

Research Article



Comparative Antioxidant Activity and Phytochemical Contents of Callus and Leaf Extracts of *Holarrhena antidysenterica* (L.)

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ABSTRACT

Indian medicinal plants are used since ancient times to treat different diseases and ailments as these natural products exert broad-spectrum actions. The present study was justifiably planned to raise the callus of valuable medicinal plant *Holarrhena anti dysenterica* L. in *in vitro* condition with various combinations/concentrations of plant growth regulators, and to compare the antioxidant and phytochemical content difference in the *in vivo* plant and *in vitro* grown callus of *Holarrhena anti dysenterica*. The callus and crude extracts were used for total phenolic contents, primary metabolite detection and antioxidant activity by (DPPH and OH radical scavenging methods). Maximum callus (88.19%) was obtained on MS medium supplemented with (2, 4-D+Kinetin) at (2.0+1.5)mg/L. Significantly higher DPPH scavenging activity (85.11%) at 280µg/ml and (58.34%) at 240µg/ml in both the extracts i.e. leaf and callus in comparison to OH scavenging activity (74.11%) at 280µg/ml and (50.30%) at 240µg/ml with phenol contents (2.11mg/g) and (1.53mg/g) were observed in leaf and *in vitro* raised callus extract respectively. The findings indicates greater amount of phenolic compounds leads to more potent radical scavenging effect as shown by leaf extracts of *Holarrhena antidysenterica* and the ability to utilize tissue culture techniques towards development of desired bioactive metabolites from *in vitro* culture as an alternative way to avoid using endangered plants in pharmaceutical purposes.

Keywords: Antioxidant activity, Crude extract, *Holarrhena antidysenterica*, *In-vitro*, *In-vivo*, Phytochemicals.

INTRODUCTION

India is one of the world's twelve leading biodiversity centre with the presence of over 45,000 different plant species, out of this about 15,000-20,000 plants have good medicinal properties of which only about 7,000-7,500 are being used by traditional practitioners. All traditional medicines have their roots in folk medicines and household remedies. WHO has listed 20,000 medicinal plants used in different parts of the world.^{1,2} WHO has estimated that 80% of the world's populations rely primarily on traditional medicine.^{3,4} In India, it is reported that traditional healers use 2500 plant species and 1000 species of plants serve as regular sources of medicine.⁵ During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. Traditional medical knowledge of medicinal plants and their use by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development in the present and future.⁵

In this study our attention has been focused on *Holarrhena antidysenterica* under the family Apocynaceae is one such plant, popularly known as "Indrajav" is a shrub, distributed throughout India. It is a deciduous shrub or small tree. The bark is rather rough, pale brownish or greyish; the leaves are opposite, sub sessile, elliptic or ovate-oblong, membranous; the flowers are white, in terminal corymbose cymes; the follicles, divaricate, cylindrical and usually white spotted; the seeds

are light brown. In Indian traditional medicine, the plant has been considered a popular remedy for the treatment of dysentery, diarrhoea, intestinal worms^{6,7} and the seeds of this plant are also used as an anti-diabetic remedy in Asian countries. Kutaja tree is popular for its numerous medicinal properties. The tree forms part of several indigenous systems of medicines, which has been used in the treatment of dysentery and diarrhoea. Several Indian tribes have used the plant in ailments like anaemia, epilepsy, stomach pain and cholera. In the Ayurvedic system of medicine, Kurchi is used as an antihelminthic, for diarrhoea and skin diseases. Recently many research works on comparative phytochemical and antioxidant activity of *in vivo* and *in vitro* raised plants are being carried out in many research laboratories.^{8,9,10} The present is justifiably planned to propagate the valuable medicinal plant *Holarrhena antidysenterica* in *in vitro* condition with various combinations/concentrations of plant growth regulators, and compare the antioxidant and phytochemical content differences in the *in vivo* and *in vitro* cultivated callus of *Holarrhena antidysenterica*.

