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Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation of spine gourd (*Momordica dioica* Roxb. ex. willd) for the assessment of phenolic compounds and biological activities

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ABSTRACT

An efficient protocol for hairy root induction of spine gourd (*Momordica dioica*) was established using *Agrobacterium rhizogenes* (KCTC 2703). This study evaluates the phenolic compound production, antioxidant and antimicrobial (antibacterial, antifungal and antiviral) activities of transgenic hairy root cultures in *M. dioica* . Hairy roots were induced from leaves, petiole, and internodal explants. Molecular analysis of PCR and gene sequencing using specific primers of *rolC* and *aux1* revealed T-DNA integration in the hairy root clones and RT-PCR analysis confirmed the expression of hairy root inducible genes (*rolC* and *aux1*). The greatest biomass accumulation of hairy roots on MS liquid medium supplemented with 3% sucrose was observed at 22 days. Ultra-HPLC was used to compare the individual phenolic compound contents of transgenic and non-transgenic roots. Moreover, transgenic hairy roots efficiently produced several phenolic compounds, such as flavonols, hydroxycinnamic acid and hydroxybenzoic acid derivatives. The total phenolic, flavonoid contents and biological (antioxidant, antibacterial, antifungal and antiviral) activities were higher in hairy roots compared to non-transformed roots. These results demonstrate the greater potentiality of *M. dioica* hairy root cultures for the production of valuable phenolic compounds and for studies of their biological activity.

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1. Introduction

Momordica dioica Roxb. ex. Willd (Family: Cucurbitaceae) is a high nutritional value of wild edible vegetable and also highly used for the traditional medicine (Bharathi et al., 2014). It is commonly known as spine gourd or small bitter gourd or teasle gourd is an annual or perennial dioecious climber. It has native of tropical regions on Africa, South America and Asia with extensive distribution in Bangladesh, China, India, Japan, and Pakistan (Thiruvengadam and Chung, 2011; Talukdar and Hossain, 2014). Spine gourd contains significant amount of alkaloids, steroids, triterpenoids, glycosides, saponins, carotenoids, polyphenols, vitamins, and other health promoting phytochemicals, these may be helpful used for antioxidant, diabetes, cancer, neurodegenerative diseases, asthma, leprosy, hepatoprotective, analgesic, postcoital anti-fertility, nematocidal, jaundice, bleeding piles, anti-allergic, anti-malarial, anti-feedant, anti-bacterial, anti-fungal and anti-viral activities (Thiruvengadam et al., 2013; Talukdar and Hossain, 2014). Seed dormancy and pre-flowering sex determination are major controlling factor of commercial cultivation in spine gourd (Bharathi et al., 2014). The production of bioactive compounds through *in vitro* culture has been important and promising aspect of modern biotechnology. To meet the increasing demand for plants utilized in the nutraceutical, pharmaceutical, and cosmetical industry, much of the recent research has focused on the development of *in vitro* tissue or hairy root culture techniques as a useful alternative to improve the yield of bioactive metabolites in spine gourd.

Hairy root cultures induced through *Agrobacterium rhizogenes*-mediated transformations have developed as potential biotechnological system because these cultures fast growth rates, ease of maintenance, genetic stability, large scale biomass production without the need for external application of phytohormones and ability to synthesize a vast array of valuable secondary metabolites (Srivastava and Srivastava, 2007; Chandra and Chandra, 2011). Till date, hairy root cultures have been studied for the production of secondary metabolites for used as pharmaceuticals, nutraceuticals, food additives and cosmetics (Srivastava and Srivastava, 2007; Chandra and Chandra, 2011). Previously, it was reported that

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hairy roots enhanced the amount of saponin in *Bacopa monnieri* (Majumdar et al., 2011), anthroquinones in *Polygonum multiflorum* (Thiruvengadam et al., 2014a), total phenolic content in *Solanum lycopersicum* (Singh et al., 2014), polyphenols in *Momordica charantia* (Thiruvengadam et al., 2014b), and glucosinolates in *Arabidopsis thaliana* (Kastell et al., 2015). However, there are no reports on the induction of hairy roots, and production of phenolic compounds and also their biological activities from hairy root cultures of spine gourd. The main goal of the present investigation was to develop a biotechnological system for producing bioactive phenolic compounds of *M. dioica* as an alternative to harvesting the wild plant. First time, we have successfully established an efficient protocol for hairy root cultures of *M. dioica* for the production of individual phenolic compound profiles (flavonols, hydroxycinnamic and hydroxybenzoic acids) using by UHPLC analysis. In addition, we optimized the different liquid nutrient media and various concentrations of sucrose on hairy root growth, in terms of fresh and dry biomass accumulation in *M. dioica* . Finally, we evaluated the total phenolic, flavonoid contents, antioxidant and antimicrobial (antibacterial, antifungal and antiviral) activities from transgenic hairy roots and non-transgenic roots of spine gourd.

2. Material and methods

2.1. Collection plant materials

Tubers of *M. dioica* Roxb. ex. Willd (one-year-old) were collected from the Semmalai hills, Western Ghats (altitude 300–600 m) and developed in the botanical garden at Kulathur, Tamil Nadu, India (Thiruvengadam et al., 2013). The explants of leaves, petiole, and internodals were washed with a detergent solution for 5 min and rinsed with running tap water for five times. After being soaked in 70% (v/v) ethanol for 1 min, then explants were rinsed in distilled water, further, sterilized with 1.0% (v/v) sodium hypochlorite solution for 10 min, and rinsed repeatedly with sterilized distilled water. The explants were cut into small pieces of leaves (~10-mm^2), petiole, and internodal (~0.5-cm long) in size.

2.2. Hairy root induction by Agrobacterium rhizogenes and proliferation of hairy root cultures

Leaves, petiole, and internodal explants were infected with bacterial culture (OD600 nm = 1.0) of *A. rhizogenes* strain KCTC 2703 (Sivakumar et al., 2005) for 30 min. Thereafter, they were blotted dry on sterilized tissue paper, co-cultured on MS solidified medium and incubated under dark conditions at 25 ± 2 °C for three days. The co-cultured explants were then washed thoroughly with sterilized distilled water and transferred to a MS solid medium supplemented with 300 mg/L cefotaxime (Duchefa Biochemie, Netherlands). Root cultures were incubated under 16 h light/8 h dark provided by 40 W white fluorescent tubes (40 μmol m^{-2} s^{-1}) at 25 ± 2 °C for 25 days. The aseptically excised roots (2–3 cm long) were subcultured individually into MS liquid medium, supplemented with 3% sucrose and 300 mg/L cefotaxime. The cultures were kept on an orbital shaker (100 rpm) and incubated under the same conditions and subcultures of roots were done by every 12 days. The cefotaxime level was gradually reduced to 200 and 100 mg/L during the second and third subculture, respectively. After third subculture, roots were transferred on MS liquid medium without cefotaxime. Non-transformed roots were excised from *in vivo* grown plants cultured on MS liquid medium.

