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ORIGINAL PAPER

In vitro propagation and biosynthesis of steroidal sapogenins from various morphogenetic stages of *Moringa oleifera* Lam., and their antioxidant potential

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Abstract *Moringa oleifera* is a highly valued medicinal plant. The present research reports callus cultures of *M. oleifera* Lam., established from seeds and nodal segments on Murashige and Skoog's (MS) medium using different concentrations and combinations of auxins and cytokinins. Best induction of callus was observed at BAP:IBA (3 mg l^{-1} each). Shooting and rooting from callus in terms of morphogenesis were observed in MS media supplemented with BAP:KN (2:0.2 mg l^{-1}) and IBA:NAA (3:0.5 mg l^{-1}), respectively. Multiple shooting was observed at treatment dose of BAP:NAA:IAA (1:1:0.2 mg l^{-1}). Regenerated shoots were rooted and mature plants were established, acclimatized, and thrived in greenhouse conditions. Over 95 % of plantlets survived after transplanting plantlets into trays with a mixture of sand and perlite (2:1) for 20 days. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of bioactive constituents of this medicinal plant. Further qualitative and quantitative production of steroidal sapogenins (diosgenin and tigogenin) from various morphogenetic stages was studied using TLC, PTLC, IR spectra, HPLC and GC–MS analysis. Steroidal sapogenins were maximum in the callus associated with rooting. Various stages were further analyzed for their antioxidant potential.

Keywords *Moringa oleifera* · Nodal segment · Morphogenesis · Steroidal sapogenins · PTLC · HPLC · GC–MS · IR spectra · Antioxidant

Abbreviations

2, 4 D	2, 4-Dichlorophenoxyacetic acid
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
IBA	Indole butyric acid
IAA	Indole acetic acid
Kn	Kinetin
BAP	Benzyl amino purine
TLC	Thin layer chromatography
PTLC	Preparative thin layer chromatography
IR-Infra	Red spectroscopy
HPLC	High performance thin layer chromatography
GC–MS	Gas chromatography and mass spectroscopy
DPPH	2,2-Diphenyl-1-picrylhydrazyl

Introduction

Moringa oleifera Lam., commonly called as '*Sahanjana*' belongs to family *Moringaceae,* which bears 14 species among which *M. oleifera* is most commonly found. The plant is native to northern India. The pods and leaves are reported to contain 2.5 and 6.7 g protein/100 g, respectively (Verma et al. 1976). The plant parts are used in folk remedies for tumors, abdominal discomfort, boils, cold, conjunctivitis, high blood pressure, hysteria, relapsing fever, skin diseases, etc. (Hartwell 1967–1971). The plant also bears some bioactivities, viz., anti-inflammatory (Sulaiman et al. 2008), anti-asthmatic (Agrawal and Mehta

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2009), antioxidant and hepatoprotective (Fakurazi et al. 2008), antimicrobial (Jabeen et al. 2008), antiurolithiatic (Karadi et al. 2008), antitumor (Guevara et al. 1999) etc. Besides scanty reports of Islam et al. (2005) and Nieves and Aspuria (2011) there are some reports (Kantharajah and Dodd 1991; Katherine et al. 2004; Benjamin et al. 2009; Marfori 2010; Saini et al. 2012) on callus cultures and organogenesis on *M. oleifera.* In the present study an attempt has been made to raise callus cultures and induce organogenesis in the in vitro grown plant. The study describes an effective method for shoot regeneration through callus culture initiated from in vitro seedlings and nodal explants using MS medium supplemented with different concentrations and combination of growth regulators. Further various steroids were isolated and characterized and their antioxidant potential was determined using established protocol.

The steroids are derivatives of Cyclopentanoperhydrophenethrene that include sterols, steroidal sapogenin, steroidal glycosides, cardiac glycosides, hormones, corticosteroids and oral contraceptives (Wall 1960; Heftman 1967, 1974; Coppen 1979). Among various steroids, diosgenin is one of the promising compounds reported from few plant species (Liu et al. 2005). Diosgenin can be absorbed through the gut that plays an important role in the control of cholesterol metabolism (Roman et al. 1995), and is also used as starting material for partial synthesis of oral contraceptives, sex hormones and other steroids (Zenk 1978). Such partial synthesis of steroids from plant-based precursors has been a boon because of the increasing demand for corticosteroids, contraceptives, sex hormones and anabolic steroids since 1960 (Hall and Walker 1991). An attempt as been made to isolate steroidal sapogenin (diosgenin and tigogenin) from different morphogenetic stages for the first time. Finally, an attempt has been made to characterize steroidal sapogenins using various spectral techniques like HPLC and GC–MS.

In recent past much attention has been given to the antioxidants obtained from plant sources because of their health benefits (Makris and Kefalas 2001). They counteract reactive oxygen species (ROS) (Lu and Foo 1995), such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non-free-radical species as H_2O_2 and singlet oxygen (O_2). In foods, ROS can cause lipid peroxidation which is responsible for the rancidity and foul smell, consequently decreasing the nutritional quality and safety of foods. The addition of antioxidant can increase the shelf life of foods (Cook and Samman 1996). Oxidative stress has been implicated in many degenerative diseases, such as atherosclerosis, coronary heart diseases, ageing and cancer (Finkel and Holbrook 2000; Valko et al. 2007). Minimising the oxidative stress thus prevents the damage of body as they inhibit the free radicals. However, there is a widespread agreement that synthetic antioxidants need to be replaced with natural antioxidants because some synthetic antioxidants have shown potential health risks and toxicity as possible carcinogenic effects (Safer and Al-Nughamish 1999). Therefore, new sources of safe and inexpensive antioxidants of natural origin to use them in foods and pharmaceutical preparations are important to replace synthetic.

So an attempt has been made to evaluate antioxidant potential of various morphogenetic stages of callus using established protocol for the first time.

Materials and methods

The plant parts were collected from the fields located in University campus and the herbarium specimen was deposited in the Botany Department, University of Rajasthan, Jaipur, India (RUBL No. 20393[1]).

Surface sterilization and inoculation

Seeds and nodal segments were initially treated with teepol reagent (1 %) followed by distilled water and then treated with antibiotic (Ciprofloxacin, 250 mg l^{-1}) prior to inoculation in order to remove any kind of microbial interactions.

