IN VITRO MICROPROPAGATION, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF ORTHOSIPHON STAMINEUS BENTH

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ABSTRACT
Plant tissue culture is a potentially useful technique for growing endangered or threatened plants, sometimes it’s difficult to discover the favorable conditions for re-establishment and sustainable growth of plants. The present study was carried out to study the in vitro callus, shoot and root induction from different explants (leaf and petiole) from medicinally important plant Orthosiphon stamineus Benth. Significantly more shoots were induced on the medium containing IBA (1.5 mg/l) than any other treatment. Sub-culturing regenerated shoots on a medium with (IBA 1.5 mg/l) induced the maximum rate of shoot multiplication. Methanol extract potentially decomposed the free radicals by in vitro antioxidant of DPPH at low concentration (30 µg/mL). In the present study, O. stamineus possessed the highest antioxidant and antibacterial activities and thus could be a potentially rich source of natural antioxidants.

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INTRODUCTION
Natural products served the significant source of drugs since, ancient times and part of today’s drugs are derived from natural sources. Pharmacological activities of secondary metabolites from the plants were previously unknown but it can be investigated comprehensively as a source of medicinal agents in recent years [1]. Plants are the reservoirs of organic compounds and they have been used as a source of medicines for many years. Herbal medicine is a part of larger therapeutic system for both traditional and folk medicine. It is necessary to evaluate the potential use of folk medicine to treat the infectious diseases and disorders [2]. Traditional herbal medicine plays a key role in developing countries to treat many infectious diseases and health care [3]. At present 80% of the World population depends on plant-derived medicine for primary health care of human beings since it has no side effects. Hence, alternative medicines are available for those who do not want conventional medicine or who cannot be helped by conventional medicine.

Orthosiphon stamineus Benth.(or) Misai Kucing is a popular medicinal herb in South-East Asia, belongs to the Lamiaceae family (Malay for “Cat’s Whiskers) are mostly present in Malaysia, Indonesia, Thailand and some part of Tropical Australia. It is an herbaceous shrub which grows to a height of 1.5 meter [4,5] and is also consumed as a healthy java tea to facilitate body detoxification in Japan [6]. The leaves are used as antidiabetic drugs used as a remedy to treat urinary tract and renal diseases [7]. In Europe, people use O. stamineus extract as a tonic to treat liver and gallbladder, kidney and bladder stone problems, urinary tract infections and also used to reduce cholesterol and blood pressure [8].

Recently there are number of commercial products derived from O. stamineus are rich in flavonoids where most of the flavonoids [9]. They exhibit excellent antibacterial, antifungal, anti-tumor and insect activities [10]. Several studies have investigated the biological and pharmacological effects of O. stamineus including its Hypertension, Diabetes mellitus, Gout, Rheumatism, Diuretic, anti-inflammatory, influenza, diuretic and anti-oxidant effects as well as its beneficial effects on hyperglycemia and altered lipid profile in diabetic rats [11,12]. Free radical contain one or more unpaired electrons which is highly unstable and cause damage to other molecules by extracting electrons in order to attain stability.

The beneficial effects of antioxidants such as carotenoids, enzymes, phenolic compounds and vitamins are due to their ability to scavenge or neutralize reactive oxygen species (ROS) such as superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (1O$_2$). The recent year’s investigations of natural products have regained the importance due to its biological consequences such as antioxidants, radical scavenging, antiproliferative activities and recognition of the origin as well as function of their structural diversity [13].

MATERIALS

Chemicals
Ascrobate, citrate, DPPH (1, 1- diphenyl- 2-picrylhydrazyl), Ferrous chloride, ferrozine, Gibberallic acid 3, Indole Butyric Acid, 1N Hcl, Mercuric chloride, Murashige and Skoog medium, Na$_2$EDTA, 1N NaOH, sucrose, Tris-HCl, Tween-20 (Sigma Chemical Co, USA).

