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Antibacterial Activity of Kauranes from *Annona squamosa* L. Pericarp

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Bioactivity directed fractionation and isolation of active principles from the pericarp of *Annona squamosa* yielded three kauranes namely, (-)-ent-kaur-17-acetoxy, 19-oic acid, 16α, 17-dihydroxy-ent-kauran-19-oic acid and (-)-ent-kaur-17, 19-dioic acid. These compounds were tested against two bacteria, *Staphylococus aureus* (MTCC 737, S.N. Saxena, *Staphlococcus* Institute of Microbial Technology) and *Escherichia. coli* (MTCC 2991, N. Goel, Enterobacteriaceae, Institute of Microbial Technology). 16α, 17-Dihydroxy-ent-kauran-19-oic acid and (-)-ent-kaur-17, 19-dioic acid at 100 μg and 500μg, respectively, showed more activity towards *Escherichia. coli* than the antibiotic, ampicillin at 500 μg and these kaurenes do not show promising activity towards *Staphylococcus aureus*, when compared with the standard drug, gentamycin. Here, we report the bioactivity directed fractionation, isolation and identification of the active principles by IR, NMR, MS and by the chemical conversions of the active compounds.

Key Words: *A. squamosa*, *Annonaceae*, Antibacterial activity, Pericarp.

INTRODUCTION

Annonaceae family composed of a large number of plants comprising about 120 genera and more than 2000 species. A large number of *Annonaceae* plants have been described as cytotoxic and are used as folk medicine for various types of tumours and cancers[1]. *Annona squamosa* is a small, more or less evergreen tree of 15-20' high bearing yellowish green fruit having 3-4 inches diameter[2]. Anti-bacterial studies on the alkaloids obtained from *Annona cherimoya* were reported[3]. Cytotoxic as well as antimicrobial activity of compounds isolated from *Annona densicoma* and *Annona Montana* were carried out[4,5]. 16α-Hydroxy-(11)-kaurenoic acid was studied for the selective cytotoxicity against cancer cells[6]. Kauranes from Homalanthus accuminatus were found to be active towards HIV virus[7].

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EXPERIMENTAL

A. squamosa (Annona, squamosa, RRL/BJ/9, Annonaceae) fruits were collected from a tree near Pappanamcode, Trivandrum, India and were identified by Mrs. Valsala, Taxonomist, University of Kerala and a voucher specimen has been deposited at Regional Research Laboratory, Trivandrum.

Method of extraction, fractionation and isolation: The shade-dried pericarp was powdered and extracted successively with hexane, chloroform and methanol. As the hexane and chloroform extracts showed similarity in TLC and in their antibacterial effects, the samples were mixed and subjected to fractionation using hexane, 80:20 hexane:ethyl acetate, 50:50 hexane:ethyl acetate, 100 % ethyl acetate and 100 % methanol. Bioactivity directed fractionation and isolation yielded three kaurane type diterpenes and their chemical structures were analyzed using IR, NMR and MS and confirmed their structures by chemical conversions.

Spectral data of (-)-ent-kaur-17,19-dioic acid

IR: (KBr, ν_{max}) 2950, 2850, 1705, 1480, 1280, 960, 750 cm^{-1}. Mass spectra: M^+ 334, 290 (11), 271 (14), 210 (35), 162 (34), 151 (50), 91 (56), 77 (25), 57 (41), 43 (31), 40 (58), 39 (100), 32 (100, 28 (100). ^1H NMR (300 MHz, $CdCl_3$/TMS)(δ): 0.93 (s, 3H, -Me), 1.04795 (s, 3H, -Me), 9.75 (s, 1H, -COOH, C-17), 9.8 (s, 1H, -COOH, C-19), 0.925-2.9 (M, 28H), ^{13}C NMR (75 MHz, $CdCl_3$/TMS) (δ_c): 41.80 (C-1), 19.3 (C-2), 38.5 (C-3), 44.2 (C-4), 57.4 (C-5), 56.7 (C-6), 22.3 (C-6), 41.6 (C-7), 44.7 (C-8), 55.5 (C-9), 41.2 (C-10), 18.9 (C-11), 33.6 (C-12), 44.3 (C-13), 40.2 (C-14), 49.4 (C-15), 45.5 (C-16), 179.06 (C-17), 29.5 (C-18), 178.31 (C-19), 15.74 (C-20).

