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Phytochemical Analysis and Quantitative Estimation of Callus Biomass, Total Phenolic, Flavonoids and Alkaloid Content in Leaves Calli of *Achyranthus aspera* L. with Various Growth Regulators and Incubation Temperature

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Abstract

Aim: Phytochemical screening was performed in different solvent extracts of leaves calli of *Achyranthus aspera* L. and Quantitative estimation of callus biomass, Total Phenolic, Flavonoids and alkaloid content in leaves calli of *Achyranthus aspera* L. was done with various growth regulators and Incubation temperature. **Methods:** leaves Explants were aseptically cultured on Murashige and Skoog (MS) medium which supplemented with different concentrations and combination of auxins and cytokinins like 2, 4-Dichlorophenoxyacetic acid (2,4-D), Indole Acetic Acid (IAA), Naphthalene Acetic Acid (NAA) and 6-Benzyl Adenine 6-BA and the preliminary phytochemical analysis of callus was performed with different chemical test. Out of twenty-two various hormonal combinations, four hormone combination which contain good yield of callus biomass, were selected for the maintaining of growth of callus under different incubation temperature 15°C, 20°C, 25°C, 30°C and 35°C. Quantitative analysis of total phenolics, flavanoid and alkaloid content were estimated in these four calli. **Result:** highest biomass, total phenolics and flavanoid content was found in leaves calli supplemented with hormone combination 2,4-D (1.6mg/L) + NAA (0.3 mg/L) at 25°C incubation temperature and highest alkaloid content was estimated in leaves calli supplemented with hormone combination 2, 4-D (1.3mg/L) + NAA (0.2 mg/L) at 30°C incubation temperature. The

preliminary phytochemical analysis of callus showed the presence of Alkaloids, Carbohydrates, Phenolic compounds, Tannins, Flavonoids, Proteins, Saponins, Steroids and Terpenoids. **Conclusion:** qualitative analysis of phtytochemical compound was done in leaves calli and attempt has been made correlation between secondary metabolites production in leaves calli with different incubation temperature and growth regulators.

Keywords
Callus biomas, Growth regulator, Incubation temperature, Flavonoid, Phenolics, Phytochemical analysis.

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1. INTRODUCTION

Free radicals are mediator of some diseases that possessing high reactivity due to the unpaired electrons. More quantity of free radicals in human will lead to oxidative stress of the cells continuously directed to the emergence of degenerative diseases such as cancer, diabetic, inflammation and cardiovascular [1, 2]. To overcome this, antioxidants are important component to protect the cells from the negative effect of free radicals [3]. However, the use of synthetic antioxidants such as butyl hydroksianisol (BHA) and butyl hydroxyl toluene (BHT) cause serious side effects which is cytotoxic for the lungs and liver and are also carcinogenic [4]. Natural antioxidant compounds derived from plant sources have more benefits than the synthetic antioxidant [5].

Achyranthus aspera L. is found in plains, forests, foot hills, waysides and roadsides. Distributed areas comprise of road sides, abandoned gardens, crops, grasslands, savannah and forest margins [6]. The plant grows in tropical and warmer regions and is found throughout tropical Asia, Africa, Australia and America [7].

Phytochemical screening of A. aspera showed that it contains alkaloids, flavonoids, tannins, anthrapuinones, saponins, glycosides and volatile oils [8]. Chemical constituents of various parts of the plant has been isolated and identified. Betaine and Achyranthine are the principal alkaloids, identified from the whole plant. Seeds contain Achyranthes saponin A and its ester, named as Aschyranthes saponin B3 [9]. The presence of ecdysterone is also reported. Shoots contain an essential oil, tannins and glycosides. Fatty acid compostion of seeds showed presence of lauric, myristic, palmitic, stearic, arachidic, behenic, oleic and linoleic acids. Certain other chemicals were also isolated and identified as strigmasta-5, 22-dien-3-β-ol, trans-13-docasenoic acid, n-hexacosanyl n-decaniate, n-hexacos-17-enoic acid and n-hexacos-11-enoic acid. A new aliphatic acid was isolated from ethanolic extracts of the roots and identified as n-hexacos-14-enoic acid. This compound is reported for the first time from any natural source [9].

