# *Notes*

## Ehretianone, a Novel Quinonoid Xanthene from *Ehretia buxifolia* with Antisnake Venom Activity

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Ehretianone (**1**), a new quinonoid xanthene, together with known sterols, was isolated from a MeOH extract of the root bark of *Ehretia buxifolia*. The structure of ehretianone was elucidated as 7-hydroxy-9aα-(3-methylbut-2-enyl)-4aα,9α-(2-methylprop-2-enyl)-4a,9a-dihydro-1,4-dioxoxanthene on the basis of spectroscopic data and X-ray crystallographic analysis. The antisnake venom activity of ehretianone against *Echis carinatus* venom in mice is also reported.

*Ehretia buxifolia* Roxb. (Boraginaceae) (syn. *E. microphylla* Lam.) is reported to be useful medicinally in the Ayurvedic and Siddha systems of medicine in India.[1] From an EtOH extract of the aerial parts of the plant bauerenol, ursolic acid and a quinonoid, microphyllone, have been reported.[2] A MeOH extract of the leaves has afforded rosmarinic acid, bauerenol, α-amyrin, *β*-amyrin, astragalin, and nicotoflorin.[3] A field survey conducted in the Chengalpattu District, Tamilnadu, India, revealed that the root bark of the plant is used by the Irula tribe as an antidote to *Echis carinatus* envenomation.[4] The root bark powder is given orally, and it is claimed to stop hemorrhaging caused by the venom. We report herein the isolation and structure elucidation of a new quinonoid xanthene, ehretianone (**1**), from a MeOH extract of the root bark of the plant. In addition to ehretianone, a sterol mixture containing *β*-sitosterol, stigmasterol, stigmastanol, α-spinasterol, campesterol, and cholesterol was also obtained.

The antisnake venom activity of ehretianone (**1**) against *E. carinatus* envenomation in mice also has been investigated.

The MeOH extract of the root bark of *E. buxifolia* was subjected to column chromatography over Si gel. Elution of the column with $C_6H_6$−EtOAc (9:1) gave ehretianone (**1**), which was crystallized from a hexane−ether mixture as red-orange crystals, mp 152 °C. It gave a green color with alcoholic $FeCl_3$ (phenol) and a pink color with alcoholic KOH (quinone). The EIMS showed a molecular ion peak at *m/z* 350, which analyzed for $C_{22}H_{22}O_4$. The IR spectrum showed hydroxyl (3350 $cm^{-1}$), quinonoid carbonyl (1670 $cm^{-1}$), olefinic double bond, and aromatic (1600, 1590, 1570, 970, 880, 855, 820, and 795 $cm^{-1}$) absorptions. The UV spectrum showed peaks at 210, 230 (sh), and 295 nm, indicating extended conjugation in the molecule. The $^1$H-NMR ($CDCl_3$ and $C_6D_6$) and $^{13}$C-NMR ($CDCl_3$) spectra (Table 1) revealed the presence of a phenolic hydroxyl ($\delta_H$ 4.83, 1H, br s, $D_2O$ exchangeable) and an isopentenyl side chain with two vinylic methyls ($\delta_H$ 1.48 and 1.66, 3H each, s; $\delta_C$ 17.90 and 25.90), a trisubstituted double-bond proton ($\delta_H$ 4.98, 1H, t, $J$ = 7.5 Hz; $\delta_C$ 117.08), and a methylene group attached to a saturated tertiary carbon atom ($\delta_H$ 2.30 and 2.51, 1H each dd, $J$ = 7.3, 14.6 Hz; $\delta_C$ 31.80). Two 1,4-quinone carbonyls appeared as singlets at $\delta_C$ 198.53 and 193.29, and a pair of ortho-coupled quinone ring protons were apparent ($\delta_H$ 6.46

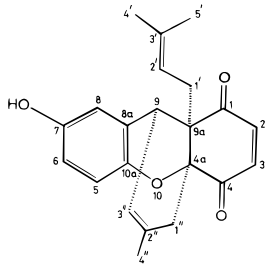


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**Table 1.** $^1$H-NMR and $^{13}$C-NMR Data of Ehretianone (**1**)

