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Original Article

EVALUATION OF ANTIOXIDANT POTENTIAL AND REDUCING POWER OF CALLUS INDUCED FROM LEAVES OF ASYSTASIA GANGETICA (L.) T.ANDERSON

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ABSTRACT

Objective: To evaluate the bioactive molecules and antioxidant potential of callus induced from leaves of *Asystasia gangetica.*

Methods: In this report, the leaves of *A.gangetica* (AG) were incubated with Murashige and Skoog (MS) medium supplemented with combinations of auxins and cytokinins for callus induction. The qualitative estimation of bioactive molecules like flavonoids, phenolics, tannins and their antioxidant potential were investigated. The ability of radical scavenging activity and reducing power of methanolic, ethanolic and aqueous extract using DPPH, FRAP and Phopshomlybdate assay were carried out.

Results: Callus was induced on MS medium supplemented with various concentration and combination of auxins and cytokinins.Maximum percentage of callusing was seen on media supplemented with 2,4-Dichlorophenoxyaceticacid 5mg/L or combination of Kinetin 2mg/L and 2mg/L Napthaleneaceticacid.The total phenolic content, flavonoids and tannins in callus were estimated in various solvents. Further, the callus showed the FRAP values of 17.67 ± 0.0, 17.30 ± 1.830 and 23.81 ± 0.945 µg AAE / mg extract for methanolic, ethanolic and aqueous extract respectively. Methanolic extract showed highest DPPH scavenging activity and reducing abilty.

Conclusion: *A.gangetica* callus had substantial amount of bioactive molecules exhibiting potent antioxidant activity and reducing ability. Development of appropriate strategies for enhancing the bioactive molecules in callus could have far-reaching implications for isolation of novel antioxidant molecules for human health.

Keywords: *Asystasia*, Callus, Phenolics, Tannins, FRAP, Reducing power, DPPH.

INTRODUCTION

Plants are considered as rich source of natural bioactive compounds that differ in terms of structure and biological properties. They are valued for their pharmacological activities and play an important role in traditional medicine across the world. It has been a common practice to harvest the plants in large quantity for the industrially important compound to be used for human welfare. This unscientific harvesting of plants has lead to destruction of natural population ultimately resulting to extinction of many species. Biotechnological and molecular tools has been utilized to enhance the qualitative and quantitative production of pharmacologically important bioactive compounds and food additives [1] .

Several authors have reviewed the beneficial uses of plant species for human welfare [2,3,4,5] and traditional use of these plants for their primary health care needs, that mainly involves use of plant extracts and their active components [6]. Plants, such as herbs have been used in folk medicine for centuries throughout the world. Asystasia gangetica (L); (Acanthaceae) is considered as traditional folk medicinal plant used by Africans as well by Indians for treating different ailments such as asthma, rheumatism, vermifuge, ear disease, diabetes, ulcers etc [7,8]. The leaves of this plant have bronchospasmolytic and anti-inflammatory properties [9, 10]. The whole plant extracts have shown to be antibacterial [11] and exhibits antifungal properties [12]. The plant is also considered to be antihyperglycemic, antihyperlipodmic [13,14], anti-helminthic, anti arthritic [15] and potential antihypertensive properties [16]. It also shows anti inflammatory, anti cancerous and antioxidant activities [7, 17].

The Plant is utilised as nutritional source and indegenous leafy vegetable [18, 19, and 20]. A number of phytochemicals including phenolics, flavonoids, alkaloids, glycosides, tannins, steroids and saponins have been reported [12, 21]. Several bioactive compounds like 5, 11-epoxymegastigmane glucoside (asysgangoside), a megastigmane glucoside has been reported in *A.gangetica* [22]. Few flavonoids like apigenins and flavone uronide has been documented by Subramanian and Nair [23]. Iridoid and flavone glycosides having antioxidant activity have also been isolated [24].

Antioxidants are compounds which delay or prevent the oxidation of lipids or other biomolecules at low concentrations by inhibiting the oxidative chain reactions and have beneficial effects on human health. As antioxidant production in plants are affected by various environmental conditions including seasonal changes [25], *in vitro* cultures are considered as alternate source for production of natural antioxidants independent of environmental fluctuations [26]. In this work, we report the induction of callus from leaves and investigated total phenolic compounds, flavonoids, total condensed tannin content and their antioxidant potential from methanolic, ethanolic and water extracts of *A.gangetica* (AG) callus utilizing more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action [27]. The present work is the first report on comprehensive study of antioxidant activity of *A.gangetica in vitro* cultures.

