

Hairy Root Cultures of *Hypericum perforatum* L.; A Promising Method for The High Scale Production of Hypericin

Montazeri M (M.Sc.)¹, Azadbakht M (Ph.D.)^{2*}, Najafi Zarrini H (Ph.D.)¹, NematZadeh GhA (Ph.D.)¹, Pakdin Parizi A (Ph.D.)¹, Davoodi A (Pharm.D.)²

1- Department of Genetic & Agricultural Biotechnology, Agricultural Sciences & Natural Resources University, Sari, Iran

2- Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

*Corresponding author: Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
Tel: +98-113-3543728, Fax: +98-113-3543084
E-mail: Azadbakht110@gmail.com

Received: 3 May 2017

Accepted: 20 May 2018

Abstract

Background: Biotechnologic methods are common for secondary metabolites production from the plants and other sources in pharmaceutical sciences. Hairy root cell lines as the biotechnologic method have been used for *in vitro* production of major plant metabolites.

Objective: In this study, hairy roots of *Hypericum Perforatum* have been prepared using the seeds and bio transformed by bacteria. Finally, the hypericin have been produced by the hairy roots.

Methods: First, the seeds have been incubated in the plant media to hairy roots produced. Then, the hairy roots have been dipped in *Rhizobium rizogenes* suspension for biotransformation of bacterial genes. Morphological and phytochemical features of hairy roots have been determined in order to select the *H. perforatum* genotypes with higher hypericin contents. The fresh and dry weight of ten lines clones were measured after 30 days. In addition to, methanolic extracts of final hairy roots have been prepared and hypericin has been isolated and assayed by High Performance Liquid Chromatography.

Results: According to the observations, one of ten hairy root lines shows 75-fold higher hypericin content (339.27 ppm) compared to non-transformed *H. perforatum* (4.58 ppm) in the same of body weight of roots. The clone with the most content of hypericin had significant development of biomass of hairy roots and increase the hypericin production. Transformed clones were varied in morphology, growth, and metabolite productivity.

Conclusion: The mentioned methods induce the production of hairy root secondary metabolites in high scale to improve the quality and the quantity of pharmaceutical compounds.

Keywords: *Hypericum perforatum*, *Rhizobium rhizogenes*, Hairy root, HPLC, Hypericin

Introduction

Many plants have been recommended for the medicinal and non-medicinal usages. In addition to, these natural resources due to different phytochemical compounds have been important [1, 2].

Hypericum perforatum L. (Hypericaceae) is one of the best-studied medicinal plants in the world [3]. *H. perforatum* is native to Europe, West Asia and North Africa. In addition to, this plant distributed in temperate regions of Iran such as Mazandaran, Golestan and Gilan provinces [4, 5]. Aerial part of *H. perforatum* included buds, young stems, sepals, leaves and especially flowers have several secretory structures and unique specialized glands. These components contain secondary metabolites especially anthraquinones and anthraquinone glycosides such as hypericin and pseudohypericin have been biosynthesized by

polyketide pathway in the plant (Figure 1) [6]. The compounds have been used in numerous analytical and pharmacological purposes in medical sciences [7, 8]. In addition to, *H. perforatum* produce the secondary metabolites with limited capacity and high amounts of the plant should be gathered to achieve the significant percentages of hypericin and pseudohypericin. Thus, laboratory methods for the secondary metabolites production in high scale are needed.

Hence, researchers almost have been discovered the methods for achieve to commercial production of these valuable compounds due to their high demand. Hairy root bioreactors, cell suspension technology and immobilized plant cell bioreactors are the modern and applicable methods for high scale production of secondary metabolites [7-11].

