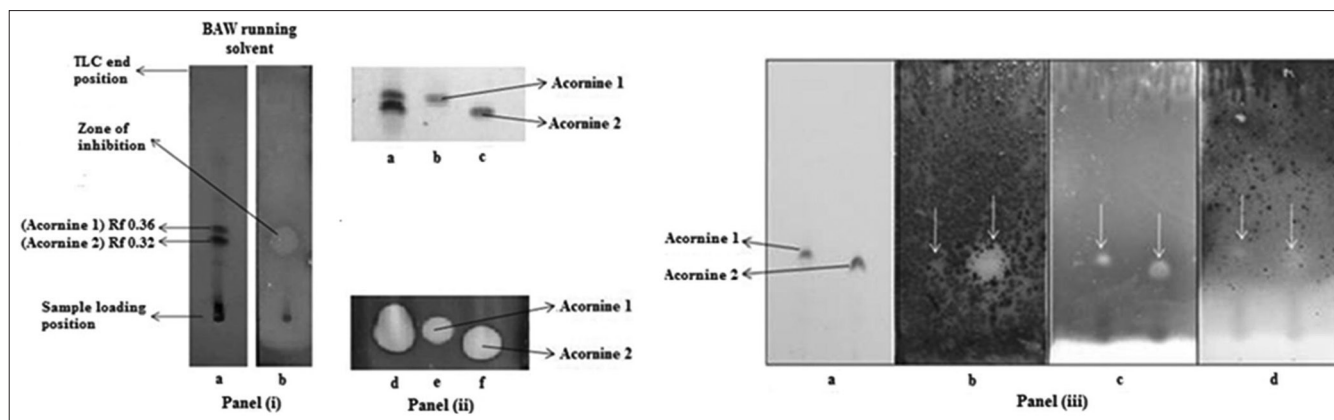


Figure 1: Antifungal activities of *A. corniculatum*. Panel (i) Lane a - TLC showing Acornine 1 and Acornine 2 in bark extract at R_f 0.36 and 0.32; Lane b - Growth inhibition of *S. cerevisiae* by R_f 0.36 and 0.32 bands. Panel (ii) - TLC (lanes a, b and c) and bioautography (lanes d, e and f) of bark extract, purified Acornine 1 and Acornine 2 respectively. Panel (iii) a: TLC of purified Acornine 1 and Acornine 2; Panels (iii) b, c and d - bioautography of Acornine 1 and Acornine 2 with *T. clypeatus*, *B. subtilis* and *B. coagulans* respectively. White arrows in Panel (iii) indicate positions of growth inhibitions

solvent system, exhibit the presence of two dominant bands at R_f of 0.36 and 0.32 [Figure 1. Panel (i), Lane a]; both these two bands show strong fungicidal activities when the same TLC plate is subjected to bioautography using *S. cerevisiae* as test organism [Figure 1. Panel (i), Lane b]. We have named the compounds representing these two bands as Acornine 1 (R_f 0.36 in BAW solvent system) and Acornine 2 (R_f 0.32 in BAW solvent system) for easy reference. We have taken advantage of the clear difference in the mobility of Acornine 1 and Acornine 2 [Figure 1. Panel (i), Lane a] in BAW solvent system for their eventual purification. Following preliminary and routine extractions of powdered bark of *A. corniculatum* using hexane, benzene and chloroform in a soxhlet apparatus and extraction in methanol, we used long-bed preparative TLC to separate Acornine 1 and Acornine 2 from each other. Then the bands were scraped off the TLC plate and the Acornine 1 and 2 were individually extracted with methanol and concentrated for further use [Figure 1. Panel (ii), Lanes b and c respectively]. Materials in the extracted bands, when tested for their fungicidal activities by bioautography technique using *S. cerevisiae*, were found to have retained the activity quite well [Figure 1. Panel (ii), Lanes e and f]. In addition, we tested the purified Acornines on *T. clypeatus*, (a basidiomycetes fungus), and *Bacillus subtilis* as well as *Bacillus coagulans*, both Gram-positive bacteria using TLC-bioautography method; both the Acornines exhibited growth inhibitory activity against the fungus *T. clypeatus* and the Gram-positive bacteria tested [Figure 1. Panel (iii), Lanes b, c and d respectively]. Acornines did not exhibit any detectable activity against Gram-negative bacteria (data not shown). Both the purified compounds were found to be white amorphous materials.

