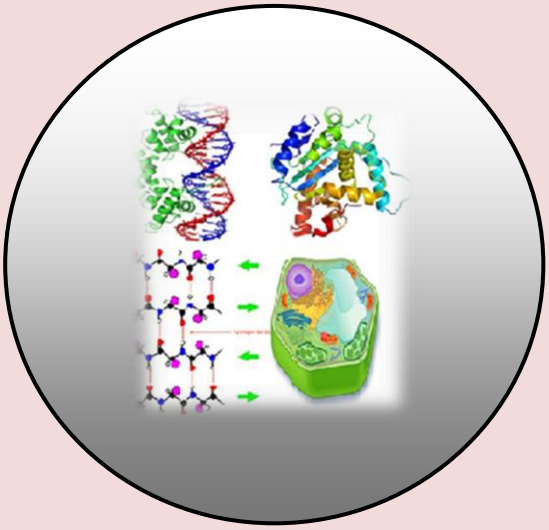
Isolation of Novel C-21 Nor Precursors of Pregnane from *Hoya longifolia*

By

Satish C. Pandey, Kuldeep Kumar Shashi Bala and Desh Deepak

ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66



J. Biol. Chem. Research
Volume 39 (2), 2022 Pages No. 158-171

Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 39, No. 2, 158-171, 2022
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 39/02/101/2022

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

Dr. Desh Depak
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

RESEARCH PAPER

Received: 09/11/2022 Revised: 28/12/2022 Accepted: 29/12/2022

Isolation of Novel C-21 Nor Precursors of Pregnane from *Hoya longifolia*

Satish C. Pandey, *Kuldeep Kumar Shashi Bala and Desh Deepak

Department of Chemistry, University of Lucknow, Lucknow 226007, India
***Department of Chemistry, Maharana Pratap Gov. P.G. College, Hardoi 241001, India**

ABSTRACT

Three novel pregnane viz Gifogenin (E), Longigenin (F) and Longin (I) were isolated from *Hoya longifolia* (family: Asclepiadaceae) and their structures were elucidated by using modern physicochemical methods and chemical transformation. Compound E, F and I were found to be C-21 nor compounds and were identified as: 2-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene, 2(3'-methoxy)-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene and 14β-hydroxy-21-nor-pregn-5-ene-3-O- β-D-Cymaropyranoside respectively.

Keywords: *Hoya longifolia*, Asclepiadaceae, Gifogenin, Longigenin and Longin.

INTRODUCTION

Biogenetics studies have revealed that pregnanes are biological precursors of Cardiac glycosides which are of importance in medical chemistry due to their digitalis like effect on cardiac muscles (Hossain et al., 2011, Khare et al., 1986, Hossain et al., 2010). Pregnanes also resembles in their structure with medicinally efficacious corticoids. These important class of compounds are abundantly present as aglycons and their glycosides in the plants of Asclepiadaceae family. Recently pregnanes have shown antitumor (Hossain et al., 2010, Deepak et al., 1989, Deepak et al., 1997, Deepak et al., 1997), anticancer (Hossain et al., 2010, Deepak et al., 1989, Deepak et al., 1997, Deepak et al., 1997, Hussain et al., 2015), cytotoxic (Babu et al., 2008), α-amylase inhibitory activities (Duong et al., 2021) lactogenic (Piacente et al., 1998, Sethi et al., 2008), BRD4 inhibitory and cytotoxic activity (Sun et al., 2021) and selective cytotoxicity (Wang et al., 2022). In our recent work on the constituents of *Hoya longifolia* (family: Asclepiadaceae), we have not isolated C-21 nor Pregnane derivative from chloroform soluble extract of plant *Hoya longifolia.*

These nor pregnane derivative with varied structure are biological precursors of pregnanes which ultimately leads to the formation of cardiac glycosides. Surprisingly these precursors also possess the digitalis like effect as exhibited by their ultimate compounds of biosynthetic pathway in cardiac glycosides. These nor derivative bind strongly to the steroid recognition sites on the cardiac glycosides receptors Na^+, K^+, ATPase. Besides having the cardio-tonic activity the C-3 glycosides exert certain potentially useful effect on heart and kidney not shared by the digitalis drugs (Neupane et al., 2017). They are also involved in regulation of vascular tone[1] and have shown significant anabolic potency. Isolation of these nor pregnane derivatives from *Hoya longifolia* (family: Asclepiadaceae) with biological activity similar to that shown by Cardinolides and pregnanes clearly relates the biogenetic pathway of cardenolides, pregnanes and nor pregnanes. This papers deals with structure elucidation of two novel nor pregnane and nor pregnane glycoside.

EXPERIMENTAL

The ^1H, ^{13}C NMR and 2D NMR spectra were recorded on 200 MHz and 300 MHz Bruker spectrometer in $CDCl_3$ using TMS as internal standard. FAB mass spectra were recorded with JEOL mass spectrometer model JMS-SX102 FAB with DA 6000 data system and JEOL mass spectrometer D-300 with IMA-2000 data system. TLC was performed on silica gel-G (Qualigens) and silica gel 60-120 mesh (Qualigens) was used for Column chromatography. Normal sugars were made visible by partridge reagent on PC.

Plant Extraction

The plant *Hoya longifolia* was collected in bulk (about 50 kg) from Sippighat, South Andaman, India and identified by Dr. M. N. Srivastava, Scientist, Botany division, Central Drug Research institute, Lucknow and its herbarium sample was submitted at CDRI, Lucknow. Shade dried, powdered plant material (10 kg) of *Hoya longifolia*was extracted and fractionated by the method used for pregnane glycosides. These extracts were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding $CHCl_3$ soluble extract (7.5 gm), $CHCl_3$-EtOH (4:1) soluble extract (4.0 gm) and $CHCl_3$-EtOH (3:2) soluble extract (3.5 gm). Repeated column chromatography of $CHCl_3$ extract using C_6H_{14}-$CHCl_3$-MeOH of varied concentration yielded two novel C-21 nor pregnanes and one novel C-21 nor pregnane glycoside namely Gifogenine, Longigenin and Longin respectively.

Longigenin (F)

m.p. 158ºC, (Found: C, 76.29%; H, 9.07% $C_{28}H_{40}O_4$ requires C, 76.36%, H, 9.09%). It gave positive Liebermann Burchard test and tetra-nitro methane test.

