Turkish Journal of Agriculture - Food Science and Technology, 3(6): 439-443, 2015

Turkish Journal of Agriculture - Food Science and Technology

www.agrifoodscience.com,
Turkish Science and Technology

Response Surface Modelling of Noradrenaline Production in Hairy Root Culture of Purslane (*Portulaca oleracea* L.)

Mehdi Ghorbani [1], Aghil Ghorbani [2], Mansoor Omidi [1], Seyed Mohammad Hashemi [1]

[1]*Department of Agronomy and Plant Breeding, University College of Agriculture and Natural Resources, University of Tehran, Iran.*
[2]*Department of Crop Biotechnology and Breeding, Ferdowsi University of Mashhad, Iran.*

A R T I C L E I N F O

Article history:
Received 29 January 2015
Accepted 19 February 2015
Available online, ISSN: 2148-127X

Keywords:
Agrobacterium rhizogenes
Hairy root culture
Noradrenaline
Portulaca oleracea L.
Response surface methodology

* Corresponding Author:
E-mail: mghorbani_90@ut.ac.ir

A B S T R A C T

Common purslane (*Portulaca oleracea* L.) is an annual plant as one of the natural sources for noradrenaline hormone. In this research, hairy root culture of purslane was established by using *Agrobacterium rhizogenes* strain ATCC 15834. In the following, Box-Behnken model of response surface methodology (RSM) was employed to optimize B5 medium for the growth of *P. oleracea* L. hairy root line. According to the results, modelling and optimization conditions, including sucrose, $CaCl_2.H_2O$, H_2PO_4 and NO_3^-/NH_4^+ concentrations on maximum dry weight (0.155 g) and noradrenaline content (0.36 $mg.g^{-1}$ DW) was predicted. These optimal conditions predicted by RSM were confirmed the enhancement of noradrenaline production as an application potential for production by hairy root cultures.

Introduction

The common purslane, *Portulaca oleracea* L. is an herbaceous annual plant belongs to family Portulacaceae usually distributed in the humid regions of Iran, which has been used as food and medicinal plant (Zargari, 1991). Antitumor effects *in vivo* (Shen et al., 2013) and *in vitro* (Tian et al., 2013) model and several powerful pharmaceutical activities for example soothing, hepatoprotective, neuropharmacological, anti-inflammatory, antidiabetic, bronchodilator, antihypertensive, antioxidant and containing the noradrenaline hormone has been reported (Amirul Alam et al., 2014). Noradrenaline and Dopamine are the major catecholamine bioactive compounds present in *P. oleracea* L. as an immune system modulator for treatment of shock (Chen et al., 2003). Over the past few years, tissue culture systems for *P. oleracea* L. has been studied in the callus induction, plant regeneration (Safdari and Kazemitabar 2009; Safdari and Kazemitabar 2010) and L-dopamine; noradrenaline content in hairy root culture (Ahmadi Moghadam et al., 2014; Ahmadi Moghadam et al., 2011; Pirian and Piri 2013; Pirian et al., 2012). The most commonly used B5 medium in experimental plant tissue culture and plant biotechnology was formulated nearly 47 years ago (Gamborg et al., 1968). Problems with up-scaling, low yields and high-priced process conditions result in an increased demand to modeling of plant *in vitro* cultures (Maschke et al., 2015). Hairy root culture medium optimization for dopamine production as a neurotransmitter precursor using response surface methodology (RSM) in *Stizolobium hassjoo* has been reported (Sung and Huang 2000). RSM is one of the most popular modelling and optimization methods used in the last two decades as a useful technique for developing and improving processes in which a response of interest is influenced by several variables (Bas and Boyacı, 2007). The present study proposes a Box-Behnken model of RSM to identify the best B5 medium composition for best growth of *P. oleracea* hairy roots and noradrenaline accumulation as hormone.

