J. Appl. Environ. Biol. Sci., 1(8)158-163, 2011

ISSN 2090 – 424X
Journal of Applied Environmental and Biological Sciences
www.textroad.com

In vitro Production of Some Phenolic Compounds from *Ephedra alata* Decne.

Ghada Abd El-Moneim Hegazi[1*] and Taghried Mohammed El-Lamey[2]

[1]Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, 11753 El-Matarya, 1 Mathaf El-Matarya St., Cairo, Egypt.
[2]Ecophysiology Unit, Plant Ecology and Range Management Department, Desert Research Center, 11753 El-Matarya, 1 Mathaf El-Matarya St., Cairo, Egypt.

ABSTRACT

The production of five medicinally important phenolic compounds from *Ephedra alata* Decne. callus cultured on MS medium supplemented with 1 mg/l of both 2,4- dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn) and different phenylalanine (L-phe) and casein hydrolysate (CH) concentrations, was investigated. The stress conditions under which the plant lives, which cause the production of these valuable secondary metabolites were also studied. The greatest amounts of phenolic compounds (chlorogenic acid, rutin, catechin, quercetin, coumaric acid) were obtained from callus maintained in the media containing CH, these media produced higher amounts of phenolic compounds as compared to that in the stem of the wild plant. This protocol could be applied to improve the yield of these compounds and is a promising alternative for direct extraction from plants grown in natural habitat, which help in the conservation of natural plant resources.

Key Words : *Ephedra alata*, phenolic compounds, callus, *in vitro*, Phenylalanine, Casein hydrolysate.

Abbreviations: CH - Casein Hydrolysate; CGA - Chlorogenic acid; 2,4-D - 2,4- Dichlorophenoxyacetic acid; Kn - Kinetin; L-Phe - L-Phenylalanine; MS - Murashige and Skoog; PGR - Plant Growth Regulator.

INTRODUCTION

Plants are a tremendous source for the discovery of new products of medicinal value for drug development. Today, several distinct chemicals derived from plants are important drugs currently used in one or more countries in the world. Phenolic compounds are secondary metabolites generally involved in plant adaptation to environmental stress conditions such as infection by microbial pathogens, mechanical wounding and excessive ultraviolet or high visible light levels (Haard and Chism, 1996). Most of these compounds have received considerable attention as potentially protective factors against human chronic degenerative diseases (cataracts, macular degeneration, neurodegenerative diseases, and diabetes mellitus), cancer and cardiovascular diseases (Scalbert et al., 2005). Also, these compounds have a number of beneficial health properties related to their potent antioxidant activity as well as hepatoprotective, hypoglycemic and antiviral activities (Farah and Donangelo, 2006). Phenolic compounds are a large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants. These phenolics are the most abundant secondary metabolites in plants and can be classified into non-soluble compounds such as condensed tannins, lignins, cell-wall bound hydroycinammic acids, and soluble compounds such as phenolic acids, phenyl propanoids, flavonoids and quinones. All these groups are involved in many processes in plants (Farah and Donangelo, 2006). Phenolics have several function in plants as antibiotics, natural pesticides, attractant for pollinator, protective agents against ultra violet (UV) light (Heldt, 1997) growth inhibition, allelopathy and protect against vascular disorders, which may be caused by oxidative damage of cell membranes. The mechanism of their action, could be related to their antioxidant function (Quartacci and Navaro-Izzo, 1992).

Ephedra alata Decne. belongs to family Ephedraceae, the common name is ephedra and the Chinese name is ma huang. There are 40 species within the family Ephedraceae that grow in China, India, Egypt, the Middle East, Europe and the Americas. They are an evolutionary primitive group of shrubs that grow on dry, rocky or sandy terrain in desert or arid areas.