MATERIALS AND METHODS

Experimental plant material

Healthy plant materials of *Holarrhena antidysenterica* were collected and the herbarium was deposited in Department of Botany, Utkal University, Bhubaneswar, Odisha, India. The voucher specimen number obtain was SK-235.



Callus induction

Leaf explants were taken from healthy plants of *Holarrhena antidysenterica* for callus induction. Callus initiation was studied with the 2, 4-D (2,4-Dichlorophenoxyacetic acid) and KIN (Kinetin; Sigma, USA) concentration ranging between 0.5 to 2.5 mgL⁻¹ in the MS medium. After inoculation with established culture, the culture flasks were sealed properly, labelled and the triplicates were maintained. Then they were transferred to the incubation room and kept in appropriate condition. After two weeks, the callus induction rate was recorded.

Preparation of callus extracts

A known quantity of one-month old callus was taken and oven dried at 60°C to constant weight. The callus was finely grinded and extracted with methanol using soxhlet apparatus for 8h and solvent was removed by distillation. The crude extract obtained was used for antioxidant activity studies.

Preparation of leaf extracts

The dried and powdered leaves (50g) were extracted successively with ethanol as organic solvent (400ml.) for 10-12 hours through Soxhlet apparatus. The collected extracts were filtered through Whatman No-1 filter paper. The extracts were evaporated to dryness under reduced pressure at 90°C by Rotary vacuum evaporator to obtain the respective extracts and stored at -18°C until used for further analysis.

Phytochemical screening

Phytochemical tests were carried out in both the leaf and callus extracts for the qualitative determination of phytochemical constituents as per the standard procedure.¹¹⁻¹⁴ The total phenolic contents of plant extracts were determined by using Folin-Ciocalteu Spectrophotometric method according to the method described Kim et al.,¹⁵ Reading samples on a UV-Vis Spectrophotometer at 650nm. Results were expressed as catechol equivalents (µg/mg).

DPPH radical scavenging assay

The antioxidant activity of the *Holarrhena antidysenterica* leaves on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined according to the method described in Brand-Williams et al.,¹⁶ with slight modification. The following concentrations of extracts were prepared 40µg/mL, 80µg/mL, 120µg/mL, 160µg/mL, 200µg/mL, 240µg/mL, 280µg/mL, 320µg/mL and 360µg/mL. All the solutions were prepared with methanol. 5 ml of each prepared concentration was mixed with 0.5mL of 1mM DPPH solution in methanol. Experiment was done in triplicate. The test tubes were incubated for 30 min. at room temperature and the absorbance measured at 517nm. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was

used as a standard and the same concentrations were prepared as the test solutions. The difference in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following equation.

$$\text{Scavenging effect (\%)} = (1 - A_s/A_c) \times 100$$

A_s is the absorbance of the sample at t = 0 min.

A_c is the absorbance of the control at t = 30 min.

Hydroxyl radical scavenging assay

2-Deoxyribose is oxidized by OH⁻ radicals that are formed by Fenton reaction and degraded to Malondialdehyde which can be detected by reacting with Thiobarbituric acid (TBA).¹⁷ 100 ml of Riboflavin solution [20 mg], 200 ml EDTA solution [12mM], 200 ml methanol and 100 ml NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50mM]. The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5 min. This is taken as control. 50 ml of different concentrations of test samples as well as standard preparation were taken and diluted up to 100 ml with methanol. To each of these, 100 ml Riboflavin, 200 ml EDTA, 200 ml methanol and 100 ml NBT was mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrophotometer. The IC₅₀ value for each compound as well as standard preparation were calculated by using the following formula

$$\% \text{ scavenging/Inhibition} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right] \times 100$$

Statistical Analysis

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The data are expressed as mean ± standard deviation.