HPLC analysis was used next for the purpose of checking the purity and suitability of the isolated Acornine 1 and Acornine 2 for subsequent analyses. Figure 2 shows the

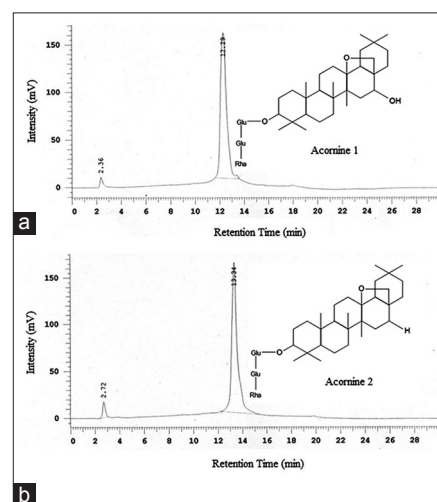


Figure 2: Analytical HPLC (c18, 5 μ M, 250 X 4.6 mm, UV detector) chromatograms of Acornine 1 (a) and Acornine 2 (b) isolated from the bark of *A. corniculatum*. Spectral data were recorded at 254 nm. Purity of both compounds was determined using the area and area% of the peaks, automatically calculated by the HPLC system software. Both Acornine 1 and 2 are in sufficient state of purity (more than 98% and 95% respectively) for structure determination

retention times of the two compounds. The larger peaks (at 12.29 min for Acornine 1 and 13.34 min for Acornine 2; Figure 2 (a) and Figure 2 (b) respectively) are that of the active compounds, each more than 95% pure, whereas very small peaks (at 2.36 min for Acornine 1 and at 2.72 min for Acornine 2) may indicate a negligible amount of contaminating phytochemicals in the otherwise pure samples. Both Acornine 1 and Acornine 2 give pinkish colouration in Liebermann-Burchard test suggesting their triterpenic nature.^[20]

Having purified the compounds Acornine 1 and Acornine 2 from the bark of *Aegiceras corniculatum*, they were tested on some pathogenic fungi having detrimental effects on

human health by determining their MIC values. MIC is the minimum concentration of the antimicrobial agent at which the growth of the microorganisms gets inhibited. The MIC values ($\mu\text{g}/\text{disc}$) of the Acornine 2 against the experimental organisms obtained are represented in Table 1. It shows that Acornine 2 is active against *Candida albicans*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The same fungi were not responsive to Acornine 1 treatments. However, there are still possibilities for Acornine 1 to be effective against some other fungi which we have not tested. Infrared (IR) spectroscopy is an excellent method for the identification of organic functional groups. It is often used to confirm the presence (or absence) of a specific functional group in a compound as well as the compound's purity. The FTIR spectra of Acornine 1 and Acornine 2 are presented in Table 2; they are almost identical to each other. It is evident that Acornine 1 and Acornine 2 in their IR spectra show a strong broad band at 3430 cm^{-1} characteristic of the stretching vibration of a free hydroxyl group. The appearance of strong bands at 1241 and 1022 cm^{-1} for stretching vibration of C-O bonding is suggestive of the presence of $\text{CH}_2\text{-O-C}$ linkage in the compound. The strong absorption at 2994 cm^{-1} is assignable to C-H stretching vibration of carbon-hydrogen frame work while the absorption at 1428 cm^{-1} may be ascribed to C-H stretching vibration of C-methyl groups in Acornine 1.