The *Ephedra* plant is strongly aromatic, with a bitter taste. The dried stem is the part of the shrub that is used. It is available in bulk herb, capsules, hydroalcoholic extract and is often found in weight loss and energy formulas. *Ephedra* is approved for diseases of the respiratory tract with mild bronchospasms (Blumenthal et al., 1998). It is commonly used as a bronchodilator and anti-asthmatic. It has been used in traditional Chinese medicine for 5,000 years to treat allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion. *Ephedra* has been a natural

***Correspondence Author:** Ghada Abd El-Moneim Hegazi, Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, 11753 El-Matarya, 1 Mathaf El-Matarya St., Cairo, Egypt. Telephone no.: (+2012) 7550486 Email: hegazighada@yahoo.com

product source of many constituents include, alkaloids, tannins, saponins, proanthocyanidins, phenolic acids, flavonoids and essential oils (O'Dowd et al., 1998). Plants-derived polyphenols are of great importance for their potential antioxidant and antimicrobial properties. Phenolic compounds exhibit a considerable free-radical scavenging (antioxidant) activity. Antioxidants are important in the prevention of human diseases; therefore, the importance of search for natural antioxidants has greatly increased in the recent years (Zollman and Vickers, 1999), because some synthetic antioxidants have shown potential health risk and toxicity ,most notably possible carcinogenic effect (Safer and Al-Nughamish, 1999). Therefore, it is of great importance to find new sources of safe and inexpensive antioxidants of natural origin in order to use them in foods and pharmaceutical preparation to replace synthetic antioxidants.

Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics. According to the literature; the callus has the potential to show secondary metabolites activity and can often be compared in this respect with the original plant. Callus culture of *Epherda* species for the induction and elevation of these important secondary metabolites are becoming increasingly important for medicine and food industry. Phenolics producing callus cultures has been reported earlier in *Ephrdra strobiliacea*, *Ephedra procera* and *Ephedra pachyclada* species (Parsaeimehr et al., 2010).

In the present paper, an experiment was conducted for a comparative determination of the production of five medicinally important phenolic compounds from *E. alata* wild plant and its callus tissue, cultured on medium supplemented with different concentrations of L-Phenylalanine (L-Phe) as a precursor, or casein hydrolysate (CH) as an elicitor, callus tissue growing on the original medium served as a comparative control. Aiming to improve the production of these compounds, which mainly act as natural antioxidants. In addition to studying the stress conditions under which the plant live, which cause the production of these valuable secondary metabolites.

MATERIALS AND METHODS

1. Ecological studies:

The mean values of climatic factors for the studied area; Wadi El-Bagha, about 5 Km south west of Wadi Sudr, in Sinai Peninsula, were obtained from the Applied Agricultural Meteorological Laboratory, Ras Sudr Experimental Station of Desert Research Center, during the period of samples collection (2008-2009). Chemical and physical analyses of the soil supporting *E. alata* plant were studied (Page, 1987).

2. Collection of plant material

Stems of *E. alata* were collected from mature plants grown in their natural habitat; Wadi El-Bagha, about 5 Km south west of Wadi Sudr, in Sinai Peninsula.

3. Plant callus cultures

3.1. Disinfection of plant material

Stem explants of *E. alata* were washed under running tap water followed by a detergent for 5 min. The surface of stems were sterilized in 20% (v/v) commercial bleach solution (Clorox) (containing 1% sodium hypochlorite) for 20 min, then rinsed 4-5 times with sterile distilled water. Subsequently, the wound sites exposed to the sterilization agent were trimmed and 0.8 ± 0.2 cm internodes were separated under a laminar air flow hood.

3.2. Culture conditions and callus induction

Sterilized stem explants of *E. alata* were produced callus on Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, the Netherlands) (Murashige and Skoog, 1962) containing 30 g/l sucrose, 2,4-dichlorophenoxy acetic acid (2,4-D), as an auxin, at a concentration of 1 mg/l and Kinetin (Kn) as a cytokinin at a concentrations of 1 mg/l [Plant growth regulators (PGRs); Sigma Cell Culture, min. 90%, St. Louis, USA]. The pH of the medium was adjusted to 5.7-5.8 with 0.1N HCL and 0.1N NaOH, and solidified with 2.5 g/l phytagel (Duchefa, Haarlem, the Netherlands) before autoclaving at a pressure of 1.06 Kg cm^{-2} and 121˚C for 15 min. Subcultures were continued every 60 days, and the callus was incubated under fluorescent light (2500-3000 lux) at 25°C ± 2 with a 16-h photoperiod.

