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In vitro Regeneration and Comparison of Phenolic Content, Antioxidant and Antityrosinase Activity of *in vivo* and *in vitro* Grown *Asparagus officinalis*

(Penjanaan Semula *in vitro* dan Perbandingan Kandungan Fenolik, Antioksida dan Aktiviti Antitirosinase *Asparagus officinalis* Ditanam Secara *in vivo* dan *in vitro*)

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ABSTRACT

Asparagus officinalis *as a valuable medicinal plant has a low multiplication rate using the conventional methods. This study was carried out to establish an efficient* in vitro *propagation protocol and also to compare some biological activities of* in vivo *and* in vitro *grown Asparagus. The nodal explants were cultured on MS medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) or kinetin (Kn) and Indolebutyric acid (IBA), under light and dark conditions. After 6 weeks of culture, the highest percentage (100%) of callus formation was found in 17 of treatments under dark condition and 3 treatments under light condition. Also between the two groups of hormones, Kn +IBA showed better results in promoting callus formation. The highest average number of shoots (4.25) of size 4 mm or more per explant, formed under dark condition using 1.5 mg/L BAP mixed with 0.05 mg/L NAA. Rooting was best induced in shoots excised from shoot cultures which were proliferated on MS medium supplemented with an optimal concentration of 0.4 mg/L IBA (2 roots per explant). In the second part of the study, the extracts of* in vivo *and* in vitro *grown plants as well as callus tissue were tested for their total phenolic and flavonoid content, antioxidant and antityrosinase activities, using two different extraction solvents (methanol and hexane). The methanol extract of* in vivo *grown plants showed a significantly higher amount of total phenolic and flavonoid content. The antioxidant activity of tested samples followed this order;* in vivo *plant > callus >* in vitro *plant.*

Keywords: Antioxidant; antityrosinase; flavonoid; phenolic; propagation

ABSTRAK

Asparagus officinalis *sebagai tumbuhan ubatan yang bernilai mempunyai kadar pembiakan yang rendah apabila dibiakkan secara konvensional. Kajian ini bertujuan untuk menghasilkan kaedah pembiakan secara* in vitro *yang cekap dan untuk membandingkan aktiviti biologi daripada* Asparagus officinalis *yang ditanam secara* in vivo *(biasa) dan* in vitro *(kaedah kultur tisu). Eksplan nodal dikultur menggunakan media MS yang ditambah kepekatan hormon 6-benzilaminopurin (BAP) dan asid 1- naftalena (NAA) atau kinetin (Kn) dan asid indolbutrik (IBA) di bawah keadaan cahaya dan gelap. Selepas 6 minggu, peratus tertinggi (100%) pembentukan kalus didapati daripada 17 rawatan yang diletakkan di bawah keadaan gelap dan 3 rawatan di bawah cahaya. Didapati daripada 2 kumpulan hormon, Kn dan IBA telah menunjukkan keputusan yang lebih baik dalam pembentukan kalus. Purata pembentukan pucuk tertinggi (4.25) bersaiz 4 mm atau lebih bagi setiap eksplan, terbentuk di bawah keadaan gelap menggunakan 1.5 mg/L BAP beserta 0.05 mg/L NAA. Pertumbuhan akar didapati terbaik apabila pucuk diambil daripada kultur yang dibiakkan dalam media MS yang ditambah dengan 0.4 mg/L IBA (2 akar setiap pucuk). Dalam bahagian kedua eksperimen, ekstrak daripada tumbuhan yang ditanam secara* in vivo, in vitro *dan juga tisu kalus telah dikaji untuk mengetahui jumlah fenolik dan kandungan flavonoid, aktiviti antioksidan serta antitirosinase menggunakan 2 pelarut (metanol dan heksan). Ekstrak metanol daripada tumbuhan* in vivo *menunjukkan jumlah fenolik dan kandungan flavonoid yang ketara dan signifikan. Aktiviti antioksidan bagi sampel yang telah dikaji adalah dalam susunan berikut: tumbuhan* in vivo> *kalus > tumbuhan* in vitro.

