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Chemical Constituents of *Indigofera Aspalathoides* Vahl. Ex. DC.

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Civarvembu/ Sivanimba is botanically equated to *Indigofera aspalathoides* Vahl ex. DC. (Leguminosae). The plant is prescribed for eczema, psoriasis, boils, burns, wounds and ulcers. The whole plant is an ingredient in Civanar vembut tailam and civanar vembut kulit tailam which are popular for skin diseases. Based on the therapeutic applications it was aimed to find out the secondary metabolites present in the whole plant. Extraction, fractionation and characterization of the solvent extracts led to the identification of ten compounds *n*-butyl ester of nanodecanoic acid, 1-octadecanol, 4-heneicosanone, *α*-amyrin, *n*-octacosanol, *β*-sitosterol, salicylic acid, erthroxydiol X, erythroxydiol Y and β-sitosterol-3β-D-glucopyranoside. All these compounds are reported for the first time from the genus *Indigofera*.

Keyword: *Indigofera aspalathoides,* Chemical Constituents, ^{13}C NMR Assignment.

1. Introduction

Civanarvembu/Iraivanvembu in Tamil is botanically equated as *Indigofera aspalathoides* Vahl.ex.DC. belonging to the family Leguminosae. In Sanskrit it is Patakohomba /Sivanimba. Its leaves, flowers and tender shoots are cooling and demulcent, they are used in the form of decoction for leprosy and cancerous affections. The leaves are also applied to abscesses. Roots are used as dentrifice, and also in mouth ulcers. The root is chewed in toothache and apathies. The whole plant is an ingredient of an oily preparation used for dandruff[1], syphilis and other skin affections. In Siddha system of medicine, the plant is prescribed for eczema, psoriasis, boils, burns, wounds, ulcers, and used also as an antidote to snake venom[2]. Civanar vembu Tailam and Civanarvembuk kulit Tailam are two popular Siddha preparations used for various types of skin diseases including leprosy[3]. It is used along with camphor for different kinds of wounds. This plant is regarded as one used in Kayakalpa drugs and in the discovery of anticancer elixir. Water soluble fraction of alcoholic extract of dried tender shoots of *I. aspalathoides* showed significant anti-inflammatory effect in experimental albino mice. As there is no record of phytochemical work of this highly potential traditional drug, present investigation is carried out and phyto chemical analysis of this plant is reported for the first time.

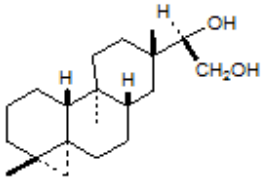
2. Materials and Methods

2.1 Collection of the Plant

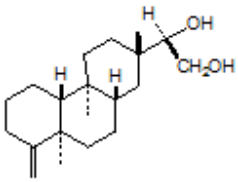
Whole plant of *I. aspalathoides* were collected from Kolli Hills, Tamil Nadu during November and was authenticated by Mr.D.Narayanappa, Chief botanist, TAMPCOL, Arumbakkam, Chennai-600 106 and the voucher specimen no N/288, WP8C is deposited in the museum of botany department of this Institute.

2.2 Extraction of the Plant

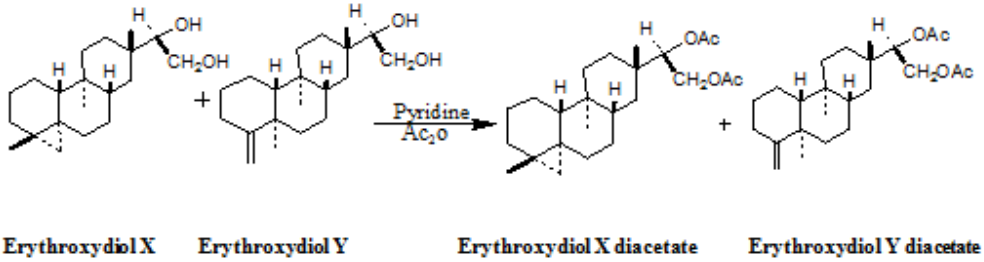
The plant material (3 kg) was shade dried, coarsely powdered and extracted with *n*-hexane, chloroform, ethyl acetate and methanol successively by cold percolation method (48hr). Nearly 80% of the solvent was removed by distillation on water bath at atmospheric pressure and the last traces were removed under reduced pressure. The extracts were then subjected to column chromatography.