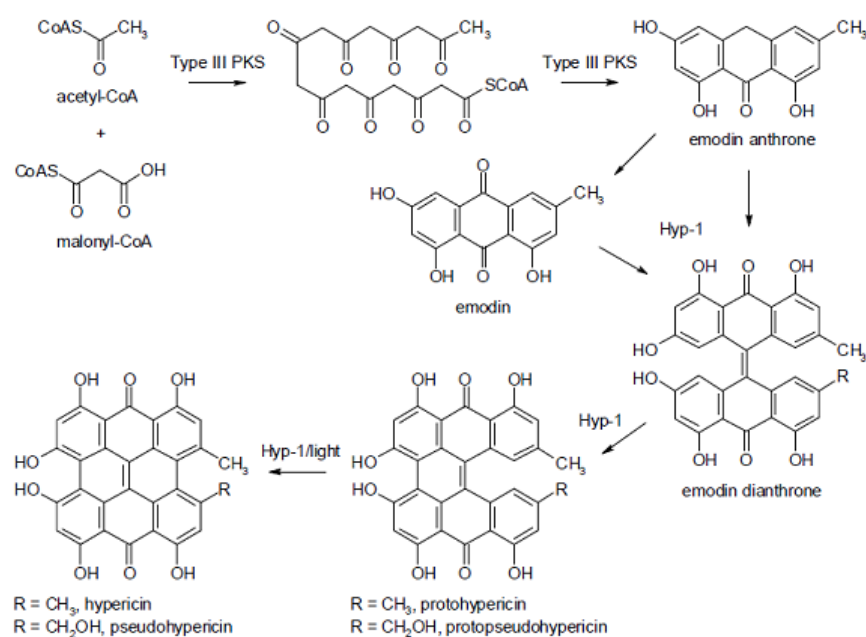


Figure 1- Biosynthesis pathway of hypericin in the plants

Hairy roots have a wide range capability to produce the secondary metabolite similar to the parent plants as well as novel compounds, are a potential systems for secondary metabolites production in large scale [12]. The roots have organized nature, fast growth rate, sustainability in metabolite production, lower sensitivity to shear stress than cell suspension cultures, and also their clones are genetically and biosynthetically stable for long periods. In addition to, secondary metabolite production induces using gene transformation by some suitable bacterial strains especially *R. rhizogenes* [13-17].

R. rhizogenes is a gram-negative soil bacterium which transfers its gene information into genetic content of host plants. Actually, host hairy roots have been modified in type and scale of their secondary metabolites production. The transformed hairy roots capable to produce the secondary metabolites in large amounts [18].

The aim of this study was evaluation of production in large quantities of hypericin through hairy root lines of *H. perforatum*. In the study, *R. rhizogenes* bacteria have been employed for gene transformation into hairy root lines. Actually, these processes induce the production of hypericin in high scales.

Materials and methods

This study was conducted to access hypericin in high amounts using hairy roots technology. The seeds of *H. perforatum* were prepared from "Isfahan Pakan Bazr" Institute (Voucher's number: E214411). The seeds have been disinfected by dipping in 70% ethanol solution for 15 seconds and washing with pure distilled water. In addition to, the seeds were

exposed in stoke of aqueous sodium hypochlorite solution 1.5 % with a drop of Tween 80 for 10 minutes. The seeds have been washed with distilled water 4 times and were transferd to Murashige and Skoog (MS) medium supplemented by soluble vitamins (according to B₅ and B₁₂), 0.8% agar and 2% sucrose at pH 5.8 before autoclaving (20 minutes at 120°C) to germination [19, 20]. Then, the sterilized seeds and the germinated plants were placed in growth chamber (25 ±2°C+16 hours light photoperiod, 60-70% relative humidity).

Inoculation, Polymerase Chain Reaction (PCR) and hairy root production

Hairy root (HR) clones have been obtained using A4 strains of *R. rhizogenes* from sterile explants leaves, buds and stems by optimized method.

Actually, direction inoculations have been transferred using *Rhizobium* strains in LB broth and MYA liquid containing 100 mg/L rifampin. Then, the medium was shaken and incubat at 28 °C with speed 160 rpm for twenty-four hours. Two mls of this culture medium was transferred to 100 ml of new bacteria culture medium with the same conditions. The bacterial suspension precipitated at 5000 rpm and 4°C after reaching 0.4-0.6 optical density (OD) in 660 nm. In addition to, sedimented bacteria were dispersed slowly in Murashige and Skoog (^{1/2}MS) liquid medium with 100 mg/l acetosyringone (AS) at pH 7.5.

The explants of germinated seeds mentioned above, have been dipped in *Rhizobium* suspension without acetosyringone

and with acetosyringone (in OD 660: 0.4-0.6), separately in 45 minutes, and have been kept for 72 hours in co-culture medium at 25 ± 2 °C and darkness to inoculation of the bacterial DNA was completed [21].

For *Rhizobium* removing, subculturing of explants was done in the culture media MS + ½ MS, B5 and ½ B5 that containing 500 mg/l cefotaxime antibiotic.

Polymerase chain reaction has been done using primers of genes rolB and rolC for determination of inoculation of T-DNA into *H. perforatum* cells. Length of duplicated pieces of sequence of the rolB and rolC genes which is available in the bacterial T-DNA are 420 bp and 612 bp, respectively. Genomic DNA of hairy roots and control samples (Non-transgenic roots) have been extracted using CTAB method and plasmid DNA of *Rhizobium* strains extracted using alkaline lysis method. In addition to, quality and quantity of the extracted DNA was studied using the results of spectrophotometry. The cycle of polymerase chain reaction was performed according to cited process. In addition to, the initial denaturing 4 min at 95°C, followed by 35 cycles with 45s at 95°C, 45s at 55°C for annealing rol BC, extension 1:30s at 72°C and final extension (5min at 72°C). Reaction mixture was prepared with 50 ng of genomic DNA, 2.0 ml of each primer (5 pmol), 0.5 ml of dNTP mix (2.5 mM each), 2.5 ml of PCR buffer, and 0.25 ml of Taq DNA polymerase (DyNazyme™ Company, 5 U/ml) and final 25 µl volume have been obtained using parenteral sterile water. Finally, electrophoresis has been carried out using 1%

gel and 5 V/cm voltage and biotransformed hairy roots were determined.

