

Full Length Research Paper

Antioxidant and antibacterial activities of ethanolic extracts of *Asparagus officinalis* cv. Mary Washington: Comparison of *in vivo* and *in vitro* grown plant bioactivities

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The antioxidant and antibacterial activities of ethanolic extracts of *in vivo* grown *Asparagus officinalis* cv. Mary Washington were investigated using superoxide dismutase, erythrocyte haemolysis and 2,2-diphenyl-1-picrylhydrazil free radical scavenging methods. The measured antioxidant and antimicrobial potential were then compared to the activities offered by the ethanolic extracts of *in vitro* grown *A. officinalis* as well as ethanolic extract of undifferentiated callus cells of *A. officinalis* produced on Murashige and Skoog medium containing 1.5 mg/l 6-benzylaminopurine combined with 0.5 mg/l naphthalene acetic acid. The highest antioxidant capacity was obtained from the *in vivo* grown plant extract followed by *in vitro* grown plant extract in all three examined assays. Although, no antibacterial activity was detected from both *in vivo* and *in vitro* grown plant extracts in the disc diffusion antimicrobial assay, ethanolic extract of *A. officinalis* offered antibacterial activity against *Bacillus cereus*.

Key words: Antibacterial activity, antioxidant activity, *Asparagus officinalis*.

INTRODUCTION

Production of secondary metabolites using *in vitro* propagation techniques has been studied since the 1970s. For example, Al-Abta et al. (1979) showed that production of phthalides, the main flavour compounds of celery, can be detected in differentiated calli of celery plant but was undetected in the undifferentiated callus tissues. Based on several investigative studies, a compound produced in an *in vivo* plant could be produced at the same or different levels or not produced at all (Verpoorte et al., 2002).

The variety of compounds produced in *in vivo* and *in vitro* plants can show different bioactivity potentials. A study by Grzegorzczak et al. (2007) showed that the

concentration of carnosic acid in acetone extract of *in vitro* grown *Salvia officinalis* shoots was higher than acetone extract of the *in vivo* plant, while amount of carnosol reduced from *in vivo* to *in vitro* plant shoot extracts. They have also reported a higher antioxidant potential for acetone extract of *in vitro* *S. officinalis* shoots when compared to *in vivo* shoot extracts using linoleic acid oxidation prevention assay.

Asparagus officinalis or vegetable asparagus has been traditionally consumed as a medicinal plant for many years (Flory, 1931). The medicinal and nutritional values of asparagus were also confirmed in different bioactivity studies as investigations showed it to be a rich source of proteins with gullible activities. For example, antifungal activity of asparagus was reported due to the presence of a novel deoxyribonuclease in its seeds (Wang and Ng, 2001). The anthocyanins isolated from spears of *A. officinalis* cv. Purple Passion showed a high antioxidant potential (Sakaguchi et al., 2008).

This study is to investigate if plant tissue culture can be a reliable tool for the mass production of pharmaceutical

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Abbreviations: MS, Murashige and Skoog medium; BAP, 6-benzylaminopurine; NAA, 1-naphthalene acetic acid.

compounds produced in a medicinal plant. Hence, the antioxidant and antibacterial effects of *in vivo* grown *A. officinalis* were measured and compared with *in vitro* grown plantlets and callus tissue of *A. officinalis*. To our knowledge, it is the first report on comparison of the bioactivity of *in vivo* and *in vitro* grown *A. officinalis*.

MATERIALS AND METHODS

Chemicals

Tween 20 (Cat.: 27,434-8), Murashige and Skoog medium (M0404), 6-benzylaminopurine (B3408), 1-naphthalene acetic acid (N0640), superoxide dismutase (SOD) determination kit (19160), 2,2-diphenyl-1-picrylhydrazil (D9132) and ascorbic acid (A4544) were purchased from Sigma-Aldrich (St. Louis, Mo), while 95% ethanol were obtained from System ChemAR® and hydrogen peroxide from UNI-CHEM® (South Kearny, NJ). Tert-butylated hydroxytoluene (34750) from Fluka (Spain) and the tetracycline antibiotic disc (9043) were purchased from Iofilchem (Italy).