2.3. Optimization of growth index in the hairy root cultures

Single hairy roots (300 mg fresh mass) were excised and cultured in MS liquid medium supplemented with 3% sucrose. Growth kinetics at different time intervals (7, 15, 22, and 30 days) was examined to optimize biomass accumulation. Full and half strength of MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), NN (Nitsch and Nitsch, 1969), and LS (Linsmaier and Skoog, 1965) media and different concentrations of sucrose (1, 2, 3 and 4%) were tested to find a combination that resulted in the highest root biomass. The cultures were kept under continuous agitation at 100 rpm in an orbital shaker and incubated at 25 ± 2 °C with a 16 h light/8 h dark (40 μmol m^{-2} s^{-1}) supplied by 40 W white fluorescent lamps. The biomass of hairy roots was assessed at 22 days of culture. The roots were separated from the media and their fresh mass (FM) was determined later they were washed with distilled water and the excess surface water blotted away. Dry mass (DM) was noted after the roots were dried at 60 °C until a consistent weight was observed. The growth ratio was determined as GR = growth ratio is the quotients of the dry mass of harvested biomass and the dry mass of the inoculum.

2.4. Molecular characterization of hairy roots

2.4.1. Polymerase chain reaction (PCR)

Genomic DNA extracted from transgenic root clones and non-transgenic roots of *M. dioica* were used the DNA isolation kit (Fermentas Life Sciences, USA). The amplification reaction was carried out in GeneAmp PCR system DNA thermal cycler (PerkinElmer, USA) using 22-mer oligonucleotides as primers. A primer pair of 5′-ATGGCTGAAGACGACCTGTGTT-3′ and 5′-TTAGCCGATTGCAAACTTGCAC-3′ was used to amplify a ~500-bp fragment of the *rolC* gene (Sivakumar et al., 2005) and 5′-CCAAGCTTGTCAGAAAACTTCAGGG- 3′ and 5′-CCGGATCCAATACCCAGCGCTTT-3′ was designed to amplify a ~815-bp fragment of the *aux1* gene (Medina-Bolivar et al., 2007). In addition, primers (5′-ATGCCCGATCGAGCTCAAGT-3′ and 5′-CCTGACCCAAACATCTCGGCT-3′), amplifying a fragment of ~338 bp were used for detecting the *virD2* gene (Medina-Bolivar et al., 2007). The reaction mixture consisted of 1 μL of 1 unit *Taq* polymerase, 2.5 μL of 100 nM dNTP, 1 μL of 20 pM primer, 1 μL of 20 ng template DNA and 2.5 μL of 10X reaction buffer plus sterile distilled H_2O for a final volume of 25 μL. PCR was performed under the following conditions for *rolC*, initial denaturation at 94 °C for 4 min, followed by 30 cycles of amplification (94 °C 1 min, 60 °C 1 min and 72 °C 2 min) and 5 min at 72 °C; for *aux1*, initial denaturation at 95 °C for 3 min, 30 cycles of amplification (95 °C 30s, 58 ° C 30 s and 72 °C 1 min) and 72 °C for 10 min; for *virD2*, initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C 30 s, 56 °C 30 s and 72 °C 45 s) and 10 min at 72 °C. PCR results were checked using agarose gel electrophoresis with *Hind* III-digested λDNAmaker, detected by ethidium bromide staining, and photographed using the gel documentation system (Bio-Rad, USA).

2.4.2. Gene sequencing

The amplified PCR product (~500 bp) was extracted using the MinElute Gel Extraction kit (Qiagen, Germany) following the manufacturer's instructions. The eluted product was commercially sequenced (Macrogen, Korea) from both the orientations of gene to confirm the presence of the *rolC* gene in transformed hairy roots.

2.4.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using an RNA isolation kit (Fermentas Life Sciences, USA) from transgenic root clones and non-transgenic roots. RT-PCR was carried out with a Revert-Aid^{TM} first strand complementary DNA (cDNA) synthesis kit (Fermentas Life Sciences, USA) following the manufacturer's instructions. The same

PCR primers for the *rolC* and *aux1* genes were used with similar conditions.

2.5. Determination of total phenolic contents (TPC)

Total phenolic content was determined by spectrophotometry according to the Folin-Ciocalteu assay previously reported by Thiruvengadam et al. (2014a,b). Lyophilized transgenic and non-transgenic root powders were extracted with methanol. A 100 μL (100 μg) of extracts were combined with 3.10 mL of distilled water, followed by addition of 0.2 mL Folin-Ciocalteu reagent. They were mixed well and added 0.6 mL of 20% sodium carbonate solution. Color developed after one hour at room temperature and the absorbance was measured at 760 nm using a UV–visible spectrophotometer (UV-2120 Optizen, Mecasys, Korea). The concentration of the TPC was calculated as mg of gallic acid equivalent by using an equation obtained from the gallic acid calibration curve.

2.6. Determination of total flavonoid contents (TFC)

Total flavonoid content of the extracts was determined by using the aluminum chloride spectrophotometric method described earlier by Thiruvengadam et al. (2014a,b). Lyophilized transgenic and non-transgenic root powders were extracted with methanol. Extracts (0.2 mL), 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL), and distilled water (4.6 mL) were mixed and incubated at room temperature for 30 min. The absorbance was measured at 415 nm using a UV–visible spectrophotometer. Quercetin was used to construct the calibration curve and calculated as mg of quercetin equivalent.

2.7. Profile of individual phenolic compounds

2.7.1. Extraction of individual phenolic compounds

Analysis of phenolic compounds in the dried root samples was performed using a modification of a prior method (Kim et al., 2012; Thiruvengadam et al., 2014a,b). Briefly, the lyophilized transgenic and non-transgenic root powders (1 g) was added to 10 mL of acetonitrile and 2 mL of 0.1 N hydrochloric acid, and the resulting mixture was extracted using a shaker at 200 rpm for 2 h at room temperature. The crude root extract was filtered through No. 42 Whatman filter paper, the filtrate was concentrated *in vacuo* at <35 °C using a vacuum evaporator. The residue was reconstituted with 80% aqueous MeOH (5 mL), and then filtered through a 0.2 μm syringe filter (17 mm, Titan, USA).

2.7.2. Analysis of individual phenolic compounds by ultra-high performance liquid chromatography (UHPLC)

The filtrate was used for analysis using an ultra-HPLC (Accela UHPLC system, Thermo Fisher, USA) with a reverse phase column (Thermo, C_{18}, 2.1 × 100 mm, 2.6 μm). Previously reported analytical conditions (Kim et al., 2012; Thiruvengadam et al., 2014a,b) were slightly modified for our UHPLC analysis. The mobile phases were 0.1% glacial acetic acid in HPLC grade water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The flow rate of the mobile phase was 0.5 mL/min and the injection volume was 4 μL. The absorbance of the phenolic compounds of the root samples was measured at 280 nm. The gradient procedure described earlier (Kim et al., 2012; Thiruvengadam et al., 2014a,b). Solutions (25, 50, 100, and 150 μg/mL) of pure gallic acid, protocatechuic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic acid, *ρ*-coumaric acid, *o*-coumaric acid, salicylic acid, gentisic acid, chlorogenic acid, vanillic acid, β-resorcylic acid, ferulic acid, veratric acid, vanillin, rutin, *t*-cinnamic acid, homogentisic acid, myricetin, catechin, quercetin, naringenin, kaempferol, biochanin A and hesperidin were used as standards (St. Louis, USA) were dissolved in methanol. Phenolic compounds of hairy roots and non-transformed root extracts were identified based on the retention time and UV spectra of authentic standards whereas the quantitative data were calculated based on the calibration curves of the individual standards (Kim et al., 2012). Results were expressed as μg/g of each compound from the total phenolic compounds.

