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Research Article

SUITABLE COMBINATION OF AUXIN AND CYTOKININ FOR CALLUS BIOMASS, TOTAL PHENOLICS, FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITIES OF LEAVES CALLI OF *ACHYRANTHES ASPERA* L.

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ABSTRACT

Objectives: Quantitative analysis of callus biomass, total phenolic and flavonoid content and determination of antioxidant activity were performed in leaves calli of *Achyranthes aspera* L. supplemented with various combinations of auxin and cytokinin hormone. Material and method: Leaves calli 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 Amino Purine (BAP) were initiated . During the maintenance of the calli, the sub-culturing was routinely done after every three week. The 60 days old callus was used to preparation of extracts. Result and conclusion: Maximum callus biomass was observed in leaves calli supplemented with 2,4-D (2.1 mg/l) + BAP (0.5mg/l). Highest total phenolics and flavonoids content were reported in leaves calli on the MS medium supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l). Highest DPPH radical scavenging activity (85.31%), H_2O_2 scavenging activity (75.31%) and ferric reducing power (25.3µg/g) were observed in calli supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l). Excellent *in vitro* antioxidant activity was given by extract of callus culture on MS medium supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l). So they can be used as natural antioxidants after isolation and purification.

Keywords: leaves calli, Phenolics, Flavonoids, Callus biomass, *In vitro* antioxidant activity

INTRODUCTION

***Achyranthes aspera* L.** is found in plains, forests, foot hills, waysides and roadsides[1] and grows in tropical and warmer regions and is found throughout tropical Asia, Africa, Australia and America[2]. It predominantly grows as a weed on road sides, in vacant agricultural land, especially in uncultivated lands and along the boundaries of the cultivated fields.

***Achyranthes aspera* L.** belongs to the family Amaranthaceae, is an annual, stiff erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides[3, 4]. Achyranthes aspera L. is a well-known plant drug in Ayurvedic, Unani-Tibbi, Siddha, Allopathic, Homeopathic, Naturopathic & Home Remedies[5]. It is an annual shrub found distributed throughout the tropical and subtropical regions. The plant is utilized in alternative system of medicine. It is helpful to induce blood flow to pelvic area and uterus. It reduce inflammation in joints and suppress the action of hormones that promote pregnancy. It is used as laxative to relieve in constipation, ecbolic to induce the contraction of uterus, abortifacient to induce miscarriage, anthelmintic to expel worms from intestine, aphrodisiac to increase libido. It is also applied as antiviral to treat viral infection, antispasmodic to remove spasm, antihypertensive to decrease blood pressure, anticoagulant to prevent blood clot, diuretic to reduce blood pressure and anti-tumor agent to treat cancer[6]. Extract of the plant is utilized as expectorant and treatment of swelling in kidneys, fistula, tuberculosis in neck, skin disease, malaria fever, impotence, asthma, piles and snake bites[7]. This plant is astringent, digestive, diuretic, laxative, purgative and stomachic. The juice of the plant is used in the treatment of boils, diarrhea, dysentery, hemorrhoids, rheumatic pains, itches and skin eruptions[8]. It is reported to contain alkaloids, flavonoids, saponins, steroids and terpenoids. Flavonoids have shown to prevent or slows the development of some cancers[9] and mostly act as an anti-oxidant and anti-inflammatory agents.

Plant tissue and cell cultures techniques are an important source to regenerate plant for the generation of great valuable secondary metabolites[10, 11]. However, an impressive advance has been made to generate and collection of secondary metabolites using plant cell cultures [12, 13]. A few techniques have been received for the improvement of bioactive metabolites generation *in vitro*; one of them is utilizing plant hormones which are an important parameter in generation of bioactive compounds. [14]. 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 [15].

Generally, the amount of reactive oxygen species (ROS) and antioxidants produced in the body are balanced, but some unavoidable circumstances, antioxidant defense mechanism proves to be insufficient to compensate the highly enhanced ROS

which is harmful for the body. Likewise, various toxicants act through the ROS like superoxide anions (O^{2-}), hydroxyl radicals ($HO^{\cdot}$) and non free radical species such as H_2O_2, singled oxygen (O_2) and nitric oxide (NO) that play a major role in initiation of degenerative processes such as cellular damage that may be related to many body ailments viz. heart diseases , cancer and aging. Antioxidants are intimately involved in prevention of cellular damage, Catalase, superoxide dismutase and glutathione peroxidases are some of the natural antioxidants found in body. They neutralize free radicals as the natural by-product of normal cell processes[16]. Since ancient time, in vitro studies on medicinal plants and vegetables have shown growing interest towards natural antioxidants from herbal sources in the form of phytochemicals which exert a protective effect against oxidative stress in biological systems[17].