Then, the obtained biotransformed clones were exposed at the same condition in a shaker incubator with 78 rpm. After the growth in darkness for 30 days and reaching to the suitable biomass, they harvested from the culture medium for extraction and evaluation of the clones morphology. The clones washed using distilled water and their fresh weight measured and maintained in oven at 40°C until reach to the constant weight and their dry weight recorded [22, 23].

Extraction of Hypericum from hairy roots

0.1 gram of various HR clones were dried in oven with 40°C temperature and crushed. The obtained powder has been dissolved in methanol and rotated using shaker incubator with 60 °C and 140 rpm for 24 hours. Then, the solution was filtered in dark condition and was evaporate by rotary evaporator with 40°C. Dried extracts of *Hypericum* hairy roots have been weighed and dissolved in 1 ml methanol. Finally, the solution was filtered through a membrane 0.2 µm (Gelman, USA) and injected and analyzed using HPLC.

Hypericin assay

The HPLC-UV method have been used for this study (Shimadzu system, Duisburg from Germany). HPLC conducted on a Hypersil reversed-phase column (RP C18, 100 Å) (LC 150 × 4.6 mm, 5 µm). In addition to, the programs and conditions have been conducted for injection contains Isocratic Elution Type, in 25°C, 1.5 ml/min flow rate and the peak was detected using UV-VIS in 590 nm.

The mobile phase was prepared with combination of 50% acetonitrile, 30% methanol and 20% buffer solution 0.1N Ammonium Acetate with pH 9.7. In addition to, total procedure time with injection 20 μ l of the extract solution of each the clones were 20 min, separately. A calibration curve was

obtained using standard methanolic solutions of hypericin (Sigma-Aldrich) with various concentrations (0-100 μ g / ml)[24, 25]. Finally, retention time values (RT) for hypericin was observed at 15.38 min (Figures 2, 3).

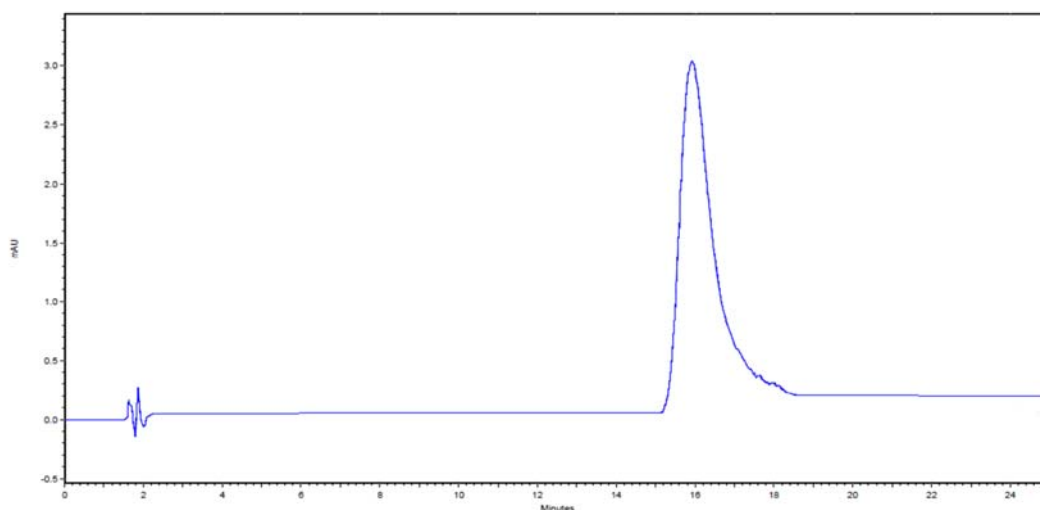


Figure 2- HPLC Chromatogram of standard solution of hypericin in concentration of 10 ppm