Kata kunci: Antioksidan; antitirosinase; fenolik; flavonoid; propagasi

INTRODUCTION

In terms of economic and medicinal values, *Asparagus officinalis* L. (garden asparagus) is the most important asparagus species, which is a very remarkable vegetable (Stajner et al. 2002). Asparagus has a low multiplication rate when conventional systems are used, so that under optimal condition and with the absence of any pest invasion in the period of one year only 2-4 new plants produce from one plant (Sarabi & Almasi 2010).

In vitro propagation systems have been expansively studied in several species of Asparagus genus that are considered as ornamental or medicinal plants such as *Asparagus officinalis* (Carmona et al. 2014; Stajner 2013), *Asparagus racemosus* (Bopana & Saxena 2008),

Asparagus maritimus (Štajner et al. 2002), *Asparagus cochinchinensis* (Jiang et al. 2013). So far, there were numerous methods established for *in vitro* regeneration of Asparagus such as direct organogenesis (Murashige et al. 1972; Pontaroli & Camadro 2005), indirect organogenesis (Reuther 1984) and somatic embryogenesis (Ghosh & Sen 1991; Reuther 1977).

In this study, a rapid and efficient protocol for the large-scale propagation of *Asparagus officinalis*, through *in vitro* culture of nodal explants gained from one month-old seedlings was described. Nodal explants were treated with different hormones, in order to obtain optimum callus, shoot and root formation.

Using the plant cell and tissue culture methods as a source of generating medicinal metabolites has a long history (Verpoorte et al. 2002). Using natural materials as sources of new antioxidant have gained lots of popularity for the past 2 decades and many studies have been carried out. Numerous researches verified the activity of traditional herbs against microorganisms (Biruhalem et al. 2011; Palombo 2011). These studies have provide a decision that we can reach new principles in current medicine based on the significant role of the plants (Brewer 2011; Dimitrios 2006).

Browning reaction during food storage is an unfavorable effect which decreases food appeal and nutritional value. Roh et al. (2004) reported that the role of tyrosinase on catalyzing the oxidation of phenol and regulating the initial step of melanin production has a primary effect in the enzymatic browning reaction in food processing. Therefore, many studies have focused on finding natural additives to reduction the browning progression in food storage (Germanas et al. 2007).

In view of the importance of decreasing both tyrosinase activity and oxidative reaction in foods to protect the nutritional components against oxidative damage, the other purpose of the present study was to investigate the capacity of *Asparagus officinalis* on tyrosinase activity and oxidative damage. Thus, the objectives of the current study were; to establish efficient regeneration system for this species from nodal explant and to explore the biological activities such as antioxidant and antityrosinase of the extracts from *in vivo* and *in vitro* grown plants with two different solvents. The novelty of this study was that the antioxidant and antityrosinase properties of *Asparagus officinalis* were compared between *in vitro* grown whole plant and also *in vitro* grown tissue (callus) with the *in vivo* grown plants, which are grown out of laboratory condition. Also the protocol described here compared the *in vitro* propagation of *Asparagus officinalis* under light and dark condition and combination of different hormones to reduce time on callus formation, shoot regeneration and root development.

Materials and Methods

Seeds of *Asparagus officinalis* cv. Mary Washington were germinated in the green house (25±1˚C and 16 h of light) at Institute of Biological Sciences, Faculty of Science, University of Malaya. The four-weeks-old plants were collected and used as explants.

Callus induction and direct organogenesis

After surface sterilization, explants were cultured for callus induction and shoot initiation on MS medium (Murashige & Skoog 1962) containing 3% sucrose and 0.28% phytogel agar with different combinations of BAP (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mg/L) and NAA (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/L) and also different combinations of Kn (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mg/L) and IBA (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/L).

The nodal parts from four-weeks-old plants were collected. Each nodal cutting was approximately the same size (0.6 × 0.4 cm). Two explants were placed into each flask containing 40 mL of medium. Each of the cultured flask was placed in the growth chamber under light condition (1000 Lux) and at a temperature of 25±1ºC. This experiment was repeated for the same treatments with the same conditions but flasks were placed in the growth chamber in the dark. After a period of 6 weeks, the media and explants were screened and the responses noted.