Preparation of plant hormones

Different concentrations and combinations of various auxins and cytokinins were dissolved in distill water and pre added in the media before autoclave. Initially auxins were dissolved with one drop of ethanol and then volume were raised with distill water while cytokinins were dissolved in one drop of ethanol and HCl and volume was make up with distill water.

Incubation

Cultured flasks were incubated in culture chamber. The temperature of chamber was maintained at 25 ± 10 °C and 1,200 lux light intensity. A photoperiod of 16 h light was provided. The cultures were observed and examined every week and final morphogenetic data were recorded.

Subculturing procedure

Explant About six explants from ten replicates including germinated seeds, nodal explants and in vitro grown seedlings were inoculated to fresh MS medium without

[1] RUBL Rajasthan University Botanical Library.

hormone and later MS medium (Murashige and Skoog 1962) supplemented with various concentrations of hormones.

Callus The primary cultures were obtained having calli were sectored in small pieces and then transferred to fresh medium after every 4–6 weeks of culture initiation till 32 weeks. The callus was also transferred to various combinations and concentrations of auxins and cytokinins for regeneration in terms of shooting and rooting.

Hardening procedure

In vitro grown plantlets were ready for transfer from aseptic culture to the field. These were prepared for further growth, hardening and acclimatization. Plantlets were removed from the parental cultures and transferred to the reduced salt concentration eliminating vitamins and growth regulators. The plantlets were then removed from cultures and were thoroughly washed with sterile water to remove agar. They were then transferred to small pre autoclaved earthen pots containing soil:vermiculture (3:1) mixture. The plantlets were watered with sterile distilled water having 1:1 solution of ammonium nitrate and potassium nitrate and covered with polythene bags to maintain humidity. These pots were kept at 25 ± 1 °C and 16 h light and then plants were steady and acclimatized, they were transferred to the field.

Growth index

The maintained calli were harvested regularly at the transfer age of 2, 4, 6 and 8 weeks. About ten replicates each of the callus samples was harvested and their growth indices (GI) calculated on fresh weight basis.

Extraction of steroidal sapogenins

Callus tissue with various morphogenetic stages (callus with shooting, multiple shooting and callus with rooting) was powdered weighed and defatted separately in Soxhlet apparatus in petroleum ether for 24 h on a water bath. Each mixture was hydrolyzed with 15 % ethanolic HCl (1 g/5 ml: w/v) for 4 h by refluxing on water bath (Tomita et al. 1970). Each hydrolysate was filtered and filtrate extracted thrice with ethyl acetate. The ethyl acetate fractions of each sample was pooled and washed to neutrality by repeated washings with distill water, dried in vacuo, reconstituted in chloroform, filtered, dried again and weighed. Each test sample was replicated thrice. Thin glass plates coated with silica gel (250 μm thick) were dried at room temperature, thereafter kept at 100 °C for 30 min to activate. The freshly prepared activated plates were used for qualitative as well as quantitative analysis.

Qualitative analysis

The crude steroidal sapogenins extract of each sample was examined on TLC, along with the reference steroidal sapogenin (diosgenin and tigogenin). The plates were developed in a solvent system of chloroform, hexane and acetone (23:5:2), air dried and sprayed with 50 % sulphuric acid and anisaldehyde reagent (composed of 0.5 ml of anisaldehyde, 1 ml of conc. sulphuric acid and 50 ml of acetic acid), separately and heated to 100 °C until the characteristics colors developed. The fluorescence response as well as permanent black zones was recorded. The times required for the initial appearance of a colour reaction, the initial colour in day light and after heating for 10 min and the colour in UV light (360 nm) were recorded. A combination of other solvent systems such as benzene and ethyl acetate (85:15; Heble et al. 1968) and acetone and benzene (1:2; Khanna and Jain 1973) were also used but solvent system of chloroform, hexane and acetone (23:5:2) was comparatively better than other solvent system. Three replicates were run and R_f values were calculated.

Quantitative analysis

Preparative thin layer chromatography (PTLC)

PTLC was used to isolate diosgenin and tigogenin from crude steroidal sapogenins extract on silica gel G plates by using solvent mixtures of chloroform, hexane and acetone (23:5:2). The spots were marked on TLC by spraying with anisaldehyde reagent, to one of the columns on each plate and spots corresponding to the standard diosgenin and tigogenin were marked and scrapped separately from the unsprayed plates/column. The PTLC was repeated until about 20 mg of the substance was obtained. Co–TLC of crystallized isolated substance along with reference marker (standard diosgenin and tigogenin) was carried out to test the purity of isolated compounds. Such chromatograms were also visualized by spraying a solution of antimony trichloride in conc. HCl (Kadkade et al. 1976). After PTLC the diosgenin was crystallized from methanol-acetone (Kaul and Staba 1968) and examined for MP, MMP and infrared spectral studies.

Spectrophotometry of diosgenin and tigogenin

The spectrophotometric method of Sanchez et al. (1972) was adopted for quantification. It includes the preparation of regression curve of the standard diosgenin and tigogenin from their stock solution (1 mg ml^{-1}) prepared in chloroform, from which different concentrations (20–200 μg)

were applied on silica gel G plates and developed along with a parallel run of blank in an organic solvent of chloroform, hexane and acetone (23:5:2), which were later on dried and exposed to iodine vapours. The resultant dark yellowish spots as also the spots corresponding in the blank were marked and the plate were heated to 100 °C for 15 min to remove iodine. Each of the marked spot was scrapped along with the adsorbent, transferred to separate test tube and eluted with 5 ml of methanol. The mixture was then centrifuged, the supernatant transferred to separate test tube and evaporated to dryness. To the dried residues, 4 ml of 80 % methanolic sulphuric acid was added and left at room temperature for about 2 h by intermittent shaking. Optical density of the reaction mixture was read at 405 nm against a blank solution (80 % methanolic sulphuric acid). Three replicates of each concentration were taken and average optical density was calculated. A regression curve between various concentrations and their respective optical density was computed which followed the Beer's law.

Each of the crude extract of tissue samples were dissolved in 5 ml of chloroform and applied (0.1 ml) on silica gel G plates along with standard steroidal sapogenins as markers, developed in solvent mixture of chloroform, hexane and acetone (23:5:2) which were later on dried and exposed to iodine vapours. The resultant dark yellowish and pink spots and corresponding spot of the standard authentic samples were marked on each plate, scrapped, eluted with methanol, dried and then treated with 80 % methanolic sulphuric acid as above. The concentrations of diosgenin and tigogenin in each case sample were worked out referring to their optical densities in the standard curve and the results were calculated on dry weight basis. Three replicates of each sample were taken and their mean values calculated.