| position | $\delta_H$ $CDCl_3$ (mult., $J$ in MHz) | $\delta_H$ $C_6D_6$ | $\delta_C$ |
|---|---|---|---|
| 1 | | | 198.53 |
| 2 | 6.46 (d, 10.7) | 5.92 | 138.10 |
| 3 | 6.80 (d, 10.7) | 6.25 | 138.87 |
| 4 | | | 193.29 |
| 5 | 6.58 (d, 8.8) | 6.67 | 117.36 |
| 6 | 6.48 (dd, 8.3, 2.9) | 6.28 | 114.31 |
| 7 | | | 149.85 |
| 8 | 6.54 (d, 2.9) | 6.42 | 114.57 |
| 9 | 3.58 (d, 6.3) | 3.63 | 37.52 |
| OH | 4.83 (br s, $D_2O$ exchangeable) | | |
| 1′ | 2.30, 2.51 (each dd, 7.3, 14.6) | 2.18, 2.42 | 31.80 |
| 2′ | 4.98 (t, 7.5) | 4.98 | 117.08 |
| 3′ | | | 131.48 |
| 4′ | 1.66 (s) | 1.37 | 25.90 |
| 5′ | 1.48 (s) | 1.33 | 17.90 |
| 1″ | 2.52, 2.80 (each d, 19.5) | 2.62, 2.72 | 35.78 |
| 2″ | | | 136.85 |
| 3″ | 5.62 (d, 6.3) | 5.38 | 122.75 |
| 4″ | 1.74 (s) | 1.58 | 22.60 |
| 4a | | | 81.00 |
| 8a | | | 127.96 |
| 9a | | | 53.93 |
| 10a | | | 144.87 |

**Table 2.** Cross-Peaks in the $^1$H-$^1$H Long-range COSY NMR Spectrum of Ehretianone (**1**) (non-aromatic region)

| $^1$H ($\delta$ ppm) | $^1$H ($\delta$ ppm) |
|---|---|
| H-4′ (1.66) | H-5′ (1.48), H-2′ (4.98), H-1′ (2.30, 2.51) |
| H-5′ (1.48) | H-4′ (1.66), H-2′ (4.98), H-1′ (2.30, 2.51) |
| H-1′ (2.30) | H-5′ (1.48), H-4′ (1.66), H-1′ gem (2.51), H-2′ (4.98) |
| H-1′ (2.51) | H-5′ (1.48), H-4′ (1.66), H-1′ gem (2.30), H-2′ (4.98) |
| H-2′ (4.98) | H-4′ (1.66), H-5′ (1.48), H-1′ (2.30, 2.51) |
| H-4″ (1.74) | H-1″ (2.52, 2.80), H-3″ (5.62), H-9 (3.58) |
| H-1″ (2.52) | H-4″ (1.74), H-1″ gem (2.80), H-3″ (5.62) |
| H-1″ (2.80) | H-4″ (1.74), H-1″ gem (2.52), H-3″ (5.62) |
| H-3″ (5.62) | H-4″ (1.74), H-9 (3.58), H-1″ (2.52, 2.80) |
| H-2 (6.46) | H-3 (6.80) |
| H-3 (6.80) | H-2 (6.46) |

and 6.80, 1H each, d, $J$ = 10.7 Hz). The coupling pattern of the three aromatic protons ($\delta_H$ 6.48, 6.54, and 6.58) in the $^1$H-NMR spectrum showed the presence of a 1,3,4-trisubstituted benzene ring. The spectral data also showed the presence of a methylprop-2-enyl bridge, which is also present in microphyllone previously reported from *E. buxifolia*.[2]

The $^1$H-NMR spectrum of ehretianone (**1**) in $C_6D_6$ was more highly resolved than when run in $CDCl_3$, with the overlapping signals well separated and the quinonoid protons moving upfield and being well separated from the aromatic protons. Similarly, the quartet due to H-1″, which was found to overlap with the double doublets due to H-1′, was also well separated. The $^{13}$C-NMR spectrum of **1** showed three methyl groups, two methylene groups, eight methine groups, and nine quaternary carbon atoms. The assignments were made by comparison with model compounds.[5,6]

The $^1$H- and $^{13}$C-NMR assignments of **1** were confirmed by $^1$H−$^1$H long-range and $^1$H−$^{13}$C one-bond COSY spectra. The cross-peaks observed between the various protons in the non-aromatic region of the $^1$H−$^1$H long-range COSY NMR spectrum of ehretianone (**1**) are given in Table 2. The assignments of the quaternary carbon atoms are based on model compounds.