Plant samples

In vitro plant samples

Seeds of *A. officinalis* cv. Mary Washington were purchased from NEW TRIO PRODUCTS (Selangor, Malaysia) and were cultivated in the green house of Institute of Biological Sciences, Faculty of Science, University of Malaya. The nodal explants from four-week old plants were collected. The explants were surfaced sterilized by first placing under running tap water for 30 min, rinsed once with sterile distilled water and immersed in 6% bleach including 1 to 2 drops of Tween 20 for 15 min. The explants were then rinsed five times with sterile distilled water and immersed in 70% ethanol for 1 min. The explants were lastly rinsed five times again with sterile distilled water and blotted on sterilized filter paper.

The explants were regenerated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1.5 mg/l 6-benzylaminopurine (BAP) combined with 0.5 mg/l 1-naphthalene acetic acid (NAA). The *in vitro* regenerated *A. officinalis* after 6 weeks of culture was used as one of the plant materials in this study.

Callus tissues of *A. officinalis* were also produced using MS medium containing 1 mg/l BAP and 0.5 mg/l NAA. The 6-week-old calli were collected as one of the *in vitro* plant materials in this study.

In vivo plant samples

The complete *A. officinalis* plants generated from the cultivated seeds were collected after four weeks of germination. The roots of the plants were removed and the aerial parts were used as *in vivo* plant material.

Ethanol extract preparation

The plant samples (*in vivo* and *in vitro* grown plant as well as callus tissues) were dried in an incubator at 40°C and were then ground to produce fine homogenous powders using an electric blender. The fine powder (3 g) was soaked in 40 ml of 95% ethanol at room temperature in the dark for three days before it was filtered through Whatman® No. 1 filter paper (Whatman International, England).

The filtered solutions were then evaporated to dryness by placing them in a water bath at 40°C overnight. The plant extracts (10 and 100 mg/ml) were dissolved in phosphate buffered saline (PBS) and kept at 4°C until required for the experiments.

Antioxidant activity

Superoxide dismutase (SOD) assay

SOD assay kit was used to determine the SOD activity of prepared extracts. The procedure was carried out based on the protocol in the kit used. Plant extracts (20 µl) of the concentration of 10 mg/ml were added to 200 µl of the kit working solution. The mixture, after a gentle shaking was incubated at 37°C for 20 min after adding 20 µl of the kit enzyme working solution. The absorbance of the mixtures was measured at 450 nm using a microplate reader (BIO-RAD Model 550, USA) and the SOD activity was calculated using the following equation (Xing et al., 2010):

$$\text{Percentage of inhibition (SOD activity)} = \frac{\{[(\text{blank 1} - \text{blank 3}) - (\text{sample A} - \text{sample A's blank 2})] / (\text{blank 1} - \text{blank 3})\} \times 100}{1}$$

where blank 1 was a mixture of the working solution (200 µl) and enzyme working solution (20 µl) containing 20 µl double distilled water (ddH₂O). Blank 2 contained the plant extract (20 µl) with working solution (200 µl) and dilution buffer (20 µl), while ddH₂O (20 µl) was added to the plant extract in the blank 3. Ascorbic acid (1 mg/ml) and 1 mg/ml of tert-butylated hydroxytoluene (BHT) were employed as the positive controls in this study.

Erythrocytes haemolysis prevention assay

This assay was based on the method described by Rafat et al. (2010a). Erythrocyte suspension was prepared by centrifugation of rabbit blood at 1000 xg and 4°C for 20 min. An equal volume of PBS was then added with the collected cells. The erythrocyte suspension (500 µl) was pre-treated using 1000 µl of the plant extract (10 mg/ml) at 37°C for 40 min before adjusting the mixture volume to 9 ml by adding phosphate buffered saline (PBS). Oxidative stress was then induced by mixing 1 ml of 10 mM hydrogen peroxide (H₂O₂). Finally, the released hemoglobins from the hemolysed erythrocytes into the supernatant of the mixtures was determined at 540 nm using a spectrophotometer. Ascorbic acid (1 mg/ml) was applied as the positive control, while the non-pre-treated erythrocyte was used as the negative control in this study. Complete erythrocyte hemolysis was acquired by using ultra pure water and labeled as 100% hemolysis, and hemolysis of other samples were stated as a percentage of this value.