2.8. Screening of biological activity

2.8.1. Antioxidant activity

2.8.1.1. Assay of DPPH- radical-scavenging activity. The DPPH free-radical-scavenging activity was measured using a prior method of Thiruvengadam et al. (2014a, b) with some slight modifications. A 0.4 mM solution of 1,1-diphenyl-2-picrylhrdrazyl (DPPH) was prepared in MeOH, and 3.0 mL of this solution was mixed with 100 μL (100 μg) aliquot of transgenic and non-transgenic roots samples. The mixture was placed in a dark room for 10 min and the absorbance was then measured using a spectrophotometer at 517 nm. The DPPH free-radical-scavenging activity was calculated as an inhibition percentage based on the following equation: Inhibition (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample aliquot.

2.8.1.2. Assay of reductive potential. The reducing power of the extracts was determined according to the method described by Thiruvengadam et al. (2014a, b). A 100 μL (100 μg) of transgenic and non-transgenic root extracts in 1 mL of distilled water were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide $[K_3Fe(CN)_6]$, and the mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloro acetic acid (TCA) was added to the mixture and centrifuged at 650 g for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. The increased absorbance of the reaction mixture indicated increased reducing power.

2.8.1.3. Evaluation of antioxidant capacity by phosphomolybdenum method. The total antioxidant capacity was evaluated using phosphomolybdenum method described previously by Thiruvengadam et al. (2014a,b). An aliquot of 0.1 mL of transgenic and non-transgenic root extracts (1 mg/mL) was combined with 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used in the sample, and it was incubated under the same conditions as the rest of the sample. For samples of unknown composition, water soluble antioxidant capacity was expressed as equivalents of α-tocopherol (μg/g of extract).

2.8.1.4. Chelating effects on ferrous ions. The chelating effects ferrous ion was determined according to the method of Praveen et al. (2012) with some modifications. Briefly, 1 mL of transgenic and non-transgenic root extracts (250 μg/mL) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated by using the following equation: Metal chelating effect (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control (the control contains $FeCl_2$ and ferrozine complex formation

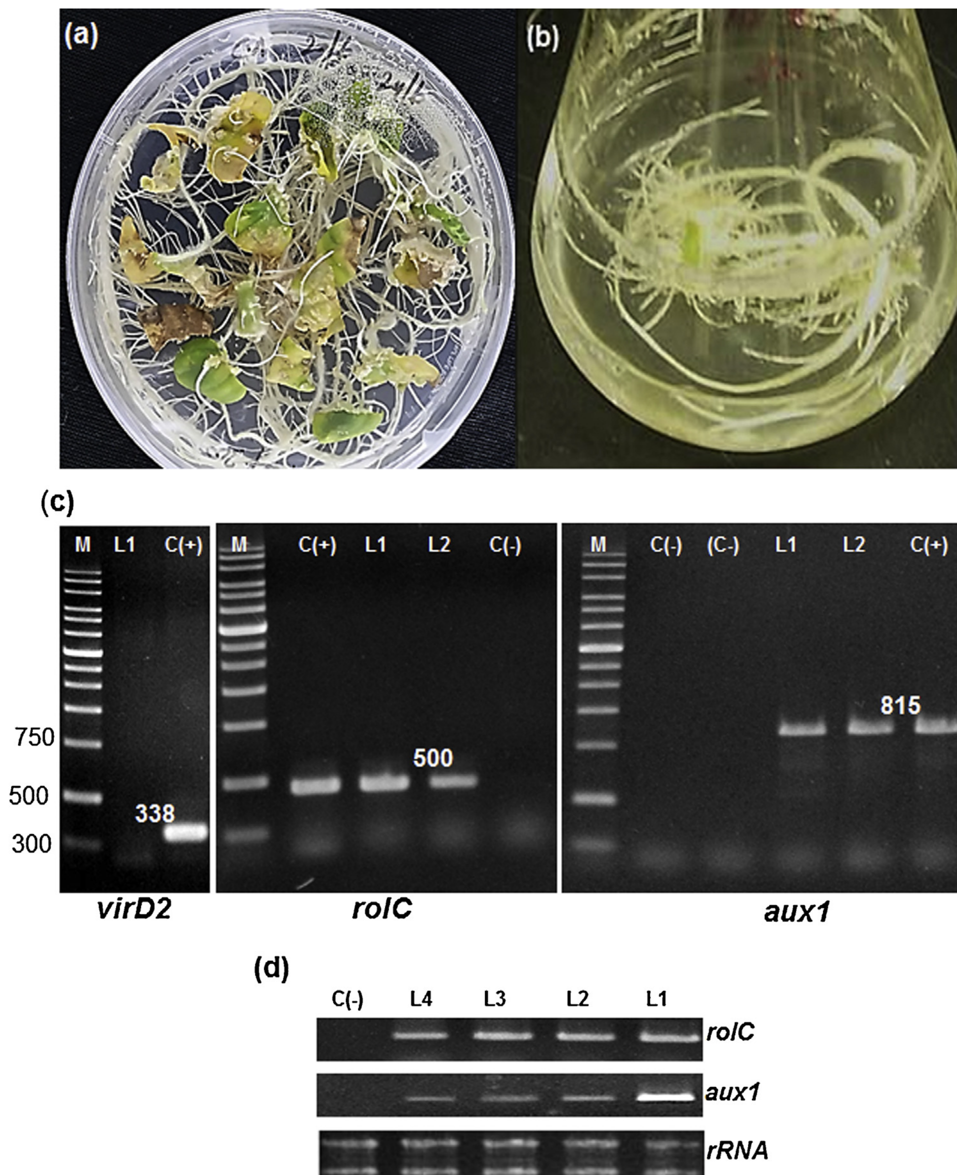

Fig. 1. *Agrobacterium rhizogenes* -mediated hairy root culture in *Momordica dioica* (a) Hairy roots induction from leaves, petiole, and internodal explants. (b) Hairy root cultures in hormone-free MS liquid medium. (c) PCR analysis of the *virD2*, *rolC* and *aux1* genes in the hairy root lines of *M. dioica* [Lane M, marker; L1–L2, root clones induced by *A. rhizogenes* ; C (+), plasmid DNA from KCTC 2703 (positive control); C (−), roots from a non-transformed control plant (negative control)]. (d) RT-PCR analysis of the *rolC* and *aux1* gene expressions using specific primers in the hairy root lines of *M. dioica* [L1–L4, root clones induced by *A. rhizogenes* ; C (−), roots from a non-transformed control plant (negative control)].

molecules), and A_1 is the absorbance of the sample aliquot. EDTA was used as a standard.