In the present study we have made an effort to determine the total phenolic and flavonoid contents as well as to establish the in vitro antioxidant activity in methanol extract of callus culture from leaf explants of ***Achyranthes aspera* L.**

MATERIAL AND METHOD

Collection of drugs

I collected Fresh Leaves of *Achyranthes aspera* L. from Herbal garden, Mewar University, Chittorgarh, India in the month of July 2017. It was authenticated by Prof (Dr.) B.L. Yadav at Department of Botany, Mewar University, Chittorgarh, India.

Surface sterilization of leaves explants of *Achyranthes 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 Bavistin 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 minute with occasional shaking; it was then washed with the sterile double distilled water for at least six times .

Culture medium and plant growth regulators

Young leaf explants (1-2 cm) were inoculated on MS medium[18] containing 3% sucrose and gelled with 0.8% agar supplemented with various combination of of IAA, 2,4-D and BAP. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121ºC for 15 lbs pressure.

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.

Physical conditions of cultures

The cultures were maintained in the culture room at 26 ± 2ºC. 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.

Sub culturing

Sub culturing was carried out at regular intervals of 21 days. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

Preparation of methanol extract of calli cultures

Callus 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 extracted with methanol in a water bath at 45°C for 3 h. Whatman filter paper No. 4 was used for filtration of liquid extract. Solvent was evaporated from collected filtrates under vacuum at 40°C. The extraction was rehashed twice. The dried residue was re-dissolved in methanol and utilized for the accumulation of phenolics and flavonoid contents and determination of antioxidant activities[19].

Determination of total phenolics content[20]

Preparation of sample: 0.5ml of extract of callus 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 minute in the dark. The absorbance of reaction mixtures were measured at 765 nm. The total phenolics content was expressed as mg Gallic acid equivalents / 100 g dry weight (d.w.) of the extract.

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 minute in the dark. The absorbance of reaction mixtures were measured at 765 nm.

Determination of total flavonoid content[20]

Preparation of sample: 0.5 ml of extract of callus culture (4mg/ml) was added to 1.5 ml methanol and mixed well and 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 to2.8 ml of Distilled water, mixed and allows standing for 30 minutes in dark. The absorbance of reaction mixtures were measured at 415 nm. The total flavonoid content was expressed as mg rutin equivalents / 100 g d.w. of the extract.

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.

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.

Determination of antioxidant activity

DPPH free radical scavenging activity

0.1 mM solution of 1, 1- diphenyl-2-picryl-hydrazyl (DPPH) solution was arranged by using methanol solvent. 3 ml of methanol extract was mixed with 1 mL of DPPH solution and incubated for 30 minutes. Discoloration reading was taken at 517 nm in triplicate. Butylated hydroxytoluene (BHT) was utilized as a positive control. The capacity to rummage the DPPH radical was estimated utilizing the formula: DPPH rummaging impact (%) = [A*DPPH* − A*S* / A*DPPH*] x100 where, A*DPPH* is the absorbance of the DPPH solution and A*S* is the absorbance of the test solution when the sample extract is used. The sample concentration giving 50% hindrance of radical-scavenging activity (IC_{50}) was estimated and communicated as mg/mL, d.w.[21]

Ferric reducing power determination

Extracts at concentration of 500 µg/mL were mixed with phosphate buffer (2.5 mL, 200 mM, pH 6.6) and 1% potassium ferricyanide (2.5 mL). Then the mixture was incubated at 50 °C for 20 min. 2.5 mL of 10% trichloroacetic acid was added to above mixture and centrifuged at 10000 rpm for 10 min. The supernatant layer of the solution (5 mL) was dissolved with distilled water (5 mL) and 0.1% ferric chloride (1 mL). The absorbance of the reaction mixture was taken at 700 nm. The final results were communicated as µg ascorbic acid equivalents / g based on dry weight of the extract[22].