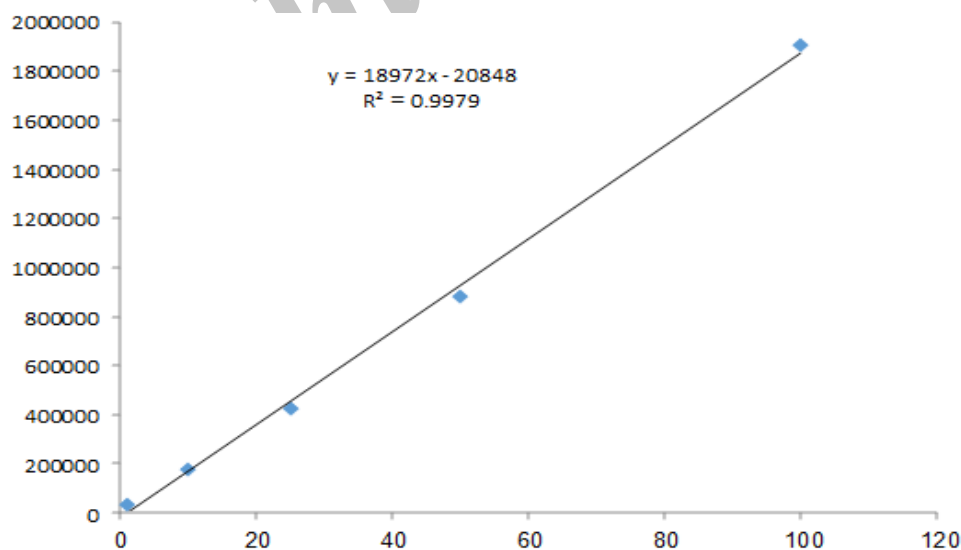


Figure 3- Standard curve of Hypericin

Data analysis

Integration of T-DNA in the genome of hairy roots with polymerase chain reaction was evaluated. In addition to, the hypericin amount in the hairy roots of *Hypericum perforatum* L. was measured. After collection of information as mean \pm SD, data analysis and charts drawing were performed using SPSS 15 and Excel 2007.

Results

Electrophoresis data of PCR products have been showed T-DNA integration into the host genome of *H. perforatum* cells, certainly (Figure 4, lane 7 and 9 have been received the T-DNA of *R. rizogenes*).

This case has been used in the next steps. Unfortunately, in the hairy roots production process, explants of *Hypericum* despite the several turning subculture in media with

different cefotaxime concentrations became afflicted to severe necrosis. Primary hairy roots have been showed in Figure 5.

Comparison of four kinds of culture media in the same conditions showed that *R. rhizogenes* at $\frac{1}{2}$ MS medium had the highest efficiency (89.58%) for hairy roots induction, so was placed in (a) class. *Rhizobium* along with acetosyringone at B₅ medium also had the lowest percentage (1.35%) of induced hairy roots, so was placed in (d) class. In addition to, the leaf area, number and density of dark glands in the leaves were different even among the clones.

After 30 days of growth, fresh and dry weight was measured in $\frac{1}{2}$ MS hormone-free medium. Comparisons of mean fresh and dry weight of clones at level of 99% have shown significant differences (Figure 6).

