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Assessment of Phytochemicals and Antioxidant Activities of Leaf and Leaf Derived Callus Extracts of *Lepianthes umbellata* (L.) Raf.

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ABSTRACT

The aim of the present work is to ascertain and compare the level of phytochemical and antioxidant activity of different solvent extracts from leaf and leaf derived callus of Lepianthes umbellata (L.) Raf. a rare medicinal plant of Western Ghats. The phytochemical analysis indicated the presence of alkaloids, terpenoids, steroids, carbohydrates, tannins, saponins flavonoids etc., DPPH, total phenolic content, total phospho molybdenum content, reducing power assay, antilipid peroxidation assay and nitric oxide scavenging assays were used to assess the antioxidant activity. In all the tested methods methanolic extract of leaf and leaf derived callus possessed the appreciable amount of antioxidant activity. In addition to this hemolytic activity of all the extracts was screened against normal human erythrocytes. All most all the tested extracts possessed minimum hemolytic activity. Results of the present study indicated both leaf and leaf derived callus contain natural antioxidant constituents which could be exploited for possible drug development.

Key words: *Antioxidant, Callus, Lepianthes umbellata, Phytochemical.*

INTRODUCTION

Medicinal plants have been used to treat human diseases from thousands of years because they have a vast and diverse assortment of organic compounds that can produce a definite physiological action on the human body. Most important of such compounds are alkaloids, tannins, flavonoids, terpenoids, saponins and phenolic compounds. Natural antioxidants are often added in foods to prevent the radical chain reactions of oxidation by inhibiting the initiation and propagation step leading to the termination of the reaction and a delay in the oxidation process[12].

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Reactive oxygen species can inactivate enzymes and damage vital cellular components, causing injury. Antioxidants may provide resistance against oxidative stress by scavenging free radicals. Therefore, compounds with antioxidative properties may be useful in the treatment of various disorders[10].

The present research work is mainly focused on the phytochemical and antioxidants present in the plant derived callus extracts of *Lepianthes umbellata* which is belonging to the family Piperaceae. Leaves are important ingredients of their medicines used for curing piles, dysentery diarrhoea, arthritis, jaundice and kidney problems, leucorrhoea, menstrual problems, ear pain, blood clot etc[25]. To the best of our knowledge, there are no reports available on comparative studies of phytochemical and antioxidant activities of leaf and leaf derived callus extracts of this species until now. Thus, this study aims to carryout phytochemical evaluation and possible antioxidant activities of leaf and leaf derived callus.

MATERIAL AND METHODS

Collection of plant material

The fresh and healthy plants of *Lepianthes umbellata* (L.) Raf was collected from Western Ghats of Karnataka, India. The plant was identified with the help of Flora of Presidency of Madras[8] and they were maintained in the medicinal plant garden of Botany department of the University of Mysore for further studies. Leaf explants were used as an experimental material.

Callus induction

Fresh leaves of *L. umbellata* were first washed with running tap water then surface sterilized with 70% ethyl alcohol for 3 min followed by treatment with 0.1% mercuric chloride for 4 min. Further the explants were washed with sterilized distilled water consecutively three times, blot dried and were inoculated on MS medium supplemented with 2,4-D, NAA, BAP, 30g/L sucrose, 9g/L agar at pH 5.8 for the production of callus according to Murashige and Skoog[16]. Then the inoculated cultures were incubated at 21°C±2 for 16h photoperiod. Induced callus was subcultured onto fresh MS medium having growth regulators alone and in combination at various concentrations before being analysed.

Preparation of extract

The collected leaves were washed in running tap water and then shade dried to complete dryness and the induced callus was harvested and dried at 50°C. Both dried leaves and callus were powdered using a mechanical blender. 30g of dried leaf and callus powder were taken in thimbles extracted with hexane, petroleum ether, chloroform, ethyl acetate and methanol successively based on their polarity with the help of Soxhlet extractor. The resulting extracts were subjected to rotary evaporator and the concentrated extracts were stored at 4°C in air tight vials[11].

Phytochemical screening

Phytochemical screening of the leaf and callus extracts of *L. umbellata* revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, sterols, tannins and terpenoids. This has been assessed as described by Trease and Evans[28].

