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Research Article

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Standardization of zygotic embryo culture from *Nerium oleander* L. and comparative analysis of biosynthesized cardiac glycosides within *in vitro* and acclimatized plants

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ABSTRACT

The primary result of our experiment revealed that the germination percentage of *N. oleander* mature seeds is only 30%. From this observation, the concept of protocol standardization for zygotic embryo culture of this plant was originated. Zygotic embryo culture was proved an efficient *in vitro* multiplication system of *N. oleander*. The maximum germination percentage (96%) of zygotic embryos was observed on ¼ MS medium with 15 gm/L sucrose, whereas the best growth medium was optimized as ½ B5 with same sucrose concentration. The second part of this study was aimed to find out the cardiac glycoside accumulation pattern in both *in vitro* and acclimatized plants. For this purpose, one-month-old *in vitro* plantlets and acclimatized plants were subjected to LC-MS analysis and 09 cardiac glycosides were detected and quantified in both systems. Most of the cardiac glycosides including odoroside A (32.71 mg/gm DW), odoroside H (4.69 mg/gm DW) and oleandrin (0.52 mg/gm DW) were found to be accumulated at the maximum level within *in vitro* plantlets. CG 840b (1.89 mg/gm DW) is the only cardiac glycoside, which was maximally accumulated in acclimatized plants. From this study, it can be concluded that zygotic embryo culture is a better choice for *in vitro* multiplication of *N. oleander* when compared to matured seeds and *in vitro* grown plantlets of this species favor cardiac glycosides biosynthesis in comparison to acclimatized plants. Therefore, all future research on the enrichment of cardiac glycosides from this plant may be conducted on zygotic embryos derived *in vitro* grown plantlets or cultures.

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INTRODUCTION

Zygotic embryo culture (ZEC), first introduced by Henning in 1906, has proven its outstanding efficiency in expanding the plant population on a laboratory scale. *In vitro* Plantlets (IVP), developed through ZEC under controlled culture conditions, can provide sterile starting materials (explants) for experimental purposes without much metabolic alteration. Such plantlets can also be efficiently used for the biosynthesis and enrichment of targeted bioactive metabolites (Grosser & Collins, 1984; Bridgen, 1994; Haslam & Yeung, 2011). However, the embryo's delicate nature requires an exact medium composition to support its germination and increased plantlet survival. On the other hand, plantlet production from mature wild-type seeds is sometimes very complicated and time-consuming due to the slow germination rate (Ramming, 2019).

Nerium oleander, a member of the family Apocynaceae, typically characterized by the presence of latex is an evergreen shrub or small tree. It is highly enriched with various classes of compounds like pragnanes, terpenoids, phenolics, tannins, chlorogenic acids, alkaloids and cardiac glycosides (CGs) (Ekalu *et al.*, 2019). CGs are medicinally useful as cardiotonic (Matsui & Schwartz, 1968), anti-proliferative, immunomodulatory, CNS depressant, anti-inflammatory and antiviral agent (Matsui & Schwartz, 1968; Newman *et al.*, 2008;

Menger *et al.*, 2013; Dogar *et al.*, 2020; Plante *et al.*, 2020; Tiwari *et al.*, 2020). CGs are the principal active constituent of Anvirzel and PBI-02504, the aqueous and supercritical CO_2 extracts of *N. oleander* respectively. Both have been submitted for phase II clinical trial due to their high potency against various types of cancer, including pancreatic and glioma (Wang *et al.*, 2000; Mekhail *et al.*, 2006; Colapietro *et al.*, 2020). Structurally, CGs have steroidal moiety attached with sucrose and are well known for the activity against Na^+-K^+ ATPase pump to produce an inhibitory effect. CGs have also been reported to involve complex cell-signal transduction mechanisms, resulting in selective control of tumors without affecting normal cell growth and division (Newman *et al.*, 2008). Numerous *in vitro* and *in vivo* studies support the anti-proliferative and apoptotic effects of CGs from *N. oleander* in several cancer cell lines, including breast, prostate, lung, leukemia, melanoma, pancreatic, neuroblastoma and renal adenocarcinoma. Recent studies have also shown the activity of Nerium's CGs against retroviruses like HIV and SARS COVID-19 (Reddy *et al.*, 2020).

However, the main drawback of performing drug development research on this class of compounds is their overall concentration within the plant, which is abysmally low. Moreover, the accumulation of these metabolites fluctuates with the plant growth stage, season and tissue type. Hence, there is a need to establish *in vitro* cultures of *N. oleander* for constant and higher production of targeted metabolites. Although, a few reports on oleandrin biosynthesis in suspension culture have been published (Ibrahim *et al.*, 2009a, 2009b) but significant enrichment of other CGs like odoroside H, odoroside A is yet to be achieved. *In vitro* culture is a well-known practice for a steady and higher accumulation of various essential metabolites under different classes. In particular, concerning the large-scale biosynthesis of CGs, the organ and whole plant cultures are considered as a better option than the callus and suspension cultures (Isah *et al.*, 2018; P. Singh *et al.*, 2019). This could be explained by the fact that organ and whole plant cultures are developmentally well-regulated like the shoot, leaf, root, etc. as compared to the undifferentiated cultures like callus or suspension (Hagimori *et al.*, 1982; Zhang *et al.*, 2011). The present study is a first-time report on developing a standardized protocol for rapid multiplication of *N. oleander* through ZEC and the subsequent identification and quantification of their CGs content. For the first time, a total of 09 CGs including the bioactive odoroside A ($C_{30}H_{47}O_7$), odoroside H ($C_{30}H_{46}O_8$), oleandrin ($C_{32}H_{49}O_9$), and others were biosynthesized in the *in vitro* model. A comparative analysis of accumulated CGs within IVP and acclimatized plants (ACP) was also made.

MATERIAL AND METHODS

Murashige and Skoog medium (MS), Schenk and Hiderbrant medium (SHM), Woody plant medium (WPM), Gamborg B5 medium (B5), sucrose, cetrimide, agar and NaOH were purchased from Hi-Media Laboratories (Mumbai, India). Extran detergent and HPLC grade methanol were procured from Merck Specialties (Mumbai, India). HCl was supplied by Ranken RFCL Ltd. (New Delhi, India) and absolute ethanol (HPLC grade) was acquired from Fisher chemical. Phytochemical standards like oleandrin, odoroside A and odoroside H were purchased from Wuhan Chem Faces Biochemical Co. Ltd., Hubei, PRC. The undehisced pods were collected freshly from healthy and actively growing fully mature plants, located at Jankipuram Extension, Lucknow during the fruiting period (September-January 2020) (Figure1A & B). The seeds of the same plant were used as starting material for ZEC. Simultaneously, the mature seeds from the same plant were also collected to know their germination vigor under *in vitro* conditions. Plant authentication was done by the corresponding author of this paper and voucher specimen No. 25294 was deposited at CSIR-CDRI Herbarium.