2.8.2. Antibacterial activity

The pathogenic microorganisms *Staphylococcus aureus* (KACC 13257), *Pseudomonas aeruginosa* (KACC 10259), and *Escherichia coli* (KACC 13821) were used to test for antibacterial activity. The pure bacterial strains were obtained from the Korean Agricultural Culture Collection (KACC), Korea. The methanolic extracts of hairy roots and non-transformed roots (1 mg/mL) were tested for antibacterial activity. Antibacterial tests were carried out by the NCCLS disc diffusion method was previously described (Thiruvengadam et al., 2014b). For the positive control, paper discs were impregnated with 50 μL of chloramphenicol and plates were incubated at 37 °C for 18–24 h. Antibacterial activity was assessed by measuring the diameter of growth inhibition zone (IZ) in millimeters.

2.8.3. Antifungal activity

The pathogenic fungi *Fusarium oxysporum* and *Aspergillus niger* were used to test for antifungal activity. The fungal strains were received from Prof. S.C. Chun, Department of Molecular Biotechnology, Konkuk University, Korea. The methanolic extracts of hairy roots and non-transformed roots (1 mg/mL) were tested for antifungal activity. Antifungal tests were carried out by the NCCLS disc diffusion method was previously described (Thiruvengadam et al., 2014b). For the positive control, paper discs were impregnated with 50 μL thymol and plates were incubated at 37 °C for 24–48 h. Antifungal activity was assessed by measuring the diameter of growth inhibition zone (IZ) in millimeters.

2.8.4. Antiviral activity

2.8.4.1. Virus and cells. DF-1 cells were grown in 25 cm^2 flasks with Dulbecco's modified Eagle medium (DMEM) (HyClone, USA) supplemented with 10% fetal calf serum (FCS) and 1% antibiotics

(penicillin, streptomycin). IBD virus (IBDV) was adapted in chicken embryo fibroblast (CEF) cultures. The DF1 cells and maintenance, IBDV propagation and harvesting procedure were described previously by Rekha et al. (2014).

2.8.4.2. Cytotoxicity assay. Cytotoxicity of sample on DF-1 cells was measured by microculture tetrazolium (MTT) assay. To investigate cellular toxicity of the hairy root and non-transgenic root extracts, DF-1 cells were grown in 96-well plate for 24 h to obtain confluence. After 24 h, the monolayer was washed with DMEM and different concentrations of hairy root and non-transgenic root extracts prepared in DMEM were added and the plate was sealed and kept at 37 °C in an atmosphere of 5% CO_2 for 24 h. Afterwards, hairy root and non-transgenic root extracts solution was removed from the flask. 50-μL of MTT solution (4 mg/mL in DMEM) was added to each well in a 96-well plate, and incubated at 37 °C for 4 h. MTT solution was removed and 200 μL of DMSO was added to dissolve MTT- formazan crystals. Then 25 μL of glycine buffer was added and absorbance was recorded at 570 nm immediately with the help of a microplate reader (VersaMax, CA, USA). The % of cell inhibition was determined using the following formula:

Cell Inhibition (%) = $A_{(sample)}/A_{(control)} \times 100$. The Inhibitory concentration required for 50% cytotoxicity (IC_{50}) value was analyzed using sigma plot software.

2.8.4.3. Quantitative assay of virus yields using plaque assay. To test inhibitory effect of hairy roots and non-transformed root extracts on IBDV yields in DF-1 cells, the cells were infected with IBDV at multiplicity of infection (MOI) of 0.1 PFU/cell and treated with hairy roots and non-transformed root extracts (0, 0.1, 0.5, 1 mg/mL) at the same time. At 24 and 48 h after inoculation, cultured supernatant from untreated IBDV infected cells was collected for measuring virus yields by plaque assay. A 10-fold serial dilution of cultured medium was added into the well DF-1 cell monolayer at 37 °C for 1 h and overlaid with DMEM medium. Viral plaques were stained with dye after three-days of incubation.

2.9. Experimental design and data analysis

All experiments were performed in triplicate ($n = 3$) and each experiment was repeated three times. The data were expressed as mean ± standard deviation (SD). One-way ANOVA analysis followed by Duncan's test was used to determine significant differences ($P \leq 0.05$). All statistical analyses were performed using the SPSS Ver. 20 statistical software package (SPSS, USA).

3. Results and discussion

3.1. Establishment of hairy root lines

Leaf explants were highly responded for induction of hairy roots (85%) compared to internodal (62.5%) and petiole (40.2%) explants to inoculation by *A. rhizogenes* . No similar root induction was observed in the control explants. Primarily, root bulges developed within 10-12 days of inoculation at the wounded edges of leaf explants, with hairy roots developing within 21 days of inoculation (Fig. 1a). In our investigation, leaf explants produced higher number of hairy roots compared to internodal and petiole explants ($P \leq 0.05$). The leaf explants was induced higher frequency of hairy root induction in *Solenostemon scutellarioides* (Saleh and Thuc, 2009), *Gymnema sylvestre* (Nagella et al., 2013), *M. charantia* (Thiruvengadam et al., 2014b) and *Stevia rebaudiana* (Fu et al., 2015). After 3 weeks of culture, the hairy roots that measured ~1.0–2.0 cm in length were excised and transferred to liquid medium for suspension culture (Fig. 1b). The established hairy roots shown typical morphological characteristics with rapid growth on phytohormone-free medium, lack of geotropism, and extensive lateral branching (Fig. 1a and b).

3.2. Molecular confirmation of hairy roots

In order to assess the molecular confirmation of the hairy roots, we used a PCR analysis that targeted the *A. rhizogenes rolC*, *aux1* and *virD2* genes. The *rolC* and *aux1* genes, located on independent T-DNAs (TL-DNA and TR-DNA, respectively) of the Ri plasmid of *A. rhizogenes* strain, are diagnostic for T-DNA integration into the host genome. The *virD2* gene, located outside the T-DNA, is diagnostic for the presence of any remaining *Agrobacteria* in the root tissue. The integration of Ri T-DNA into the genome of plant cells caused the formation of hairy roots, in which *rol* and *aux* genes were harbored (Gai et al., 2015). The *rol* and *aux* genes are essential for the induction of hairy roots and it act as a potential activator of secondary metabolites in several plants (Bulgakov, 2008; Park et al., 2011; Nagella et al., 2013; Gai et al., 2015). PCR amplification of the *rolC* and *aux1* genes in *A. rhizogenes* induced roots was confirmed by the stable and typical hairy root phenotype of the cultured roots (Fig. 1c). Four randomly selected fast growing transgenic hairy root lines, non-transgenic roots (negative control) and *Agrobacterium* cultures (positive control) were used. Integration of the *A. rhizogenes* Ri plasmid T-DNA into the *M. dioica* genome was confirmed by PCR, RT-PCR and sequencing for the *rolC* and *aux1* genes. The primers for *rolC*, *aux1* and *virD2* were used according to the method earlier described (Sivakumar et al., 2005; Medina-Bolivar et al., 2007). PCR and RT-PCR analysis using *rolC* and *aux1* specific primer with fragments of ~500 bp and 815 bp confirmed the transgenic roots. The fragment for *rolC* and *aux1* were observed in the amplified DNA from all the four hairy root lines and positive control (Fig. 1c and d). No such amplification product was found in the DNA isolated from non-transformed control roots (Fig. 1c and d). Furthermore, the *virD2* gene was used to verify the complete absence of *A. rhizogenes* in the hairy roots lines of *M. dioica* (Fig 1c). This result indicates that pRi T-DNA fragments of *A. rhizogenes* were successfully integrated into the genome of *M. dioica* without bacterial residues. The sequence of the eluted PCR product was obtained and submitted to NCBI GenBank. Sequence analysis in BLASTn indicated alignments with the *rolC* gene, thereby confirming its integration in the transgenic hairy roots. The obtained full length coding sequence of *rolC* gene (543 nucleotides) encodes 181 amino acids. The use of PCR combined with DNA sequencing as a instead of Southern blotting for the characterization of transgenic plants has the advantage that the newly inserted genes can be detected at an earlier stage with less DNA and less plant material (Vergauwe et al., 1996; Gangopadhyay et al., 2010; Thiruvengadam et al., 2014b).