Materials and Methods

Establishment of hairy root cultures

Seeds of *P. oleracea* L. were surface sterilized with 70% (v/v) ethanol for 30 s and 2.5% (v/v) sodium hypochlorite solution for 3 min then rinsed in sterilized distilled water. The seeds were germinated on B5 medium at 25ºC in the culture room (16:8h light-dark). 12-days old explants (Cotyledonary leaves and stem) were co-cultivated with *A. rhizogenes* strain ATCC 15834 on 0.7% agar phytohormones-free B5 medium containing 100 µM acetosyringone and then were transferred in dark culture room (48h at 25°C). *A. rhizogenes* was removed

by culturing in new B5 medium supplemented with 500 $mg.L^{-1}$ Cefotaxime. Uninfected sterile leaf and stem explants were grown on the declared medium as the control. After two weeks, the emerging hairy roots (3-5 cm) were excised from explants and transferred to solidified phytohormones-free B5 medium at 25°C in dark condition for more growth. DNA from samples was extracted using AccuPrep® GMO DNA Extraction Kit, Cat. No.: K-3031 (Bioneer, Korea). The transgenic nature of hairy roots was confirmed by PCR with *rolC* gene specific primers (F: 5'-CTCCTGACATCAAACTCGTC-3', R: 5'-TGCTTCGAGTTATGGGTACA-3'). To confirm the transgenic-free from any residual bacterial contamination, *virD* gene (F: 5'-ATGTCGCAAGGACGTAAGCCGA-3', R: 5'-GGAGTCTTTCAGCATGGAGCAA-3') specific primers were used. GelRed™ -stained PCR products were examined by electrophoresis on 1% (w/v) agarose gel.

Design of experiments

Box-Behnken design of RSM was employed to optimize the liquid B5 medium compositions. Experimental procedure was designed by using Design-Expert® software (Stat-Ease, Inc. Trial version 9). Transgenic hairy root line H3 was selected for the liquid medium optimization studies during four weeks. The B5 medium formulations were tested at in vitro conditions without phytohormones (Table 1). All media were supplemented with 100 $mg.L^{-1}$ Myo-inositol. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min. For determination of the noradrenaline, 50 mg lyophilized-powdered root was immersed with 1 mL 0.1 M HCl solution at room temperature in a 1.5 mL tube followed by ultrasonic extraction for 1.5 h at room temperature. The extract was centrifuged at 10000 g at room temperature for 10 min. Supernatant was transferred to a new tube. The samples were filtered through the syringe membrane filter (PTFE, 0.22 µm), and 15 µL was injected into the HPLC (Knauer, PLATIN blue system, Germany). Analytical column was C18 column (Eurosphere, 250 mm × 4.6 mm, 5 µm). The mobile phase consisted of 0.02 M KH_2PO_4-Acetonitrile (95:5 v/v). The flow rate was 1 $mL.min^{-1}$ with UV detection wavelength at 280 nm and the column temperature was 25°C. The quantification of the noradrenaline was carried out using an external standard calibration method (Chen et al. 2003).

Results and Discussion

Establishment of Portulaca oleracea L. root cultures

The transgenic hairy roots obtained were verified by PCR with the specific primer pairs of *rolC* gene and genomic DNA from hairy roots amplified the expected amplicon of 629 bp. On the subject of non-transformed root, no amplified fragment of the *rolC* gene was distinguished. Contamination confirmation was also performed by PCR analysis using *virD* (438 bp) gene specific primers, no amplicon was found in the hairy root under study, indicating the absence of bacterial contamination (Fig. 1). In this work, control explants showed natural hairy root induction, which were emerged from the stem and cotyledonary leaf control explants showed necrosis on the terminal tips (Fig. 2). Our results for control explants were not consistent with the results of Ahmadi Moghadam and Colleagues (Ahmadi Moghadam et al., 2011).

Analysis of experimental design

In this study different factor treatment, including Sucrose, $CaCl_2.H_2O$, H_2PO_4 and NO_3^-/NH_4^+ on dry weight and noradrenaline content of *P. oleracea* hairy roots were studied (Table 1). During the investigated time period (three weeks), the noradrenaline content of the treated cultures were measured. Noradrenaline was identified using comparison of its retention time and absorption spectrum of its standard solution. The retention time value for noradrenaline was at 9 minutes (Fig. 3). Chromatographic data confirmed the results, which have been reported by Chen and Colleagues (Chen et al., 2003). According to the results (Table 2), modeling and optimal conditions, including 30-40 g. L^{-1} sucrose, the minimum concentration of $CaCl_2.H_2O$, 150 $mg.L^{-1}$ H_2PO_4 and 3 g. L^{-1} $NO3^-/NH_4^+$ on maximum dry weight (0.155 g) and noradrenaline content (0.36 $mg.g^{-1}$ DW) was evaluated (0.927 < desirability < 0.934). In relation to dry weight and secondary metabolites production, hairy root cultures of *P. oleracea* L. has been studied through elicitation technique. Methyl Jasmonat (MJ) elicitor treatment resulted in 4.3 fold Dopamine concentration compared with the control hairy roots (Ahmadi Moghadam et al., 2014). In addition, it has been reported that MJ increased the production of noradrenaline (Pirian et al., 2012).