Explant Preparation for ZEC

Fruits were washed thoroughly under running tap water for 30 minutes and then treated with a 10% aqueous solution of extran (detergent) with gentle stirring. After 20 minutes, explants were transferred under running tap water to remove all traces of soap. These clean, dirt-free explants were dipped in 3% w/v cetrimide (aq) for 10 minutes to get rid of any unwanted microbial load and then washed thoroughly by double distilled water (DDW). Further steps were done under a laminar airflow hood, where the pods were treated with 0.2% w/v $HgCl_2$ for 2 minutes with continuous shaking, followed by 3-5 times rinsing with sterile DDW. After surface sterilization, the pods were opened with a sterilized scalpel under aseptic conditions. The fresh immature seeds were dissected out carefully, followed by the isolation of zygotic embryos and their incubation in a culture media plate (twenty per plate). After germination, the plantlets were transferred into culture bottles containing different media with variable salt strength and sucrose concentrations.

Optimization of ZEC

To investigate the effect of media, zygotic embryos were cultured on one fourth (1/4), half (1/2) and full-strength MS (Murashige & Skoog, 1962), WPM (McCown, 1981), SHM (Schenk & Hildebrandt, 1972) and B5 (Gamborg *et al.*, 1968) media, each with three levels of sucrose (0,15 and 30 g/l). All the media were solidified with 0.8% (w/v) agar (Bacteriological grade). The pH of all media was adjusted to 5.8 and autoclaved for 20 min at 121°C and 15 psi. Isolated embryos were placed horizontally on the medium in culture plates, which were maintained at 25 ±2°C in the dark for germination and then transferred to 16/8 h of dark/light photoperiod after one week. (Figure 1 C). Before the optimization of culture media for ZEC, efforts were made to know the germination percentage of mature seeds from the wild plants under *in vitro* condition.

Plant Acclimatization

The 4-week-old *in vitro* germinated plantlets were recovered from the culture bottles and washed gently with sterile DDW to remove all the media traces and then transferred to plastic pots containing soilrite: vermiculite (1:1). Pots were covered with

plastic bags and maintained under controlled environmental conditions. After four weeks, plants were transferred to earthen pots containing garden soil and kept in normal daylight conditions in the partially shaded field. Each experiment set was repeated thrice (Figure 1D-G).

Harvesting and Sample Preparation for CGs Profiling

Four-week-old IVP, grown under different culture media and the ACP were washed and dried in a traditional hot air oven at 40°C. Samples were then finely ground and 50 mg of each sample was extracted with 5 ml of HPLC grade methanol for 72 hrs. The supernatant was filtered out and passed through a 0.2 μm membrane filter (Millex-GV, PVDF, Merck Millipore, Darmstadt, Germany) and 1 ml of each extract was submitted for LC-MS analysis.

Qualitative Analysis of CGs

Qualitative analysis of the CGs was done using (UPLC-MS/ MS) ultra-performance liquid chromatography with electrospray ionization-triple quadruple mass spectrometer (Waters USA) system. For analysis, Waters BEH C18 (100 × 2.1 mm, 1.7 µm) reverse-phase column at, mobile phase CH_3CN and 5 mM $NH_4CH_3CO_2$ buffer was used at a flow rate of 0.250 mL/min. 2 µL of extracts were injected with the elution of solvent started with 5 % CH_3CN (A) and increased up to 80 % over 24 minutes. A linear gradient was applied for CGs elution from 5-30 % A (6 min), 30-60 % A (12 min), 60-60 % A (20 min), 60-80 % A (24 min) and moves to the initial chromatographic condition of 80-5 % A (30 min). Electrospray ionization (ESI) source operated in the ES ± ion mode and N_2 used as nebulizing and drying gas at flow rates of 50 and 650 L/h, respectively. ESI source parameters were capillary voltage at 3.5 kV and cone voltage at 30 V. Source and desolvation temperature was set at 120 and 350 °C, respectively. The data were acquired scan range of m/z 150- 1500 Th. Argon was used as collision gas and collision-cell energy ramping from 30-5 eV leads to achieving 5-30 % relative intensity of each precursor ion. MassLynx (vs. 4.1) software was used for data acquisition and processing. Similarly, accurate mass measurement of CGs was recorded on Thermo Orbitrap Velos pro hybrid Mass Spectrometer (USA). It was equipped with an Accela UHPLC system with an ESI source. Thermo Accucore C18 (150 × 2.1 mm, 2.6 µm) reverse-phase column was used with the same linear-gradient mobile phase as above.

Data Analysis

All the experiments were performed in triplicate and results were calculated as mean ± SE. The data from comparison of metabolite accumulation in the IVP vs. ACP was statistically analyzed by analysis of variance performed using statistical software SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA) at $p = 0.05$.

RESULT

Optimization of Germination Media

Considering the overall germination percentage of zygotic embryos, MS medium was found to be the best, followed by WPM, SHM and B5 media. ¼ MS medium with 15gm/l sucrose showed the best result with ~97 % germination rate, followed by~93% germination in the full as well as ½ strength of WPM also with 15 gm/l sucrose. Full-strength MS medium with the 15gm/l sucrose concentration also showed a good germination percentage, i.e., ~90%. Whereas ~87% germination was observed on sucrose-free full-strength MS medium, ½ MS medium with 15 gm/l sucrose, ¼ MS medium with 30 gm/l sucrose, full-strength WPM medium with 30 gm/l sucrose and ¼ WPM medium with 15 gm/l sucrose. ½ SHM and the full-strength B5 with 15 gm/l sucrose and the sucrose-free ¼ WPM media also showed more than 80% germination rate (Table 1). On the other hand, the maximum germination percentage of mature wild seeds under *in vitro* conditions was noticed only 30% (data not shown).