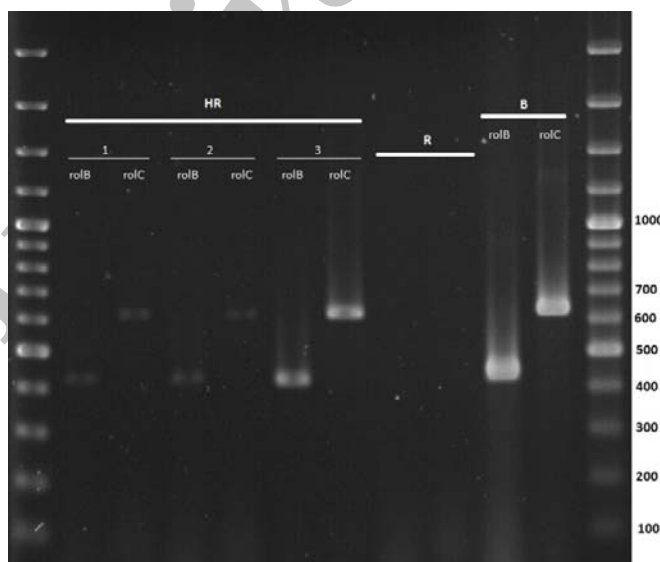


Figure 4- PCR analysis of *H. perforatum* hairy root cultures



Figure 5- Primary hairy roots

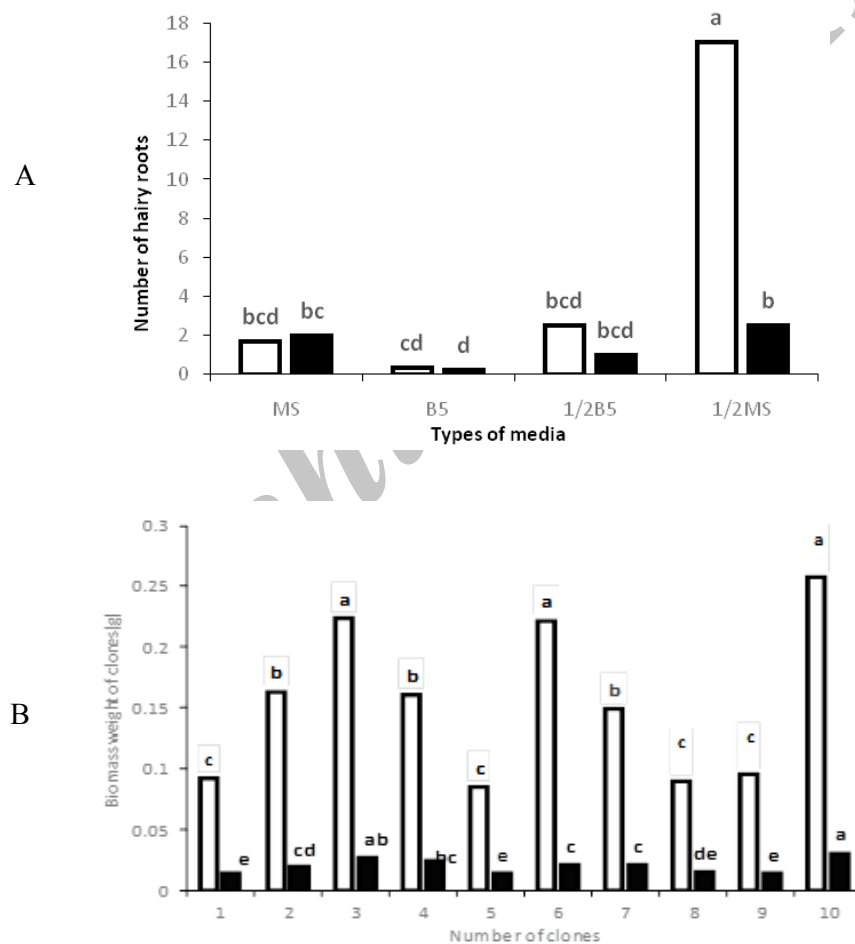


Figure 6- (A) The effect of different culture medium on induction of hairy roots (□: Inductional hairy roots with A4 strains, ■: Inductional hairy roots without A4 strains). (B) Figure 6- Comparison of average fresh and dry weight of hairy roots clones (□: Fresh Weight, ■: Dry Weight).

The clone's numbers 3, 6 and 10 with the highest fresh weight were placed in (a) class. The clone's number 10 (with highest dry weight) and clone 3 (with dry weight less than clone 10) were placed in (a) and (ab) class, respectively (Table 1).

In addition to, perfect plant with flowers spontaneously was regenerated in the majority of clones at hormone-free medium. The transgenic roots had regeneration to complete plants capability. The numerous morphological and physiological changes such as wizened and small leaves, decrease fertility, reduce apical dominance, shorter internodes; more picks of gland were created in them but

as same as original plants are stable, genetically.

HPLC screening of methanol extracts showed a large variation in hypericin content of clones (Figure 7). In the best case (Line 3) was observed 75-fold higher hypericin content (339/27 ppm) compared to nontransformed *H. perforatum* (4.58 ppm) in the same of concentration (Figure 8). Fresh and dry weight of 10 lines clones were measured after 30 days. Furthermore, the line 3, with highest hypericin, had significant development of biomass. This clone had significant production of hypericin compound towards before researches.

Table 1- Phenotypic comparison of the hairy roots clones

Number of Clones	ST.ERROR	ST.DEV	Dry weight (g)	ST.ERROR	ST.DEV	Wet weight (g)
Clone 1	0.002	0.0015	e0.0145	0.014	0.0045	c0.0928
Clone 2	0.002	0.00057	cd0.0203	0.014	0.0255	b0.1633
Clone3	0.002	0.0061	ab0.0266	0.014	0.002	a0.224
Clone 4	0.002	0.00152	bc0.0243	0.014	0.0065	b0.1613
Clone 5	0.002	0.00332	e0.0141	0.014	0.0035	c0.0855
Clone 6	0.002	0.0034	c0.0211	0.014	0.0397	a0.2216
Clone 7	0.002	0.0036	c0.021	0.014	0.019	b0.149
Clone 8	0.002	0.00264	de0.015	0.014	0.012	c0.09
Clone 9	0.002	0.0002	e0.014	0.014	0.015	c0.095
Clone 10	0.002	0.00378	a0.0306	0.014	0.0548	a0.258

