Medicinal & Aromatic Plants
ISSN: 2167-0412

Al-Hawamdeh et al., Med Aromat Plants 2013, S1
DOI: 10.4172/2167-0412.S1-001

Research Article Open Access

In vitro Production of Silymarin from *Silybum marianum* L.

Fayha M. Al-Hawamdeh[1], Rida A. Shibli[1]* and Tamara S. Al-Qudah[2]

[1]*Faculty of Agriculture, Department of Horticulture and Agronomy, University of Jordan, Amman, Jordan*
[2]*Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Amman, Jordan*

Abstract

Silybum marianum L. is a wild medicinal herbal plant in Jordan. It is widely used in folk medicine due to its high content of silymarin. *In vitro* production of silymarin was experimented at different concentrations (0.0, 0.4, 1.0, 1.6 or 2.0 mg/l) of growth regulators (6-furfurylaminopurine (kinetin), 6-benzylaminopurine (BA), or 6-(gamma,gamma-Dimethylallylamino) purine (2ip)) and different concentrations (15, 30,45,60 mg/l) of carbon sources (glucose, fructose, and sucrose). HPLC (High Performance Liquid Chromatography) analysis was used to identify silymarin components. *In vitro* grown *S. marianum* on MS (Murashige and Skoo 1962) medium supplemented with (1.6 mg/l) kinetin and (0.1 mg/l) 1-Naphthaleneacetc acid (NAA) gave the highest silymarin content of (0.84%) silybin and (0.49%) silydanin as compared with cultures grown on hormone-free MS media which contained (0.36%) silybin and (0.30%) silydanin. *In vitro* grown *S. marianum* on MS medium supplemented with (2.0 mg/l) BA and 0.1 mg/l NAA yielded (0.67%) silybin and (0.37%) silydanin, while MS media supplemented with (1.0 mg/l) of 2iP gave (0.72%) silybin and (0.24%) silydanin. Otherwise, the *in vivo* (wild) grown shoots of *S. marianum* gave (1.07%) for silybin and (0.46%) sildyanin. Among carbon source, glucose at (45 g/l) gave (1.63%) of silymarin content. Results indicated a significant use of *in vitro* grown cultures for silymarin production.

Keywords: Carbon source; HPLC; *In vitro*; Plant growth regulators (PGR); Silymarin

Introduction

Medicinal plants comprise about 485 species of wild plants which are categorized according to their families and genera. The importance of these wild plants resides because of their secondary products which are used in folk medicine and pharmaceutical industries [1]. Medicinal plants are distributed in all arid and semi-arid regions.

Milk thistle *(Silybum marianum* L.) is an important medicinal plant that has volatile oils and other secondary metabolites. The most common compound in milk thistle is silymarin, which is an isomeric mixture of flavonolignans (silybin, silychristin, and silydanin). Silymarin acts as a strong anti-hepatotoxic, which has been used for chronic inflammatory liver disease and liver cirrhosis [2].

In vitro production of plant secondary metabolites has been considered as a good solution for economic production of these chemicals because it alternated conventional methods with a high-yielding cell culture and secondary metabolites [3]. Plant secondary metabolites which have been extracted from *in vivo* grown plants were used for a long time but the general trend new is to extract these secondary metabolites from *in vitro* grown plants [4,5]. We cannot ignore the importance of using tissue culture techniques which gives the ease of almost all of plant researches [6], also there are so many other reasons such as protection from weather [7] and from diseases, pests, soil problems and obtaining mass quantities with low cost [8]. Plant-derived chemicals can be obtained from callus, cell and cell suspension cultures [9-11] as well as from plant leaves or flowers.

Wichtl [12] reported that silymarin compounds were used in liver disorders treatment for more than 2000 years. Silymarin extract is now an important hepatoprotective agent, and is used in the treatment of hepatitis and other liver diseases, as well as liver damage due to alcohol and drug abuse [13]. Silymarin is formed by an oxidative coupling reaction between the flavonoid taxifolin and a phenylpropanoid, and belongs to a group of compounds termed as flavonolignans. Silybin and silydanin of mature seeds from Jordanian wild plants contents were determined against external reference standards using high performance liquid chromatography (HPLC) [14]. Two pairs of diastereoisomeric flavonolignans, silybin A, silybin B, isosilybin A, and isosilybin B, were successfully separated from *Silybum marianum* by sequential silica gel column chromatography, preparative reversed-phase HPLC, and recrystallization [15].