Radical scavenging capacity assay

DPPH* (2,2-diphenyl-1-picrylhydrazil) free radical scavenging capacity assay was obtained using the protocol described by Rafat et al. (2010b). DPPH* (950 µl), a concentration of 90 µM was mixed with 50 µl of the plant extracts (10 mg/ml) and the volume was adjusted to 4 ml using 95% ethanol before incubation at room temperature in the dark for 120 min. Scavenging of DPPH* reduced the colour of the solution and was measured using a spectrophotometer at 515 nm. Comparison of the reduction of colour in the examined samples with the blank (solution without plant extract) was used to measure the potential of scavenging capacity of our plant extracts using the following equation (Rafat et al., 2010b):

$$\text{Radical scavenging capacity (\%)} = \frac{[(\text{Blank} - \text{Sample A}) / \text{Blank}] \times 100}{1}$$

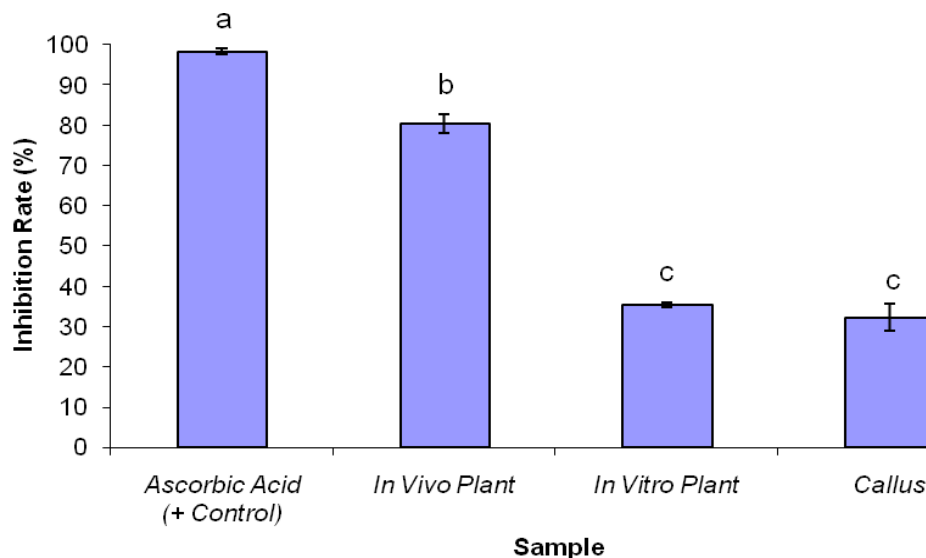


Figure 1. Antioxidant activity of examined plant extracts (*in vivo* plant, *in vitro* plant and callus) of *Asparagus officinalis* measured using superoxide dismutase (SOD) assay are presented as percentage of inhibition rate. Ascorbic acid (1 mg/l) was used as the positive control. The data were analyzed by one-way ANOVA and the inhibition rate means of samples were compared using Duncan's multiple comparison test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

Antibacterial activity assay

The antibacterial potential of *A. officinalis* ethanolic extracts was studied using the paper disc diffusion method of Kil et al. (2009). Two gram-negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram-positive pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were obtained from the Microbiology Division of Institute of Biological Sciences, University of Malaya and then grown in nutrient broth medium to yield a final concentration of 10^7 colony forming unit (CFU)/ml. The test bacteria (0.1 ml) were streaked on Mueller Hinton medium plates using sterile cotton swab. Sterilized filter paper discs were soaked in ethanolic extracts (100 mg/ml) and were then placed in the center of test bacteria plates. The plates were incubated for 24 h and the diameters of the inhibition zones were measured. Tetracycline disc (30 μ g) and PBS were used as the positive and negative controls, respectively.

Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version 15. The means were compared with Duncan's multiple comparison test (DMCT) and $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Antioxidant activity

SOD assay

Based on the result of SOD assay (Figure 1), *in vivo* plant

extract had the highest inhibition rate (80.44%) among all three examined plant extracts which is significantly different with both *in vitro* plant and callus extracts. Although, *in vitro* plant extract inhibition rate (35.42%) was slightly higher than callus extract (32.24%), there was no significant difference between them. All extracts used showed significantly lower inhibition rates compared to 1 mg/l ascorbic acid (98.35%) positive control.

Erythrocytes haemolysis assay

The result of the rabbit erythrocytes haemolysis assay is shown in Figure 2. The negative control (without pre-treatment) obtained a mean of 62.8% haemolysis, while ascorbic acid (1 mg/l) could reduce the haemolysis percentage to 6.46% in positive control. All plant extracts examined in this study could reduce the erythrocytes haemolysis significantly when compared to the negative control. *In vivo* plant extracts (14.47%) showed the highest erythrocytes haemolysis prevention when compared to the *in vitro* (53.19%) and callus (54.47%) extracts. There was no significant difference between the rates of hemolysis reduction by *in vitro* plant and callus extracts, while both of them were significantly different with *in vivo* plant extract.

DPPH* scavenging activity assay

Based on the DPPH* scavenging activity assay results

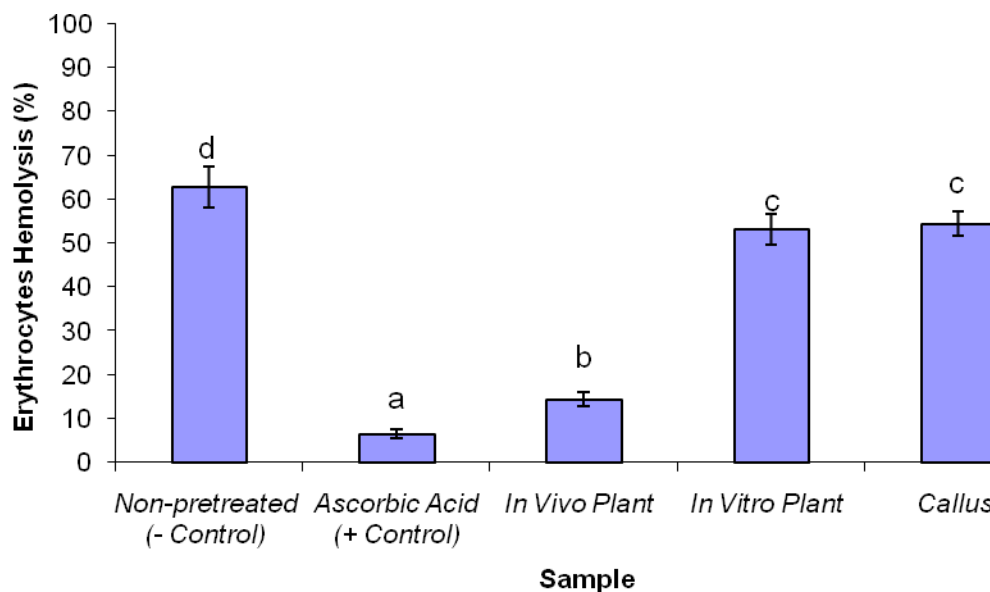


Figure 2. Antioxidant activity of examined plant extracts (*in vivo* plant, *in vitro* plant and callus) of *A. officinalis* measured using rabbit erythrocytes haemolysis assay are stated as percentage value. Ascorbic acid (1 mg/l) was used as the positive control. The data were analyzed by one-way ANOVA and the haemolysis percentage means of samples were compared using Duncan's multiple comparison test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

(Figure 3), the highest scavenging capacity was obtained from *in vivo* plant extract (62.67%) followed by *in vitro* plant extract (61.33%), while there was no significant difference between these two samples. Callus extract (54.67%) showed significantly lower scavenging potential when compared to the other plant extracts. Although all examined plant extracts showed significantly lower DPPH* scavenging activities when compared to 1 mg/l of ascorbic acid (82.67%), all samples showed significantly higher free radical scavenging potential when compared to 1 mg/l BHT (33.67%).

