Research Article

Antipseudomonal Ergosteryl Triterpenes from the Paste of *Spondias pinnata* kruz. Bark, Pre-Treated with Curd-Brew

Tapan Kumar Lai[1*], Krishnendu Acharya[2], Soumya Chatterjee[2], Nima Lamu Sherpa[2], Anindita ghosh[3]
[1]Department of Chemistry, Vidyasagar Evening College, 39, Sankar Ghosh Lane, Kolkata 700006, India.
[2]Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, India.
[3]Department of Chemistry, University of Calcutta, 92, A. P. C. Road, Kolkata 700009, India.
***Corresponding author's E-mail:** tapan_leo1@rediffmail.com

Accepted on: 06-07-2014; **Finalized on:** 31-08-2014.

ABSTRACT

Two new ergosteryl triterpenes were isolated from ethnopharmacological lead of Indian origin consisting of a paste of *Spondias pinnata* barks, pre-treated with curd brew. The chemical structures of the newly isolated compounds were established from detailed IR, 1H NMR, 13C NMR, GCMS and HRMS spectroscopic analyses. The triterpenes were evaluated for their antipseudomonal activities against a tetracycline and ampicillin-resistant strain of *Pseudomonus aeruginosa* by disc diffusion method. One of the compounds exhibited significant protection against the test microbe, comparable to streptomycin.

Keywords: Antimicrobial, Column chromatography, Ethnomedicine, *Pseudomonus, Spondias pinnata,* Spectroscopy.

INTRODUCTION

Pseudomonas aeruginosa is intrinsically resistant to a large range of antimicrobials and is one of the most common pathogens in nosocomial infections as well as microbial substitutions. The growing instances of antimicrobial resistance in *P. aeruginosa* severely compromise the selection of appropriate antibacterial treatment and are therefore associated with significant morbidity and mortality[1]. Reason for such resistance lies in the extraordinary capacity of this microorganism to undergo mutation under any applied condition. The resistance problem demands that a renewed effort be made to seek antibacterial agents effective against pathogenic strains resistant to current antibiotics. Unfortunately, in recent years, little progress has occurred in developing novel anti pseudomonal agents that can overcome multi drug resistance in *P. aeruginosa*.

Herbal medicines have been the basis of treatment for various ailments in many world cultures since ancient times. According to World Health Organization (WHO) traditional medicines are relied upon by 65 – 80% of the World's population for their primary healthcare needs. In addition to that, emergence of multiple drug resistant strains of microorganisms due to indiscriminate use of antibiotics has generated a renewed interest in herbal medicine[2], which has culminated in the discovery of several excellent antimicrobials[3]. In our endeavour of finding newer antimicrobials from nonconventional sources, a curd-brew cultured paste of *Spondias pinnata* Kruz. barks (synonym *Spondias mangifera* Willd., family Anacardiacea) was selected for systematic phytochemical as well as biological evaluation. This particular concoction is valued as a medicine for healing ulcerated wounds in some aboriginal cultures across India. Bark paste of *S. pinnata* is applied externally to treat articular and muscular rheumatism. Other parts of this plant find use in various ailments too, e.g. fruits, leaves, bark as astringent, antidysenteric, antispeptic, and antiscorbutic. Roots are used for regulating menstruation. *The Ayurvedic Pharmacopoeia of India* recommends stem bark in haemorrhagic diseases[4]. Leaves of this plant contain antiviral caffeoyl esters[5]. A related species *S. mombin*, however, finds extensive uses in uterine stimulation and abortive action[6], as muscle relaxant[7], as anti-anxiety, sedative and anti-convulsant agent[8], for antioxidant, anti-inflammatory and anti-microbial activities[9], for cytotoxic and anticancerous activities[10]. However, there is no scientific record of any sort whatsoever, on this medicinal preparation till now. Current study was aimed at the isolation and characterization of active compounds from this cultured paste and evaluation of its antimicrobial activities. Two new triterpenoid molecules were isolated and characterized through detailed spectroanalytical studies. One of the compounds exhibited significant *in vitro* antipseudomonal activity against a moderately resistant strain of *P. aeruginosa*.

