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Neurological Studies of Novel Compounds from *Swertia Chirayita*

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Abstract

Natural plants exhibit different activities due to different biologically active constituents that's why it is the need that isolated compound from the plant must be fully characterized in a biological, pharmacological sense and biochemical sense. *Swertia chirayita* is a well known traditional Ayurvedic plant and is considered most important for its medicinal properties. It has been shown by the literature that plant are the rich sources of informative molecules like xanthones, seco-irridoid glycosides etc, main aim of this research is to extract, isolate and characterized the active principles mainly novel compounds and do their biological activity two compounds have been isolated which have not been reported yet and their pharmacological studies (CNS) has been performed.

Key words: *Swertia chirayita*, xanthones, Gentianaceae, CNS, anti-depressants, ALD_{50}

Introduction

Swertia chirayita (Roxb.ex.flem) Karsh grows abundantly in the temperate regions of the Himalayas and belongs to the family Gentianaceae. It has been shown in the literatures that several biological activities attributed to *Swertia chirayita*. The entire plant is used for medicinal properties. It has been used in the Ayurvedic system of medicines as a bitter stomachic,febrifuge,anthelmenthic,diuretic antiepileptic[1-2] and for certain type of mental disorders. In the earlier chemical investigation of *Swertia chirayita* number of xanthones 1, 5,8-trihydroxy-3-methoxyxanthone; 1-hydroxy-3,5,8-trihydroxyxanthone; 1-hydroxy-3,7,8-trimethoxyxanthone; 1,8-dihydroxy-3,5-dimethoxyxanthone; 1,8-dihydroxy-3,7dimethoxy

xanthone; 1,3,6,7-tetrahydroxyxanthone-2-β-D-glucosides(mangiferin); 1,3,8-trihydroxy-5-methoxyxanthone; 1-3,5,8-tetrahydroxyxanthone,1,3,7,8-tetrahydroxyxanthone, dimeric xanthone(chiratanin), a number of triterpine including swertanones and the alkaloids gentiatine, gentiocrucine and enicloflavine were isolated [3-7] The present investigation was carried out to find out the some novel constituents and characterized and identified them chemically and biologically. We reported the isolation and characterization of two novel compounds with xanthones 1,8-dihydroxy-3,5-dimethoxyxanthone,1,8-dihydroxy-3,7-dimethoxyxanthone reported earlier from the plant [8-12].

The total compounds isolated were seven out of which we got two novel compounds with xanthones and were studied in detailed.

Material and Methods

Aerial part of the plant material were collected in the month of October from the sub-Himalayan region of west Bengal, India, and identified from the CDRI,Lucknow.

Preparation of the extract:
Air dried plant material 4 Kg was placed in the glass percolator and was kept over night for 24h. Percolate was collected and concentrated in rota vapour at 45°C.The weighing of the ethanolic extract was observed as 195.5gm

Fractionation of crude ethanolic extract:
After the extraction the crude ethanolic extract was fractionated with different solvent as shown in the Table 1.

Table: 1: Fractionation of extract with different solvents

Solvent used	Amount of solvent (ml)	Weight of extract obtained (gm)
Hexane	500 (4 times)	24.50
Ethyl acetate	500 (4 times)	53.23

Isolation and the characterization:
Isolation was done through column chromatography. Column was packed with silica gel (60-120 mesh).

Silica gel used for packing was 20-40 times of the crude product of hexane fraction. Column was setup for every fraction and column was eluted with solvents in the increasing order of polarity (10% $CHCl_3$ in hexane, 15% $CHCl_3$ in hexane).

Fractions were collected and examined and pooled according to their TLC Pattern. TLC chromatograms were visualized in iodine vapours by spraying of 5% dilute sulphuric acid followed by heating at 105°C Pooled fractions were concentrated under vacuum and purified by recrystallization with different solvents.

Chemistry:
IR studies of isolated compounds were conducted with FTIR Perkin Elmer. Mass studies were done by MS JOEL D 300 and NMR studies of compounds were conducted with Bruker avance DRX 300.

