

Determination of Hypericin Content in Callus and Cell Suspension Cultures of *Hypericum triquetrifolium* Turra

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Abstract *Hypericum triquetrifolium* Turra. is a valuable medicinal plant owing to source of many bioactive compounds. Hypericin is one of the significant components among these compounds. The aim of this study was to develop an efficient method allowing to improve hypericin production from calli and cell suspension cultures of *Hypericum triquetrifolium* Turra. Callus formation was obtained from axenic leaf explants grown in Murashige and Skoog (MS) salts supplemented with 1 mg l⁻¹ 6-benzyl adenine (BA) and 2 mg l⁻¹ α -naphthaleneacetic acid (NAA) and 1 mg l⁻¹ (BA) + 0.4 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D). Growth medium of cell suspension culture was the same with callus medium but devoid of agar. The increase of biomass in cell suspension culture was obtained between 6.31-6.28 fold compared to the first day of culture, on the 20th day. We assayed hypericin content in methanolic extracts of calli and cell suspension cultures of *H. triquetrifolium* Turra. Hypericin contents of the samples were measured at 589 nm by a UV-VIS spectrophotometer. Analysis of hypericin contents showed that levels found in callus were 0.0527 and 0.0485 mg g⁻¹, while in cell suspension cultures these rates were 0.0018 and 0.0016 mg g⁻¹, suggesting that the accumulation of this compound in cell suspension needs further modifications.

Keywords *Hypericum triquetrifolium* Turra., Hypericin, Callus Culture, Cell Suspension Culture, UV-VIS Spectrometer

1. Introduction

The genus *Hypericum* belongs to the Clusiaceae (Guttiferae) family which encompasses approximately 460 species accommodate in 36 sections throughout the world [1]. The genus *Hypericum* includes both annual and perennial herbaceous plants, as well as woody, shrubs and small trees. *H. triquetrifolium* Turra., which is important plant of *Hypericum* genus, widespread in warm-temperate areas

throughout the world and well represented in the Mediterranean and in the Near East area. *H. triquetrifolium* is a herbaceous perennial plant, and it usually grows on dry stony or sandy places [2]. The genus are of great economical and medicinal importance for containing of phytochemical constituents [3].

Hypericum species carry secretory structures in above-ground organs [4,5], which are sites of synthesis and accumulation of biologically active substances [6,7], primarily hypericin, pseudohypericin and their derivatives protohypericin and protopseudohypericin [8]. Hypericin and pseudohypericin, which are the basic chemicals of *Hypericum* extracts, are formed in sepals, petals, and stamens of *Hypericum* species [9-11], and these compounds have photodynamic, antiviral, antibacterial, antidepressant and antitumoral activities [12,13].

Properties of callus tissue are an important factor in the establishment of cell cultures. These features allow to form single or clusters of cells in liquid culture and are further increased quality of cell suspension culture. Such cells dispersed in the liquid medium have a potential to synthesize any of the compounds normally associated with the intact plant [14]. Division of cells in suspension cultures was much higher and more affected by changing media conditions in comparison with cells in callus culture [15].

Production of secondary metabolites via plant cell and tissue cultures has various advantages such as obtaining desirable secondary metabolites and large scale production [16]. In recent years, plant cell culture techniques has been commonly applied to the production of many useful secondary metabolites, including pharmaceuticals, pigments, and other bioactive compounds [17,18]. Several valuable studies have been performed on the production of useful secondary metabolites in plant cell cultures and organ cultures of some *Hypericum* species. The relationship between biosynthesis of hypericin compounds and *in vitro* development of differentiated structures in *Hypericum* plants has also been investigated [19-22].

The aim of this work was to develop an efficient procedure for the establishment of callus and cell suspension cultures

from *H. triquetrifolium* and determine hypericin production of these cultures. The role of plant growth regulator treatments has been particularly studied.