RESULTS

Callus Induction from Nodal Explants in MS Medium

Callus formed at the cut surfaces of nodal explants when grown on MS medium supplemented with different concentrations of 2, 4-D in combination with Kinetin within 7-15 days of culture maximum callus (88.19±0.09%) yield was obtained in MS medium fortified with 2.0 mg/L of 2,4-D and 1.5 mg/L of Kinetin, after five successive subcultures and the color of the callus was whitish green (Figure 1). The callus was regularly sub cultured with the same medium in regular interval of about two weeks for the revival of the initial callus. It was noticed that by increasing the concentration of 2, 4-D and Kinetin (2.0+1.5mg/L) formation of callus was decreased gradually (Table-1 & Figure 2). It may be concluded that up to (2mg/l) of 2, 4-D appreciable growth of callus was found in *Holarrhena antidysenterica* and further increase

may have a negative impact on callus development. Similarly Kinetin (1mg/l) gave the best result in callusing and brownish callus was obtained at the same concentration, which may have more phenol contents.

The same callus medium was used for callus ignition and subcultured (30d). The growth rate of callus was determined at regular intervals (10, 15, 20, 30 and 45d) (Figure 3). The callus growth expressed three distinct phases. A lag phase of 10d where 10% growth in callus

mass was noticed followed by exponential or linear phase with a rapid and significant increase (65%) in formation of callus during 10-15d of culture representing early linear phase. It recorded a further growth rate of 15% during late linear growth phase extending from 15th and 30thd of culturing. The growth declined after 30d representing log phase. Depletion of nutrients, accumulation of toxic products, and other limiting factors might have led to cell death and eventually decline in growth (Figure 1).

Table 1: Effect of different concentrations of auxin and cytokinin on callus induction in *Holarrhena antidysenterica* W. from nodal explants in MS medium

Growth regulators (mg/L) 2,4-D+ KINETIN	Percentage of callus induction. (Mean ± SEM)	Type of callus	Degree of callusing at the end of 4 weeks
0.5+0.5	34.01±0.13	light yellow	+
1.0+1.0	45.15±0.11	light yellow and friable	+
1.5+1.5	62.65±0.28	whitish yellow	+
0.5+1.0	62.83±0.31	whitish yellow	++
1.0+1.5	64.37±0.16	white and friable	+
2.0+1.0	67.60±0.22	Light pink	+++
2.0+1.5	88.19±0.09	Whitish green	++++
2.0+2.0	61.41±0.20	brownish & friable	++
2.5+2.0	60.26±0.21	brownish	++
2.5+2.5	58.12±0.19	Dark brown	+

+ = POOR; ++ = FAIR; +++ = GOOD; ++++ = VERY GOOD; Data represents the mean of 10 replicates for each treatment; Data were recorded after 4 weeks of culture.

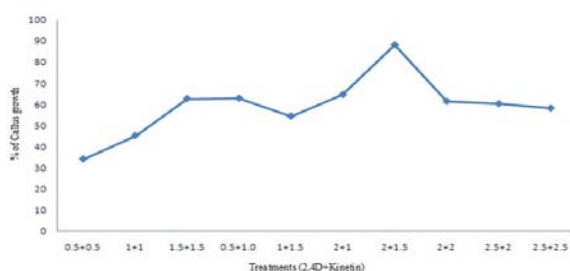


Figure 1: Effect of different concentration of 2,4-D and Kinetin on callus induction in *H. antidysenterica*

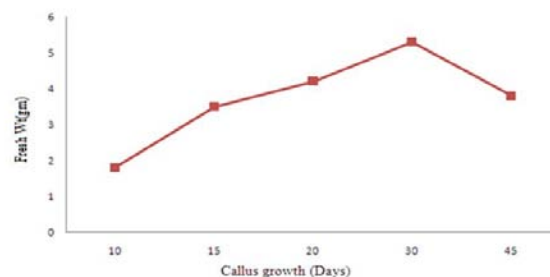


Figure 2: Callus growth curve of *Holarrhena antidysenterica* in Different days of culture