Antibacterial activity assay

The results of antibacterial activity assay (Table 1) showed that bacterial growth inhibition was shown only in callus extract (100 mg/ml) against only one of the test pathogenic bacteria *Bacillus cerus*. The inhibition zone produced by callus extract (14 mm) was significantly smaller than 30 µg tetracycline (40 mm). The rest of the extracts showed no antimicrobial activity in the same concentration of 100 mg/ml against any of the tested pathogenic bacteria.

DISCUSSION

The present study is in agreement with the previous reports on high antioxidant activity of *A. officinalis* (Makris

and Rossiter, 2001; Rodríguez et al., 2005; Sun et al., 2007). Antioxidant activities of the methanol, acetone and water extracts of *A. officinalis* were examined by Sun et al. (2007) and acetone extract showed the highest antioxidant capacity, while water extract had the lowest activity. The high solubility of antioxidant compounds in ethanol in different plants, such as *Doispyros kaki* cv. Fuyu (Jang et al., 2010) and *Cajanus cajan* (L.) Millsp (Wu et al., 2009), was reported. Rodríguez et al. (2005) used ethanol to extract the antioxidant compounds from different cultivars of *A. officinalis*. The results of this study also showed that ethanol can be a suitable solvent to extract the antioxidants of *A. officinalis*. In terms of antimicrobial activity of the plant extracts, Jana and Shekhawat (2010) reported that ethanol extract of different parts of *Anethum graveolens* showed higher antimicrobial activity than aqueous extracts of the plant parts.

Since application of only one antioxidant activity evaluation assay may not give a reliable result, using few methods is recommended (Rafat et al., 2010). Hence, three different techniques for investigation of antioxidant activity in this present study were employed. The results showed that all applied assays are in agreement with the fact the antioxidant capacity of *in vivo* grown *A. officinalis* was higher than both *in vitro* grown plant and callus tissues. Perhaps these results show that the amount of antioxidants produced in *in vivo* grown *A. officinalis* is higher than the *in vitro* grown ones. This result confirmed the study of Tanwer et al. (2010) when they showed that