3.3. Precursor feeding and elicitation

An amino acid precursor; L-phenylalanine (L-Phe) (Sigma Cell Culture, St. Louis, USA) at concentrations of 12.5, 25, 50 and 100 mg/l, and an elicitor; casein hydrolysate (CH) (OXOID, OXOID Limited, Hampshire, England) at concentrations of 0.5, 1, 1.5 and 2 g/l, were selected in order to improve the yield of the phenolic compounds in the callus cultures. The control medium was made containing 1 mg/l of both 2,4-D and Kn, without additives. Growth of the callus

tissue and phenolics content were measured. For the measurement of callus growth, callus tissues were oven dried at 35˚C for 48 hours, and the fresh and dry weights were recorded (Hegazi and El-Lamey, 2011).

3.4. Extraction and determination of phenolic compounds

Phenolic compounds were extracted from dry powdered samples of callus and stem of *E. alata* in 70% methanol (HPLC grade), and shaken for 15 min at room temperature. After centrifugation (3,000 rpm, 5 min), the supernatant was filtered on 0.45 µm Whatman filter paper. The pellet was then re-extracted with 5 ml of 70% methanol and finally rinsed with 5 ml of 100% methanol. All three supernatants were pooled together before removal of the methanol under vacuum with a rotary-evaporator at 70˚C. When the methanol has been evaporated, the extract was concentrated and resuspended in 70% methanol. Samples were analyzed by high performance liquid chromatography (HPLC) (Agilent 1100 series), coupled with UV-Vis detector G1322A and G1315B DEGASSER, using a method based on that described by Rispail et al. (2005). Samples were chromatographed on a ZORBAX-EclipseXDB-C_{18} column (4.6 x 250 mm, particle size 5 µm) using methanol/acetic acid (5%) gradient from 0 to 100% methanol for 50 min at a flow rate of 2 ml/min. Substances responsible for peaks detected at 210 nm were identified by comparison of retention times and UV spectra with those of authentic compounds.

RESULTS AND DISCUSSION

1. The ecological conditions of Wadi El-Bagha

E. alata plant was collected from Wadi El-Bagha, the third tributary of Wadi Wardan. It starts from the regional water divided with an east-west channel, which reflects downstream towards the northwest until it joins with Wardan main trunk. Data presented in table 1 and 2 indicated that *E. alata* associated soil had generally loamy sand to sand loamy texture. Soil reaction ranged between slightly alkaline and alkaline, where pH values varied from 7.7 to 8. Soil pH is determined by the concentration of hydrogen ions in the soil solution, which is affected by the relative abundance of basic cations. Soil rich in these cations has higher pH values than those with lower levels. Water percolating down through the soil, tends to take the basic cations with it, leaving H^+ ions in their place. Cations were dominated by Na^+ followed by Ca^{+2} and Mg^{+2}, while K^+ was the least. Ca^{+2} plays a very important role within the cell, it is hugely important in primary cell wall structure (Ridge, 2002). The anions were dominated by Cl^- and followed by HCO^{-3} and SO^{-4}. The soil extract electric conductivity (EC) showed a tendency to decrease with depth.