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), Gallic acid, Quercetin, Catechin, 2, 4, 6-tripyridyl-s-triazine (TPTZ), $FeCl_3.6H_2O$, 2,4-Dichlorophenoxy acetic acid (2,4-D) were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade purity and procured from Himedia, Bombay.

Media and Culture conditions for callus induction

Young leaves of *Asystasia gangetica* (0.2-1 cms) were used as explants. Surface sterilization of the explants were performed by washing with Tween -20 for 45 mins,then in 70% alcohol for one minute.The explants were later sterilized with 0.1% $HgCl_2$ for 5-8 mins.The surface sterilized explants were thoroughly washed with sterile water to remove the traces of the sterilent and finally inoculated onto the culture media. MS basal medium [28] supplemented with various combinations of phytohormones viz; Auxins such as Naphthaleneaceticacid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA) and Cytokinins such as Kinetin (KIN), 6-Benzylaminopurine (BAP) in

different combinations and concentration ranging from (0.5-10mg/L) were used for callus induction. The media was fortified with 3% sucrose as carbon source. Agar 0.8% (w/v) was used as geling agent and the PH was adjusted to 5.8 by adding 1N NaoH or 1N HCL prior to autoclaving for 20 mins at 121 ^{0} C for 15psi.The cultures were maintained in a culture room under regular cycle of 14 hrs light and 10 hrs dark at 25±2 ^{0}C. The entire callus was maintained through regular sub culture after every 7 weeks.

Preparation of callus extract for Phytochemical Analysis

About 30 gms of 30 days old matured green friable leaf calli inoculated on MS+KIN+NAA(2+2mg/Lt) were collected and ground into powder in liquid nitrogen.The powder was suspended in 100 mL of methanol / ethanol respectively and kept at room temperature for 24 hrs with periodic shaking. The aqueous extract was prepared by boiling the call us at 100 ^{0} C for 10 mins in water bath and the solution were filtered using whatman No 1 filter paper, the filtrate were centrifuged at 4000rpm for 15 mins.The pooled extracts were concentrated by keeping it in a hot air oven at 38±2 ^{0}C, later the condensed extract was weighed and reconstituted in minimum volume of methanol/ethanol/water and stored at 4^{0}C until further use. Preliminary qualitative phytochemical screening was performed using standard methods described by Horborne [29] for alkaloids, anthocyanins, flavonoids, phenols, triterpenes, steroids, saponins, and anthroquinones.

Estimation of Total phenolic content (TPC)

The total phenolic content in the callus extracts was determined using Folin-Ciocalteu method [30], with some modifications. Briefly, 0.5 ml of callus extract was mixed with 0.2 ml of Folin- Ciocalteu's reagent and allowed to stand at room temperature for 5 min. 2 ml of sodium carbonate (Na_2CO_3, 7.0 %, w/v) was added and the reaction mixture was made up to 5 ml with sterile distilled water.The reaction was allowed to stand for another 90 min in dark with intermittent shaking. Then the absorbance of the blue color that developed was measured at 725 nm using spectrophotometer (Elico SA 165 spectrophotometer) against the blank. The experiment was carried out in triplicates. The total phenolic compounds concentration in callus extracts was expressed as micrograms of gallic acid equivalent per mg (µg GAE/mg) of extract.

Estimation of Total Flavonoids

The total flavonoid content in the methanolic/ethanolic/aquoeus extract of callus tissue was determined by aluminum chloride method [31] with slight modifications. Briefly 0.5 ml of callus extract was mixed with 2.5 ml of 95% ethanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water was added to the mixture to bring the final volume up to 9 ml. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Elico spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank (r2= 0.9934). The final absorbance of each sample was compared with a standard curve plotted from Quercetin. The total flavonoid content was expressed in µg of Quercetin per mg of extract.