Traditionally, the plant is used in asthma and cough. The plant possesses diverse pharmacological activities like antiperiodic, antiasthmatic, hepatoprotective, anti-allergic, expectorant, stomach tonic, laxative, antihelmintic, diuretic, anti-inflammatory, anticataract, antifungal, antibacterial, hypoglycemic, antihyperlipidemic and haematinic and various other important medicinal properties [7]. Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and cutaneous diseases [10]. For snake bites the ground root is given with water until the patient vomits and regains consciousness [7]. Inhaling the fume of *Achyranthes aspera* mixed with *Smilax ovalifolia* roots is suggested to improve appetite and to cure various types of gastric disorders. Ash of the plant is applied externally for ulcers and warts. The crushed leaves rubbed on aching back to cure strained back [7]. A fresh piece of root is used as tooth brush. Paste of the roots in water is used in ophthalmia and opacities of the cornea. Paste of fresh leaves is used for allaying pain from bite of wasps [6]. The plant is useful in liver complaints, rheumatism, scabies and other skin diseases. It also possesses tranquillizing properties [11, 12].

Plant cell cultures are an attractive alternative source to whole plant for the production of high value secondary metabolites [13, 14]. However, a considerable progress has been made to stimulate production and accumulation of secondary metabolites using plant cell cultures [15, 16]. Several strategies have been adopted for the enhancement of bioactive metabolite production in *in vitro* cultures; one of them is using growth regulators which are often a crucial factor in secondary product

accumulation [17]. Auxin appears to be the primary factor controlling growth and morphology of roots, while the effects of cytokinin vary depending on secondary metabolites and species concerned [18].

In the present study we have made an effort to determine the total phenolic and flavonoid contents with different incubation temperature and growth regulators as from leaves calli of *Achyranthus aspera* L.

2. MATERIAL AND METHOD

2.1 Reagents and equipment

Folin-Ciocalteu reagent (SIGMA-ALDRIC H, US) and sodium carbonate (POC H, PL) were used for total phenolic contents estimation. Rutin and gallic acid (SIGMA-ALDRIC H, US) were used as standards for calibration curves. WAS 100/X analytical balance (RA DWAG, PL), ML 147 water bath (AJ L ELECTRONIC, PL) were applied to prepare extracts. U1800 UV spectrophotometer (HITACHI, JP) was used for measuring absorbance. The EXCEL calculating sheet (MICROSOFT, US) was used for calculation.

2.2 Collection of Fresh Leaves Sample

Fresh Leaves of *Achyranthus aspera* L were collected from herbal garden, Jamia Hamdard University, New Delhi, India in the month of Nov 2016. It was authenticated by Department of Botany, Jamia Hamdard, New Delhi, India.

2.3 Surface sterilization of leaf explants of *Achyranthus aspera* L.

The disease free, young and healthy leaf explants were selected for carrying out the study as young cells are supposed to have retained their totipotency. The explants (immature leaves) were washed under running tap water in glass beaker for 15 minutes, followed by soaked in the soap solution (10%) for 1 minutes, then washed with double distilled water for many time in order to wash off the external dust/contaminants.

The washed explants were kept in Bavistine solution (1% w/v) for 30 minutes to check fungal growth and then treated with Savlon (1% v/v) for one minute as cleansing agent to decrease the surface tension between epidermis and upper fatty layer. The Savlon treated explants were washed thrice with sterile double distilled water and transferred to alcohol 70% for 30 seconds, which itself is a sterilizing agent and penetration enhancer.

Finally, it was treated with 1% concentrations of mercuric chloride for 2 minutes with occasional shaking, it was then washed with the sterile double distilled water for at least six times.

2.4 Culture medium

Young leaf explants (1-2 cm) were inoculated on Murashige and Skoog medium (18) containing 3% sucrose and gelled with 0.8% agar supplemented with combination of various concentrations of auxin and cytokinin. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121°C for 15 lbs pressure.

2.5 Inoculation of explants

After being sterilized, leaves explants were placed on a sterilized petridish with a sheet of millimeter graph paper underneath to allow accurate sizing of explants during dissection. The different explants were cut into 5-10 mm pieces with the help of a sterile and flamed forceps. The explants were transferred to the culture tubes containing nutrient agar media supplemented with different growth hormones for the induction of callus. All the above-mentioned processes starting from the sterilization of explants to inoculation were done in the inoculation room under a laminar air flow cabinet.