Characterization and spectral analysis

Compound *1 (26-Hydroxy tritriacontan-16-one):*

Chloroform solvent eluted white solid which was crystallized from acetone. Melting Range: 77-79°.C; IR (cm^{-1}): 3450cm^{-1}(OH), 2945 cm^{-1}(CH stretching of methyl group),1710 cm^{-1}(acylic ketone).; ^{1}H NMR in $CDCl_3$ at 200 MHz displayed the triplet at δ 0.84 (6.6Hz), 0.88 (6.4Hz) which were assigned to the protons of terminal methyl at C-1 and C-33.Three broad signals at δ 1.25 (36 H) and at δ 1.58 (18 H) were accounted to the 27 methyl group. One signal appear at δ 3.49 was assigned to the proton of carbon attached to the OH group. Two multiplets δ 2.35 and δ 2.38 were ascribed to the methylene group at C-17 and at C-19 respectively, adjacent to carbonyl functional group. Its Electron Spray Ionization Mass spectrum showed molecular ion peak at m/z 495 (M+1) corresponding to molecular formula $C_{33}H_{66}O_2$.The ion peak at m/z 365, 241, 255 supported the carbonyl group at supported the carbonyl group at C-18.Mass spectrum and its fragmentation pattern showed the ion peak at m/z 495 (M+1) m.wt 494, having molecular formula $C_{33}H_{66}O_2$.

On the basis of above evidences, it was found that structure of isolated phytoconstitutent compound has been elucidated as 26-Hydroxy tritriacontan-16-one [13].
$CH_3(CH_2)_{13}CH_2COCH_2(CH_2)_7CH_2CHOHCH_2(CH_2)_5CH_3$**.**

Compound 2 *(Pentacosanoic acid):*

Ethyl acetate fraction yielded the white solid eluted from the 15% methanol:chloroform from the column. Melting Range: 70-73°C.; IR (cm^{-1}):2921 $cm^{-1}$and 2852 cm^{-1}(CH stretching of methylene group), 1725 cm^{-1} (C=O stretching of aliphatic acid).Two bands arising from the C=O stretching vibration and O-H bending of COOH at 1216 $cm^{-1}$and at 1435 cm^{-1}.; ^{1}HNMR in ($CDCl_3$) at 300 MHz showed the presence of 50 protons supported by the ^{1}HNMR.One broad signal appeared at δ 1.27 ppm was assigned to about 42 protons of methylene group. Chemical shift of the proton on the methylene carbon adjacent to COOH functional group in aliphatic compound appeared at δ 2.39 and at 2.36 ppm corresponding to methylene protons. One triplet appeared at δ 0.92, 0.10 and at 0.87 was assigned to protons of terminal methyl group. ^{13}CNMR in $CDCl_3$ at 300MHz justifies the presence of carbonyl carbon of COOH group at δ 179.23 ppm.The peak left to the solvent peak is of olefinic carbon of COOH and to right are of aliphatic carbon. DEPT 135 showed the presence of 25 CH_2 carbons down. Peak appeared at δ 22.69 and at 24.69 for CH_2 carbon at δ 33.91 ppm and 31.93 ppm. It showed the absence of CH carbon. Electron Spray Ionization Mass spectrum showed the molecular ion at m/z 382$[M]^+$ corresponding to molecular formula $C_{25}H_{50}O_2$ as supported by ^{1}HNMR ^{13}CNMR DEPT 135 and DEPT 90.

On the basis of these analysis the compound is elucidated as pentacosanoic acid. CH_3 CH_2 $(CH_2)_{21}CH_2COOH$ [13]**.**

Compound 3 *(1, 8-dihydroxy-3, 5-dimethoxyxanthone):*

It was obtained as yellow solid which responded positively to the xanthone specific test. Melting Range: 184-186°.C; IR(cm^{-1}): 3450 cm^{-1}(OH), 3101 cm^{-1} (aromatic C-H stretching), 2920,2849 cm^{-1}(methyl C-H stretching), 1244 cm^{-1} (asymmetric C-O-C stretching) and symmetric C-O-C stretching at 1053 cm^{-1}, 1497 cm^{-1} 1502 cm^{-1}(C=C stretching).; A singlet at δ 3.64 in ^{1}H NMR was assigned to the protons of the methoxyl groups integrating for 6 protons of CH_3 group. A broad peak appearing at δ11.39, 11.99 was assigned to the hydroxyl proton present at C-1 position. Two doublets at δ 6.34 and at δ 6.54 corresponded for the presence of two single protons present in meta position at H-2 and H-4

respectively. Two doublets at δ 6.69 and at δ 6.673 and each for ortho coupled protons as shown in the Table 2