Hydrogen peroxide scavenging activity

Solution of H_2O_2 (43 mM) was arranged in phosphate buffer (0.1 M, pH 7.4). 50 µg/mL Extract solutions were mixed in 3.4 mL phosphate buffer and 600µL H2O2 solution. The absorbance of the reaction mixture was taken at 230 nm. BHT at concentration 50µg/mL utilized as positive control and percentage H_2O_2 rummiging impact was estimated as $[A_{Control} - A_{Sample} / A_{Control}]$ x 100 where $A_{control}$ is the absorbance of the control (blank, without extract), and A_{Sample} is the absorbance of the sample extract. The extract concentration giving 50% of H_2O_2 rummiging action (IC_{50}) was estimated and communicated as µg/mL based on test dry weight [23].

Statistical analysis

The experiments were performed utilizing Completely Randomized Design (CRD). Triple readings were taken in each experiment. Data are given as means ± standard deviation (SD). Analysis of variance and significant differences among means were tried by one-way ANOVA using the COSTAT computer package (Cohort Software, 1989). The least significant difference (LSD) at $P \leq 0.05$ level was estimated. Correlation coefficients (R^2) from regression analysis between total phenolic, flavonoid contents and antioxidant activities were also estimated.

RESULTS

Effect of various PGRs on callus growth and morphology of callus

Different combinations of auxin and cytokinin were tried for callus initiation and ten hormone combinations were found better for callus initiation (Table 1). Figure 1 shows morphology and texture of calli with different combinations of auxin and cytokinin. Intensity of callus, callus biomass and callus response with different combination of auxin and cytokinin were shown in Table 1. Maximum calli biomass (2.30 gram/tube) was obtained when the medium was supplemented with 2,4-D (2.1mg/l) + BAP (0.5mg/l).

Total phenolics and flavonoid content in shoot culture of leaf explant

One-way ANOVA analysis showed significant differences ($p \leq 0.05$) in total phenolics and flavonoid content among the ten different combinations of auxin and cytokinin. Quantitative estimation of total phenolic and flavonoids content from different combination of auxin and cytokinin were shown in Figure 2. Highest phenolics content (476 mg/g d.w.) and flavonoid (280 mg/g) were observed in methanol extract of callus culture supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l).

DPPH free radical scavenging activity in leaves calli culture of *Achyranthes aspera* L.

The antioxidant activity of leaves calli of ***Achyranthes aspera* L.** and of the standard antioxidant BHT was determined using the DPPH method. Table 3 shows highest radical scavenging activity (85.31 %) with IC50 value (0.880 mg/ml) was observed in methanol extract of leaves calli supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l).

DPPH free radical scavenging activity 89.31 % with IC50 value (0.920 mg/mL) were observed by BHT as positive control.

Ferric reducing power determination in leaves calli culture of *Achyranthes aspera* L.

Involvement of reducing compound such as antioxidant substances in samples results the reduction of the Fe3+ ferricyanide complex to the ferrous form. The change of iron (III) to iron (II)-reducing property in the shoot culture of methanol extracts was communicated as µg ascorbic acid equivalent/g sample based on dry weight. Table 3 shows Highest reducing power activity (25.3 µg/g d.w.) was observed in methanol extract of leaves calli supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l) as compared to the activity of control (27.3 µg/g d.w.).

Hydrogen peroxide scavenging activity in leaves calli culture of *Achyranthes aspera* L. Hydrogen peroxide can cross membranes and may slowly oxidize a number of compounds. Thus, removing of hydrogen peroxide as well as superoxide anion is very important for protection of food systems. Table 3 shows 2,4-D (1.5 mg/l) + IAA (0.5mg/l) gave the highest H_2O_2 scavenging activity (75.31 %) with IC_{50} value 82.34 µg/ml in callus culture.

DISCUSSION

2, 4- D was superior to other auxins in the callus induction of *Centella asiatica* [24,25]. This was because 2,4-D, a synthetic auxin, increases callus induction at a higher rate compared to other types of auxin. 2,4-D was better and exhibited 50% higher induction rate, compared to NAA in orchardgrass (*Dactylis glomerata* L.)[26].