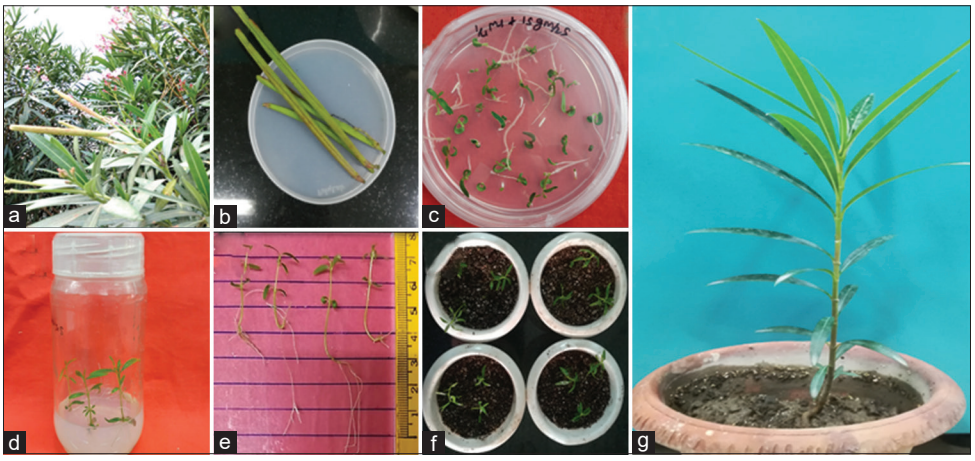

Figure 1: Standardization of zygotic embryo culture from *N.oleander* (a) fruiting twigs (b) immature fruits (c) germination of zygotic embryos (d) *in vitro* plantlets in culture bottles (e) measurement of *in vitro* plantlets (f) *in vitro* plantlets under controlled environmental conditions (g) complete acclimatized plant

Optimization of growth media

Vegetative growth of IVP was found to be controlled by all the three factors like type and strength of culture media as well as the concentration of sucrose. These factors played their role either independently or in combination. Sucrose supplementation was supposed to exhibit a significant impact on seedling growth. Under sucrose-free conditions, the vegetative growth of IVP in all tested media was suppressed and ranged between 1.3 to 3.8 cm. However, when sucrose was added to the media in 15 and 30 gm/l concentration, IVP height was increased, ranging between 2.4 to 4.8 cm depending upon the media type and strength used. Further, when the role of the media type and their strength on the growth of IVP was studied, it was observed that ½ B5 medium with 30 and 15 gm/l sucrose was the best, followed by ¼ MS medium with 30 gm/l sucrose. The IVP height in these media was 4.8, 4.7 and 4.5 cm, respectively (Table 2).

Identification and Quantification of CGs in IVP

Out of around 72 CGs reported from various parts of this plant globally, 09 were identified as per our previously established method (Figure 2, Table 3) (Tripathi *et al.*, 2013; Pandey *et al.*, 2016; Singh *et al.*, 2021) using MS/MS fragmentation pattern, accurate mass measurement (ESI-HRMS) and earlier published CGs structures from this plant (Supplementary Information, Figure S1-S9). The mass spectrometry data of detected CGs in IVP and ACP have been shown in Table 3. From the quantitative analysis, it was observed that the accumulation of all the CGs was found to be varied significantly in IVP depending on growth media, salt strength and sucrose concentration. Full-strength SHM medium with 15 gm/l sucrose was found optimum for the accumulation of three CGs *viz*. CG 840a (1.61mg/gm DW), CG 842 (2.28mg/gm DW) and CG 840b (1.89mg/gm DW). The same medium with 30 gm/l sucrose and sucrose free full-strength WPM medium also favored the accumulation of CG 840 a (1.51 mg/gm DW) and CG 840b (1.84 mg/gm DW) respectively. Sucrose free full-strength SHM medium showed the best effect on the accumulation of CG 901 (0.34 mg/gm DW) in comparison to others, which was followed by the full-strength WPM with 15 gm/l sucrose and sucrose-free conditions (0.29 and 0.28 mg/gm DW). Maximum accumulation of odoroside A (32.71 mg/gm DW) was found in ½ SHM media with 30 gm/l sucrose, whereas its accumulation (32.39 mg/gm DW) was also comparable in sucrose-free ¼ SHM medium. MS medium, with its different strength and sucrose concentrations, favored the accumulation of 03 CGs. CG 738 was accumulated at maximum level (2.89 mg/ gm DW) in sucrose-free, full-strength MS medium. Optimum accumulation of oleandrin was noticed on ½ MS medium with 15 gm/l sucrose (0.52 mg/gm DW) and sucrose-free (0.51 mg/gm DW) conditions. Odoroside H was found to be accumulated in maximum quantity (4.69 mg/gm DW) on sucrose-free ¼ MS medium, whereas ½ MS medium either with sucrose-free condition or with 15 gm/l sucrose also favors its accumulation (4.67 mg/gm DW, 4.57 mg/gm DW). Full-strength sucrose-free B5 medium was found to favor the accumulation of only CG 754 (1.6 mg/gm DW) (Figure 3).

Table 1: Germination percentage of zygotic embryos of ***N. oleander*** on different germination media

Germination Media (salt strength gm/l)	% Germination (mean± SE) (1 week) n=60		
	30 gm/l Sucrose	15 gm/l Sucrose	0 gm/l Sucrose
MS	80.00± 11.54	90.00±5.77	86.66 ±6.66
1/2 MS	66.60 ± 6.6	86.66±6.66	73.33±6.66
1/4 MS	86.60 ±3.33	96.66±3.33	56.66 ±3.33
WPM	86.60± 6.66	93.33±6.66	60.00±11.54
1/2 WPM	76.60± 8.81	93.33±6.66	73.33± 11.54
1/4 WPM	53.30 ±6.66	86.66±6.66	80.00±11.54
SHM	66.60± 13.33	70.00±9.99	63.33±12.01
1/2 SHM	66.60 ± 6.66	83.33±3.33	66.66±6.66
1/4 SHM	73.33 ±6.66	76.66±3.33	70.00±9.99
B5	30.00 ±5.77	80.00±9.99	73.33±6.66
1/2 B5	50.00 ± 5.70	76.33±8.81	76.66±8.81
1/4 B5	70.00 ± 5.70	73.33±6.66	66.66±3.33

Table 2: Growth of *in vitro N. oleander* plantlets on various growth media

Growth Media (salt strength gm/l)	Growth cm (mean± SE) (1 month) n=60		
	30 gm/l Sucrose	15 gm/l Sucrose	0 gm/l Sucrose
MS	2.9 ± 0.36	3.1±0.15	1.3±0.17
1/2 MS	3.6± 0.44	4.2±0.14	1.9±0.30
1/4 MS	4.5 ± 0.15	4.2±0.15	3.4±0.46
WPM	2.9 ± 0.08	3.1±0.08	2.3±0.20
1/2 WPM	4.0 ± 0.48	2.5±0.09	2.6±0.19
1/4 WPM	3.2 ± 0.45	3.3±0.49	2.9±0.23
SHM	3.9 ± 0.13	2.5±0.13	2.1±0.16
1/2 SHM	4.3 ± 0.33	3.8±0.31	2.7±0.17
1/4 SHM	3.8 ± 0.30	2.8±0.52	3.8±0.26
B5	2.4 ± 0.24	4.0±0.61	2.6±0.23
1/2 B5	4.7 ± 0.75	4.8±0.26	2.8±0.55
1/4 B5	3.5 ± 0.29	3.7±0.63	3.1±0.05