2. Materials and Methods

2.1. Plant Material and Culture Conditions

The seeds of *H. triquetrifolium* were collected in September 2008 from Diyarbakir, Turkey. The seeds were surface sterilized by immersion in a 70% (v/v) ethanol solution for 30 s, followed by immersion in a 53% aqueous solution of 5% sodium hypochlorite in water for 10 min and five times rinses with sterile distilled water. After sterilization process, the seeds were germinated on Murashige and Skoog basal MS medium [23] devoid plant growth regulator in Magenta vessels (GA-7). The pH of the media was adjusted to 5.8 and it was supplemented 0.7 % agar for media solidification prior to autoclaving at 1 atm 121°C for 25 min. The cultures were incubated under a photoperiod of 16 h light/8 h darkness in a growth chamber at 25 ± 2°C. After 4 weeks initiation of *in vitro* cultures, plantlets obtained from the germinated seeds were transferred to an MS basal medium containing 1 mg l⁻¹ BA, agar 0.7% (w/v) and 3% (w/v) sucrose and they were grown in this medium for proliferation.

2.2. Callus Induction

To induce callus formation, leaves of *in vitro* grown plantlets were used (Fig. 3a). Leaf explants excised from seedlings approximately 4 cm high were divided into four parts and aseptically transferred with the abaxial side-down to the culture medium. In order to test the effect of different growth regulators on calli induction, the different combinations of auxins (NAA and 2,4-D) and cytokinins (BA and Kin) were tested and the frequency of callus induction and growth rates was evaluated 4 weeks after culture initiation (Table 1). The cultures of calli were regularly subcultured every 28 days.

2.3. Establishment of Cell Suspension Cultures

For initiation of cell suspension cultures, calli obtained from *in vitro* grown leaves were used (Fig. 3b). After 3-5 subcultures, calli (1g F.W.) were inoculated in 100 ml flasks containing 20 ml MS medium and maintained on a rotary shaker at 100-105 rpm. Medium used for suspension culture was the same of callus medium but without agar. Initial subcultures of plant cell cultures performed in 100 ml erlen flasks containing 20 ml of fresh liquid media but the next subcultures were maintained in 500 ml erlen flasks containing 50 ml of fresh media and for 500 ml erlen flasks were used 100 ml fresh media (Fig. 3d). After 15 days in liquid culture, the cells released from calli were transferred

into fresh liquid media (Fig. 3c). During the each subculture, microscopic observations were carried out to observe presence of single cells (Fig. 3e) and small cell clusters (Fig. 3f) and morphological appearance of the cells in suspension cultures after filtering through sieves of mesh sizes 500, 250 and 100 µm. Each subcultures were performed every 2 weeks.

2.4. Growth Parameters of Cell Suspension Cultures

Cell growth of two media containing MS medium supplemented with 1 mg l⁻¹ BA + 2 mg l⁻¹ NAA and 1 mg l⁻¹ BA + 0.4 mg l⁻¹ 2,4-D was measured by determining the packed cell volume (PCV/100), fresh weight (FW/100) and dry weight (DW/100) at 4, 8, 12, 16, 20 days. The values of PCV, FW and DW were obtained by compressing the cell mass in a given volume of cell suspension culture (10 ml). Each point on the growth curve represents the mean of three independent measurements ± S.E.

Table 1. Effects of medium compositions on callus induction

Media(mg ml ⁻¹)	Callus formation (%)	Callus appearance
MS+BA (1) + NAA (2)	92.3 ±4.63	green, friable
MS+BA (1) + NAA (0.5)	81.7 ±6.21	green, compact
MS+BA (1) + 2,4-D (0.4)	56.2 ±3.42	yellowish, friable
MS+BA (0.06) + 2,4-D (0.1)	43.8 ±4.39	yellowish, compact
MS+Kin (0.9) + 2,4-D (3)	27.5 ±2.91	brownish, compact
MS+Kin (0.4) + 2,4-D (2)	14.4 ±1.73	brownish, compact

