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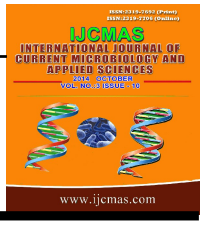

Original Research Article

In Vitro Preliminary Phytochemical and Antioxidant Activity of *Alangium salviifolium* linn.

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A B S T R A C T

Keywords

Alangium salviifolium, antioxidant activity, callus, Alangiaceae, DPPH

Medicinal plant *Alangium salviifolium* Linn. is commonly known as Akoul and belongs to Alangiaceae family. In present study we attempted to screen preliminary phytochemical and antioxidant activities in callus. MS medium supplemented with different concentrations of 2, 4- D (2, 4-dichlorophenoxy acetic acid) and IAA (Indole-3- Acetic Acid) for callus induction. Leaf explants showed maximum callus formation on 2, 4- D at the concentration 2.26µM/liter. Callus obtained from 2, 4- D (2.26µM/liter) was further evaluated for primary metabolite estimation and antioxidant activity. Soluble sugar and starch were found maximum in 2week and 6week old callus respectively. Proteins were found maximum in 4week old callus. The maximum concentration of lipid and total phenolic contents were found in 8 week old callus (40.0±0.45 and 53.8±1.60 respectively). The highest DPPH radical scavenging activity (90.76±1.14%) and superoxide radical scavenging activity (73.6±1.45%) were recorded in 8week old callus.

Introduction

To cure the harmful effects of free radicals an antioxidant compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate is used (Halliwell, 1995; Halliwell and Gutteridge, 2007). Although synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and tertbutylhydroquinone have been commonly used as antioxidants in foods for years, their safety has long been questioned (Branen, 1975; Ito et al., 1983). In recent decades, interest in chemopreventive plant natural products has grown rapidly. The etiology of several degenerative and aging-related diseases has been attributed to oxidative stress and numerous studies have been undertaken to search for the most effective antioxidants (Halliwell, 1995; Soobrattee et al., 2005). This has led to an increased interest in natural antioxidants (Lim et al., 2002; Braca et al., 2002).

Medicinal plants have been the subjects of man's curiosity since time immemorial. For the production of medicinal metabolites the use of plant cell and tissue culture

methodology has a long history (Rout et al., 2000; Verpoorte et al., 2002). Since plant cell and tissue culture emerged as a discipline within plant biology, researchers have endeavored to utilize plant cell biosynthetic capabilities for obtaining useful products and for studying the metabolism (Verpoorte et al., 2002). Cultured plant cells synthesize, accumulate and sometimes exude many classes of metabolites. Numerous metabolites like alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been reported from *in vitro* cultures and reviewed several times (Misawa, 1994; Verpoorte et al., 2002; Vanisree and Tsay, 2004).

Alangium salviifolium Linn. (Akoul) belongs to Alangiaceae family. It is endemic and endangered species used for timber, fuel and fodder for its good nutritional value in summer season some of West Himalayan areas in India (Samant and Dhar, 1997). Different parts of this plant are reported to possess acrid, astringent, emollient, anthelmintic, emetic, antiprotozoa, hypoglycemic, diuretic and purgative properties. It is also used externally in acute case of rheumatism, leprosy and inflammation. Fruits are sweet, cooling and purgative and used as a poultice for treating burning sensation and heamorrhage period (Kritikar and Basu, 1993).

However no callus cultures were established in *Alangium salviifolium* to date and no study in *in vitro* raised cells had been studied. So we attempted to screen preliminary phytochemical and antioxidant activities in callus.

Material and Methods

Collection of plant material

Plant material collected from pot cultivated plants in Rajasthan University campus. Plant material was authenticated by Herbarium, Department of Botany, Rajasthan University, Jaipur, Rajasthan, India. The callus was shade dried and powdered with pestle mortar.