Difficulty in assembling the above groups with the correct stereochemical details led to the study of ehretianone (**1**) by X-ray crystallography. The structure of

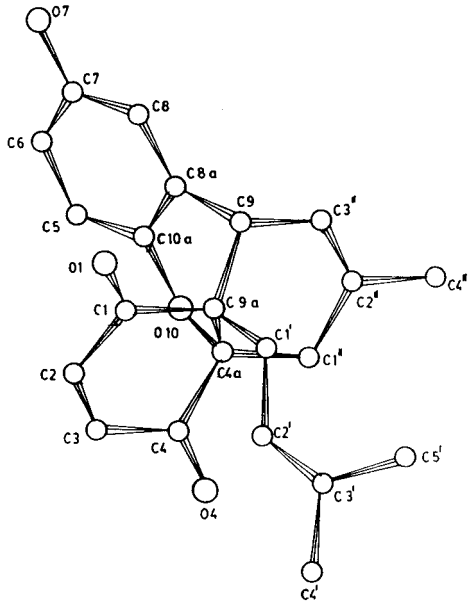


**Figure 1.** Perspective view of the molecular structure of ehretianone (**1**).

ehretianone (**1**) was thereby elucidated as 7-hydroxy-9aα-(3-methylbut-2-enyl)-4aα,9α-(2-methylprop-2-enyl)-4a,9a-dihydro-1,4-dioxoxanthene. The perspective view of the molecule as obtained by the single-crystal X-ray analysis of ehretianone (**1**) is shown in Figure 1. The pyran ring is puckered, with the total puckering amplitude being 0.664(7) Å. The puckering causes the pyran ring to be distorted into a sofa conformation. The molecule is folded at both the ring junctions C-9a−C-4a and C-8a−C-10a. The quinone ring adopts a twist conformation. The twist runs through the atoms C-2 and C-4a such that the dihedral angle between the two halves of the ring is 28.2(5)°. The bridge connecting C-9 and C-4a is α-linked and adopts a sofa conformation. Final positional parameters and equivalent isotropic temperature factors of non-hydrogen atoms are given in Table 3. In the packing of the molecules, intermolecular hydrogen bonding was absent, and the molecules were held together by van der Waals forces of attraction.

The antisnake venom activity of ehretianone (**1**) was determined using the $LD_{50}$ of *E. carinatus* venom in mice by the subcutaneous route. The $LD_{50}$ of the venom used was 6.65 mg/kg. The compound did not show any toxic effect up to 100 mg/kg. In vitro neutralization studies showed that compound **1** gave significant protection at a dosage level of 3.75 mg/kg in mice when challenged with the $LD_{50}$ of the venom (Table 4). The prophylactic and curative activities (compound given after venom injection) are given in Table 5. In the prophylactic treatment, compound **1** was administered 30 min before venom injection and the mortality was reduced by 35.0% as compared with controls. In the curative study, the same dosage of the compound gave significant protection up to 5 min after venom injection.

In preliminary studies, the total MeOH extract of the root bark of the plant also showed all three of the above modes of activity (in vitro neutralization, prophylactic activity, and curative activity). Other potential active principles besides ehretianone (**1**) are yet to be identified.

**Table 3.** Final Positional Parameters and Equivalent Isotropic Temperature Factors (Å$^2$) of the Non-Hydrogen Atoms with Estimated Standard Deviations in Parentheses

| atom | *x/a* | *y/b* | *z/c* | $B_{eq}$ (Å$^2$)[a] |
|---|---|---|---|---|
| C-1 | −0.1709(8) | 1.0023(6) | 0.3889(2) | 3.4(1) |
| O-1 | −0.2614(6) | 1.0521(4) | 0.4244(2) | 4.4(1) |
| C-2 | −0.1142(10) | 1.0832(6) | 0.3417(3) | 4.2(2) |
| C-3 | −0.0252(11) | 1.0305(6) | 0.3006(3) | 4.5(2) |
| C-4 | 0.0326(9) | 0.8817(6) | 0.3007(2) | 3.9(2) |
| O-4 | 0.0766(8) | 0.8261(5) | 0.2595(2) | 5.5(2) |
| C-5 | 0.2952(8) | 1.0689(6) | 0.4354(2) | 3.2(2) |
| C-6 | 0.2868(8) | 1.1405(6) | 0.4840(2) | 3.3(1) |
| C-7 | 0.1713(9) | 1.0998(6) | 0.5234(2) | 3.4(1) |
| O-7 | 0.1544(10) | 1.1695(6) | 0.5720(2) | 4.7(2) |
| C-8 | 0.0586(8) | 0.9882(6) | 0.5145(2) | 3.0(1) |
| C-9 | −0.0690(8) | 0.8078(6) | 0.4500(2) | 2.7(1) |
| O-10 | 0.1991(5) | 0.8835(4) | 0.3786(1) | 3.3(1) |
| C-10a | 0.1854(8) | 0.9562(5) | 0.4271(2) | 2.9(1) |
| C-4a | 0.0426(7) | 0.8158(6) | 0.3566(2) | 2.9(1) |
| C-8a | 0.0651(8) | 0.9160(5) | 0.4666(2) | 2.8(1) |
| C-9a | −0.1223(8) | 0.8475(5) | 0.3917(2) | 3.0(1) |
| C-1′ | −0.2906(9) | 0.7630(7) | 0.3741(3) | 3.6(2) |
| C-2′ | −0.3358(10) | 0.7630(6) | 0.3152(3) | 4.4(2) |
| C-3′ | −0.3837(9) | 0.6548(6) | 0.2869(3) | 4.5(2) |
| C-4′ | −0.4294(18) | 0.6670(12) | 0.2272(4) | 7.0(4) |
| C-5′ | −0.4017(18) | 0.5141(8) | 0.3088(4) | 7.3(3) |
| C-1″ | 0.0767(10) | 0.6621(6) | 0.3545(3) | 3.7(2) |
| C-2″ | 0.0686(9) | 0.5950(5) | 0.4091(3) | 3.4(2) |
| C-3″ | 0.0051(8) | 0.6616(5) | 0.4519(2) | 3.2(1) |
| C-4″ | 0.1288(12) | 0.4469(7) | 0.4126(4) | 4.7(2) |