2.5. Extraction and Hypericin Analysis Of Samples

The samples of 100 mg (DW) were placed in vials containing chloroform (10 ml) and extracted in a sonicator to remove their chlorophyll contents. Following that, the chloroform was removed in a vacuo. After removing the chloroform, the samples were re-extracted with methanol (10 ml) in the sonicator for 5 min with methanol, the extraction process was repeated three times and then, the methanol was removed from the vacuo. The final samples (supernatants) were dissolved in methanol and 1 ml of each methanolic extract was placed in a test tube for the determination of hypericin content and spectrometric analysis were performed. Absorbance was measured at 589 nm by a spectrometer (Shimadzu UV/Vis-T80+).

2.6. Statistical Analysis

The spectrometer analysis was performed by using an eight point calibration curve generated with pure hypericin (Fig. 1). Experiments were repeated 3 times using a complete randomized block design. Analysis of samples was performed and differences between the means of the treatments were determined by Duncan's Multiple Range test at p 0.05 and 0.01. All calibration curves showed good

linear regression ($R^2=0.992$) within the test range.

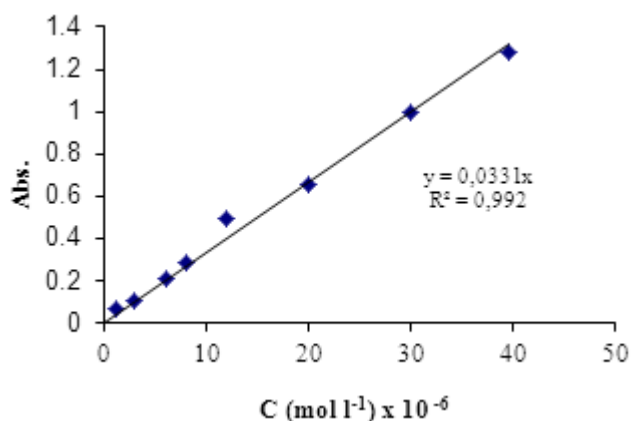


Figure 1. Calibration curve of pure hypericin standard at 589 nm.

3. Results

Initiation of callus and cell suspension cultures

All media tested formed callus but calli obtained showed different features in terms of both appearance and growth rate. Although the calli obtained kinetin in combination with 2,4-D showed undesired properties due to weak growth and the calli formation rate, BA in combination with 2,4-D and NAA had desired properties (Table 1). In MS media containing 1 mg l⁻¹ BA in combination with 2 mg l⁻¹ NAA and 1 mg l⁻¹ BA + 0.4 mg l⁻¹ 2,4-D was the most convenient properties (Table 1). Calli grown in medium containing 1 mg l⁻¹ BA and 2 mg l⁻¹ NAA was green, friable and more rapid in terms of growth (Fig. 3b). The calli grown in medium containing 1 mg l⁻¹ BA and 0.4 mg l⁻¹ 2,4-D was yellowish, rapid and friable. These calli grown in different media were used for establishment of cell suspension cultures. Growth medium of cell suspension cultures was the same with callus growth medium but without agar.

3.1. Growth Parameters of Cell Suspension Cultures

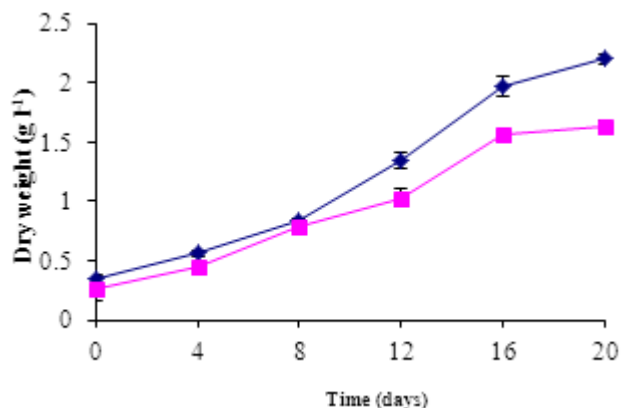


Figure 2. Changes in biomass values of the cell suspension cultures from day 0 to 20. Data are the mean of 3 different measurement