Chemicals: All the chemicals and growth regulators were used are analytical grade and purchased from Hi Media Pvt. Ltd., Mumbai, India.

Callus induction: Explants (leaf and nodal segments) were surface sterilized by 1 % Teepol for 15 min followed by immersion in 70 % ethanol for 1 min and in 0.1 % mercuric chloride for 10 min, and then rinsed thoroughly with sterile distilled water. The explants were inoculated in the MS medium10 fortified with different concentrations of 2, 4-D (2, 4-dichlorophenoxy acetic acid) and IAA (Indole-3- Acetic Acid). The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg cm-2 and 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25 ± 2°C, relative humidity 55 ± 5%, and 16-h photoperiod. 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals. The friable callus cultures could be maintained by frequent subculture in the same medium.

Preparation of plant extract: Callus of *A. salviifolium* was successively extracted by pure methanol with the help of Soxhlet apparatus till the residue remains colorless. The obtained extract was concentrated using rotary evaporator under vacuum and reduced pressure at 40°C and the residue was used for further studies.

Preliminary phytochemical screening and Primary metabolite estimation

These extracts were then subjected to preliminary phytochemical screening for detection of various plant constituents. Each of these extracts was processed further to evaluate the presence of carbohydrates, proteins, tannins, flavonoids and alkaloids following the established protocols (Kokoshi et al., 1949). The powder was treated with acids like 1N HCl, H_2SO_4, HNO_3, Acetic acid and alkaline solutions like 1N NaOH and ammonia. The callus of different age of *Alangium salviifolium* L. were evaluated quantitatively to estimate the total levels of soluble sugars, starch, proteins, lipids and phenols following the established methods for the sugars, starch (Dubois et al., 1951), lipid (Jayaraman, 1981), protein (Lowry et al., 1981) and phenol (Bray and Thorpe, 1954). All experiments were repeated five times for precision and values were expressed in mean ± standard deviation in terms of air dried material.

Determination of total phenolic contents

Total phenols were determined by Folin Ciocalteu reagent (McDonald et al., 2001). A dilute extract of each plant extract (0.5 ml of 1:10 g ml-1) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L-1 solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g −1 of dry mass), which is a common reference compound.

Antioxidative assay

The antioxidative activity of the extracts was elucidated by 2, 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging assay (Prachayasittikul et al., 2008). Experiments were initiated by preparing a 0.1 mM solution of DPPH in methanol. Two ml of this solution was added to a sample solution (0.1ml, 1mg/ml in methanol). After 30 min, absorbance at 515nm was measured and the percentage of radical scavenging activity was calculated from the following equation:

% Radical scavenging = (1-Abs.sample/Abs.control)×100

Abs. control is the absorbance of the DPPH solution without sample and Abs. sample is the absorbance of the tested sample.

The superoxide radical scavenging capacity of plant extract was analyzed using a modified method of Beauchamp and Fridovich (1971) as described by Zhishen et al (1999). The 2ml of reaction mixture containing 3x10-6 mol/l riboflavin, 1x10-2 mol/l methionine and 1x10-4 mol/l nitrobluetetrazolium (NBT) in 0.05 M phosphate buffer (pH 7.8) was illuminated with two 20W fluorescent lamps at 25ºC for 25min in an aluminium foil-lined box. The photochemically reduced riboflavin generated O^{2-} which reduced NBT to blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance (A) was measured at 560nm. The plant extracts (0.1 ml, 1mg/ml in methanol) were added to the reaction mixture, which scavenged O^{2-} generation, thereby inhibiting the NBT reduction. Absorbance (A1) was measured and the decrease in O^{2-} was calculated by A–A1. The degree of the scavenging was calculated by the following equation:

Scavenging (%) = (A – A1 / A) × 100%

Result and Discussion

Callus induction

MS medium supplemented with different concentrations of 2, 4- D and IAA for callus induction. Leaf explants showed maximum callus formation on 2, 4- D at the concentration 2.26µM/liter (Table 1; Figure 1). Callus was fragile and yellowish green colored. However, IAA showed direct root induction from leaf and nodal explants at all the concentrations used. Callus obtained from 2, 4- D (2.26µM/liter) was further evaluated for primary metabolite estimation and antioxidant activity.