The methyl ester is produced by reacting with diazomethane and the product was crystallized from hexane: ethyl acetate mixture and spectral studies were carried out and finally confirmed the structure of the compound as (-)-ent-kaur-17,19-dioic acid with m.p. 222ºC (Fig. 1).

Spectral data of (-)-ent-kaur-17-acetoxy-19-oic acid

IR: (KBr, ν_{max}) 3398, 2950, 1728, 1300, 1250, 1150, 980 cm^{-1}. Mass spectra: M^+ 362 (15.3), 304 (14), 105 (11), 107 (14), 97 (30), 79 (39), 57 (59.5), 43 (100), 40 (100), 32 (100), 28 (100). ^1H NMR (300 MHz, $CdCl_3$/TMS): 0.9354 (s, 3H, -Me, H-20), 1.2313 (s, 3H, -Me), 3.9-4.15 (AB quartet, $-CH_2O-CO-CH_3$, 2H, AB quartet, 11Hz, H-17), 2.175 (s, 3H, $-CO-CH_3$), 0.800-2.2 (M, 31 H). ^{13}C NMR (75 MHz, $CdCl_3$/TMS) (δ_c): 41.1 (C-1), 18.1 (C-2), 37 (C-3), 42.6 (C-4), 55.9 (C-5), 21.2 (C-6), 42.6 (C-7), 43.4 (C-8), 55.4 (C-9), 39.8 (C-10), 18.1 (C-11), 25.0 (C-12), 36.3 (C-13), 38.6

(C-14), 49.3 (C-15), 38.1 (C-16), 65.2 (C-17), 28.0 (C-18), 180.7 (C-19), 14.5 (C-20), 20.0 (C-21), 170.9 (C-22).

With the above data the structure of the compound (Fig. 2) was confirmed as (-)-ent-kaur-17-acetoxy-19-oic acid, with m.p. 200ºC (40 mg).

Spectral data of 16α, 17-dihydroxy-ent-kauran 19-oic acid

IR: (KBr, ν_{max}) 3419, 3255, 2933, 2871, 1698, 1449, 1234, 1161, 1025, 873 cm^{-1}. Mass spectra: M^+ 335 (20), 317 (11), 305 (26), 287 (13.8), 259 (27.68), 121 (27), 93 (56.9), 81 (38), 79 (60), 67 (51), 55 (66), 42 (84.6) 41 (100). ^{1}H NMR (300 MHz, DMSO/TMS) (δ): 0.963 (s, 3H, -Me, H-20), 1,21 (s, 3H, -Me, H-18), 3.86 (DD, AB quartet, 11Hz, -OH, H-17), 4.34 (DD, AB quartet, 11Hz, -OH, H-16), 11.78 (BRS, 1H, -COOH, H-19), 0.963-2.013 (M, 26H). ^{13}C NMR (75 MHz, DMSO/TMS) (δ_c): 41.1 (C-1), 19.8 (C-2), 38.7 (C-3), 43.9 (C-4), 57.0 (C-5), 23.0 (C-6), 42.8 (C-7), 45.0 (C-8), 56.3 (C-9), 40.1 (C-10), 19 (C-11), 26.8 (C-12), 45.9 (C-13), 37.8 (C-14), 53.9 (C-15), 81.29 (C-16), 65.82 (C-17), 28.88 (C-18), 179.69 (C-19), 15.49 (C-20).

The identification of the compound was further confirmed by its chemical conversion to its acetate and by its ^{1}H NMR, ^{13}C NMR. Thus the strucutre of the compound Fig. 3 was confirmed as 16α,17-dihydroxy-ent-kauran-19-oic acid.

