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Normal and genetically transformed tissue cultures of *Calotropis procera* (Ait) R.Br. for production of secondary metabolites

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Abstract

Calotropis procera (Ait) R.Br (Family: Asclepiadaceae), is an important medicinal plant, described in traditional system of medicines, *viz*., Ayurveda and Unani has been used in treatment of various ailments. Present investigation provides a detailed insight on the development of normal callus, suspension and morphogenetic as well as genetically transformed cultures of *C. procera* and was further examined for the presence of phenolics and flavonoids by spectrophotometric methods. Results obtained suggested that MS medium supplemented with 6-BA (0.5 ppm) + 2, 4-D (2.0 ppm) was the most suitable combination for the development of independent calli of leaf and stem. Co-cultivation with *A. tumefaciens* (MTCC 2250) for 15 min was found to be quite effective for the crown gall formation whereas incubation of *in vitro* raised shoots for 30 min with *A. rhizogenese* (MTCC 2364), resulted in hairy roots from the leafy explants of the plant. Preliminary phytochemical screening of *in vitro* cultures showed the presence of almost all the phytoconstituents similar to that of natural plant with significant amount of phenolics and flavonoids in leaf callus, followed by crown galls led to conclude that *in vitro* culture produce secondary metabolites similar to natural plant and, hence, these cultures can be used as an alternative source of medicines.

Key words: *Calotropis procera* (Ait) R.Br., callus culture, suspension, genetic transformation, hairy roots

1. Introduction

Calotropis procera (Ait) R.Br (Family: Asclepiadaceae), a giant milk weed, known for its pharmacological importance for centuries, possesses anticancerous, ascaricidal, schizonticidal, antimicrobial, anthelmintic, insecticidal, anti-inflammatory, antidiarrheal, larvicidal potential with many other beneficial properties (Sharma *et al*., 2012). The plant is distributed in tropical and sub-tropical Asia and Africa and occurs almost throughout the India from Punjab and Rajasthan in the north to Kannya Kumari in the south, extending into West Bengal, Assam in the east (Anonymous, 1992). Plant is described as a golden gift for human kind as it consists of cardiac glycosides like calotropin, calotoxin and calactin (Bruschweiler *et al*., 1996b), flavonoids (Tiwari *et al*., 1978), rutin (Lal *et al*., 1985), milk-clotting enzyme (Aworh and Nakai, 1986), phenols (Marimuthu and Kothari, 1986), saponins (Gupta *et al*., 2003) as its important phytoconstituents.

Due to immense potential as the plant is the rich source of metabolites such as flavonoid (rutin) and cardiac glycosides (calotropin and calotoxin). The present investigation has been taken up to carry out the development of *in vitro* cultures as plant tissue culture finds its potential applications in the mass production of plant-derived pharmaceuticals by providing them with the environment for the well-controlled production and overcoming the limitations of natural factors such as geographical location and seasonal variations (Ahmad *et al.*, 2013). Moreover, only few of the reports were available on tissue culture of *C. procera* (Dhir,1984; Suri and Ramawat 1995; Suri and Ramawat, 1996) including induction of calli and the development of laticifer cells in callus (Suri and Ramawat, 1997) added by fewer ones on genetically transformed cultures of the plant, hence, it was considered worthwhile to develop normal and genetically transformed cultures of the plant on normal leaf, stem, root, leaf callus, stem callus, root callus, leaf suspension, stem suspension, root suspension, regenerated shoot, regenerated root, *A. tumefaciens* mediated crown gall, crown gall suspension, normal root culture and *A. rhizogenese* induced hairy roots and to compare the presence of phytoconstituents in them with that of normal plant.

2. Materials and Methods

Six to eight month old whole plant (roots, stem and leaves with flowers) of *C. procera* (Ait) R.Br. was collected in the month of March-April from the species, grown in Herbal Garden of Jamia Hamdard, New Delhi which were identified by Dr. H. B. Singh, Head, Department of Raw Material Herbarium and Museum,

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All the chemicals, *viz.*, growth hormones and culture media, Murashige and Skoog (MS) medium and Gamborg B5 medium and solvents toluene, ethyl acetate were of analytical grade and obtained from Merck India Company.