MATERIALS AND METHODS

General

Melting points were determined on a capillary melting point apparatus from Sunvic, UK. IR spectra were recorded on a Perkin Elmer 300E FTIR instrument. ^{1}H and ^{13}C NMR spectra were obtained on a 300 MHz Bruker AV-300 spectrometer, with a 5 mm inverse probe using residual solvent signal as internal standard. The GCMS analyses were performed on a GCMS-SHIMADZU-QP 5050A mass spectrometer (GasPro fused silica column 30m x 0.32mm, helium as carrier gas, at 1ml/min from 90°C to 300°C at 5°C/min coupled to a 70 eV ionization source). Column chromatography (CC) was carried out

with silica gel (230 – 400 mesh, Merck) on a large glass column (8.5 cm x 170 cm), and analytical chromatography was performed using silica gel 60 Art. 5553 (0.20 mm, Merck). All the solvents were purified before use.

The plant material

The barks of the plant *Spondias mangifera* i.e parts of its epididermis and cortex were procured from local suppliers in Calcutta and identified by Dr. K Acharya, Associate Professor of Botany and a senior taxonomist of the University of Calcutta. The barks were mashed and mixed with sufficient curd brew in order to form moulds. The shade dried moulds were ground and 310 g of this material was subjected to extraction with chloroform-methanol mixture. Concentration of the extract under reduced pressure yielded 75 g residue. It was purified through column chromatography in silica-gel using a mixture of hexane and ethyl acetate as eluent starting from hexane only to a ratio of 1:4.

Anti pseudomonal studies

Test organisms- The test organism *Pseudomonas aeruginosa* MTCC 8158 was obtained from Microbial Type Culture Collection, Chandigarh, India. The culture was maintained in nutrient agar medium by subculturing for subsequent use.

In vitro susceptibility test for antipseudomonal activity

In vitro susceptibility tests were performed by agar disc diffusion method to evaluate minimal inhibitory concentrations (MICs) of both these compounds. MICs were determined with the adjusted inoculum suspension of 2×10^6 cfu/mL by diluting it 100 times with nutrient broth medium to get a final inoculum concentration of 2×10^4 cfu/mL. Sterilized discs were saturated with different concentrations (12.5-200 µg/disc) of the compound **SP-40** in 20% *v/v* dimethyl sulfoxide and used for evaluation of antipseudomonal activity. Standard antifungal susceptibility test discs containing ampicillin, tetracycline and streptomycin from Himedia Laboratories Pvt. Ltd, Mumbai, India were used as positive control. 20% aqueous DMSO was used as negative control. The plates were incubated at 37°C for 24 h and the MIC values were recorded as the lowest concentration at which significant inhibition of growth was observed.

RESULTS AND DISCUSSION

Phytochemical analysis

The combined extracts were concentrated, washed with water, dried and then loaded onto a large glass column of silica gel (8.5 x 170 cm). Elution of the column over a hexane-ethyl acetate gradient yielded two major fractions, A and B; which upon further purification afforded two analytically pure compounds I, or SP-40 and II, or SP-60.

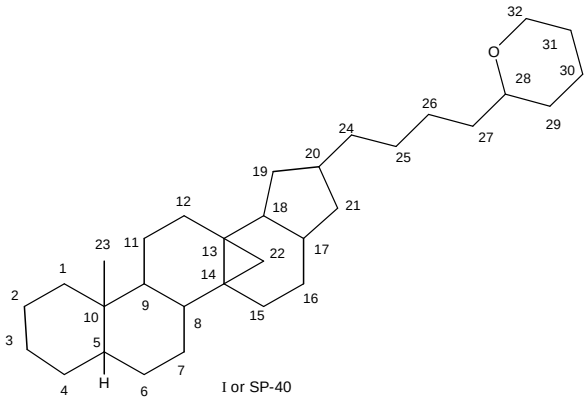
SP-40, melting point 141°C, was obtained as white amorphous powder from the eluate at 12% ethyl acetate in hexane fractions followed by crystallization from acetone. The molecular mass of SP-40 was settled to be 452.5 from GCMS peak at *m/z* 452.50. The molecular formula $C_{32}H_{52}O$ was thus obtained by comparing with its calculated value (Molecular mass calcd. for $C_{32}H_{52}O$ is 452.45) requiring seven double-bond equivalents. Indication for a lanostane-type structure was obtained from the GCMS fragment peak at *m/z* 311.3. Other fragment peaks of significance were observed at *m/z* 438.3 $(M+H -CH_3^{\cdot})^+$, 424.4 $(M - C_2H_4)^+$, 420.4 $(M - CH_3OH)^+$, 410.5 $(M - C_3H_6)^+$,396.4 $(M - C_4H_8)^+$, 392.3 $(M - C_3H_7OH)^+$ and 368.3 $(M - C_5H_8O)^+$. The thin film IR spectrum (KBr disc) showed no absorbance around 3400 cm^{-1} indicationg the absence of OH group. However, it exhibited C – H stretch bands at 2919 – 2849 cm^{-1}, a strong CH_2 bending absorbance at 1468 cm^{-1}, a very feeble CH_3 bending absorbance at 1390 cm^{-1}. The C – O stretch band at 1061 cm^{-1} indicates the presence of a C – O – C fragment. Another weak absorption at 1010 cm^{-1} was later on, attributed to a cyclopropane moiety.