Study of ^1H NMR spectra of Acornine 1 and Acornine 2 has been done with a view to secure important information about the proton environments of the molecules. Accordingly, the ^1H NMR spectra were studied in deuterated methanol (MeOD) with a 300 MHz instrument. Tetramethyl silane (TMS) was employed as the internal standard. The chemical shift values along with their probable assignments are tabulated in Table 3. From the Table 3 it appears that the ^1H NMR spectra of Acornine 1 and Acornine 2 are almost similar and furnish a lot of valuable information which may help to establish the structures of these compounds. Thus the proton signals at δ 0.896, 0.921, 0.945, 1.22, 1.25, 1.28 and δ 1.30 in the ^1H NMR spectrum of Acornine 1 and the signals at δ 0.868, 0.926, 0.947, 1.23, 1.25, 1.28 and δ 1.30 in the ^1H NMR spectrum of Acornine 2 indicate the presence of seven tertiary C-methyl groups in these two triterpene saponins. The doublet of three protons around δ 1.65 ($J = 10\text{ Hz}$) in both the spectra is attributable to a secondary (CH-CH_3) protons in these two compounds. The presence of an epoxy linkage ($\text{CH}_2\text{-O-}$) in these triterpenoids is obvious from the appearance of a two proton singlet at δ 3.30. The three doublet signals at δ 3.98 ($J = 7\text{ Hz}$), 4.02 ($J = 7\text{ Hz}$) and δ 4.07 ($J = 7\text{ Hz}$) in the ^1H NMR spectra of both these compound reveal the presence of three anomeric protons of glycosidic moiety. The appearance of a singlet of one proton at δ 4.87 suggests that presence of a carbinol

Table 1: MIC ($\mu\text{g}/\text{disc}$) for antifungal activity of compound, Acornine 1 and Acornine 2 from *A. corniculatum*

Microorganisms	MIC ($\mu\text{g}/\text{disc}$)	
	Acornine 1	Acornine 2
<i>Candida albicans</i>	ND ^a	25-50
<i>Trichophyton mentagrophytes</i>	ND	25-50
<i>Trichophyton rubrum</i>	ND	50-100
<i>Saccharomyces cerevisiae</i>	37.5-75	4.67-9.38
<i>Termitomyces clypeatus</i>	ND	75-150

^aNot detected

Table 2: Infrared absorption spectrum of Acornine 1 and Acornine 2

Characteristic absorption band cm^{-1} (Acornine 1/Acornine 2)	Intensity	Probable assignment
3430/3430	s	O-H stretching vibration of OH group
2994/2994	s	C-H stretching vibration of carbon hydrogen frame work
1428/1428	vs	C-methyl group
1241/1241	s	$\text{CH}_2\text{-O-C}$ stretching vibration
1022/1022	s	C-O stretching vibration

Table 3: ^1H NMR proton signals of Acornine 1 and Acornine 2 in MeOD at 300 MHz

Chemical shift in δ (Acornine 1/ Acornine 2)	Number of proton	Multiplicity	Probable assignment
0.896/0.868	3	s	Tert C-Methyl
0.921/0.926	3	s	Tert C-Methyl
0.945/0.947	3	s	Tert C-Methyl
1.22/1.23	3	s	Tert C-Methyl
1.25/1.25	3	s	Tert C-Methyl
1.28/1.28	3	s	Tert C-Methyl
1.30/1.30	3	s	Tert C-Methyl
1.65/1.65	3	d ($J=10\text{ Hz}$)	Secondary C-methyl (CH-CH_3)
1.70-1.91/ 1.70-1.91	Number of protons	Series of peaks	Triterpenoid fingerprint (Methylene proton)
3.30/3.30	2	s	$\text{CH}_2\text{-O}$ linkage
3.98/3.98	1	d ($J=7\text{ Hz}$)	Anomeric proton
4.02/4.02	1	d ($J=7\text{ Hz}$)	Anomeric proton
4.07/4.07	1	d ($J=7\text{ Hz}$)	Anomeric proton
4.87/4.87	1	s	Carbinol methane (CH-OG)

methine of the glycosidic linkage (CH-OG). A series of short peaks appearing in the region δ 1.70 to 1.91 in the spectra of Acornine 1 and Acornine 2 are considered to be the triterpenoid fingerprint. Thus, the essential structural features of Acornine 1 and Acornine 2 that are revealed from the ^1H NMR spectra may be summarized as follows: The presence of (i) Seven tertiary C-methyl groups,

(ii) One secondary C-methyl group (CH-CH₃), (iii) Three anomeric protons of glycosidic linkage, iv) One carbinol methine as >CHOH and v) one epoxy linkage. The above structure of Acornine 2 is also in agreement with the high resolution ¹³C NMR spectral data described in Table 5. ¹³C NMR data of Acornine 1 is practically completely identical with that of Acornine 2 excepting the carbon signal at C16 (data not shown) and confirms the proposed structure of Acornine 1.