Antibacterial activity analysis

Using cultures of *E. coli* and *S. aureus* the antibacterial studies of naturally occuring kauranes were done. The antibacterial activity was assayed using disc diffusion technique.

Mueller Hinton agar was sterilzed and poured into sterile petridishes and allowed to solidify to form a layer. Organism inoculated in MH agar was overlaid on the solidified media. Two concentrations of the test samples, compound 1, 2 and 3 (10 mg/mL and 50 mg/mL) were dissolved in methanol. 100 filter paper discs were placed in 1 mL each of the test sample so that they contained 100 μg and 500 μg, respectively. 100 discs were placed in 1 mL of methanol to be treated as the control. The numbers 1-6 were marked on the bottom of the petridishes. 1000 μg disc of Ampicillin and 850 μg disc of Gentamycin were used as standard. They were placed in the medium in the petridishes and labeled as 1 and 2, respectively. The control disc was also kept. Plates were prepared for each organism similarly and incubated at 37 ± 0.5ºC for 24 h. The experiments were repeated thrice and the mean values were taken. The zone of inhibition, which indicated the antibacterial activity, was measured. The results were summarized in Table-1.

TABLE-1
ANTIBACTERIAL ACTIVITY OF KAURANES FROM *Annonaceae*

Sample	Concentration (μ g)	*S. aureus* inhibition zone (mm)	*E. coli* inhibition zone (mm)
Ampicillin	500	-	10
Gentamycin	500	16	-
16α,17dihydroxy-ent-	100	4	10.5
kauran-19-oic-acid	500	9	17.5
(-)-Ent-kaur-17-	100	6	8
acetoxy,19-oic acid	500	8.5	10
(-)-Ent-kaur17,19-	100	6	8.5
dioic acid	500	9	17

RESULTS AND DISCUSSION

The fruit of *Annona squamosa* is an edible one having thick fruit rind. The results of the study enhanced the medicinal property of the fruit. Bioactivity guided fractionation and isolation yielded three crystalline compounds. With the help of IR, ^{1}H NMR, ^{13}C NMR, DEPT and EIMS, the compounds were identified as (-)-ent-kaur-17-acetoxy,19-oic acid, 16α,17-dihydroxy-ent-kauran-19-oic acid and (-)-ent-kaur-17,19-dioic acid. The antibacterial activities of the pure kauranes were also studied and the results are summarized in the Table-2. From spectral data and by chemical conversion, the structure of the compounds was established.