This study was conducted to determine the effect of multiple growth regulators and different carbon sources on silymarine content in *Silybum marianum in vitro.*

Materials and Methods

Milk thistle seeds were germinated on hormone free MS [16] (Murashige and Skoog 1962 medium), after surface sterilization as described by Al Hawamdeh [17], then shoots were multiplied on MS medium supplemented with 6-furfurylaminopurine (kinetin) at 0.5 mg/L and 0.1 mg/L of 1-Naphthaleneacetc acid (NAA). Cultures were transferred to growth room and maintained under daily light regime of 16-h (photosynthetic photon flux density (PPFD)=40-45 µ mol. m^{-2} sec^{-1}) light, 8-h dark at 24 ± 1°C.

In vitro cultured plants were cultured on the surface of MS media supplemented with different cytokinin (6-furfurylaminopurine (kinetin), 6-benzylaminopurine (BA), or 6-(gamma,gamma-Dimethylallylamino) purine (2ip)) concentrations (0.0, 0.4, 1.0, 1.6 or 2.0 mg/L), and 0.1 mg/L NAA. Plant samples with five replicates were taken from each treatment at each concentration to perform chemical analysis for silybin and silydanin.

***Corresponding author:** Rida A. Shibli, Faculty of Agriculture, Department of Horticulture and Agronomy, University of Jordan, Amman, Jordan, Tel: 962 6 535 5000; Fax: 962 6 535 5577; E-mail: r.shibli@ju.edu.jo

Received January 30, 2013; **Accepted** March 13, 2013; **Published** March 15, 2013

Citation: Al-Hawamdeh FM, Shibli RA, Al-Qudah TS (2013) *In vitro* Production of Silymarin from *Silybum marianum* L.. Med Aromat Plants S1: 001. doi:10.4172/2167-0412.S1-001

In another experiment, microshoots were subcultured on MS media supplemented with different carbon sources (sucrose, glucose, fructose) at (15, 30, 45, 60) g/L. Data were recorded after one month for silymarin (silybin and silydanin) % content.

Wild *Silybum marianum* samples were collected from Jerash-Jordan, in January 2011. Silymarin content was tested from whole plant; this test was carried out to compare silymarin content for both *in vivo* and *in vitro* samples.

Silymarin analysis

Approximately 100 mg of finely groundeach sample of *in vitro* microshoots and in vivo samples of milk thistle were weighed. The powder was transferred into an extraction thimble and subjected to continuous-extraction with hexane. Then the sample was diluted to final volume (50-ml) with methanol. Each sample was diluted 1:1 with HPLC grade methanol and filtered into an HPLC vial.

The HPLC column was YMC-Pack™ ODS-A C18, 5 µm, 4.6×150 mm and detection is UV at 288 nm. Column temperature: 40°C, Flow rate: 1.0 ml/minute, Mobile phase: gradient. Eluent A, Methanol (200 ml) was mixed with 800 mL of water and 5 mL of phosphoric acid was added into 995 ml of the MeOH:Water (20:80). Eluent B, Methanol (800 ml) was mixed with 200 ml of water and 5 mL of phosphoric acid was added into 995 ml of the MeOH:Water (80:20). Individual "Flavonolignan"%=(C)(FV)(D)(100%)/(W), where: C=individual silymarin concentration (mg/ml) as determined from extrapolation of the linearity curve. FV=final volume of the sample preparation (ml). D=dilution factor of the sample preparation (if needed). W=sample weight (mg).

Growth Regulator concentration (mg/L)	Silybin content % (w/w)	Silydanin content % (w/w)	Silymarin content % (w/w)
Kinetin			
C1 [x]	0.36 d[z]	0.30 d	0.66 d
C2 [x]	0.35 d	0.29 d	0.64 d
0.4	0.59 c	0.40 c	0.99 c
1.0	0.66 b	0.44 b	1.09 b
1.6	**0.84 a**	**0.49 a**	**1.33 a**
2.0	0.64 bc	0.42 bc	1.06 b
BA			
C1 [x]	0.29 e[z]	0.12 d	0.41 e
C2 [x]	0.27 e	0.14 d	0.41 e
0.4	0.36 d	0.21 c	0.57 d
1.0	0.47 c	0.28 b	0.75 c
1.6	0.58 b	0.31 b	0.89 b
2.0	**0.67 a**	**0.37 a**	**1.04 a**
2iP			
C1 [x]	0.47 d[z]	0.14 c	0.60 d
C2 [x]	0.50 cd	0.12 c	0.61 d
0.4	0.59 bc	0.21 ab	0.80 bc
1.0	**0.72 a**	**0.24 a**	**1.00 a**
1.6	0.67 ab	0.19 b	0.86 ab
2.0	0.62 ab	0.13 c	0.74 b

[x]Control treatment (C1 and C2 represent control treatments (without and with 0.1 mg/L NAA) respectively). [z]Means within columns for each cytokinin having different letters are significantly different according to Tukey HSD at P ≤ 0.05

Table 1: Effect of different kinetin, BA, or 2ip concentrations on silybin, silydanin, and total silymarin content of *in vitro* grown *Silybum marianum* L.