Acornine 1 and Acornine 2 were subjected to electron impact mass spectral studies next. Various mass peaks obtained from these two triterpenoidal saponins and the probable interpretations of the genesis of these mass peaks are elaborated as follows: The mass spectrum [Table 4] of Acornine 1 exhibits molecular ion peak at m/z 928 which is in complete harmony with the molecular composition, C₄₈H₈₀O₁₇. The appearance of significant peaks in the mass spectrum at m/z 678 and at m/z 249 resulting from retro-Diels-Alder collapse around ring (C) under electron impact reveals the presence of oleanane type of pentacyclic triterpenoid skeleton in the compound.^[21] However, the absence of any olefinic proton signal in its ¹H NMR spectrum as well as the presence of an epoxide functionality in Acornine 1 suggests the presence of an epoxide group in the compound. This assumption is further supported by the appearance of ion peaks containing ring D and E at m/z 249 and 219 [249-30(-CH₂-O)] in the mass spectrum. Further, the display of ion peak at m/z 201 (219-H₂O) by the mass spectrum indicates the presence of a hydroxyl function in the ring (D) probably at C-16 position. It may be mentioned that C-16 H proton signal probably overlapped in the region around δ 3.20. Once again, ion peaks at m/z 782, 620, 458 and at m/z 457 in the mass spectrum is suggestive of the presence of gluc-gluc-rha type of glycosidic linkage. The appearance of the peak at m/z 678 in the mass spectrum generated by retro-Diels-Alder cleavage represents the rings A/B. The mass value of this ion peak indicates the presence of the glycosidic linkage in the rings A/B portion and its location at C3 is highly probable from biogenetic background. The mass spectrum of Acornine 2 [Table 4] is almost identical in all respects with that of its congener Acornine 1 excepting in the mass values of molecular ion peaks which appears at 16 mass units less than that of Acornine 1 is at at m/z 912 as well as of other ion peaks. As expected it exhibits ion peaks at m/z 912 (M⁺), 678, 233, 203, 766, 604, 442 and m/z 441. From the analyses of different spectral characteristics described above the most probable structures of Acornine 1 and Acornine 2 may be elaborated as shown in Figure 2. The nomenclature of the isolated compounds may be designated as follows:

Oleanane 13,28 epoxy-16 hydroxyl-3-glycoside (Acornine 1)
Oleanane 13,28 epoxy-3-glycoside (Acornine 2).

Table 4: Mass spectrum of Acornine 1 and Acornine 2

Acornine 1/Acornine 2	
Characteristic absorption band (m/z)	Probable assignment
678 and 249/678 and 233	retro-Diels-Alder collapse around ring (C) reveals the presence of oleanane type of pentacyclic triterpenoid skeleton
201 (only in Acornine 1)	[219-18 (H ₂ O)], presence of hydroxyl functional group in the ring (D) probably at C-16 position
219/203	[249-30 (-CH ₂ -O)]/[233-30 (-CH ₂ -O)]
782/766	[928-146 (Rha)]/[912-Rha]
620/604	[928-146+162(Rha+Gluco)]/[912-(Rha+Gluco)]
458/442	[928-146+162+162 (Rha+Gluco+Gluco)]/[912-(Rha+Gluco+Gluco)]

Rha: Rhamnose, Gluco: Glucose

Table 5: ¹³C NMR (600 Mhz) data of Acornine 2

Triterpene portion		Sugar portion	
Carbon atom	Chemical shift (δ)	Carbon atom	Chemical shift (δ)
C-1	38.9	C-1	100.15
C-2	26.08	C-2	77.50
C-3	89.50	C-3	78.03
C-4	38.00	C-4	73.50
C-5	57.82	C-5	76.6
C-6	18.05	C-6	180.6
C-7	30.91	C-1'	100.30
C-8	48.73	C-2'	79.00
C-9	48.87	C-3'	76.02
C-10	36.00	C-4'	71.03
C-11	23.90	C-5'	80.00
C-12	32.65	C-6'	65.07
C-13	86.56	C-1''	100.40
C-14	44.18	C-2''	75.30
C-15	28.09	C-3''	77.10
C-16	25.08	C-4''	68.99
C-17	32.00	C-5''	69.50
C-18	49.41	C-6''	16.91
C-19	40.00		
C-20	40.03		
C-21	30.03		
C-22	36.18		
C-23	25.08		
C-24	16.50		
C-25	20.03		
C-26	18.12		
C-27	18.94		
C-28	72.17		
C-29	72.38		
C-30	24.83		