Table no 2: NMR data compound 3 in cdcl$_3$ at 300 MHz, δ in ppm, j in hz.:

Carbon	δ ppm
C-1	161.4
C-2	97.87
C-3	166.1
C-4	93.03
C-4a	156.3
C-4b	152.7
C-5	138.5
C-6	120.2
C-7	109.2
C-8	144.06
C-8a	107.9
C-8b	101.5
C-9	183.2

^{13}C NMR ($CDCl_3$): It depicts the presence of 15 carbons in the structure as supported by its molecular formula revealed through ESIMS. The ^{13}C NMR showed the presence of carbonyl carbon and two methoxyl carbons.

Distortion Enhancement for Polarization Transfer (DEPT) spectrum distinguishes between the CH_3, CH_2 and CH group. DEPT 90 justifies the presence of 4 CH carbons. DEPT 135 showed the peak. CH_3 and CH carbon up and CH_2 carbon down. It showed the absence of CH_2 carbon. The peaks to the left of the solvent peak are olefinic and to the right are aliphatic. A signal at δ 56 and 54.64 ppm was assigned to the methoxyl carbon. A signal at δ 96.59 and 91.75 ppm was assigned to the carbon attached to the OH group adjacent to the CH carbon. Carbonyl carbon appears at δ 183.2 0 ppm. DEPT 90 showed CH carbon at δ 93.03, 97.87, 109.24 and 129.27 ppm.

Thus ^{13}C NMR, DEPT 135, DEPT 90 justifies the value of chemical shift corresponding to that carbon positions. ESIMS showed peak at 289 $(M+H)^+$,290 $(M+2)^+$ which is consistent with its molecular formula $C_{15}H_{12}O_6$ as revealed by its ^{1}H-NMR as well as ^{13}C NMR spectral data.On the basis of above spectroscopic evidences, the compound 2 was elucidated as 1, 8-dihydroxy-3, 5-dimethoxyxanthone which was further conformed by the co-TLC and by comparison of its physicochemical data those reported in literature.

Compound 4 ***(1,5,8-trihydroxy-3-methoxyxanthone)****:*
Yellowish green color amorphous solid crystallized from the ethyl acetate fraction. Melting Range : 264-266°**C** Electron Spray Ionization Mass Spectroscopy showed the molecular ion peak at m/z 273 $(M-1)^+$ corresponding to the molecular formula $C_{14}H_{10}O_6$.; IR (cm^{-1}): 3440 cm^{-1}(OH group), 1246 cm^{-1} (C=C stretching), 600-900 cm^{-1}(CH out of plane bending)
^{1}HNMR ($CDCl_3$): δ 12.06, 11.24, 8.79 (1Hs,3×OH), 7.27 (1H, d, *J*=6Hz), 6.64 (1H,d,*J*=6Hz),6.56(1H,d,*J*=2Hz),6.33(1H,d,*J*=2Hz).

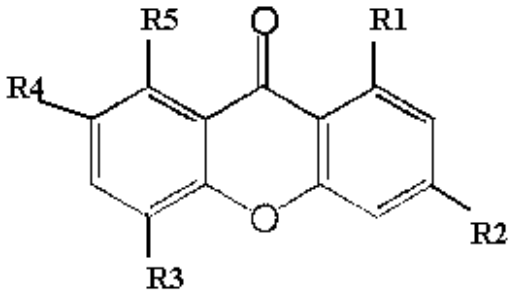

Figure: 1: Basic Skeleton of the isolated compounds (3 to 6) called Xanthone

Compound 3 = R_1=R_5=OH,R_2=R_3=OCH_3
Compound 4 = R_1=R_3=R_5=OH,R_2=OCH_3
Compound 5 = R_1=OH,R_2=R_4=R_5=OCH_3
Compound 6 = R_1=R_5=OH,R_2=R_4=OCH_3