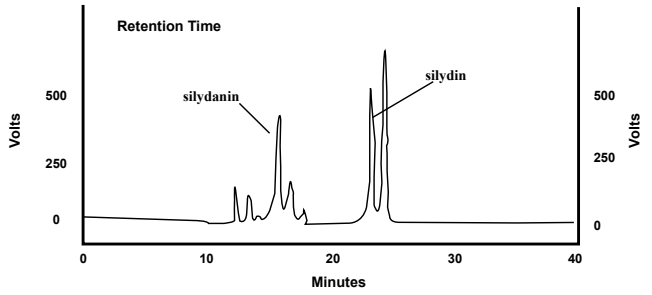

Figure 1: HPLC chromatogram for the silymarin content from microshoots of *in vitro* grown *Silybum marianum* on MS media supplemented with 1.0 mg/L kinetin and 0.1 mg/L NAA.

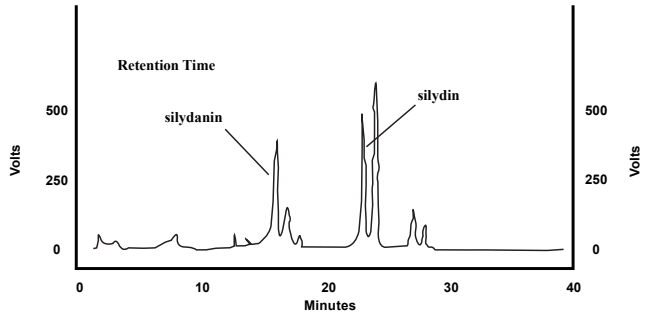

Figure 2: HPLC chromatogram for the silymarin content from microshoots of *in vitro* grown *Silybum marianum* on MS media supplemented with 2.0 mg/L BA and 0.1 mg/L NAA.

Satistical analysis

Complete Randomized Design (CRD) was used to arrange all treatments involved in silymarin content. Each treatment was replicated five times. Observations were averaged within the experimental unit. The obtained results were statistically analyzed by using SPSS analysis system. Analysis of variance (ANOVA) was used to analyze the obtained results. Mean separation was done with probability level of 0.05 according to the Tukey's HSD (honestly significant difference) test.

Results

Table 1 and figure 1 show the effect of kinetin on the content of silymarin compounds. MS medium supplemented with 1.6 mg/L kinetin and 0.1 mg/L NAA gave the maximum percentage for both silybin (0.84%) and silydanin (0.49%) and total silymarin content (1.33%). Increasing kinetin concentrations more than 1.6 mg/l reduced the production of silymarin, control treatment did not give any significant difference in silymarin production. Silybin and silydanin (silymarin content) increased with increased BA concentration as compared to the control as shown in table 1 and figure 2. MS medium supplemented with 2.0 mg/L BA and 0.1 mg/L NAA gave the highest silybin (0.67%), silydanin (0.37%), and silymarin (1.04%) content.

For 2iP, the maximum percentages for both silybin (0.72%) and silydanin (0.24%) were obtained at 1.0 mg/L 2iP and 0.1 mg/L NAA. Table 1 and figure 3 show the effect of 2iP on the content of silymarin compounds. Higher concentration of 2iP more than 1.0 mg/L reduced the production of all silymarin compounds.

Glucose was found to be a better carbon source than sucrose and fructose (Table 2) in terms of their effects on silymarin production.