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

Hormone code	Hormone Combination (Mg/L)			Intensity of Callus	Nature of callus	Callus Biomass (Gram/Tube)	Response (%)
	IAA	BAP	2,4-D				
H_1	_	0.3	1.0	++	Dark green compact	1.68 ± 0.02^{C}	75
H_2	_	0.4	1.6	+++	Light green nodular	2.10 ± 0.04^{a}	100
H_3	_	0.5	2.1	+++	Yellowish green compact	2.30 ± 0.01^{d}	100
H_4	_	1.6	3.0	+++	Light green nodular	1.90 ± 0.08^{C}	100
H_5	_	1.7	4.0	++	Dark green nodular	1.45 ± 0.06^{a}	78
H_6	0.5	_	1.2	++	Yellowish green compact	1.58 ± 0.02^{a}	67
H_7	0.3	_	1.5	+++	Light brown friable	2.08 ± 0.03^{C}	100
H_8	0.4	_	2.0	++	Light green copact	1.48 ± 0.02^{C}	69
H_9	1.2	_	3.0	++	Yellowish green friable	2.16 ± 0.06^{a}	80
H_{10}	1.5	_	4.0	+	Dark green friable	1.62 ± 0.04^{b}	75

(+++) appreciable amount; (++) moderate amount; (+) trace amount, 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 2. Evaluation of in vitro antioxidant activity of methanol extracts of leaves calli with different hormone combination of auxin and cytokinin

Hormone Code	*DPPH scavenging activity (%, mean ± SD)	*IC50 values (mg/mL, d.w.) of DPPH scavenging activity(%, mean ± SD)	*Ferric reducing power(µg/g, dw) (%, mean ± SD)	*Hydrogen peroxide scavenging activity (%, mean ± SD)	*IC50 values (µg/mL, d.w.) of H_2O_2 scavenging activity (%, mean ± SD)
H_1	79.63 ± 0.176	0.793 ± 0.034	23.4 ± 0.163	71.63 ± 0.176	81.54 ± 0.143
H_2	82.31 ± 2.685	0.790 ± 0.084	22.3 ± 0.182	70.31 ± 0.585	80.34 ± 0.353
H_3	72.7 ± 0.463	0.669 ± 0.024	18.6 ± 0.124	64.32 ± 0.213	71.70 ± 0.463
H_4	74.31 ± 0.585	0.754 ± 0.031	21.2 ± 0.113	68.63 ± 0.176	65.8 ± 0.393
H_5	71.89 ± 2.224	0.721 ± 0.074	21.7 ± 0.193	70.89 ± 0.224	73.5 ± 0.273
H_6	69.63 ± 0.176	0.513 ± 0.034	15.4 ± 0.163	67.63 ± 0.176	66.54 ± 0.143
H_7	85.31 ± 2.685	0.880 ± 0.084	25.3 ± 0.182	74.31 ± 0.585	85.34 ± 0.353
H_8	68.7 ± 0.463	0.749 ± 0.024	18.6 ± 0.124	66.32 ± 0.213	72.70 ± 0.463
H_9	61.31 ± 0.585	0.654 ± 0.031	22.2 ± 0.113	62.63 ± 0.176	63.8 ± 0.393
H_{10}	63.89 ± 2.224	0.611 ± 0.074	20.9 ± 0.193	62.89 ± 0.224	60.5 ± 0.273
Control	89.31 ± 2.685	0.920 ± 0.084	27.3 ± 0.182	78.31 ± 0.585	85.34 ± 0.353

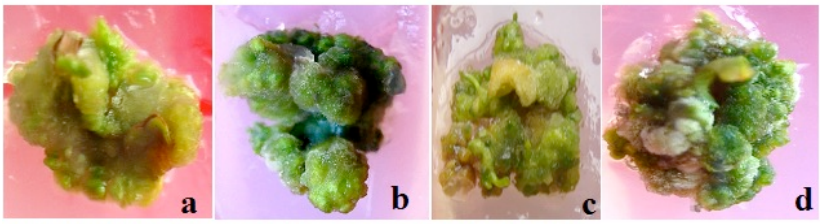

Figure 1: Growth of callus from leaves explants after 6 weeks of inoculation.

a) Callus mainted on MS media supplemented with 1.6 mg/L 2,4-D+ 0.4 mg/l BAP
b) Callus mainted on MS media supplemented with 2.1 mg/L 2,4-D+ 0.5 mg/l BAP
c) Callus mainted on MS media supplemented with 3.0 mg/L 2,4-D+ 1.6 mg/l BAP
d) Callus mainted on MS media supplemented with 1.5 mg/L 2,4-D+ 0.3 mg/l IAA