2.6 Physical conditions of cultures

The cultures were maintained in the culture room at 26 ± 2°C. The cultures were placed under light, provided by Phillips fluorescent tubes (40 W, 220 V) with a light intensity of 1400-3300 lux at the culture level. The relative humidity (RH) was kept within the normal range (i.e. 60-70%).

After inoculation, culture tubes were kept in B.O.D. incubator at 25º C ± 2º C and the culture tubes were exposed to light for 16 hours (having light intensity of 1600 lux) and kept in the dark for 8 hours, alternatively. These inoculated cultures were observed for any growth and in those cultures, growth was found further subculture in to same hormone combinations. The remaining cultured tubes were observed for 45 days.

2.7 Incubation temperatures on callus culture

The callus which produced good callus biomass were sub cultured on MS medium and they were kept under different incubation temperatures such as 15, 20, 25, 30 and 35°C. The callus tissues from various temperatures were used to analyze the various parameters.

2.8 Callus biomass

After 30 days of subculture the fresh weight of callus from each treatment was measured. The fresh weight of callus and callus morphology such as colour and texture were noted after 30 days of subculture. Based on the initial (FW1) and final (FW2) fresh weight of callus the relative growth rate (RGR) was calculated according to the following formula: RGR = (FW2 – FW1)/Number of days.

2.9 Preparation of plant extracts

Shoot cultures derived from leaves explants were air dried at room temperature and ground in a mortar. 0.5 g of the dried powder obtained from each hormonal combination of auxin and cytokinin was refluxed with methanol in a water bath at 45°C for 3 h. The extracts were filtered through Whatman filter

paper No. 4. The collected filtrates were dried under vacuum at 40°C. The extraction was repeated twice. The resulting residue was re-dissolved in methanol and used for the determination of phenolics, flavonoid contents and alkaloid content [19].

3.0 Phytochemical analysis

Preliminary phytochemical screening was perfomed. The presence of phytoconstituents such as Tannins, Saponins, Phenolic, Terpenoids, Steroids, Phytosterol Anthraquinone Glycosides and Flavanoid were confirmed by the following procedure [20, 21].

3.1 Test for tannins

About 2 ml of filtered extract was taken in a test tube and 2 ml of ferric chloride added. The presence of blue-black coloured precipitate indicates the presence of tannins.

3.2 Test for saponins

To 0.5 ml of extract was added 5ml of distilled water in a test tube. The solution was shaken vigorously and observed for stable persistent forth. Test for terpenoids (salkowski test) to 0.5 ml each of the extract was added 2 ml of chloroform. Concentrated Sulphuric acid 3 ml was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

3.3 Test for cardiac glycosides

To 2 ml of extract 1 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layed with 1 ml of sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

3.4 Test for anthraquinone glycoside

0.5 ml of the extract was boiled with 10 ml of sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette in to another test tube observed for colour changes.

3.5 Test for flavonoids

Dilute ammonia 5 ml was added to the extract. Concentration sulphuric acid 1 ml was added. A yellow colouration that disappears on standing indicates the presence.

3.6 Test for steroids

To 1 ml extract 10 ml of chloroform was added. 10 ml of concentrated sulphuric acid was added carefully to form coloured layer. Upper layer turns red. Sulpuric acid layer forms yellow with green fluorescence, indicates the presence of steroids.

3.7 Test for phytosterol

1 ml of extract was dissolved in 10 ml of chloroform and 10 ml concentrated sulphuric acid along the side of the test tube. Brown ring indicates presence of phytosterol.

3.8 Test for phenolic

2 ml of extract 1 ml ferric chloride was added, a blue or green colour indicates presence of phenolic.

3.9 Determination of Alkaloids

3.5 gram of the callus was prepared in a beaker and 200 ml of 10 % acetic acid in ethyl alcohol was added to the extract sample. The mixture was covered and allowed to stand for 4 h. The mixture then filtered, and the extract was allowed to become concentrated in a water bath until it reached to one quarter of the original volume. Concentrated ammonium hydroxide was added until the precipitation was complete. The whole solution was allowed to settle, and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed [22].