Estimation of Total condensed tannins

Determination of total condensed tannins (content of proanthocyanidins) was based on the procedure [32]. In brief, to 50 µL of diluted sample, 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated HCl were added. The mixture was incubated at room temperature for 15 min, and absorption was measured at 500 nm against methanol as a blank. Total content of proanthocyanidins was expressed in terms of catechin equivalent, CAE (standard curve equation: y = 0.0101 x + 0.1209, R^2=.9984), µg of CAE/mg of extract. All samples were analyzed in triplicate.

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability:

Radical scavenging activities of *A.gangetica* callus were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay [33]. The extract (20µl) was added to 1 ml of 50µM DPPH solution in methanol. The extracts tested ranged from 0–250µg/ml.The mixtures were mixed well and incubated in the dark for 30 min. The reduction of DPPH absorption was measured at 517 nm. Ascorbic acid was used as the positive control. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Percentage inhibition} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

From the obtained values, the EC_{50} (defined as the concentration of sample at which 50% of maximum scavenging activity was recorded) was calculated for each sample.

Total antioxidant capacity by Phosophomolybdenum Assay

Total antioxidant activity of the extract was evaluated by the phosphomolybdate method [34] using ascorbic acid as a standard. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank. Ascorbic acid equivalents were calculated using standard graph of AA (r^2= 0.9945). The experiment was conducted in triplicates and values were expressed as equivalent of ascorbic acid per mg of extract.

Ferric reducing ability of plasma (FRAP) Assay

The antioxidant activity based on the ferric reducing ability of *Asystasia* callus extracts was estimated based on the assay [35] with some modifications. A working reagent was prepared fresh by mixing 10 ml of 300 mM acetate buffer with 1 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM of hydrochloric acid (HCl) and 1 ml of 20 mM $FeCl_3.6H_2O$. The freshly prepared FRAP reagent was pre-warmed at 37°C after which a blank reading was taken at 595 nm. Subsequently, 30 µl of sample / standard was added to 900 µl of the FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent and again at 30 min after the start of the reaction. The change in absorbance in the 30 min reaction was calculated by comparison to the absorbance changes of ascorbic acid against a standard curve tested in parallel. Results were expressed as micromoles of ascorbic acid equivalents (AAEs) per milligram of extract (µmol of AAEs/mg). All experiments were carried out in triplicate.

Reducing power assay

The reducing power was determined according to the method [36] with modifications. Various concentrations of callus extracts (1 ml) were mixed with 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50^{0}C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml deionised water and 0.5 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration that gave 0.5 absorbance (EC_{50}) was calculated from a graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as the standard.

Statistical Analysis

All the experiments were carried out in triplicates (n=3) and the results were expressed as mean ± standard deviation (SD). Statistical tests as well as mean and SD calculations were performed using Graph Pad Prism v 5.

RESULTS

Influence of auxins and cytokinins on Induction of callus from leaf

The induction of callus was observed in leaf explants of *A.gangetica* innoculated on MS basal media supplemented with various Plant Growth Regulators (PGR) as shown in table 1. Callus formation was

observed to be initialy started at the margins in explants and robust callus was developed within four weeks without any shoot regeneration. Further, no callus formation was observed during the culture period in the control (MS medium without growth regulators) in all explants. Initiation of callus was observed after 10-12 days of inoculation with various concentrations of 2, 4-D and Kinetin. When 2, 4-D alone was used (Fig 1 A and B) a yellowish green friable callus (1-3 mg/L) was formed but the callus was whitish friable at higher concentration (5-10 mg/L) and the response for callusing was slow.

When Kin (1-6mg/L) alone was used as growth regulator, the callus was compact darkgreen and rhizogenic. At higher concentration of Kin (8-10mg/L) the callus was very compact and showed browning. When explants were innoculated on MS medium with combination 2, 4-D (2mg/L) and Kin (1-6mg /L), maximum response of the callus formation was seen in all combinations as shown in table, but at higher concentration of Kinetin, callus underwent necrosis with browning. Similar observation was seen when combination of 2,4-D and NAA was used. But the morphology of the callus was slightly different when combination of Kin and NAA was used.The callus proliferation was robust without any rhizogenesis (Kin 1-6mg/L + NAA 2mg/L). Higher concentration of kinetin with NAA combination had negative effect with slow growth and finally leading to necrosis. When BAP either used alone (Fig. 1E) or in combination with Kin (Fig.1 D), the leaf explant formed shooty callus without any regeneration. To maintain the proliferation status the calli was subcultured on MS medium with 2mg/L Kin and 2mg/L NAA.