3.3. Growth index of hairy root culture

Previously, it was demonstrated that hairy root induction and variations of the growth level during different growth stages in *Nasturtium officinale* (Park et al., 2011), *G. sylvestre* (Nagella et al., 2013) and *M. charantia* (Thiruvengadam et al., 2014b). The biomass increased slowly and reached peak of 99.05 g/l FM and 10.35 g/l DM at the end of 22 days of culture. A 9.7-fold increment was evident when compared with initial inoculum fresh biomass (Fig. 2). Nagella et al. (2013) stated that the FM (0.5 g) of *G. sylvestre* hairy roots increased ~9.4-fold after 25 days of culture in MS liquid medium without phyto-hormones. The exponential growth stage during the 22 day was followed by the stationary phase during the 15–25 days (Fig. 2). Profuse adventitious root growth was achieved after 21 days of growth reached its maximum up to 35 days of culture in *Picrorhiza kurroa* (Verma et al., 2007). These results suggest

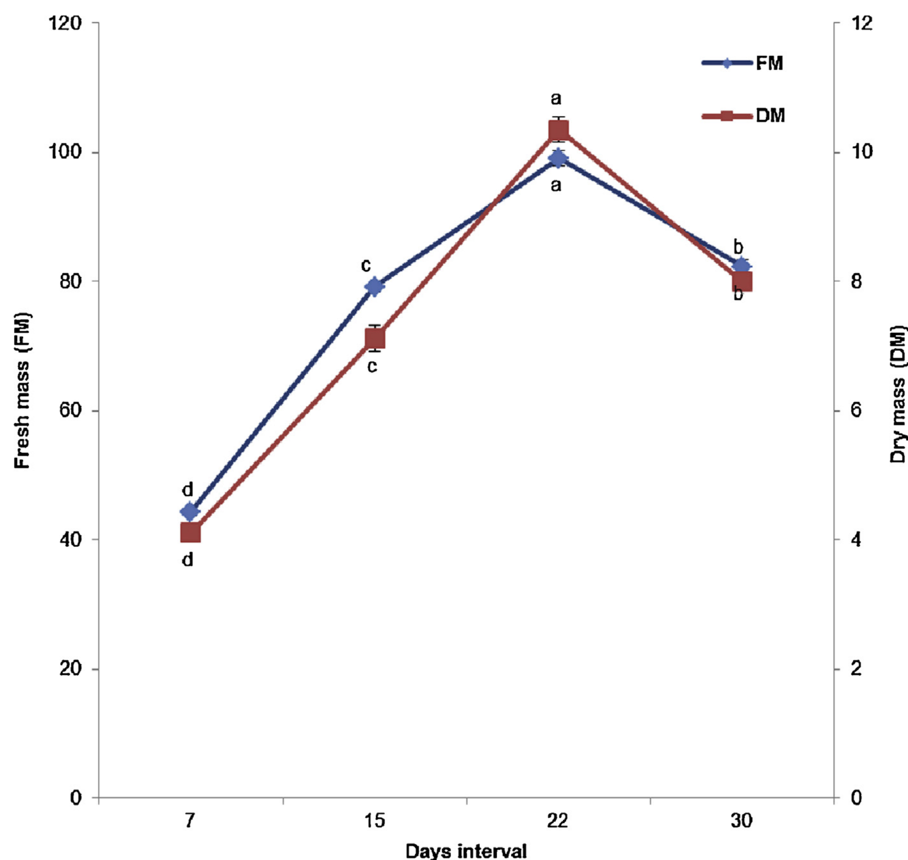

Fig. 2. Time schedule of hairy root growth kinetics in liquid cultures of *Momordica dioica*. Three hundred milligrams of roots were cultured in MS liquid medium supplemented with 3% sucrose. Means ± standard deviation of three replicates followed by the same letters are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

Table 1
Effects of various growth media and different sucrose concentrations of biomass accumulation in *Momordica dioica* hairy root cultures.

Media	Fresh mass (g/L)	Dry mass (g/L)	Growth ratio
Full MS	99.05[a]	10.35[a]	9.70[a]
Half MS	77.12[b]	7.85[b]	7.75[b]
B5	77.45[b]	7.90[b]	7.78[b]
NN	66.00[c]	6.65[c]	6.20[c]
LS	58.50[d]	5.82[d]	5.21[d]
Sucrose concentration (%)	**Fresh mass (g/L)**	**Dry mass (g/L)**	**Growth ratio**
1	45.25[d]	4.50[c]	4.11[c]
2	75.51[b]	7.65[b]	7.15[b]
3	99.05[a]	10.35[a]	9.70[a]
4	70.15[c]	7.22[b]	6.95[b]

Means ± standard deviation of three replicates ($n = 3$) within column followed by the same letters are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

that hairy root cultures of *M. dioica* are promising for large-scale biomass production in liquid cultures.

3.4. Effects of sucrose concentration and different media on biomass accumulation

Sucrose is the most significant carbon source for plant tissue cultures and helps as the chief energy source and an important constituent in secondary metabolite biosynthesis (Nagella et al., 2013). The amount of sucrose usually affects the accumulation of secondary metabolites in cultures. We examined the effects of sucrose concentration (1–4%) in MS medium on the growth of hairy roots (Table 1). In the present study, 3% sucrose produced 99.05 g/l FM and 10.35 g/l DM ($P \leq 0.05$). Hairy root growth dramatically decreased in media containing concentrations of sucrose either above or below the 3% level. Similarly, hairy root cultures of *M. charantia* have also been cultured in 3% sucrose supplemented MS medium (Thiruvengadam et al., 2014b). Suitable type of culture medium was helpful for cell/organ growth and also secondary metabolite production (Gai et al., 2015). In the present study, different media, full and half strength MS, B5, NN, and LS were employed in hairy root culture and the results shown that MS medium was superior for biomass accumulation (Table 1). The highest accumulation of biomass was found in the full strength MS medium, followed by B5 medium ($P \leq 0.05$). It has been stated that MS medium was suitable for biomass accumulation and metabolite production in hairy roots of *G. sylvestre* (Nagella et al., 2013) and *M. charantia* (Thiruvengadam et al., 2014b).