CGs Quantification in ACP and Their Comparison with IVP

All the 09 identified CGs could also be detected in ACP, which were quantified and compared with their maximum accumulation within IVP. It was revealed that ACP accumulated a lower concentration of almost all the CGs. A significant decrease of odoroside A was observed from 32.71 mg/gm DW to 11.71 mg/gm DW, whereas the concentration of odoroside H was almost reduced to half from 4.69 mg/gm DW to 2.12 mg/gm DW in ACP. Oleandrin accumulation was also found to be diminished to almost nil, i.e., from 0.52 to 0.01 mg/gm DW. Other CGs *viz*. CG 840a, CG 754 and CG 842 showed a significant reduction in their quantities from 1.89, 1.62 and 2.27 mg/gm DW in IVP to 0.97, 0.43 and 1.83 mg/gm DW respectively in ACP. CG 901, which was accumulated in low quantity i.e., 0.29 mg/gm DW in IVP, was further reduced to 0.15 mg/gm DW in ACP. CG 738 was also slightly reduced from 2.88 to 2.30 mg/gm DW. CG 840b is the only CG, which was found to be accumulated in higher quantity within ACP (2.93 mg/gm DW) with compare to IVP (1.61 mg/gm DW) (Figure 4).

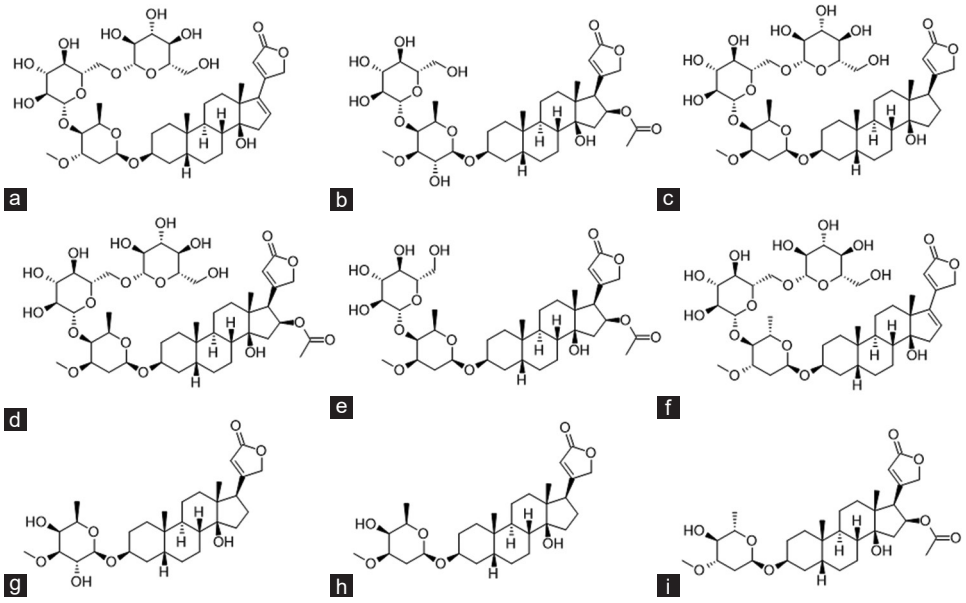

Figure 2: Identified structures of cardiac glycoside in *in vitro* grown and acclimatized plantlets
(a) CG840a (b) CG754 (c) CG842 (d) CG901 (e) CG738 (f) CG840b (g) CG534 (h) CG518 (i) CG576

Table 3: Identification of CGs in *in vitro* grown and acclimatized plantlets

S. No.	RT	Analyte code	Compound name	ESI-HRMS	MF	MW	CGs product-ions in ESI+ ion mode (*m/z* % relative intensity)
1.	2.28	CG 840a	Δ^{16}Anhydrogitoxigenin-β-D-sar-β-D-glu-β-D-glu	$[M+NH_4]^+$858.4476	$C_{42}H_{64}O_{17}$	840	373(35), 325(32), 275(29), 163(25), 145(47), 113(100), 95(31)
2.	2.85	CG 754	Oleandrigenin-β-D-digi-β-D-glu	$[M+NH_4]^+$772.4100	$C_{38}H_{58}O_{15}$	754	593(15), 433(100), 373(15), 355(12), 323(4), 161(5)
3.	3.40	CG 842	Digitoxigenin-β-D-dig-β-D-glu-β-D-glu	$[M+NH_4]^+$860.4614	$C_{42}H_{66}O_{17}$	842	631(13), 437(11), 375(31), 325(32), 275(29), 163(20), 145(35), 113(100), 95(34)
4.	3.55	CG 901	Oleandrigenin-β-D-dig-β-D-glu-β-D-glu	$[M+NH_4]^+$918.4688	$C_{44}H_{72}O_{19}$	901	433(95), 373(5), 355(9), 325(12), 275(28), 163(20), 145(22), 113(100), 95(25)
5.	4.23	CG 738	Oleandrigenin-β-D-dig-β-D-glu	$[M+NH_4]^+$756.4166	$C_{38}H_{58}O_{14}$	738	433(68), 373(18), 145(62), 113(100)
6.	4.75	CG 840b	Δ^{16}Anhydrogitoxigenin-β-D-ole-β-D-glu-β-D-glu	$[M+NH_4]^+$858.4474	$C_{42}H_{64}O_{17}$	840	373(19), 325(13), 275(17), 163(23), 145(40), 113(100), 95(24)
7.	5.40	CG 534	Digitoxigenin-β-D-digi (Odoroside H)	$[M+H]^+$ 535.3266	$C_{30}H_{46}O_8$	534	375(31), 357(22), 339(24), 87(100), 161(8)
8.	8.40	CG 518	Digitoxigenin-β-D-dig (Odoroside A)	$[M+H]^+$ 519.3321	$C_{30}H_{47}O_7$	518	375(73), 357(8), 339(18), 145(95), 113(100)
9.	9.10	CG 576	Oleandrigenin-L-ole (Oleandrin)	$[M+H]^+$ 577.3375	$C_{32}H_{49}O_9$	576	517(22), 433(34), 373(85), 355(36), 337(10), 145(62), 113(100)

RT= Retention time, MF= molecular formula, MW= molecular weight, and *m/z*= mass to charge ratio