^{1}H NMR

δ0.83 (s, 3H, H-18), δ0.88 (s, 3H, H-19), δ1.33 (d, 3H, J= 7.5 Hz, H-20), δ1.61 (m, H-16), δ2.31 (m, H-17), δ3.67 (s, 3H, -OCH_3 at C-3), δ4.69 (s, 3H, -OCH_3 at C-3'), δ4.94 (m, 1H, H-3), δ5.34 (m, H-6), δ5.81 (m, H-2), δ7.12 (m), δ7.26 (m), δ7.52 (s) and δ7.55 (m) for aromatic protons.

FAB-MS

m/z 480 $[M+K+H]^+$, m/z 474 $[M+Na+H]^+$ and m/z 440 $[M]^+$, m/z 423[M-OH], m/z 361[M-79], m/z 285[361-C_6H_4], m/z 269[M-171], m/z 254[269-CH_3], m/z 239[254-CH_3], m/z 224[239-CH_3], m/z 166[M-274], m/z 274[M-166], m/z 167[274-107], m/z 136[167-31].

$NaOCH_3$ hydrolysis (F)

The compound **(F)** (1mg) was taken into MeOH in round bottom flask and added pieces of sodium metal portion wise. No change was observed over the TLC. This indicates that the compound **F** does not have ester function into it.

Acetylation of compound F

Compound F (1mg) was dissolved in anhydrous pyridine (0.5ml) and mixed with acetic anhydride (0.5ml). The mixture was heated on a water bath at 100 ºC for 1 hour. But no change was observed over the TLC, thus indicating the absence of acetylable hydroxyl group.

Gifogenin (E)

m.p. 154.5ºC, (Found: C, 78.98%; H, 9.24% $C_{27}H_{38}O_3$ requires C, 79.02%, H, 9.26%). It gave positive Liebermann Burchard test and tetra-nitro methane test.

^{1}H NMR

δ0.82 (s, 3H, H-18), δ0.88 (s, 3H, H-19), δ1.33 (d, 3H, J= 7.6 Hz, H-20), δ1.62 (m, H-16), δ2.31 (m, H-17), δ3.66 (s, 3H, -OCH_3 at C-3), δ4.92 (m, 1H, H-3), δ5.34 (m, H-6), δ5.34 (m, H-6), δ5.78 (m, H-2), δ7.11, δ7.11, δ7.14, δ7.52 and δ7.54 for aromatic protons.

FAB-MS

m/z 473 $[M+Na+K+H]^+$, m/z 393[M-OH], m/z 317[M-OC_6H_5], m/z 285[M-171], m/z 245[M-2x-OCH_3, -OH], m/z 230[M-OC_6H_5-OCH_3-OH-3xCH_3], m/z 231[230+H], m/z 253[230+Na], m/z 269[230-K], m/z 166[M-244], m/z 244[M-166], m/z 213[244-OCH3], m/z 120[213-OC_6H_5].

$NaOCH_3$ hydrolysis (E)

The compound **(E)** (1mg) was taken into MeOH in round bottom flask and added pieces of sodium metal portion wise. No change was observed over the TLC. This indicates that the compound **F** does not have ester function.

Acetylation of compound E

Compound E (1mg) was dissolved in anhydrous pyridine (0.5ml) and mixed with acetic anhydride (0.5ml). The mixture was heated on a water bath at 100 ºC for 1 hour. But no change was observed over the TLC, thus indicating the absence of acetylable hydroxyl group.

Longin (I)

m.p. 84.5ºC, (Found: C, 72.31%; H, 9.79% $C_{27}H_{44}O_5$ requires C, 72.32%, H, 9.82%). It gave positive Liebermann Burchard, xanthydrol, Kellar- Killani and tetra-nitro methane test.

^{1}H NMR

δ0.80 (s, 3H, H-18), δ1.077 (s, 3H, H-19), δ1.61 (m, 2H, H-16), δ2.36 (m, 1H, H-17), δ3.70 (m, 2H, H-3), δ3.40 (s, 3H, -OCH_3), δ3.06 (m, 1H, H-3), δ4.52 (1H, H-1', dd, J= 1Hz, J= 8.5Hz), δ2.65 (m, H-2' equatorial), δ1.42 (d, 6H, J=6.2Hz, H-20 and H-6'), δ1.77 (m, H-2' axial), δ5.406 (m, H-6),

FAB-MS

m/z 471 $[M+Na]^+$, m/z 448$[M]^+$, m/z 304[M-145], m/z 290, m/z 182[290-138], m/z 138[290-182], m/z 162[M-304], m/z 145, m/z 117, m/z 86, m/z74.

$NaOCH_3$ hydrolysis (I)

The compound **(I)** (1mg) was taken into MeOH in round bottom flask and added pieces of sodium metal portion wise. The change was observed over the TLC. This change indicates that the ester linkage is present in the compound **I**.

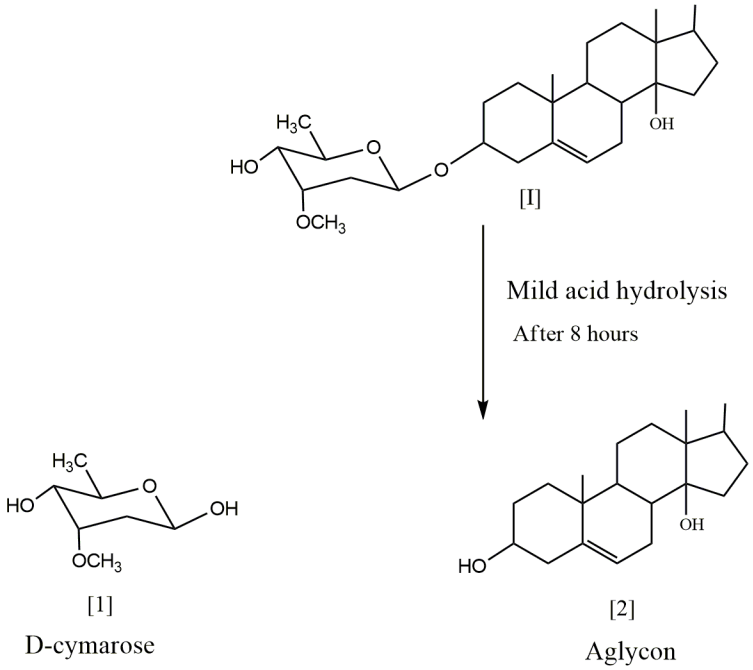
Mono-O-Acetyl Longin

Substance (I) (5mg) was dissolved in anhydrous pyridine (0.5ml) and mixed with acetic anhydride (0.5ml). The mixture was heated on a water bath at 100ºC for 1 hour. Pyridine and excess acetic anhydride was then removed under reduced pressure.

