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Research Article

COMPARATIVE PHYTOCHEMICAL SCREENING AND ANTIOXIDANT PROPERTIES OF *IN VITRO* AND *IN VIVO* PROPAGATED *ORTHOSIPHON STAMINEUS* BENTH (LAMIACEAE)

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ABSTRACT

Objectives: Present study aims in *in vitro* propagation of the valuable medicinal plant, *Orthosiphon stamineus* in various concentrations of plant growth regulators and to compare the antioxidant activity and phytochemical contents of wild and *in vitro* cultivated *O. stamineus*.

Methods: Explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyl amino purine (BAP) and kinetin (KIN). The multiple shoots were subcultured in BAP (1.0 mg/l) and KIN (2.0 mg/l). The leaf powder of wild and *in vitro* plants was subjected to sequential extraction using hexane, chloroform, and ethanol. Preliminary phytochemical analysis and antioxidant activity of the leaf extracts of wild and *in vitro* cultivated plants were carried out. Antioxidant assay was performed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and hydrogen peroxide scavenging methods.

Results: Rapid multiple shoot growth (90%) was observed in MS medium with 1.0 mg/l BAP and 2.0 mg/l KIN. The results showed that the ethanol extract of the *in vitro* regenerated plantlets and the wild plant contained carbohydrates, tannins, quinones, phenols, and coumarin. Chloroform extract of the *in vitro* regenerated plantlets showed strong DPPH radicals scavenging activity with % of inhibition of 81.10 µg/mL compared to the wild plant extracts with % of inhibition of 69.22 µg/mL. Similarly, the ethanol extract of the *in vitro* regenerated plantlets showed strong scavenging activity with % of inhibition of 65.14 µg/mL compared to the wild plant extracts with % of inhibition of 40.46 µg/mL.

Conclusions: The results indicated that *in vitro* regenerated plantlets exhibited excellent free radical scavenging activity and reducing activity compared to the extracts obtained from wild plants.

Keywords: *Orthosiphon stamineus, In vitro* propagation, Phytochemical analysis, Antioxidant assay, 1, 1-diphenyl-2-picryl hydrazyl free radical scavenging, Hydrogen peroxide scavenging.

INTRODUCTION

Medicinal plants play an essential role in the human health care since the ancient period. They possess many active principles which are used as antibacterial [1], antioxidant [2], antiulcer [3], anti-inflammatory [4], and anticancer agents [5]. They are the main sources of medicines for different types of illnesses in the developing countries where infectious diseases are prevalent and health services and sanitation facilities are scarce. Estimations made by the World Health Organization (WHO), revealed that 80% of people who live in developed countries generally use traditional medicine [6].

Orthosiphon stamineus is a widely distributed plant in Africa and Southeastern Asia [7]. *O. stamineus* is a perennial herb that belongs to *Lamiaceae* family and is usually known as "cat's whiskers." The plant grows in temperate and tropical areas such as India, Malaysia, China, Australia, and the Pacific [8]. This plant is a well-known therapeutic herb in South-East Asia [9]. *O. stamineus* has been used as antihypertensive, anti-inflammatory, anti-allergic, and anti-cancer drug [10]. It is also believed that *O. stamineus* leaves have diuretic properties and has been used to remove uric acid stones from the kidneys [11]. It is also extensively useful in conventional medicine to cure rheumatism, fever, hepatitis, gallstones, hypertension, diabetes, epilepsy, and eruptive [12].

Several herbs contain antioxidant compounds which protect the cells against the damaging effects of reactive oxygen species (ROS) [13]. ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide play a key role in the growth of various diseases [14]. Antioxidants from plant materials terminate the action of free radicals and protect the body from various diseases [15]. There is a growing interest all over the world for discovering the unexploited reservoir of medicinal plants. Hence, the increasing utility of herbal plants has increased the need for alternative methods to cultivate them.

Biotechnological tools play a vital role in the reproduction and genetic enrichment of the therapeutic plants by adopting techniques such as *in vitro* regeneration and genetic transformation [16]. Plant tissue culture provides new possibilities for *in-vitro* propagation and manipulation of plants and also recognized as a capable tool for fast clonal propagation [17].

