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IN VITRO AND BIOTRANSFORMATIONAL STUDIES OF *ALOE BARBADENSIS* MILL.

ZARREEN BADAR, SAIFULLAH KHAN*, SAIFULLAH, KASHIF ALI, SYED GHULAM MUSHARRAF AND MUHAMMAD IQBAL CHOUDHARY

H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.
Corresponding author's e-mail: drsaif65@gmail.com

Abstract

Tissue culture technology can play an important role in the yield improvement of active ingredients of medicinal plants. In the present study, the potential of regeneration system of *Aloe barbadensis* along with biotransformational ability was explored. The maximum calli (5.65±1.90; fresh weight**)** were induced under the dark condition on MS (Murashige & Skoog) medium supplemented with 2.0 mg/L of NAA (α-naphthaleneacetic acid), as compared to light. The highest number of shoots (12.725) were proliferated on MS regeneration medium, containing 1.0 mg/L of BAP (6-Benzyl Aminopurine) and 0.1 mg/L of IBA (Indole-3-Butyric Acid) incubated at 22 ± 2ºC and 16/8 hr photoperiod provided by white fluorescent tube lights. These plantlets were then transferred onto root inducing medium and maximum number of roots (8.0 ± 0.70) with longer length (6.38 ± 0.34 cm) acquired at 1.0 mg/L of IBA within 14-20 days. The regenerated plants were shifted to green house for acclimatization. Effect of plant growth regulators and light was also assessed on callus cultures produced from conventionally propagated and *in-vitro* regenerated *A. barbadensis* plants. Biotransformation ability of *Aloe barbadensis* cell suspension culture was studied by incubation with (+)-adrenosterone (1), which afforded three products; Δ^{1-2}-dehydroadrenosterone (2), 5α-androst-1-ene-3, 11, 17-trione (3) and 17β-hydroxyandrost-4-ene-3, 11-dione (4). These metabolites were structurally characterized on the basis of spectroscopic techniques.

Introduction

The genus *Aloe* belongs to the family Liliaceae comprises of over 300 species of herbs, shrubs, and trees, spread worldwide. *Aloe*, native to East and South Africa, is also known as 'lily of the desert' and 'plant of immortality'. *Aloe* has a long history of use as a multipurpose folk remedy. For many years, products of *Aloe vera* have been used for medicinal and cosmetic purposes. *Aloe vera* gel has been reported to demonstrate wound healing, anti-fungal, anti-bacterial, anti-inflammatory and immune stimulating activities (Reynolds, 1999; Eli Harlev *et al*., 2012; Ibrari *et al*., 2013). It has been used in the treatment of peptic ulcers, diabetes mellitus, and also found to be active against cancer (Langmead *et al*., 2004; Harlev *et al*., 2012). Research on the medicinal properties of *Aloe* has expanded in scope during the last three decades and the production or transformation of value-added compounds, which are medicinally important, still needs to be explored.

Plant tissue culture is a promising technology, especially for the multiplication and production of novel and improved plants species and for an increased biosynthesis of products of industrial and medicinal value from vegetative resource (Vanisree *et al*., 2004). Plant cell cultures have also been extensively used for the production of biologically important secondary metabolites (Mulabagal *et al*., 2004) and especially for bioconversion of organic compounds into pharmaceuticals (Pras *et al*., 1995). There have been many attempts made towards the *in-vitro* culture of *Aloe* species; Natali and his coworkers in 1990 reported the *in-vitro* culture of *Aloe barbadensis* Mill., from vegetative meristems. Tissue culture of *Aloe arborescens* was reported (Kawai *et al*., 1993) in which sucrose, kinetin, and NAA, were important factors for callus induction from stalks. Alternatively, seeds (Abrie & Van Staden, 2001) were also used as explants for plant regeneration. Regeneration was also achieved through somatic organogenesis using young immature inflorescences, in which efficient regeneration was initiated on MS medium supplemented with BA or TDZ (Velcheva *et al*., 2005).