Compound 5 *(1-Hydroxy-3, 7, 8-trihydroxyxanthone)*
Ethyl acetate fraction yielded the yellow needles from the eluting solvent chloroform. Melting Range: 163-166°.C Electron Spray Ionization Mass Spectroscopy showed the m/z 303 $[M+1]^+$ corresponding to molecular formula $C_{16}H_{14}O_6$ supported by IR and NMR data.
IR (cm^{-1}): OH (3376), 2919, 2852, 2362, C-H stretching of methyl group C=C stretching of methyl group at 1437, 1198.; ^{1}HNMR ($CDCl_3$) at 300MHz: δ 3.97 (9H,s,OMe), 13.19(1H,s,OH), 6.73(1H,d,*J*=9Hz), 7.20 (1H,d,*J*=9Hz),6.33 (1H,d,*J*=9Hz),6.49 (1H,d,*J*=9Hz) [15].

Compound 6 *(1,8-dihydroxy-3,7-dimethoxyxanthone):*
Ethyl acetate fraction yielded the yellow solid from the eluting solvent chloroform. Melting Range: 196-198°C.; ESIMS showed the molecular ion peak at 288 $[M]^+$,289$[M+1]^+$and at 313 $[M+Na+1]^+$ corresponding to molecular formula $C_{15}H_{12}O_6$.; IR (cm^{-1}): OH (3657), 2919, 2851 (C-H stretching methyl group) [14].; ^{1}H NMR ($CDCl_3$) 300 MHz: δ 11.98 (1H,s,OH), 11.39 (1H,s,OH), 3.96, 3.89 (6H,s,2OMe), 6.75 (1H,d,*J*=9Hz), 6.70 (1H,d,*J*=9Hz), 6.35 (1H,d,*J*=9Hz), 6.36 (1H,d,*J*=2Hz).

Compound 7 (*β-sitosterol)*
It was obtained as white amorphous solid which was crystallized from methanol. It responded positively to the Lieberman-Buchard test for steroid.; IR(cm^{-1}): 3435 (OH), 2941cm^{-1} (C-H stretching of methyl group) 1597 cm^{-1} (indicating the presence of unsaturation in the compound).; ^{1}H NMR spectrum of the compound displayed the presence of protons of two tertiary methyl, one primary methyl and three secondary methyl group. Two tertiary methyl signal appearing at δ 0.93(6H), 0.84(3H) were assigned to the protons of secondary methyl.Olefinic proton appearing at δ 5.36(1H,m, H-6) as shown in the Table 3. ^{13}C NMR spectra of compound justified the presence of 29 carbons in the molecule.

DEPT justifies the presence of CH and CH_3 carbon up and CH_3 down. DEPT 90 showed the presence of 9 CH carbons and DEPT 135 revealed the presence of 11 CH_2 down and 18 carbons (CH and CH_3) up. It showed the CH_2 signal at δ 21.07, 23.05, 24.28, 26.06, 28.23, 31.89, 33.93, 37.24, 39.76 and 42.48 ppm.

Table no 3: NMR data compound 7 in $CDCL_3$ at 300 MHz, δ in ppm.

No.	δ1H(J(Hz))	δ13C Value	Multiplicity
1		31.67	CH_2
2	2.29 (m, 2H)	37.28	CH_2
3	3.53 (m, 1H)	71.79	CH
4		42.33	CH_2
5		140.78	Q
6	5.35 (m, 1H)	121.67	CH
7		28.23	CH_2
8		29.22	CH
9		45.88	CH
10		36.52	Q
11		21.09	CH_2
12		39.80	CH_2
13		50.20	Q
14		56.79	CH
15		24.36	CH_2
16		25.38	CH_2
17		56.11	CH
18	0.87 (s,3H)	11.98	CH_3
19	1.02 (s, 3H)	12.04	CH_3
20		36.15	CH
21	0.94 (d, 3H)	19.38	CH_3
22		33.98	CH_2
23		28.88	CH_2
24		50.18	CH
25		29.22	CH
26	0.70 (d, 3H)	19.79	CH_3
27	0.70 (d, 3H)	19.05	CH_3
28		26.17	CH_2
29	0.84 (m, 3H)	18.78	CH_3