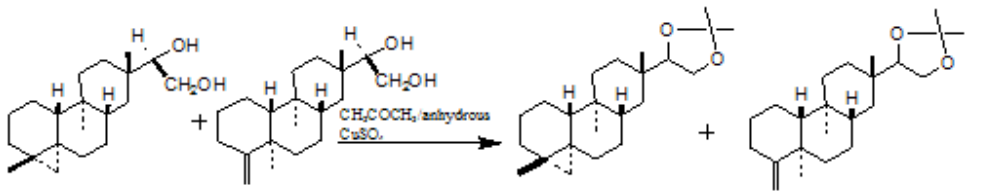
Erythroxydiol X



Erythroxydiol Y



Erythroxydiol X **Erythroxydiol Y** **Erythroxydiol X diacetate** **Erythroxydiol Y diacetate**



Erythroxydiol X **Erythroxydiol Y** **Erythroxydiol X acetonide** **Erythroxydiol Y acetonide**

OCOCH3 170.54, 170.75 and –OCOCH3 20.69, 20.78 for both the compounds.

2.3 Isolation of Compounds

n-Hexane extract (15g) was chromatographed through a column of silica gel (100g, acme 100-200 mesh). The column was eluted with solvents of increasing polarity in the order of *n*-hexane, benzene, benzene, chloroform and ethyl acetate and mixtures of these solvents. Six compounds namely *n*-butyl ester of nanodecanoic acid (100 mg, m.p. 80°C), 1- octadecanol (80 mg, m.p. 58°C), 4-heneicosanone (120 mg, m.p. 72°C), α-amyrin 115 mg, m.p. 184-187°C, $(\alpha)_D$ + 78° C (C=2, $CHCl_3$), *n*-octacosanol (100 mg, m.p. 83° C)and *β*-sitosterol (250 mg, m.p. 131°C) were isolated and characterized.

2.4 Isolation of compounds from Chloroform Extract

Chloroform extract (10 g) of the plant was subjected to column chromatography over silica gel. Earlier fractions yielded *n*-octacosanol and *β*-

sitosterol. Further elution of the column with chloroform-ethyl acetate (9:1) gave a solid which was crystallized from chloroform to get colourless needles (90 mg, m.p.141°C). It produced brisk effervescence with sodium bicarbonate solution and gave violet color with alcoholic ferric chloride. It was identified as salicylic acid by comparison with an authentic sample.

2.5 Isolation of Erythroxydiol X and Y

The column was further eluted with chloroform-ethyl acetate (4:1). Similar fractions were combined and washed with acetone to give a colourless solid, which was crystallized from methanol to get erythroxydiol X and erythroxydiol Y (400 mg, m.p. 116°C).

The compound formed an acetonide with dry acetone and anhydrous copper sulphate. The acetonide showed two distinct spots on TLC in silica gel impregnated with silver nitrate (10%) in the solvent system *n*-hexane: benzene (1:1). The acetonide mixture on column chromatography over silica gel impregnated with silver nitrate (10%) afforded the acetonides of erythroxydiol X and erythroxydiol Y in pure form, in about equal proportion. *n*-hexane: benzene (4:1) eluates yielded the acetonide of erythroxydiol X while *n*-hexane: benzene (1:1) eluates gave the acetonide of erythroxydiol Y.

Methanol extract on column chromatography yielded *β*-sitosterol-3-*β*-D-glucopyranoside (130 mg, m.p. 295°C).

2.6 Acetylation of Erythroxydiol X and Y

Erythroxydiol X and Y (150 mg) in dry pyridine (8 drops) was treated with freshly distilled acetic anhydride (2.5 ml) at room temperature and kept overnight. The mixture was worked up in the usual way and the diacetate was purified by passing through a short column of silica gel. Elution with benzene-chloroform (4:1) yielded a solid which on crystallization from chloroform-methanol mixture afforded the diacetate (100 mg) m.p. 92-94°C.

2.7 Periodate Oxidation of Erythroxydiol X and Y

Erythroxydiol X and Y (150 mg) was dissolved in methanol (5 ml) and a solution of sodium meta periodate (36 mg) in water (7 ml) was added to this solution. The mixture was kept aside at room temperature for 3 hrs. Methanol was removed in vacuum and the solution was extracted with chloroform. The aldehyde formed was purified by column chromatography over silica gel. Benzene eluates yielded the aldehyde as a gum (70 mg).

2.8 Acetonide of Erythroxydiol X and Y

Erythroxydiol X and Y (200 mg) was dissolved in 60 ml of dry acetone and mixed with anhydrous copper sulphate (500 mg). The mixture was kept standing at room temperature for 3 days. After filtering the copper sulphate, acetone was evaporated to give the mixture of acetonides which showed two distinct spots in TLC (silica gel impregnated with 10% silver nitrate) in *n*-hexane-benzene (1:1).