HPLC for diosgenin

For diosgenin quantification a HPLC Hewlett Packard instrument model HP-1100 (Palo Alto, CA, USA) equipped with the software version ChemStation A.06.01, a diode array detector (DAD-UV) was used; in addition, a Hypersil ODS C18 column (250 × 4.0 mm, 5 µm) and a 20 µl Rheodyne manual injector were used.

Gas chromatography and mass spectroscopy (GC–MS)

The extract and the standard samples were analyzed by GC–MS of Hewlett-Packard 6890/5973 operating at 1,000 eV ionization energy, equipped with a HP-5. Capillary column (phenyl methyl siloxane, 25 m × 0.25 mm i.d.) with Helium (He) was used as the carrier gas with split ratio 1:5. Oven temperature was 100 °C (3 min) to 280 °C at 1 to 40 °C min^{-1}; detector temperature, 250 to 280 °C; carrier gas, He (0.9 ml min^{-1}). Retention indices were determined by using retention times of samples that were injected under the same chromatographic conditions. The components of the standard and plant samples were identified by comparison of their mass spectra and retention time with those given in literature and by comparison with the mass spectra of the Wiley library or with the published mass spectra.

DPPH radical scavenging assay

Different fractions were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. (2,2-Diphenyl-1-picrylhydrazyl; Brand-Williams et al. 1995) assay. Plant extract (0.75 ml) at different concentrations ranging from 10 to 100 µg ml^{-1} were mixed with 1.5 ml of a DPPH methanolic solution (20 mg l^{-1}). Pure methanol was taken as control and ascorbic acid (vitamin C), vitamin A, E was used as a reference compounds. The absorbance was measured at 517 nm after 20 min of reaction. The percent of DPPH decoloration of the sample was calculated according to the formula

$$\text{Decoloration } \% = [1 - (\text{Abs}_{\text{SAMPLE}}/\text{Abs}_{\text{CONTROL}})] \times 100$$

The decoloration was plotted against the sample extract concentration and a logarithmic regression curve was established in order to calculate the IC_{50}. The results are expressed as antiradical efficiency (AE), which is 1,000-fold inverse of the IC_{50} value ($AE = 1{,}000/IC_{50}$, Parejo et al. 2002).

Statistical analysis

About ten replicates of each experiment were performed to calculate for statistical analysis

Values are given as mean ± SEM (standard error of the mean) and were compared using one-way ANOVA to judge the difference between various groups. Values of $p < 0.05$ were considered statistically significant

The statistical error of mean was calculated by the following formula:

$$\text{SE} = \frac{\sigma}{\sqrt{n}}$$

where, σ standard deviation, n number of observations.

The test of significance (t test) was calculated by the following formula:

$$t = \frac{m_1 - m_2}{\sqrt{(\text{SEM}_1)^2 + (\text{SEM}_2)^2}}$$

where, m_1 mean of one set of values, m_2 mean of second set of values, SEM_1 standard error of the first set of values, SEM_2 standard error of the second set of values.

The probability p for obtaining t value of at least as great as the calculated one for a given number for the degree of freedom was found in the Fisher's table.

The p values were signified according to the following conventions.

$p < 0.05$ difference was almost significant, $p < 0.01$ difference was significant, $p < 0.001$ difference was highly significant.

Results

Callus induction

Seed germination of *M. oleifera* started after 8–10 days. In vitro grown seedlings were also used as explants. Nodal explants showed initial swelling followed by initiation of callus from the cut ends within 1st week of inoculation on media [MS_7] supplemented with BAP:IBA (3:3 mg l^{-1}). Best response in terms of undifferentiated mass of callus (about 78 %) was obtained on media [MS_7] supplemented with BAP:IBA (3:3 mg l^{-1}). Best induction of callus from seeds was observed on media supplemented with BAP:IBA (3:3 mg l^{-1}; MS_7) and 2, 4-D: Kn (5:0.02 mg l^{-1}; MS_3). The texture of callus became brown, compact and hard when media [MS_3] was supplemented (5.0:0.02 mg l^{-1}) with 2, 4-D: Kn and green when media were supplemented with BAP:NAA (1.0:2.0 mg l^{-1}; MS_9:1:0.2 mg l^{-1}; MS_{10}). When media [MS_{11}] was supplemented with IAA:IBA (1.0:0.3 mg l^{-1}) the texture of callus changed from green to grey. Callus turned brown and proliferated from nodal segments grown on media (MS_4) supplemented with BAP + IAA (1.0:0.2 mg l^{-1}). Callus induction from in vitro grown seedlings was observed when media [MS_5] was supplemented with BAP:IAA (3:3 mg l^{-1}). Growth of the callus was slow during first 4 weeks and later the proliferation of callus increased for next 2 weeks and finally from 7th week the growth became stationary and then declined. During 8th week the media started to turn brown, which might be due to leaching out of phenols and accumulation of phenolic compounds in the medium. The probability of survival and proliferation of *M. oleifera* callus tissue on subculturing the callus during 5–6th week was higher in comparison to subculturing after 7–8th week (Table 1).

Morphogenesis

Plant regeneration via shoot and root morphogenesis was observed when callus was transferred on media with various combinations and concentrations of hormones. When media [MS_{26}] was supplemented with BAP:NAA (1.0:0.5 mg l^{-1}) initiation of little shoots from callus was observed. When media [MS_{32}] was supplemented with BAP:Kn:NAA (2.0:1.0:0.2 mg l^{-1}) callusing along with multiple shooting was observed. Shoot organogenesis was observed from callus on media [MS_{23}] supplemented with BAP:Kn (2.0:1.0 mg l^{-1}) and on media [MS_{28}] supplemented with Kn:IAA (2.0:1.0 mg l^{-1}). Best shooting was observed on media [MS_{22}] supplemented with BAP:Kn (2.0:0.2 mg l^{-1}). Callus associated with shoots were observed on explants subcultured from in vitro grown shootings grown on (MS_9) supplemented with BAP:NAA (1.0:2.0 mg l^{-1}) treatment dose. Callusing and shooting simultaneously were observed (67 %) when (MS_{24}) supplemented with BAP:NAA (1.0:0.1 mg l^{-1}) treatment dose. Rooting developed from callus on media [MS_{36}] containing IAA:IBA (0.5:3.0 mg l^{-1}). Maximum rooting from callus was observed on media supplemented [MS_{41}] with IBA:NAA (3.0:0.5 mg l^{-1}). When MS media was supplemented with IAA and IBA, media MS_{36} recorded the lowest growth of shoots and roots from callus with 39 % compared to media MS_{34} with 51 %.