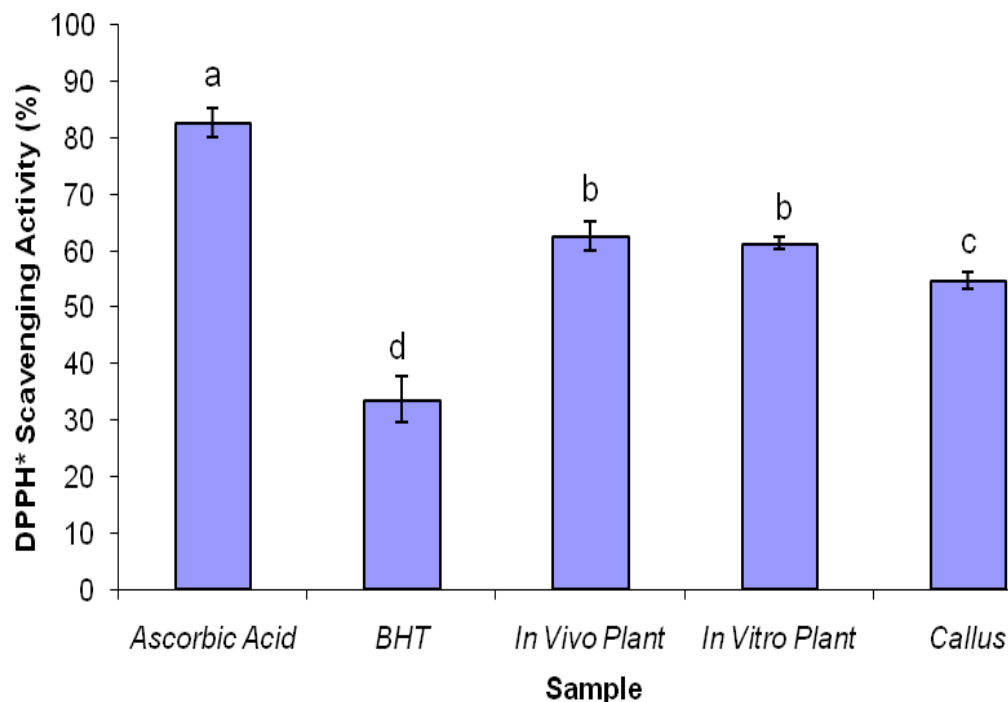


Figure 3. Antioxidant activity of examined plant extracts (*in vivo* plant, *in vitro* plant and callus) of *Asparagus officinalis* measured using DPPH* scavenging activity assay presented as percentage value. Ascorbic acid (1 mg/L) and butylated hydroxyl toluene (BHT) (1 mg/L) were applied as the positive controls. The data were analyzed by one-way ANOVA and the DPPH* scavenging activity percentage means of samples were compared using Duncan's Multiple comparison test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

antioxidant activity of methanolic extract of the *in vivo* grown *Spilanthes acemella* is higher than the *in vitro* grown callus. In the present study, the lowest antioxidant potential obtained from all three tests belonged to the callus extract but it was not significantly different from *in vitro* plant extract in superoxide dismutase (SOD) and erythrocyte haemolysis assays.

Poyrazoğlu (2009) studied the antimicrobial activity of different extracts of *A. officinalis* against some microorganisms and showed that ethanolic extract of the plant could only inhibit the growth of *Pseudomonas fluorescens* among all the examined yeasts and bacteria. However, the results of this study showed that the callus extract used can inhibit the growth of only one gram-positive test bacteria (*B. cereus*). Based on the statistical analysis, this inhibition is significantly smaller than positive control (30 μ M of tetracycline). The other *in vitro* and *in vivo* grown *A. officinalis* extracts showed no antimicrobial activity against both groups of gram-negative and -positive bacteria.

The results of well diffusion antimicrobial activity carried out by Jana and Shekhawat (2010) also showed that the ethanolic extract of *A. graveolens* callus inhibited the growth of *Bacillus subtilis* more than the ethanolic extract of the plant roots. The antibacterial activity of the callus extract might be either related to the production of a

compound produced in only undifferentiated callus cells or may be produced in higher amounts in these cells when compared to differentiated cells. Several quantitative estimations and studies showed that the production of biocompounds can vary between differentiated and undifferentiated plant cells. For example, Tanwer et al. (2010) reported that calli of *S. acemella* produced a higher amount of sugars when compared with stem, leaves and roots of the plant. Jana and Shekhawat (2010) also showed that the callus cells of *A. graveolens* produced saponins, while the *in vitro* leaf cells are not able to produce the same compound.

Conclusion

This study showed the antioxidant and antimicrobial activities of *A. officinalis* and also showed that these bioactivities differ between *in vitro* and *in vivo* grown plants. Total antioxidant capacity of *in vivo* grown plant was higher than *in vitro* grown plant, while the only antimicrobial activity was obtained from *in vitro* callus tissue against *B. cereus*. Some phytochemical studies are required to investigate the production of antioxidant and antimicrobial compounds in differentiated and undifferentiated callus cells of *A. officinalis*.

Table 1. Inhibition effect of 100 mg/ml of *A. officinalis* ethanolic extracts (*in vivo* plant, *in vitro* plant and callus) against the growth of four pathogenic bacteria.

Bacteria	Inhibition zone (mm)			
	<i>In vivo</i> plant	<i>In vitro</i> plant	Callus	Tetracycline (30 µg)
<i>Escherichia coli</i>	- _b	- _b	- _b	42 ± 3.00 ^a
<i>Staphylococcus aureus</i>	- _b	- _b	- _b	20 ± 2.64 ^a
<i>Pseudomonas aeruginosa</i>	- _b	- _b	- _b	11 ± 2.00 ^a
<i>Bacillus cereus</i>	- _c	- _c	12 ± 1.00 ^b	40 ± 3.60 ^a

-: No inhibition; The data were analyzed by one-way ANOVA and the inhibition means of samples were compared using Duncan's multiple comparison test (DMCT). Mean of different samples labeled with different letters are significantly different in each row ($p < 0.05$).

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