DISCUSSION

In the present study, the germination percentage of *N. oleander* zygotic embryos was found much higher than the wild mature seeds in the culture medium, which might be due to the presence of various inhibitors in the seed coat (Kaveri & Rao, 2015). However, some other direct (Karrunakaran & Shri, 2010; Hatzilazarou *et al.*, 2019) and indirect *in vitro* micropropagation techniques are reported from this plant (DH Paper, 1989; Santos *et al.*, 1994; Vila *et al.*, 2010) yet no ZEC and CGs profiling of acclimatized and *in vitro* raised plants found to be reported in our knowledge. ZEC depends on many factors including the source of carbon and other nutrients used, which helps in the germination, survival and growth of the delicate IVP. In the present study, we achieved maximum germination percentage of *N. oleander* zygotic embryos at a lower sucrose concentration (15 gm/l), whereas the higher (30 gm/l) and the sucrose-free conditions did less favor. A similar observation was reported in *Schisandra chinensis*, where lower concentrations of sucrose (2%) in the medium increased the germination percentage (Chen *et al.*, 2010). On the contrary, in some other cases like *Givotia rottleriformis* and *Boswellia serrata* a higher germination percentage of zygotic embryos was noticed at 30 gm/l sucrose than its lower concentrations (7.5 & 15 gm/l) (Rambabu *et al.*, 2006; Ghorpade *et al.*, 2010). Such types of contradictory results indicate that sucrose concentration for ZEC is very much species-specific. Sucrose in the media serves as a primary carbon source and most importantly, it controls the

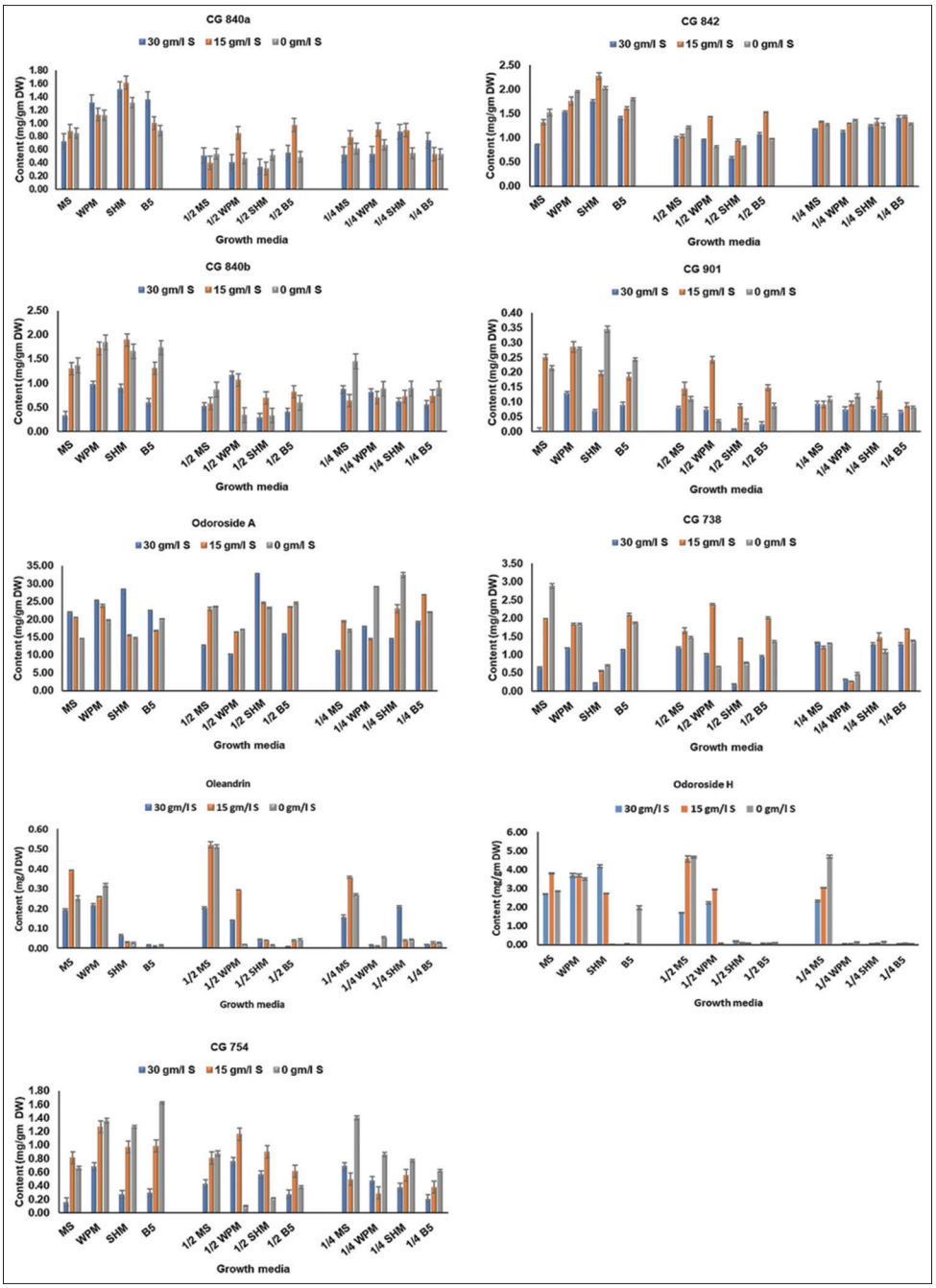

Figure 3. CGs accumulation in different types and strength of culture media with various sucrose concentrations

osmotic balance (Bridgen, 1994; Raghavan, 1977, 2003). In our study, both lower (15 gm/l) and higher (30 gm/l) concentrations of sucrose were found to support the growth and proliferation of IVP. Such positive effects of sucrose on plantlets growth under *in vitro* conditions are well known and have already been documented earlier (Mills, 2009; Thorpe & Yeung, 2011). Along with sucrose, the source and concentration of nitrogen in the media are also responsible for the germination of zygotic embryos. So, it becomes necessary to optimize the media type and their strength. In our experiment, we observed that the best medium for germination of zygotic embryo of *N. oleander* was ¼ MS, which was immediately followed by the full strength and ¼ WPM media. In all these media, the common source of nitrogen is ammonium nitrate and its concentration ranges from 200 to 400 mg/l, which is far less than the full MS and other media. This observation is supported by an earlier work, where the reduced concentration of ammonium nitrate as a primary source of nitrogen favored the zygotic embryo culture of *Datura* sp. (MATSUBARA, 1964; Umbeck & Norstog, 1979; George *et al.*, 2008). For the growth of IVP, B5 media was found to be the best at both 30 and 15 gm/l sucrose levels. A similar observation was also reported during *in vitro* seedling establishment of *Jatropha curcas* (Warakagoda & Subasinghe, 2009). Regarding CGs content within *in vitro* grown plantlets of *N. oleander*, 05 CGs were found to be accumulated at maximum level in the plantlets, grown on SHM medium.