Explant materials for the rooting study were obtained from plantlets derived from the multiplication trial experiments. Uniform cuttings were performed aseptically and placed vertically in flasks containing 40 mL of medium supplemented with eight different concentrations of IBA (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0 and 1.5 mg/L) to determine the optimal root development. Each of the eight treatments was replicated five times. Data was recorded with respect to the number of roots after six weeks of culture.

Phytochemical Screening Activity

Extract preparation

Two different solvents (methanol or hexane) were applied for extraction of the soluble compounds from the samples. After drying the plant samples (*in vivo*, *in vitro* and callus) (at 40˚C) using incubator they were ground by electric blender to produce fine powders. Three grams of powder was soaked in 40 mL of methanol or hexane and kept in dark place at room temperature for 72 h then it was filtered using filter paper (Whatman No.1). The filtered solutions were then placed in water bath at 40˚C for evaporating to dryness.

Determination of total phenolic and flavonoid content

The method reported by Kaurinovic et al. (2012) was followed for assessment of total phenolic content. The extracts were prepared in three different concentrations of 0.125, 0.25 and 0.5 mg/mL. Gallic acid with different concentrations (0.50 to 100 µg/mL) was used as the standard. The results were presented as a mean value of triplicate tests. The value of total phenolic was represented

as mg gallic acid per gram of dry extract. According to Pourmorad et al. (2006) to determine the total flavonoid content, the aluminum chloride colorimetric method was used. Plant extracts were prepared in different concentrations of 1.0, 2.0 and 4.0 mg/mL, while rutin solutions as a standard were prepared in concentration range from 0.50 to 100 µg/mL. All experiments were repeated three times and the mean values of total flavonoid content, calculated according to the standard calibration curve were expressed as µg of rutin equivalents per gram of dry extract.

DPPH ASSAY

Free radical scavenging capacity or DPPH (2,2-diphenyl-1-picrylhydrazil) assay was done according to the method described by Rafat et al. (2010). Plant extract (50 µL) was added to 950 µL of DPPH in concentration of 90 µM, the volume of solution was adjusted to 4 mL using 95% methanol. The resulted solution was kept in a dark place at room temperature for 2 h. By using a spectrophotometer, the colour reduction of solution (scavenging of DPPH*) was measured at 515 nm. Scavenging capacity potential of the extracts calculated with comparison of colour reduction between blank (solution without plant extract) and examined plant extracts using the follow equation (Rafat et al. 2010):

(%) Radical scavenging capacity = ((Blank - Sample A) / Blank) × 100).

ERYTHROCYTE HEMOLYSIS MEDIATED BY PEROXYL FREE RADICALS

This assay was based on the method described by Cheung et al. (2003). Male rabbit was used to obtain the blood for this assay. Erythrocytes detached from plasma and the buffy coat were washed with 10 mL of 10 mM phosphate buffer saline (PBS) for three times at pH7.4 (prepared by mixing 10 mM of NaH2PO4 and Na2HPO4 and 125 mM of NaCl in 1 L of distilled water) and centrifuged (EBA-20, Zentrifugen, Germany) for 5 min at 1500 g. The erythrocytes were gained during the last washing when centrifugation done at 1500 g for 10 min.

Suspension of erythrocytes (0.1 mL) (20%) in PBS was mixed with 0.2 mL of 200 mM 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH) solution (in PBS) and 0.1 mL of selected plant extracts with different concentrations (5, 10, 15 and 20 mg/mL). The resulted solution was kept in a shaking incubator at 37˚C for 3 h. The reaction mixture was centrifuged at 1041 g for 10 min after dilution with 8 mL of PBS. The absorbance of obtained supernatant was then measured by a spectrophotometer (Absorbance A) at 540 nm. Similarly, to achieve complete hemolysis the reaction mixture was treated with 8 mL of distilled water instead of PBS, then the absorbance of the obtained supernatant (Absorbance B) was measured at 540 nm. The percentage of hemolysis inhibition was calculated by using the following equation. The positive control in this study was Ascorbic acid in concentration of 1 mg/mL.