The calli (30 day old) raised in different hormone combinations were initially screened for flavonoids and phenolic content during the rapid growth phase of the culture cycle and were found to have relatively same amount of bioactive substances except for the rhizogenic and shooty callus. For further analysis of antioxidant and scavenging activities, the callus grown under the combination of Kin 2mg/L + NAA 2mg/L were utilised.

Table 1: Effect of auxins and cytokinins (mg/L) in MS medium for callus induction of *A. gangetica (L) leaves*.

Phytohormone (mg/L)				Response percentage (%)	Intensity of callus formation	Color,texture/morphology
2,4-D	**KIN**	**NAA**	**BAP**			
0.5	0.0	0.0	0.0			
1.0	0.0	0.0	0.0	50	++	Yellowish-Green friable and sticky callus
2.0	0.0	0.0	0.0	80	+++	Yellowish-Green friable and sticky callus
3.0	0.0	0.0	0.0	80	+++	Yellowish-Green friable and sticky callus
5.0	0.0	0.0	0.0	100	+++	Whitish friable
8.0	0.0	0.0	0.0	50	++	Whitish friable
10.0	0.0	0.0	0.0	20	+	Whitish friable
0.0	0.5	0.0	0.0	40	+	
0.0	1.0	0.0	0.0	100	+++	Green,Compact,rhizogenic
0.0	2.0	0.0	0.0	100	+++	Green,Compact,rhizogenic
0.0	3.0	0.0	0.0	100	+++	Green,Compact,rhizogenic
0.0	5.0	0.0	0.0	100	+++	Green,Compact, rhizogenic
0.0	8.0	0.0	0.0	50	+	Dark green Compact
0.0	10.0	0.0	0.0	00	-	Slow response with necrosis
2.0	2.0	0.0	0.0	100	+++	Pale Green, friable granular
2.0	4.0	0.0	0.0	100	+++	Pale Green, friable granular
2.0	6.0	0.0	0.0	50	+++	Pale Green, friable granular
2.0	10.0	0.0	0.0	00	-	Explants turned brown
2.0	0.0	2.0	0.0	100	+++	Light green
2.0	0.0	4.0	0.0	100	+++	Light green
2.0	0.0	6.0	0.0	70	++	Light green
2.0	0.0	8.0	0.0	50	++	Light green
2.0	0.0	10.0	0.0	00	-	Explants turned brown
0.0	2.0	2.0	0.0	100	+++	Light green callus
0.0	4.0	2.0	0.0	80	+++	Light green callus
0.0	6.0	2.0	0.0	50	++	Slow growing brown callus with necrosis
0.0	8.0	2.0	0.0	00	-	No response
0.0	0.0	0.0	1.0	70	++	Green, compact Slimy
0.0	0.0	0.0	2.0	75	++	Green, compact Slimy
0.0	0.0	0.0	3.0	50	+	Green, compact Slimy
0.0	0.0	0.0	4.0	100	++	Green, compact Slimy
0.0	0.0	0.0	6.0	100	++	Green, compact Slimy
0.0	0.0	0.0	8.0	100	+	Green, compact Slimy
0.0	0.0	0.0	10.0	100	+	Green, compact Slimy

+++: Profuse callus, ++: Moderate callus, +: Poor callus, -: No response

Table 2: Preliminary Qualitative phytochemical screening of callus extracts of *A. gangetica*

S. No.	Phytochemicals	Ethanolic extract	Methanolic extract	Aqueous extract
1	Tannins	+	+	-
2	Saponins	+	+	+
3	Terpenoids	-	+	+
4	Glycosides	+	+	+
5	Anthroquinones	+	+	+
6	Flavanoids	+	+	+
7	Steroids	-	-	-
8	Phytosterols	-	+	+
9	Phenolics	+	+	+

Here, (+) = present and (–) = absent.

Phytochemical screening

Callus extract in various solvents yeilded approximately 436, 540 and 469 mg / 30 gms of fresh callus for methanolic, ethanolic and water extract respectively. Phytochemical screening of above callus extracts demonstrated the presence of anthraquinones, glycosides, flavonoids, saponins, phytosterols, tannins and terpenoids as shown in table 2. Steroids were not present in any of the callus extract.