2.1 Tissue culture studies

2.1.1 Preparation of stock solution

The stock solutions of major, minor and vitamins were prepared separately. The stock solutions (01 mg mL^{-1}) of auxins were prepared by dissolving indole acetic acid (IAA), naphthalene acetic acid (NAA), indole 3-butyric acid (IBA) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) in alcohol whereas cytokinins like kinetin, benzyl adenine were prepared by dissolving them in doubled distilled water, containing few drops of 0.1 N hydrochloric acid. All the stock solutions were stored in refrigerator at 5-6°C, however, brought back to the ambient temperature before using them for the media preparation.

2.1.2 Preparation of culture media

MS basal media with appropriate combination and concentration of different hormone with varying concentrations was used and added into the culture tubes after dividing desired volumes from stock media and the pH was adjusted to 5.6. Agar (1%) was added to the medium with gentle heating and divided equally into all of the culture tubes and kept at 15 lb/ $inch^2$ pressure for 15 min at 120°C.

2.1.3 Induction of cultures

Explants were washed thoroughly under running tap water for 30 min, soaked in 10% soap solution for 15 min, followed by washing with tap water and finally with double distilled water and then they were transferred into laminar flow for assisting the surface sterilization carried out by using 0.1 % w/v solution of mercuric chloride. Explants were then inoculated on a medium under strict aseptic conditions and incubated in the culture room maintained at 25±2°C, under fluorescent light with an intensity of 60 UE/M2/sec maintaining the photoperiod of 16 h and keeping relative humidity to 65-75% with the help of an air cooling system. Incubation conditions were maintained at constant rate throughout the experiment. MS (Murashige and Skoog, 1962) and Gamborg B5 (Gamborg *et al*., 1968) basal medium supplemented with different concentrations of plant growth hormones, were used for initiation and development of leaf and stem callus of *C. procera*. The cultures were incubated at 25 ± 2°C with alternate light (16 h) and dark (8 h) cycle.

2.1.4 Development of culture

The initiated cultures were subcultured for the further development and maintenance of *in vitro* cultures, *viz.* callus, suspension and morphogenetic as well as genetically transformed cultures using MS basal media (Murashige and Skoog, 1962), supplemented with plant growth hormones. These were subcultured at regular intervals and growth was monitored at the interval of 20 d. up to period of 60 d. Strains of *Agrobacterium* were used for affecting the genetic transformation of *C. procera* cultures.

2.1.5 Shoot and root callus from seedling of *C. procera*

The study was carried out to find out the correct hormonal combination for initiation of callus from shoot and root explants of seedling of *C. procera*. The shoot and root explants from 10-12 d. old aseptically germinated seedlings were inoculated on MS medium supplemented with various phytohormones either alone or in combination and the selected ones get utilized further for callusing.

2.1.6 Leaf, stem and root suspension cultures of *C. procera*

Friable callus (1- 2 weeks old) derived from the leaf, stem and root developed on MS medium, supplemented with callus inducing hormone, were used for the establishment of suspension cultures. Cultures were developed in 250 ml flask containing 60 ml of MS medium, supplemented with similar hormonal combination. In the subsequent cell subcultures, 0.1% pectinase was added to the basal MS liquid medium to reduce cell adhesion.

2.1.7 Crown gall tumor / hairy root induction

The precultured shoots with calli subjected to cocultivation with two strains of *Agrobacterium rhizogenese* MTCC 532 and MTCC 2364 at different time intervals to induce hairy roots and while on the other hand, *A. tumefaciens* strains MTCC 431 and MTCC 2250 were used for inducing the crown gall tumours. The wounded explants were immersed in bacterial cultures and kept on rotary shaker for different time intervals (5, 10, 15, 20 and 30 min.) to facilitate the infection of the wounds. After shaking for the given period of time, the explants were taken out in aseptic conditions and washed with sterilized double distilled water and dried with sterilized tissue papers. The explants were then inoculated on hormone free MS, MS/2 and B5 medium, containing cefotaxime (200-250 µg /ml). All the cultures were kept in incubator at 24 ± 2°C for the further observations.