Multiple shooting and rooting

Variety of auxins and cytokinins concentrations gave effect on shoot production with or without callus. The lower auxin concentration enhanced multiple shoots production. Nodal segments inoculated in the media [MS_{13}] supplemented with BAP:NAA:IAA (1:1:0.2 mg l^{-1}) gave long multiple shoots. These shoots were rooted in media without any growth regulators. Profuse rooting was seen in vitro regenerated shoots grown in the media [MS_{19}] supplemented with IBA (3.0 mg l^{-1}) and when NAA also added in the media [MS_{17}] with IBA:NAA (2:0.2 mg l^{-1}), rooting from in vitro shoots were observed (Table 1).

Hardening procedure

After successful root development, in vitro grown rooted plantlets were taken out from the culture vessels, without causing any damage to the delicate root system, gently washed with sterile distilled water. Three plantlets were then transferred to small pre-autoclaved earthen pots having a mixture of sterile vermiculite and soil in the ratio of 1:3. Immediately after transplantation, the pots were kept in growth chamber for 15 days at 25 ± 1 °C and 16 h light intensity for acclimatization. In order to maintain high humidity, the pots were covered with inverted glass beakers. These plantlets were irrigated two times daily with sterilized water containing 1:1 solution of ammonium and potassium nitrate. After 2 days of transplantation, wilting and yellowing started in leaves of 35 % plantlet. After 1 week wilting increased in remaining plantlet and about 40 % plants were wilted in next 1 week, remaining plantlets continued to grow for 20 days. After 20 days, plantlets

Table 1 Morphogenetic response of *M. oleifera* on MS medium supplemented with various treatment doses of growth hormones

Growth hormones	Modified MS Media	Concentration (mg l^{-1})	Explant used					
			Nodal segment			Seeds and in vitro seedlings		
			Frequency (in %)	Morphological appearance	Nature of callus	Frequency (in %)	Morphological appearance	Nature of callus
(i) Without hormones								
MS	MS_{00}		78	–	–	–	–	–
(ii) Callus induction								
MS + 2,4-D + KN	MS_1	1.0 + 0.5	25	BN	FG	31	BN	FG
	MS_2	2.0 + 0.5	37	BN	FG	82	BN	FG
	MS_3	5.0 + 0.02	21	BN	FG	86	BN	FG
MS +BAP + IAA	MS_4	1.0 + 0.2	19	BN	FG	21	BN	FG
	MS_5	3.0 + 3.0	17	BN	FG	67	BN	FG
MS + BAP + IBA	MS_6	1.0 + 0.2	43	BN	FG	52	BN	FG
	MS_7	3.0 + 3.0	78	BN	FG	–	BN	FG
	MS_8	3.0 + 4.0	52	BN	FG		BN	FG
MS + BAP + NAA	MS_9	1.0 + 2.0	19	GN	FG	54	GN	FG
	MS_{10}	1.0 + 0.2	65	GN	FG	–	GN	FG
MS + IAA + IBA	MS_{11}	1.0 + 0.3	61	GR	FG	–	GN	FG
			Shoots and Roots					
(iii) Direct regeneration	MS_{12}	1.0 + 0.5 + 0.2	19	–	–	–	–	–
MS + BAP + NAA + IAA	MS_{13}	1.0 + 1.0 + 0.2	75	–	–	–	–	–
	MS_{14}	3.0 + 2.0	65	–	–	–	–	–
MS + BAP + KN	MS_{15}	2.0 + 0.7	71	–	–	–	–	–
	MS_{16}	2.0 + 0.2	19	–	–	47	–	–
MS + IBA + NAA	MS_{17}	0.5 + 2.0	32	–	–	–	–	–
	MS_{18}	0.5 + 3.0	45	–	–	–	–	–
MS + IBA	MS_{19}	3.0	82	–	–	–	–	–
(iv) Differentiation			Shoots and Roots from Callus					
MS + BAP + KN	MS20	1.0 + 0.1	28	–	–	–	–	–
	MS21	1.5 + 0.2	30	–	–	–	–	–
	MS22	2.0 + 0.2	79	–	–	–	–	–
	MS23	2.0 + 1.0	55	–	–	–	–	–
MS + BAP + NAA	MS24	1.0 + 0.1	67	–	–	–	–	–
	MS25	1.0 + 0.2	53	–	–	–	–	–
	MS26	1.0 + 0.5	56	–	–	–	–	–
MS + KN + IAA	MS27	1.0 + 0.5	28	–	–	–	–	–
	MS28	2.0 + 1.0	–	–	–	–	–	–
	MS29	3.0 + 1.0	32	–	–	–	–	–
	MS30	4.0 + 2.0	–	–	–	–	–	–
MS + BAP + KN + NAA	MS31	1.0 + 0.1 + 0.1	–	–	–	–	–	–
	MS32	2.0 + 1.0 + 0.2	53	–	–	–	–	–
	MS33	2.0 + 0.3 + 0.3	66	–	–	–	–	–
MS + IAA + IBA	MS34	1.0 + 1.0	51	–	–	–	–	–
	MS35	1.0 + 2.0	42	–	–	–	–	–
	MS36	0.5 + 3.0	39	–	–	–	–	–
	MS37	1.0 + 4.0	46	–	–	–	–	–

Table 1 continued

Growth hormones	Modified MS Media	Concentration (mg l^{-1})	Explant used					
			Nodal segment			Seeds and in vitro seedlings		
			Frequency (in %)	Morphological appearance	Nature of callus	Frequency (in %)	Morphological appearance	Nature of callus
MS + IBA + NAA	MS38	2.0 + 5.0	45	–	–	–	–	–
	MS39	1.0 + 2.0	35	–	–	–	–	–
	MS40	2.0 + 3.0	41	–	–	–	–	–
	MS41	3.0 + 0.5	82	–	–	–	–	–
	MS42	3.0 + 0.3	76	–	–	–	–	–