4. Determination of total phenolics content [23].

4.1 Preparation of sample: 0.5ml of extract of shoot culture (8mg/ml) was added to 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 1M Na_2CO_3 solution, mixed and allowed to stand for 15 minutes in the dark. The absorbance of reaction mixtures was measured at 765 nm. The total phenolics content was expressed as mg Gallic acid equivalents / 100 g dry weight (d.w.) of the extract.

4.2 Preparation of standard: 0.5ml of standard dilution (10µg, 20 µg, 50 µg and 100 µg) was added to 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 1M Na_2CO_3 solution, mixed and allowed to stand for 15 minutes in the dark. The absorbance of reaction mixtures was measured at 765 nm.

4.3 Determination of total flavonoid content [23].

4.3.1 Preparation of sample: 0.5 ml of extract of shoot culture (4mg/ml) was added to 1.5 ml methanol and mixed well. After that 0.1 ml of $AlCl_3$ (0.1mg/ml) and 0.1 ml of 1M CH_3COONa reagents were added to above solution. This reaction mixture was added to 2.8 ml of Distilled water, mixed and allows standing for 30 minutes in dark. The absorbance of reaction mixtures was measured at 415 nm. The total flavonoid content was expressed as mg rutin equivalents / 100 g d.w. of the extract.

4.3.2 Preparation of Standard: 0.5 ml of standard dilution (10µg, 20 µg, 50 µg and 100 µg) was taken and added to 1.5 ml methanol and mixed. After that 0.1 ml of $AlCl_3$ (0.1mg/ml) and 0.1 ml of 1 M CH_3COONa reagents were added to above solution. This reaction mixture was added to 2.8 ml of Distilled water, mixed and allows standing for 30 minutes in dark. The absorbance of reaction mixtures was measured at 415 nm.

4.3.3 Blank solutions: 2 ml of methanol was added to 0.1 ml of $AlCl_3$ and 0.1 ml of CH_3COONa reagents and then added to 2.8 ml Distilled water and mixed.

4.4 Statistical analysis

The experiment was conducted using Completely Randomized Design (CRD). All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Analysis of variance and significant differences among means were tested by one-way ANOVA using the COSTAT computer package (Cohort Software, 1989). The least significant difference (LSD) at $P \leq 0.05$ level was calculated. Correlation coefficients (R^2) from regression analysis between total phenolic or total flavonoid contents and antioxidant activities were also calculated.

5. RESULT AND DISCUSSION:

5.1 Effect of growth regulator on callus culture

The auxins 2, 4-D, IAA and NAA are commonly used to induce callus from explants inoculated on culture medium. Auxin, mainly 2, 4-D has been reported to be as most effective plant growth regulator for callus induction and growth of callus [24] and [25]. The auxins 2, 4 -D, NAA and IAA were added to MS basal medium for callus induction. Colour and texture of callus depends on the type of growth regulator. A plant growth regulator is a key factor responsible for callus initiation and development in plant cell cultures. When the medium is fortified with low concentrations of NAA leaf explants did not induce callus. The effectiveness of high concentrations of NAA has been demonstrated in callus induction *in Luppia junelliana* [26] and *Quercus suber* [27]. High concentrations of NAA induced callus formation from explants *Lippia alba* [28]. The low concentrations of NAA are insufficient to induce callus formation while high concentrations of 2, 4-D exert a depressive effect.

About twenty-two different hormone combination of auxin and cytokinin were tested for callus formation. Callus response, callus morphology, callus response time and callus intensity were given in **Table 1**. This table shows there are four important hormone combination 2,4-D (1.6mg/L) +BA (0.4 mg/L), 2,4-D (0.3 mg/L) + BA (0.4 mg/L) + IAA (0.1 mg/L), 2,4-D (1.3mg/L) + NAA (0.2 mg/L) and 2,4-D (1.6mg/L) + NAA (0.3 mg/L) which gave high intensity callus formation with 100 % response. No callus induction was noted from explants placed on the control medium.

These four-hormone combinations were selected to induce calli which growth were maintained with different incubation temperature. Total phenolics, flavonoid and total alkaloid were also quantitatively estimated with different incubation temperatures.