Total annual rainfall, in the site under which the plant lives, was 21.09 and 45.86 mm during the studied period of 2008 and 2009, respectively. The dry period extended to seven months, from April to October, during 2008. The seasonal maximum temperature was recorded in July and August in both years, and relative humidity ranged between 46.6 and 76.88% during 2008, and between 46.6 and 71.62 during 2009. Reviewing these data indicate that the climatic conditions of the studied habitat are of the arid type with high temperature especially during the dry period. Severe weather conditions tend to increase the content of phenolic compound in the plant. The phenolic metabolism in higher plants is induced in response to environmental stress conditions such as infection by microbial pathogens, mechanical wounding, and excessive UV or high visible light levels (Herrmann, 1995; Haard and Chism, 1996). Thus, information about the influence of climatic factors on the production of secondary compounds must be taken into consideration for a better production of biological active constituents to select suitable habitats for the culture of these plants (Wijesekera, 1991) and to improve the drought and/or salt tolerance of crop plants.

Table 1. Granulometric analysis of soil supporting *E. alata* at Wadi El-Bagha.

Wadi El-Bagha	Soil Depth (cm)	$CaCO_3$ %	Soil particles distribution (%)				Texture Class
			Coarse sand (1 – 0.5 mm)	Fine sand (0.25 – 0.1 mm)	Silt (0.05 - 0.002 mm)	Clay < (0.002 mm)	
Upstream	0-30	55.73	58.50	25.45	4.91	11.14	Loamy sand
	30-60	51.94	34.43	55.39	2.83	7.35	Sand loamy
Midstream	0-30	59.72	57.24	29.95	2.94	9.87	Loamy sand
	30-60	56.40	39.36	61.15	3.35	6.14	Sand loamy
Downstream	0-30	57.32	52.49	28.93	8.05	10.53	Loamy sand
	30-60	52.48	23.74	62.34	7.59	6.33	Loamy sand

Table 2. Chemical analysis of soil supporting *E. alata* at Wadi El-Bagha.

Wadi El-Bagha	Depth (cm)	pH	EC (ds/m)	Soluble anions (meq/ 100 g)				Soluble cations (meq/ 100 g)			
				CO^{-3}	HCO^{-3}	SO^{-4}	Cl^-	Ca^{+2}	Mg^{+2}	Na^+	K^+
Upstream	0-30	7.8	1.21	0	1.31	1.01	2.72	1.87	0.68	2.38	0.11
	30-60	7.8	0.63	0	0.78	0.37	2.73	0.92	0.58	2.31	0.07
Midstream	0-30	7.7	1.55	0	1.35	1.07	2.68	1.83	0.72	2.41	0.14
	30-60	7.8	0.71	0	0.82	0.41	2.71	0.89	0.63	2.33	0.09
Downstream	0-30	8.0	9.30	0	0.98	0.73	0.51	1.28	0.28	0.61	0.03
	30-60	7.8	5.80	0	1.39	0.58	0.87	1.10	0.39	0.77	0.04

2. *In vitro* production of phenolic content from *E. alata*

Five phenolic compounds were detected in callus tissue of *E. alata* (Table 3). Among these, rutin was the major compound, followed by catechin. Comparison of phenolics production from callus tissue indicated that larger amounts of rutin, quercetin and coumaric acid were obtained from callus tissues maintained on the medium containing 0.5 mg/l CH as a supplement, and the highest concentration of catechin was observed on the medium containing 1.5 mg/l CH. CH is an organic nitrogen source containing amino acids mixture, and is an elicitor for secondary metabolites production. Hegazi and El-Lamey (2011) found that CH stimulated the bioaccumulation of ephedrine from callus of *E. alata*. Also, CH has promoted alkaloids accumulation in callus of *Catharanthus roseus* (Ahmed et al., 2000) and *Stephania tetrandra* (Kuo et al., 2011). The only phenolic compound that showed its highest amount on a medium containing L-phe (100 g/l) was chlorogenic acid, as L-phe is a precursor of phenolics. Plant phenolic compound are synthesized from phenylalanine and tyrosine *via* the shikimic acids pathway (Farah and Donangelo, 2006). The control medium containing 1 mg/l of both 2,4-D and Kn, without additives produced phenolic compounds, but in small amounts (except quercetin). This is due to the fact that plant growth regulators not only regulate plant growth and development, but may influence differently the secondary metabolism in different higher plants (da Rocha et al., 2005).