The chemical modifications in the medicinally important compounds carried out by living cells, organs or enzymes are known as biotransformation, which has a great potential to generate novel products of high medicinal importance. These compounds can be chemically synthesized but the process might be of high cost and labor intensive (Schulze & Wabbolts, 1999). Biotransformation can be done by using variety of living cells including microbial (Ward & Singh, 2000), fungal (Ramachandra & Ravishankar, 2000), plant (Saifullah *et al*., 2013; Choudhary *et al*., 2006), animal (Novais 1998), or even isolated enzymes (Franssen & Walton, 1999) can also be used. The use of microbial and fungal cell for the biotransformation has been extensively applied but the enzyme system of these sources is limited for certain types of chemical modifications (Ward & Singh, 2000) while the animal cells or the isolated enzymes are highly sensitive and difficult to handle (Franssen & Walton, 1999).

Adrenosterone (1) is a hormone that is secreted by the adrenal gland in the human body. It is also produced in the testes of some fish like Salmon and Talpia, along with another major hormone; androgen 11-ketotestosterone. Its function in human is to modulate the presence of Cortisol (a vital hormone for efficient fuel processing under stress conditions) in certain body fluids.

In the present study, attempts were made to explore the potential of a highly efficient multiplication method based on callus induction and regeneration of *A. barbadensis.* The effect of various factors such as light/dark conditions and different types and concentrations of plant growth regulators on the efficiency of the multiplication system was evaluated. In another experiment, the potential of cell

suspension culture for biotransformation of Adrenosterone (1) was studied.

Materials and Methods

Preparation of explant material and culture media: Young axillary buds of 2.0-2.5 inches size of *Aloe barbadensis* were collected from the garden of H.E.J. Research Institute of Chemistry, University of Karachi, as an ex-plant material. The stalks were carefully separated from the axillary buds and their surface was sterilized by immersing them in 0.12% mercuric chloride for 12 minutes, followed by 3-4 times washing with sterile autoclaved distilled water to remove the traces of mercuric chloride. The sterilized explants were transferred onto simple MS medium (full strength) for the establishment of *A. barbadensis* shoots cultures. After 20 days, the *In vitro* grown shoots were used for the callus induction experiment. Murashige & Skoog (MS) basal agar media (1962), supplemented with various concentrations and combinations of plant growth regulators were used for callus induction and plant organogenesis. The pH of the media was adjusted to 5.7 by 1 M NaOH and/or 1 M HCl and the media was gelled with 8.0 mg/L w/v agar. 25 ml of media were then distributed into the glass-jars of 250ml. The sterilization of the media was performed by auto claving at 121ºC for 15 minutes under 15 psi pressure.

Callus induction: Each *In vitro* grown plantlet was excised aseptically into small sections of about 1 cm x 1 cm, which were then placed into culture jars containing MS medium supplemented with various concentrations (0.0-8.0 mg/L) and combinations of BAP (6-Benzyl amino purine) and kinetin (0-2 mg/L) with NAA (α-Naphthaleneacetic acid), IBA (Indole-3-butyric acid), and 2, 4-D (2, 4-Dichlorophenoxy acetic acid). Half of the cultures were incubated at 22±2ºC in dark and the rest in illumination with a 16/8 hrs photoperiod at photon flux density of 75 µmol m^{-2} s^{-1}, provided by cool white fluorescent tube lights. The cultures were incubated for duration of four weeks and then the explants were sub-cultured on the same fresh nutrient media. Data on the callus induction with reference to their description was recorded after four weeks of incubation in both dark and light conditions.

Organogenesis: After four weeks of incubation, calli were excised into small pieces of about 1.0 gm FW (Fresh Weight) and cultured on MS medium supplemented with different concentrations and combinations of BAP and IBA to investigate their potential for organogenesis. All of the cultures were incubated at 22±2ºC in 16/8 hrs photoperiod. After shoots had developed from calli, their tips were excised and sub-cultured on the same regeneration media for multiplication. Regenerated shoots were transferred to the medium containing different concentration of rooting hormones; MS medium enriched with indole butyric acid (IBA) and naphthallene acetic acid (NAA) were used ranging from 0.25 to 1.5 mg/L for this purpose and to select the best concentration of the hormone.