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of both leaf and callus extracts were determined by DPPH method Blois[5]. All the collected extracts were subjected for DPPH free RSA at 50, 100, 200, 400, 800 and 1600µg/ ml. The reaction mixture containing 3 mL of extract and 1 mL of DPPH (300 µM) in methanol was incubated at 37±2º C for 30 min in dark and absorbance was measured at 517 nm using UV-Visible spectrophotometer (Beckman coulter DU 730, Made in Germany). The reaction mixture containing ascorbic acid used as a standard and the DPPH solution alone served as a control. The experiment was carried out in triplicates and percent RSA was calculated using a formula

$$\text{Radical scavenging activity (\%)} = [A_{control} - A_{sample} / A_{control}] \times 100 \quad \text{- Equation (1)}$$

where Asample is the absorbance of the solution when the sample extract has been added at a particular concentration and Acontrol is the absorbance of the DPPH solution. Asample is the absorbance of the extracted sample. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of RSA percentage against extract concentration.

Total phenolic content

Total phenolic content of the extracts of both l eaf and callus was determined spectrophotome trically according to Folin–Ciocalteu colorimetric method[26]. Sample concentration was 50µg/ml and the absorbance was measured at 765 nm using a spectrophotometer. A calibration curve (0.0–250µg/mL) was plotted using Gallic acid and total phenolic content was expressed as Gallic acid equivalents (GAE).

Total antioxidant capacity (phosphomolybdenum method)

Total antioxidant capacity was determined by phosphomolybdenum method[20]. 0.1 mL of sample was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95° C for 90 min and then cooled to room temperature. Absorbance was measured at 695 nm. Antioxidant capacity of each sample was expressed as Gallic acid equivalent.

Reducing power Assay

Reducing power was determined by the method of Oyaizu[18]. The reaction mixture contains extract of different concentrations (20-120µg/ml) was mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of potassium ferri cyanide (1%) and incubated at 50°C for 20min. Then 2.5ml of 10%TCA was added to the reaction mixture Centrifuged at 3000rpm for 10mins. The supernatant (2.5ml) was mixed with 2.5ml double distilled water and 0.5ml of 0.1%ferric chloride and the absorbance was measured at 700nm. The reducing power capacities of the plant and callus extracts were expressed graphically by plotting absorbance against concentration. BHT was used as a standard antioxidant.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was measured spectrophotometrically according to the method described by Garrat[9]. About 1 ml of sodium nitroprusside (5 mM) in phosphate buffer (pH 7.4, 0.1 M) was mixed with different concentrations of all the solvent extracts of leaf and leaf derived callus (200 - 1000 µg/ml). The tubes were then incubated at 25°C for 2 h. After incubation 1.5 ml of Greiss reagent was added and measured spectrophotometrically at 546 nm. A control tube was maintained with all chemicals excluding test extracts. The experiment was carried out in triplicates. % nitric oxide radical scavenging was calculated by using equation[1]. Percentage of inhibition was linearized against the concentration of each extract and standard ascorbic acid.IC_{50} an inhibitory concentration for each extract required to reduce 50% of nitric oxide formation was also determined.

Antilipid peroxidation assay

Inhibition of lipid peroxidation was carried out by TBARS (Thiobarbituric acid reactive species) assay[17] using egg yolk homogenate as lipid rich medium. Egg homogenate (0.5ml of 10% v/v) and 0.1ml of each extract were added to a test tube and made the volume to 1ml with distilled water. Then 0.05ml of $FeSO_4$ (0.07M) was added to induce lipid peroxidation and incubated for 30min. Then 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5ml of 0.8% TBA in 1.1% sodium dodecyl sulphate and 0.5ml of 20% TCA were added and the resulting mixture was vortexed and then heated at 95°C for 60min. Then 5ml of butanol was added to each tube after cooling and centrifuged at 3000rpm for 10min. The absorbance of the supernatent was measured at 532nm. The experiment was carriedout in triplicates. % antilipid peroxidation was calculated by using equation[1].

Hemolytic Assay

Hemolytic activity was carried out by spectrometric method[27]. From a normal healthy individual 5ml of blood was taken and centrifuged at 1500rpm for 3min and the pellet

of blood was washed thrice in sterile phosphate buffer saline solution (pH 7.2). The pellet was re-suspended in 0.5% saline solution. A volume of 0.5 ml of various concentrations of extracts (100, 200,300,400,500µg/mL in saline) were added in 0.5ml cell suspension. After incubation of the mixture at 37°C for 30min, it was centrifuged at 1500rpm for 10min. Hemolytic activity was assessed by measuring the absorbance at 540nm. Distilled water was used as positive control and phosphate buffer was used as negative control.

% Hemolysis was calculated by using the formula = [As-An/Ac-An] X 100

where As is the absorbance of extracts, An is the absorbance of phosphate buffer saline and Ac is the absorbance of sterile distilled water.

Statistical Analysis

All the experiments were carriedout in three replicates and the results are expressed as mean ± standard error. The data was subjected to analysis of variance (ANOVA) using SPSS Inc. 16.0 by Tukey's Honestly Significant Differences (HSD) test with F value ($p \leq 0.05$).