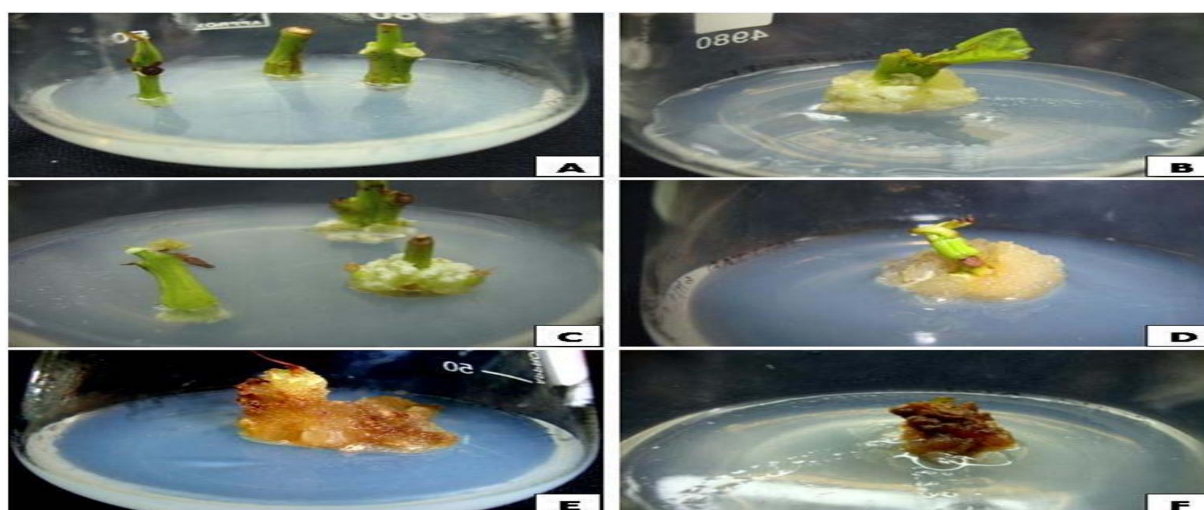


Figure 3: (A) - Freshly inoculated *Holarrhena antidysenterica* nodal explants (B) - Initiation of whitish callus in from *H. antidysenterica* explants (C) - Greenish callus of *H. Antidysenterica* (D) - Light pink coloured callus of *H. Antidysenterica* (E) - Brownish callus of *H. antidysenterica* (F) - Black and compact mass of callus of *H. antidysenterica*

Screening and qualitative comparison of phytochemicals

Both the crude and callus extracts were subjected to preliminary chemical tests¹⁸ to detect the presence and absence of various phytochemicals like alkaloids, flavonoids, steroids, triterpenes, saponins, phenolics, tannins etc (Table 2).

- **Alkaloid:** The ethanolic extracts of leaf extracts of *Holarrhena antidysenterica* responded positively to Mayers, Wagners and Dragendorffs reagent test indicating the presence of alkaloids, while the callus extract did not show precipitation indicating the absence of alkaloids.
- **Steroid:** The ethanolic extracts of leaf extract only have shown positive response to Salkowski and Leibermann Burchards test indicating the presence of sterols.
- **Phenolics:** The ethanol extracts of both the leaf and callus of *Holarrhena antidysenterica* displayed positive response, but the leaf extract showed the strong result in comparison to callus extract.
- **Triterpenes:** The ethanol extracts of both the extracts leaf and callus responded negatively to Salkowski, Leibermann and Tschugajiu tests.
- **Flavonoids:** The ethanolic extracts responded positively to flavonoids *in vivo* and *in vitro* raised callus of *Holarrhena antidysenterica*.
- **Saponins:** Both the ethanol extracts leaf and callus of extracts crude and callus of *Holarrhena antidysenterica* responded negatively to foam tests indicating the absence of saponins.
- **Tanins:** The ethanolic extracts of leaf extract of *Holarrhena antidysenterica* showed strong result while the callus extract did not show any coloration in ferric chloride test which indicated the absence of tannin in callus extract.