Total Phenolics

The content of phenolic compounds (µg / mg) in callus extract were determined using regression equation of callibration curve (r^2= 0.979) and expressed as gallic acid equivalents (GAE) was found to be 14.33 ± 0.0799 µg (GAE) / mg in methanolic extract,and 7.473 ± 0.4504 µg (GAE) / mg in callus extracted in ethanol. The aqueous extract had 26.95 ± 1.2540 µg (GAE) / mg as shown in table 3. These phenolic compounds are likely to contribute to the radical scavenging activity of callus.

Flavonoids

Flavonoids, the low molecular weight, secondary plant phenolic compounds have antioxidant activity and are ubiquitously found in plants. The content of flavonoid compounds (µg/mg) in different extracts of callus was determined using the regression equation of callibration curve (r^2= 0.9934) and expressed as Quercetin Equivalent (QE). The total flavonoids in methanolic and ethanolic extract of callus was determined spectrophotometrically as 25.13 ± 0.0173 µg (QE) / mg and 30.87 ± 3.466 µg (QE) / mg of extract respectively. The aqueous extract had 25.73 ± 1.626 µg / mg of extract as shown in table 3.

Condensed tannins

Tannnins, the water soluble phenolics have been considered as both primary and secondary antioxidants. Proanthocyanidins, also called condensed tannins, are group of secondary plant metabolites having substantial antioxidant activity. The content of proanthocynidin compounds (µg/mg) in different extracts of callus was determined (table 3) using the regression equation of callibration curve (r^2= 0.9984) and expressed as Catechin Equivalent (CE). The condensed tannins in methanolic and ethanolic callus extract was determined spectrophotometrically as 14.93 ± 1.234 µg (CE) / mg and 19.03 ± 1.321 µg (CE) / mg of extract respectively. The aqueous extract had 19.00 ± 0.99 µg (CE) / mg of extract.

Table 3: Total phenolics, flavonoid and condensed tannins of various callus extracts of *gangetica*.

Plant extract	Total phenolics µg gallic acid equivalent/mg extract	Total flavonoids µg Quercetin equivalent/mg extract	Condensed tannins µg catechin equivalent/mg extract
Methanol extract	14.33 ± 0.0799	25.13 ± 0.0173	14.93 ± 1.234
Ethanol extract	7.473 ± 0.4504	30.87 ± 3.466	19.03 ± 1.321
Aqueous extract	26.95 ± 1.254	25.73 ± 1.626	19.00 ± 0.990

Ferric reducing antioxidant power (FRAP) Assay

The FRAP assay measures the antioxidant effect of the substance in the reaction medium as reducing ability. Antioxidant potential of the callus of *A.gangetica* was estimated for their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex.

The antioxidant capacities of callus extract in methanol and ethanol did not vary significantly (Table 4). The methanol and ethanol extract showed FRAP values of 17.67 ± 0.0 µmol of AAEs / mg and 17.30 ± 1.830 µmol of AAEs / mg respectively. Whereas the aqueous extract showed slightly higher reducing ability with FRAP value of 23.81 ± 0.945 µmol of AAEs / mg.

Phosphomolybdenum assay

The phosphomolybednum quantitative method was utilised to evaluate the total antioxidant capacity of the callus extract. The callus extract exhibited different degrees of activity as shown in Table 4. The reducing power of a compound is associated with electron donating capcity and seves as an indicator of antioxidant activity [58].

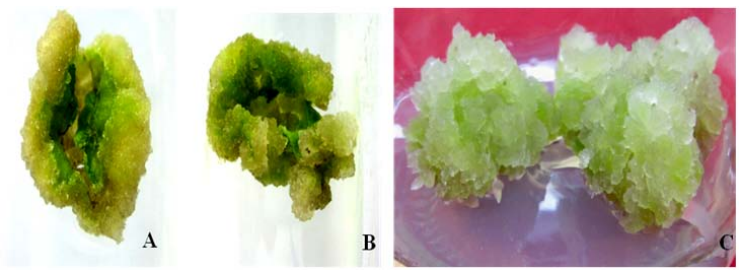
The results indicate that methanolic extract had comparitively higher antioxidant activity with differences in the degree of Mo reduction between three types of extracts used. Results indicated that methanolic extracts showing higher degree (58.032 ± 1.220 µg AAE /mg extract) of antioxidant capacity than the ethanolic extract (45.229 ± 1.383µg AAE / mg extract). However the antioxidant activity noticed in aqeuous extract was comparatively less with 36.229 ± 1.708 µg AAE /mg extract.