5.2 phytochemical analysis of different solvent extracts from leaves calli of *Achyranthus aspera* L.

Table 2 shows the presence of phenolic compounds, flavonoids, terpenoids, alkaloids, protein and saponin glycosides in **solvent extracts** of **leaves calli of *Achyranthus aspera* L.** particularly, methanol and chloroform extracts of calli were good sources of different classes of compounds. This indicates that these solvents are effective to isolate active biological compounds due to their high polarity. Flavonoids were detected in chloroform, acetone and methanol extracts of calli except hexane extract. Flavonoids belong to the group of polyphenolic compounds and are typically known for health promoting properties such as antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties [29]. They exist widely in the plant kingdom and displayed positive correlation between increased consumption of flavonoids and reduced risk of cardiovascular and cancer diseases [30]. Correspondingly, these extracts also tested positive for phenolic compounds. The phenolic compounds are aromatic secondary metabolites that impart colour, flavour and associated with health benefits such as reduced risk of heart and cardiovascular diseases [31, 32]. Terpenoids such as triterpenes, sesquiterpenes and diterpenes have been referred to as antibiotics, insecticidal, anthelmintic and antiseptic in pharmaceutical industry [33]. The alkaloid was observed in chloroform, Benzene and methanol extracts except hexane extract. Alkaloids have been reported to possess analgesic, antispasmodic and bactericidal, antimalarial and analgesic activities [34].

5.3 Effect of different incubation temperature and growth regulator on callus biomass and callus morphology

Temperature is one of the climatic factors which act as major abiotic environmental stressor that limit plant growth and development. Incubation temperature affects the cell culture techniques for cell growth, morphology and secondary metabolites production. Plant cells acquire biochemical and metabolic changes to alterations in the incubation temperature. Different in growth and callus morphology of rice calli were observed when they were cultured in incubation temperatures (25±2°C and 30±2°C) [35].

The callus grown under various incubation temperatures and four different hormonal combinations listed in table 3 show difference in callus biomass and slight variation in callus morphology.

Table 3 shows that callus biomass in all the concentrations was reduced than the control and out of these four hormone combination highest callus biomass (1.99g/tube) with the relative growth rate (0.077gram/day) was reported in 2,4-D (1.6mg/L) + NAA (0.3 mg/L) at 25°C and the lowest callus biomass(0.10 gram/tube) with the relative growth

rate (0.003gram/day) was reported in 2,4-D (0.3 mg/L)+BA (0.4 mg/L) + IAA (0.1 mg/L) at 15°C. Results shown in table 3 shows that callus biomass with relative growth rate was increased in each hormone combination from incubation temperature 15°C to 25°C and it was decreased with relative growth rate from incubation temperature 25°C to 35°C.

Under each incubation temperature the texture of the callus was compact but there was insignificant variation in the colour. Greenish brown callus was observed less at 15°C incubation temperature. When the incubation temperature was increased from 15°C to 25°C, callus was changed in brown colour. Brownish Callus was turned into blackish brown callus at incubation temperature 30°C and 35°C (Table 3 and Figure 1).

5.4 Effect of different incubation temperature and growth regulator on total phenolics, flavanoid and total alkaloid content in calli

Four different hormone combinations with high callus intensity shown in table 1 were selected to estimate total phenolics, flavonoids and total alkaloid content with different incubation temperature. Table 4 shows that highest total phenolics, flavanoid and total alkaloid content were quantitatively estimated in the calli which growth was maintainted at 30°C and supplemented with 2,4-D (1.3mg/L) + NAA (0.2 mg/L) whereas Minimum amount of total phenolics, flavanoid and total alkaloid content were quantitatively estimated in the calli which growth was maintainted at 15°C and supplemented with 2,4-D (1.6mg/L) +BA (0.4 mg/L).

Over twofold of callus growth and threefold of isoflavone production were demonstrated at 32±2ºC from callus cultures of *Pueraria candollei*. [36].

Table 1: Effect of different concentration of auxin with cytokinin on growth of callus from leaf explant of *Achyranthus aspera* L.