Table 1 Coded levels chosen for Box-Behnken design.

Variables	Units	-1 Level	+1 Level
A- Sucrose	$g.L^{-1}$	10	50
B- $CaCl_2.H_2O$	$mg.L^{-1}$	50	250
C- $H2PO4^-$	$mg.L^{-1}$	50	200
D- NO_3^-/NH_4^+	$g.L^{-1}$	1.5	3.5

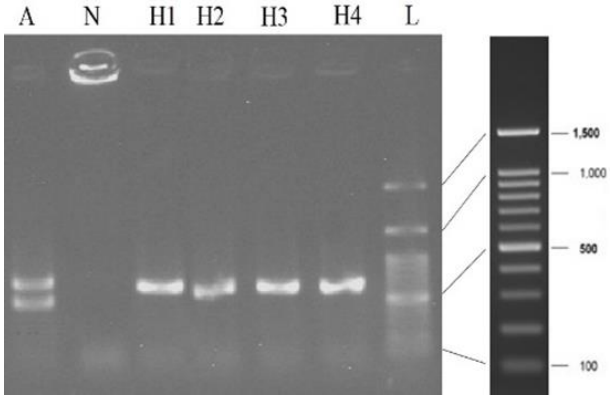


Fig. 1 Electrophoretogram analysis of hairy roots for confirmation of *rolC* gene integration and *virD* gene. A: Ri-plasmid of *Agrobacterium rhizogenes* strain ATCC 15834, N: Natural root sample, H1-H4: Transgenic hairy root lines, L: DNA Ladder (1500-100 bp, SinaClone, Iran).

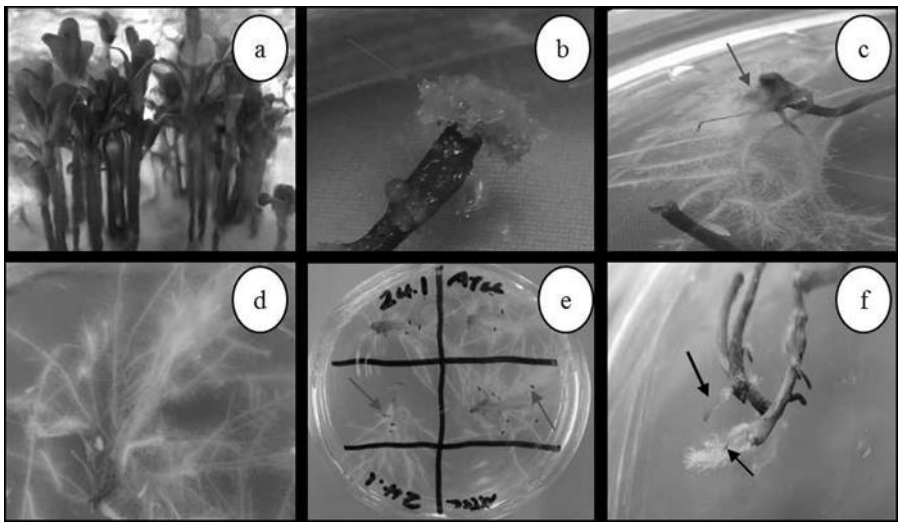


Fig. 2 *Agrobacterium rhizogenes* mediated transformation of *Portulaca oleracea* L. 12-days old seedlings of purslane (a). Callus induction in cotyledonary leaf (b) and stem (c) explants after one week of inoculation. Growth of four hairy root lines on solid B5 medium after one week. Arrows indicate the first site of the growth (d and e). Natural root emerged from control stem explant after three days in phytohormones-free solid B5 medium. Arrows indicate necrosis signs on the terminal tips in natural root (f).

Table 2 Experimental and results of Box-Behnken design for dry weight of hairy roots and noradrenaline content.