DPPH Radical Scavenging activity

The DPPH radical scavenging property of the compounds extracted from *A.gangetica* callus is shown in Fig 2. The effective concentrations at 50% inhibition (EC_{50}) of methanolic and ethanolic extract were in the range of 65.775 ± 2.302 µg / ml and 86.823 ± 3.178 µg / ml respectively. The EC_{50} (table 4) values of methanolic and ethanolic extract were relatively high compared to positive control of ascorbic acid (EC_{50} 4.2096 ±.0891µg/ml) implying the low potency in free radical scavenging activity. The scavenging activity of aqueous extract was 3 fold less compared to other extracts.

Reducing Power

In the reducing power assay, the antioxidant compounds convert the oxidation form of iron (Fe+3) in ferric chloride to ferrous (Fe+2). To support the antioxidant activity exhibited by callus extracts from various solvents in free radical scavenging assay, reducing power of the callus extracts of *A.gangetica* were evaluated. Fig 3 indicates a dose dependent increase in activity of callus. The increased absorbance at 700 nm due to the reduction of potassium ferricyanide / ferric chloride complex indicates the presence of reducing power in all the three extracts tested including the standard antioxidant ascorbic acid. The result of this assay showed that the reducing power of the AG methanolic extract was slightly more (EC_{50} = 600 ± 16.091 µg/ml) compared to ethanol (EC_{50} = 699 ± 13.781 µg/ml) extract. However, the reducing power increased drastically at higher concentration of methanol extract (2mg/ml). The reducing power of the aqueous extract was less when compared to other extract (EC_{50} > 2000 µg/ml).



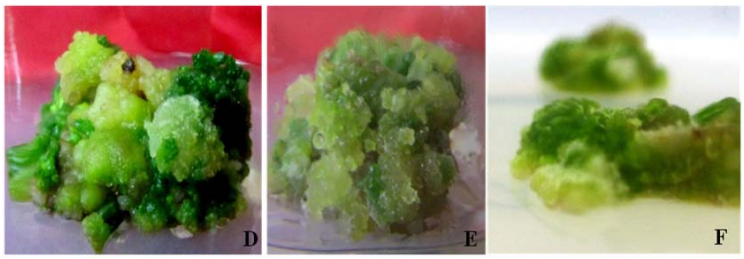


Fig. 1: Different types of callus obtained using auxins and cytokinins A. 2,4-D (4mg/Lt) 3 weeks old callus, B. 2,4-D (1mg/Lt) 3 weeks old callus, C. NAA+KIN (2+2mg/Lt), D. KIN+BAP (1+2mg/Lt) 8 weeks old callus, E. BAP (2mg/Lt) 8 weeks old callus, F. KIN (5mg/Lt) 3 weeks old callus

Table 4: Quantitative estimation of antioxidant, radical scavenging and reducing power of *A.gangetica* callus extract

Extract	Phosphomolybdenum assay µg AAE / mg extract	FRAP assay µg AAE / mg extract	Reducing power (EC_{50} [a])	DPPH radical scavenging assay (EC_{50} [b])
Methanolic extract	58.032 ± 1.220	17.67 ± 0.0	600 ± 16.091	65.775 ± 2.302
Ethanolic extract	45.229 ± 1.383	17.30 ± 1.830	699 ± 13.781	86.823 ± 3.178
Aqueous extract	36.229 ± 1.708	23.81 ± 0.945	> 2000	202.587±11.209

[a]EC_{50} (µg/ml): effective concentration at which the absorbance is 0.5

[b]EC_{50} (µg/ml): effective concentration at which 50% of DPPH radicals are scavenged