Primary metabolite estimation

The 2 week old callus recorded maximum concentration of sugars (54.1±1.14 mg/gdw) due to its easily availability in culture medium and these cells are in highly proliferating stage so they used more primary metabolites (Singh et al., 2011). The maximum concentration of lipid and phenols found in 8 week old callus (40.0±0.45 and 53.8±1.60 respectively) (Table 2; Graph 1).

Antioxidant activity

The antioxidant activity of callus and other plant parts of *Alangium salviifolium* was measured using 2, 2`-diphenyl-1-picryhydrazyl (DPPH) free radical and superoxide radical scavenging assays. The results showed that all the parts exhibited antioxidative activity. The highest DPPH radical scavenging activity (90.76±1.14%) and superoxide radical scavenging activity (73.6±1.45%) were recorded in 8week old callus. (Table 4; Graph 2)

In our study also different plant parts as well as callus had showed superoxide radical scavenging activity according to their phenolic contents since presence of phenolic contents supports antioxidant status of the callus as also reported in Indian herbal tea (Naithani et al., 2006).

Phytochemical Screening

The 8 week old callus was shade dried and subjected to sequential extraction in petroleum ether, benzene, chloroform, ethanol and water (Table 3). This investigation depicted that petroleum ether extract contains steroid, benzene extract contains alkaloids and steroids, chloroform extract contains protein, alkaloids and flavonoids, ethanol extract contains alkaloids, steroids, flavonoids carbohydrates and proteins, aqueous extract contains alkaloids, tannins, flavonoids, carbohydrates and proteins. The maximum extractive value found in aqueous extract (1.233%).

In today's environment hyper physiological burden of free radical causes imbalance of homeostatic phenomenon between oxidants and antioxidants in the body. This imbalance leads to oxidative stress that is being suggested as the root cause of aging and various human diseases like arteriosclerosis stroke, diabetes, cancer and neurodegenerative disease such as Alzheimer's and Parkinsonism (Jain, 2010).Thus free radical scavenging is very essential for preventing organ injury. Plants and plant products are known to possess excellent antioxidant properties and play a significant role in preventing the conditions due to the excessive free radicals. The presence of flavonoids and tannins in all the plants is likely to be responsible for the free radical scavenging effects observed.

Table.1 *Percentage of the callus induction from Alangium salviifolium L. leaves under different levels of 2, 4-D and IAA after 8 weeks of culture.*

S. No	Growth regulators	Concentration (µM/liter)	Percentage of the callus induction	Nature of callus
1	2,4-D	2.26	95.20±0.47	Greenish yellow, compact
		4.52	65.43±1.10	Greenish yellow, compact
		6.78	51.3±0.73	Greenish yellow, compact
		9.05	20.65±1.05	Greenish yellow, compact
2	IAA	2.85	0.0±0.0	-
		5.71	11.20±.55	Greenish yellow, compact
		8.56	7.0±1.14	Greenish yellow, compact
		11.41	0.0±0.0	-

Data are presented as mean ± S.E.M (n=20)

Table.2 Estimation of primary metabolites (mg/gdw) in callus of *Alangium salviifolium Linn.* harvested at different age

Callus age	Sugar	Starch	Protein	Lipids
2 weeks	54.1±1.14	17±1.30	24.5±1.41	9.8±0.71
4 weeks	49.2±1.58	23.3±1.64	27.6±0.49	14.5±0.84
6 weeks	43.7±1.71	27.2±1.41	21.8±1.64	28.7±0.81
8 weeks	39.0±1.41	25.0±1.09	18.4±1.02	40.0±0.45