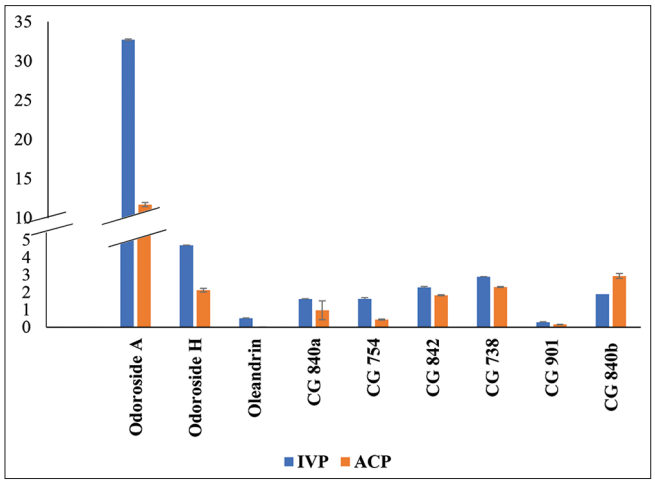

Figure 4. Comparative analysis of CGs accumulation in *in vitro* (IVP) vs acclimatized plants (ACP)

The plantlets on the rest of the media also accumulated these CGs albeit at lower concentrations. SHM medium supports the higher accumulation of maximum CGs, possibly due to its low nitrogen and high phosphate concentrations. One earlier worker also made a similar observation during enhanced oleandrin biosynthesis from the same plant (Ibrahim *et al.*, 2009b). Following the SHM, MS medium was found to elicit the accumulation of 03 other CGs, which is similar to an earlier report on higher accumulation of periplocin in *Periploca sepium* on MS medium (Zhang *et al.*, 2011). Further, when the quantity of CGs accumulated within IVP and ACP were compared, a prominent distinction was observed and the IVP were found far superior. This observation strongly suggests that IVP are the better option for CGs biosynthesis and enrichment-related research as compared to naturally grown plants.

CONCLUSION

The complete study was planned in two parts. In the first part, *in vitro* multiplication protocol of *N. oleander* through ZEC was standardized and plantlets were acclimatized under natural conditions. In the second part, accumulated CGs in both *in vitro* grown and ACP were identified and quantified through LC-MS. Optimum germination medium was observed as ¼ MS with 15 gm/l sucrose, whereas the best growth medium was standardized as ½ B5 with both 30gm/l and 15 gm/l sucrose. A total of 09 CGs could be identified and quantified in both *in vitro* as well as ACP, of which 08 were found maximally accumulated within IVP. On the other hand, ACP were found to favor the maximum accumulation of only 01 CG. From the overall experiments, it can be concluded that the ZEC is a quick and efficient method for micropropagation of *N. oleander*, whereas for CGs accumulation *in vitro* grown plantlets are the better option.

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SUPPLEMENTARY FILE

Supporting Information

CG840a

RT: 2.28; Δ^{16}Anhydrogitoxigenin-β-D-sar-β-D-glu-β-D-glu (Liu *et al.*, 2019); observed ions ESI+ *m/z* 841 $[M+H]^+$, 858 $[M+NH_4]^+$, ESI- *m/z* 839 $[M-H]^-$, 899 $[M+CH_3COO]^-$, M.W. 840 Da.

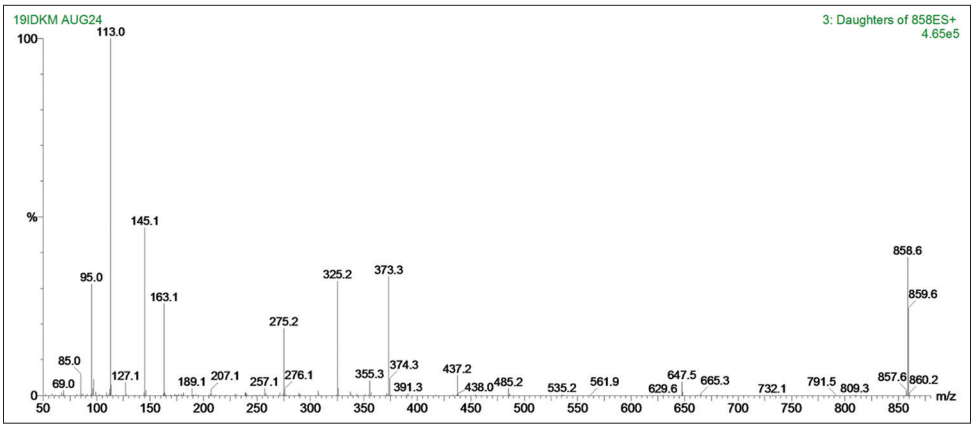

Figure S1. MS/MS spectra of Δ^{16}Anhydrogitoxigenin-β-D-sar-β-D-glu-β-D-glu.

CG754

RT: 2.85; Oleandrigenin-β-D-digi-β-D-glu(Zhao *et al.*, 2011); observed ions ESI+ *m/z* 755 $[M+H]^+$, 772 $[M+NH_4]^+$, ESI- *m/z* 753 $[M-H]^-$, 813 $[M+CH_3COO]^-$, M.W. 754 Da.

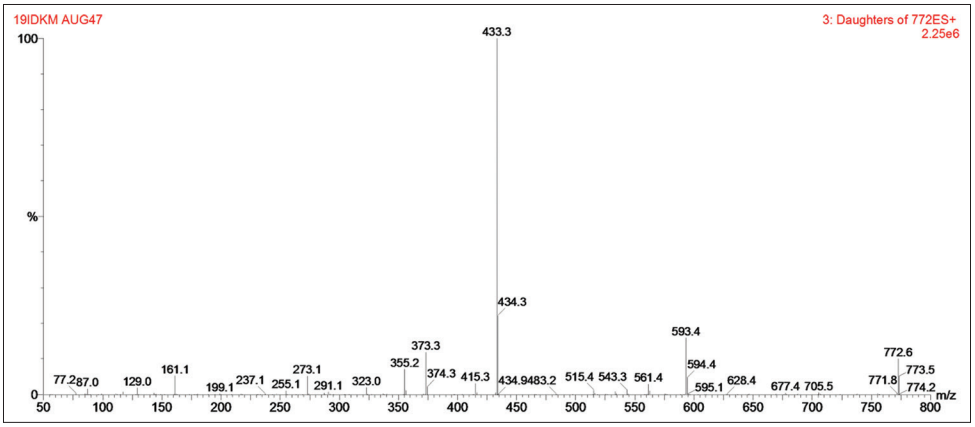

Figure S2. MS/MS spectra of Oleandrigenin-β-D-digi-β-D-glu.