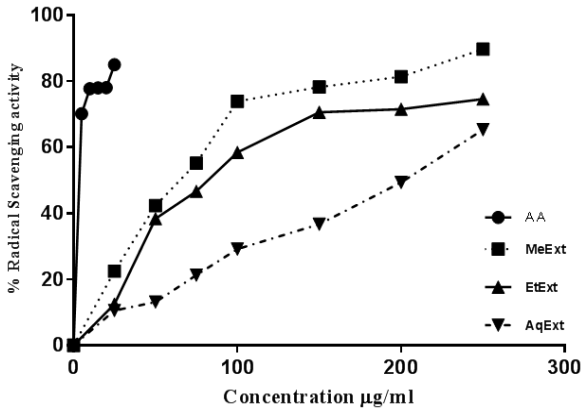


Fig. 2: DPPH scavenging effect of *A.gangetica* callus extracts. (AA-Ascorbic acid,MeExt-Methanolic extract,EtExt-Ethanolic extract,AqExt-Aquoeus extract)

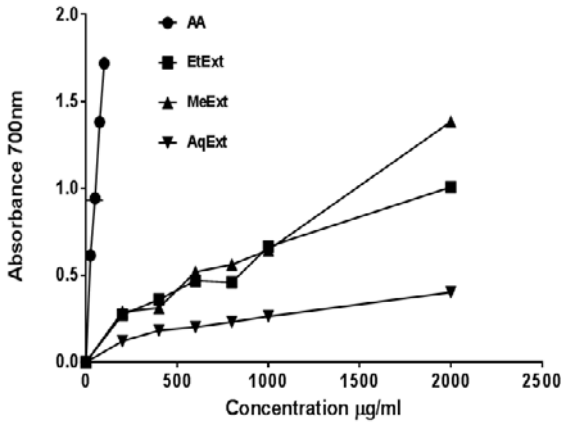


Fig. 3: Reducing power of *A.gangetica* callus extracts. (AA-Ascorbic acid, MeExt-Methanolic extract, EtExt-Ethanolic extract, AqExt-Aquoeus extract)

DISCUSSION

The continuing interest in screening of medicinal plants for new bioactive molecules having natural antioxidant activity has immense beneficial effects to human health in circumventing cancer and acting as cardio protective agents through antioxidant activity [37]. Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the illeffects caused by oxidative stress [38]. These bioactive molecules are produced in plants in less quantity, which is largely influenced by environmental factors.This has lead to looking for alternate resources like callus and suspension cultures where the synthesis of bioactive molecules can be enhanced either with PGRs or elicitors [39]. Callus, the unorganized cell mass could be induced from any part of plant under *invitro* conditions [40] and that the balance between two plant hormones i.e. auxin and cytokinin, determines the state of differentiation and dedifferentiation [41] with an intermediate ratio of auxin and cytokinin is believed to promote callus induction. Callus has been widely used in both basic research and industrial applications [42]. In the present investigation we have developed an efficient invitro callus induction system for *Asystasia gangetica* using MS media. When auxins like 2,4-D was used alone or with combination of NAA maximum callusing percentage with friable callus was seen at 2-6mg /L but higher concentration had negative effect. Similar effect has been reported in several other plant species [43,44]. When 2,4-D was used in combination with kinetin,it showed maximum response at 2-4 mg/L. However, higher concentration of Kn in combination with 2,4-D resulted in slow proliferation and necrosis. The synergistic effect of auxins and cytokinins observed in callus induction of this plant is similar to *Achyranthes aspera* [45,46].

The phytochemical investigations of callus showed the presence of saponins, tannins, flavonoids, phenolics, phytosterols, glycosides, terpenoids and anthroquinones. Among these, flavonoids and phenolics are considered as primary antioxidant bioactive molecules. Table 1 shows the quantity of total flavonoids and total phenolic content. Maximum phenolic content was observed in aqueous extract (26.95 ± 1.254 µg GAE/mg) and the lowest in ethanolic extract (7.473 ± 0.4504 µg GAE/mg),whereas the flavonoids were maximum in ethanolic extract (30.87 ± 3.466 µg QE/mg) and the amount was almost same in methanolic and aqueous extract (≈25 µg QE/mg). These compounds have been reported to have multiple biological effects, including antioxidant