The viscous residue was taken in $CHCl_3$ (1 ml) and it was washed in sequence with 2N-HCl (2 x 1 ml), ice cold 2N-$NaHCO_3$ (2 x 1 ml) and finally with H_2O (2 x 1 ml). The $CHCl_3$ layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the amorphous acetate (5.1 mg).

Mild acid hydrolysis of substance I with acid

To a solution of (I) (10 mg) in 1,4-dioxane (1ml) was added 0.1 N H_2SO_4 (1ml) and the solution was warmed for 30 minutes at 50^0 C. Hydrolysis was completed in 8hrs. The reaction mixture showed two new spots which were the sugar (1) and aglycon (2). Dioxane was removed under reduced pressure. The aqueous portion was repeatedly extracted with $CHCl_3$. The aqueous hydrolysate was neutralized with freshly prepared Ba_2CO_3 filtered and concentrated under reduced pressure to afford sugar. The TLC analysis of compound I with the sugar was identified as D-cymarose by comparison with authentic samples (TLC, PC) (Scheme1). It was further characterized by its oxidation with bromine water to yield the corresponding lactone, which on treatment with phenyl hydrazine gave a known D-Cymaronic acid phenyl hydrazide (mp152-154°C). Hence, the glycon was deduced to be D-cymarose ($[\alpha]_D$+53.5°).



Scheme 1. Mild acid hydrolysis of Longin (I).

RESULTS AND DISCUSSION

Substance F (Longigenin)

Compound F, $C_{28}H_{40}O_4$, m.p. 158.5ºC, showed positive Liebermann-Burchard (Zemplen and Kiss, 1927) and tetra-nitro methane tests (Abisch and Reichstein, 1960, Tschesche et al., 1953, Nagata et al., 1957) suggesting it to be the steroidal moiety with double bond in it. The FAB mass spectrum of F showed molecular ion peak M^+ at m/z 440 along with the quasimolecular ion peak at m/z 480 $[M+K+H]^+$ confirming the molecular formula of longigenin as $C_{28}H_{40}O_4$ and molecular weight as 440.

^{1}H NMR spectrum of compound F exhibited the characteristic signals of pregnane derivatives but it lacked the signals for –$COCH_3$ and $CH_3CH(OH)$- side chain generally present at C-17 position of the pregnane. However, it contained a doublet at δ1.33 (d, 3H, J=7.5Hz, H-20) for secondary CH_3 group which gives a cross peak with a multiple at δ2.31(m, H-17) assigned to C-17 methine proton in its ^{1}H-^{1}H COSY spectrum. This methine proton further gives a cross peak at δ1.61(m) assigned to C-16 methylene protons suggesting that the compound F may be a norpregnane derivative at C-21. The ^{1}H NMR spectrum also contained a singlet at δ3.67 along with a multiplet at δ4.94 suggesting the presence of methoxy group at C-3 of pregnane moiety, while the presence of another methoxy group at δ4.69 accompanied by aromatic protons along with downfield shifted methine proton at δ5.81 suggested the presence of methoxy substituted benzoxy group at C-2 of F. the position of –OCH_3 group in benzoxy was assigned as meta by the pattern of ^{1}H NMR of aromatic protons protons i.e. δ7.12(m), δ7.26(m), δ7.52(s) and δ7.55(m). The multiplet at δ5.34 could be assigned to the presence of a double bond between C-5 and C-6 of the compound F. Other protons signals of Longigenin are given in the table 1.

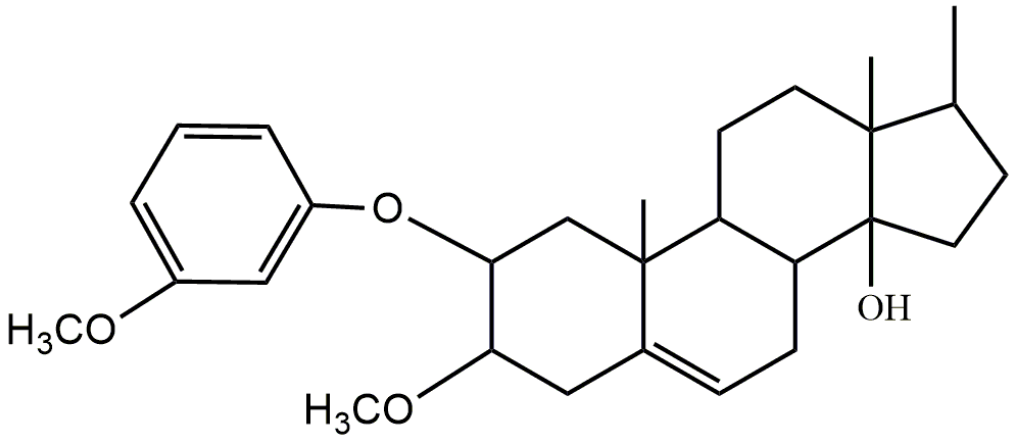
Table 1. ^{1}H NMR data of Compound F and E at 300MHz and Compound I at 200 MHz in $CDCl_3$

