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ENHANCED ANTIOXIDANT AND PHYTOCHEMICAL PROPERTIES OF *IN VITRO* GROWN *ASPARAGUS RACEMOSUS*

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Abstract

Asparagus racemosus is a valuable medicinal plant having antioxidant property and various phytochemical constituents. The aim of the study was to compare the difference in antioxidant property and phytochemicals constituents between in vitro propagated *Asparagus racemosus* and its in vivo grown counterpart. Considering this Asparagus racemosus was propagated in vitro under controlled conditions with varying concentration and combination of plant growth regulators. The antioxidant property of both the plants were analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) scavenging assays. The estimation of total phenolic, flavonoid and carotenoid contents were performed by spectrophotometric analysis. Surprisingly, the antioxidant property and phytochemical constituents in in-vitro propagated plants were substantially higher in comparison to the in vivo grown plants. In vitro plant extract (100µg/ml) exhibited the highest DPPH (A_{517}=76.08 ±0.571) and NO (A_{540}=80.20 ±0.46) radical scavenging activities respectively. Moreover, In vitro plant extract had the highest phenolic (738 µg/ml), flavonoid (594.5 µg/ml) and carotenoid contents (393 µg/ml). This is the first study showing that the secondary metabolite production can be better under controlled in vitro conditions. In conclusion, this technique harbors significant potential for the large-scale production of various therapeutically important compounds in medicinal plants.

Key words: Asparagus racemosus, Medicinal plant, Antioxidant, Phytochemicals, Plant tissue culture.

Introduction

Medicinal plants have been used as therapeutic agents in traditional medicine such as Ayurveda, and Unani medicine from ancient time (Firenzuoli and Gori, 2007; Petrovska, 2012; Nankar *et al*., 2017). In the under developed regions of the world where patients still do not have access to modern medicine people still heavily rely on various plant parts for the treatment of their ailments (Kaur *et al*., 2016; Sharma, 2016; Gaikwad *et al*., 2018). It is quite evident that the Indian people have unabated belief in medicinal plants for improving memory and immunity; and treatment of plethora of diseases including muscular dystrophy (Ganguly, 2019; Gautam *et al*., 2009; Mootooswamy, 1889; Penmetsa and Pitta, 2019; Vahia, 1963; Kaur *et al*., 2016a, 2016b).

Asparagus racemosus is one of the most crucial medicinal plants of tropical and sub-tropical India (Malik *et al*., 2013; Malik *et al*., 2016; Rather *et al*., 2018). Its medicinal benefits have been reported in various pharmacopoeias and ethno-medicinal practice across the globe. The genus *Asparagus* includes consists of 300 species and it is considered to have significant medicinal value due to the presence of numerous phytochemicals in various parts of the plant. Even today various ayurvedic practitioners use the different concoctions and formulations made of *Asparagus racemosus* to treat menstrual irregularities. Various products containing *A. racemosus* are available in the market for different medicinal usage (Arathip, 2001; Chakraborty *et al*., 2015; Saxena *et al*., 2018). One of the important components present the Asparagus as the phytoestrogenic compound which have different therapeutic uses. Various studies have shown that it has several pharmaceutical activities like antioxidant, antistress, anti-diarrhoeal, adaptogenic action, anti-dyspepsia, anti-ulcerogenic action and cardio protective activity (Karuna *et al*., 2018; Pandey *et al*., 2018; Venkatesan *et al*., 2005; Singla and Jaitak, 2014; Prasher *et al*., 2018).

The antioxidant property is one of the most significant properties of *A. racemosus* (Karuna *et al*., 2018). Antioxidants scavenge various free radical and prevent the oxidation of various other biomolecules. (Chauhan *et al*., 2017; Sivakumar *et al*., 2011; Prabhakar *et al*., 2013) Various metabolic reactions in our body generate free radicals, which can eventually start different reactions that will damage the cells. Oxidative damages have been implicated in the cause of many diseases such as Alzheimer's disease and different type of cancer (Kashyap *et al*., 2019; Mitra *et al*., 2012; Sharma *et al*., 2019). The present study shows the antioxidant effects of various compounds present in both the aqueous as well as crude extracts of *Asparagus racemosus*. The concentration of antioxidant compound in *Asparagus racemosus* was comparable to that of the established antioxidants such as glutathione and ascorbic acid. *In vitro* study has shown that the extracts of this plant showed a potent antioxidant activity in mitochondrial membranes of rat liver (Kamat *et al*., 2000; Saxena *et al*., 2016; Sharma, 2016; Chauhan *et al*., 2017; Mishra *et al*., 2018). These studies corroborate the hypothesis that *Asparagus racemosus* is rich source of antioxidant compounds and can be used for the treatment of oxidative stress caused due to different metabolic and pathological reasons.