(% Hemolysis inhibition = (1 - A/B) × 100%)

DETERMINATION OF TYROSINASE ACTIVITY

According to Chu et al. (2009) the tyrosinase inhibitory activity was determined with a degree on inhibition on tyrosinase-catalysed oxidation of L-DOPA. All the experiments were performed in 25 mM sodium phosphate buffer (pH6.8). The reaction mixture consisting of sample, 0.8 mL sodium phosphate buffer (25 mM) (pH6.8), 0.1 mL of mushroom tyrosinase (250 Units/mL) and L-DOPA (3.8 mM) was added in this order to measure the initial rate as a linear increase in the absorbance at 475 nm for 5 min. The reaction was achieved at 25˚C. The value in the absence of sample was represented as a control. The inhibition of tyrosinase activity was calculated with the following formula:

Inhibition (%) = (1– (OD 475 in sample/OD 475 in control)) × 100%.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version 17. The means were compared with Duncan's multiple comparison test (DMCT) and $p<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

MICROPROPAGATION

Different morphogenic responses were found in explants of *Asparagus officinalis* cultured on MS basal medium supplemented with different plant hormones. Callus was induced in many of the 97 media under light and dark conditions starting from the second week of inoculation. The calli proliferated very fast to form compact masses that ranged from pale yellow to green color under light condition and white color in dark condition. After 6 weeks, callus was initiated in most of the cultures. Tables 1 and 2 exhibits the different cultures with percentage of callus formation.

From comparison of all treatments with control, it was determined that combination of cytokinin and auxin hormones are needed for callus formation of *Asparagus officinalis* and the amount of cytokinin should be more than auxin.

Inagaki et al. (1980) reported the optimal callus formation (72%) for *Asparagus officinalis* on MS medium supplemented with 1.0 mg/L BAP and 5.0 mg/L NAA. Harada and Yakuwa (1983) reported hardly observed of callus induction from Cladophylls of *Asparagus officinalis* with both NAA and IBA hormones. They reported a fairly high percentage (51 to 90%) of callus induction in a

TABLE 1. Percentage of callus formation in MS media with different concentrations of BAP + NAA after 6 weeks

NAA mg/L	BAP mg/L 0.0	0.2	0.4	0.6	0.8	1.0	1.5
0.0	0 c	12.5 bc	0 c	12.5 bc	0 c	12.5 bc	0 c
	0 b	0 b	0 b	25 ab	25 ab	25 ab	0 b
0.05	0 c	75 a	37.5abc	37.5abc	50 abc	37.5abc	37.5abc
	0 b	50 ab	0 b	50 ab	25 ab	0 b	25 ab
0.1	0 c	50 abc	75 a	50 abc	75 a	50 abc	50 abc
	25 ab	75 ab	75 ab	25 ab	25 ab	25 ab	100 a
0.2	0 c	37.5abc	50 abc	50 abc	25 abc	50 abc	62.5 ab
	0 b	100 a	50 ab	25 ab	50 ab	25 ab	50 ab
0.3	0 c	12.5 bc	62.5 ab	62.5 ab	50 abc	75 a	62.5 ab
	0 b	100 a	25 ab	75 ab	25 ab	25 ab	75 ab
0.4	0 c	25 abc	37.5 abc	75 a	0 c	37.5 abc	25 abc
	0 b	50 ab	25 ab	50 ab	25 ab	75 ab	50 ab
0.5	0 c	75 a	37.5 abc	37.5 abc	25 abc	62.5 ab	0 c
	50 ab	50 ab	75 ab	50 ab	50 ab	25 ab	25 ab

□ Light condition ■ Dark condition
The means were compared with Duncan's multiple comparison test (DMCT) at $p<0.05$, the means with same small letter are not significantly different

TABLE 2. Percentage of callus formation in MS media with different concentrations of Kn + IBA after 6 weeks