Statistical analysis: Data on shoot regeneration were recorded after four weeks and analyzed using two-way ANOVA program and the means were compared using Duncan's Multiple Range Test at p=0.05. Rooting data were also recorded, and their means and standard deviation were calculated.

Establishment of plant cell culture: Cell suspension cultures were derived from static cultured calli in Erlenmeyer flasks (500 ml), each containing 200 ml of the MS liquid medium, supplemented with ingredients as callus induction medium, except agar. After 15 days of preculturing on a gyratory platform shaker at 100 rpm and 22±2°C with a 16 hr photoperiod.

Growth curve analysis: Freshly grown calli (5 gm) were transferred into 24 Erlenmyer flasks (250ml) containing 50 ml of liquid MS medium as similar as cell suspension culture. All the flasks were kept at 22±2ºC in light conditions same as mentioned above, with an agitation on platform shaker (100 rpm). After every five days, three flasks were harvested and their mean Fresh Weight (FW) was calculated.

Biotransformation of compound 1: During the biotransformation experiment, 800ml of a 15 days old suspension was inoculated with 200 mg of substrate (Compound 1). The suspension was kept on shaker with continuous shaking at 100 rpm incubated at 22±2ºC with 16/8 hrs photoperiod. After every five days, the degree of conversion was measured using TLC. After twenty days, as the TLC shows no spot of Compound 1, the cells were separated from the nutrient medium by filtration. The cells were washed with CH_2Cl_2 (200 ml) and the filtrate was extracted with CH_2Cl_2 (3 × 200 ml). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, which afforded a brown gum (0.41 g), which after repeated CC (petroleum ether/ AcOEt gradient) yielded compounds: 1 (51.4 mg; with petroleum ether/AcOEt 72: 28), 2 (16.2 mg; with petroleum ether/AcOEt 61: 39), and 3 (81.7 mg; with petroleum ether/AcOEt 57: 43).

Two Controls were also run along with the test. One is to check the substrate's stability (only the media for suspension culture was fed with the substrate, without any calli) and the other to identify the metabolites produced by the plant cell itself (Cell suspension culture without any substrate feeding). TLC indexing was performed to nullify the spots appear due to substrate degradation or plant metabolites, if any.

Results and Discussion

Callus induction: The primary objective was to identify the optimum conditions for the growth of callus of *Aloe barbadensis* plant. The establishment of cell suspension cultures for biotransformational studies was also targeted. In this regard, along with the yield, the texture of the callus is of great importance since it ensures the equal distribution of the plant cells in the medium. For the biotransformational studies, the callus should be friable,

which ultimately results in the establishment of a very effective suspension culture i.e. having an equal or uniform distribution of single plant cells or micro-clumps of calli.

Effect of light: The effect of light was studied for the induction of callus in *Aloe barbadensis*. Previous results show that callus induction in another *Aloe* i.e., *Aloe arborescens*, requires light conditions and the presence of both auxins and cytokinins (Kawai *et al*., 1993). Our results completely differ from these results as in our case the maximum percentage of friable callus induction and growth was achieved under the dark conditions (Fig. 1a and 1b, Tables 1 and 2). The retarded growth of callus was observed in light, it may be because of light induces increased production of phenolic compounds, which interfere with growth regulator activity. Based on these observations, it can be easily postulated that light is not essential requirement for the callus induction in *Aloe barbadensis.*

Table 1. Effect of BAP and NAA on callus formation in *Aloe barbadensis* under light condition.

Media codes	BAP (mg/L)	NAA (mg/L)	Fresh Weight[a] (gm)	Description of callus	
				Color	Texture
C 1	0.0	0.0	0.0	-	-
C 2	0.0	2.0	4.3 ± 1.1	Off-white	Compact
C 3	0.0	4.0	5.2 ± 1.7	White	Compact
C 4	0.0	8.0	5.5 ± 2.1	Light brown	Compact
C 5	1.0	0.0	0.0	-	-
C 6	1.0	2.0	2.4 ± 0.78	Light green	Loosely Compact
C 7	1.0	4.0	3.7 ± 1.64	Greenish	Compact
C 8	1.0	8.0	4.7 ± 1.45	Greenish Brown	Compact
C 9	1.5	0.0	3.5 ± 1.72	Green	Compact
C 10	1.5	2.0	4.2 ± 2.0	Green	Loosely compact
C 11	1.5	4.0	4.4 ± 2.1	Green	Compact
C 12	1.5	8.0	5.4 ± 2.3	Greenish Brown	Compact
C 13	2.0	0.0	0.0	-	-
C14	2.0	2.0	3.7 ± 1.65	Greenish Brown	Loosely Compact
C15	2.0	4.0	4.1 ± 1.87	Greenish off-white	Loosely compact
C 16	2.0	8.0	4.3 ± 1.98	Dark green	Compact