FG fragile, *GN* green, *GR* grey, *BN* brown, '–' no response

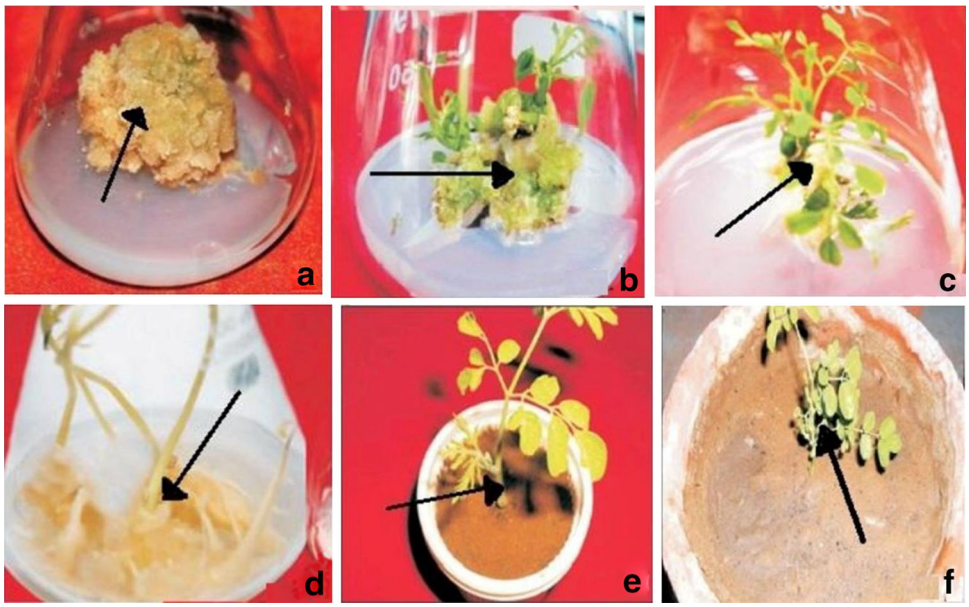

Fig. 1 **a** Callus formation from nodal segment grown on (MS_7) supplemented with BAP:IBA treatment dose (3 mg l^{-1}) each. **b** Shooting and greening of proliferated calli from nodal segments grown on MS media (MS_{10}) supplemented with BAP (1.0 mg l^{-1}): NAA (0.2 mg l^{-1}). **c** Clonal propagation by multiple shoots using nodal explants grown on MS media (MS_{13}) supplemented with BAP (1.0 mg l^{-1}): NAA (1.0 mg l^{-1}): IAA (0.2 mg l^{-1}). **d** Rooting of clonally propagated plantlets grown on (MS_{00}) media without growth regulators. **e** Regenerated plants transferred to plastic pots. **f** Plants in pots kept in field condition

were transferred to the soil where they continued to grow normally. In all, about 95 % plantlets successfully survived and were transferred to the soil (Fig. 1a–f).

During subculture, age of tissue was calculated by growth index which is calculated as follows.

Growth and maintenance

The callus grown on media [MS_7] supplemented with BAP:IBA (3:3 mg l^{-1}) gave best results. The callus cultures were maintained for 6 months by periodic subculturings. Callus was harvested at the time intervals of 2, 4, 6 and 8 weeks of fresh subculturing to determine growth index (GI). The GI value on fresh weight basis depicted a sigmoid pattern from 2 to 8 weeks. Maximum GI was observed in 6 weeks (3.06) old callus tissue, which declined further (Fig. 2)

$$\text{Growth index} = \frac{\text{Final weight of tissue} - \text{Initial weight of tissue}}{\text{Initial weight of tissue}}$$

Steroidal sapogenins (diosgenin and tigogenin)

Qualitative

On thin layer chromatography, isolated sapogenins gave fluorescent spots in solvent system of Chloroform:Hexane:Acetone (23:5:2) under UV lamp. Green and pink colored

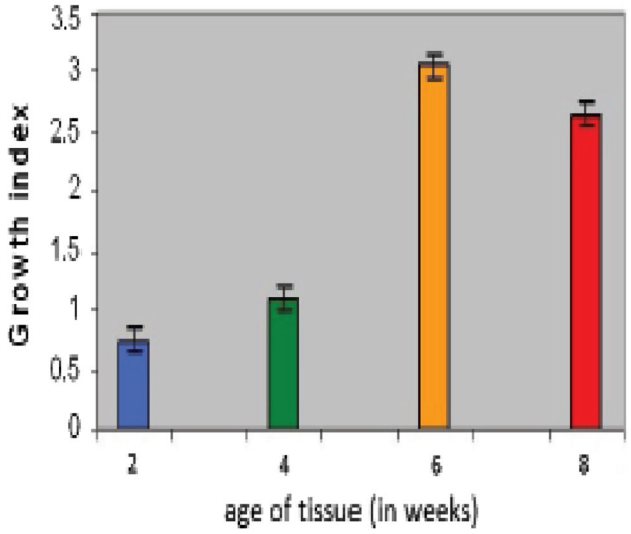

Fig. 2 Growth index (GI) of *M. oleifera* grown on modified MS medium

Table 2 Chromatographic behavior and physico-chemical characteristics of isolated steroidal sapogenins

Isolated compound	In UV	R_f value		Color after spray		Melting point (°C)
		S_1S_2		R_1R_2		
Diosgenin	BR-BL	0.58	0.57	GN	GN	203–205
Tigogenin	BL	0.52	0.68	Orange	Pink	207–208

S_1 chloroform:hexane:acetone (23:5:2), S_2 benzene:ethyl acetate (85:15), R_1 50 % H_2SO_4, R_2 anisaldehyde reagent, *GN* green, *BR* bright, *BL* blue

spots were observed after spraying of these developed plates with anisaldehyde reagent and with 50 % sulphuric acid. Spot having R_f 0.58 coincided with that of standard diosgenin and R_f 0.52 with that of tigogenin. Isolated fraction was purified and subjected to crystallization when subjected to determination of melting points, corresponded with that of respective diosgenin (203–205 °C) and tigogenin (207–208 °C). The characteristic peaks of IR spectra of isolated diosgenin and tigogenin also superimposed with IR spectra of reference compounds (Table 2; Figs. 3, 4).

Quantitative

Various in vitro grown morphogenetic stages were analysed for its diosgenin and tigogenin content showed that callus associated with rooting had maximum diosgenin and tigogenin (3.13 ± 1.36 and 2.79 ± 1.01 mg gdw^{-1}) followed by callus associated with shooting (2.58 ± 1.02 and 2.34 ± 0.98 mg gdw^{-1}) and minimum (1.58 ± 0.73 and 1.19 ± 0.27 mg gdw^{-1}) in multiple shooting (Fig. 5).