[a] $B_{eq} = (8\pi^2/3)\Sigma_i\Sigma_j U_{ij} a_i^* a_j^* a_i a_j$.

**Table 4.** *In Vitro* Neutralization Effect of Ehretianone (**1**) against the $LD_{50}$ (6.65 mg/kg) of *E. carinatus* Venom in Mice

| dose of **1**[a] (mg/kg) | percentage mortality | | |
|---|---|---|---|
| | test | | control |
| 1.25 | 43.3 | ($p > 0.05$) | 50.0 |
| 2.50 | 26.7 | ($p < 0.05$) | 48.3 |
| 3.75 | 13.3 | ($p < 0.001$) | 51.7 |

[a] Ehretianone (**1**) and the venom were incubated at 37 °C for 30 min before administration.

**Table 5.** Effect of Ehretianone (**1**) (3.75 mg/kg) on the Lethality of *E. carinatus* Venom When Administered to Mice at Various Time Intervals, before and after Administering the $LD_{50}$ (6.65 mg/kg) of the Venom

| time of administration (min) | percentage mortality | | |
|---|---|---|---|
| | test | | control |
| before administration of venom | | | |
| 30 | 16.7 | ($p < 0.001$) | 51.7 |
| after administration of venom | | | |
| 5 | 21.7 | ($p < 0.001$) | 53.3 |
| 30 | 38.3 | ($p > 0.05$) | 55.0 |
| 45 | 45.0 | ($p > 0.05$) | 48.3 |

## Experimental Section

**General Experimental Procedures.** IR spectra were recorded on a Perkin-Elmer 337 grating spectrophotometer using KBr disks. The UV spectra were taken on a Shimadzu 160 A instrument in MeOH. The mass spectrum was recorded on a Shimadzu GC-MS QP 1000A spectrometer at 70 eV. The sterols were analyzed by GC−MS using a Hewlett-Packard 5972 mass selective detector hooked with a Series II plus 5980 gas chromatograph equipped with electronic control flow. A J & W capillary column (30 m × 0.25 mm i.d.) containing 5% diphenylpolysiloxane and 95% dimethylpolysiloxane, of 0.25-$\mu$m film thickness, was used. The temperature program was 80 to 280 °C, 8 °C/min, at an operating voltage of 70 eV. The $^1$H-NMR and $^{13}$C-NMR spectra were recorded in $CDCl_3$ or $C_6D_6$ on a JEOL GSX instrument at 400 MHz and 75 MHz, respectively, using TMS as the internal standard. The 2D COSY NMR studies were performed on a Bruker AMX 500 spectrophotometer in $CDCl_3$. The $^1$H−$^1$H long-range COSY NMR spectrum was recorded with 2048 and 512 data points in the $t_2$ and $t_1$ dimensions, respectively, on the reverse detection probe. The data sets were processed with zero filling in $t_1$ to give a 1 k × 1 k contour map. Magnitude calculation COSY was used. The one-bond $^1$H-$^{13}$C COSY NMR spectrum was obtained using a 5-mm broadband probe. The data were accumulated with 2 k and 512 k points in the $t_2$ and $t_1$ dimensions, respectively. Zero filling in the $t_1$ dimension was used during processing to give a 1 k × 1 k contour map. The experiment was run in the phase-sensitive mode using the TPPI method.[7] X-ray analysis was carried out on an Enraf-Nonius CAD-4 diffractometer with the $\omega/2\theta$ scan mode, $\lambda$(MoK$\alpha$) = 0.71069 Å.