Compound F	Compound E	Compound I
δ0.83 (s, 3H, H-18)	δ0.82 (s, 3H, H-18)	δ0.80 (s, 3H, H-18)
δ0.88 (s, 3H, H-19)	δ0.88 (s, 3H, H-19)	δ1.077 (s, 3H, H-19)
δ1.33 (d, 3H, J=7.5Hz, H-20)	δ1.33 (d, 3H, J=7.6Hz, H-20)	δ1.61 (m, 2H, H-16)
δ1.61 (m, H-16)	δ1.62 (m, H-16)	δ2.36 (m, 1H, H-17)
δ2.31 (m, H-17)	δ2.31 (m, H-17)	δ3.70 (m, 2H, H-3)
δ3.67 (s, 3H, OCH_3 at C-3)	δ3.66 (s, 3H, OCH_3 at C-3)	δ3.40 (s, 3H, OCH_3)
δ4.69 (s, 3H, OCH_3 at C-3')	δ4.92 (m, 1H, H-3)	δ3.06 (m, 1H, H-3)
δ4.94 (m, 1H, H-3)	δ5.34 (m, H-6)	δ4.52 (d, H-1', dd, J=1Hz, J= 8.5Hz)
δ5.34 (m, H-6)	δ5.78 (m, H-2)	δ2.65 (m, H-2' equatorial)
δ5.81 (m, H-2)	-	δ1.42 (d, 6H, J=6.2Hz, H-20 & H-6')
δ7.12 (m), δ7.26 (m), δ7.52 (s) and δ7.55 (m) for aromatic protons	δ7.12 (m), δ7.26 (m), δ7.52 (s) and δ7.55 (m) for aromatic protons	δ1.77 (m, H-2' axial)
δ0.83 (s, 3H, H		δ5.406 (m, H-6)

From the results obtained from ^{1}H- ^{1}H COSY spectrum of compound F it was confirmed that Longigenin belongs to neither category of nor pregnanes. Besides the ^{1}H- ^{1}H COSY correlations of H-20, H-17 and H-16, the ^{1}H- ^{1}H COSY of F also confirms the connectivity of H-3 and H-2 protons. The H-3 multiplet present at δ4.94 showed a cross peak with H-2 proton present at δ5.81 (m-H-2) on one side and simultaneously it also shows connectivity with CH_2 proton of C-4. This correlation of C-3 and C-2 protons confirms that the 3-O-methyl benzoxy and methoxy groups are present at C-2 and C-3 in A ring of the C-21 nor pregnane and are flanked by two methylene groups of C-1 and C-4.

The ^{13}C NMR spectrum of compound F showed a total of 28 carbon signals, of which 20 were assigned to the C-21 nor pregnanes and remaining 8 were assigned to the two substituents at C-2 and C-3. In the ^{13}C NMR spectrum the presence of C-5 and C-6 double bond was confirmed by the signals at δ147.1 and δ119.1 respectively. The signal present at δ76.4 was assigned to C-14. The signals present at δ70.2 and δ79.8 were assigned for C-3 and C-2 respectively. The signals of two methoxy groups present at C-3 and C-3' were observed at δ51.8 and δ56.2 respectively. Other carbon signals of Longigenin are given in the table 2.

The FAB mass spectrum of Longigenin further confirmed the assigned structure. The highest mass ion peak recorded at m/z 480 and m/z 440 were for quasimolecular ion peak (M+K+H) and molecular ion M^+ thus confirming the molecular weight of the compound F as 440. The mass spectrum contains the ion peak at m/z 423 assigned to the loss of –OH group present at C-14. The mass ion peak at m/z 423 fragmented to give mass ion peak at m/z 361 which is attributed to the loss of the two-OCH_3 groups. The loss of 74 a.m.u. from m/z 361 gave mass ion peak at 285 which corresponds to the loss of $–C_6H_4$. The fragment at m/z 285 further looses 16 a.m.u for oxygen atom to give ion peak at m/z 269. The mass ion peak at m/z 269 further fragmented to give the mass ion peaks at m/z 254, 239 and 224 which aroused after the consecutive losses of three CH_3 groups present at C-18, C-19 and C-20 respectively. The characteristic retro diels alder fission (Zemplen and Kiss, 1927) of C_5- C_6 double bond observed in pregn-5-enes gave peak at m/z 166 thus confirming the presence of hydroxyl group at C-14. The complimentary ion peak at m/z 274 led support to the presence of methoxy and methoxy substituted benzoxy groups in the A ring of the C-21 nor pregnane. Further, the loss of 107 and 31 from m/z 274 giving mass ion peaks at m/z 167 and m/z136 confirmed the presence of methoxy substituted benzoxy and methoxy groups in the ring A. In the light of the foregoing evidences the structure of **Longigenin (F)** was established as **2(3'-methoxy)-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene**