Wahidullah *et al.*,^[13] isolated and characterized an oleanane triterpenoid oligoglycoside having antifungal activity from this plant which they named as corniculatonin. The deduced structure for corniculatonin^[13] has similarities with that of Acornine 1 and Acornine 2. Both corniculatonin

and acornines have oleanane triterpenoid backbones. The points of major differences are however, the (i) existence of only three sugar units in cases of Acornine 1 and Acornine 2 as opposed to five in case of corniculatonin and (ii) absence of hydroxyl group (OH) in Acornine 2. In addition, Wahidullah *et al.*,^[13] did not report any Gram-positive antibacterial activities for corniculatonin. Because we do not have corniculatonin we cannot determine the relative efficiency of this compound compared to the Acornines. Acornine 2 appears to be more efficient than Acornine 1 where antifungal activities are concerned. It is not clear at this time whether the absence of the –OH group in Acornine 2 is responsible for the observed differences in antifungal activities between themselves. Also, the backbone of the three compounds (corniculatonin and acornines) remaining similar to each other, yet the observation that Acornine 1 shows reduced activities compared to Corniculatonin and Acornine 2 may shed lights on the roles of side carbohydrate moieties in the observed antifungal activities and may help in the elucidation of their mechanism of action. Mention must be made here that Wahidullah *et al.*,^[13] collected their plant samples from the west coast of India whereas our plant samples were collected from Sundarban estuarine areas in the east coast of India. Our findings suggest the presence in *A. corniculatum*, collected from Sundarban regions of West Bengal state of India, of two naturally occurring compounds having antifungal activities that are structurally similar to corniculatonin but not identical to it. When the bark extracts of our plant samples were run following the conditions described by Wahidullah *et al.*,^[13] in a TLC experiment, we have not seen any component having the mobility characteristic even close to that of corniculatonin described by them (data not shown). Added to this, when one considers the structural similarities of corniculatonin and Acornines, it prompts us to opine that Acornine 1 and 2 may be different metabolic manifestations of corniculatonin expressed differently under different microenvironments. From that viewpoint, Acornine 1 and 2 may be regarded as novel compounds. We have named these two compounds as Acornine 1 (Oleanane 13,28 epoxy-16 hydroxyl-3-glycoside; R_f 0.36 in BAW solvent system) and Acornine 2 (Oleanane 13,28 epoxy-3-glycoside; R_f 0.32 in BAW solvent system) to distinguish them from corniculatonin described by Wahidullah *et al.*^[13]. Acornine 2, in particular, was effective as antifungal against several pathogenic fungi tested and also against some Gram-positive bacteria.

CONCLUSION

We isolated two compounds having notable antifungal activities from the bark of *Aegiceras corniculatum*, a mangrove

plant, found in the Sundarban estuary of India. We named the two compounds as Acornine 1 (Oleanane 13,28 epoxy-16 hydroxyl-3-glycoside) and Acornine 2 (Oleanane 13,28 epoxy-3-glycoside). Acornine 2 was effective as antifungal against several pathogenic fungi tested and also against some Gram-positive bacteria.

Wahidullah *et al.*,^[13] collected plant samples from western coast of India whereas plant samples used in this study were collected from the Sundarban areas in the east coast of India. The differences in the microenvironments and biotic as well as abiotic stress factors in two geographically distinct coastal areas may be the reasons why the plant is synthesizing the compounds having antifungal activities in different ways. Differential gene expression under changed environments may have enabled the synthesis of slightly different compounds. Each of these compounds (corniculatonin and Acornines) may be endowed with better ability that is suited best in the particular environment in which the plant is growing for combating differential threats (biotic and abiotic) existing in two coastal areas of India having different climatic and stress conditions. Additionally, our finding strengthens the well-known belief that (i) environmental factors dictate the secondary metabolites profile of medicinal plants and (ii) the same plant species growing in different natural habitats around the world may have different metabolite profiles with respect to these compounds. Acornine 1 and Acornine 2 are previously unreported compounds having antifungal activities. Presence of both antifungal and Gram-positive antibacterial activities indicates necessity of further studies of these two compounds.

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