HPLC of diosgenin

The hydrolysis of the *n*-butanol extracts with the enzyme naringinase produced diosgenin as well as other sapogenins. The use of the enzyme hydrolysis reduced significantly diosgenin degradation as well as the production of artifacts and this might increase the diosgenin yields in this study. The diosgenin recovery using the method reported here was 97 %, which is higher than that obtained by the acid hydrolysis of *n*-butanol extracts from other plant sources (Oleszek 2002).

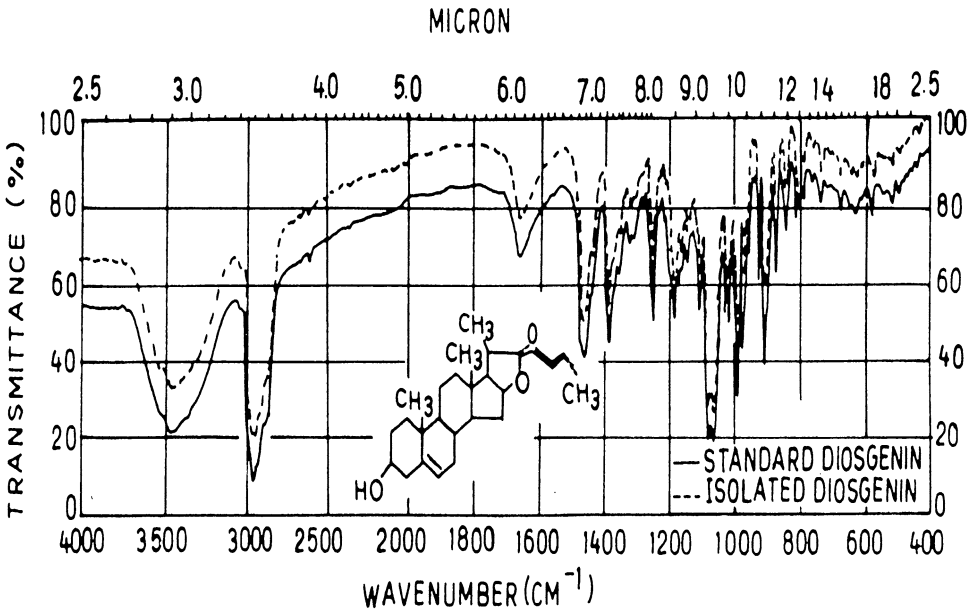

Fig. 3 Infrared spectra of isolated and standard diosgenin

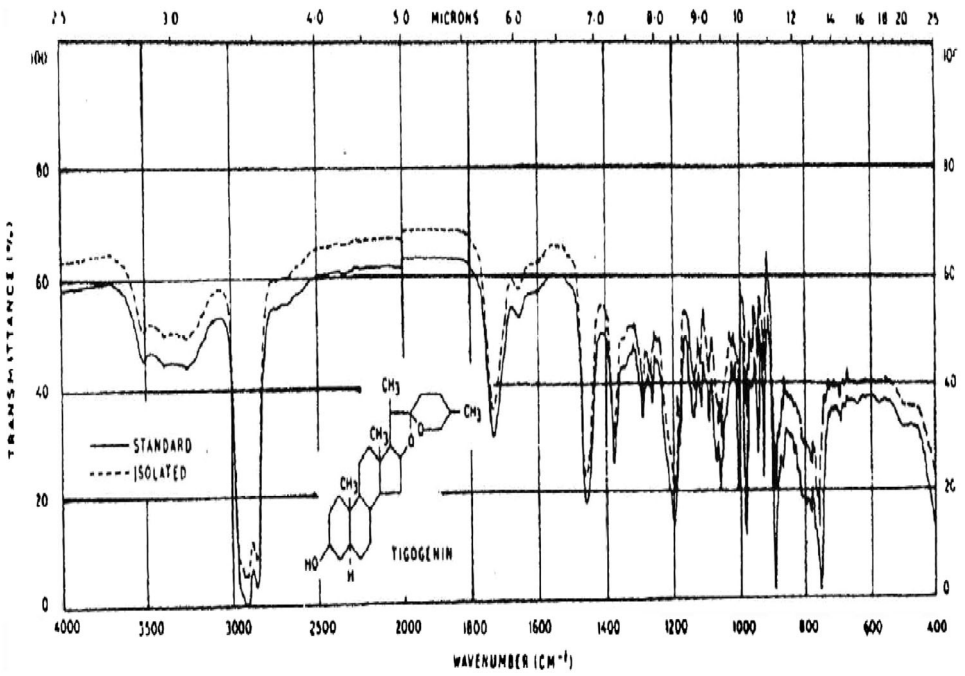

Fig. 4 Infrared spectra of isolated and standard tigogenin

The experimental conditions used for diosgenin quantification by HPLC in the different *M. oleifera* extracts gave a highly reproducible retention time (t_R) equal to 11.8 ± 0.05 min. All samples studied gave the same chromatographic pattern. The diosgenin peak in gas chromatography–mass spectrometry (GC–MS) of some plant extracts displayed similar fragmentation patterns as diosgenin standard (data not shown). The results of diosgenin quantification by HPLC are given. The percentages of diosgenin obtained on this work are in the range from 0.02 to 2.64 %, which is significant since there are several literature data where the diosgenin contents are very low.

GC–MS

The GC–MS studied showed that the retention time and peaks of the isolated steroidal sapogenin were comparable with that of the standard, and 41 new compounds were identified in plant sample as shown. Phytocompounds with their biological activities identified through the GC–MS study are presented in table (Table 3).