2(3'-methoxy)-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene

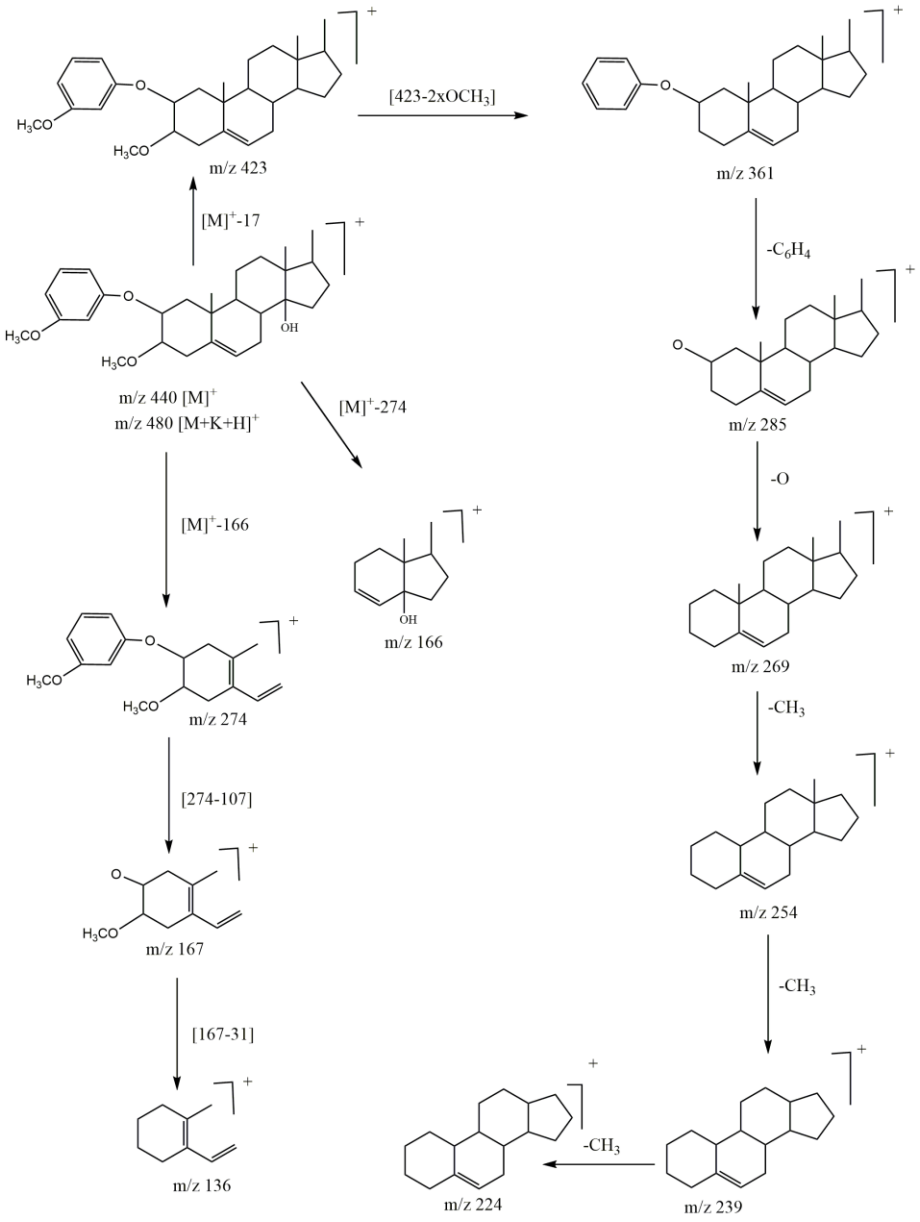


Figure. FAB mass fragmentation of compound Longigenine(F).

Substance E (Gifogenin)

Compound E, $C_{27}H_{38}O_3$,m.p. 154.5ºC,showed positive Liebermann- Burchard (Zemplen and Kiss, 1927) and tetra-nitro methane tests (Abisch and Reichstein, 1960, Tschesche et al., 1953, Nagata et al., 1957) suggesting it to be the steroidal moiety with double bond in it. The FAB mass spectrum of E showed the highest mass ion peak as quasimolecular ion peak at m/z 473 which could be assigned for $[M+Na+K+H]^+$. Thus confirming the molecular weight of Gifogenin as 410 and molecular formula $C_{27}H_{38}O_3$.

The pattern of ^1H NMR spectrum of compound E showed the structural resemblance with earlier isolated longigenin and also lack the signals for $-COCH_3$ and $CH_3CH(OH)$- side chain generally present generally at C-17 position of the pregnane.

However, likewise longigenin it contains a -CH_3 group(C-20) doublet at δ1.33 (d, 3H, J=7.5Hz) which gives a cross peak with a methine proton of H-17 δ2.31(m) which further gives a cross peak with the multiplet of C-16 methylene proton at δ1.62 suggesting that the compound E may be a nor pregnane derivative at C-21. The ^{1}H NMR spectrum also contained a singlet at δ3.66 for methoxy group but give aromatic protons at δ7.11, δ7.12, δ7.14, δ7.52 and δ7.54 for benzoxy group protons. These signals were accompained by methine proton at δ4.92 and δ5.78 which could be assigned to H-3 and H-2 methine protons suggesting the position of -OCH_3 and -OC_6H_5 group at positions H-3 and H-2 respectively. A multiplet at δ5.34 could be assigned for the presence of a double bond between C-5 and C-6 of the compound E. Other proton signals of Gifogenin are given in the table 1.

Table 2. ^{13}C NMR data of Compoumd E, F and I at 200MHz in $CDCl_3$.

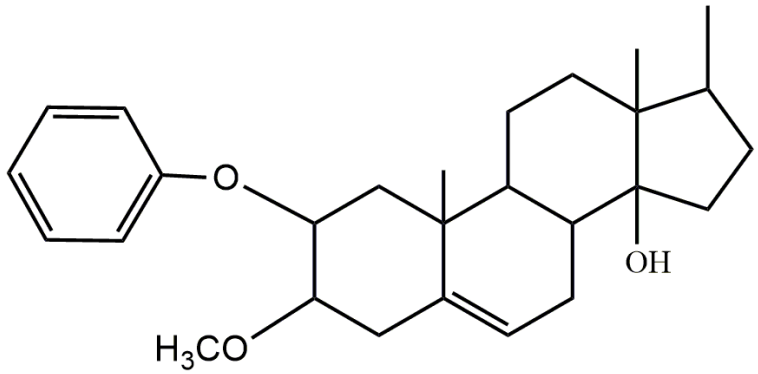
Carbon	Compound F	Carbon	Compound E	Carbon	Compound I
1	34.5	1	35.2	1	39.5
2	79.8	2	79.9	2	30.5
3	70.2	3	69.2	3	78.2
4	34.4	4	34.9	4	42.2
5	147.1	5	147.5	5	140.5
6	119.1	6	119.5	6	126.2
7	29.2	7	29.7	7	29.0
8	31.4	8	32.3	8	37.4
9	31.9	9	34.8	9	45.5
10	34.8	10	37.5	10	32.3
11	30.2	11	31.8	11	29.7
12	29.1	12	29.6	12	30.0
13	37.1	13	34.2	13	50.6
14	76.4	14	76.8	14	80.2
15	30.0	15	30.6	15	30.5
16	24.7	16	25.4	16	24.5
17	29.4	17	30.0	17	30.0
18	14.1	18	14.4	18	14.5
19	14.2	19	14.6	19	15.2
20	22.6	20	23.1	20	20.3
1’	147.6	1’	148.1	1’	98.6
2’	138.6	2’	124.8	2’	31.8
3’	129.7	3’	139.0	3’	78.2
4’	124.4	4’	148.0	4’	70.8
5’	123.9	5’	138.8	5’	67.3
6’	138.4	6’	124.3	6’	17.2
OMe	56.2	OMe	55.5	OMe	56.1
OMe	51.8				

The results obtained from ^{1}H-^{1}H COSY spectrum of compound E confirmed that Gifogenin belongs to category of substituted nor pregnanes.

The ^{1}H- ^{1}H COSY correlations shows the connectivity of H-20, H-17 and H-16 and also confirms the substitution of $–OCH_3$ and $–OC_6H_5$ group at H-3 and H-2 positions. The H-3 multiplet present at δ4.92 showed a cross peak with H-2 proton present at δ 5.78 (m, H-2) and simultaneously it also shows connectivity with CH_2 proton of C-4. The H-2 proton at δ5.78 also showed cross peak with H-3 proton at δ 4.92 (m,1H,H-3) and CH_2 proton of C-1 on other side. This correlation of C-3 and C-2 protons confirms that the two substituent groups present in A ring of the C-21 nor pregnane are adjacent to each other and are flanked by two methylene groups of C-1 and C-4.