As shown in Figure 2, biomass values of the suspension cell cultures cultured with MS medium containing 1 mg l⁻¹

BA and 2 mg l⁻¹ NAA enhanced with the elongation of culture time from day 0 to 20. Total biomass showed a sharp increase between 8-16th days of cultures. The rate of increase in the culture biomass reached the maximum level on the 20th day. Compared to the first day, on the 20th day, a 6.28-fold increase in the biomass of culture was reported. The medium containing 2,4-D realized sharp increases between 12-16 days and compared to the first day growth rate was 6.31 -fold.

3.2. Quantification of Hypericin Compounds

Calli and cell suspensions were analysed for quantification of hypericin compounds and the data was compared in terms of hypericin contents. Hypericin contents of the samples are shown in Table 2. The level of hypericin in cell suspension cultures was very low compared to callus cultures.

4. Discussion

The calli grown in medium containing 1 mg l⁻¹ BA and 2 mg l⁻¹ NAA (% 92.3±4.63) and 1 mg l⁻¹ BA and 0.4 mg l⁻¹ 2,4-D (% 56.2±3.42) were used for the initiation of cell suspension due to friable and granular structure calli.. Biomass in cell suspension culture was higher in medium containing 2,4-D (6.31 fold) compared to that of NAA (6.28 fold). [24] reported that MS basal media containing BA in combination with 2,4-D showed the highest calli formation, while Walker et al. (2002) [25] reported that BA in combination with 2,4-D was efficient for calli formation. Ayan et al. (2005) [26] obtained that the highest frequency of callus induction obtained from the leaf discs cultivated on the medium supplemented with of 2,4-D and kinetin. According to previous reports, in combinations with 2,4-D and kinetin or BA proved to be effective plant growth regulators for callus induction and cell suspension cultures from *H. perforatum*. But our findings showed that the combinations of NAA and BA was most efficient for calli induction and establish cell suspension cultures for *H. triquetrifolium*.

Hypericin content of calli in MS medium containing 2,4-D were 0.0527 ± 0.0043 mg g⁻¹, while this rate was 0.0018 ± 0.00026 mg g⁻¹ in suspension cells. Hypericin content of calli in the medium containing NAA was 0.0485 ± 0.0077 and in suspension cells, this rate was 0.0016 ± 0.00021. Several studies have been reported on callus and shoot cultures of *H. triquetrifolium*. A similar study on hypericin content in callus cultures of *H. triquetrifolium* Turra. conducted by Oluk et al. (2010) [27] and they detected 0.048 mg g⁻¹ amount of hypericin in calli of *H. triquetrifolium*. In another study, Santarem and Astarita (2003) [28] reported that the level of hypericin in callus of *H. perforatum* was very low, representing only 0.11% of the amount found in the field-grown plants. Kartnig and Göbel (1992) [29] reported that cell cultures of *H. perforatum*, *H. Maculatum* and *H. tomentosum* produced hypericin and pseudohypericin.

Moreover, it has also been reported that in cell cultures of some *Hypericum* species were detected presence of hypericin, but this rate was very low in comparison with *ex vivo* plants [30,31]. It has been reported that callus initiated from stamens of *H. perforatum* showed only traces of hypericin or pseudohypericin [32]. Another result we obtained Although biomass in the MS medium containing NAA was higher than 2,4-D (Fig. 2), in cell suspension culture, hypericin production of medium containing 2,4-D and NAA was close to each other (Table 1). Pasqua et al, (2003) [33] reported that although undifferentiated calli and suspension cultures in MS medium containing 1.105 mgI^{-1} 2,4-D, 0.215 mgI^{-1} Kinetin and 0.186 mgI^{-1} NAA was not produce but callus with vegetative buds produced hypericin (0.015 %) and pseudohypericin (0.050 %).