FABMS (Fast atom bombardment Mass spectroscopy) showed [M]+ at m/z 413 (M-1)+ corresponding to molecular formula $C_{29}H_{50}O$ supported by its ^{1}HNMR and ^{13}CNMR data and its FAB-MS also showed the peak [M-142]+ at m/z 271 appeared due to the loss of side chain isopropyl group. On the basis of these spectroscopic evidences the structure of compound was established as β-sitosterol as shown in the figure 2 which was further confirmed by derivitization and by the comparison of its physiochemical data as those reported in the literature.

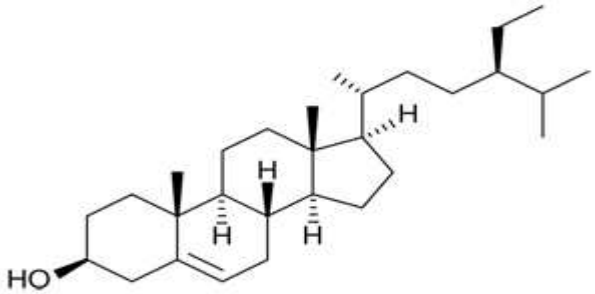

Figure 2: Compound 7: β-sitosterol

As it has already been shown in the literature the plant are the rich sources of informative molecules like xanthones,sterols,glycosides etc so the present isolation resulted in the elution of 7 compounds and from the spectroscopic analysis it was found that the compounds 3,4,5,6 and 7 has already been reported and fully identified in pharmacological and biochemical sense. On the basis of literature study and spectroscopic characterization it was found that compound 1 and 2 have not been reported yet.

These two compounds which has been elucidated as 26-hydroxytritriacontain-16-one i.e. $CH_3(CH_2)_{13}CH_2COCH_2(CH_2)_7CH_2CHOHCH_2(CH_2)_5CH_3$ and another compound 2 has been elucidated as Pentacosanoic acid i.e.$CH_3CH_2(CH_2)_{21}CH_2COOH$ are taken for further pharmacological studies as depicted in the further C.N.S studies undertaken.

Pharmacological studies

i) CNS Activity

The present study was carried out on mice of either sex weighing 20-30gm. The animals were allowed food and water ad libitum.Compounds were administered in dose of 20 mg/kg (i.p) as an aqueous suspension in gum acacia and results were compared as those of imipramine gross behaviour on motor, sensory and autonomic system were studied in mice after administration of test compounds. Compounds were screened for the effects on gross behavior, antidepressant and anti-parkinsonian activity [15].

Behavioral effects

Spontaneous motor activity (SMA), awareness, posture gait, reflexes and autonomic symptoms (respiration, salivation etc) were observed before and 3h after administration of test compounds as shown in the Table 4.

Table 4: Behavioural studies- swimming despair test

Compound	Control Mean ± S.E (in sec)	Immoblity Test Mean ± S.E (in sec)
I	188 ± 24.8	108.0 ± 4.8
II	162 ± 28.2	130.2 ± 25.94
Imipramine	----------	120.±43.2

Activity of above compounds is as compared to standard drug Imipramine

ii) Antidepressant activity using a) **Swimming Despair Test model**

Each mouse was subjected to this test 24 h prior to (control)and 2 h after drug treatment (Table: 4 and Table: 5) This test was performed according to method of Porsolt et al. 1978 [15].

Table 5: Anti-depressant activity of compounds administered with dose of 100 mg/Kg i.p.