The ^{13}C NMR spectrum further supported the derived structure of compound E which contained 27 carbon signals out of which 20 were not assigned to the C-21 nor pregnane and remaining 7 were present as $–OCH_3$ and $–OC_6H_5$ at C-2 and C-3. In the ^{13}C NMR spectrum the presence of C-5 and C-6 double bond was suggested by the signals at δ147.5 and δ119.5 respectively. The signal present at δ79.9, δ76.8 and δ69.2 could be assigned to C-2, C-14 and C-3 respectively. The carbon of methoxy groups was observed at δ55.5. Other carbon signals of Gifogenin are given in the table 2.

The FAB mass spectrum of Gifogenin further confirmed the assigned structure. The highest mass ion peak recorded at m/z 473 which could be assigned for quasimolecular ion peak $[M+ Na+K+H]^+$ thus confirming the molecular weight of the compound E as 410. The FAB mass spectrum contains the ion peak at m/z 393 which could be due to the loss of –OH group present at C-14. The mass ion peak at m/z 410 fragmented to give mass ion peak at m/z 317 which resulted from the loss of the benzoxy group $[M-OC_6H_5]$. The loss of 31 a.m.u. from m/z 317 leading to the mass ion peak at m/z 286 corresponded to the loss of $–OCH_3$. The fragment ion peak at m/z 410 further loses 125 a.m.u giving mass ion peak at m/z 285due to the loss of methoxy and benzoxy from the molecular ion peak. The fragment ion peak at m/z 285 further fragmented to give the mass ion peaks at m/z 240 corresponding to the loss of three CH_3 groups. The characteristic retro diels alder fission[15] of C_5- C_6 double bond observed in pregn-5-enes gave peak at m/z 166 thus confirming the presence of hydroxyl group at C-14. The complimentary ion peak at m/z 244 confirmed the presence of methoxy and benzoxy groups in the A ring of the C-21 nor pregnane. Further, the loss of 31 and 93 from ion peaks at m/z 244 gave mass ion peaks at m/z 213 and m/z120 confirming the presence of benzoxy and methoxy groups in the ring A. In the light of the foregoing evidences the structure of **Gifogenin (E)** was established as **2-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene**



2-benzoxy-3β-methoxy-14β-hydroxy-21-nor-pregn-5-ene

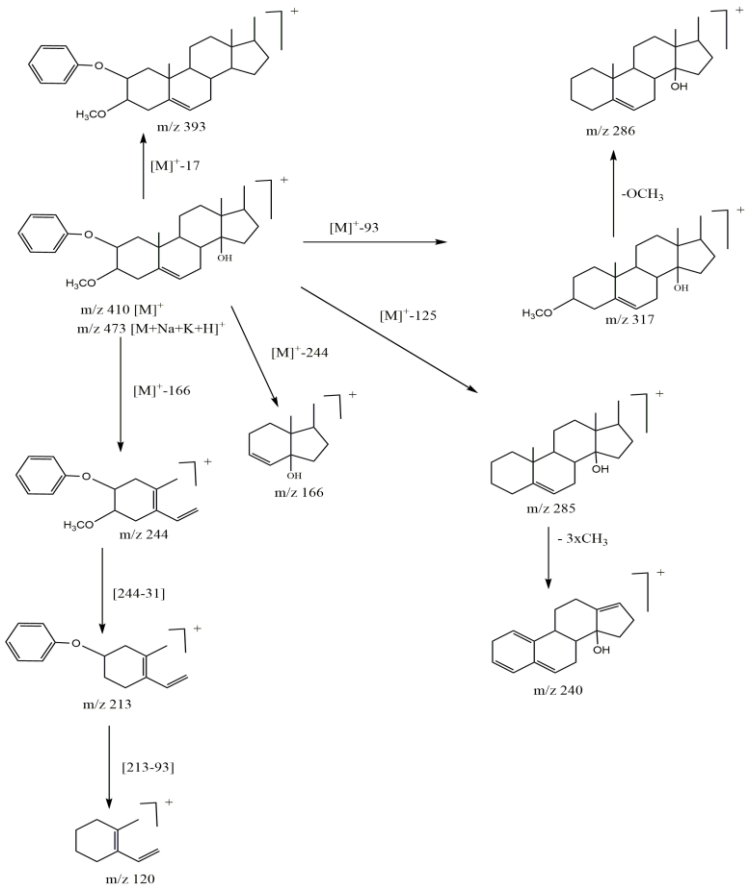


Figure. FAB mass fragmentation of compound Gifogenine (E).

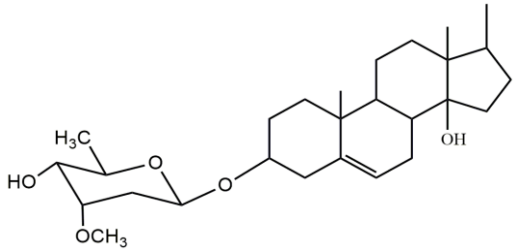
Substance I (Longin)

Compound I, $C_{27}H_{44}O_5$,m.p.84.5ºC showed positive Xanthydrol (Partridge and Westall, 1948), Keller-Kiliani (Rangaswami et al., 1949, Krasso et al., 1963, Warshina et al., 1994) Liebermann-Burchard and tetra-nitro methane tests suggesting it to be the steroidal glycoside of 2,6 dideoxy sugars and also the presence of a double bond in it. The FAB mass spectrum of I showed the highest mass ion peak at m/z 471 and 448 which could, be assigned for $[M+Na]^+$ and $[M]^+$ confirming the molecular formula of longin as $C_{27}H_{44}O_5$ and molecular weight as 448. It showed one anomeric carbon at δ98.6 in its ^{13}C NMR spectrum and one anomeric proton as double doublet at δ4.52 in its $^{1}HNMR$ spectrum at 300MHz spectrum.Besidesthe anomeric proton signal, it also contains a doublet at δ1.42 for secondary methyl of cymarose, suggesting it to be a monoglycoside of 2-6 dideoxy sugar. The chemical shift of anomeric signal in the ^{13}C and ^{1}H NMR spectrum suggested that the sugar present in Longin may be D-cymarose. To confirm the genin and sugar in compound I, Longin was subjected to mild acid hydrolysis with TLC and PC monitoring, using method of Mannich and Siewart. The hydrolysis was completed in eight hrs showing two spots which on column chromatography afforded two pure products vizglycon1and aglycon 2. The sugar was confirmed as D-cymarose by co- chromatography with authentic sample (TLC, PC) (Scheme 1). It was further characterised by its oxidation with bromine water to yield the corresponding lactone, which on treatment with phenyl hydrazine gave a known D-Cymaronic acid phenyl hydrazide (mp152-154°C).