Table 2: Qualitative Detection of Ethanolic Extracts of Leaf and Callus of *Holarrhena antidysenterica*

Phytochemicals	Leaf extracts	Callus extracts
Alkaloids	++	-
Antraquinone glycosides	+	+
Gums mucilage	+	-
Proteins	+	+
Amino acids	++	+
Tanins	++	-
Phenolic compound	++	+
Phlobatannins	-	-
Triterpenoids	-	-
Steroids	+	-
Sterols	-	-
Saponins	-	-
Flavones	+++	-
Flavonoids	+++	+
Thiol group	-	-

- = Absent; + = weakly present; ++ = moderately present; +++ = strongly present

DPPH radical-scavenging activity

The reduction capability of DPPH radical is determined by the decrease in its absorbance at 517nm induced by antioxidants. The scavenging effect increased with increasing concentrations of leaf and callus extracts. The percentage inhibitions of Ascorbic acid (taken as standard), leaf and callus extract is shown in Table-3, showing that the leaf extract is able to inhibit the DPPH 85.11% at 280µg/ml and the callus is able to inhibit 58.34% at the concentration of 240µg/ml. (Table 3).

Table 3: (%) of DPPH inhibition in leaf and callus extracts with respect to Ascorbic acid as standard

Concentration (µg/ml)	Ascorbic acid % of inhibition	Leaf extract % of inhibition	Callus extract % of inhibition
40	43.35±0.34	35.36±1.14	13.52±1.22
80	51.52±0.32	46.25±0.05	18.25±2.15
120	58.55±0.11	55.56±0.46	38.56±1.40
160	70.85±0.25	67.12±0.55	48.36±0.63
200	78.41±0.13	74.58±1.21	53.12±2.24
240	86.50±0.56	78.45±1.22	58.34±0.37
280	90.74±0.67	85.11±0.54	57.40±0.30
320	94.20±0.08	83.14±0.32	54.15±0.14
360	97.32±0.75	80.47±0.11	50.24±1.08

OH-scavenging activity

Among the oxygen radicals, hydroxyl radical is one of the most reactive species and induces severe damage to adjacent bio-molecules. Formation of a highly reactive tissue damaging species like hydroxyl radical is caused by the interaction of iron ions with hydrogen peroxide in biological systems. In Hydroxyl scavenging assay the leaf extract is able to inhibit the OH[•] 74.11% at 280µg/ml and the callus is able to inhibit 50.30% at the concentration of 240µg/ml (Table 4).

Table 4: (%) of OH inhibition in leaf and callus extracts with respect to Ascorbic acid as standard

Concentration (µg/ml)	Ascorbic acid % of inhibition	Leaf extract % of inhibition	Callus extract % of inhibition
40	37.25±1.18	31.66±0.56	9.25±1.53
80	49.53±0.15	37.21±0.15	10.40±0.21
120	58.44±0.25	47.32±1.33	32.23±1.35
160	65.41±1.36	53.74±0.41	37.41±0.16
200	72.66±0.12	65.62±0.20	42.11±0.45
240	76.40±0.45	70.75±1.35	50.30±0.33
280	82.11±1.25	74.11±1.34	48.22±0.24
320	87.12±0.12	70.13±0.18	45.20±1.40
360	95.52±1.32	68.44±0.14	43.40±1.35

IC₅₀ Values

Significantly lower IC₅₀ values were observed in comparison to *in vivo* grown plant and *in vitro* raised callus. From the (Fig-4), it is found that IC₅₀ values of the leaf extracts of *Holarrhena antidysenterica* is 110µg/ml and 155µg/ml (DPPH and OH method respectively) where as the IC₅₀ value of callus extract is 196µg/ml and 240µg/ml (DPPH and OH method respectively).



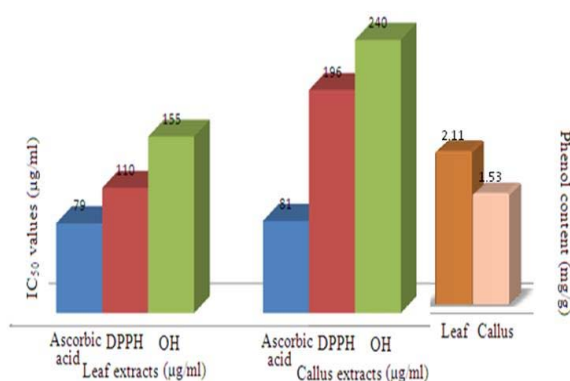


Figure 4: IC₅₀ value and Phenol content of *Holarrhena antidysenterica* in leaf and callus extracts