2.1.8 Phytochemical screening

The alcoholic, chloroform and aqueous extracts of leaf; stem; root; leaf callus; stem callus; shoot callus; root callus; leaf, stem, root suspension; regenerated shoots; regenerated roots; root culture; crown gall cultures and hairy root cultures of *C. procera* were subjected to phytochemical screening procedures by using standard chemical tests (Ahmad, 2007) to ascertain the presence of phytocontituents in them, using for alkaloids, carbohydrates, glycosides, phenolic compounds, tannins, flavonoids, proteins and amino acids, saponins, mucilages, steroids and terpenoids.

2.1.9 Estimation of total phenolic and total flavonoids

Spectral and absorbance measurements were carried out on a SHIMADZU UV-2401 spectrophotometer by using 1.0 cm quartz cells for the estimation of total phenolic and flavonoid contents (Miliauskas *et al*., 2004)

Total phenolic contents: The content of total phenolic compounds in methanolic extracts was determined by Folin-Ciocalteu phenol reagent (Folin and Ciocalteu, 1927). The calibration curve was plotted by using 1ml aliquots of 0.025, 0.050, 0.075, 0.100, 0.150, 0.200 and 0.250 mg/ml ethanolic gallic acid solutions, mixed with 5.0 mL Folin-Ciocalteu reagent (diluted ten-fold) and 4 mL of sodium carbonate (1M). The absorption was read after 30 min at 20°C at

765 nm and the calibration curve was drawn. 1 ml of methanolic plant extract (5 g/l = 5 mg/ml) was mixed with the same reagents as described above, and the absorption was read after 1h for the determination of plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c * V/m$$

where: C-total content of phenolic compounds (mg/g) plant extract in GAE; c-the concentration of gallic acid established from the calibration curve (mg/ml); V-the volume of extract (ml); m-the weight of pure plant methanolic extract (g).

Total flavonoid content: The content of flavonoids was determined using rutin as a reference. Sample solution was prepared by mixing 1 ml of plant extract in methanol (10g/l = 10 mg/ml), 1 ml aluminum trichloride in ethanol (20 g/l), diluted with ethanol to 25 ml. Blank solution consisted of 1 ml of plant extracts with 1 drop acetic acid diluted to 25 ml and standard solution comprised of 0.05 g of rutin. The absorption was read at 415 nm after 40 min at 20°C for all the solutions and the determinations were carried out in triplicate. The amount of flavonoids in rutin equivalents (RE) was calculated by using the following formula:

$$X = (A * m^0 * 1000)/ A_0 * m$$

where: X-flavonoid content, mg/g plant extract in RE; A-the absorption of plant extract solution; A_0-the absorption of standard rutin solution; m-the weight of plant extract, g; m_0-the weight of rutin in the solution in g.

2.2 Statistical Analysis

Statistical analysis was done using Dunnet's t test and $p < 0.05$ is considered as statistically significant.

3. Results and Discussion

3.1 Tissue culture studies

The MS basal medium supplemented with 6-BA (0.5 ppm) + 2,4-D (2.0 ppm) and 2, 4-D (4.0 ppm) showed prominent callus initiation in leaf and stem in 7-10 d. (Figures 1A, 2A, 2B). The maintenance of independent calli up to 6th passage on MS medium, supplemented with 6-BA (0.5 ppm) + 2, 4-D (2.0 ppm) (Figure 1B, 2G), yielded significant amount of biomass of leaf and stem calli. The growth cycle of callus was having relatively short lag phase (12-14 d.), followed by a long duration of exponential growth till 91-98 d. afterwards cell entered in stationary period preceded by senescent phase after 112 d.

The MS basal medium, supplemented with the Kin (0.5 ppm) + 2, 4-D (2.0 ppm) exhibited prominent callus initiation on shoot and root seedlings in 07-10 d while MS medium, supplemented with NAA + 6-BA (1.0 ppm each) showed callusing in roots only. The growth kinetics study of 15 d. old calli of leaf and stem on MS medium, supplemented with Kin (0.5 ppm) + 2,4-D (2.0 ppm), resulted in maximum growth of shoot and root callus in 8th week.

MS medium, supplemented with 6-BA (1.5 ppm), caused regeneration of shoots from the *in vitro* germinated seedlings (Figure 1 C), followed by rooting also after few days (Figure 1E) whereas supplementation with NAA + 6-BA (0.5 ppm each) affected the formation of thick shoots and fine roots (Figure 1D).