The current study was carried out to propagate the important medicinal plant *O. stamineus in vitro* with various combinations or concentrations of plant growth regulators, and to compare the phytochemical content, antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, and hydrogen peroxide scavenging capacity using H_2O_2 assay in the *in vivo* (wild plants) and *in vitro* cultivated plant of *O. stamineus.*

METHODS

Collection of plant

O. stamineus plants were collected from the Irula tribal women's welfare society, Thandarai, Chengalpattu, identified by Dr. G. Jeya Jothi, Taxonomist, Loyola College, Chennai and grown in nursery green house.

In vitro regeneration

Nodal explants of *O. stamineus* were collected from the wild plants. The nodal segments were repetitively washed with running tap water to remove adhering particles of living and non-living. This was followed with rinsing in 5% (v/v) liquid detergent solution (Teepol) for 7 minutes at room temperature. After thorough rinsing in sterile distilled water, they were washed in 70% (v/v) ethanol for 30 seconds and finally surface sterilized in 0.1% (w/v) HgCl2 solution for

3 - 4 minutes. The explants were then rinsed in sterile double distilled water seven times.

The explants were trimmed and cultured on Murashige and Skoog (MS) basal medium [18] with 3% (w/v) sucrose, and 0.8% (w/v) agar (Himedia). For multiple shoot induction, the basal medium was supplemented with different concentrations of BAP (0.5, 1.0, 2.0, and 2.5 mg/l) and KIN (0.5, 1.0, 2.0, and 2.5 mg/l). The response was 90% with 1 ml/L BAP and 2 ml/L of KIN. All the cultures were maintained by subculturing in the same medium.

Crude extract preparation

Fresh leaves collected from wild plants and *in vitro* propagated plants were shade dried and ground into a fine powder. The powder (100 g) was sequentially soaked in threefold quantity (W/V) of hexane, chloroform, and ethanol for 72 h and filtered using Whatman No.1 filter paper. The filtrate was concentrated under reduced pressure using rotary vacuum evaporator and air dried to yield 0.16 g of hexane, 1.12 g of chloroform, and 1.88 g of ethanol extracts. Similarly, the *in vitro* fresh leaves were collected from our plant tissue culture lab and extracted using the same procedure. The extractive yield was 0.033 g of hexane, 0.35 g of chloroform, and ethanol 0.56 g extracts.

Qualitative phytochemical test

The qualitative phytochemical analysis was done for carbohydrates [19], tannins [20], saponins [21], flavonoids, cardiac glycosides, terpenoids, phlobatannins, anthraquinones [22], Alkaloid [23], quinones, phenols, coumarins [24], glycosides [25], and steroids and phytosteroids [26].

Antioxidant activity - DPPH radical scavenging assay

DPPH is a stable free radical with purple color, the intensity of which is measured at 517 nm spectrophotometrically. Antioxidants reduce DPPH to 1, 1-diphenyl-2-picryl hydrazine, a colorless compound. The ability of the extracts to annihilate the DPPH radical (DPPH) was investigated by the method described by [27]. The stock solution of leaf extracts was prepared to the concentration of 1 mg/ml. 100 µg of each extracts were added, at an equal volume, to methanolic solution of DPPH (0.1 mM). The reaction mixture is incubated for 30 minutes at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid was used as a standard control. The annihilation activity of free radicals was calculated in % inhibition according to the following formula.

% of inhibition = (A of control – A of Test)/A of control X 100
Where A= Absorbance at 517nm

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of [28]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (8500 II, BioCrom GmbH, Zurich, Switzerland). Extracts (200–1000 µg) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after ten minute against a blank solution containing in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of the extracts and standard compounds was calculated using the following equation:

% of inhibition = (A of control − A of test)/A of control × 100
Where A = Absorbance at 230 nm

RESULTS

Shoot induction from nodal explants in MS medium

In vitro propagation technique is a powerful tool for plant germplasm conservation. Hence, tissue culture is the only rapid process for the mass propagation of plants. The ability to generate plants directly for explants is fundamental to clonal multiplication of elite germplasm via micropropagation [29]. In the present study, to raise stock culture, nodal explants were taken from the field growing wild plants. The axiliary bud of *O. stamineus* was initiated on the MS basal medium supplemented with 1 ml/L BAP and 2 ml/L of KIN. The medium composition gave good yielding results (Fig. 1).