IBA mg/L	Kn mg/L 0.0	0.2	0.4	0.6	0.8	1.0	1.5
0.0	0 f	0 f	0 f	0 f	0 f	0 f	0 f
	0 c	0 c	0 c	0 c	0 c	0 c	25 bc
0.05	0 f	12.5 ef	0 f	62.5abcd	25def	50 bcde	50 bcde
	0 c	0 c	75 ab	50 abc	25 bc	75 ab	25 bc
0.1	0 f	50 bcde	37.5cdef	75 abc	87.5 ab	62.5abcd	75 abc
	0 c	75 ab	100 a	100 a	100 a	100 a	100 a
0.2	0 f	62.5abcd	87.5 ab	100 a	75 abc	75 abc	62.5abcd
	25 bc	75 ab	100 a	100 a	75 ab	50 abc	100 a
0.3	0 f	75 abc	100 a	62.5abcd	87.5 ab	62.5abcd	75 abc
	0 c	75 ab	50 abc	100 a	50 abc	75 ab	75 ab
0.4	0 f	12.5 ef	37.5cdef	100 a	87.5 ab	37.5cdef	62.5abcd
	0 c	50 abc	75 ab	50 abc	100 a	100 a	100 a
0.5	0 f	75 abc	75 abc	87.5 ab	87.5 ab	50 bcde	62.5abcd
	0 c	25 bc	75 ab	75 ab	100 a	100 a	75 ab

□ Light condition ■ Dark condition
The means were compared with Duncan's multiple comparison test (DMCT) at $p<0.05$, the means with same small letter are not significantly different

combined addition of auxins (NAA or IBA) and cytokinin (BAP). In their research, callus formation was not observed in the MS medium without either auxins or cytokinins. It was in agreement with the finding of the present study that both cytokinin and auxin were needed for callus induction in *Asparagus officinalis* explants and the amount of cytokinin should be more than auxin.

Among the two groups of hormone, Kn + IBA was found to be more effective than BAP + NAA in promoting callus formation. Also, between dark and light condition, dark condition was found to be more efficient than light condition in promoting callus growth. However, the callus that was formed under dark condition was yellow in color, but the callus formed under light condition was green color. After 6 weeks of culture, the mean number and length of shoots per explant were recorded. The best treatment with the highest average number of shoots (4.25) of size 4 mm or more per explant was obtained on the MS media supplemented with 1.5 mg/L BAP and 0.05 mg/L NAA under dark condition but the shoots were less developed, with unusual thickness, yellow color compared with the shoots formed under light condition. In light condition, the highest average number of shoots (3.63) of size 4 mm or more per explant was found on the MS medium supplemented with 0.8 mg/L BAP alone, without NAA (Tables 3 and 4).

Growth regulators especially cytokinins are one of the most significant factors affecting the response of shoot proliferation. Sarabi et al. (2010) reported highest average number of shoot proliferation of size 3 mm or more per explant in *Asparagus officinalis* on MS medium supplemented with 0.5 mg/L BAP and 0.015 mg/L NAA after 3 months of culture under light condition. In contrast to the current study the best results with highest average number of shoot proliferation of size 4 mm or more per explant was obtained on MS medium supplemented with 1.5 mg/L BAP and 0.05 mg/L NAA after 6 weeks under dark condition. Several studies have concluded that the major problem of Asparagus micropropagation protocol was not the establishment of shoot culture and shoot multiplication, but difficult root initiation.

Chin (1982) reported considerable improvement of shoot and root development in *Asparagus officinalis* with ancymidol. The most efficient auxins for rooting are IBA and NAA (Uddin et al. 2005). Kar and Sen (1985) reported that lower concentration of IBA added to the MS medium failed to induce roots in *Asparagus racemosus*. This was true of *Asparagus adscendens*, where IBA did not induce rhizogenesis. However, Sarabi and Almasi (2010), reported root initiation in *in vitro* regenerated shoots of *Asparagus officinalis* on MS supplemented with 1.5 mg/L of IBA. Similarly, in the present study among the various concentrations tested for rooting, treatments with 0.4 mg/L IBA showed the best result (2 roots per explant) (Table 5) where roots initiated after 6 weeks of culture.