Thin shoots and roots were regenerated from the *in vitro* germinated seedlings at MS basal medium, supplemented with NAA (0.5 ppm) + 6- BA (2.0 ppm) (Figure 1F), however, fortifying the medium with NAA + IBA (1.0 ppm each), resulted in bunch of thin roots (Figure 1G).

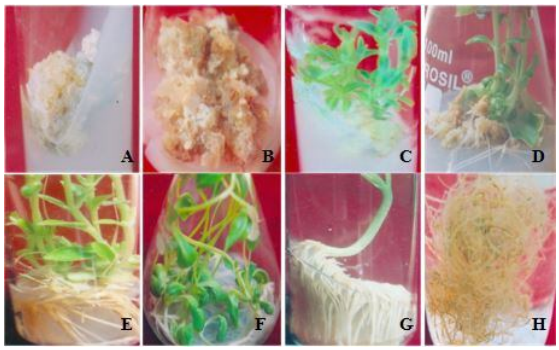

Figure 1: Cultured leaf explants of different basal medium supplemented with different hormone combinations at different stages

Legends : [A: Initiated callus from the leaf explant on MS basal medium supplemented 6-BA (0.5 ppm) + 2,4-D (2.0 ppm); B: 90 d. old callus on MS basal medium supplemented 6-BA (0.5 ppm) + 2,4-D (2.0 ppm) C: Shoot regeneration from *in vitro* germinated seedling at MS basal medium supplemented at 6-BA (1.5 ppm); D: Regeneration of thick shoots and fine roots at MS basal medium + NAA + 6- BA (0.5 ppm each) each; E: Rooting and shooting from the *in vitro* germinated seedlings at MS + 6- BA (1.5 ppm) + IBA (0.5 ppm); F: Regeneration of thin shoots and fine roots in *in vitro* germinated seedlings at MS basal medium with NAA (0.5 ppm) + 6- BA (2.0 ppm); G: A bunch of thin roots from seedlings at MS +NAA + IBA (1.0 ppm each); H: Hairy roots maintained on hormone free MS/2 medium supplemented with 250 µg of cefaotaxime].

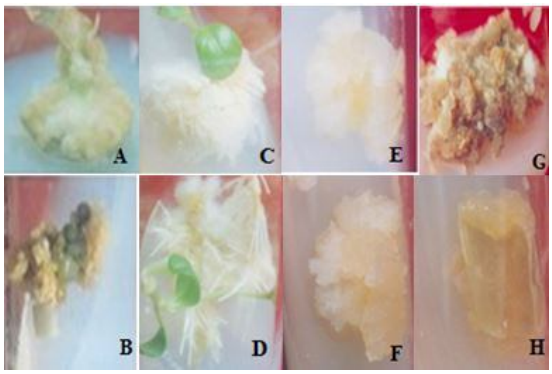

Figure 2: Cultured stem explants of different basal medium supplemented with different hormone combinations at different stages

Legends : [A: Initiated callus from the stem explant on MS basal medium supplemented 6-BA (0.5 ppm) + 2,4-D (2.0 ppm); B: 10 d. old callus on MS basal medium supplemented with 2,4-D (4.0 ppm); C: Induction of hairy roots on precultured shoots by cocultivation with MTCC 2364 on hormone free MS/2 medium infected with cefotaxime; D: Hairy roots on precultured shoots by cocultivation with MTCC 2364 on hormone free MS/2 medium treated with 250 µg cefatoxime; E: 28 d. old crown gall maintained on MS/2 on hormone free medium F: 42 d. old crown gall maintained MS/2 on hormone free medium; G: 90 d. old stem callus maintained on MS +6- BA (0.5 ppm) + 2,4-D (2.0 ppm) ; H: Four weeks old crown galls by co-cultivation with MTCC 2250 on hormone free MS/2 medium treated with 250 µg cefotaxime].

Friable leaf and stem callus developed on MS medium, supplemented with 6-BA (0.5 ppm) + 2,4-D (2.0 ppm) and root callus on MS with Kin (0.5 ppm) + 2,4-D (2.0 ppm) along with their suspension cultures at the respective hormone combinations were subjected to growth kinetic studies that in turn revealed 3.55, 3.53 and 3.71 folds (approximately) increase in biomass of leaf, stem and root cells, respectively at the end of growth period (28 d).