Asparagus plants propagated from the seed show variations in their quality and quantity. There is dire need to establish an effective and rapid method for the asexual reproduction of plants without any variations in their medicinal characteristics. Plant tissue culture is an important tool for multiplication and enhancement of the medicinal plants. Studies have shown that the *in vitro* propagation of plants provide a remarkable opportunity for the synthesis of high quality plant derived medicines (Saxena and Bopana, 2009; Prabhakar *et al*., 2020). This technique can also be used for the production of different secondary metabolites by using plants as bioreactors. This study aims to advance tissue

culture technique for the *in vitro* propagation of plants with medicinal value from numerous explants and improve the secondary metabolites production (Kaur *et al*., 2020).

Generally, plant tissue cultures are performed using apical meristem, lateral shoot and shoot tips of the asparagus plants. However, this avenue has certain limitations like less number and insufficient amount of explant material for the mass propagation. For the commercial production of secondary metabolite from *A. racemosus* a large number of explant will be required. To solve these problems, with this study, we used cladophylls attached with nodal segment of asparagus and carried out the *in vitro* culture of the cladophylls attached with nodal segments of asparagus, which were developing in numbers of tens of hundreds from only a single plant (Singh *et al*., 2019).

Next, we aim to evaluate the antioxidants and phytochemical constituents of *in vivo* and *in vitro* grown *Asparagus racemosus.* Currently, there is a great interest in identifying and evaluating antioxidant compounds due to their various therapeutic values. Here, we have shown that crude extract of *in vitro* grown *Asparagus racemosus* has better antioxidant and phytochemical properties.

Materials and Methods

Media Preparation

For the propagation of *Asparagus racemosus* Murashige and Skoog's (MS) media was used. The MS powder was dissolved in double distilled water and pH of the prepared media was maintained at 5.8. In sterile culture vessel 50 mL media was poured and autoclaved at standard conditions to sterilize the media. Then, the autoclaved bottles with MS media were stored for future use under aseptic condition in media storage facility.

Explant selection and sterilization

Axillary nodes, internodes, and young leaves of Asparagus were used as explants. The explants were washed under running tap water to remove any dust and dirt particles. Then the explants were immersed in 0.3% bavistin and 0.03% streptomycin solution for 10 min. Following that the explants were washed twice. Treated explants were submerged in aqueous solution of savlon for 10 minutes and again washed twice thoroughly with autoclaved water. Following this, the washed explants were surface sterilized with 0.01% mercuric chloride solution for 5 minutes and re-washed thoroughly with autoclaved distilled water.

Initiation of culture

From the sterilized explants dead and undesirable parts were removed and transferred to the culture media asceptically with the help of a sterilized forceps. Then the lid of the culture bottles were closed and sealed with Klin film. For all the explants same procedure was followed. Finally, the culture bottles were incubated under cool white fluorescent light at 25±2ºC, with a photoperiod of 16 h daylight and 8 h night break.

Establishment of cultures

The bud-proliferated explants were transferred to new culture vessels with fresh MS medium. After incubating the explants for 21-25 days the initiated explants were taken out and transferred to fresh semi-solid MS media. Then, the culture bottles with semi- solid media were incubated under white fluorescent light at 25±2ºC for 8-16 h of day and night period.

Auxiliary shoot proliferation

The explants were transferred to MS medium supplemented with different plant regulators at varying concentrations. The shoot multiplication were performed by repeated sub-culturing in MS media with auxin and cytokinin. The pH of growth regulator media was maintained at 5.8 and the culture bottles were incubated at 25±2ºC. These steps were repeated after an interval of 25 days.

Nitric oxide scavenging assay

The plant materials were collected, dried and then powdered with the help of mechanical grinder. Then the powder was dissolved in ethanol for 3 hours at 25^0 C for obtaining the extract. Nitric oxide (NO) was generated from Sodium nitroprusside. 10 mM Sodium nitroprusside in phosphate buffer saline (PBS) was mixed with varying concentration of the plant extract (100, 300, 500, 700 & 900 µg/ml). Then this mixture was treated with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). The absorbance was measured at 540 nm and for the positive control ascorbic acid was used. The percentage inhibition was measured using the following formula:

$$\% \text{ Inhibition} = (A_0 - A_t) / A_0 \times 100$$

Where A_0 is absorbance of control (blank) and A_t is the absorbance in presence of extract.