CG842

RT: 3.40; Digitoxigenin-β-D-dig-β-D-glu-β-D-glu (Odoroside A gentiobioside)(Y. Bai *et al.*, 2011); observed ions ESI+ *m/z* 843 $[M+H]^+$, 860 $[M+NH_4]^+$, ESI- *m/z* 841 $[M-H]^-$, 901 $[M+CH_3COO]^-$, M.W. 842 Da.

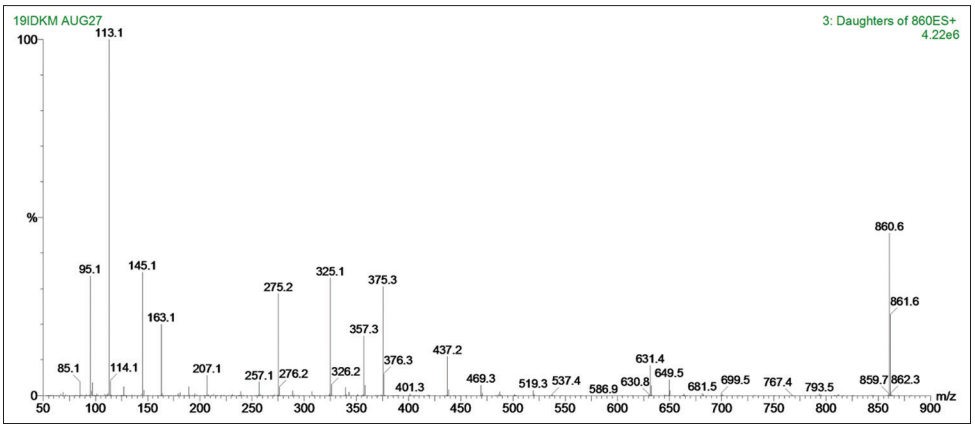

Figure S3. MS/MS spectra of Digitoxigenin-β-D-dig-β-D-glu-β-D-glu (Odoroside A gentiobioside).

CG901

RT: 3.55; Oleandrigenin-β-D-dig-β-D-glu-β-D-glu(Hanada *et al*., 1992), observed ions ESI+ *m/z* 902 $[M+H]^+$, 919 $[M+NH_4]^+$, ESI- *m/z* 900 $[M-H]^-$, 960 $[M+CH_3COO]^-$, M.W. 901 Da.

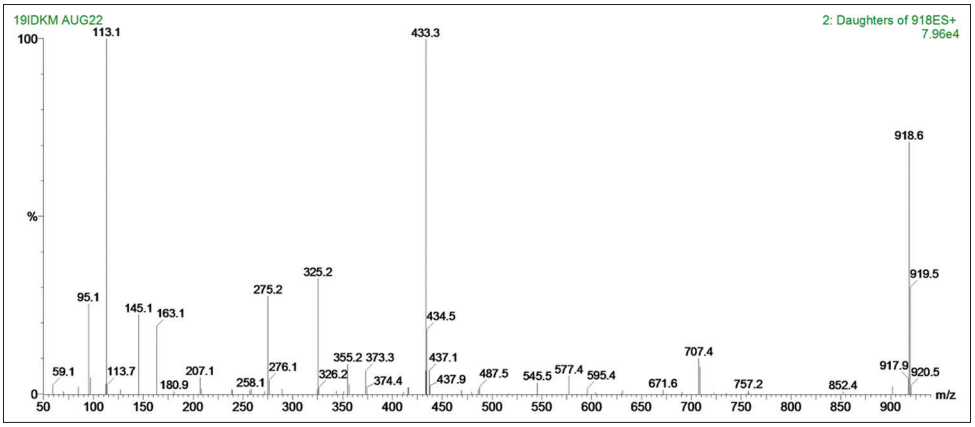

Figure S4. MS/MS spectra of Oleandrigenin-β-D-dig-β-D-glu-β-D-glu.

CG738

RT: 4.23; Oleandrigenin-β-D-dig-β-D-glu (Hanada *et al*., 1992), observed ions ESI+ *m/z* 739 $[M+H]^+$, 756 $[M+NH_4]^+$, ESI- *m/z* 737 $[M-H]^-$, 797 $[M+CH_3COO]^-$, M.W. 738 Da.

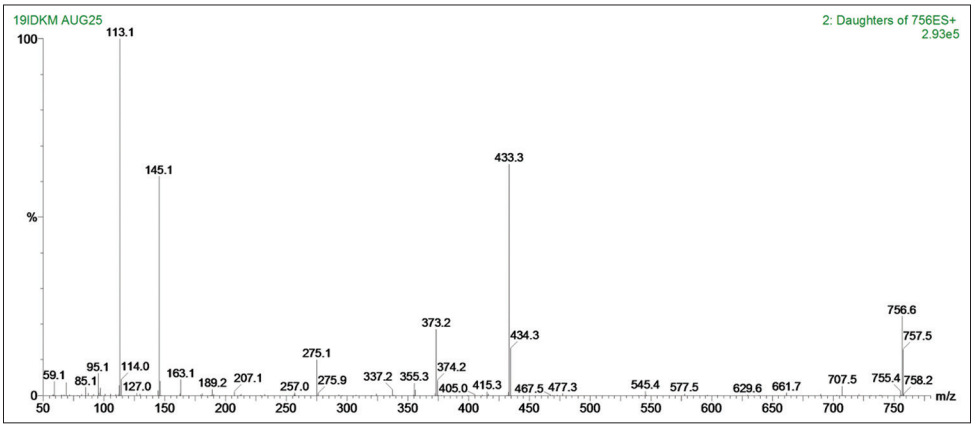

Figure S5. MS/MS spectra of Oleandrigenin-β-D-dig-β-D-glu.

CG840b

RT: 4.75; Δ16Anhydrogitoxigenin-β-D-ole-β-D-glu-β-D-glu(Y. Singh *et al.*, 2021); observed ions ESI+ *m/z* 841 $[M+H]^+$, 858 $[M+NH_4]^+$, ESI- *m/z* 839 $[M-H]^-$, 899 $[M+CH_3COO]^-$, M.W. 840 Da.