The ^1H NMR spectrum showed two low field triplets at δ_H 3.64 (1H, t, *J*=6.6 Hz) and at δ_H 2.35 (2H, t, *J*=7.5 Hz) attributable to hydrogens attached to oxygen bearing carbons of the heterocyclic ring i.e. H–C(28) and H–C(32). Another pair of complex triplets were observed at δ_H 1.61 (2H, t, *J*=7.0 Hz) and at δ_H 1.57 (2H, t, *J*=7.2 Hz) and were ascribed to the ß-carbons to the oxygen of the heterocyclic ring i.e. H–C(29) and H–C(31) respectively. In both these cases, one or more of the neighboring protons did not participate in coupling as they might have fallen in the non-coupling dihedral range of 72° to 108° in the pyran ring. The remaining part of the ^1H NMR spectrum was packed with overlapping signals indicative of simple non-functionalized aliphatic or alicyclic system.

The ^{13}C NMR spectrum and DEPT Experiments accounted for altogether thirty two carbon resonances, comprising one methyl, twenty one methylene, seven methine and three quaternary carbons. Only two low-field signals were observed at δ_C 78.5 ppm (CH) and 63.1 ppm (CH_2) ascribable to the two oxygen bearing carbons, i.e. C(28) & C(32). Other notable features include a large number of carbon resonances at δ_C 29.7 ppm, a surplus of CH_2 signals, out of which one was observed at unusually high field region at δ_C 22.7 ppm and a single methyl signal at δ_C 14.1 ppm. The CH_2 signal at δ_C 22.7, coupled with a high degree of DBE in the molecular formula was accommodative of a cyclopropane moiety. On this basis a triterpene structure I was proposed for the molecule.

SP-60, melting point 188°C, was obtained as colourless needles from the eluate at 25% ethyl acetate in hexane fractions followed by crystallization from $CHCl_3$-MeOH. The molecular mass of SP-60 was settled to be 414.4 from GCMS peak at *m/z* 414.40. The molecular formula $C_{28}H_{46}O_2$ was thus obtained by comparing with its calculated value (Molecular mass calcd. for $C_{28}H_{46}O_2$ is 414.35). This formula requires six double-bond equivalents. Indication for a lanostane type structure was obtained from the GCMS fragment peak at *m/z* 303.3.

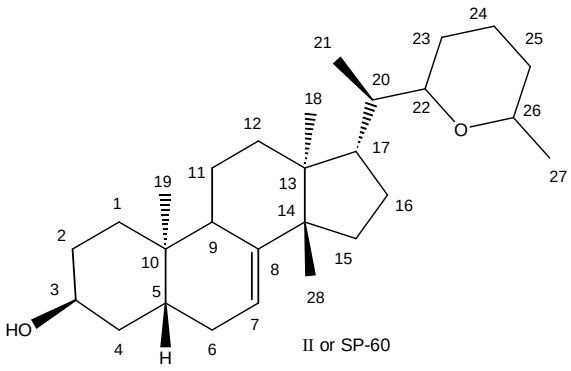
Other notable fragment peaks were observed at *m/z* 396.4 $(M - H_2O)^+$, 399.4 $(M - CH_3)^+$, 329.3 $(M - CH_3–C_5H_{10})^+$and 289.3 $(M - C_8H_{13}O)^+$.