Phytochemical analysis

The extracts of the wild plant and the *in vitro* regenerated plantlets were subjected to the preliminary phytochemical analysis (Table 1). The results showed that the ethanol extract of the *in vitro* regenerated plantlets and the wild plant contains carbohydrates, tannins, quinones, phenols, and coumarin. The hexane extracts of both wild and *in vitro* contain flavonoids, alkaloids, phenol, and coumarin. The chloroform extract of both wild and *in vitro* contains tannins, flavonoids, alkaloids, phenol, and coumarins.

DPPH assay

The assay was carried out for the extracts from the wild plant and the *in vitro* regenerated plantlets. The results of the assay are expressed in percentage (%) of inhibition of DPPH free (Table 2, Figs. 2 and 3). The analysis of that the radical scavenging activity of the extracts of *O. stamineus* extracts of *in vitro* regenerated and wild plant increases with increasing in concentration. The chloroform extract of the *in vitro* regenerated plantlets showed strong activity on scavenging DPPH radicals with (%) of inhibition of 81.10 µg/mL when compared to the wild plant extracts with (%) of inhibition of 69.22 µg/mL. Similarly, the hexane extract of the *in vitro* regenerated plantlets showed strong activity on scavenging DPPH radicals with (%) of inhibition of 52.10 µg/mL when compared to the wild plant extracts with (%) of inhibition of 27.50 µg/mL. Whereas, the ethanol extract of the wild plant extracts shows strong activity on scavenging DPPH radicals with (%) of

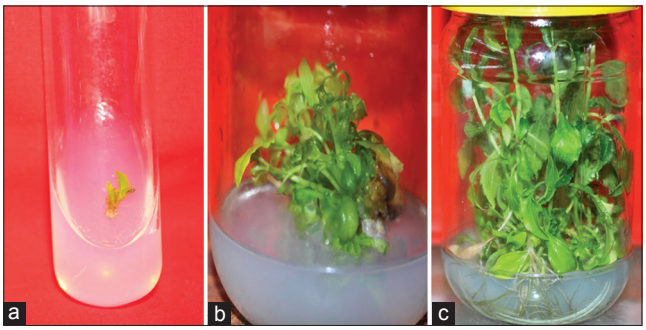

Fig. 1: Multiple shoot induction from nodal explants of *Orthosiphon stamineus,* (a) Initiation of nodal explants, (b) shoot multiplication on MS medium supplemented with 1.0 mg/L 6-benzyl amino purine, (c) Proliferation of shoot on the same medium after 8 weeks of culture

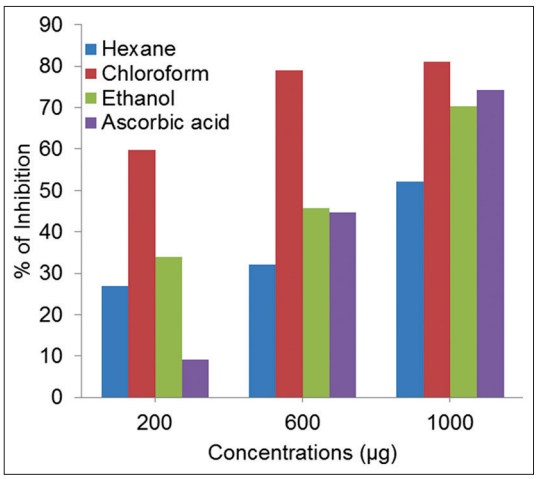

Fig. 2: 1, 1-diphenyl-2-picrylhydrazyl scavenging activity of *Orthosiphon stamineus* extracts of *in vitro* regenerated plantlets

Table 1: Phytochemical analysis of *in vitro* regenerated plantlets and wild plants of *O. stamineus*

Plant	Extract	Phytochemicals													
		ALK	AQN	CGI	CHO	COUM	FLV	GLY	PHE	PTAN	QN	SAP	STER	TAN	TERP
In vitro regenerated plants	Hexane	+				+	+		+						
	Chlor.	+			+		+							+	
	Ethanol			+	+				+		+			+	
Wild plants	Hexane	+			+	+	+		+						
	Chlor.	+				+			+					+	
	Ethanol			+	+				+		+			+	

(+) indicates the presence and (-) indicates absence, Chlor.: Chloroform, ALK: Alkaloid, AQN: Anthraquinones, CGI: Cardiac glycosides, CHO: Carbohydrates, COU: Coumarins, FLV: Flavonoids, GLY: Glycosides, PHE: Phenols, PTAN: Phlobatannins, QN: Quinones, SAP: Saponins, STER: Steroids, TAN: Tannins, TERP: Terpenoids, *O. stamineus: Orthosiphon stamineus*