**Plant Material.** The plant material was collected in Chengalpattu District, Tamilnadu, India, during May 1993. It was identified by Dr. S. Usman Ali, Central Research Institute (Siddha), Madras, and a voucher specimen has been deposited in the herbarium of Captain Srinivasa Murti Drug Research Institute for Ayurveda, Madras.

**Extraction and Isolation.** The shade-dried and coarsely powdered root bark (800 g) was extracted with MeOH by the cold percolation method (48 h). The extract was filtered and concentrated *in vacuo* (yield, 95 g).

**Sterol Mixture.** The MeOH extract was subjected to column chromatography over Si gel (100−200 mesh). Elution of the column with $C_6H_6$−EtOAc (9:1) gave a sterol mixture. The mixture was subjected to GC−MS analysis. The sterols present were: stigmasterol (39.0%), stigmastanol (29.0%), $\beta$-sitosterol (19.0%), campesterol (5.15%), $\alpha$-spinasterol (2.5%), cholesterol (1.5%), and an unidentified sterol (7.0%).

**Ehretianone (1).** Further elution of the column with the same solvent mixture gave the new quinonoid xanthene, ehretianone (**1**). The compound (50 mg, yield 0.00625%) was crystallized from hexane-ether as red-orange crystals, mp 152 °C. IR (KBr) $\nu$ max 3350, 2990, 2940, 2880, 1670, 1660, 1600, 1590, 1570, 1550, 1380, 1350, 1330, 1300, 1260, 1250, 1200, 1110, 1100, 970, 880, 855, 820, 795 cm$^{-1}$; UV (MeOH) $\lambda$ max 210, 230 (sh), 295 nm; $^1$H NMR and $^{13}$C NMR data, see Table 1; EIMS (70 eV) *m/z* [M]$^+$ 350 (64), 283 (6), 282 (31), 267 (7), 265 (9), 264 (9), 247 (11), 237 (9), 235 (12), 221 (8), 161 (11), 158 (11), 138 (9), 128 (8), 115 (14), 93 (57), 92 (7), 91 (13), 77 (16), 69 (100), 65 (17), 55 (29). *Anal.* Calcd for $C_{22}H_{22}O_4$ C, 75.42; H, 6.28. Found: C, 74.77; H, 6.78.

**X-ray Crystallography of 1.** Suitable crystals of ehretianone (**1**) were obtained from a $CHCl_3$−MeOH mixture by slow evaporation. The crystal belonged to the orthorhombic system, space group $P2_12_12_1$ with $a$ = 7.563(1) Å, $b$ = 9.655(1) Å, $c$ = 24.604(1) Å, $Z$ = 4, $V$ = 1796.47(2) Å$^3$, $D_c$ = 1.307 g cm$^{-3}$, and $\mu$(MoK$\alpha$) = 0.08 mm$^{-1}$. Of the 1675 reflections collected, only 1265 reflections with $I > 3\sigma(I)$ were used for structural analysis. The structure was solved by direct methods and refined by the full matrix-least squares procedure to a final $R$ = 0.044 and $Rw$ = 0.044 where $w$ = 1.2601

($\sigma^2$ ($F$) + 0.000822$F^2$). The positions of the hydrogen atoms were identified by difference Fourier methods. Crystallographic calculations were performed on microvax 11 computer by using the SHELXS 86 structure-solving package.

**Antisnake Venom Activity of Ehretianone (1).** *E. carinatus* venom was purchased from the Irula Snake Catchers Industrial Cooperative Society, Mahabalipuram, Chengalpattu District, Tamilnadu, India. The $LD_{50}$ of the venom was determined in Swiss albino mice (20−22 g), six groups, eight in each. The venom was dissolved in 0.9% saline before use and administered by the subcutaneous route. The animals were observed for 6 h, and the number that died within 24 h was recorded. The $LD_{50}$ was calculated by the method of Miller and Tainter.[8] Ehretianone (**1**) was administered as a fine suspension in water containing Tween 80.

The in vitro neutralization activity of ehretianone (**1**) was determined by incubating the $LD_{50}$ of *E. carinatus* venom with various doses of the test compound at 37 °C for 30 min before subcutaneous administration to mice.

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