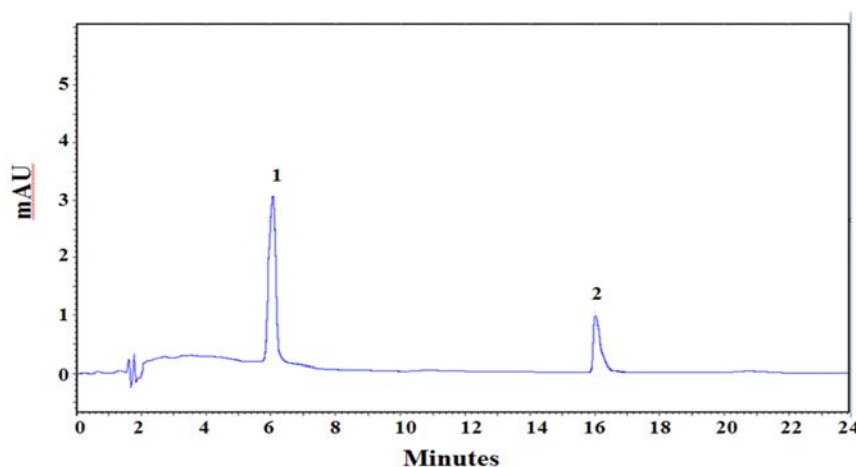


Figure 7- HPLC chromatogram of *H. perforatum* hairy root extract (1 = pseudohypericin, 2 = hypericin)

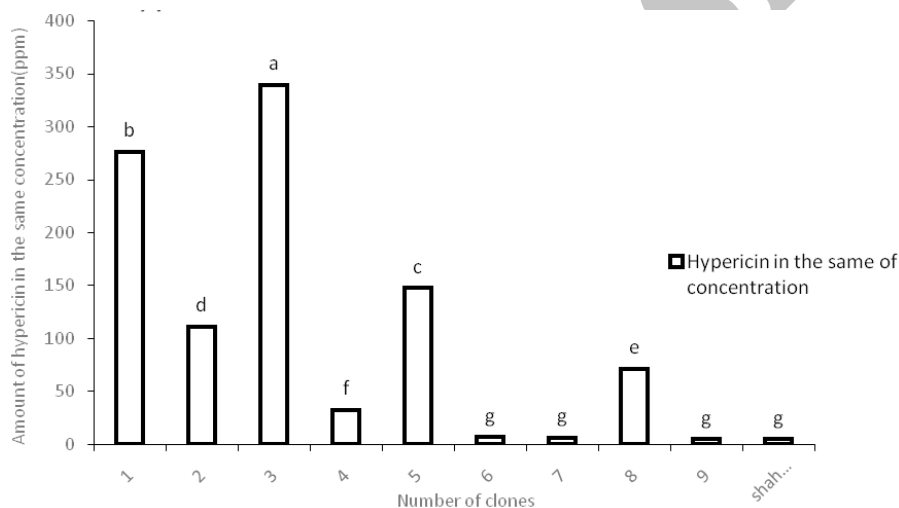


Figure 8- Comparison between amounts of hypericin in extract clones and in the same concentration of hypericin at control sample.

Discussion

Different factors have been influenced in hairy roots preparation included types of co-cultivation medium, exposure time with co-cultivation medium, insemination methods, types of *Rhizobium* strains, different explants and select of appropriate antibiotics to remove bacteria [26]. In addition to, several experiments have been performed for

optimizing induction conditions and establishment of plant hairy roots in the world.

Bivadi et al. (2014) have optimized the culture conditions of hairy roots of *H. perforatum* which contains three strains of *Rhizobium* (A4, ATCC15834, 11325) and three types of explants including leaves, stems and buds [21].

In this study, the maximum number of the hairy roots have been obtained by treatment of *H. perforatum* explants with A4 strains of *R. rizogenes* for 45 minutes inoculation in 72 hours co-cultivation using $1/2$ MS co-cultivation medium.

Various species of the plants have different sensitivity to infection with *R. rhizogenes*, *R. tumefaciens* and other bacterial species [27]. In the different studies, inoculation methods immersion in *R. rhizogenes* suspension was compared with direct injection of *R. rhizogenes* in the same conditions. Direct injection was more efficient than immersion method in *Rhizobium* suspension. Among leaves, stems and terminal buds explants, stem was the most suitable explant in the both insemination methods. In addition to, hairy roots almost have been induced in the stem of explant [27].

Different tissues of *H. perforatum* such as leaves, stems, flowers and roots generally are containing secondary metabolites such as hypericin and hyperforin [28].

Onelli et al. during his studies showed that hypericin will accumulate in glandular structures of *Hypericum* leaves [29]. In addition to, Zobayed et al. Studies showed that concentrations of hypericin and pseudohypericin in the species are dependent on the number, size and surface area of dark glands [23].

In addition to, Kornfeld et al. have been explained that may be other different parts of the plant such as the peripheral cells of leaves were related to the production of hypericin using electron microscope techniques (SEM). In cases where secondary metabolites

accumulate only in aerial parts of a plant, hairy roots culture of them is able to synthesize and accumulate of metabolites [30]. For example hairy roots of *Artemisia*, like its aerial parts, can produce artemisinin [31].