Table 3. Phenolics production from callus tissue of *E. alata* cultured on MS medium containing 1 mg/l of both 2,4-D and Kn, in addition to different concentrations of L-Phe or CH.

L-phe conc. (mg/l)	CH conc. (g/l)	Conc. of phenolic compounds (µg/g dry weight)				
		Chlorogenic acid	Rutin	Catechin	Quercetin	Coumaric acid
-	-	0.070	3.690	1.49	Not detected	0.044
12.5	-	Not detected	16.58	0.82	Not detected	0.240
25	-	Not detected	17.31	0.96	Not detected	0.390
50	-	Not detected	11.46	0.53	0.39	0.190
100	-	0.170	1.800	Not detected	Not detected	Not detected
-	**0.5**	Not detected	21.60	1.54	3.18	0.650
	1.0	0.093	4.160	0.93	Not detected	0.300
-	**1.5**	0.140	7.870	2.05	Not detected	0.580
-	**2**	0.077	4.030	0.43	Not detected	0.250

The five detected phenolic compounds have many health benefits; chlorogenic acid (CGA) is a hydroxycinnamic acid, a polyphenol and a member of a family of naturally occurring organic compounds (Clifford et al., 2003). It is an important biosynthetic intermediate. This compound, long known as an antioxidant and an inhibitor of the tumor promoting activity of phorbol esters, and might therefore contributes to the prevention of Type 2 Diabetes Mellitus (Paynter et al., 2006) and cardiovascular disease, also slows the release of glucose into the bloodstream after a meal (Johnston et al., 2003). It is claimed to have antiviral (Jassim and Naji, 2003), antibacterial (de Sotillo et al., 1998) and antifungal (Bowels et al., 1994) effects with relatively low toxicity and side effects, alongside properties that do not lead to antimicrobial resistance. Potential uses are suggested in pharmaceuticals, foodstuffs, feed additives, and cosmetics. In plant CGA play an important role in plant stress adaptation. It may contribute to the control of seed germination and cell growth, through regulation of the levels of indole acetic acid (Clifford, 1985).

Rutin (quercetinrutinoside), is a glycoside of the flavonoid quercetin. Both quercetin and rutin are used in many countries as medications for blood vessel protection and are ingredients of numerous multivitamin preparations and herbal remedies (Guardia et al., 2001; Shen et al., 2002). They are also antioxidants (Jung et al., 2007). Furthermore, they have been shown to control some cancers (Luo et al., 2008). Quercetin is capable of interaction with carcinogens in the gastroinerstinal tract, thereby reducing their bioviability.

Catechin is a natural phenol antioxidant plant secondary metabolite. It prevents human plasma oxidation (Lotito and Cesar, 1998). Catechin also has ecological functions. It is released into the ground by some plants to hinder the

growth of their neighbors, a form of allelopathy (Broz and Vivanco, 2006). Catechin could be used also as a scavenger for indoor air pollutants such as volatile organic compounds to adapt for instance as filters to air conditioners or to air purifiers (Takano et al., 2008).

Coumaric acid is a hydroxycinnamic acid, an organic compound that is a hydroxy derivative of cinnamic acid. It is a major component of lignocellulose. *p*-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer (Ferguson et al., 2005) by reducing the formation of carcinogenic nitrosamines.

Comparing the highest amounts of phenolics in callus tissue with their content in the stem of the wild *E. alata* plant (Table 4), revealed that callus tissue grown on the medium containing CH attained the ability to produce higher amounts of all phenolic compounds, except chlorogenic acid, which has more or less the same concentration in both callus and stem of the intact wild plant.

Table 4. A comparison between the content of phenolics in the stem of wild *E. alata* plant and its derived callus tissue.