Run order	Sucrose ($g.L^{-1}$)	$CaCl_2.H_2O$ ($mg.L^{-1}$)	$H_2PO_4^-$ ($mg.L^{-1}$)	NO_3^-/NH_4^+ ($g.L^{-1}$)	Dry Weight (mg)		Noradrenaline ($mg.g^{-1}$ DW)	
					Actual	Predicted	Actual	Predicted
1	30.00	250.00	125.00	1.50	83.00	79.08	0.0936	0.12
2	30.00	150.00	125.00	2.50	131.00	132.40	0.1969	0.26
3	50.00	150.00	125.00	1.50	120.00	115.96	0.2336	0.24
4	50.00	150.00	50.00	2.50	134.00	125.92	0.2841	0.27
5	50.00	150.00	200.00	2.50	119.00	119.92	0.3984	0.33
6	10.00	150.00	200.00	2.50	90.00	93.250	0.2166	0.21
7	30.00	150.00	125.00	2.50	130.00	132.40	0.3074	0.26
8	30.00	50.00	50.00	2.50	142.00	134.13	0.3344	0.32
9	30.00	150.00	125.00	2.50	138.00	132.40	0.2871	0.26
10	10.00	150.00	50.00	2.50	80.00	74.250	0.0520	0.15
11	30.00	150.00	200.00	1.50	121.00	120.13	0.3447	0.35
12	30.00	150.00	200.00	3.50	147.00	142.79	0.4076	0.39
13	30.00	50.00	125.00	1.50	156.00	154.42	0.2931	0.27
14	30.00	150.00	125.00	2.50	132.00	132.40	0.2063	0.26
15	30.00	250.00	200.00	2.50	110.00	111.29	0.2212	0.25
16	30.00	250.00	125.00	3.50	150.00	146.75	0.2311	0.24
17	50.00	50.00	125.00	2.50	132.00	137.96	0.3005	0.33
18	30.00	150.00	125.00	2.50	131.00	132.40	0.2873	0.26
19	10.00	50.00	125.00	2.50	92.000	96.790	0.0972	0.11
20	30.00	150.00	50.00	3.50	123.00	135.29	0.4065	0.40
21	10.00	250.00	125.00	2.50	64.000	69.46	0.0962	0.06
22	30.00	50.00	200.00	2.50	140.00	139.63	0.3442	0.39
23	50.00	150.00	125.00	3.50	142.00	140.63	0.2331	0.29
24	30.00	250.00	50.00	2.50	110.00	103.79	0.2172	0.19
25	50.00	250.00	125.00	2.50	100.00	106.62	0.1023	0.09
26	30.00	150.00	50.00	1.50	99.000	114.63	0.2056	0.22
27	30.00	50.00	125.00	3.50	131.00	130.08	0.4094	0.36
28	10.00	150.00	125.00	1.50	85.000	79.790	0.0931	0.05
29	10.00	150.00	125.00	3.50	101.00	98.460	0.2053	0.22

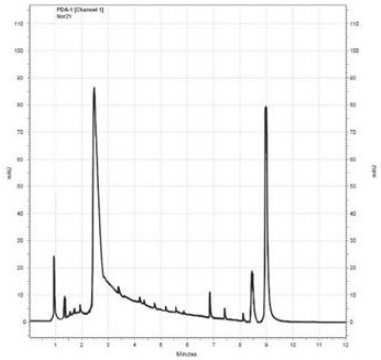
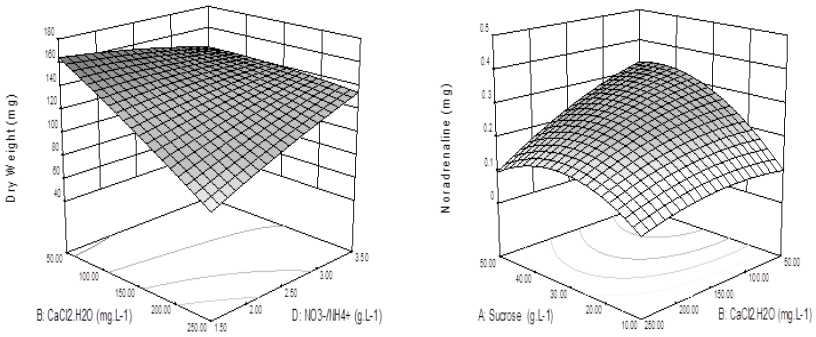


Fig. 3 Chromatogram of *Portulaca oleracea* L. sample hairy root extract (mAU on the Y-axis versus the time in Minute on the X-axis). NA: Noradrenaline



(a) (b)

Fig. 4 3D response surface plot of *P. oleracae* hairy root dry weight (a) and noradrenaline content (b) in B5 liquid medium after 3-weeks.