Compounds	Awareness	Gait	Steriotype	Piloerection	Ptosis mean score	ALD_{50} (mg/kg i.p)
I	4+	N	3+	1+	4.0	> 1000
II	3+	N	3+	1+	4.0	< 1000
Imipramine	4+	N	3+	1+	4.0	

NA = Not aware, A=Aware, N=Normal, AB=Abnormal

b) **Reserpine Reverse Activity**
Anti-depressant activity effect was observed by testing the compound for reserpine reverse test [16] .Reserpine (5mg/kg i.p) was administered 15 min prior to the compounds to be tested and the following parameters were observed for 2 h after test compounds administration.

c) **Locomotor activity:**
It was measured by placing each mouse in photoactometer for 5 minutes and the total count was recorded.

d) **Ptosis**: Intensity of ptosis was graded according to method of Rubin.et.al 1957 [17]

e) **L-Dopa Potentiation test:**
It was performed of placing each mouse in photo-actometer and observing locomotor activity.

iii) Anti-Parkinsonian Activity
The study was carried out on the mice weighing 20-30 gm of either sex. The animals were fed food and water ad libitum.The number of animals in each group was 5. The two compounds were administered as dose of (100mg/kg i.p) as shown in the Table: 6 and 7

Table 6: CNS profile of compounds

Compounds	Control Mean ± S.E	Hypokinesia Mean ± S.E	Control Mean ± S.E	Locomotor activity Mean ± S.E
I	42.2 ±11.4	20.4 ± 3.20	36.0 ± 4.85	86.4 ± 2.20
II	104.4 ± 16.25	29 ± 5.08	112 ± 40.9	32.8 ± 15.09

Hypokinesia = reserpine reversal test $p < 0.001$
Locomotor activity. L-Dopa potentiation test $p < 0.001$

Table 7: Anti-Parkinsonian activity of the compounds:

Compounds	Oxytremorine (0.5 mg/kg i.p) Tremor index	Rigidity (%)	Hypokinesia (%) count	ALD_{50}
I	1	2	21± 3.16	> 500
II	2	2	35± 11.6	>500
Imipramine	0	0	79 ± 36.0	--------

a) *Tremor:* Tremor was induced by oxytremorine (0.5 mg/kg i.p) in mice 45 minutes after pretreatment with test compound. After 5 min of oxytremorine injection tremors were assessed usually and scored as 0 = no tremor, 1 = occasional tremor, 2 = intermittent tremor,3 = continuous tremor according to Coward et al [18] each animal of a group was scored and tremor index (mean score of each group) was determined.

b) *Rigidity:* Reserpine (5mg/kg i.p) was administered in rats to produce rigidity and after 15 min test compound was injected. The degree of resistance was measured according to method of Goldstein et al, 1975 [19].
0 = no resistance, 1 = normal resistance, 2 = complete resistance. A score of 2 was selected as criteria for rigidity and expressed as percentage of animal showing rigidity in group.

c) *Hyperkinesia*: It was produced by administering reserpine (5mg/kg i.p) in rats. Locomotor's activity was measured after 2h by placing each group of rats in photoactometer for 15 min and total counts were recorded. The test compound was administered 15 min after reserpine.The percentage increase or decreases in counts were calculated after 15 min of reserpine administration.

Approximate Lethal Dose: (ALD_{50})

It was estimated that the compound were investigated for their acute neurological toxicity. Mice (either sex) weighing 20-25 gm were used for the study.
ALD_{50} values were determined by observing mortality with in 24 hrs after drug administration.

Results and Discussion

The isolated compounds were studied for their CNS activity to asses behavioural effects. The CNS activity profile of the compounds are given in the (Table 5-7) an increase in awareness was found and decrease in immobility time was also recorded in Swimming Despair test (Table 4) and the above compounds exhibited decrease in immobility time and were further studied for anti-parkinsonian activity. From reserpine reversal test it was observed that compounds antagonized the reserpine induce ptosis showing similarity with imipramine. Compounds also potentiated the L-Dopa effects thus showing anti-depressant activity (Table 6-7) as far as anti-parkinsonian activity is concerned against reserpine reversal test reavealed that compounds exhibited hyperkinesia showing similarity with anti-depressant test. In L-Dopa potentiation test compounds exhibited L-Dopa effect. In Oxytremorine test compounds exhibited very less tremors as compounds to test compounds.

Acute neurological studies revealed that all compounds exhibited ALD_{50} values more than 1000 mg/kg.

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