TOTAL PHENOLIC, ANTIOXIDANT AND ANTITYROSINASE ACTIVITY

A significant correlation between antioxidant activity and phenolic content of plant extracts have been reported

TABLE 3. Mean number of shoots per explant in MS media with different concentrations of BAP + NAA after 6 weeks

		BAP mg/L						
		0.0	0.2	0.4	0.6	0.8	1.0	1.5
NAA mg/L	0.0	1 abc	2.75 abc	0.62 bc	1.25 abc	3.62 a	0.87 abc	1.75 abc
		0.75ab	2.75 ab	3.5 ab	2 ab	0.75 ab	2 ab	3.5 ab
	0.05	0 c	1.25 abc	2.25 abc	2.75 abc	1.75 abc	2.5 abc	1.5 abc
		0.75 ab	0.75 ab	2.75 ab	1.5 ab	3.25 ab	3 ab	4.25 a
	0.1	0 c	2.62 abc	0.37 c	1.62 abc	0.5 c	1.37 abc	1.37 abc
		1.5 ab	1.25 ab	0.75 ab	1.75 ab	1.5 ab	1.75 ab	0 b
	0.2	0 c	1.87 abc	0.75 bc	0.62 bc	1.62 abc	0.62 bc	1 abc
		0 b	0 b	1.5 ab	2.75 ab	2.25 ab	0.5 ab	1 ab
	0.3	0 c	0.87 abc	1 abc	0.87 abc	0.75 bc	0.25 c	0.25 c
		0 b	0 b	0.5 ab	1.5 ab	2.75 ab	3.5 ab	1.75 ab
	0.4	0 c	3.37 ab	1.62 abc	0.37 c	1.75 abc	1.87 abc	1.5 abc
		0 b	2.25 ab	1.75 ab	0.5 ab	2.25 ab	0.5 ab	1 ab
	0.5	0 c	1.12 abc	1.62 abc	1.25 abc	1.25 abc	0.75 bc	2.5 abc
		1.25 ab	1.25 ab	0.75 ab	1.5 ab	1.25 ab	1.25 ab	0.75 ab

□ Light condition ■ Dark condition

The means were compared with Duncan's multiple comparison test (DMCT) at $p<0.05$, the means with same small letter are not significantly different

TABLE 4. Mean number of shoots per explant in MS media with different concentrations of Kn + IBA after 6 weeks

IBA mg/L	Kn mg/L 0.0	0.2	0.4	0.6	0.8	1.0	1.5
0.0	0.87 b	0.87 b	1.62 a	0 b	1.37 b	0 b	0.25 b
	0.25 ab	0 b	1.25 ab	2.25 a	0 b	1 ab	0 b
0.05	0.12 b	1.37 b	0.12 b	0 b	1.12 b	0 b	0.25 b
	0 b	0 b	0.75 ab	0 b	1.25 ab	2.25 a	1.5 ab
0.1	0 b	0.25 b	1.12 b	0 b	0 b	0.75 b	0.37 b
	0 b	0.75 ab	0 b	0 b	0 b	0 b	0 b
0.2	0 b	0 b	0.37 b	0 b	0.37 b	0.75 b	1.25 b
	0 b	0.25 ab	0 b	0 b	0.25 ab	0.75 ab	0 b
0.3	0 b	0 b	0 b	0.37 b	0.5 b	0.5 b	0.25 b
	0 b	0 b	0 b	0 b	1 ab	0.25 ab	0.75 ab
0.4	0 b	0 b	0.25 b	0 b	0.12 b	1 b	0.37 b
	0 b	0.75 ab	0.25 ab	0.5 ab	0 b	0 b	0 b
0.5	0 b	0 b	1.12 b	0.12 b	1 b	1.25 b	0.37 b
	0 b	0.5 ab	0 b	0.25 ab	0 b	0 b	0.5 ab

□ Light condition ■ Dark condition
The means were compared with Duncan's multiple comparison test (DMCT) at $p<0.05$, the means with same small letter are not significantly different

TABLE 5. Mean number of roots per explant in MS media containing different concentrations of IBA, after 6 weeks

IBA concentration mg/L	0.1	0.2	0.3	0.4	0.5	0.75	1	1.5
Mean number of roots	0.16	0.37	1.29	2	0.76	0.18	0.74	0.1

by Erkan et al. (2008). Phenols are very important plant constituents because of their function as scavengers of alkoxyl radicals and intermediate peroxyl, myocardial infraction, cancer, processes of aging, arteriosclerosis and chelating agents for metal ions (Tosun et al. 2009). As shown in Table 6, the total content of phenolics in the plant extract (expressed as mg GAE per g of dry extract) was relevant with the extraction solvent. For all samples the methanol extract showed higher phenolic content than the hexane ones. Among the tested samples, the largest content of phenols was found in the methanol extract of *in vivo* grown plants ($p<0.05$).