Table 2: DPPH Scavenging activity of *O. stamineus* extracts of *in vitro* regenerated and wild plants

Plant	Extract	% of inhibition		
		Concentration (µg)		
		200	600	1000
In vitro regenerated plants	Hexane	26.8490	32.1175	52.1023
	Chloroform	5.8784	79.0020	81.1043
	Ethanol	33.9412	45.8206	70.3140
Wild plants	Hexane	6.6869	10.5876	27.5076
	Chloroform	52.9635	61.3475	69.2249
	Ethanol	56.5096	69.1995	80.5724
Ascorbic acid (reference control)		9.0678	44.7821	74.1641

O. stamineus: Orthosiphon stamineus, DPPH: 1, 1-diphenyl-2-picrylhydrazyl

Table 3: Hydrogen peroxide scavenging of *O. stamineus* extracts of *in vitro* regenerated plantlets and wild plants

Plant	Extract	% of inhibition		
		Concentration (µg)		
		200	600	1000
In vitro regenerated plants	Hexane	9.33	14.52	18.25
	Chloroform	35.26	44.6	58.29
	Ethanol	38.79	52.69	65.97
Wild plants	Hexane	7.26	11.2	17.63
	Chloroform	35.68	49.17	31.95
	Ethanol	25.1	31.95	40.46
Ascorbic acid (reference control)		45.64	65.97	87.34

O. stamineus: Orthosiphon stamineus

inhibition of 80.57 µg/mL when compared to the *in vitro* regenerated plantlets extracts with (%) of inhibition of 70.31 µg/mL.

Hydrogen peroxide scavenging

The *in vitro* regenerated plantlets and wild plant extracts of *O. stamineus* showed a significant dose-dependent hydroxyl radical scavenging activity (Table 3, Figs. 4 and 5). The hexane extract of the *in vitro* regenerated plantlets showed strong scavenging activity with (%) of inhibition of 18.25 µg/mL compared to the wild plant extracts with (%) of inhibition of 17.63 µg/mL. Similarly, the ethanol extract of the *in vitro* regenerated plantlets showed strong scavenging activity with (%) of inhibition of 65.14 µg/mL compared to the wild plant extracts that showed (%) of inhibition of 40.46 µg/mL. The Chloroform extract of the wild plant showed strong scavenging activity with (%) of inhibition of 58.92 µg/mL compared to the extracts of *in vitro* regenerated plantlets, which showed (%) of inhibition of 58.29 µg/mL.

DISCUSSION

Plants are endowed with various phytochemical molecules such as, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, vitamins, terpenoids, and other metabolites, which are rich in antioxidant activity [30]. The amount of phytochemical substances varies considerably from species to species and even from plant to plant, depending on the age and various ecological and climatic factors [31]. Most of the natural products are secondary metabolites and about 12,000 of such products have been isolated so far. In the present study, the phytochemical analysis of the *in vitro* regenerated plantlets and wild plants were performed. The phytochemical screening of *in vitro* and wild plants extract indicates the presence of most of the secondary metabolites. The pharmacological exploration of *in vitro* derived secondary metabolites may be helpful for the production of natural antioxidants.

A number of methods are available for the determination of free radical scavenging activity, but the assay employing the stable DPPH has received the maximum attention owing to its ease of use and its convenience

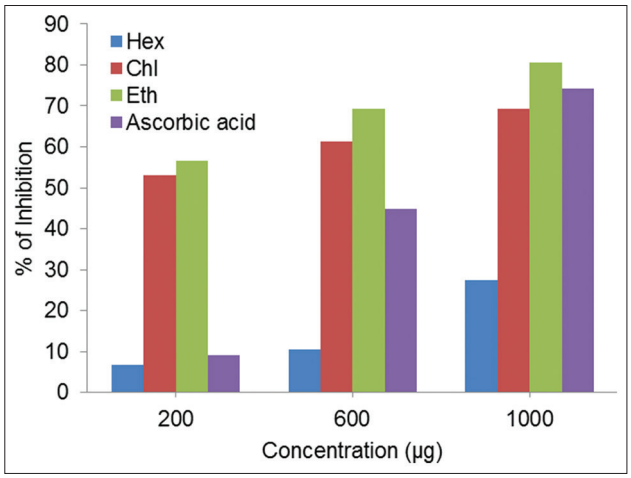

Fig. 3: 1, 1-diphenyl-2-picrylhydrazyl scavenging activity of *Orthosiphon stamineus* extracts of wild plant