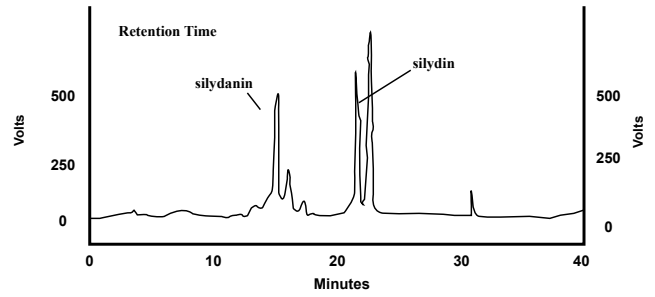

Figure 3: HPLC chromatogram for the silymarin content from in microshoots of *in vitro* grown *Silybum marianum* on MS media supplemented with 1.0 mg/L 2iP and 0.1 mg/L NAA.

concentration (mg/L)	Silybin content % (w/w)	Silydanin content % (w/w)	Silymarin content % (w/w)
Glucose			
15	0.33 d[z]	0.30 d	0.63 d
30	0.91 b	0.55 b	1.46 b
45	**0.99 a**	**0.64 a**	**1.63 a**
60	0.46 c	0.41 c	0.87 c
Sucrose			
15	0.42c[z]	0.30 c	0.72 d
30	0.75 b	0.57 a	1.32 b
45	**0.81 a**	**0.59 a**	**1.40 a**
60	0.46 c	0.38 b	0.84 c
Fructose			
15	0.40 c[z]	0.29 c	0.69 c
30	0.87 a	**0.55 a**	1.42 a
45	**0.91 a**	0.51 ab	**1.42 a**
60	0.51 b	0.48 b	0.99 b

[z]Means within columns having different letters for each carbon source are significantly different according to Tukey HSD at P ≤ 0.05.

Table 2: Effect of different carbon sources on silybin, silydanin, and total silymarin content of *in vitro* grown *Silybum marianum* L.

Both of silybin and silydanin gave the maximum amounts of 0.99% silybin and 0.64% of silydanin, and 1.63% of total silymarin content at 45g/L of glucose. On the other hand, fructose gave 0.91% at 45 g/L of silybin and 0.55% of silydanin at 30 g/L, and 1.42% of total silymarin content at both concentrations 30 g/l and 45 g/l. In addition, sucrose gave 0.81% for silybin and 0.59 % for silydanin, and 1.40% of total silymarin content.

In vivo (wild) *Silybum marianum* samples which were collected from Jordan in January 2011 showed higher percentage of silymarin yield than *in vitro* samples. Silymarin content which was obtained from *in vivo* grown leaves gave a high percentage yield (1.07%) for silybin and (0.46%) for silydanin (Figure 4). For *in vitro* grown microshoots, the sample which was grown on solid MS media supplemented with 1.6 mg/L kinetin and 0.1 mg/L NAA showed the highest yield (0.84%) for silybin and (0.49%) for silydanin and (1.33%) of silymarin as compared to *in vivo* samples.

Discussion

In the literature, the effect of different cytokinins on plant secondary metabolites production has been studied. In a review published in 2004, kinetin found to be the most common growth regulator used for secondary metabolites production in more than 30 different plant species [18]. Also, Sudria et al. [19] reported that auxin and cytokinin incorporated into culture medium have a marked influence on the production of secondary metabolites. Arikat et al. [4] found that the percentage yield of the oil of *Salvia fructicosa* Mill which was grown on MS medium containing (0.02 mg/L NAA and 0.5 mg/L BA and 0.03 mg/L GA_3) was (0.7%). Furthermore, Al-Qudah et al. [20] indicated that *in vitro T. polium* which was grown on solid MS media supplemented with 0.5 mg/L BA and 0.1 mg/L NAA showed a high percentage oil yield of 0.40% (w/w). Besides that, Harpagoside (an anti-inflammatory iridoid glucoside) content was maximized in micropropagated shoots of *Scrophularia yoshimurae* on MS medium supplemented with 10 mg/L BA and 0.2 mg/L NAA [18]. In *Withaina somnifera*, the active ingredient (withaferin) was maximized in the *in vitro* shoots grown on MS medium supplemented with 1.0 mg/L BA and 3% sucrose [21]. Also, In *Mentha arvensis*, the active ingredient terpenoid was maximized in the *in vitro* shoots grown on MS medium supplemented with 5.0 mg/L BA and 0.5 mg/L NAA [22]. The content of gentiopicroside and swertiamarin was detected in the aerial and underground parts of the *in vitro* shoots of *Gentiana davidii* grown on MS medium supplemented with 2.0 mg/L BA and 0.2 mg/L NAA [18]. Al-Qudah et al. [20] explained that microshoots of *Teucrium polium* L. showed a greater amount of β-caryophyllene than *in vivo* plants, which may be related to the significant effect of BA in the culture medium on the biosynthesis and the level of β-caryophyllene. Tawfik et al. [23] and Arikat et al. [4] found that BA gave significant effect in the production of camphor in the *in vitro* grown shoots of *Salvia officinalis* and *Salvia fructosia* Mill, respectively. In higher plants, growth regulators were used to produce secondary metabolites via tissue culture, 2iP was used in *Simmondsia chinensis* to produce fixed oil [24].