Table 1: ^{13}C NMR Assignment of erythroxydiol X and Y

Carbon*	Erythroxydiol X	Erythroxydiol Y
1(t)	34.02	37.95
2(t)	32.34	33.16
3(t)	23.22	28.76
4(s)	17.22	160.68
5(s)	37.95	36.73
6(t)	19.63	21.23
7(t)	26.22	28.61
8(d)	41.53	42.11
9(s)	40.71	40.71
10(d)	49.91	56.45
11(t)	33.6	35.01

12(t)	29.03	29.11
13(s)	37.34	38.14
14(t)	36.73	36.85
15(d)	81.37	81.44
16(t)	62.67	62.67
17	22.28(q)	102.08(t)
18	24.7(t)	21.52(q)
19(q)	11.57	12.34
20(q)	18.87	19.03

2.9 Separation of Erythroxydiol X and Y acetonides

40 g of Silica gel (column chromatography) was made into slurry with 4 g of silver nitrate in methanol. Silica gel was dried in an oven for 1 hr. The acetonide mixture was chromatogrpahed through a long column of silica gel impregnated with silver nitrate (10%). *n*-hexane-benzene (4:1) eluates afforded crystals of erythroxydiol X acetonide which was recrystallised from methanol (46 mg) m.p. 88-90°C. *n*-hexane-benzene eluates (1:1) yielded the crystals of erythroxydiol Y acetonide which was recrystallised from methanol (53 mg), m.p. 144-145°C.

Table 2: ^{13}C NMR Assignment of erythroxydiol X and Y diacetate

Carbon*	Erythroxydiol X diacetate	Erythroxydiol Y diacetate
1(t)	33.97	37.91
2(t)	32.13	33.13
3(t)	23.17	28.72
4(s)	17.23	160.54
5(s)	40.47	40.67
6(t)	19.6	21.21
7(t)	26.08	28.47
8(d)	41.39	41.97
9(s)	37.22	38.03
10(d)	49.87	56.4
11(t)	33.41	34.81
12(t)	28.82	28.82
13(s)	36.64	36.64
14(t)	36.64	36.75
15(d)	79.4	79.4
16(t)	63.13	63.13
17	22.25(q)	102.15(t)
18	24.67(t)	21.48(q)
19(q)	11.51	12.29
20(q)	19.18	19.32

3. Results and discussion

All the known compounds namely *n*-butyl ester of nanodecanoic acid, 1-octadecanol, 4-heneicosanone, α-amyrin, *n*-octacosanol, salicylic acid, β-sitosterol and its glycoside were identified by IR, UV and NMR data. The compound erythroxydiol X and Y had IR absorption bands at 3400(hydroxyl), 2924, 2853, 1634, 891 (exomethylene), 1050 (cyclopropane) cm^{-1}. It formed a diacetate m.p. 92-94°C with pyridine and acetic anhydride showing the presence of two hydroxyl groups. ^{1}H NMR spectrum showed the presence of six methyl groups at δ 0.72, 0.73, 0.87, 0.89, 0.98 and 1.00 (s each, 6xCH_3). Two one proton doublets at a high field δ 0.12 and 0.55(J=4.1Hz) indicated the presence of a tetra