Sample	Conc. of phenolic compounds (µg/g dry weight)				
	Chlorogenic acid	Rutin	Catechin	Quercetin	Coumaric acid
Wild plant	0.22	3.77	0.77	1.05	0.03
Callus	0.17	21.6	2.05	3.18	0.65

In conclusion, these results demonstrates that callus cultures of *E. alata* could be potentially a rich source of natural antioxidants, due to its ability to produce some phenolic compounds of antioxidant activity, and the manipulation of medium by adding plant growth regulators, and CH can enhance the production of phenolics from callus cultures. The amount of most of the studied phenolic compounds produced from callus was greater than that present in the stems of the intact plant. Although wild plant genotype is exposed to wide range of environmental stresses and these stresses cause the increase of secondary metabolites. Our results indicated the ability to utilize plant biotechnology techniques towards development of desired bioactive metabolites in *in vitro* culture instead of using the wild plant itself in pharmaceutical purposes.

Therefore, this protocol could be applied to improve the yield of these compounds and is a promising alternative for direct extraction from plants grown in natural habitat, which help in the conservation of natural plant resources. Further studies should be conducted to produce these compounds in a large scale through cell suspension cultures, with a focus on their application in health-related research.

REFERENCES

Ahmed, F.A., O.M. Abdel-Fatah, M.I. Kobeasy and O.K. Ahmed, 2000. Factors affecting growth and indole alkaloids content of catharanthuscalli (*Catharanthus roseus* L.): amino acids, casein hydrolysate and irradiation. Biotechnology, 5: 379-384.

Bowels, L. Bobby, and A.J. Miller, 1994. Caffeic acid activity against clostridium botulinum spores. Journal of Food Science (Blackwell Publishing), 59 (4): 905.

Blumenthal, Mark, et al., 1998. The complete German commission E monographs. American Botanical Council, Texas, pp: 125-126.

Broz, A.K. and J.M. Vivanco, 2006. Secondary Metabolites and Allelopathy in Plant Invasions: A Case Study of Centaureamaculosa.

Clifford, M.N. 1985. Chlorogenic acids. In: Coffee. Vol. 1. Chemistry (eds R.J. Clarke and R. Macrae). Elsevier Applied Science Publication, London, UK.

Clifford, M. N., K. L. Johnston, S. Knigh and N. Kuhnert, 2003. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. Journal of Agriculture and Food chemistry, 51(10): 2900–2911.

da Rocha, L.K., A.J.B. de Oliveira, C.A. Mangolin and M.P. Machado, 2005. Effect of different culture medium components on production of alkaloid in callus tissues of *Cereus peruvianus* (Cactaceae). Acta Scientiarum, Biological Sciences, 27(1): 37-41.

de Sotillo, D.R., M. Hadley and C. Wolf-Hall, 1998. Potato peel extract a nonmutagenic antioxidant with potential antimicrobial activity. Journal of Food Science, 63 (5): 907.

Farah, A. and C.M. Donangelo, 2006. Phenolic compounds in coffee. Brazilian Journal of Plant Physiology, 18(1).

Ferguson L.R., Z. Shuo-tun and P.J. Harris, 2005. Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29. Molecular Nutrition and Food Research, 49(6): 585–693.

Guardia, A.O. Juarez and L.E. Pelzer, 2001. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Il Farmaco, 56 (9): 683–7.

Haard, N.F. and G.W. Chism, 1996. Characteristics of edible plant tissues. In: Food Chemistry, 3rd edition (ed O.W. Fennema) pp: 944-1011. Marcel Dekker Inc., New York.

Hermann, K.M. 1995. The shikimate pathway as an entry to aromatic secondary metabolism. Plant Physiology, 107: 7-12.

Hegazi, Gh.A. and T.M. El-Lamey, 2011. Callus induction and extraction of ephedrine from *Ephedra alata* Decne. cultures. American-Eurasian Journal of Agricultural and Environmental Science, 11(1): 19-25.