DPPH radical scavenging assay

Free radicals are reactive species with an unpaired electron. Antioxidants are able to reduce free radicals by donating an electron or hydrogen atom to the free radical. The HAT (hydrogen atom transfer) activity of plant extracts was studied using the DPPH· free radical and its reaction with a phenolic antioxidant can be written as:

$$\mathrm{DPPH}\cdot + \mathrm{ArOH} \rightarrow \mathrm{DPPH2} + \mathrm{ArO}\cdot$$

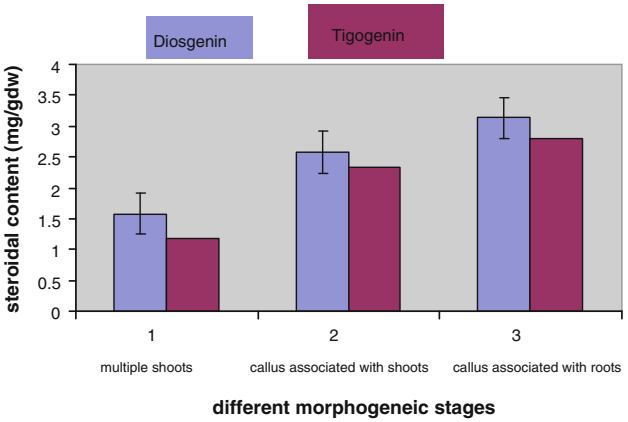

Fig. 5 Steroidal content at different in vitro morphogenetic stages of *M. oleifera*

Antioxidant compounds present in aqueous plant extracts found to be able to donate hydrogen atoms to DPPH· were also able to reduce ferric iron via single electron donation. In DPPH radical scavenging assay Antiradical Efficiency (AE) ranged from 24.54 to 48.16 (Table 4). The callus associated with rooting showed the highest AE (48.16 ± 1.58) while multiple shoots gave lowest (24.54 ± 0.94). The AE of diosgenin and tigogenin was also evaluated. It was observed that AE was found to be lower than callus associated with rooting but higher than rest of other parts (Table 5).

Discussion

Internal hormonal levels, blocked direct regeneration in the experimental plant. Hussey (1975 have improved

Table 3 Activity of phyto-components identified from callus associated with rooting cultured from *M. oleifera* by GC–MS

Peak#	R. time	Area %	Name of compound identified	Biological activity
1	4.857	0.20	Heptadecane	Enhancement of fertility
2	6.648	4.40	Methane Sulfinyl	Antimicrobial
3	7.368	0.19	Heneicosane	Antifungal
4	9.077	1.48	6-Octen-1,l, 3,7-dimethyl	Antifungal
5	9.644	0.06	Pentadecane	Antimicrobial
6	9.949	0.20	Eicosane	Antimicrobial, larvicidal
7	10.294	0.11	Docosane	**New compound**
8	10.966	0.07	Hexadecane	Bioremediation
9	12.268	0.38	2-Pyridinecarboxylic acid, ethyl ester	Antihypertensive
10	12.379	0.14	Heptadecane, 8-methyl	Anticancerous
11	12.856	0.14	1-Tricosene	**New compound**
12	13.472	0.14	Octadecane	Larvicidal
13	14.782	0.86	Heneicosane	Antifungal
14	15.552	0.78	1-Octadecanethiol	Antioxidant
15	16.411	2.81	Tetracosane	Tetracosane
16	18.509	7.53	Celidoniol, deoxy	**New compound**
17	19.576	1.59	1-Tricosene	**New compound**
18	19.976	0.50	9-Octadecenoic acid ethyl ester	Cytotoxic
19	20.518	11.30	Heneicosane	Antifungal
20	20.803	0.56	Ethyl (9z,12z)-9,12-octadecadienoate	Antioxidant
21	21.269	0.63	Nonacosane	Antiviral
22	22.084	12.97	Heneicosane	Antifungal
23	22.825	1.01	Tricosane	Antifungal
24	23.543	3.64	Heneicosane	Antifungal
25	23.767	9.61	Nonacosane	Antiviral
26	25.768	8.44	Tetratetracontane	Antimalarial, Antibacterial
27	26.858	0.46	Docosane	**New compound**
28	27.245	1.02	Ethyl docosanoate	Antimicrobial
29	28.219	7.56	Tetratetracontane	Antimalarial, Antibacterial
30	29.596	0.45	Celidoniol, deoxy	**New compound**
31	30.407	1.43	Tetratetracontane	Antimalarial, Antibacterial
32	31.279	3.86	Tetratetracontane	Antimalarial, Antibacterial
33	33.334	0.77	4,8,13,17,21-Pentamethyl-4,8,12, 16,20-docosapentaena	Anti-inflammatory
34	33.900	0.81	Heptadecanoic acid, ethyl ester	**New compound**
35	35.195	2.47	Tetratetracontane	Antimalarial, Antibacterial
36	35.968	1.47	Octadecanoic acid	Chemical defense against fouling organisms
37	37.339	3.01	1-Hexacosanol	Antibacterial
38	37.892	1.35	Bis(2-ethylhexyl) Phthalate	**New compound**
39	40.188	2.10	Hexacosane	Antioxidant
40	44.272	0.74	*n*-Butan-di-amide	Antifouling
41	46.591	1.25	Tetratetracontane	Antimalarial, Antibacterial

differences in callus production between different types of explants of Hyacinth. It seems that one of the important reasons why the rate of produced callus in scale explants was higher than other of plant was because the existence of meristemoid-like cell is in basal plate of bulb that is in better situation for callus. The effects of different types of auxin on tissue culture of hyacinth have been compared and the existences of differences affecting of IAA, NAA and 2.4-D (Hussey 1975) IBA and IAA (Kim et al. 1981), NAA and IBA (Hussey 1975) have been reported.

The variations in regenerative characteristics among explants are sometimes attributable to difference in explant

Table 4 Antiradical efficiency (AE) of various morphogenetic stages of *M. oleifera* using DPPH radical scavenging activity

Fractions	AE of various morphogenetic stages of *M. oleifera*		
	Multiple shoots	Callus associated with shooting	Callus associated with rooting
MF	24.54 ± 0.94	31.82 ± 0.70	48.16 ± 1.58

Table 5 Antiradical efficiency (AE) of various steroids isolated from *M. oleifera* using DPPH radical scavenging activity

Fractions	AE of various steroids isolated from *M. oleifera*	
	Diosgenin	Tigogenin
MF	34.54 ± 0.94	33.47 ± 0.70

physiological age and differentiation among the constituent cells (Murashige 1974). Thus, attention must be paid to the existence structural of differences in this connection.