Serial no.	Concentration of plant hormone (mg/L)				No. of explants inoculated	Response percentage	Intensity of callus formation	Time for response in days	Nature of callus
	IAA	BA	2,4-D	NAA					
1.	0.0	0.4	1.6	0.0	10	100	++++	11	Dark green compact
2.	0.0	0.6	1.8	0.0	10	80	+++	14	Light green nodular
3.	0.0	1	1	0.0	10	60	++	17	Yellowish green compact
4.	0.1	0.4	0.3	0.0	10	100	++++	11	Light green nodular
5.	1.0	2.0	1	0.0	10	50	+	18	Dark green nodular
6.	0.0	1.0	3	0.0	10	45	+	20	Yellowish green compact
7.	1.0	4.0	3	0.0	10	60	++	18	Light brown friable
8.	1.0	0.5	4	0.0	10	30	+	22	Light green compact
9.	1.0	0.5	1.5	0.0	10	20	+	23	Yellowish green friable
10.	0.5	3.0	2	0.0	10	40	+	19	Dark green friable
11.	0.0	0.3	1	0.0	10	0	+	20	No callus
12.	0.0	0.4	1.5	0.0	10	60	++	18	Green compact
13.	0.0	0.4	2.0	0.0	10	60	++	18	Green friable
14.	0.0	0.6	4	0.0	10	40	+	20	Light green compact
15.	0.0	0.7	5	0.0	10	0	+	18	No callus

16.	0.0	0.0	1.3	0.2	10	100	++++	11	Dark green friable
17.	0.0	0.0	3.0	1.0	10	70	++	13	White friable
18.	0.0	0.0	2.0	0.6	10	65	++	15	Green compact
19.	0.0	0.0	1.6	0.3	10	100	++++	11	Green friable
20.	0.0	0.0	2.0	0.2	10	80	+++	12	White friable
21.	0.0	0.0	4.0	0.9	10	0	++	20	No callus
22.	0.0	0.0	4.5	0.5	10	0	++	21	No callus

(+++) appreciable amount; (++) moderate amount; (+) trace amount and (-) completely absent

Table 2: Effect of different incubation temperature and plant growth regulator on callus biomass and callus morphology from leaf explants of *Achyranthus aspera* L.

		Plant growth regulator concentration (mg/L)											
		2,4-D (1.6mg/L) +BA (0.4 mg/L)			**2,4-D (0.3 mg/L) +BA (0.4 mg/L) + IAA (0.1 mg/L)**			**2,4-D (1.3mg/L) + NAA (0.2 mg/L)**			**2,4-D (1.6mg/L) + NAA (0.3 mg/L)**		
Serial number	Incubation temperature (˚C)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)
1	15	0.13 ± 0.03^{c}	0.004	cream /soft	0.10 ± 0.02^{c}	0.003	White cream /soft	0.15 ± 0.02^{c}	0.005	cream /soft	0.18 ± 0.02^{c}	0.004	White cream /soft

2	20	0.53 ± 0.01[c]	0.009	Cream brown/ soft	0.63 ± 0.02[c]	0.007	Yellowish cream brown/ soft	0.43 ± 0.03[c]	0.009	Cream brown/ soft	0.73 ± 0.02[c]	0.008	Yellowish cream brown/ soft
3	25(control)	1.82 ± 0.02[a]	0.058	cream brown/ soft	1.94 ± 0.01[a]	0.067	Brown cream / soft	1.79 ± 0.04[a]	0.057	cream brown/ soft	1.99 ± 0.02[a]	0.077	Brown cream / soft
4	30	0.63 ± 0.03[b]	0.015	Dark brown cream/ soft	0.67 ± 0.01[b]	0.017	Dark brown cream/ soft	0.83 ± 0.02[b]	0.018	Dark brown cream/ soft	0.93 ± 0.02[b]	0.014	Dark brown cream/ soft
5	35	0.54 ± 0.01[c]	0.005	Dark brown cream/ soft	0.44 ± 0.02[c]	0.006	Dark brown cream/ soft	0.49 ± 0.01[c]	0.007	Dark brown cream/ soft	0.64 ± 0.03[c]	0.008	Dark brown cream/ soft
5	35	0.54 ± 0.01[c]	0.005	Dark brown cream/ soft	0.44 ± 0.02[c]	0.006	Dark brown cream/ soft	0.49 ± 0.01[c]	0.007	Dark brown cream/ soft	0.64 ± 0.03[c]	0.008	Dark brown cream/ soft