DPPH radical scavenging assay

Different concentrations (200 µl) of sample solution and 50 µl of DPPH (0.659 mM) solution were mixed and stored in dark condition at room temperature for 20 min. Following the incubation the absorbance was monitored spectrophotometrically at 517 nm and percentage inhibition of DPPH was evaluated (Sudhakar *et al*., 2015).

Estimation of phenolic compounds

5 g of samples were homogenised in acetone and stored overnight. The supernatant was centrifuged and filtered to remove debris. The purified supernatant was used to estimate the pheolic content in the acetone extract. To the extract ferric ammonium-sulphate was added and incubated at 27^0C. Finally potassium ferri-cynide was added and absorbance was measured spectrophometrically at 720 nm.

Flavonoid content estimation:

5 g of powered sample was treated with sulphuric acid and followed by neutralization with sodium hydroxide. To the acid hydrolyzed sample ethyl acetate was added. After evaporating the ethyl acetate, dry residue was mixed with methanol and analyzed for the total flavonoid flavonoids content determination by adding aluminum chloride and measuring the absorbance at 430 nm (Tanwar and Modgil, 2012).

Carotenoids content estimation:

After homogenization of 5g of sample in acetone, repeated filtration was performed until extract was free from pigment. The filtrate was then portioned with equal volume of ether thrice. Then, the ether phase was evaporated and residue was reconstituted in ethanol. Then, potassium

hydroxide was added and the carotenoid content was estimated by measuring absorbance at 450 nm.

Results

Micro propagation of *Asparagus racemosus*

After incubating the explants with bud proliferation under the optimum condition in MS media, the shoot formation was observed as shown in figure 1.

Fig. 1: *In vitro* grown *Asparagus racemosus*

In vitro grown plant extract exhibited the higher DPPH and NO radical scavenging activities when compared to *in vivo* grown plants of *A. racemosus*

The antioxidant property of the *A. racemosus* was analyzed by calculating the percentage inhibition of DPPH free radical Higher the antioxidant activity lower will be the absorbance at 530nm (Vyas *et al*., 2017; Bashir *et al*., 2019) The antioxidant activity of *A. racemosus* extract is increased with the increasing concentration of the extract. (Kar *et al*., 2018; Singh and Thakur, 2018; Mishra *et al*., 2019) The comparison of the free radical inhibition by *in vivo* plant extract and *in vitro* plant extract is demonstrated in Figure 2. (Vyas, *et al*. 2019) We observed that the *in vitro* plant extract inhibited 76.08 ±0.571 % of the DPPH and the *in Vivo* plant extract is able to inhibit 66.77 ± 0.72% DPPH.

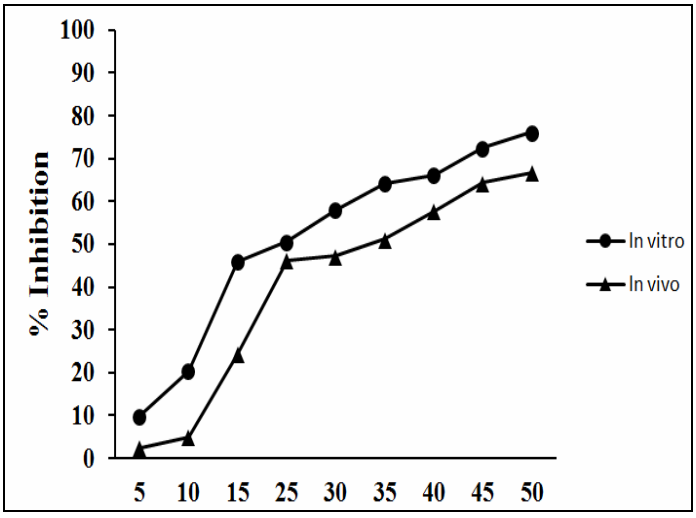

Fig. 2: Percentage inhibition of DPPH by *in vivo* and *in vitro* propagated *A. racemosus* plant extract