Carbon sources (sugars; such as sucrose, glucose and fructose) had been studied previously for their effects on secondary metabolites production in plants. For example, Rosmarinic acid (RA) was extracted from *in vitro Salvia fruticosa* Mill and it was maximized at 40g/L of sucrose [4]. A study of the effect of sucrose concentration and inoculums size in *Perilla frutescens* showed that the highest pigment production of 5.8 g/L was attained after 10 days of cultivation at an initial sucrose concentration of 45 g/ L [25]. These amounts of cell mass and anthocyanin pigments were 3.3 and 24 times higher than those at an initial sucrose concentration of 15 g/L and inoculum size of 15 g wet cells/L, respectively [26]. In a research carried out to show the effect of ABA and sugar on anthocyanin formation in grape berry cultured *in vitro*, results have shown that the effect of carbon source on using 10% of sucrose, fructose, glucose and rhamnose was more significant on increasing anthocyanin production than effect of combination of these sugars with ABA [27].

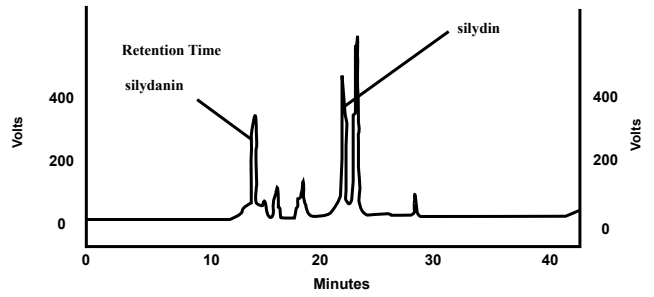

Figure 4: HPLC chromatogram for the silymarin content from *in vivo* grown plant sample of *Silybum marianum*.

In vivo (wild) *Silybum marianum* samples have different percentages of silymarin yield than *in vitro* samples. Both silybin and silydanin content were differing between different wild populations of *Silybum marianum* [14]. Silybin contents varied between the populations and it ranged from 0.05% to 1.14% as presented by Zatimeh [14]. Shokrpour et al. [27] in Iran compared silybin in wild milk thistle populations and introduced varieties. They indicated that the native accessions had highest silybin concentrations (ranged between 0.24-1.10% w/w) than the foreign varieties. Al-Rifaee [28] found that hypericin content significantly varied (0.03 to 0.14%) among wild populations of *Hypericum triquetrifolium*.

Conclusions

Silybin and silydanin content analysis indicated that silymarin from *in vivo* grown shoots showed higher percentage than *in vitro* samples. *In vitro* samples which were grown on solid MS media supplemented with 1.6 mg/L kinetin and 0.1 mg/L NAA showed higher percentage of silybin and silydanin than the other *in vitro* sample which was grown on hormone-free MS media. In studying carbon source effect on the silymarin content, glucose gave the largest amount of silymarin content (1.63%). *In vitro* grown *Silybum marianum* L. can be considered for silymarin production if cultured on MS medium supplemented with suitable growth regulators.

Acknowledgement

Authors would like to thank the Deanship of Research at University of Jordan for funding this study.