Table 3 ANOVA table for dry weight.

Source	SS	df	MS	F Value	P-value
Model	20299.1	14	1449.9	8.152	0.0001
A-Sucrose	6210.7	1	6210.7	34.92	< 0.0001
B-$CaCl_2.H_2O$	6210.7	1	6210.7	34.92	< 0.0001
C-$H_2PO_4^-$	0.75	1	0.75	0.004	0.9491
D-NO_3^-/NH_4^+	720.7	1	720.7	4.05	0.0637
AB	0.25	1	0.25	0.001	0.9706
AC	100.0	1	100.0	0.56	0.4658
AD	9.00	1	9.00	0.051	0.8253
BC	484.0	1	484.0	2.72	0.1213
BD	1444.0	1	1444.0	8.12	0.0129
CD	42.25	1	42.25	0.24	0.6335
A^2	4461.0	1	4461.0	25.08	0.0002
B^2	32.1	1	32.11	0.18	0.6773
C^2	1092.0	1	1092.0	6.14	0.0266
D^2	39.73	1	39.73	0.22	0.6437
Residual	2489.8	14	177.8	-	-
Lack of Fit	2207.0	10	220.7	3.12	0.1420
Pure Error	282.8	4	70.70	-	-

SS: Sum of Square, df: Degree of freedom, MS: Mean of square

Table 4 ANOVA table for noradrenaline content.

Source	SS	df	MS	F Value	P-value
Model	0.27	14	0.019	7.25	0.0003
A-Sucrose	0.052	1	0.052	19.95	0.0005
B-$CaCl_2.H_2O$	0.056	1	0.056	21.26	0.0004
C-$H_2PO_4^-$	0.016	1	0.016	5.97	0.0284
D-NO_3^-/NH_4^+	0.033	1	0.033	12.61	0.0032
AB	0.0097	1	0.0097	3.71	0.0745
AC	00063	1	0.0006	0.24	0.6306
AD	0.0031	1	0.0031	1.21	0.2893
BC	0.0000	1	0.0000	0.003	0.9556
BD	0.0001	1	0.0001	0.043	0.8388
CD	0.0047	1	0.0047	1.82	0.1988
A^2	0.044	1	0.044	16.66	0.0011
B^2	0.0052	1	0.0052	2.01	0.1779
C^2	0.022	1	0.022	8.34	0.0119
D^2	0.0035	1	0.0035	1.37	0.2607
Residual	0.037	14	0.0026	-	-
Lack of Fit	0.026	10	0.0026	0.99	0.5532
Pure Error	0.011	4	0.0026	-	-

SS: Sum of Square, df: Degree of freedom, MS: Mean of square

In this study, choice of these ranges for optimization was carried out by investigating the effect of one factor on the dry weight and noradrenaline, separately. The final equations obtained in terms of the actual factors for culture conditions are:

$$\text{Dry Weight (mg)} = +23.781 + 5.281 \times (\text{Sucrose}) - 0.456\,(CaCl_2.H_2O) + 0.19 \times (CaCl_2.H_2O) \times ({NO_3}^-/{NH_4}^+) - 0.065 \times (\text{Sucrose})^2 - 0.0023 \times ({H_2PO_4}^-)^2$$

$$\text{Noradrenaline } (mg.g^{-1}\,DW) = -0.2468 + 0.0238 \times (\text{Sucrose}) + 0.0008 \times (CaCl_2.H_2O) - 0.00066 \times ({H_2PO_4}^-) + 0.02652 \times ({NO_3}^-/{NH_4}^+) - 0.00020 \times (\text{Sucrose})^2 + 1.03148 \times ({H_2PO_4}^-)^2$$

Fig. 4 shows the results obtained for dry weight and noradrenaline content. The dry weight, quadratic model fitness was expressed with the coefficient of determination; R^2, which was calculated to be 0.89, indicates that 89% of the variability in the biomass could be explained by the model. The model F-value of 8.15 implies the model for dry weight is significant (Table 3). Experiments are carried out with each of the constituents of the culture medium to examine noradrenaline content, simultaneously. The values of the determination coefficients R^2 equal to 0.87 indicated that only 13% of the total variation for noradrenaline accumulation was not explained by the model. In the range of studied concentrations of each factor except $CaCl_2.H_2O$, the biomass and the noradrenaline accumulation go through a maximum. Notwithstanding supplementation of high concentration of KNO_3, to keep ${NO_3}^-$ level constant will produce an accumulation of ${K}^+$ which can lead to toxicity, contrast to in this work, increasing of the KNO_3 concentration caused more biomass and noradrenaline content (Mairet et al., 2009).