[a]Sign represents mean fresh weight ± standard deviation

Table 2. Effect of BAP and NAA on callus formation in *Aloe barbadensis,* under dark condition.

Media codes	BAP (mg/L)	NAA (mg/L)	Fresh Weight[a] (gm)	Description of callus	
				Color	Texture
C 1	0.0	0.0	-	-	-
C 2	**0.0**	**2.0**	5.65 ± 1.90	Off-white	Friable
C 3	0.0	4.0	5.23 ± 1.87	White	Loosely compact
C 4	0.0	8.0	5.32 ± 1.92	White	Compact
C 5	1.0	0.0	2.11 ± 1.45	Light green	Compact
C 6	1.0	2.0	3.12 ± 1.62	Off-white	loosely Compact
C 7	1.0	4.0	3.87 ± 2.32	Light brown	Compact
C 8	1.0	8.0	3.96 ± 2.02	Brown	Compact
C 9	1.5	0.0	2.24 ± 1.57	Greenish Brown	Compact
C 10	1.5	2.0	2.85 ± 1.69	Off-white	Compact
C 11	1.5	4.0	3.36 ± 1.83	Light brown	Loosely compact
C 12	1.5	8.0	4.21 ± 1.09	Brown	Compact
C 13	2.0	0.0	2.01 ± 0.97	Greenish	Friable
C14	2.0	2.0	2.32 ± 0.95	Greenish	Compact
C15	2.0	4.0	2.38 ± 1.04	Light brown	Compact
C 16	2.0	8.0	3.12 ± 1.65	Brown	Compact

[a]Sign represents mean fresh weight ± standard deviation

Fig. 1. Callus formation in *Aloe barbadensis*, (a) and (b) under illumination conditions, (c) and (d) under dark.

Effect of hormones: The effect of various types of plant growth regulators were also evaluated for their potential to induce calli in *Aloe barbadensis*. MS medium, containing 2 mg/L of NAA (α-naphthalene acetic acid), showed maximum callus formation (Table 2, and Fig. 1c and 1d). The maximum callus obtained had friable texture and off-white color with fresh weight of 5.65 gm under dark condition. MS medium, supplemented with 4 mg/L of NAA, also induced white colored callus under light condition. At the same concentration of NAA but under light the growth was bit retarded having 4.3 gm and compactly textured. It was reported that callus induction in *Aloe arborescens*, required the presence of both auxins and cytokinins (Kawai *et al*., 1993), but in our case the maximum percentage of friable callus induction and growth was achieved by only using the auxin i.e., NAA. Our results showed that the combination of both auxin (NAA) and BAP (6-benzyl amino purine), and cytokinin, retarded the callus growth. Concentration of NAA higher than 2 mg/l induced callus but that was bit compactly structured which is not obviously suitable for suspension culture. Based on these observations, it can be postulated that for callus growth in this species, only auxin (NAA) is sufficient with no light requirements. Calli induced in this system, were used to establish the cell suspension cultures, to study the growth curve and biotransformation ability of *Aloe barbadensis*.

Effect of hormones on shoots and roots: Highest regeneration was observed when MS medium was supplemented with a combination of 1.0 mg/L BAP and 0.1 mg/L IBA (Table 3). On this media, shoot formation and elongation were enhanced and healthy plantlets, measuring 2.475 inch in height, were obtained along with maximum number of multiple shoots i.e., 12.725 (Fig. 2a and 2b). BAP alone was unable to induce shoots but when it was used in combination with IBA even with smaller concentration it resulted in vigorous shoot growth as can be observed in Fig. 2. At higher concentration of IBA number of

shoots and height were considerable but not as inferred in case of IBA with 0.1 mg/L. Number of multiple shoots and average number of leaves was comparable between R2 and R5 media but they differ from each other on the basis of average height of plants. However, here an increase in concentrations of both cytokinin/auxin did not show exponential plantlet regeneration because a balance between auxin and cytokinin growth regulators is mostly required for the formation of adventitious shoots. The requisite concentration of each type of regulator differs greatly according to the plant being cultured (George 1993).