Bertoli et al. (2008) are studied morphological and phytochemical features of hairy roots lines in order to select *H. perforatum* genotypes with higher valuable bioactive compounds [32]. In our study at the same conditions and free of any environmental changes (culture medium and light, temperature, oxygenation and purveyance speed at shaker incubator), hypericin contents were different in all clones. Numerous studies in transgenic plants or hairy roots have been shown the diversity in metabolite production, for example, these observations were accordance with the results obtained by other studies. In the Bertoli study, one of the twelve lines of hairy root, have been contained 10-fold higher hypericin content (0.25 mg/g DW) compared to the quantities that reported in literature [32]. The results of Koperda'kova indicated that plants obtained from clone B had the most number and density of dark glands on their leaves and its hypericin has been the amount of 1/5 up to 3 fold higher than other studies [22].

In our research, clones have been obtained from various strains or one strain have significantly difference in the rate of growth, development of biomass, the amount of regeneration and hypericin production among the clones. This phenomenon could be due to the diversity in transform process. For example, the presence different doses of T-DNA in primary transgenic cells of each

clone and different sites insertion of foreign T-DNA in plant genome or in other cases, gene silencing involved at the secondary metabolism that causes blocking and inhibiting production of specific compounds [33].

Cui et al. Investigate adventitious roots of *H. perforatum* in balloon type airlift bioreactors (3l capacity) and after 6 weeks of culture, an approximately 50-fold increase in biomass was recorded at an inoculum density of 3 g l⁻¹ and an aeration volume of 0.1 V/Vm [14, 15]. Plant roots can synthesize, store, and secrete a vast array of compounds and transformed root cultures have a wide range of biosynthetic capacities. The use of high

potential of hairy roots between different methods that are stimulants biosynthetic pathway of these compounds considerable success provides in the commercialization of valuable compounds. In order to gaining economic interest recommended its cultivation in the optimal condition of bioreactors.

Conclusion

We attempted to produce the hypericin in high scale using transformed hairy root cell lines. Finally, the methods have been used in the study suggest high potential of hairy roots to improve the quality and the quantity of pharmaceutical compounds.

References

1. Azadbakht M, Marston A, Hostettmann K and Ramezani M. Biological activity of leaf extract and phenol glycoside arbutin of *Pyrus boissieriana*. *J. Med. Plants* 2004; 3 (10): 9-14 (Persian).
2. Azadbakht M, Safapour S, Ahmadi A, Ghasemi M and Shokrzadeh M. Anti-diabetic effects of aqueous fruits extract of *Diospyros lotus* L. on streptozotocin-induced diabetic rats and the possible morphologic changes in the liver, kidney and heart. *J. Pharmacognosy and Phytotherapy* 2010; 2 (2): 10-16.
3. Hou W, Shakya P and Franklin G. A Perspective on *Hypericum perforatum* Genetic Transformation. *Front Plant Sci.* 2016; 7 (1): 879-86.
4. Saroglou V, Marin P, Rancic A, Veljic M and Skaltsa H. Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochem. Syst. Ecol.* 2007; 35 (3): 146-52.
5. Crockett SL, Schaneberg B and Khan IA. Phytochemical profiling of new and old world *Hypericum* (St. John's Wort) species. *Phytochem. Anal.* 2005; 16 (6): 479-85.
6. Karioti A and Bilia AR. Hypericins as potential leads for new therapeutics. *International J. Molecul. Sci.* 2010; 11 (2): 562-94.
7. Ciccarelli D, Andreucci A and Candpagni AM. Translucent glands and secretory canals in *Hypericum perforatum* L. (Hypericaceae): morphological, anatomical and histochemical studies during the course of ontogenesis. *Ann. Bot.* 2001; 88 (1): 637 - 44.
8. Renato B and Gianni S. Factors affecting polyphenol biosynthesis in wild and field grown St. John's Wort (*Hypericum perforatum* L. Hypericaceae/Guttiferae). *Molecules* 2009; 14 (1): 682- 25.
9. Murthy HN, Lee EJ and Paek KY. Production of secondary metabolites from cell

and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell Tissue Organ Cult.* 2014; 118 (1): 1-16.

10. Giri CC and Zaheer M. Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell Tissue Organ Cult.* 2016; 126 (1): 1-18.

11. Amiri S, Kazemitabar SK, Ranjbar GA and Azadbakht M. In vitro propagation and whole plant regeneration from callus in *Datura* (*Datura stramonium* L.). *African J. Biotech.* 2011; 10 (3): 442-48.

12. Parr AJ and Hamill JD. Relationship between *Agrobacterium rhizogenes* transformed hairy roots and intact, uninfected *Nicotiana* plants. *Phytochem* 1987; 26 (12): 3241-45.