In our study, spectrophotometric analysis of *in vitro* cultures of *H. triquetrifolium* showed that hypericin contents was different from each other, suggesting that this *in vitro* culture system can be improvement for selection of plants with high levels of hypericin. Some researchers reported the utilization of elicitors influenced the hypericin production in calli and cell suspension cultures. Xu et al. (2011) [34] reported that ozone treatment to cell suspension cultures enhanced hypericin production about 4-fold. presence of salicylic acid in calli and suspension cells, hypericin production enhanced 2-fold [35]. Xu et al (2005) [36] reported that nitric oxide elicitation enhanced hypericin production.

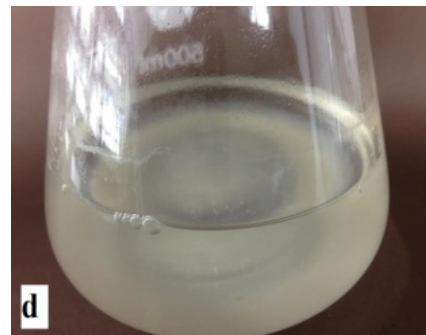
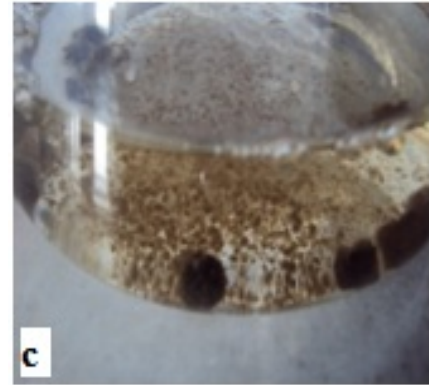
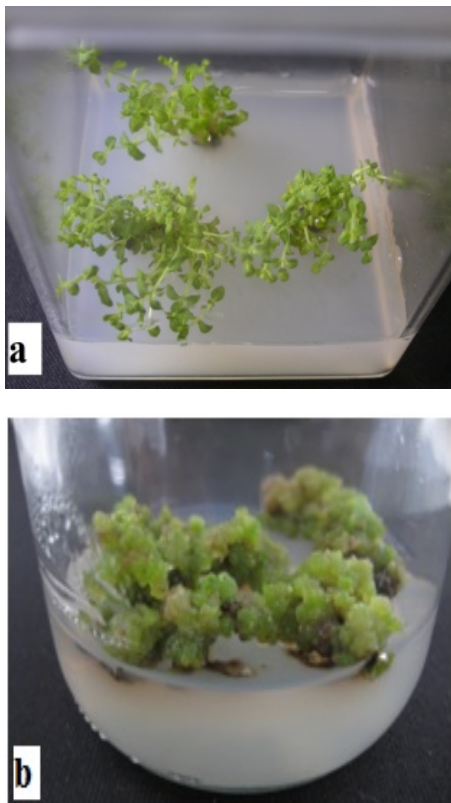


Figure 3. *In vitro* cultures of *H. triquetrifolium*: **a**, axenic leaf explants grown in Murashige and Skoog (MS) salts supplemented with 1 mg l^{-1} 6-benzyl adenine (BA) **b**, granular callus initiation from leaf explants of *H. triquetrifolium* **c**, Initiation of cell suspension cultures **d**, Maintaining of cell suspension cultures **e**, enlarged single cell **f**, small cell cluster.

5. Conclusions

The present study has also shown that cell cultures of *H. triquetrifolium* provide an alternative for hypericin production due to presence of hypericin. However, the commercial application of cell culture for hypericin is limited due to the low production. It needs optimization of the used culture media. To perform this, it needs elisitation of the media.