METHODS

Collection and Identification of plant material
The whole plant of Orthosiphon stamineus was collected from Tropical Botanical garden and Research institute, Pallode, Kerala, India. It can grow on any soil types including sandy soil like bris, tin tailings and needs an average rainfall of 180 - 200 cm to grow well, can withstand waterlogged conditions. The plant has a dry, salty, bitter taste. The O. stamineus was identified by Prof. K. Murugesan, Professor, CAS in Botany, University of Madras, Guindy campus, Tamil Nadu, India. The herbarium No.781 was maintained in our campus.

Preparation of Murashige and Skoog medium
Stock solutions were prepared and stored in brown bottle to prevent photolysis at 4ºC. The solution 1 of MS media was taken in the amount of 50 mL; 2, 3 and 4 MS media was taken in the amount of 5 mL, respectively and suspended in 935 mL of distilled water, 3% sucrose was added and pH of the medium was adjusted to 5.6 using 1N HCl or 1N NaOH. Before autoclaving, the medium was solidified by adding 0.8 % agar and it was poured into culture flasks and sterile plant hormones were added [14].

Micropropagation
The collected plant materials were cut (1-2 cm length) using sterile plant cutter and were used as explants. The explants were washed in 1.0% (v/v) Tween-20 (Sigma Chemical Co, USA) for 10 minutes after then washed in running tap water for 15 minutes. Further, they were immersed in a solution containing 50 mg/l of ascorbate and citrate to remove the phenol exudation from the explants for 2 minutes. Finally the surface was sterilized by passing through 0.1% (w/v) mercuric chloride for 7 minutes. After rinsing five times with sterile distilled water, the explants were planted in MS medium (Murashige and Skoog, 1962) supplements with auxin (IBA) and GA3 containing 8.0 g/l for 4-5 weeks and incubated in growth chamber (Sciogenics Biotech, model Orbitek), at 25±1º C for 7-30 days. The inoculated tissue culture flasks were regularly observed for the initiation of callus, shoot and root.
Preparation of extract

Orthosiphon staminues was collected from the field and washed thoroughly with running tap water and rinsed in sterile distilled water by following the method of [15]. The washed plant materials were shade dried at room temperature for 10 days. The shade dried plant parts were made into a coarse powder it was extracted with methanol in a Soxhlet apparatus for 8 to 16 h. The samples were concentrated using a rotary evaporator (Heidolph laborata, Germany) at various temperature under reduced pressure.

Antioxidant activity

The measurement of the antioxidant activity with different concentration of methanol extract was carried out using 1, 1-diphenyl- 2-picrylhydrazyl (DPPH) [16]. Assay was performed in 3 mL reaction mixtures containing 2.0 mL of 0.1 mM DPPH in ethanol solution, 0.9 mL of 50 mM Tris-HCl buffer (pH 7.4), methanol in different concentrations extracts (5 -30 µg/mL). After 30 minutes incubation at room temperature, the absorbance of reaction mixtures was taken at 517 nm. The free radical-scavenging activity (FRSA) was calculated using the following formula

\[ FRSA = \left( \frac{Ac - As}{Ac} \right) \times 100 \]

Where Ac - absorbance of control, As - absorbance of sample.

Determination of chelating effects of ferrous ions

The chelation of ferrous ions by the extracts were estimated by the method of [17]. Briefly, 50 μL of 2 mM FeCl₂ was added to the extracts (0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL). The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 minutes. Absorbance of solution was thereafter measured at 562 nm. Na₂EDTA was used as positive control. The percentage inhibition of ferrozine Fe²⁺ complex formation was calculated using

\[ \left( \frac{A_0 - As}{As} \right) \times 100, \]

A₀ - absorbance of control; As - absorbance of sample.

ANTIBACTERIAL ACTIVITY

Sterility test of plant extract

Methanol extract was tested for any growth or contaminants. This was carried out by inoculating 1 mL of extract on sterile Mueller Hinton Agar plates and incubated at 37°C for 24 h and the plates were observed. After incubation absence of growth in the extracts indicated that they were sterile and different extracts were then assessed for antimicrobial activity.