Heldt, H.W. 1997. Plant biochemistry and molecular biology. Oxford University Press, pp: 352-414, 501.

Jassim, S.A.A. and M.A. Naji, 2003. Novel antiviral agents: a medicinal plant perspective. Journal of Applied Microbiology, 95(3): 412–427.

Johnston, K.L., M.N. Clifford and L.M. Morgan, 2003. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. American Journal of Clinical Nutrition, 79 (4): 728–733.

Jung, C.Y., C.H. Cho, Kim and C. Jong, 2007. Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. Archives of Pharmacal Research, 30(12): 1599–1607.

Kuo, C.L., J.Y. Chang, H.C. Chang, S.K. Gupta, H.S. Chan, E.C.F. Chen and H.S. Tsay, 2011. *In vitro* production of benzylisoquinoline from *Stephania tetrandra* through callus culture under the influence of different additives. Botanical Studies, 52: 285-294.

Lotito, S.B. and G. Cesar, 1998. Fraga (+)-Catechin prevents human plasma oxidation. Free Radical Biology and Medicine, 24(3): 435-441.

Luo, King, M. Sarah, Chen and Y. Charlie, 2008. Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids. Nutrition and Cancer, 60(6): 800–9.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

O'Dowd, N.A., G. McCauley, J.A.N. Wilson, T.A.K. Parnell and D. Kavanaugh, 1998. *In vitro* culture, micropropagation and the production of ephedrine and other alkaloids. In: Biotechnology in Agriculture and Forestry (ed Y.P.S. Bajaj) pp: 41. Springer: Berlin, Germany.

Page, A.L. 1987. Methods of soil analysis, part 2. Chemical and microbiological properties-agronomy monograph. No. 9. American Society of Agronomu Inc., Madison, pp: 167-179.

Parsaeimehr, A., E. Sargsyan and K. Javidnia, 2010. A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra. Molecules, 15: 1668-1678.

Paynter, P. Nin, H.C. Yeh, S. Voutilainen, M.I. Schmidt, G. Heiss, A.R. Folsom, F.L. Brancati and W.H.L. Kao, 2006. Coffee and sweetened beverage consumption and the risk of type 2 diabetes mellitus. American Journal of Epidemiology (Oxford Journals), 164(11): 1075–1084.

Quartacci, M. F. and F. Navaro-Izzo, 1992. Water stress and free radical mediated changes in sun flower seedling. Journal of Plant Physiology, 139: 621-625.

Ridge, I. 2002. Plants. Oxford Unv. Press Inc., New York, pp: 179-181

Rispail, N., P. Morrand and K.J. Webb, 2005. Phenolic compounds: extraction and analysis. In: Lotus Japonicas Handbook (ed A.J. Márquez) pp: 349-355.

Safer, A.M. and A.J. Al-Nughamish, 1999. Hepatotoxicity induced by the anti-oxidant food additive butylated hydroxyl toluen (BHT) in rats: An electron microscopical study. Histology and Histopathology, 14: 391-406.

Scalbert, A.; I.T. Johnson, and M. Saltmarsh, 2005. Polyphenols: antioxidants and beyond. The American Journal of Clinical Nutrition, 81(suppl.): 215S-217S.

Shen, H.Y. Lin, H.C. Huang, C.H. Ko, L.L. Yang and Y.C. Chen, 2002. *In vitro* and *in vivo* inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E2 production. European Journal of Pharmacology, 446 (1–3): 187–94.

Takano, T., T. Murakami, H. Kamitakahara and F. Nakatsubo, 2008. Mechanism of formaldehyde adsorption of (+)-catechin. Journal of Wood Science, 54 (4): 329-331.

Wijesekera, R.O.B. 1991. The medicinal plant industry. CRC Press, Boca Roton, Ann Arbor, Boston, London, pp: 33-76.

Zollman, C. and A. Vickers, 1999. Complementary medicine and the patient. British Medical Journal, 319: 1486–1494.