Hence, the glycon was deduced to be D-Cymarose ($[\alpha]_D$+53.5°). The ^{1}H NMR spectrum of compound I contains the characteristic signals of pregnane glycoside but it lacks the signals for –$COCH_3$ and $CH_3CH(OH)$- side chain generally present generally at C-17 position of the pregnane. Instead it shows a doublet at δ1.42 for secondary -CH_3 group which gives a cross peak with a multiplet at δ 2.36 (m) assigned to C-17 methine proton in its ^{1}H-^{1}H COSY spectrum. Further, this C-17 methine proton gives a cross peak with the signal at δ1.61 (m) assigned to C-16 methylene protons suggesting that the compound I may be a norpregnane derivative at C-21. As the ^{13}C NMR spectrum of the compound I contains 27 carbons, 20 carbon atoms could be due to the aglycon and the remaining seven carbons could correspond to the deoxy sugar cymarose. In the^{1}HNMR of compound I the multiplet of one at δ3.06 was assigned to the methine proton of the –OH group present at C-3 of the nor-pregnane moiety. A multiplet signal present at δ5.40 confirmed the presence of a double bond between C-5 and C-6 of the C-21 nor pregnane. It also contained the methyl resonances at δ0.80(s, 3H, H-18) and δ1.07 (s, 3H, H-19). The ^{1}H NMR spectrum also showed the signals for 2-6 dideoxy sugar which exhibited axial and equatorial proton as multiplets in the region at δ1.77 and at δ 2.65 respectively. The large coupling constants of the double doublet of anomeric proton showed the presence of 2-6 dideoxy sugar in $^4C_1$ (D) conformation linked through β-glycosidic linkage. A methoxy group signal appeared at δ3.40 (s, 3H).Other proton signals of Longin are given in the table 1.

Besides the anomeric carbon signal in the ^{13}CNMR at δ98.6, two signals at δ70.8 and δ78.2 were assigned to two carbons in the sugar substituted by hydroxyl groups. The signal present at δ56.1 was due to the methoxy group present in cymarose. The signal present at δ80.2 was due to C-14 carbon containing the hydroxyl group. The presence of C-5 and C-6 double bond was further confirmed by the signal at δ140.5 and δ126.2. Other important carbon signals of Longin are given in the table 2.

The FAB mass spectrum of compound I showed the highest mass ion peak recorded at m/z 471 for [M^++Na] confirming the molecular weight as 448. The fragment ion peak at m/z 304 was due to the loss of 145 a.m.u of sugar moiety. The mass ion peak at m/z 304 further fragmented to give mass ion peak at m/z 290 corresponding to the loss of methyl from the aglycon. The characteristic retro diels alder fission[18] of C_5-C_6 double bond observed in pregn-5-enes gave peak at m/z 182 thus confirming the presence of hydroxyl group at C-14. The complimentary ion peak at m/z 138 confirmed the presence of another hydroxyl group in the ring A of the norpregnane which is involved in glycosidation with cymarose. The FAB mass spectrum of I showed the ion fragments for sugar component at m/ 162 besides the other mass fragments characteristic of sugar at m/z 145, 117, 74 and 86. In the light of the foregoing evidences the structure of **Longin (I)** was established as **14β-hydroxy-21-nor-pregn-5-ene-3-O-β-D-Cymaropyranoside**



14β-hydroxy-21-nor-pregn-5-ene-3-O-β-D-Cymaropyranoside

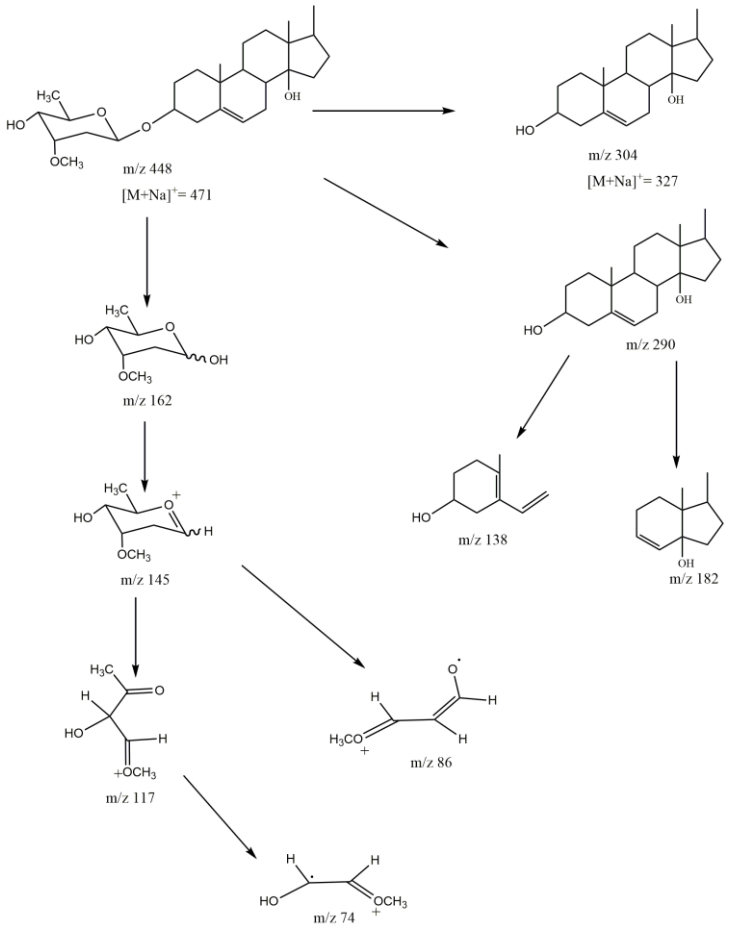


Figure. FAB mass fragmentation of compound Longin (I).