References

1. Al-Eiswai D, El-Oqlah A, Oran S, Laham J (1994) Plant biodiversity in Jordan. In: Plant genetic resources of Jordan, Jaradat AA, (ed.). International Plant Genetic Resources Institute (IPGRI). Proceeding of National Seminar. Amman, Jordan. 2: 13-51.
2. Corchete P (2008) *Silybum marianum* (L.) Gaertn: the Source of Silymarin. In: Bioactive Molecules and Medicinal Plants, Ramawat KG, Mérillon JM (eds.) 123-148.
3. Gopi C, Vatsala TM (2006) *In vitro* studies on effects of plant growth regulators on callus and suspension culture biomass yield from *Gymnema sylvestre R.Br.* African Journal of Biotechnology 5: 1215-1219.
4. Arikat NA, Jawad FM, Karam NS, Shibli RA (2004) Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill). Sci Hortic 100: 193-202.
5. Lila MA (2005) Valuable secondary products from *in vitro* culture. Plant Development and Biotechnology. CRC Press 285-289.
6. Conger BV (1981) Cloning agricultural plants via *in vitro* techniques. CRC Pess, Inc, Boca Raton, Florida 273.
7. Pierik RLM (1997) *In vitro* Culture of Higher Plants. Kluwer Academic Publishers, Netherlands.
8. Shibli RA, Ajlouni MM, Abu-Ein A (1994) Production of virus free 'Spunta' (*Solanum tuberosum*) potato via tissue culture: Factors affecting minituber production. Damascus University Journal 10: 27-38.
9. Shibli RA, Smith MAL, Kushad M (1997) Headspace ethylene accumulation effects on secondary metabolite production in *Vaccinium pahalae* cell culture. Plant Growth Regulation 23: 201-205.
10. Shibli RA, Smith MAL, Shatnawi MA (1998) Pigment recovery from encapsulated-dehydrated *Vaccinium pahalae* (ohelo) cryopreserved cells. Plant Cell Tissue Organ Cult 55: 119-123.
11. Al-Sane KO, Shibli RA, Freihat NM, Hammouri MK (2005) Cell suspension culture and secondary metabolites production in African violet (*Saintpaulia ionantha* Wendl.). Jordan Journal of Agricultural Sciences 1: 84-92.
12. Wichtl M (1994) Herbal drugs and phytopharmaceuticals. Boca Raton, FL, CRC Press.
13. Martin RJ, Lauren DR, Smith WA, Jensen DJ, Deo B, et al. (2006) Factors influencing silymarin content and composition in variegated thistle (*Silybum marianum*). New Zealand Journal of Crop and Horticultural Science. 34: 239-245.
14. Zatimeh A (2010) Diversity analysis and conservation of Milk thistle (*Silybum marianum* (L.) Gaertn. Populations in Jordan. Ph.D., Dissertation. University of Jordan, Amman, Jordan.
15. Lee DY, Liu Y (2003) Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, isolated from *Silybum marianum* (milk thistle). J Nat Prod 66: 1171-1174.
16. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.
17. Al Hawamdeh F (2012) *In vitro* propagation and silymarin production of Milk thistle *Silybum marianum* (L.) Gaertn. Msc.Thesis. University of Jordan, Amman, Jordan.
18. Vanisree M, Lee CY, Shu-Fung L, Nalawade SM, Yih Lin C, et al. (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot Bull Acad Sin 45: 1-22.
19. Sudria C, Pinol MT, Palazonn J, Cusido RM, Vila R, et al. (1999) Influence of plant growth regulators on the growth and essential oil content of cultured Lavandula dentate plantlets. Plant Cell Tissue Organ Cult 58: 177-184.
20. Al-Qudah TS, Shibli RA, Alali FQ (2011) *In vitro* propagation and secondary metabolites production in wild germander (*Teucrium polium* L.). *In Vitro* Cell Dev Biol Plant 47: 496-505.
21. Ray S, Jha S (2001) Production of withaferin A in shoot cultures of *Withania somnifera*. Planta Medica 67: 432-436.
22. Phatak SV, Heble MR (2002) Organogenesis and terpenoid synthesis in *Mentha arvensis*. Fitoterapia 73: 32-39.
23. Tawfik AA, Read PE, Cuppet S (1991) Stimulation of Growth and Monoterpene Production of Sage (*Salvia officinalis*) by benzyladenine *in vitro*. Plant Growth Regulators 20: 201-222.
24. Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. Journal of Medicinal Plants Research 3: 1222-1239.
25. Zhong J, Yoshida T (1995) High-density cultivation of *Perilla frutescens* cell suspensions for anthocyanin production: Effects of sucrose concentration and inoculum size. Enzyme Microb Technol 17: 1073-1079.
26. Hiratsuka S, Onoderaa H, Kawaia Y, Kuboa T, Itohb H, et al. (2001) ABA and sugar effects on anthocyanin formation in grape berry cultured *in vitro*. Sci Hortic 90: 121-130.
27. Shokrpour M, Moghaddam M, Mohammadi SA, Ziai SA, Javanshir A (2007) Genetic properties of milk thistle ecotypes from Iran for morphological and flavonolignans characters. Pak J Biol Sci 10: 3266-3271.
28. Al-Rifaee M (2008) Diversity and conservation of *Hypericum triquetrifolium* turra and its potential cultivation in Jordan. Ph. D, Dissertation, University of Jordan, Amman, Jordan.

This article was originally published in a special issue, **Natural Medicinal Products and Secondary Metabolites** handled by Editor(s). Dr. Chandan S Chanotiya, CSIR-Central Institute of Medicinal and Aromatic Plants, India