The total content of flavonoids, expressed as µg RE/g dry extract, are shown in Table 7. The methanol extract of *in vivo* grown plants showed the considerable total flavonoids content. While the hexane extract of *in vitro* sample showed the smallest quantity of these compounds.

DPPH radical scavenging is the common used assay to investigate the antioxidant potential of plant extract. In this study, the extracts of *in vivo*, *in vitro* and callus tissue of *Asparagus officinalis* were tested for their free radical scavenging activities. DPPH scavenging activities, due to its reduction by different selected extracts are shown in Figure 1. All the six examined extracts were able to scavenge the stable DPPH radicals. The maximum inhibition percentage (68±0.43) were found in the methanol extract (25 mg/mL) of *in vivo* grown plants, followed by the minimum inhibition percentage (18.6±0.66) for the hexane extract (5 mg/mL) of the callus samples. Overall, the radical scavenging activities of methanol extracts were higher than that of hexane extracts for the selected plant in tested concentration. Although after comparison of DPPH scavenging activities of examined plant extracts with positive control (vitamin C), it was found that the antioxidant properties of all extracts were lower than the positive control. Based on the results obtained, it can be observed that there was a positive correlation between total phenolic content and DPPH scavenging activity of the plant extract.

One of the pathways that lead to membrane hemolysis is chain oxidation of its lipids and proteins which is induced by free radicals produced by AAPH which is basically a peroxy radical initiator. These occurred through APPH thermal decomposition (Cheung et al. 2003). In this study, the extracts of callus tissue, *in vivo* and *in vitro* grown *Asparagus officinalis* were investigated for their protective effect on hemolysis by peroxyl radical scavenging activity. The examined extracts were treated

TABLE 6. Concentration of total phenolics (mg GAE/g dry extracts ± SD) in methanol and hexane extracts of callus, *in vivo* and *in vitro* grown *Asparagus officinalis*

Plant samples	Methanol extract	Hexane extract
In vivo grown plants	21.15 ± 0.54 [a]	20.37 ± 0.77 [a]
In vitro grown plants	15.36 ± 1.10 [b]	12.42 ± 1.36 [c]
Callus	17.55 ± 0.33 [b]	15.80 ± 0.54 [b]

TABLE 7. Amount of total flavonoid content (µg RE/g dry extracts ± SD) in methanol and hexane extracts of callus, *in vivo* and *in vitro* grown *Asparagus officinalis*

Plant samples	Methanol extract	Hexane extract
In vivo grown plants	11.35 ± 0.76 [a]	8.91 ± 0.72 [a]
In vitro grown plants	7.20 ± 0.86 [b]	5.25 ± 0.35 [b]
Callus	10.15 ± 0.46 [a]	9.18 ± 0.67 [a]

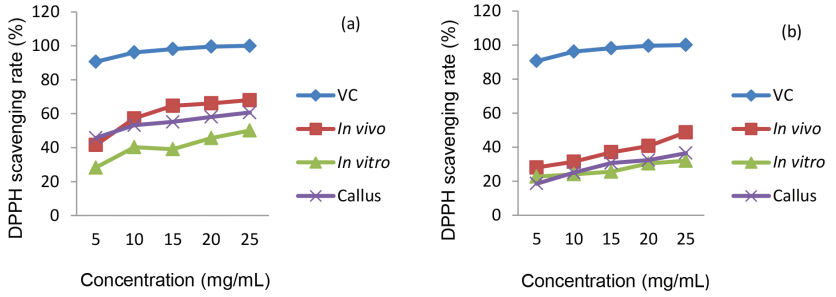

FIGURE 1. Scavenging activity (%) of DPPH radical of methanol extracts (a) and hexane extracts (b) of callus, *in vivo* and *in vitro* grown *Asparagus officinalis*. Ascorbic acid was used as the positive control ($p \leq 0.05$, $n=3$)