substituted cyclopropane ring. Exomethylene protons appeared as a broad singlet at δ 4.48. A doublet of triplets (2H) at δ 3.48 with J values 3.6 and 10.2 and two broad doublets at 3.28 (2H, J= 9.5 Hz) and 3.72(2H, J=11Hz) suggested the presence of two primary and two secondary alcoholic groups. ^{13}C NMR spectrum of the compound also revealed these functionalities viz. quartets at δ 22.3, 21.5, 18.9, 12.3, 11.6 ppm which would be attributed to six tertiary methyl groups. Triplet at 24.7 ppm could be ascribed to the cyclopropane methylene carbon. A singlet at 160.7 and triplet at 102.1 ppm (=CH_2) confirmed the presence of exomethylene group. Doublets at 81.37 and 81.44 (>CHOH) and the triplet at 62.7 ppm (-CH_2OH) accounted for the secondary and primary alcoholic groups. In addition ^{13}C NMR (DEPT) spectrum showed the presence of 19 methylene groups which suggested the possibility of the compound being a diterpenoid and not a steroid nor triterpenoid. ^{13}C NMR spectrum accounted for a total of 36 carbons. All these features suggested the compound to be a mixture of closely related isomers. The same conclusion was arrived at from ^{1}H and ^{13}C NMR spectra of the diacetate. Cyclopropane protons appeared as doublets in the high field region 0.1 and 0.5 ppm (each d, J=4.2Hz). Six tertiary methyl as three proton singlets were observed at δ 0.70, 0.71, 0.95, 0.96, 0.97 and 0.98. Two acetate methyls appeared as singlets at δ 1.99 and 2.05 ppm. Broad singlet at 4.45 ppm accounted for exomethylene protons. In the diacetate, >CHOAc proton was shifted to δ 4.7 (dt, J=9.22 and 2.3Hz). One of the –CH_2OAc protons appeared as a multiplet at 3.98 and the other as a broad doublet at 4.38 ppm (J=11.8Hz). Corresponding carbon signals for the various moieties were observed in the ^{13}C NMR spectrum of the diacetate which accounted for 42 carbons. In the diacetate also, 19 methylene carbon signals were seen. Homo decoupling studies on the diacetate showed that the hydroxyl groups are vicinal. Irradiation of the signal at δ 4.7(>CHOAc) resulted in the collapse of the multiplet at 3.98 into a double doublet and sharpening of the broad doublet at 4.38. Similarly irradiation of the multiplet at 3.98 (>CH_2OAc) caused a collapse of the broad doublet at 4.38 into a broad singlet and the double triplet at 4.7(>CHOAc) into a doublet. In the same way, on irradiation of the other >CH_2OAc proton at 4.38, the signals at 3.98 and 4.7 were much affected. Hence the two hydroxyl groups are adjacent in the compound. Irradiation of one of the doublets at 0.5 resulted in the collapse of the doublet at 0.1 into a singlet and vice versa (cyclopropane protons). Thus it can be inferred that it is probably a mixture of diterpene diols in about equal proportions, one having a cyclopropane moiety and the other having an exomethylene unit. Biogenetically >CHOH-CH_2OH is placed at C-13 of the diterpene. Absence of methyl carbon signals around δ 28 ppm and above ruled out the presence of gem dimethyl at C-4. Therefore one isomer has an exomethylene at C-4 and the other has a cyclopropane ring at positions 4 and 5. Except this difference, the other structural features are one and the same in both the compounds.

Earlier researchers isolated erythroxydiols X and Y as a mixture from the trunk wood of *Erythroxylon species* [4-8]. These two diterpenoids are based on the enantiorosane skeleton. The compound could therefore be a mixture of erythroxydiol X and Y or a mixture of diterpenoids having methyl group at C-8 instead of C-13.

Mass spectral analysis showed the molecular ion peak at m/z 306(M^+). There were mass fragments at m/z 291, 275, 245, 189, 175, 163, 149, 135, 121, 107, 95, 81, 61 and 48. The mass fragment at m/z 291 was formed by the loss of methyl group (M^+-CH_3). Loss of –CH_2OH resulted in the mass fragment m/z 275(M^+ -31). Cleavage of side chain yielded the fragment of mass m/z 245[306(-CHOH-CH_2OH)].

Periodate oxidation of the diol yielded the corresponding aldehyde. In the ^{1}HNMR spectrum of the aldehyde, the aldehydic protons of the two diols appeared as singlets at δ 9.38 and 9.39 ppm. Of the six methyl groups, two methyl proton signals were shifted from 0.87 and 0.89 to 1.00 and 1.01ppm in the oxidation product. Other methyl proton shifts were unaltered. Hence it can be concluded that the methyl group is at C-13 instead of C-8 in the diols, and the compound is

therefore a mixture of erythroxydiol X and erythroxydiol Y.

Lanthanide induced shifts on the ^{13}C NMR of the compound and its diacetate were studied after the addition of increasing amounts of $Eu(fod)_3$ $-d_{27}$. Table-1 shows the carbon assignments for the two diols and the shifts obtained for the various carbons. Analysis of the LIS data showed that there has been gradual decrease in the extent of downfield shift of signals for carbon from the point of coordination (diol and its diacetate). The extent of downfield shift for C-13 itself was however small. For the diacetate, the extent of downfield shifts for the various carbons was of lesser magnitude, probably due to weak coordination. LIS data could not provide much information regarding the position of methyl group at C-13 as a few quaternary carbon signals were buried under other large signals.