*mg/ gdw- milligram per gram dry weight

Data are presented as mean ± S.E.M (n=3)

Table.3 *Preliminary Phytochemical Test in 8 week old callus of Alangium salviifolium L.*

Tests	Extractives				
	Petroleum ether	Benzene	Chloroform	Ethanol	Aqueous
Extractive value (%w/w)	0.137	0.173	0.124	0.945	1.233
Color and Consistency	Yellowish green viscous	Yellowish green sticky	Yellowish green viscous	Yellowish green sticky	Brownish powder
Proteins	_	_	+	++	++
Carbohydrates	_	_	_	+	++
Tannins	_	_	_	_	+
Flavonoids	_	_	+	++	++
Alkaloids	_	+	+	+	++
Steroids	+	+	_	+	_

Relative intensities of tests = +/++; **No activity** = ⁻

Total ash value = 4.213; **Total % of extractives** = 2.612

Table.4 Total phenolic contents, DPPH Radical scanvenging assay and Superoxide radical scavenging assay of Alangium salviifolium Linn. at different age

Callus age	Total phenolic content GAE/gmDW	DPPH radical scavenging assay (%)	Superoxide radical scavenging assay (%)
2 week	11.8±1.45	59.24±1.14	64.0±1.14
4 week	29.3±0.49	68.47±1.09	68.3±1.02
6 week	37.5±1.64	88.97±1.64	71.6±1.58
8 week	53.8±1.60	90.76±1.14	73.6±1.45