I or SP-40

The thin film IR spectrum showed a broadened band around 3436 cm^{-1} and a matching absorption at 1057 cm^{-1} indicating the presence of hydroxyl group. Two sharp absorptions at 1460 cm^{-1} and 1376 cm^{-1} were ascribed to CH_2 bend and CH_3 bend vibrations respectively. The ^1H NMR spectrum was not much informative. However, a very interesting structural aspect was clarified from the septet like deshielded signal at δ_H 3.45. Careful inspection of the pattern revealed that it was actually a broadened doublet of quintet (1H, dq, *J*=10 & 5 Hz), a bit overlapped and was ascribed to H–C(3). One of the *trans* couplings resulting in the doublet with a relatively large coupling constant and other four coupling constants, including that for the O–H, accidentally matched giving it a quintet pattern. This confirmed that the dimethyl groups of the lanostane moiety at C(4) are actually absent here. Another notable feature of the ^1H NMR spectrum is the doublet signal at δ_H 5.28 (1H, d, *J*=5 Hz), ascribable to the vinylic proton, H–C(7), coupled with one of the adjacent protons at C–6.

It also revealed the presence of altogether five methyl groups; three singlets at δ_H 1.18, δ_H 0.94, δ_H 0.78 ppm along with two doublets at δ_H 0.74 (3H, d, *J*=6.8 Hz) and δ_H 0.62 (3H, d, *J*=5.4 Hz) respectively. The protons in the vicinity of another oxygen atom, i.e. H–C(22) and H–C(26) appeared as complex multiplets at around δ_H 2.20 ppm. The ^{13}C NMR spectrum and DEPT experiments accounted for all the twenty-eight carbon resonances, comprising five methyl, eleven methylene, eight methine and four quaternary carbons. The presence of an endocyclic double bond was supported by the low field quaternary carbon signal at δ_C 140.8 and another low field methine carbon signal at δ_C 121.7. The deshielded methine carbon signals at δ 56.9 and 56.1 were ascribed to two carbon atoms attached to the oxygen atom, i.e. C(22) and C(26). Another couple of relatively deshielded CH signals at δ_C 50.2 and δ_C 45.9 were ascribed to the allylic carbon at C(9) and homoallylic carbon at C(5) respectively. The C(3) resonance at δ_C 71.8 confirmed a typical 3-oxygenated steroidal skeleton. Based on all these observations, the triterpene structure II has been proposed for the compound SP-60.



II or SP-60

A complete assignment of all the hydrogen and carbon resonances of I & II has been given in Table 1.

Table 1: ^1H (300 MHz) and ^{13}C (75.5 MHz) NMR Spectral Data for Triterpenes SP-40 and SP-60 in $CDCl_3$

	SP-40		SP-60	
Position	**δ_C**	**δ_H, multi, (*J* in Hz)**	**δ_C**	**δ_H, multi, (*J* in Hz)**
1	29.7	1.25, m	26.2	1.19, m & 2.16, m
2	29.4	1.22, m	39.8	1.42, m
3	29.7	1.30, m	71.8	3.45, dq (10, 5)
4	29.7	1.32, m	42.3	
5	33.8	1.27, m	45.9	1.92, m
6	24.7	1.25, m	34.0	1.92, m
7	25.7	1.25, m	121.7	5.28, d (5)
8	30.0	1.25, m	140.8	
9	30.0	1.28, m	50.2	1.79, m
10	29.8		31.9	
11	22.7	1.25, m	24.3	1.20, m
12	29.7	0.90, m	31.7	1.20, m
13	29.1		31.9	
14	29.4		36.5	
15	29.2	1.25, m	28.2	1.38, m
16	29.7	1.23, m	21.1	1.44, m
17	29.9	1.25, m	36.1	1.10, m
18	29.8	1.25, m	19.8	0.76, s
19	29.7	1.29, m	19.0	0.78, s
20	29.7	1.31, m	29.2	2.20, m
21	29.7	1.30, m	12.0	0.70, d (5)
22	22.7	0.88, s	56.9	2.20, m
23	14.1	1.25, s	37.3	0.84, m
24	29.7	1.24, cm	23.1	1.78, m & 2.22, m
25	29.7	1.25, m	29.7	0.76, m
26	29.7	1.25, m	56.1	2.20, m
27	33.7	1.54, m	18.8	0.75, d (6)
28	78.5	3.64, t (6.6)	19.4	0.94, s
29	31.9	1.61, t (7)	--	--
30	29.7	1.25, cm	--	--
31	32.8	1.57, t (7.5)	--	--
32	63.1	2.35, t (7.5)	--	--