CONCLUSION

In summary, we conclude that three novel pregnaneviz Gifogenin (E), Longigenin (F) and Longin (I) were isolated and reported for the first time from any plant. Their structures were elucidated with the help of chemical transformation, chemical degradation and spectroscopic technique like ^{1}H, ^{13}C and 2 D NMR spectroscopy along with FAB mass spectroscopy.

ACKNOWLEDGEMENTS

Authors are thankful to RSIC, CDRI, Lucknow for successful running of NMR and Mass spectrum.

REFERENCES

Hossain, E., Rawani, A., Chandra, G., Mandal, S.C. and Gupta, J.K. (2011). Larvicidal activity of *Dregea volubilis* and *Bombaxmal abaricum* leaf extracts against the filarial vector *Culexquinquef asciatus. Asian Pacific Journal of Tropical Medicine.* Vol. 4, 6: 436-441.

Khare, N. K., Kumar, R., Khare, M. P. and Khare, A. (1986). Sarcogenin, a pregnane derivative from Pergulariapallida and Sarcostemmabrevistigma, *Phytochemistry* 25, 491.

Hossain, E., Sarkar, D., Maiti, A., Chatterjee, M., Mandal, S.C. and Gupta, J.K. (2010). Anti-inflammatory effect of a methanolic extract of leaves of *Dregea volubilis.* J *Ethnopharmacol*, 132 (2), pp. 525-528.

Deepak, D., Khare, A. and Khare, M.P. (1989). Plant pregnanes. *Phytochemistry*. 28:3255–3263.

Deepak, D., Srivastava, S. and Khare, A. (1997). Progress in the Chemistry of Natural Products, Pregnane glycosides. Springer Wien NewYork Vol-71, 170-325.

Deepak, D., Srivastava, S. and Khare, A. (1997). Pregnane glycosides. ProgCh Org Nat Prod. 69: 170–325.

Hussain, H., Raees, M.A., Rehman, NUr., Rawahi, A.A., Csuk, R., Khan, H.R., Abbas, G., Al-Broumi, M.A., Green, I.R., Elyassi, A., Mahmood, T. and Al-Harrasi, A. (2015). Nizwaside: a new anticancer pregnane glycoside from the sap of Desmidorchisflava.

Babu, K.S., Rao, V.R.S., Radhakrishnan, S.V., Rao, J.M. and Rambabu, S.S. (2008). A new pregnane steroid from the stems of *Carallu maumbellata*. *Journal of Asian Natural Products Research* 10: 1013–1016.

Duong Thi Hai Yen, Do Thi Trang, Bui Huu Tai, Vu Van Doan, Pham Hai Yen, Nguyen Xuan Nhiem, Chau Van Minh, Manh Hoang Duc, SeonJu Park, Jae Hyuk Lee, Sun Yeou Kim, Seung Hyun Kim and Phan Van Kiem (2021). Four new pregnane glycosides from Gymnema latifolium and their α-glucosidase and α-amylase inhibitory activities, *Natural Product Research*, 35, (22).

Piacente, S., Belisario, M.A., Delcastillo, H., Pizza, C. and DeFeo, V. (1998). Croton ruizianus: platelet pro aggregating activity of two pregnane glycosides. *J Nat Prod*. 61:322–328.

Sethi, A., Paswan, S., Srivastava, S., Khare, N.K., Bhatia, A., Kumar, A., Bhatia, G., Khan, M.M., Khanna, A.K. and Saxena, J.K. (2008). A new pregnane glycoside from *Marsdeniaroylei*as potential antioxidant and antidyslipidemic agents. *J Asian Nat Prod Res*. 10: 1023–1028.

Sun, Y., Ding C., Wang F., Zhang Y, Huang W, Zhang H., Li Y., Zhang D. and Song X. (2021). Pregnane alkaloids with BRD4 inhibitory and cytotoxic activities from *Pachysandra terminalis*, *Phytochemistry letters*, 45, 63-67.

Wang Y., Zhao D., Su S., Chen G., Wang and Pei Y. (2022). Twelve New Seco-Pregnane glycosides from *Cynanchum taihangense*. *Molecules*, 27, 5500, 1-17.

Neupane, P.B., Srivastava, A., Khare, N.K., Khare, A. and Deepak, D. (2017). Structure elucidation of a novel compound Ichnoside isolated from *Ichnocar pusfrutiscens*; *Chemistry and biology interface*, 7, 6, 351-358.

Zemplen, G. and Kiss, D. (1927). Degradation of D-glucose and α-D-glycol pentose. *Ber Deutsch ChemGes*. 60:165–170.

Abisch, E. and Reichstein, T. (1960). Preliminary chemical analysis of some Apocyanaceae. *HelvChemActa*. 43:1844–1861.

Tschesche, R., Grimmer, G. and Seehofer, F.R. (1953). UberpflanzlicheHerzgifte, XXIV. Mitteil.: Die quantitative Trennung und ¨ Identifizierung von Herzgiftglykosidenaus Digitalis purpurea und lanatadurch “echteVerteilungschromatographie an Papier”, ChemBer 86 (1953), 1235–1241. *Ber* 1953, 86, 1235-1241.

Nagata, W., Tamm, C. and Reichstein, T. (1957). The glucosides of Erysimumcrepidifolium H. G. L. Reichenbach. Glycosides and Aglycones 169. Communication.

Partridge, S.M. and Westall, R.G. (1948). Filtwpaper partition chromatography of sugars--l. General description and application to the qualitative analysis of sugars in apple juice, egg white, and fetal blood of sheep. *J. Biochem*. 42 (1948) 238-250.

Ostromisslensky, I. (1910). Remark on a werner message 'To zrage to the relations between colour and constitution'. *BerDtschChemGes*. 43:197–198.

Krasso, A.F., Weiss, E.K. and Reichstein, T. (1961). Isomerisierung eines Zuckerderivates bei der Dehydrierung mit CrO_3,-Pyridin. Ibid, 46, 1961, (1963).

Warshina, T. and Noro, T. (1994). Steriodial glycosides and Cardenolides glycosides from Asclepiasfruticosa. *Phytochemistry* 37, 217(1994).

Corresponding author: Dr. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow-226007 (U.P.), India
Email: deshdeepakraju@rediffmail.com