DISCUSSION

Plants containing phenolic compounds have been reported to possess strong antioxidant properties.¹⁹ Antioxidative activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals.²⁰ Many phenolic compounds particularly flavonoids exhibit a wide range of biological effects including antioxidant activity, antibacterial, antiviral, anti-inflammatory, anti allergic, anti-thrombotic, vasodilatory actions and the ability to lower the risk of coronary heart diseases.²¹

In present study we investigated the qualitative phytochemical screening and antioxidant activity of *Holarrhena antidysenterica* in *in vivo* and *in vitro*. The phytochemical screening of leaf and callus extracts indicates presence of most of the secondary metabolites. The phytochemicals like alkaloid, flavonoids, saponins, steroids and phenolic compounds present in the leaf and callus extracts are responsible for many biological activities.²² The pharmacological exploration of *in vitro* derived secondary metabolites may be helpful for production of natural antioxidants.

The ethanolic extracts of leaf and callus were investigated for antioxidant properties by DPPH and OH scavenging assays. The stable radical DPPH has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials. DPPH is a stable free radical with purple color. The antioxidants scavenge DPPH radical by donating hydrogen atoms leading to a non-radical with yellow color.²³ In Hydroxyl radical assay OH[•] radicals degrade 2-deoxyribose to malodialdehyde. The oxidized products from the reaction from complexes with TBA and show a pink color.^{24,25} The antioxidants of crude extract decreased formation of oxidized product of 2-deoxyribose leading to less formation of thiobarbituric acid reactive substances (TBARS).

In the present investigation higher DPPH scavenging activity (85.11%) at 280µg/ml and (58.34%) at 240µg/ml in both the extracts i.e. leaf and callus in comparison to OH scavenging activity (74.11%) at 280µg/ml and (50.30%) at 240µg/ml. with phenol contents (2.11mg/g)

and (1.53mg/g). The result of the present study doesn't show a significant difference in DPPH and OH scavenging activity. But these differences can be explained by understanding the nature and generation of free radicals as well as studying the differences in physical and chemical properties of the naturally occurring antioxidants.^{26,27} The stable radicals like DPPH reacts stoichiometrically with antioxidants which are good hydrogen donors.²⁶ But antioxidants which are chelators of transition metal ions may contribute differently to the antioxidant response in hydroxyl radical inhibition assay compared to the assays involving stable radicals, as Fe²⁺/Fe³⁺ is the active redox couple in Fenton's reactions. The differences in antioxidant activity using different stable radicals are related to different redox potentials and steric properties of the free radicals antioxidants.^{22,26,27}

Several studies are focused on the relationship between antioxidant activities of phenolic compounds, as hydrogen donating free radical scavengers, and their chemical structures. The antioxidant activity may be due to the presence of phenolic hydroxyl or methoxyl groups, flavones hydroxyl, keto groups, free carboxylic groups and other structural features.²⁸⁻³⁰ The higher antioxidant activity in the leaf extracts in comparison to callus extracts may be due to presence of higher phenol content in the leaves.

The antioxidant capacity is also expressed as 50% inhibitory concentration (IC₅₀). A lower IC₅₀ value means a higher antioxidant capacity of the sample. Significantly lower IC₅₀ values were observed in leaf extracts in comparison to *in vitro* raised callus extracts (Figure 4). The remarkable lower IC₅₀ value may be due to the presence of phenolic compounds.

CONCLUSION

The results of the present study revealed that the leaf extract of *Holarrhena antidysenterica* which contain highest amount of phenolic compounds, exhibited the greatest antioxidant activity in comparison to callus extract. Nevertheless, this work provides basic information for mass production of secondary metabolites as natural source of antioxidants in *Holarrhena antidysenterica*. The callus culture of medicinal plants can be used as a potent source of desired bioactive metabolites without destroying the rare/endangered wild plant resources.

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