3.5. Comparison of individual phenolic compounds in transgenic hairy roots and non-transgenic roots

Phenolic compounds had shown a widespread of physiological properties, for example anti-inflammatory, anti-oxidant, anti-cancer and anti-microbial effects (Oueslati et al., 2012). The qualitative and quantitative analysis of phenolic compounds from *M. dioica* hairy roots and non-transformed root extracts were investigated using Ultra-HPLC (Table 2). The phenolic compounds in the *M. dioica* extracts were identified by comparisons of the retention time and UV spectra of authentic standards and the quantitative data were calculated from calibration curves. Both transgenic and non-transgenic roots contained flavonols, hydroxycinnamic hydroxybenzoic acids, and other phenolic compounds (Table 2). Hairy roots contained higher amounts of flavonols (2529.53 μg/g),

Table 2
Major phenolic compounds identified in *Momordica dioica* transgenic hairy root and non-transgenic root extracts by ultra-high-performance liquid chromatography (UHPLC) analysis.

No	Phenolic compounds	Concentration (μg/g)	
		Non-transgenic roots	Transgenic hairy roots
	Flavonols		
1	Myricetin	$255.25 \pm 2.00^{f,y}$	$219.45 \pm 4.10^{g,z}$
2	Quercetin	$499.10 \pm 2.00^{c,z}$	$525.12 \pm 4.00^{cd,y}$
3	Kaempferol	$338.25 \pm 3.20^{ef,z}$	$364.25 \pm 2.50^{fg,y}$
4	Catechin	$652.10 \pm 3.45^{a,z}$	$735.12 \pm 3.15^{a,y}$
5	Rutin	$510.10 \pm 2.50^{bc,z}$	$555.10 \pm 2.10^{b,y}$
6	Naringenin	$115.12 \pm 2.24^{i,y}$	$110.32 \pm 2.22^{i,z}$
7	Biochanin A	$24.50 \pm 0.70^{l,y}$	$20.17 \pm 0.51^{l,z}$
	Total	2394.40 ± 2.00^{z}	2529.53 ± 2.50^{y}
	Hydroxycinnamic acid		
8	Caffeic acid	$515.25 \pm 1.30^{b,y}$	$501.50 \pm 1.00^{d,z}$
9	*p*-Coumaric acid	$105.10 \pm 1.25^{ij,z}$	$115.02 \pm 1.55^{i,y}$
10	Ferulic acid	$160.25 \pm 2.75^{g,z}$	$199.05 \pm 2.10^{gh,y}$
11	*o*-Coumaric acid	$21.0 \pm 0.50^{l,y}$	$18.00 \pm 0.55^{l,z}$
12	Chlorogenic acid	$437.10 \pm 3.00^{d,z}$	$495.00 \pm 4.21^{de,y}$
13	*t*-Cinnamic acid	$9.55 \pm 0.21^{m,y}$	$9.12 \pm 0.22^{m,z}$
	Total	1248.25 ± 1.50^{z}	1337.69 ± 2.00^{y}
	Hydroxybenzoic acid		
14	*p*-Hydroxybenzoic acid	$135.25 \pm 1.25^{h,z}$	$141.50 \pm 1.50^{h,y}$
15	Gallic acid	$375.15 \pm 1.55^{e,z}$	$394.25 \pm 2.00^{f,y}$
16	Protocatechuic acid	$39.21 \pm 1.00^{k,y}$	$27.00 \pm 2.20^{kl,z}$
17	β-Resorcylic acid	$25.15 \pm 0.40^{l,y}$	$20.25 \pm 0.51^{l,z}$
18	Vanillic acid	$15.00 \pm 0.50^{m,y}$	$11.00 \pm 0.50^{m,z}$
19	Syringic acid	$55.10 \pm 1.25^{j,z}$	$87.46 \pm 1.20^{j,y}$
20	Gentisic acid	$465.85 \pm 2.15^{cd,z}$	$538.15 \pm 2.25^{c,y}$
21	Salicylic acid	$411.00 \pm 2.52^{de,z}$	$485.00 \pm 3.00^{e,y}$
	Total	1521.71 ± 2.00^{z}	1704.61 ± 2.10^{y}
	Other Phenolic compounds		
22	Vanillin	$25.21 \pm 0.51^{l,z}$	$27.25 \pm 0.75^{kl,y}$
23	Veratric acid	$128.25 \pm 0.40^{gh,z}$	$126.14 \pm 0.25^{hi,y}$
24	Hesperidin	$47.58 \pm 1.00^{j,y}$	$45.58 \pm 1.00^{jk,z}$
25	Homogentisic acid	$37.55 \pm 1.00^{k,y}$	$34.75 \pm 0.70^{k,z}$
	Total	238.59 ± 1.50^{y}	233.76 ± 1.00^{z}

Means ± standard deviation of three replicates ($n = 3$) within a column^{a-m}, or row^{y-z} followed by the same letters are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

hydroxycinnamic acid (1337.69 μg/g) and hydroxybenzoic acid (1704.61 μg/g) when compared to non-transgenic plants ($P \leq 0.05$), which produced lower amounts of flavonols (2394.40 μg/g), hydroxycinnamic acid (1248.25 μg/g) and hydroxybenzoic acid (1521.71 μg/g). Consistently, phenolic profiles increased in hairy roots than non-transgenic roots of *Hypericum perforatum* (Park et al., 2012). Catechin was the dominant flavonols in hairy roots (735.12 μg/g) and non-transformed roots (652.10 μg/g) followed by rutin, quercetin, kaempferol, myricetin, naringenin and biochanin A. In our study, catechin, rutin, quercetin and kaempferol were higher in transgenic hairy roots than that in non-transgenic roots of *M. dioica* ($P \leq 0.05$). Constantly, catechin content was higher in hairy roots of *M. charantia* (Thiruvengadam et al., 2014b). Transformed roots yielded higher levels of catechin and epicatechin than untransformed roots of *H. perforatum* (Tusevski and Simic, 2013). Kim et al. (2009) reported that the rutin and epicatechin contents in hairy roots were several-fold higher than those in non-transgenic roots of *Fagopyrum tataricum* . Kaempferol, myricetin, naringin, quercetin and rutin have antimicrobial activity against human pathogenic microorganisms with some mechanisms of action such as inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolisms (Cushnie and Lamb, 2005). *AtMYB12* transformed hairy roots of *Fagopyrum esculentum* produced rutin content was higher compared to non-transgenic plants (Park et al., 2012). Caffeic and chlorogenic acids were major hydroxycinnamic acid derivatives in hairy roots (501.50 and 495.00 μg/g) and non-transformed roots (515.25 and 437.10 μg/g) compared to ferulic, *p*-coumaric, *o*-coumaric and *t*-cinnamic acids. Caffeic acid induced inhibition of adventitious root formation in mung bean (Singh et al., 2009). Consistent with our results, caffeic acid content level decreased in hairy roots compared to non-transformed roots. Chlorogenic acid containing plant materials have been shown to have antiviral, antifungal, and strong antibacterial activities (Karunanidhi et al., 2013). Gentisic and salicylic acids were predominant hydroxybenzoic acid groups in hairy roots (538.15 and 485.00 μg/g) and non-transformed roots (465.85 and 411.00 μg/g) followed by gallic, *p*-hydroxybenzoic, syringic, protocatechuic, β-resorcylic and vanillic acids. The amount of *p*-coumaric, ferulic, chlorogenic, *p*-hydroxybenzoic, gallic, syringic, gentisic, salicylic, and veratric acids were higher in transgenic hairy roots than that in non-transgenic roots of *M. dioica* ($P \leq 0.05$). Similarly, the content of chlorogenic, protocatechuic, ferulic acids were higher in transgenic hairy roots than non-transgenic roots of tomato (Singh et al., 2014). The present study exhibited that hairy roots contained higher amount of individual phenolic compounds compared to non-transgenic plants. Similarly, phenolic acid derivatives such as chlorogenic acid and quercetin were presented higher amount in hairy roots when compared with wild type roots of *F. tataricum* (Kim et al., 2009).