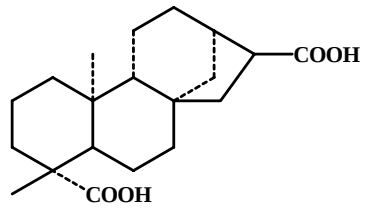

Fig. 1. (-)Rnt-kaur-17,19-dioic acid

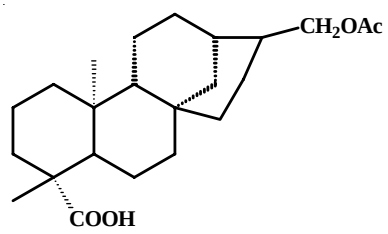

Fig. 2. (-)-ent-kaur-17-acetoxy,19-oic acid

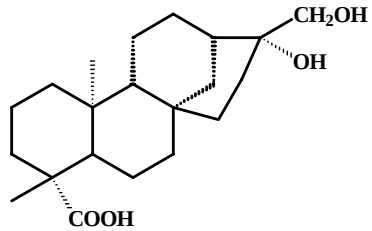

Fig. 3. 16α,17-dihydroxy-ent-kauran-19-oic acid

Compounds 16α,17-dihydroxy-en-kauran-19-oic acid, (-)-ent-kaur-17-acetoxy,19-oic acid and (-)-ent-kaur-17,19-oic acid obtained from the fruit pericarp of *Annona squamosa* were renamed as compound 3, compound 2 and compound 1 and screened for their antibacterial activity. For *Staphylococcus aureus*, gentamycin was used as the standard drug. All the three compounds showed almost same activity against *S. aureus*. Compound 2 and Compound 1 showed better activity than compound 3. Ampicillin was used as the standard antibiotic against *E. coli*. Compound 1 showed better activity against *E. coli* than the other kauranes. Compound 2 was also having similar activity. But compound 1 showed less activity compared to compound 1. The zone of inhibition produced by the compounds on comparison with the standards found to be very significant. The cytotoxic effect of *Annonaceae* and its significance in folk medicine is already established. Hence further studies make these compounds more significant in the field of medicine. In this study antibacterial effect of Kauranes from *Annona squamosa* were undertaken. *Escherichia coli* and *Staphylococcus aureus* were used to check the antibacterial effect. A compariative analysis of kauranes was undergone with the standard antibiotic discs of ampicillin and gentamycin. Distinct inhibition zones were obtained. This observation is an indicative of the role of kauranes as an antibacterial agent. Of the three compounds isolated from the pericarp of *Annona squamosa*, compound 2 and 1 showed very good antibacterial effect against *E. coli* even at very low concentration than the widely used antibiotic, ampicillin. But *S. aureus* showed very little activity compared to the antibiotic gentamycin (Table-1).

S. Aureus and *E. coli* and highly pathogenic organisms. The difference in the activity of the plant extract in the experimented organisms might be due to the difference in their cell wall constituents. *Staphylococcus aureus* is Gram-positive rods where as *E. coli* is Gram negative. Gram-negative bacteria have an outer covering outside the peptidoglycan layer. Braun's lipoprotein and Murein lipoprotein are present in it. Most important constituent in the outermembrane is the Lipopolysaccaharide. Porins is also present in the gram-negative cell wall. They protect the cell from lysis and external shock. The Kauranes are antibacterial compounds. But the mechanism is not yet clearly elucidated. However, it is known that the binding of Kauranes to the outer membrane of Gram-negative bacteria is a prerequisite to exert its bacterial activity.

E. coli produces a polysaccharide capsule. A number of filamentous protein structures resembling fimbrae are also present in *E. coli*. This causes a mannose resistant haemagglutination and this is good evidence that they play a major role in pathogenic diarrhoeal infection and urinary tract infection. Strains of *E. coli* possess a battery of virulence diterminants. The

polysaccharide of the O and K antigen protects the organism from the bactericidal effect of complement and phagocytosis in the absence of specific antibody. But the isolated compound of kauranes, compound 2 and compound 1 has inhibited the growth and proliferation of *E. coli* much more effectively than the standard antibiotic, Ampicillin. This forces us to believe in the fact that the normal function of O and K antigen might be disrupted by the active metabolites of plant orgin, kauranes. From this we can come to the conclusion that the compounds isolated, 16α,17-dihydroxy-ent-kauran-19-oic acid and (-)-ent-kaur-17,19-dioic acids at 100 μg and 500 μg is more effective than the antibiotic ampicillin (100 μg). Ampicillin has many side effects. Allergic reactions are relatively common side effects of ampicillin. This can range from rashes to anaphylaxis, a severe condition in which there may be shock and even death. Nausea, vomiting, loss of appetite, diarrhea and abdominal pain are commonly reported gastrointestinal side effects during ampicillin therapy. Diarrhoea occurs more frequently with ampicillin than with the other pencillins. In spite of the advances of modern medicine, medicinal world is eagerly waiting for more potent medicine from naturally occuring plants, as they have no side effects. This experiment showed that the compounds isolated, 16α,17-dihydroxy-ent-kauran-19-oic acid and (-)-ent-kaur-17,19-dioic acid at 100 μg and 500 μg is more effective than the antibiotic used at present. The strong invitro antibacterial activity of kauranes suggests that the compound might find wide pharmaceutical uses. This study achieved the goal of isolation, identification and invitro studies of an effective natural drug against the most common and dreadful pathogens, without any side effects from our surroundings at a low cost.

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