13. Namdeo AG, Jadhav TS, Rai PK, Gavali S and Mahadik KR. Precursor feeding for enhanced production of Secondary metabolites: A review. *Pharmacogn. Rev.* 2007; 1 (2): 227-31.

14. Cui XH, Chakrabarty D, Lee EJ and Paek KY. Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. *Bioresour. Technol.* 2010; 101 (12): 4708-16.

15. Cui XH, Murthy HN, Jin YX, Yim YH, Kim JY and Paek KY. Production of adventitious root biomass and secondary metabolites of *Hypericum perforatum* L. in a balloon type airlift reactor. *Bioresour. Technol.* 2011; 102 (21): 10072-79.

16. Kirakosyan A, Sirvent TM, Gibson DM and Kaufman PB. The production of hypericins and hyperforin by in vitro cultures

of St. John's wort (*Hypericum perforatum* L.). *Biotechnol. Appl. Biochem.* 2004; 39 (1): 71-81.

17. Amiri S, Kazemitabaar SK, Ranjbar GA and Azadbakht M. The effect of different carbon sources and concentrations, and growth regulators on anther culture and embryogenesis of *Datura stramonium* L. *J. Plant Prod.* 2011; 18 (2): 77-92.

18. Stojakowska A and Malarz J. Bioactive phenolics from in vitro cultures of *Lactuca aculeata* Boiss. et Kotschy. *Phytochem. Lett.* 2017; 19 (1): 7-11.

19. Gamborg OL, Miller RA and Ojima K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 1968; 50 (1): 151-8.

20. Murashige T and Skoog F. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Acta Physiol. Plant.* 1962; 15 (1): 473-97.

21. Bivadi V, Asghari Zakaria R, Zare N and Yazdani B. Effects of different tissue culture conditions in Hairy roots induction in *Hypericum perforatum* L. *International J. Agri. Cr. Sci.* 2014; 7 (9): 597-604.

22. Koperdaková J, Komarovská H, Košuth J, Giovannini A and Čellárová E. Characterization of hairy root-phenotype in transgenic *Hypericum perforatum* L. clones. *Acta Physiol. Plant.* 2009; 31 (2): 351-58.

23. Zobayed SMA, Afreen F, Goto E and Kozai T. Plant-environment interactions: Accumulation of hypericin in dark glands of *Hypericum perforatum*. *Ann. Bot.* 2006; 98 (4): 793-804.

24. Fakhri M, Davoodi A, Parviz M, Sadeghi Ghadi Z, Mousavinasab SN, Farhadi R and et

- al. Characterization and HPLC analysis of Manna from some *Cotoneaster* Species. *International J. Pharm. Sci. Res.* 2017; 8 (12): 5360-66.
25. Borghi Pangoni FB, Junqueira MV, Silva LL and Luciano Bruschi M. HPLC Method for Determination of Hypericin Contained in Polymeric System: Analysis of the Regression Significance, Lack of Fit, and Applicability. *Latin American J. Pharm.* 2016; 35 (10): 2125-31.
26. Ligang Z, Junjian W and Chongren Y. Progress on plant hairy root culture and its chemistry. induction and culture of plant hairy roots. *Nat. Prod. Res. Develop.* 1998; 3 (1): 19-25.
27. Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LM, Yang W and et al. Gene transfer to plants by diverse species of bacteria. *Nature* 2005; 433 (7026): 629-33.
28. Yuan Z, Edlind M, Liu P, Saenkham P, Banta L, Wise A and et al. The plant signal salicylic acid shuts down expression of the vir regulon and activates quorum-quenching genes in *Agrobacterium*. *Proc. Natl. Acad. Sci. USA.* 2007; 104 (28): 11790-5.
29. Onelli E, Rivetta A, Giorgi A, Bignami M, Cocucci M and Patrignani G. Ultrastructural studies on the developing secretory nodules of *Hypericum perforatum*. *Flora-Morph. Distr. Funct. Ecol. Plants* 2002; 197 (2): 92-102.
30. Kornfeld A, Kaufman PB, Lu CR, Gibson DM, Bolling SF, Warber SL and et al. The production of hypericins in two selected *Hypericum perforatum* shoot cultures is related to differences in black gland structure. *Plant. Physiol. Biochem.* 2007; 45 (1): 24-32.
31. Hu ZB and Du M. Hairy root and its application in plant genetic engineering. *J. Integ. Plant Biol.* 2006; 48 (2): 121-127.
32. Bertoli A, Giovannini A, Ruffoni B, Guardo AD, Spinelli G, Mazzetti M and et al. Bioactive constituent production in St. John's Wort in vitro hairy roots. Regenerated plant lines. *J. Agric. Food Chem.* 2008; 56 (13): 5078-82.
33. Giri A and Narasu ML. Transgenic hairy roots. recent trends and applications. *Biotechnol. Adv.* 2000; 18 (1): 1-22.