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REFERENCES

- [1] N. K. B. Robson. Studies in the genus *Hypericum* L. (Clusiaceae). Section 9. *Hypericum sensu lato* (part 3): subsection 1. *Hypericum* series 2. *Senanensia*, subsection 2. *Erecta* and section 9b. *Graveolentia*. Systematics and Biodiversity. 4:19–98, 2006.
- [2] N. K. B. Robson. Studies in the genus *Hypericum* L. (Guttiferae). 1. Infrageneric classification, Bulletin of the British Museum of Natural History (Botany), 5, London British Museum, London :291–355, 1977.
- [3] B. A. M. Messina Herbal Supplements: Facts and Myths-Talking to Your Patients About Herbal Supplements. Journal of Peri Anesthesia Nursing 21: 268-278, 2006.
- [4] P. H. Davis. Flora of Turkey and the East Aegean Islands. 10, Edinburgh University Press, Edinburgh, 1988.
- [5] J. Soelberg, L.B. Jørgensen, A. K. Jäger. Hyperforin accumulates in the translucent glands of *Hypericum perforatum*. Annals of Botany 99:1097–1100, 2007.
- [6] D. Ciccarelli, A. C. Andreucci, A. M. Pagni. Translucent glands and secretory canals in *Hypericum perforatum* L. (Hyperiacaceae): morphological, anatomical and histochemical studies during the course of ontogenesis. Annals of Botany 88:637–644, 2001.
- [7] E. Onelli, A. Rivetta, A. Giorgi, M. Bignami, P. G. Cocucci. Ultrastructural studies on the developing secretory nodules of *Hypericum perforatum*. Flora 197: 92–102, 2002.
- [8] E. Bombardelli, P. Morazzoni. *Hypericum perforatum*. Fitoterapia 66: 43–68, 1995.
- [9] Repcak M, Martonfi P (1997) The localization of secondary substances in *Hypericum perforatum* flower. Biologia 52: 91-94
- [10] R. B. Fornasiero, A. Bianchi, A. Pinetti. Anatomical and ultrastructure observations in *Hypericum perforatum* L. leaves. J Herbs Spices Med Plant 5:21–33, 1998.
- [11] S. M. A. Zobayed, F. Afreen, E. Goto, T. Kozai. Plant–environment interactions: accumulation of hypericin in dark glands of *Hypericum perforatum*. Annals of Botany 98:793–804, 2006.
- [12] P. Agostinis, A. Vantieghe, W. Merlevede, P. A. M. De Witte. Hypericin in cancer treatment: more light on the way. Int J Biochem Cell Biol 34: 221-241, 2002.
- [13] R. C. Guedes, L. A. Eriksson. Theoretical study of hypericin. J Photochem. Photobiol. A-Chem 172:293–299, 2005.
- [14] E. Allan Plant cell culture. In: Stafford A, Warren G (eds), Plant Cell and Tissue Culture, pp. 1-23. Chichester: John Wiley and Sons, 1996.
- [15] G. C. Phillips, J. F. Hubstenberger, E. E. Hansen. Plant regeneration by organogenesis from callus and cell suspension cultures. In:Gamborg OL, Phillips GC (eds), Plant Cell, Tissue and Organ Culture, pp. 67-78. Heidelberg: Springer and Verlag, 1995.
- [16] S. M. A. Zobayed, S. J. Murch, H. P. V. Rupasinghe, J. G. De Boer, B. W. Glickman, P. K. Saxena. Optimized system for biomass production, chemical characterization and evaluation of chemo-preventive properties of *Scutellaria baicalensis* Georgi. Plant Sci 167:439-446, 2004.
- [17] R. Verpoorte, A. Contin, J. Memelink. Biotechnology for the production of plant secondary metabolites. Phytochemistry Rev 1: 13-25, 2002.
- [18] F. Bourgaud, A. Gravot, S. Milesi, E. Gontier Production of plant secondary metabolites: a historical perspective, Plant Sci. 161:839-851, 2001.
- [19] A. Coste, L. Vlase, A. Halmagyi, C. Deliu, G. Coldea. Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tissue Organ Cult 106:279-288, 2011.
- [20] A. Kirakosian, D. Gibson, T. A. Sirvent. Comparative survey of *Hypericum perforatum* plants as sources of hypericins and hyperforin. Journal of Herbs Spices and Medicinal Plants 56:813-821, 2003.
- [21] X. N. Liu, X. Q. Zhang, J. S. Sun. Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. Plant Growth Regul 53:207–214, 2007.
- [22] C. D. Palmer, W. A. Keller Plant regeneration from petal explants of *Hypericum perforatum* L. Plant Cell Tiss. Organ Cult. 105:129-134, 2011.
- [23] T. Murashige, F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473–497, 1962.
- [24] F. R. Pretto, E. R. Santarém. Callus formation and plant regeneration from *Hypericum perforatum* leaves. Plant Cell Tiss. Organ Cult 62:107-113, 2000.
- [25] T. S. Walker, H. P. Bais, J. M. Vivanco. Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John’s wort). Phytochem 60:289-293, 2002.
- [26] A. K. Ayan, C. Çırak, K. Kevseroğlu, A. Sökmen. Effects of Explant Types and Different Concentrations of Sucrose and Phytohormones on Plant Regeneration and Hypericin Content in *Hypericum perforatum* L. Turk J Agric For