and chelating properties [47]. Different types of callus is known to have distinct gene expression profiles [48] and various combinations and concentration of PGRs would result in accumulations of phytochemicals like isoflavone, flavonoids, phenolics in callus [49,50,51]. Further, callus derived from distinct explants like root, stem or leaves produces varied accumulation of bioactive compounds [49,52]. When the AG calli was grown under the influence of different PGRs, the amount of phenolics and flavonoids didn't vary significantly except for shooty and rhizogenic callus. Tannins, particularly proanthocynidins are believed to act as antinutrients [53] and are considered as new natural antioxidants [54]. The methanolic callus extract had lowest tannin content 14.93 ± 1.234 µg Catechin equivalents /mg extract compared to 19.00 µg Catechin equivalent /mg in ethanolic and aqueous extracts. In the absence of specific bioactive antioxidant molecules being identified, the combined effect of phenolics, flavonoids and tannins are believed to effect the antioxidant property of the callus extract.

Several antiradical protocols are utilized for evaluation of antioxidant activity. DPPH, a stable free radical accepts hydrogen from a corresponding donor and its solutions lose the characteristic deep purple (λmax 515–517 nm) colour to yellow. DPPH is very popular for the study of natural antioxidants [55]. The callus exhibited various degree of scavenging activity which was dose dependent (fig 3). The EC_{50} of various extract were in the order: Methanolic EC_{50} (65.775 ± 2.302 µg/ml) < Ethanolic EC_{50} (86.823 ± 3.178 µg/ml) < Aqueous extract EC_{50} (202.587±11.209 µg/ml) indicating maximum free radical scavenging activity in methanolic callus extract in spite of having low amount of flavonoids and condensed tannins. Similar enhanced radical scavenging has been noticed in stem induced callus of *Justicia gendarussa* [56] and leaf induced callus of *Hildegardia populifolia* [57]. However, the EC_{50} of all the extracts were comparatively more than that of Ascorbic acid (EC_{50} 4.2096 ±.0891µg/ml).

The antioxidant power of the crude callus extract was also measured using ferric reducing ability of plasma (FRAP) assay, which is non-specific, measures the ability of antioxidant compounds to reduce complex (Fe(III)-TPTZ) to (Fe(II)-TPTZ).This assay is used for analysis of single antioxidant and total antioxidant power of antioxidant extracts [58]. As shown in the table (2), the reducing ability of crude extracts had highest activity in aqueous extract. The FRAP values were in the order: aqueous (23.81 ± 0.945) > methanolic (17.67 ± 0.0) > Ethanolic (17.30 ± 1.830). Further, the reducing capacity of the callus was also measured by potassium ferricyanide reduction method. The presence of antioxidants (reductants) in the callus extract causes the reduction of the Fe^{3+} / Ferricyanide complex to ferrous form which can be monitored at 700 nm. Fig (4) shows the reducing capacities of different fractions of callus extract. All the extracts showed dose dependent reducing activity. Aqueous extract showed the lowest reducing capacity. However the methanolic and ethanolic extracts showed same reducing capacity at lower concentration but at higher concentration, methanolic extract showed maximum activity.

Finally, total antioxidant capacity was evaluated quantitatively using phosphomolybdenum assay which is based on reduction of Mo(VI) to Mo(V) by the analytes with the formation of green phosphate / Mo V at low PH. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity [59]. These results indicated methanol had higher total antioxidant activity with the order: Methanol (58.032 ± 1.22) > Ethanol (45.229 ± 1.383) > aqueous (36.229 ± 1.708). Several *in vito* cultures have shown increased total antioxidant activity compared to in vivo tissues [60, 61].

CONCLUSION

In summary, callus induced from *A.gangetica* leaves had substantial amount of flavonoids, phenolics and tannins. The callus was found to be an effective antioxidant in different invitro antioxidant assays including DPPH, FRAP, Phosphomolybdenum assay and reducing power. Development of suspension cultures from callus and utilization of elicitors to increase the bioactive compounds would not only ameliorate the basic callus research having promising downstream application potentials but can also be manipulated for isolation of novel bioactive antioxidant molecules. Although the antioxidant activities found in the *in vitro* experiment were only indicative of the potential health benefits, these results remain important and are the first step in screening the comprehensive antioxidant activity of the *Asystasia gangetica* leaves and isolation of bioactive molecules.

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CONFLICT OF INTERESTS

Declared None

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