The two diols could not be separated by column chromatography, as they showed only a single spot in TLC (silica gel). Erythroxydiol X is saturated and Erythroxydiol Y has only one exomethylene double bond. Though they could not be separated by HPLC using UV detector, complete carbon assignment for the compound and its diacetate was possible. Table-2 shows the carbon assignment of erythroxydiol X and Y. The ^1H NMR and ^{13}C NMR assignment were confirmed by 2D δ_C/δ_H correlation studies, on the compound and its diacetate. In the compound, cyclopropane proton signals at 0.12 and 0.55 ppm correlated with carbon signal 24.7 (C-18). The broad singlet at 4.48 (exomethylene protons) correlated with carbon signal at 102.08 ppm (C-17). The broad doublet at 3.28 ppm (>CHOH) showed correlation with the doublets at 81.37 and 81.44 ppm thereby confirming the assignments. Similarly both the signals at 3.48 and 3.72 correlated with the carbon signal at 62.67 ppm for $-CH_2OH$.

In the diacetate also similar correlation could be observed. The doublets at 0.1 and 0.5 showed correlation with the carbon signal at 24.67 ppm (C-18). The acetate methyl signals (1.99 and 2.05) correlated with the quartets at 20.69 and 20.78 ppm. There was correlation between the broad singlet at 4.45 and the carbon signal at 102.15 ppm ($=CH_2$). The signal at 4.7 showed correlation with the doublet at 79.4 (>CHOAc) and signals at 3.98 and 4.38 correlated with triplet at 63.13 ($-CH_2OAc$). Thus 2D δ_C/δ_H correlation studies further confirmed the ^1H NMR and ^{13}C NMR assignments of erythroxydiol X and Y.

Erythroxydiol X and Y were separated as their acetonides. In the ^1H NMR spectrum of erythroxydiol X acetonide, two one proton doublets appeared at δ 0.1 (J=4.3Hz) and 0.52 ppm (J=4.0Hz) for the cyclopropane methylene protons. There was no broad singlet at 4.9 ppm for exomethylene proton as expected thereby confirming the purity of erythroxydiol X acetonide. Similarly in the ^1H NMR spectrum of erythroxydiol Y acetonide, the doublets at 0.1 and 0.5ppm were absent showing the absence of cyclopropane moiety. Instead a broad singlet (2H) was observed at δ 4.49 ppm for exomethylene protons at position 4 of the diterpenoid. The acetonide methyl signals were observed at δ 1.34 and 1.41 ppm (each singlet) for both the compounds. Three proton singlets at δ 0.76, 0.92 and 1.02 ppm accounted for the three tertiary methyl groups in erythroxydiol X acetonide. The three tertiary methyl groups in erythroxydiol Y acetonide appeared as three proton singlets at δ 0.75, 0.89 and 1.06 ppm. The $-CH_2$-O- protons of the acetonide moiety appeared as a two proton multiplet at δ 3.75 ppm in both the compounds. A double doublet at δ 3.88ppm (1H, J=3.6 & 8.32 Hz) in erythroxydiol X acetonide could be ascribed to the >CH-O- proton of the acetonide moiety. In erythroxydiol Y acetonide, the double doublet for the >CH-O- proton appeared at δ3.87 ppm with J values of 4.1 and 8.2Hz. ^{13}C NMR spectra of two acetonides accounted for all the 23 carbons for both the compounds. Table-3 shows the ^{13}C assignment for both the acetonides. The compound is therefore a mixture of the diterpenoid isomers erythroxydiol X and erythroxydiol Y in equal proportion. This is the second report of occurrence in nature. This happens to be the first report of isolation of erythroxydiol X and Y from the genus *Indigofera* and from the family Leguminosae.

Table 3: ^{13}C NMR Assignment of erythroxydiol X and Y acetonides

Carbon*	Erythroxydiol X diacetate	Erythroxydiol Y diacetate
1(t)	33.96	37.41
2(t)	32.35	33.13
3(t)	23.31	28.74
4(s)	17.13	160.73
5(s)	38.22	35.52
6(t)	19.63	21.91
7(t)	26.29	28.34
8(d)	41.39	41.95
9(s)	41.39	40.64
10(d)	49.94	56.34
11(t)	33.54	34.90
12(t)	28.60	29.69
13(s)	36.35	37.86
14(t)	35.63	36.27
15(d)	84.97	84.84
16(t)	64.72	64.69
17(q)	22.39	102.05
18	24.73 (t)	21.51(q)
19(q)	11.58	12.34
20(q)	19.10	19.36
21(s)	108.48	108.49

*Multiplicities are given in brackets.

4. Conclusion

Extraction, fractionation and characterization of the solvent extracts led to the identification of ten compounds n-butyl ester of nanodecanoic acid, 1-octadecanol, 4- heneicosanone, α-amyrin, *n*-octacosanol, β-sitosterol, salicylic acid, erythroxydiol X, erythroxydiol Y and β-sitosterol-3β-D-glucopyranoside. All these compounds are reported for the first time from the genus *Indigofera*.

5. Acknowledgement

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