cm = complex multiplet, dq = doublet of quintet

Antipseudomonal screening

The antipseudomonal activities of compounds **SP-40** and **SP-60** were studied by agar disc diffusion method against a moderately resistant strain of *Pseudomonas aeruginosa* MTCC 8158. The test organism was completely resistant to ampicillin at 10 µg/disc concentration and tetracycline at 30 µg/disc concentration and exhibited a nominal inhibition zone of 15 mm against streptomycin at 100 µg/disc concentration. **SP-40** exhibited an inhibition zone of 20 mm, better than streptomycin, at comparable concentrations (Table 2). **SP-60**, however, did not show any antimicrobial activity against this organism up to a concentration of 200 µg/disc. The MIC values of **SP-40**, thus works out to be inbetween 25 and 12.5 µg/disc. Consequently, **SP-40** may be considered as a potential new candidate for antipseudomonal treatment, laden with immense promise.

Table 2: *In vitro antipseudomonal activity against Pseudomonas aeruginosa* MTCC 8158

Compound	Concentration (µg/disc)	Inhibition zone (mm)
SP-40	200	25±2
	100	20±2
	50	14 ± 1.5
	25	10±1.5
	12.5	NZ*

**NZ means no significant zone of inhibition. Values are mean ± SD of three separate experiments, each in triplicate.*

CONCLUSION

Traditional wisdom recommends this particular cultured product for healing ulcerated wounds. Present study has revealed the chemical composition of the organic extract of this ethnomedicinal preparation. At least one of the triterpenes isolated from it exhibited significant *in vitro* antipseudomonal activity. These results provide better understanding of *Spondias pinnata* pharmacology and its positive health impacts in general. Knowledge of extensive human application, together with the edible nature of all the raw materials suggests that this particular mixture may be non-toxic in nature. However, further pharmacological and toxicological investigations are essential for complete understanding of its medicinal application.

Acknowledgement: The authors want to thank the financial supports from the University Grants Commission, New Delhi, India under Grant sanction no. F-PSW-039 of 07-08 to TKL.

REFERENCES

1. Juan C, Zamorano L, Pe´rez Jose´ L, Ge Y, Oliver A, Activity of a New Antipseudomonal Cephalosporin, CXA-101 (FR264205), against Carbapenem-Resistant and Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Strains, Antimicrobial Agents and Chemotherapy, 54, 2010, 846–851.
2. Chopra I, Hodgson J, Metcalf B, Psote G, The search for antibacterial agents effective against bacteria resistant to multiple antibiotics, Antimicrobial Agents and Chemotherapy, 41, 1997, 497–503.
3. Iwu MW, Duncan AR, Okunjii CO, New Antimicrobials of Plant Origin, In Perspectives on new crops and new uses, Edited by Janick, ASHS Press, Alexandria, Virginia, 1999, 457-462.
4. Khare CP, Indian Medicinal Plants - an illustrated dictionary, first ed, Springer-Verlag, Berlin, 2007, 623-624.
5. Corthout JPL, Claeys M, Vanden Berghe D, Vlietinck A, Antiviral caffeoyl esters from *Spondias mombin*, Phytochemistry, 31, 1992, 1979-1981.
6. Offiah VN, Anwanyu II, Abortifacient activity of an aqueous extract of *Spondias mombin* leaves, Journal of Ethnopharmacology, 26, 1989, 317-320.
7. Akubue PI, Mittal GC, Aguwa CN, Preliminary pharmacological study of some Nigerian medicinal plants, Journal of Ethnopharmacology , 8, 1983, 53-63.
8. Ayoka AO, Akomolafe RO, Iwalewa EO, Akanmu MA, Ukponmwan OE, Sedative, antiepileptic and antipsychotic effects of *Spondias mombin* L. (Anacardiaceae) in mice and rats, Journal of Ethnopharmacology, 103,2006, 166-75.
9. Calderon AI, Angerhofer CK, Pezzuto JM, Farnsworth NR, Foster R, Condit R, Gupta MP, Soejarto DD, Forest plot as a tool to demonstrate the pharmaceutical potential of plants in a tropical forest of Panama, Economic Botany, 54, 2000, 278-294.
10. Idu M, Ataman JE, Akhigbe AO, Ucho OG, Akinbo SF, Idu FK, Studies on the nutritional value and anti-tumour property of the bark of *Spondias mombin* L., Journal of Medicine and Biomedical Research, 1, 2002, 223-228.

Source of Support: Nil, **Conflict of Interest:** None.