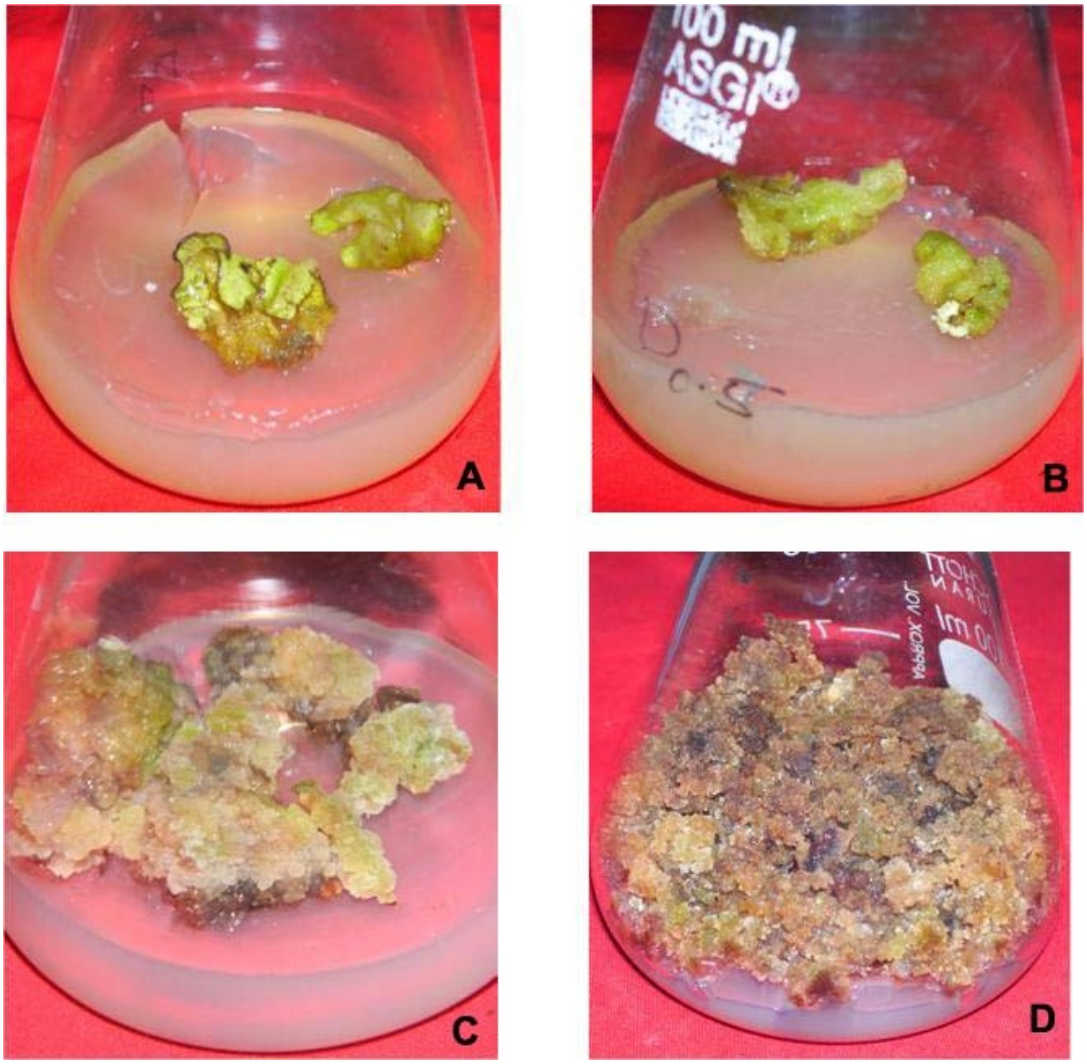
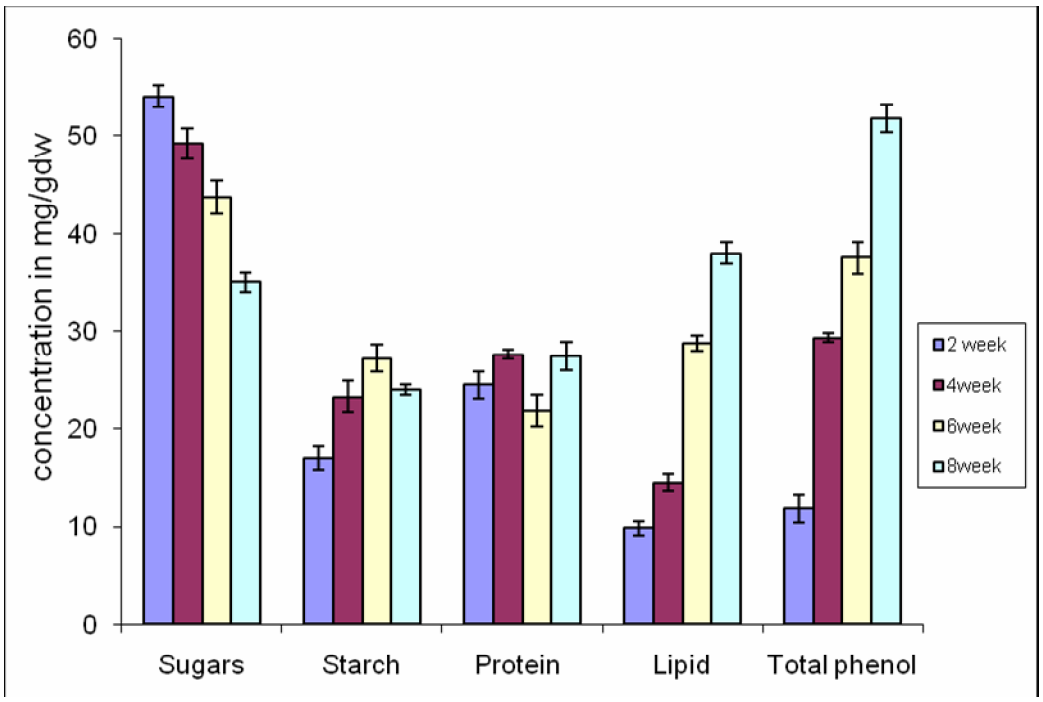
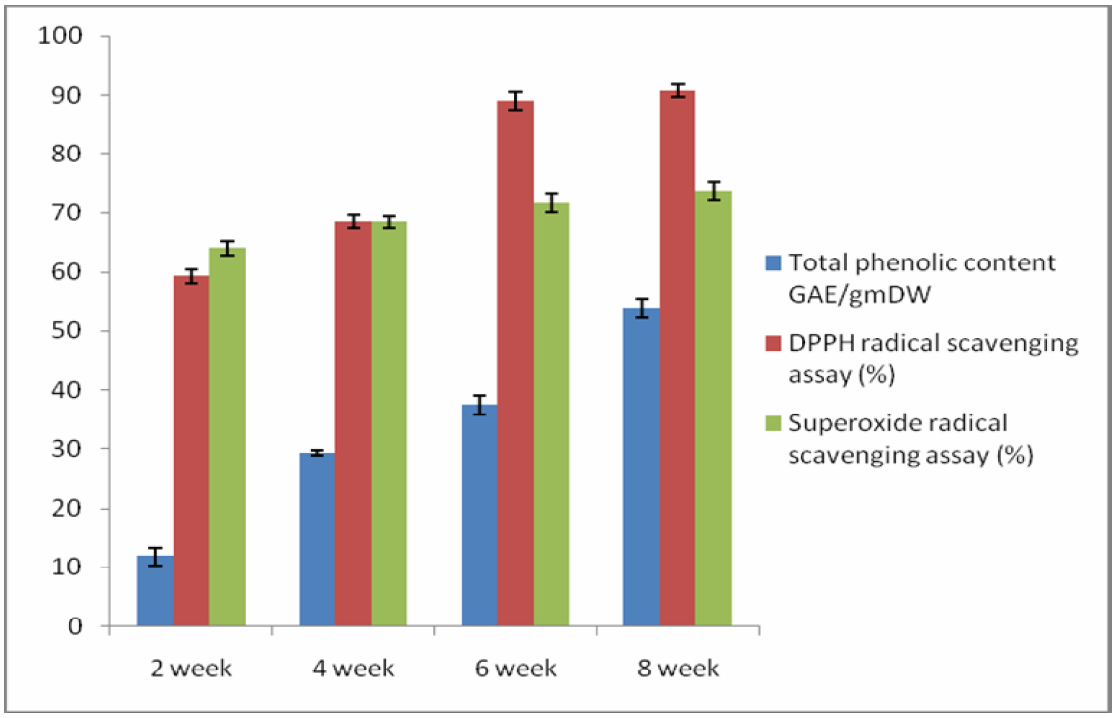