Preparation of inoculum

Overnight broth culture of the respective bacterial strains were adjusted to turbidity equivalent to 0.5 McFarland standards. 0.2 mL culture of organisms was dispensed into 20 mL sterile nutrient broth and incubated for 24 h and standardized at 10⁷-10⁸ CFU/mL adjusting the optical density to 0.1 at 600 nm.

Antibacterial activity of O. staminues was determined using agar well diffusion method [18]. The human pathogenic bacteria namely Bacillus subtilis, (MTCC441), Escherichia coli (MTCC443), Klebsiella pneumoniae (MTCC109) and Micrococcus luteus (MTCC4698) were procured from Microbial type culture collection (MTCC) center, IMTECH, Chandigarh, India. When the media solidified, 0.1 mL of active growth culture was poured over feeder layer and spread evenly by sterile spreader. The 6 mm diameter well was made by using a sterile cork borer. Each well received different concentration (50, 100 and 150 µg/ mL) of crude extract. They were dissolved in 0.4% DMSO (Dimethyl sulfoxide) and were incubated at 37°C for 48 h, appropriate control was maintained. After incubation the inhibition zone was measured by millimeter.

Statistical analysis

All the experiments were repeated three times and the data were represented as mean ± SE using Prism Graph Pad version 6.0 for windows (Graph Pad Software, San Diego, California, USA, and http://www.Graphpad.com/).

RESULTS

Callus induction

The MS medium (Murashige and Skoog) was supplemented with different concentrations of Auxin (IBA) 0. 5 to 1.5 mg/l and GA3 (0.5 to 1.5 mg/L. The callus development from leaf explants was observed in 2 week old cultures. Development of callus from leaf and petiole explants was observed after 1 week of culture on all media tested. Callus induction frequency in response to different combinations of auxin and cytokinin in MS medium (Fig. 1a and Table. 1).
Shoot induction

To induce adventitious shoot formation, the explants were cut into pieces 0.5–1 cm and cultured on MS basal medium supplemented with Auxin (IBA) 0.5–1.5 mg/l. The cultures were incubated at 25±1°C under a 12 h photoperiod. Growth of shoots on MS medium containing auxin (IBA) resulted in callusing at the base of the shoots. Regenerated shoots were separated and sub-cultured on MS medium for further shoot proliferation and growth. The number of adventitious shoots formed were evaluated after 60 days (Fig. 1b).

Root induction

Continuous culturing of shoot tips provide a good shooting system and it promoted root induction. IBA and GA3 is clearly promoting root induction than NAA or IAA (Table 1). The rooting system contained IBA and GA3 1.5 mg/l, on which the regenerated shoots developed roots within 45 days. Well-developed plantlets were hardened with low soil containing medium and transferred to plastic pots in a greenhouse. Cow dung and garden soil (2:2) was found to be effective for survival of in vitro rooted plantlets (Fig. 1c & d).

Table 1. Different concentrations of plant hormones for micropropagation.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Concentration mg/ml</th>
<th>Average no. of shoot per explant</th>
<th>Percentage</th>
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<tr>
<td></td>
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<td></td>
<td>Shooting</td>
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<tr>
<td>IBA</td>
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<td>50</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50</td>
<td>24</td>
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<td></td>
<td>1.5</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>GA3</td>
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<td>50</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>IBA+GA3</td>
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<td>50</td>
<td>8</td>
</tr>
<tr>
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<td>1.0</td>
<td>50</td>
<td>12</td>
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<tr>
<td></td>
<td>1.5</td>
<td>50</td>
<td>16</td>
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</table>

Fig 1. Micropropagation of Orthosiphon stamineus.

a) O. stamineus 7th day, b) O. stamineus 15th day, c) Root induction O. stamineus d) Hardening of O. stamineus
Free radical scavenging assay

The antioxidant potential of methanol extract of *O. stamineus* was carried out using 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), it was observed that FRSA was increased with increased concentrations of the methanol extract; it was only 25% at 5 µL while the activity was 75% in 30 µL when compared to that of control (Table. 2 & Fig. 2).