RESULTS AND DISCUSSION

Induction of callus

Callus initiation was observed on the surface as well as at the cut ends of the leaf explants after 10 days of inoculation. The best callus induction response for leaf explants of *L. umbellata* was observed on MS medium supplemented with 2,4-D, NAA and BAP individually with an average of 59-92% explants.MS medium having 0.5mg/L BAP was found to be the best for callus induction and decreases successively in NAA and 2,4-D at the same concentration (Table 1). This results are in agreement with Santos *et al.*[24] who studied *in vitro* propagation and plant regeneration of *Datura stramonium* and found that the presence of 2, 4-D is necessary for callus induction from leaf explants since the treatments lacking 2, 4-D failed to induce callus or produced scanty callus. Increase in concentration from 1mg/L to 2.5mg/L of these growth regulators the response was in reduced degree sequentially. Premjet *et al.*[19] reported the optimum response of callus production in *Barleria prionitis* Linn was found in MS medium supplemented with NAA (1.0mg/L) and BAP (0.5mg/L). Likewise Balbuena *et al.*[4] have shown that at higher concentrations of auxins and cytoninins there is a decrease in inducing and proliferating the callus. Use of other hormones like kinetin, IAA and IBA there is either scanty or no production of callus at various concentrations tried.

Phytochemical Analysis

The results of the phytochemical screening indicated the presence of different types of active phytoconstituents such as alkaloids, carbohydrates, flavonoids, glycosides, protein, resins, phenols, saponins, sterols, tannins and triterpens in both leaf and leaf derived callus extracts (Table: 2). Several recent reports have confirmed that phytochemicals including alkaloids, glycosides, terpenoids, saponins, phenols and steroids have enormous antioxidant and free radical scavenging activities[2,7].

DPPH Radical Scavenging Activity

Based on the results the highest scavenging capacity was observed from *in vitro* induced callus extract and *in vivo* plant extract showed significantly lower scavenging activities when compared to 1mg/ml of ascorbic acid. The results indicated that the extracts were dose dependent as the activity increased with increase in the concentration of extract and they are expressed in percentage (%) of inhibition of DPPH free radicals (Figure:4). Among the tested extracts, methanolic and ethyl acetate extracts of leaf and leaf derived callus exhibited highest inhibition when compared to the standard ascorbic acid while the same was as not observed with the other extracts (Figure:2) Similar results were observed in different parts and callus of *Gynura procumbens* reported by Krishnan *et al*[15]. IC_{50} values are inversely related to the extracts activity.

The highest activity was found in methanol, decreases successively in ethyl acetate, chloroform, petroleum ether and hexane as compared to standard ascorbic acid. .The IC_{50} values of *in vivo* leaf and leaf derived methanolic callus extracts were 3.61 and 3.42µg/ml respectively where as the standard ascorbic acid exhibited IC_{50} of 2.54. In accordance with our results Vinothini *et al.*[30], have also reported the highest activity of free radical scavenging in methanolic extract of callus than compared to leaf extract.

Total phenolic content

The total phenolic content of *in vitro* induced callus extract of *L. umbellata* (L.) Raf was considerably higher than that of *in vivo* leaf extracts. The total phenolic content of all the solvent extracts of both leaf and leaf derived callus are tabulated in Table 3. The methanolic extract of the callus had the highest amount of phenolic compounds, decreases successively in ethyl acetate, chloroform, petroleum ether and hexane extracts. The results of the analytical method were validated by a linear correlation between the concentration and absorbance of gallic acid and an R^2 value of 0.997 was obtained (Figure 3). It is well-known that plant phenolic compounds are highly effective free radical scavengers and antioxidants[1]. Similar results were also observed where a methanolic extract of *in vitro* plant and callus cultures of *Plectranthus barbatus* gave the highest value of total phenol reported by Ibrahim *et al*[13].

Total phosphomolybdenum activity.

In this experiment, Mo (VI) was reduced to Mo (V) by the antioxidant potential of the extracts in a concentration dependent manner. The leaf extracts exhibited comparatively less activity than callus. The antioxidant capacity of the extract and different fractions were in the order of methanol > ethyl acetate > chloroform> petroleum ether> hexane. The total antioxidant values for the methanol and ethyl acetate extracts of callus were 49.5µg/ml and 38.75 µg/ml, respectively at a concentration of 50mg (table 4). The results of the total phosphomolybdenum were validated by a linear correlation between the concentration and absorbance of gallic acid and an R^2 value of 0.994 was obtained (Figure 4). These findings are in accordance with the finding of *Asphodelus tenuifolius by* Eddine *et al.*[6] where the methanolic extracts of leaf callus exhibited the highest total antioxidant capacity.