Rooting of the shoots regenerated using tissue culture technology is an important task to acquire for tissue cultured plantlets. IBA and NAA are both employed for this objective at different percentages usually alone and in some cases in combinations. Both of the auxins were utilized to select the best hormonal concentration for *A. barbadensis* rooting *in-vitro*. The days to grow the rooting were ranging from 14 to 19 and no more growth of the roots was observed after 25 which suggested that minimum 14 days would be required to induce rooting in this specie using IBA and after 25 days, rooting medium has to be replaced with fresh one. According to the data obtained (Table 4), maximum average number of the roots was 8.0 with average length of the root of 6.38 cm at 1.0 mg/L of IBA with MS medium and 95% of the shoots were rooted at this concentration in 14-20 days. This is the best datum in all using concentrations of the hormones recruited. At other concentration of the IBA no considerable number and length of roots were observed. At the same concentration of IBA, NAA did not responded as same as IBA because average no. of root was 4.8 and average length of roots was 5.26 cm, although the percent of rooted shoots was 90% and it took almost similar time period as IBA.

Table 3. Effects of BAP and IBA on plantlet regeneration from calli in *Aloe barbadensis*.

Media codes	BAP (mg/L)	IBA (mg/L)	Number of multiple shoots[a]	Number of leaves	Height of plants (inch)
R 1	1.0	0.0	0 ^{efg}	0 ^{efg}	0 ^{efg}
R 2	1.0	0.1	12.725 ^{a}	1.75 ^{a}	2.475 ^{a}
R 3	1.0	0.5	10.95 ^{abc}	1.45 ^{abc}	1.15 ^{c}
R 4	2.0	0.0	0 ^{e}	0 ^{e}	0 ^{e}
R 5	2.0	0.1	12.5 ^{ab}	1.5 ^{ab}	1.475 ^{ab}
R 6	2.0	0.5	11.625 ^{bcd}	1.15 ^{bcd}	1.15 ^{cd}

[a]Means with the same letters are not significantly different at p=0.05 (DMRT)

Table 4. Effect of Auxins on Root formation from Regenerated Plantlets.

Hormone	Concentration	No. of roots[a]	Length of root (cm)	% of rooted plantlet	Rooting period (days)
IBA	0.25	1.8 ± 0.83	3.53 ± 0.33	60	17-22
	0.5	2.4 ± 1.10	4.35 ± 0.26	70	15-20
	0.75	3.4 ± 0.54	5.43 ± 0.14	85	15-20
	1.0	8.0 ± 0.70	6.38 ± 0.34	95	14-20
	1.5	3.2 ± 0.83	5.34 ± 0.17	75	20-25
NAA	0.25	2.2 ± 0.83	3.54 ± 0.61	60	15-21
	0.5	2.8 ± 1.48	3.32 ± 0.15	70	18-24
	0.75	3.0 ± 0.70	4.46 ± 0.09	80	16-22
	1.0	4.8 ± 0.44	5.26 ± 0.18	90	15-20
	1.5	3.2 ± 0.83	4.82 ± 0.83	85	19-24

[a]Each treatment is comprised of 5 replicate and is given in mean ± Standard deviation

Suspension culture and growth curve analysis: The same medium was used for the growth curve analysis and the cultures were kept under the same conditions as mentioned above. The mean fresh weight (FW) was calculated after every fifth day and a graph was ploted showing the change in FW of the suspension with respect to culture time. As Fig. 3 shows, the lag phase of the growth curve was last for at least 5 days. The cells were grown exponentially from seventh to twenty days of inoculation and after that the growth of the suspension culture was entered in the stationary phase. As the cells are in maximum of their functionality and growth during the log phase, the starting of the log phase (fifth day) was selected for the inoculation of substrate to the suspension culture for biotransformation.