Antibacterial activity

In vitro antibacterial activity of methanol extract exhibited maximum activity against gram negative (*Bacillus subtilis, E. coli, K. pneumoniae* and *M. luteus*) bacteria at concentrations (50-150 µg/mL). Antibacterial activity of *O. stamineus* with methanol (50-150 µL) exhibited significant antibacterial activity which ranged from 7-16 mm; the inhibition of bacterial growth is inversely proportional to the concentration of the methanolic extract (Fig. 3). Among the bacteria *M. luteus* is susceptible and *K. pneumoniae* is resistant.
DISCUSSION

The findings from this micropropagation study differs from those which were done by Lee and Chan in 2004. In their study, the highest number of regenerated shoots were obtained from stem nodal segments of O. stamineus, on [14] medium supplemented with 6.7 µM benzyl adenine with the formation of an average of 6.1 shoots per explants over a period of 4 weeks. Stem explants were found to be the best explants of O. stamineus in callus, root and shoot induction. The callus, shoot and root induction was successively achieved by using Murashige and Skoog basal medium with hormonal concentration of IBA (0.5 – 1.0 mg/L), GA3 (0.5 - 1.5 mg/L) and both IBA + GA3 (0.5 – 1.5). Higher concentrations of IBA displayed the organogenic callus formation. IBA of concentration 1.5 mg/L was found to increase the induction of the callus, shoot and root per explants more than IBA of concentration 1.0 mg/L.

Based on the results obtained on shoot induction from leaves and stems, plant growth hormone (GA3) was found to induce shoot at different rates for stem explants and combination of both IBA and GA3 was found to induce the root of O. stamineus. The findings from this study differ from those done by [19]. The results revealed best callus, shoot and root induction observed in stem explants cultured on MS medium. During the collection of sample it’s rarely available in the site. Hence, the plant sample was introduced to micropropagation.

Among the different root inducing plant growth regulators, IBA alone or in combination with GA3 was found to be most effective, particularly in the solid medium. Similar results were noticed in a number of other plant species [20, 21]. Many plants have been mass propagated in liquid medium. Liquid culture is ideal in micropropagation for reducing plantlet production costs and for automation.

The methanol extract was found to have antioxidant activity in 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity assay. The highest activity of about 74.56% has been observed in this present study. The findings from this study differ from [22] because the highest activity of about 38.27% has been observed in methanol extract. The value was measured to be 0.286 mg/mL. Therefore these results were discussed while increasing concentrations increased the antioxidant activity up to 0.745. DPPH assay provides basic information on antiradical activity of extracts and its results can indicate the presence of phenolic and flavonoid compounds in plant extracts [23]. Phytochemical constituents in the plant samples are known to be biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer. [24,25].

The broad-spectrum antibacterial activities of the plant extract, possibly due to the identified alkaloids, further confirm its use as a health remedy in folklore medicine. Bio active substance from this plant can therefore be employed in the formulation of antimicrobial agents to treat various bacterial infections. Natural sources has received much attention for searching the antimicrobials and efforts have been put in to identify compounds that can act as suitable antimicrobial agent to replace synthetic ones. Therefore, medicinal plants are finding their way into food supplements, nutraceutical and pharmaceuticals.

CONCLUSION

The present findings provide practical explanations for the growth of O. stamineus by in vitro. In conclusion, the in vitro techniques offer a powerful tool for mass-multiplication of this threatened plant species. O. stamineus contained considerable amount of flavonoids and polyphenols that exhibited significant antioxidant activity by effectively scavenging DPPH free radicals and ferrous ions giving evidence for the antioxidant activity. Further investigations with O. stamineus may also yield useful drug formulation as well as treatments for a number of ailments. Therefore, O. stamineus has promising compounds to be tested as potential antioxidant drugs for treatment of diseases resulting from oxidative stress.

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