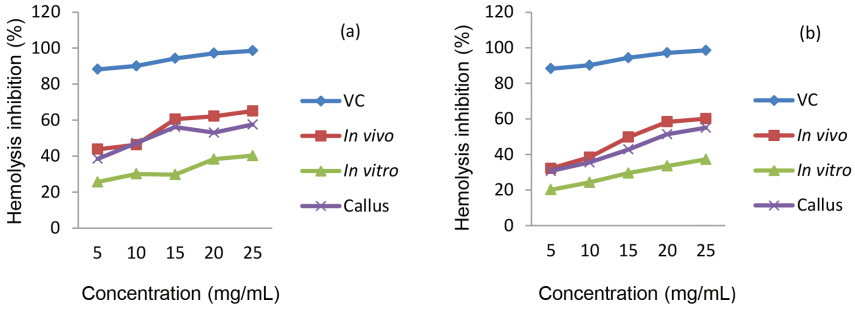

FIGURE 2. Hemolysis inhibition (%) of methanol extract (a) and hexane extract (b) of callus, *in vivo* and *in vitro* grown *Asparagus officinalis*. Ascorbic acid was used as the positive control ($p \leq 0.05$, $n=3$)

on cell membranes of erythrocytes from rabbit, to check the protection against the oxidative damage induced by AAPH. The inhibition percentages of hemolysis are shown in Figure 2. A significantly higher inhibition percentage (65.2±1.36) of hemolysis was detected in methanol extract (25 mg/mL) of *in vivo* grown plants, than that of other extracts at the same concentration. The obtained results indicated that methanol extract of *in vivo* grown plant has better protective effect against hemolysis of erythrocytes, compared with callus tissue and *in vitro* grown plant. From the results, it can also be suggested that the relatively higher inhibition percentage in methanol extract of *in vivo* grown plant is probably due to its higher phenolic content.

In addition, the tyrosinase inhibitory activities of methanol and hexane extracts of callus, *in vivo* and *in vitro* grown *Asparagus officinalis* are shown in Figure 3. In the range of 0.5-1.25 mg/mL the maximum and minimum inhibitory effects on tyrosinase activity belong to the methanol extract of *in vivo* grown plants (39.1±1.32) and methanol extract of callus samples (11.8±1.56), respectively.

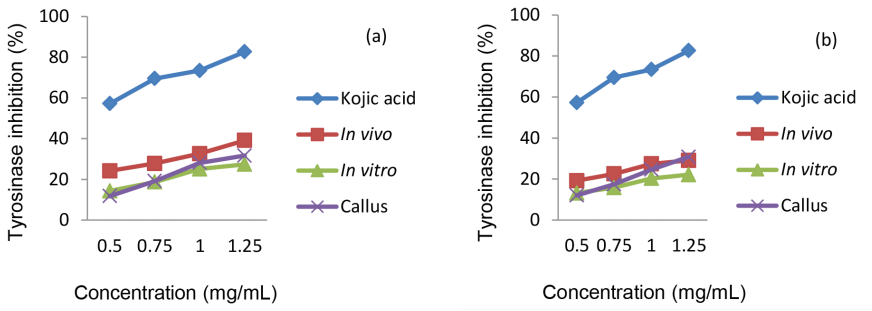

FIGURE 3. The tyrosinase inhibitory effects of methanol extract (a) and hexane extract (b) of callus, *in vivo* and *in vitro* grown *Asparagus officinalis*. Kojic acid was used as the positive control ($p \leq 0.05$, $n=3$)

As mentioned previously, tyrosinase catalysis the oxidation of L-DOPA to dopaquinone and then o-quinones undergone further oxidation to melanin in a series of enzymatic and non-enzymatic reactions. In fact, quinones being harmful electrophiles can interact with biological molecules including proteins to form Michael addition products and therefore lower the digestibility of the protein and the bioavailability of essential amino acids (Chu et al. 2009). According to the results, the extract of *in vivo* grown plants showed better inhibition for tyrosinase activity, so it means that this extract virtually decreased the intermediates and therefore prevent further transformation of o-quinone to melanin. Thus, it can reduce unfavorable changes in nutritive values of food and melanin formation on the skin.

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