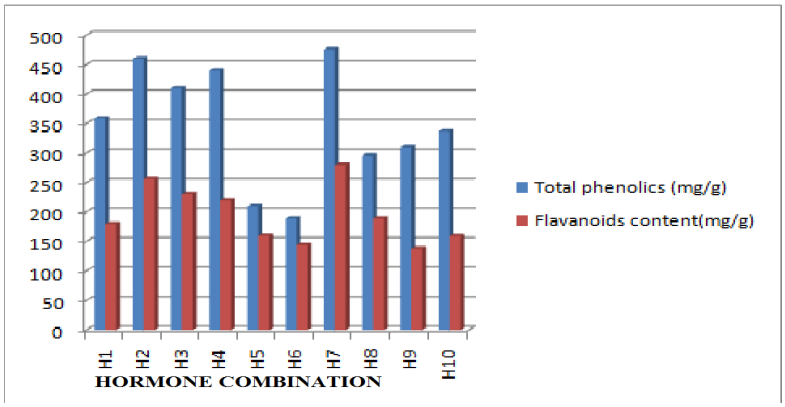

Figure: 2 Quantitative estimation of total phenolics and flavanoid of calli from *Achyranthus aspera* L. with presence of different combination of auxin and cytokinin

Frequency of callusing increased from 54.12 to 96.43% with the increasing concentrations of auxin from 0.2 to 2.0 mg/ L[27]. When the auxin concentration surpassed 2.0 mg/L decreased level of callus induction was observed due to a high concentration of 2,4-D which caused toxicity to the explants and/or calli after four weeks in culture [28]. Efficient tissue culture protocol in their study on *Juncus effusus* L., which produced 90.48% callus formation in the MS medium containing 4 mg/ L 2,4-D and 0.5 mg/L BAP. However, this study was conducted under light conditions and resulted in efficient callogenesis[29].

In most batches of cell cultures, flavonoid accumulation tends to increase at the end of the rapid cell division in the growth cycle. However, in some cases, the production of secondary products did not show a positive correlation with the maximal growth rate of the culture [30]. Various reports have indicated that maximum production of flavonoid's achieved during the active growing stage of the cells[31,32,].

The previous research suggested that the combination of auxin and cytokinin always gave good callus proliferation and maintenance [33,34]. The great amount of antioxidants in the extract of callus culture from *Achyranthes aspera* L. would be resulted in the reduction of Fe3+and Fe2+ by providing an electron. The amount of $Fe3^{+}$and $Fe2^{+}$ can be indicated by the Perl's blue colour appearance and determined by the absorbance at 700nm[35]. There is the significant correlation between the phenolic compounds, antioxidant and reducing power[36]. Reducing power has mainly caused by the presence of the high phenolic compounds of the plant[37]. Highest Ferric reducing power of callus cultures of *Achyranthes aspera* L. is given by 2,4-D (1.5 mg/l) + IAA (0.5mg/l) due to the presence of highest phenolic content and flavonoids.

The high DPPH activity could be correlated with high phenolics content. Absorbance of DDPH radical decrease with high phenolic content. DDPH acts as a stable free radical in methanol solution that easily accepts an electron or hydride radical and converted to a stable diamagnetic molecule. By reacting with suitable reducing agents DPPH radicals formed into the corresponding hydrazine. In this present study, 2,4-D (1.5 mg/l) + IAA (0.5mg/l) showed more prominent DPPH scavenging activity than other extracts of media combination.

Hydrogen peroxide, although not a radical species plays a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally-occurring iron complexes inside the cell believed to react with H_2O_2 *in vivo* to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects[38]. Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells[39]. Thus, removal of H_2O_2 is very important for protection of food systems. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. Highest capacity for scavenging of hydrogen peroxide is given by the methanol extracts of callus culture supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l).

CONCLUSION

Based on the results obtained in the present study, it was concluded that regenerated culture from leaf explants of ***Achyranthes aspera* L.** supplemented with 2,4-D (1.5 mg/l) + IAA (0.5mg/l) contain higher quantity of total phenolics and flavanoids than intact plant leaves. They give significant antioxidant activities to hydroxyl radical, superoxide radical, and DPPH radical. Methanol extracts of leaves calli with different hormonal combination possess different antioxidant activities. There was found a significant and linear relationship between the antioxidant activity and the content of flavonoids. Thus, the extract from callus culture of ***Achyranthes aspera* L.** could be used as an antioxidant herb for adjuvant therapy. As the synthetic antioxidant Butyl Hydroxy Toluene was forbidden being used in food due to its side effects on human, development of the natural antioxidants was meaningful and prospective. In this study, antioxidant activities of leaves calli from ***Achyranthes aspera* L.** was found more than extract of intact leaves of plant. So they can be used as natural antioxidants after isolation and purification.

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