Reactive nitrogen species are very harmful as they can affect the structure of various biomolecules, impair various cellular components and eventually affect different metabolic activities. (Mishra *et al*., 2019) Compound with antioxidant activity will help to alleviate these damages. Extract of both *in vivo* and *in vitro* propagated *Asparagus racemosus* shows nitric oxide reduction. The comparison of nitric oxide scavenging property of *in vivo* and *in vitro* grown plant is shown in Figure 3. We observed that the *in vitro* extract inhibited 80.20 ±0.46 % nitric oxide and the *in vivo* plant extract inhibited 72.24 ±0.25 % nitric oxide. ***In vitro* grown plant extract exhibited higher phenolic, flavonoid and carotenoid contents when compared to *in vivo* grown plants of *A. racemosus.*** The comparison of phenolic content in *in vivo* and *in vitro* propagated plant extract is demonstrated in Figure 4. The average concentration of the phenolics in *in vitro* plant extract was 738 µg/ml while the phenolic content in *in vivo* plant extract was 705.5 µg/ml.

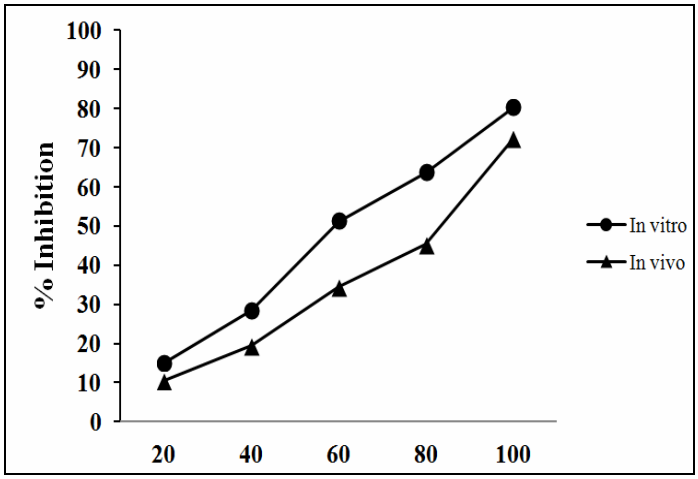

Fig. 3: Percentage inhibition of Nitric oxide by *in vivo* and *in vitro* propagated *A. racemosus* plant extract.

The comparison of flavonoids content in the extract of the *in vivo* and *in vitro* propagated plant is shown demonstrated in Figure 4. The average concentration of the flavonoid content in *in vitro* plant extract was 594.5 µg/ml, while the flavonoid content in *in vitro* plant extract was 544 µg/ml.

The comparison of carotenoids content in the extract of the *in vivo* and *in vitro* plant is demonstrated in Figure 4. The average concentration of the carotenoids content in *in vitro* plant extract was 393µg/ml, while the carotenoid content in *in vivo* plant extract was 341 µg/ml (Kumar *et al*., 2019).

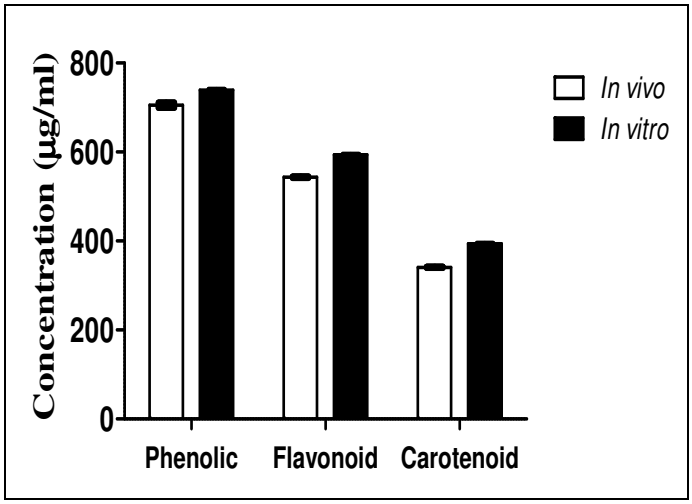

Fig. 4: Comaparative concentration of phytochemicals in *in vivo* and *in vitro* grown *Asparagus racemosus*

Discussion

This study shows an efficient way to propagate various plants of medicinal importance. We compared the antioxidant property and phytochemical constituent differences in the *in vivo* and *in vitro* propagated plant of *Asparagus racemosus* which might be very helpful for the efficient production of

Asparagus racemosus at industrial level for secondary metabolites production. This is the first study showing that the production of secondary metabolites can be better under controlled *in vitro* condition. This study shows that the antioxidant property and phytochemical constituents of *in vitro* propagated plant was relatively higher than the *in vitro* propagated plant. In conclusion, this technique harbors significant potential for the large-scale production of various therapeutically important compounds in medicinal plants.

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