[32]. The previous study indicated that the high antioxidant activity of *O. Stamineus* was due to the high content of phenolic components. It is reported that the total phenolic content of *O. stamineus* was 294.3 mg (gallic acid equivalents) per gm of extracts, which was in accordance with the DPPH result [33]. In the present study, the chloroform extract of the *in vitro* regenerated plantlets showed strong DPPH radicals scavenging activity with inhibition of 81.10 µg/mL compared to the wild plant extracts which showed inhibition of 69.22 µg/mL. Similarly, the hexane extract of the *in vitro* regenerated plantlets showed strong activity on DPPH radical scavenging with inhibition of 52.10 µg/mL compared to the wild plant extracts with % of inhibition of 27.50 µg/mL.

Hydrogen peroxide is a weak oxidizing agent and is not very reactive. It can cross biological membranes. This property places hydrogen peroxide in a more prominent role to initiate cytotoxicity than its chemical reactivity. Thus, removing H_2O_2 is very important for the

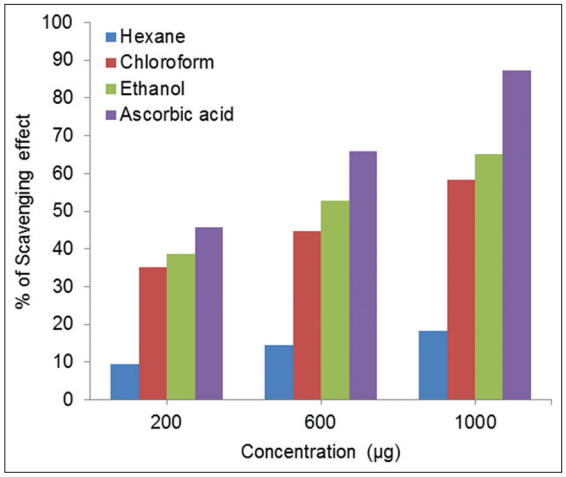

Fig. 4: Hydrogen peroxide scavenging activity of *Orthosiphon stamineus* extracts of *in vitro* regenerated plantlets

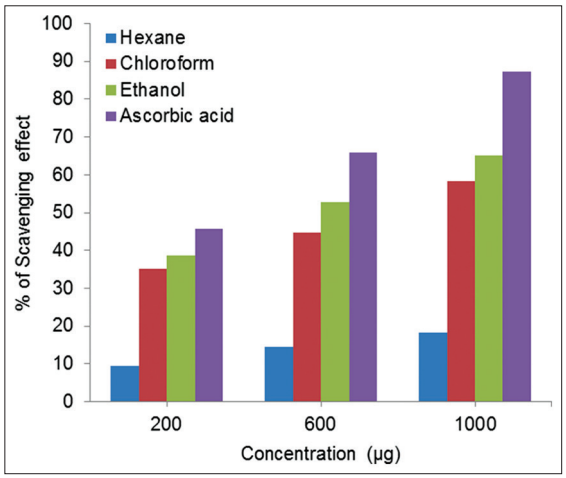

Fig. 5: Hydrogen peroxide scavenging activity of *Orthosiphon stamineus* extracts of wild plant

protection of living systems [34]. The earlier research indicates that the H_2O_2 production have established significant positive correlations with total flavonoids and total phenolics, indicating that increase in H_2O_2 might be involved in the up-regulation of the secondary metabolites production under low light condition in *O. stimaneus* [35]. *O. stamineus* extracts scavenged hydrogen peroxide which may be attributed the presence of phenolic groups that could donate electrons to hydrogen peroxidase, thereby neutralizing it into water. In the present study, the ethanol extract of the *in vitro* regenerated plantlets showed strong scavenging activity with % of inhibition of 65.14 µg/mL compared to the wild plant extracts with % of inhibition of 40.46 µg/mL.

CONCLUSION

Results of the current study clearly showed that the extracts of *in vitro* propagated *O. stamineus* contained flavonoids and phenols thereby presented significant antioxidant activity. The results also indicated that the *in vitro* regenerated plantlets exhibited excellent free radical scavenging activity compared to the wild plant extracts. Therefore *in vitro* propagated *O. stamineus* can be used as a potent source of desired bioactive compounds without destroying the wild plant resources.

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