Figure 1. **Callus induction from leaf explants on MS medium supplemented with 2,4-D (2.26 M/liter) after A. 2 Weeks B. 4 weeks C. 6 weeks D. 8 weeks**



Graph 1 **Primary metabolites in different age of callus of *Alangium salviifoilum (in mg/gdw)***



Graph 2 **Total phenolic contents, DPPH Radical scanvenging assay and Superoxide radical scavenging assay of *Alangium salviifolium***

Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Ayoola et al., 2008).

The reducing ability of a compound generally depends on the presence of reductants (Duh, 1999), which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990).

It can be concluded that callus could be induced from petiole explants of *in vitro* plantlets of *A. salviifolium* on MS medium supplemented with 2.26µM/liter 2,4-D. The antioxidant activity of *A. salviifolium* callus extract was found to be increasing in a dose dependent manner. The correlation between total phenolic content and DPPH scavenging activity was significantly high. From our study the antioxidant activity of the callus extracts is mainly due to the presence of phenolic compounds in the extract.

In conclusion, it can be concluded that the methanol extract of *Alangium salviifolium* possesses the antioxidant substances which may be potentially responsible for its anti-inflammatory and chemoprotective mechanism (Tanwer and Vijayvergia, 2012) as well as using this plant's extract as folkloric remedies.

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