3.6. Comparison of total phenolic and flavonoid contents (TPC and TFC) in transgenic hairy roots and non-transgenic roots

Biological activities related to antimicrobial and antioxidant activities may be correlated with total polyphenol and flavonoid contents (Daglia, 2011). The total phenolic and flavonoid contents were higher in hairy roots compared to non-transformed roots of *M. dioica* ($P \leq$ 0.05; Fig. 3a and b). The total phenolic content of hairy roots was 21.51 mg/g GAE and their total flavonoid content was 2.75 mg/g QE; total phenolic content of non-transgenic roots was 15.10 mg/g GAE and their total flavonoid content was 1.91 mg/g QE. Similarly, total phenolic and flavonoid contents were higher in hairy roots compared to non-transgenic roots of *Dracocephalum moldavica* (Weremczuk-Jezyna et al., 2013), *P. multiflorum* (Thiruvengadam et al., 2014a) and *M. charantia* (Thiruvengadam et al., 2014b).

3.7. Comparison of antioxidant activity in transgenic hairy roots and non-transgenic roots

Natural phytochemicals have been reported to possess a wide range of biological activities including antioxidant, antimicrobial and anti-inflammatory properties (Hendra et al., 2011). The antioxidant potential of hairy roots and non-transformed roots were determined using free radicals scavenging, reducing potential, phosphomolybdenum assays and metal chelating activity. DPPH is a stable radical, which could be easily used for the detection of antioxidant properties of different compounds in term of hydrogen donating ability. The highest antioxidant activity was exhibited in hairy roots (67.15%) compared to non-transformed roots (61.50%; $P \leq 0.05$) (Fig. 3c). The present investigation on the reducing capacity of extracts suggests that hairy roots were more potential when compared to non-transformed roots ($P \leq 0.05$) (Fig. 3d). The antioxidant capacity through phosphomolybdenum method of the hairy roots and non-transformed root extracts were measured spectrophotometrically, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte, and the subsequent formation of green phosphate/Mo (V) compounds. The antioxidant capacity of the hairy root extract was 2.21 mg/g and non-transformed root extract was 1.55 mg/g (Fig. 3e). Chelating agent may inhibit radical generation by stabilizing transition metals, consequently reducing free radical damage. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents complex (red color) formation is interrupted and as a result, the red color of the complex is

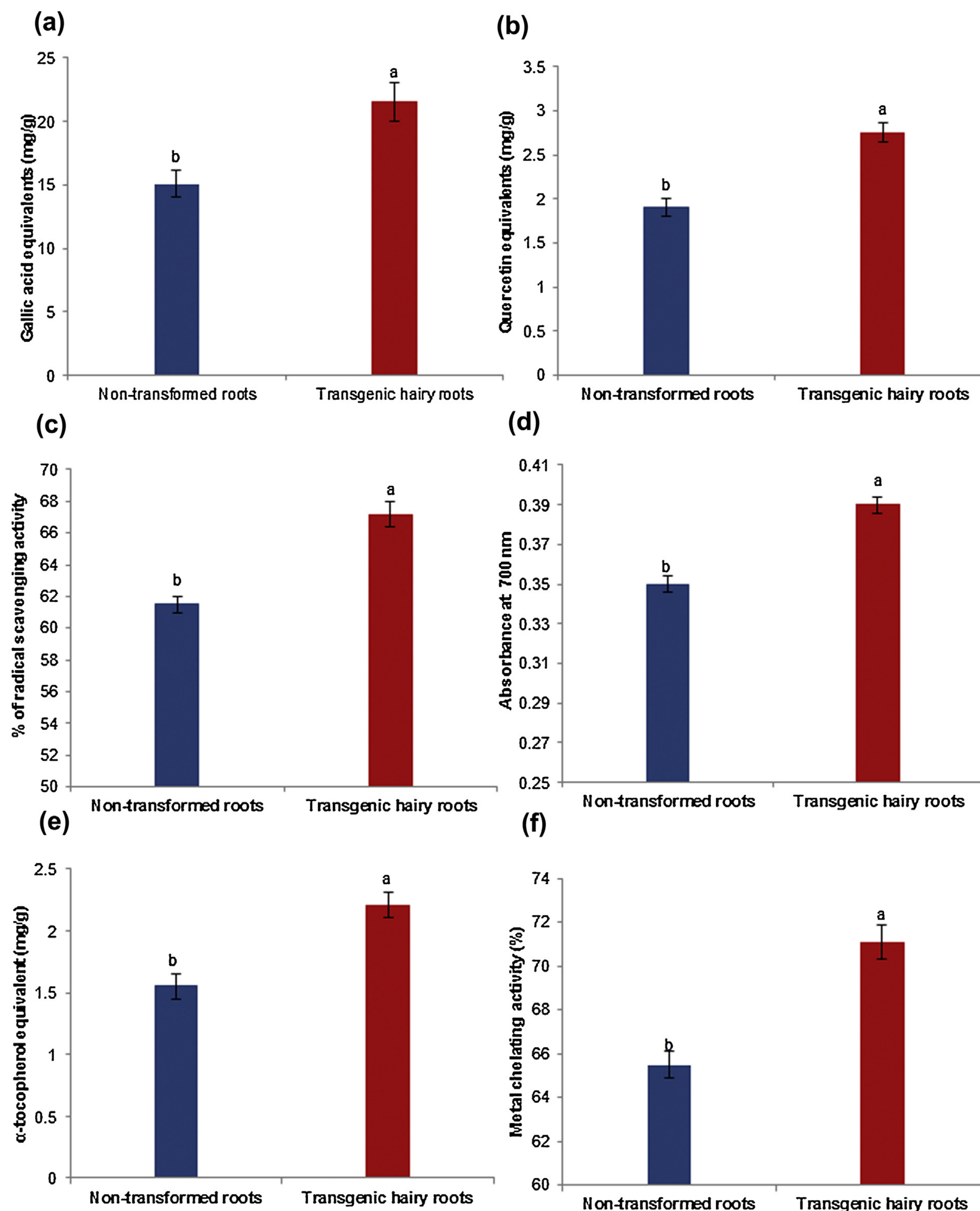

Fig. 3. Evaluation of total phenolic, flavonoid contents and antioxidant activities of transgenic hairy roots and non-transgenic roots in *Momordica dioica*. (a) Total phenolic contents. (b) Total flavonoid contents. (c) Free radical-scavenging activity by the DPPH method. (d) Reducing power. (e) The phosphomolybdenum method. (f) Metal ion chelating activity. Means ± standard deviation of three replicates followed by the same letters are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