Fresh weight of callus and callus morphology were noted after 30 days of subculture.
Values for callus biomass represent mean ± standard error of three replicates.
Mean values followed by different letters are significantly different from each other at P < 0.05 level comparison by Duncan's multiple range test (DMRT)

Table 3: Effect of different incubation temperature and plant growth regulator on callus biomass and callus morphology from leaf explants of *Achyranthus aspera* L.

Serial number	Incubation temperature (°C)	Plant growth regulator concentration (mg/L)											
		2,4-D (1.6mg/L) +BA (0.4 mg/L)			2,4-D (0.3 mg/L) +BA (0.4 mg/L) + IAA (0.1 mg/L)			2,4-D (1.3mg/L) + NAA (0.2 mg/L)			2,4-D (1.6mg/L) + NAA (0.3 mg/L)		
		Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)	Callus Biomass (Gram/Tube)	Relative Growth (Gram/day)	Callus Morphology (Colour and texture)
1	15	0.13 ± 0.03[c]	0.004	cream /soft	0.10 ± 0.02[c]	0.003	White cream /soft	0.15 ± 0.02[c]	0.005	cream /soft	0.18 ± 0.02[c]	0.004	White cream /soft
2	20	0.53 ± 0.01[c]	0.009	Cream brown/ soft	0.63 ± 0.02[c]	0.007	Yellowish cream brown/ soft	0.43 ± 0.03[c]	0.009	Cream brown/ soft	0.73 ± 0.02[c]	0.008	Yellowish cream brown/ soft
3	25(control)	1.82 ± 0.02[a]	0.058	cream brown/ soft	1.94 ± 0.01[a]	0.067	Brown cream / soft	1.79 ± 0.04[a]	0.057	cream brown/ soft	1.99 ± 0.02[a]	0.077	Brown cream / soft

4	30	0.63 ± 0.03[b]	0.015	Dark brown crea m/ soft	0.67 ± 0.01[b]	0.017	Dark brown crea m/ soft	0.83 ± 0.02[b]	0.018	Dark brown crea m/ soft	0.93 ± 0.02[b]	0.014	Dark brown crea m/ soft
5	**35**	0.54 ± 0.01[c]	0.005	Dark brown crea m/ soft	0.44 ± 0.02[c]	0.006	Dark brown crea m/ soft	0.49 ± 0.01[c]	0.007	Dark brown crea m/ soft	0.64 ± 0.03[c]	0.008	Dark brown crea m/ soft

Fresh weight of callus and callus morphology were noted after 30 days of subculture.
Values for callus biomass represent mean ± standard error of three replicates.
Mean values followed by different letters are significantly different from each other at P < 0.05 level comparison by Duncan's multiple range test (DMRT)

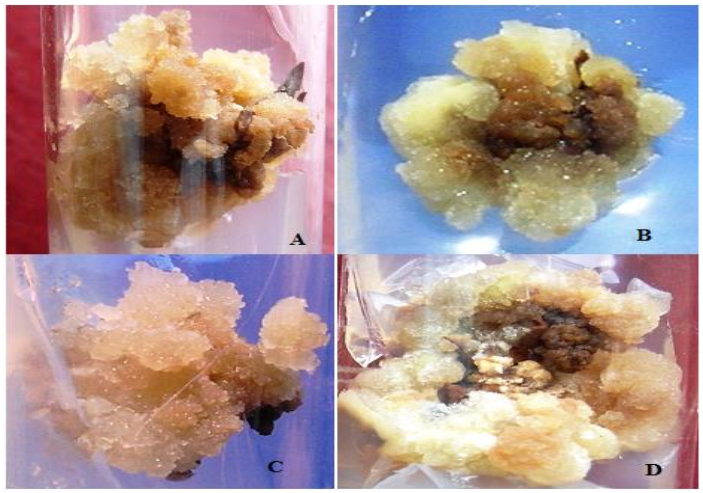

Figure 1: Influence of various incubation temperatures on callus morphology of *Achyranthus aspera* L.