Results obtained from the transformation studies showed that the cocultivation time of 15 min with *A. tumefaciens* strain MTCC 2250 was quite effective for the formation of crown galls (Figure 2E). Galls were maintained for 4 to 6 weeks on hormone free MS/2 medium treated with 250 µg/ml of cefotaxime under natural light/ dark conditions (Figures 2F, 2H), on the other hand MTCC 431 did not show any response under the same conditions. The full strength MS and B5 medium also failed to show the response with both of the strain, *i.e.*, MTCC 2250 and MTCC 431. Growth kinetics studies of crown gall revealed approximately 18-fold increase in biomass of calli within 12 weeks, which was better as compared to leaf callus (14 folds) (Figure 3).

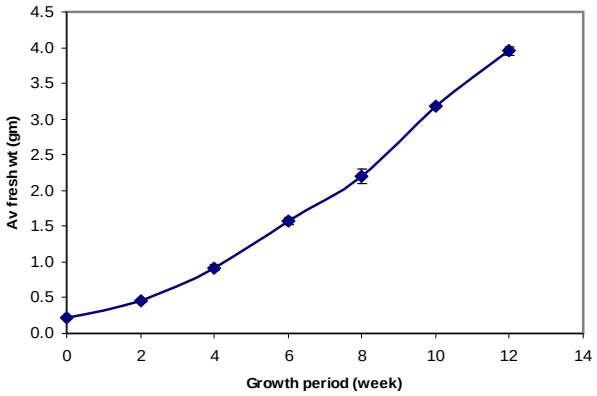

Figure 3 : Growth kinetics of crown galls in terms of fresh weight

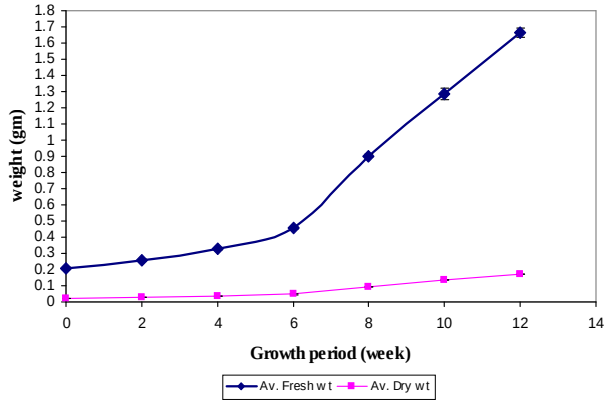

Figure 4 : Growth kinerics of hairy root culture in terms of fresh and dry weight

In parallel to above the cocultivation of 30 min with *A. rhizogenese* strain MTCC 2364, caused induction of hairy roots on precultured shoot of the plant on hormone free MS/2 medium treated with 250 µg/ml of cefotaxime in natural light/dark condition (Figures 2C, 2D) and the same was maintained up to 8-12 weeks (Figure 1H) whereas strains MTCC 532 showed zero response. The growth kinetics study of the hairy roots revealed 7.9 and 7.8 fold increase in fresh and dry weight, respectively and their growth was remained continued even after 12 weeks (Figure 4). The studies conducted were found to be in accordance with previously established protocols where genetic transformations affected the production of crown galls and transformed roots in number of medicinal plants and eventually resulted in rapid growth of the biomass and the content of active metabolites (Zafar *et al.*, 2001).

3.2 Analytical studies

3.2.1 Phytochemical screening

All the samples tested showed the presence of alkaloids, reducing sugars, non-reducing sugars, flavonoids, phenolics, tannins, amino acids, saponins, resins sterols and cardiac glycosides while anthraquinone glycosides were found absent in all the samples. Results obtained were tabulated in Table 1.

3.2.2 Phenolic and flavonoid contents

As phenolics may affect the efficacy of drugs resulting in serious consequences (Dandiya *et al.*, 1959) that is why the total phenolic and flavonoids content was determined by using UV spectrophotometer (Hajaji *et al.*, 2010; Singh *et al.*, 2012). The maximum of total phenolic content was found in methanolic extract of natural leaf (37.95 mg/g), followed by *in vitro* regenerated shoot (33.98 mg/g) and natural stem (32.13 mg/g) whilst minimum was reported in root callus (15.25 mg/g) preceded by shoot callus (16.55 mg/g) and hairy root culture (16.56 mg/g) (Table 2).