In the present study callus cultures in *M. oleifera* were raised from seeds, nodal segments and in vitro grown seedlings. These observations are in agreement with Thorpe and Patel (1984) that tissue or organs used as source of explants can also be determinant for the success of plant tissue culture (Khan et al. 2006; Ali et al. 2007; Akbas et al. 2008). It has been observed that juvenile and actively dividing plant responded effectively in vitro condition due to vigorous vegetative development stage and absence of reproductive structure formation. Even in juvenile stage, tissue and organ regeneration had been more with the younger and actively dividing tissues (Endress 1994; Reinert and Bajaj 1977). In the present investigation callus was raised from seeds, in vitro seedlings and nodal segments on various combination doses of BAP:IBA (3:3 mg l^{-1}), BAP:NAA (1:0.2 mg l^{-1}), BAP:IAA (1:0.2 mg l^{-1}) unlike other reports mentioned above. The presence of auxins and cytokinin in the culture medium regulates various aspects of dedifferentiation and differentiation (Woodward and Bartel 2005) at cellular levels. Generally, auxins have been used for callus induction and proliferation, and both cytokinins and auxins were required for redifferentiation of callus into organized cell (Wang et al. 2008).

In the present investigation, regeneration (organogenesis) in unorganized callus through shoot and root formation was observed on various concentrations and combinations of BAP:Kn (2:0.2 mg l^{-1}) and NAA:IBA (2:1 mg l^{-1}), respectively. Torrey (1966) reported various ways of regeneration of plants either by somatic embryogenesis or through adventitious shoots. In the present study multiple shoots were obtained in media supplemented with cytokinins only and without auxins. Increasing the concentration of kinetin enhanced multiple shoots associated with callus proliferation. This observation was not in agreement with findings of Handley and Chambliss (1979) who reported multiple shoots in hormone free media for *Cucumis sativus.* The root formation in isolated shoot without growth regulators in *Dyckia agudensis* was similar to the observations found in *D. macedoi* (Mercier and Kerbauy 1993) and *D. distachya* where the rooting percentage was increased with lower auxins concentration, especially NAA 1.1 μM and 0.1 mg l^{-1}, respectively (Mercier and Kerbauy 1992, 1993). However, in *Vriesea fosteriana* the addition of 0.54 μM of NAA was necessary to stop lateral shoots proliferation as well as to restabilize apical growth and rooting was easily induced (Mercier and Kerbauy 1992).

In the present investigation profuse rooting from nodal segments and in vitro grown shoots was observed when media was supplemented with IBA at 3 mg l^{-1} (MS_{19}), which are in agreement with above reports. The present investigation also shows that it is possible to propagate *M. oleifera* through tissue culture by establishing roots in vitro with or without growth regulators, followed by transition to glasshouse conditions over several weeks. Most of the in vitro explants with roots did not appear hyperhydric and it was opined that hyperhydricity was a reversible condition which gave changes to the growth environment (Kevers et al. 2004) such as in the growth regulator-free medium. It is most probable that the lack of hyperhydricity in the in vitro rooted plantlets lead to the success of their acclimatization to the glasshouse. The success of shoots rooted in vitro using IBA to re-establish ex vitrum may have been due to the massive callus growth and consequent good vascular connection between roots and shoots. Microcuttings collapsed within days after transfer ex vitro, which may have been due to a number of factors relating to the conditions in the fogging environment and ability of the cuttings to control water loss (Offord and Campbell 1992).

The results of these experiments demonstrated the viability of the micropropagation technique for the mass reproduction and it can be useful as a tool for in vitro germplasm conservation of the *Moringa* species.

There is no uniform and clear definition of growth of plant cell cultures and dry weight or fresh weight methods have been in use for determining GI, because of its preciseness, accuracy in observing variation (Grossmann 1988). Several workers have established the unorganized static cultures of different plants on different medium and observed the sigmoid growth pattern of the callus culture (Staba 1980; Endress 1994). In the present study a sigmoid pattern of growth curve was observed in *M. oleifera*. The maximum growth index was achieved at the 6th week of subculture indicating the exponential growth phase. Minimum growth index was observed at 2nd week of subculture. An increase in GI after supplementation of various

growth regulators finds support from the observations that the growth of tissue, some time depends upon the culture medium and also controlled by the environmental and biological factors like pH, dose and combinations of growth regulators used (Barz et al. 1977; Schripsema et al. 1990).

The type and concentration of auxins and cytokinins, and their relative ratio in the culture medium also controls the biosynthesis and accumulation of secondary metabolites. The accumulation of phenolics, coumarins, flavonoids, steroids and lignans were stimulated in the presence of low auxin levels, especially NAA (King 1976; Sugano et al. 1975). However, increasing the auxin concentration inhibited phenolic and steroids production (Ibrahim and Edgar 1976; Sohai and Shuler 1984).

Plant cell cultures may serve as an alternative industrial source of phytochemicals. The study of compartmentation mechanism together with metabolic studies, improvement of culture media and selection of cell lines is particularly relevant in order to increase the production of phytochemicals. It has been emphasized that secondary metabolites have not been systemically assayed in the culture medium (Petiard and Courtois 1983). The kinetics of cell death compared to the kinetics of metabolite excretion in most cases showed the excretion of phytochemicals contributing to the overall dynamics of the metabolites. Secondary metabolites are usually not distributed uniformly within the whole plant (Wiermann 1981). Some are restricted to specific organs, others to specific tissues. The knowledge of the biosynthetic pathways of secondary compounds and precise sites of accumulation is still scanty.

Antioxidant reacts with DPPH free radical, the electron becomes paired off and bleaching of the colour stoichiometrically in methanol solutions that depends on the number of electrons taken up. This diversity in such methods is due to the complexity of analyzed substrate. In the present study, various morphogenetic stages using DPPH radical scavenging assay were analysed and observed that callus associated with rooting showed better AE. Present findings are in agreement with the reports of several workers who observed antioxidant potential of in vivo and in vitro grown tissue cultures of several other medicinal plants (Badmis et al. 2003).

Conclusion

The cell differentiation follows specific biochemical and morphological principles. The formation of secondary plant products taken an integrated part of differentiation process. The synthesis and accumulation of secondary metabolites can be endogenously controlled, development dependent differentiation process or can be regulated by various exogenous factors. In some plants, initiation of morphological differentiation represents the triggering signal and different metabolites require degrees of tissue differentiation together with preservation of the level of biosynthetic activity for production of valuable secondary metabolites in laboratory conditions.

Author contribution Manas Mathur: The Research Fellow. Sunita Yadav: Helped in antioxidant activities. Pawan K. Katariya: Extraction of steroids, TLC, PTLC, Text and figures formatting. Prof. Raka Kamal: The Research Supervisor.

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Conflict of interest The authors declare that there are no conflicts of interests.

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