A= Callus at 35°C C= Callus at 20°C
B= Callus at 30°C D= Callus at 25°

Table 4: Effect of incubation temperature and different plant growth regulator on total phenolics, flavanoid and alkaloid content of *Leaves* calli of *Achyranthus aspera* L.

S. no.	Incubation temperature (◦C)	Plant growth regulator concentration (mg/L)											
		2,4-D (1.6mg/L) +BA (0.4 mg/L)			2,4-D (0.3 mg/L)+BA (0.4 mg/L) + IAA (0.1 mg/L)			2,4-D (1.3mg/L) + NAA (0.2 mg/L)			2,4-D (1.6mg/L) + NAA (0.3 mg/L)		
		Total Phenolics (µg/g)	Flavonoid content (µg/g)	Total alkaloid (µg/g)	Total Phenolics (µg/g)	Flavonoid content (µg/g)	Total alkaloid (µg/g)	Total Phenolics (µg/g)	Flavonoid content (µg/g)	Total alkaloid (µg/g)	Total Phenolics (µg/g)	Flavonoid content (µg/g)	Total alkaloid (µg/g)
1	15	3200± 0.24^{c}	230± 0.24^{c}	460± 0.24^{c}	3400± 0.24^{c}	290± 0.24^{c}	520 ± 0.24^{c}	3760 ± 0.24^{c}	310± 0.24^{c}	509± 0.24^{c}	3545 ± 0.24^{c}	240± 0.24^{c}	489 ± 0.24^{c}
2	20	3520± 0.15g	290± 0.24^{c}	570± 0.24^{c}	3670± 0.24^{c}	390± 0.24^{c}	670 ± 0.24^{c}	3960 ± 0.24^{c}	390± 0.24^{c}	618± 0.24^{c}	3670 ± 0.24^{c}	290± 0.24^{c}	540 ± 0.24^{c}
3	25	3628± 0.24^{c}	380± 0.24^{c}	640± 0.24^{c}	3610± 0.24^{c}	470± 0.24^{c}	770 ± 0.24^{c}	4020 ± 0.24^{c}	410± 0.24^{c}	798± 0.24^{c}	4300 ± 0.24^{c}	370± 0.24^{c}	610 ± 0.24^{c}
4	30	3600± 0.24^{c}	430± 0.24^{c}	697± 0.24^{c}	3620± 0.24^{c}	560± 0.24^{c}	820 ± 0.24^{c}	4530 ± 0.24^{c}	590± 0.24^{c}	890± 0.24^{c}	4350 ± 0.24^{c}	480± 0.24^{c}	596 ± 0.24^{c}
5	35	3410± 0.24^{c}	300± 0.24^{c}	590± 0.24^{c}	3480± 0.24^{c}	210± 0.24^{c}	650 ± 0.24^{c}	2360 ± 0.24^{c}	321± 0.24^{c}	547± 0.24^{c}	2650 ± 0.24^{c}	230± 0.24^{c}	501 ± 0.24^{c}
5	35	3410± 0.24^{c}	300± 0.24^{c}	590± 0.24^{c}	3480± 0.24^{c}	210± 0.24^{c}	650 ± 0.24^{c}	2360 ± 0.24^{c}	321± 0.24^{c}	547± 0.24^{c}	2650 ± 0.24^{c}	230± 0.24^{c}	501 ± 0.24^{c}

Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according. ANOVA and DMRT test (P < 0.05) to ANOVA and DMRT test (P < 0.05).

6. CONCLUSION

Phenolic compounds, flavonoids, terpenoids, alkaloids, protein and saponin glycosides were reported in solvent extracts of leaves calli of *Achyranthus aspera* L. The callus grown under various incubation temperatures was studied for their biomass, total phenolic, flavonoids and total alkaloid content. The callus biomass was increased from 15°C to 25°C control. In callus morphology there was no difference in the texture but the callus colour among the treatments show slight variations. There was no positive correlation between the callus growth and total phenolic content among the various treatments. However, the callus produced at 30°C and supplemented with 2, 4-D (1.3mg/L) + NAA (0.2 mg/L) shows maximum amount of total phenolic, flavanoid and total alkaloid content.

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