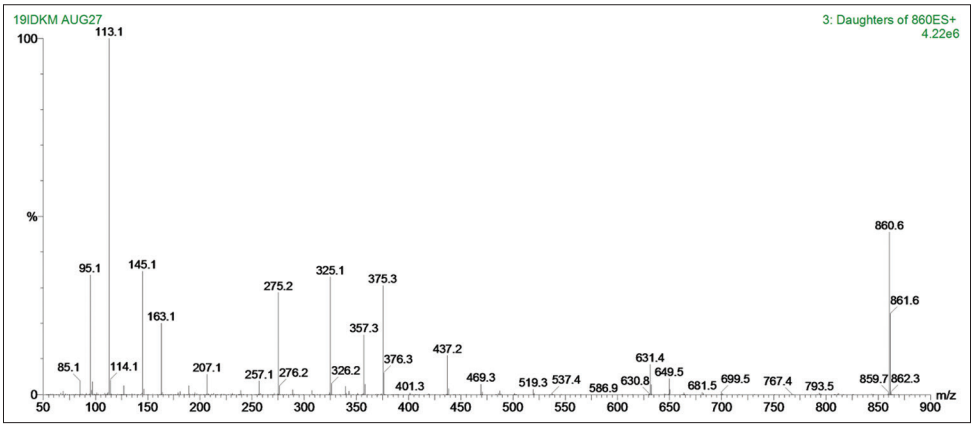

Figure S6. MS/MS spectra of Δ[16]Anhydrogitoxigenin-β-D-ole-β-D-glu-β-D-glu.

CG534

RT: 5.40; Digitoxigenin-β-D-digi (Odoroside H)(L. Bai *et al.*, 2011) ; observed ions ESI+ *m/z* 535 $[M+H]^+$, 552 $[M+NH_4]^+$, 557 $[M+Na]^+$, ESI- *m/z* 533 $[M-H]^-$, 593 $[M+CH_3COO]^-$, M.W. 534 Da.

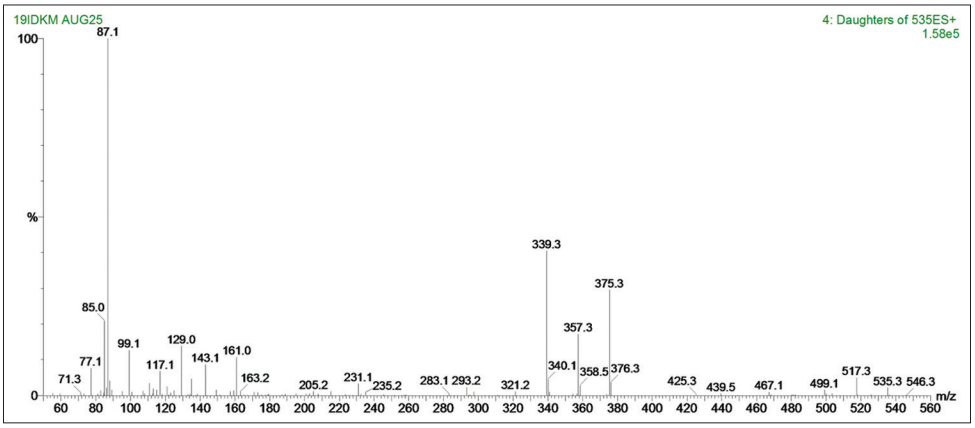

Figure S7. MS/MS spectra of Digitoxigenin-β-D-digi (Odoroside H).

CG518

RT: 8.40; Digitoxigenin-β-D-dig (Odoroside A)(Cao *et al.*, 2018); observed ions ESI+ *m/z* 519 $[M+H]^+$, 536 $[M+NH_4]^+$, 541 $[M+Na]^+$, ESI- *m/z* 517 $[M-H]^-$, 577 $[M+CH_3COO]^-$, M.W. 518 Da.

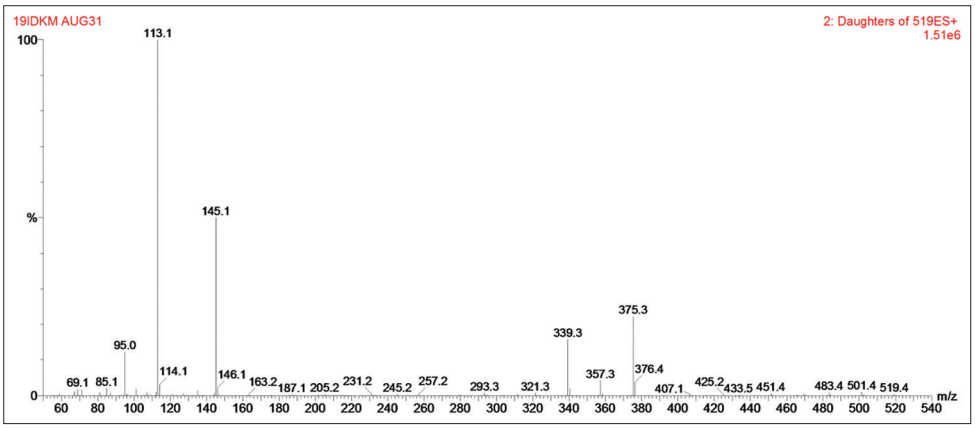

Figure S8. MS/MS spectra of Digitoxigenin-β-D-dig (Odoroside A).

CG576

RT: 9.10; Oleandrigenin-L-ole (Oleandrin)(Siddiqui *et al*., 2012); observed ions ESI+ *m/z* 577 $[M+H]^+$, 594 $[M+NH_4]^+$, 599 $[M+Na]^+$, ESI- *m/z* 575 $[M-H]^-$, 635 $[M+CH_3COO]^-$, M.W. 576 Da.

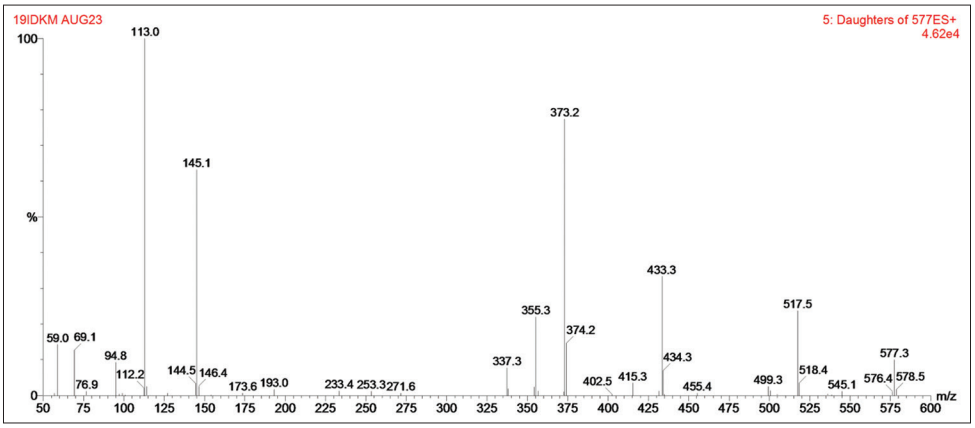

Figure S9. MS/MS spectra of Oleandrigenin-L-ole (Oleandrin).