decrease. Fig. 3f shows the percentage of metal scavenging capacity of transgenic roots (71.10%) was higher than non-transgenic roots (64.25%). The present investigation, hairy roots showed higher antioxidant activity compared to non-transformed roots ($P \leq 0.05$). Consistently, hairy roots were exhibited of higher antioxidant activity in *D. moldavica* (Weremczuk-Jezyna et al., 2013), *P. multiflorum* (Thiruvengadam et al., 2014a), *M. charantia* (Thiruvengadam et al., 2014b), and *Isatis tinctoria* (Gai et al., 2015).

Table 3
Antimicrobial activity of *Momordica dioica* hairy root extracts against bacteria and fungus.

Microorganisms	Zone of inhibition mm at 100 mg/disc		Zone of inhibition mm at 100 mg/disc	
	Positive control (antibiotics)		Non-transgenic roots	Transgenic hairy roots
	Chloramphenicol	Thymol		
S. aureus	$27.5 \pm 0.5^{c,x}$		$20.5 \pm 0.2^{a,z}$	$25.1 \pm 0.2^{a,y}$
P. aeruginosa	$28.8 \pm 0.7^{b,x}$		$19.2 \pm 0.5^{b,z}$	$23.0 \pm 0.5^{b,y}$
E. coli	$29.0 \pm 0.9^{a,x}$		$19.5 \pm 0.6^{b,z}$	$22.8 \pm 0.5^{c,y}$
A. niger		$26.1 \pm 0.6^{a,x}$	$18.5 \pm 0.4^{c,z}$	$22.0 \pm 1.0^{c,y}$
F. oxysporum		$24.2 \pm 0.5^{b,x}$	$16.5 \pm 0.5^{d,z}$	$20.0 \pm 0.5^{d,y}$

Means ± standard deviation of three replicates ($n = 3$) within a column$^{a–d}$, or row$^{x–z}$ followed by the same letters are not significantly different according Duncan's multiple range test at $P \leq 0.05$.

3.8. Comparison of antimicrobial (antibacterial, antifungal and antiviral) activities in transgenic hairy roots and non-transgenic roots

The hairy roots and non-transformed roots of *M. dioica* revealed varying antibacterial and antifungal activity, as exposed by the growth inhibition zones (Table 3). The results from the disc diffusion method indicated that both hairy roots and non-transformed root extracts had comparable antibacterial effects against Gram positive and Gram-negative bacteria. Hairy roots exhibited highest activity with both Gram-positive and Gram-negative bacteria compared to non-transformed roots of *M. dioica* ($P \leq 0.05$). Gram-positive (*S. aureus*) bacteria was exhibited greater inhibition compared to Gram-negative (*P. aeruginosa* and *E. coli*) bacteria. Table 3 depicts the results from the disc diffusion method against the fungal strains. It can be seen that extracts of *M. dioica* hairy roots and non-transformed roots exhibited good antifungal activity. These results were compared with the standard drugs of chloramphenicol and thymol for bacterial and fungal activity ($P \leq 0.05$). Previously, it was reported that the antimicrobial spectrums of the hairy root extracts was more effective against gram-positive bacteria than gram-negative bacteria (Jain et al., 2008; Wang et al., 2012). Previously many reports confirmed that hairy roots exhibited higher antibacterial and antifungal activity compared to non-transformed roots (Jain et al., 2008; Wang et al., 2012; Thiruvengadam et al., 2014b). Flavonoid derivatives have also been reported to possess antiviral activity against a wide range of viruses such as HSV, HIV, Coxsackie B virus, coronavirus, cytomegalovirus, poliomyelitis virus, rhinovirus, rotavirus, poliovirus, sindbis virus, and rabies virus (Özçelik et al., 2011). Cytotoxicity activity of hairy and non-transgenic root extracts were carried out against IBDV at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay. Results of different concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 mg/mL) of hairy root and non-transgenic root extracts. MTT assay of hairy and non-transgenic root extracts shows the significant effect on IBDV cell in concentration range between 10–1 mg/mL compared with control. The highest cytotoxicity of this extract against IBDV cell was found in 5 and 2.5 mg/mL concentration with 86.12 and 80.19% of cell growth inhibition in hairy roots and 78.50 and 71.09% in non-transgenic root extracts. It was found that the percentage of growth inhibition to be increasing with increasing concentration of test compounds. To detect inhibition of virus yield in DF-1 cells by extract of hairy roots and non-transformed roots, virus titers in cultured supernatants for IBDV infected DF-1 cells with or without treatment were measured at 24 and 48 h after infection, using plaque assay of hairy roots and non-transformed roots showed dose-dependent inhibition of IBDV replication in DF-1 cells, but no time-dependent inhibitory effect on IBDV production *in vitro* . Particularly, hairy roots and non-transgenic roots (1 mg/mL) showed virus yield reduced (42 and 58%) respectively after 24 h incubation. Previously, *M. charantia* was reported to possess several antiviral activities including hepatitis B virus, dengue virus, and human immunodeficiency virus (HIV) and influenza A subtypes including H1N1, H3N2, H5N1 (Pongthanapisith et al., 2013). Consistent with our reports hairy roots have potential antiviral activity compared with non-transgenic roots in *Phylanthus amarus* (Bhattacharyya and Bhattacharya, 2004) and *Daucus carota* (Luchakivskaya et al., 2012). Our results recommended transgenic hairy roots can be effectively used for the healing of bacterial, fungal and viral diseases.

4. Conclusion

Transgenic hairy roots grew rapidly than non-transgenic roots in standardized liquid culture conditions and produced greater amount of biomass and phenolic compounds. The phenolic groups like flavonols, hydroxycinnamic and hydroxybenzoic acids were higher in transgenic roots when compared to non-transgenic roots. The total phenolic, flavonoid contents and biological (antioxidant, antibacterial, antifungal and antiviral) activities were higher in hairy roots than non-transformed roots. The higher amount of polyphenolic compounds possibly contributes to greater biological activity of hairy roots in *M. dioica*. The genetic and biochemical stability of the hairy roots as well as its high productivity offers an effective platform for further studies on the biosynthetic pathways of phenolic compounds.

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