Conclusion

When metabolite production is expected, first we need to improve productivity of the biomass by using the optimized media. When an optimum and best culture conditions are achieved, the production of the targeted compounds can be improved by altering the composition for fed-batch processes in next steps (Mairet et al., 2009; Srivastava and Srivastava 2012).

The major objective of this research was the development of a statistical approach to modeling and optimization of hairy root growth for producing plant secondary metabolite noradrenaline as a natural hormone. This research is the first report on modeling and optimization of medium in hairy root cultures of *P. oleracea* L. Our results, altogether, delineate a promising avenue regarding to the improvement of culture condition for noradrenaline production, as a pivotal hormone in clinical applications.

References

Ahmadi Moghadam Y, Piri K, Bahramnejad B, Ghiyasvand T. 2014. Dopamine production in hairy root cultures of *Portulaca oleracea* (Purslane) using *Agrobacterium rhizogenes*. Journal of Agricultural Science and Technology 16: 409-420.

Ahmadi Moghadam Y, Piri K, Bahramnejad B, Habibi P. 2011. Hairy roots induction in purslane (*Portulaca oleracea* Linn.) using *Agrobacterium rhizogenes*. Planta Medica 77: PB24.

Amirul Alam M, Juraimi A, Rafii MY, Hamid A, Kamal Uddin M, Alam MZ, Latif MA. 2014. Genetic improvement of Purslane (*Portulaca oleracea* L.) and its future prospects. Molecular Biology Reports 41: 7395-7411.

Bas D, Boyacı IH. 2007. Modeling and optimization II: Comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction. Journal of Food Engineering 78: 846-854.

Chen J, Shi YP, Liu JY. 2003. Determination of noradrenaline and dopamine in Chinese herbal extracts from *Portulaca oleracea* L. by high-performance liquid chromatography. Journal of Chromatography A 1003: 127-132.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50: 151-158.

Mairet F, Sierra J, Glorian V, Villon P, Shakourzadeh K, Boitel-Conti M. 2009. A new approach to define optimized range of medium composition for enhancement of hairy root production in fed-batch process. Bioprocess and Biosystems Engineering 32: 257-265.

Maschke RW, Geipel K, Bley T. 2015. Modeling of plant in vitro cultures: Overview and estimation of biotechnological processes. Biotechnology and Bioengineering 112: 1-12.

Pirian K, Piri K. 2013. Influence of yeast extract as a biotic elicitor on noradrenaline production in hairy root culture of *Portulaca oleracea* L. International Journal of Agronomy and Plant Production 4: 2960-2964.

Pirian K, Piri K, Hamedan I. 2012. Effect of methyl jasmonate and salicylic acid on noradrenaline accumulation in hairy roots of *Portulaca oleracea* L. International Research Journal of Applied and Basic Sciences 3: 213-218.

Safdari Y, Kazemitabar S. 2009. Plant tissue culture study on two different races of purslane (*Portulaca oleracea* L.). African Journal of Biotechnology 8: 5906-5912.

Safdari Y, Kazemitabar SK. 2010. Direct shoot regeneration, callus induction and plant regeneration from callus tissue in mose rose (*Portulaca grandiflora* L.). Plant Omics 3: 47-51.

Shen H, Tang G, Zeng G, Yang Y, Cai X, Li D, Zhou N. 2013. Purification and characterization of an antitumor polysaccharide from *Portulaca oleracea* L. Carbohydrate Polymers 93:395-400.

Srivastava S, Srivastava AK. 2012. Azadirachtin production by hairy root cultivation of Azadirachta indica in a modified stirred tank reactor. Bioprocess and Biosystems Engineering 35: 1549-1553.

Sung LS, Huang SY. 2000. Medium optimization of transformed root cultures of *Stizolobium hassjoo* producing L−DOPA with response surface methodology. Biotechnology Progress 16: 1135-1140.

Tian JL, Liang X, Gao PY, Li DQ, Sun Q, Li LZ., Song SJ. 2013. Two new alkaloids from *Portulaca oleracea* and their cytotoxic activities. Journal of Asian Natural Products Research 16: 259-264.

Zargari A. 1991. Medicinal Plants. University of Tehran Press.