Biotransformation of adrenosterone: Cell suspension culture of *Aloe barbadensis*, maintained at the same temperature, light and agitation conditions as stated above, showed three types of reactions when compound 1 was incubated with cell suspension culture of *A. barbadensis* included the formation of C=C moiety at C-1/C-2 and yielded $\Delta^{1\text{-}2}$-dehydroadrenosterone (2), reduction of C-4/C-5 olefinic double bond resulted the formation of 5α-androst-1-ene-3,11,17-trione (3) and the reduction of C-17 keto group, which gave 17β-hydroxyandrost-4-ene-3,11-dione (4) (Scheme-1). Structures of transformed metabolites were elucidated through comparison of their reported data (Musharaf *et al*., 2002). Compounds 2 and 4 were previously reported as metabolites of 1, with various fungal biotransformation (Musharaf *et al*., 2002; Greca *et al*., 1997).

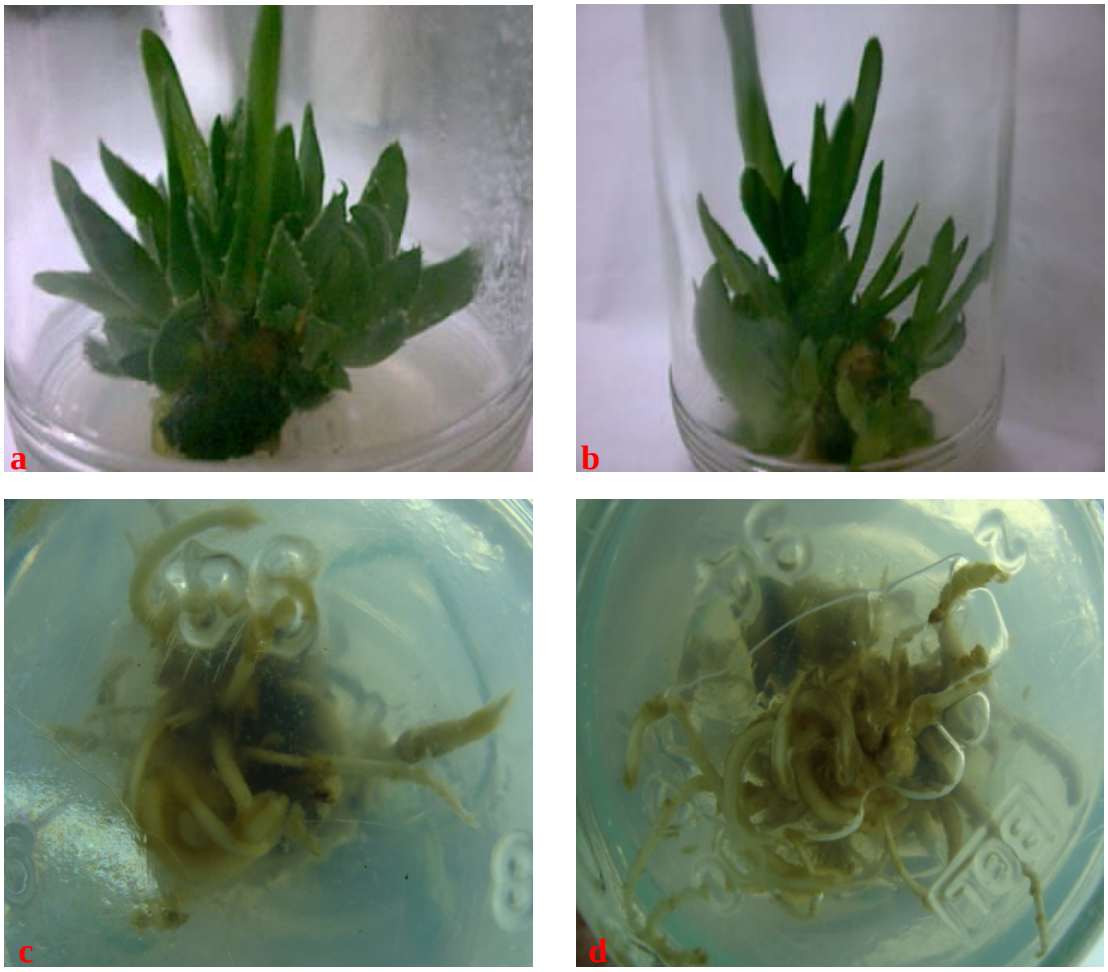
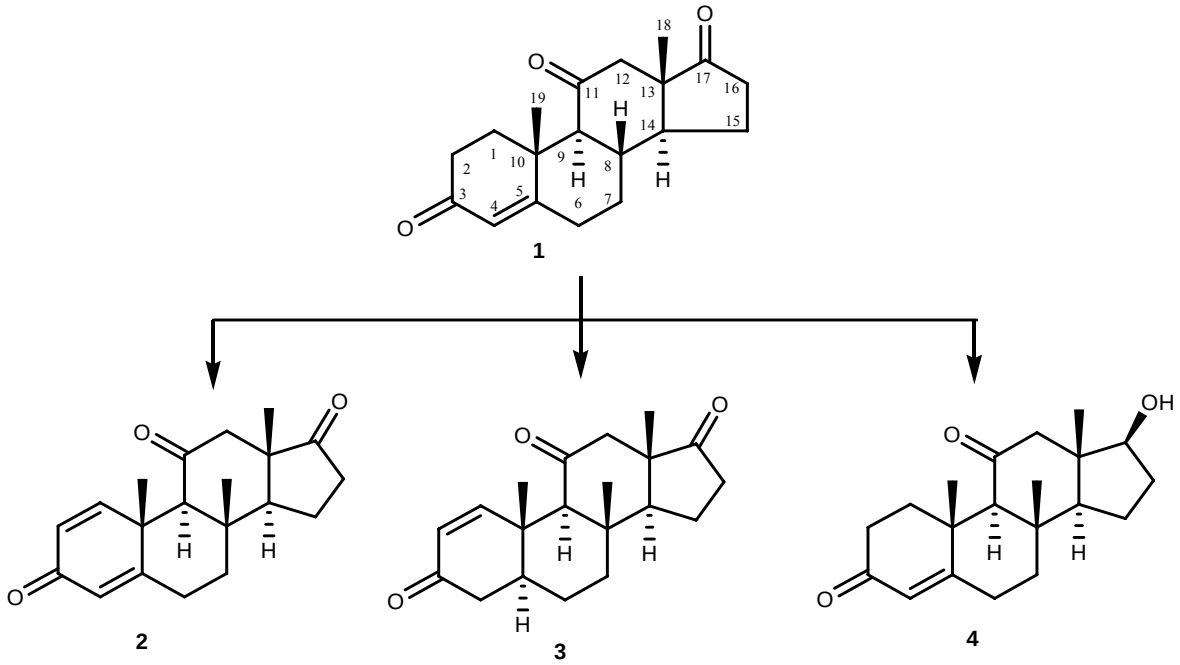


Fig. 2. Regenerated shoots from calli in *Aloe barbadensis* (a) & (b) and newly immersed roots (c), mature roots (d).



Scheme 1. Biotransformation of compound 1 by Cell suspension culture of *Aloe barbadensis.*

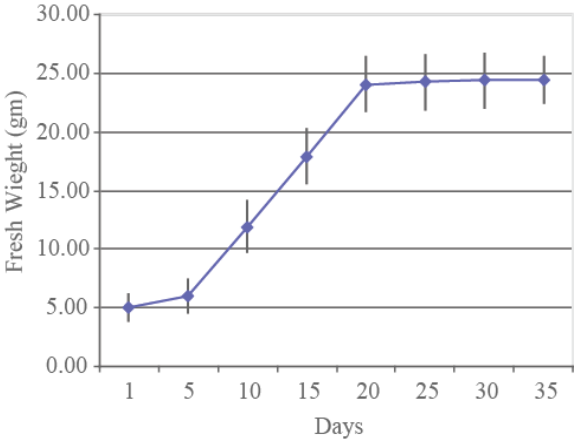


Fig. 3. Growth curve analysis in the suspension culture of *Aloe barbadensis.*

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