The highest amount of total flavonoid content was found in methanolic extract of crown galls (5.51 mg/g) culture, followed by leaf callus (5.56 mg/g), shoot callus (5.05 mg/g) and natural leaf (4.54 mg/g) whilst lowest was reported in natural root (0.96 mg/g), followed by stem callus (1.02 mg/g), root callus (1.30 mg/g) and natural stem (1.59 mg/g) (Table 3).

4. Conclusion

The present work incorporated the different methods for the establishment of *in vitro* cultures for the production of secondary metabolites. The results of phytochemical screening showed that the developed cultures consists of all the enzymes and coenzymes systems as that of natural plant, hence, they can be utilized as an alternative source of medicines and for the *in vitro* production of secondary metabolites. The techniques used in the present investigations can play a crucial role for continuous *in vitro* production of phytochemicals present in *C. procera*, which can be further explored on industrial scale.

Acknowledgement

Authors are thankful to UGC, Government of India for providing financial assistance for conducting the study.

Conflict of interest

We declare that we have no conflict of interest

Table 1 : Presence / absence of various metabolites in natural plant parts and its *in vitro* cultures

Metabolites	Samples															
	Natural			Callus culture			Suspension				Regenerated		Transformed culture			
	L	S	R	LC	SC	RC	SHC	LS	SS	RS	Reg SH	Reg R	CGC	CGS	HRS	HRL
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Reducing sugars	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nonreducing sugars																
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenolics	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amino acids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Resins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sterols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anthraquinones	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-

Legends : where L = Natural leaf, S = Natural stem, R= Natural root, LC = Leaf callus, SC= Stem callus, SHC = Shoot callus from seedling, LS = Leaf callus suspension, SS = Stem callus suspension, RS = Root callus suspension, Reg SH = Regenerated shoot, Reg R = Regenerated root, CGC = Crown gall callus, CGS = Crown gall suspension, HRS = Hairy roots in solid media, HRL = Hairy roots in liquid media.

+ Presence of metabolites; – Absence of metabolites

Table 2 : Total phenolic content in natural plant parts and *in vitro* cultures (n = 3)

Extracts	Absorbance ± SEM	Content of GAE from cal. curve (mg/ml)	Total phenolic content mg/g of plant extract
Leaf	2.391 ± 0.030	189.762	37.952
Stem	2.025 ± 0.019	160.687	32.137
Root	1.364 ± 0.012	108.254	21.651
Leaf callus	1.471 ± 0.004	116.719	23.344
Stem callus	1.332 ± 0.009	105.687	21.137
Root callus	0.961 ± 0.004	76.2963	15.259
Shoot callus	1.043 ± 0.038	82.751	16.550
Regenerated shoot	2.141 ± 0.022	169.947	33.989
Regenerated root	1.203 ± 0.007	95.449	19.090
Hairy root culture	1.044 ± 0.008	82.830	16.566
Crown gall culture	1.187 ± 0.034	94.233	18.847

$p<0.05$ (Leaf callus, Shoot callus, Reg Shoot and Crown gall culture Vs Leaf; Stem callus Vs stem; Root callus, Reg root and Hairy root culture Vs Root)

Table 3: Total flavonoid content in natural plant parts and *in vitro* cultures

Drug	OD of Drug (D)	OD of Blank (B)	OD (D-B)	Content of flavonoid mg/g
Standard Rutin	0.096	0.008	0.088	-
Leaf	3.215	3.135	0.08	4.545
Stem	0.249	0.221	0.028	1.591
Root	0.063	0.046	0.017	0.966
Leaf callus	0.192	0.094	0.098	5.568
Stem callus	0.183	0.165	0.018	1.022
Root callus	0.061	0.038	0.023	1.307
Shoot callus	0.186	0.097	0.089	5.057
Regenerated shoot	2.763	2.692	0.071	4.034
Regenerated root	0.071	0.04	0.031	1.761
Hairy root culture	0.091	0.023	0.068	3.865
Crown gall culture	0.198	0.081	0.097	5.511

$p<0.05$ (Leaf callus, Shoot callus, Reg Shoot and Crown gall culture Vs Leaf; Stem callus Vs stem; Root callus, Reg root and Hairy root culture Vs Root)

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