- 29:197-204, 2005.
- [27] E. A. Oluk, S. Orhan, Ö. Karakaş, A. Çakır, A. Gönüz. High efficiency indirect shoot regeneration and hypericin content in embryogenic callus of *Hypericum triquetrifolium* Turra. African Journal of Biotechnology 9:2229-2233, 2010.
- [28] E. R. Santarem, L. V. Astarita. Multiple shoot formation in *Hypericum perforatum* L. And hypericin production. Brazilian journal of plant physiology, 15:43-47, 2003.
- [29] T. Kartnig, I. Göbel. Hypericin and pseudohypericin in cell cultures of various *Hypericum* species and chemotypes. Planta Med 58: 579, 1992.
- [30] T. Kartnig, A. Brantner. Secondary constituents in cell cultures of *Hypericum perforatum* and *Hypericum maculatum*. Planta Medica 56: 634-637, 1990.
- [31] T. Kartnig, I. Göbel, B. Heydel. Production of hypericin, pseudohypericin and flavonoids in cell cultures of various *Hypericum* species and their chemotypes. Planta Medica 62:51-53, 1996.
- [32] A. B. Kirakosian, R. R. Vardapetyan. Charchoglyan A. G.. The content of hypericin and pseudohypericin in cell cultures of *Hypericum perforatum*. Russian Journal of Plant Physiology, 47: 270-273, 2000.
- [33] G. Pasqua, A. Pinarosa, B. Monacelli, A. R. Santamaria, M. P. Argenti. Metabolites in cell suspension cultures, calli, and in vitro regenerated organs of *Hypericum perforatum* cv Topas. Plant Science 165:977-982, 2003.
- [34] M. Xu, B. Yang, J. Dong, D. Lu, H. Jin, L. Sun, Y. Zhu, X. Xu. Enhancing hypericin production of *Hypericum perforatum* cell suspension culture by ozone exposure. Biotechnology Progress 27:1101-1106, 2011.
- [35] S. Gadzovska, S. Maury, A. Delaunay, M. Spasenoski, D. Hagege, D. Courtois, C. Joseph. The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphthodianthrones and phenylpropanoids in *Hypericum perforatum* L. Plant Cell Tiss Organ Cult 113:25-39, 2013.
- [36] M. Xu, J. Dong, G. Zhang. Enhancement of hypericin production and cell growth of *Hypericum perforatum* L. suspension culture by nitric oxide. Chin J Biotechnol 21:66-70, 2005.