Reducing power assay

The results showed that there was an increase in reducing the power of the callus extract as the extract concentration increases. The reducing power of the extract was in an order of BHT >methanol> ethyl acetate> chloroform> petroleum ether and hexane (Figure: 5). These findings are directly in line with the previous findings of Umesh *et al*.[29] in *Asystasia gangetica* (L.) methanolic callus extract exhibited highest reducing power assay as compared to other solvent extracts of leaf and callus. Similarly, Ribeiro *et al.*[22] have also reported the highest reducing power assay in methanolic callus extracts of *Hovenia dulcis.*

Nitric oxide scavenging assay

From the results, it was observed that all the extracts of both leaf and leaf derived callus scavenged nitrite radical in a dose dependent manner. Based on the IC_{50} values, the scavenging capacity of leaf extract was significantly lower than that of the callus extract. Among all the tested extracts methanolic extract of leaf derived callus exhibited highest nitric oxide scavenging activity followed by a methanolic extract of the leaf. Percentage of nitric oxide scavenging was plotted against the concentration (Figure 6). The IC_{50} value of ascorbic acid was found to be 1.254µg/mL. This results in ties very well with the previous studies of Sahoo *et.al*.[23] where in the methanolic callus extract of *Alpinia nigra* exhibited the highest nitric oxide scavenging activity.

Antilipid peroxidation assay

The results showed that approximately all the tested samples, dose dependently, inhibited lipid peroxidation. The callus extracs had a comparable inhibition in all the extracts at higher concentrations when measured and compared with the known standard for anti lipid peroxidation. However, methanol and

ethyl acetate extract of callus showed the highest scavenging potential while petroleum ether and hexane extracts of leaf showed the least anti-lipid peroxidation activity (Figure 7). The IC_{50} values obtained for the methanolic extract of callus was found to be 1.76 µg/mL and 1.63 µg/mL for ascorbic acid. Similar finding was also reported by Bajpai *et al*.[3] in *Coleus forskohlii* where methanolic callus extract exhibited highest lipid peroxidation activity as compared to other solvent extracts of leaf and callus.

Hemolytic assay

Results exhibited a differential pattern of hemolytic effect towards human erythrocytes. It indicated that the methanol extract of callus (at 100µg/mL) exhibited minimum hemolytic activity (0.78±0.02%) where as hexane extract of the leaf (at 500µg/mL) possessed the highest hemolytic activity (4.53±0.08%). Lysis of erythrocytes was found to be dose dependent which increased with an increase of extract concentration (Figure 8). These results are in line with the report of *Achyranthes aspera* where it possessed very low hemolytic activity towards human erythrocytes[21]. The reports of *Lantana camara* are also consistent with our results where its various solvent fractions also exhibited low to moderate hemolytic activity towards human erythrocytes[14].

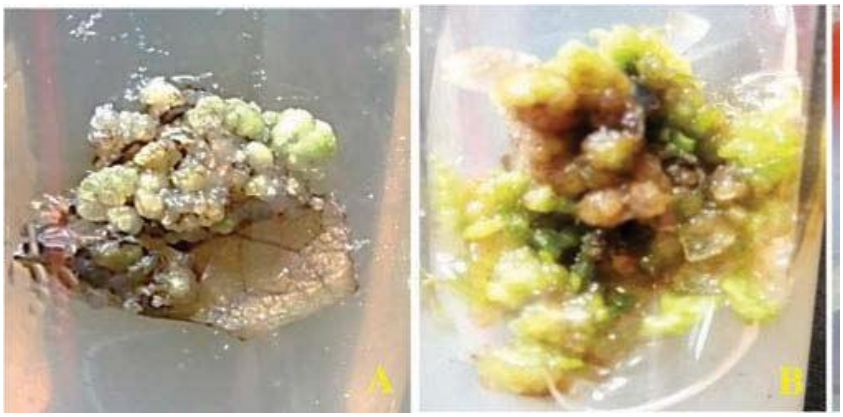

Fig. 1: A. Callus development on abaxial side of leaf explant, B. Development of globular embryoids leaf callus

Table 1: Effect of auxin and cytokinin type on callus induction from leaf explants of *Lepianthes umbellata* (L.) Raf

Plant growth regulator	Concentration mg mL^{-1}	Callus Induction (%)
Control	00.0 ± 0.0	00.0 ± 0.0
2,4-D (Dichlorophenoxyaceticacid)	0.5	72.6 ± 0.56
	1.0	67.8 ± 0.87
	1.5	63.4 ± 0.70
	2.0	61.2 ± 0.53
	2.5	59.1 ± 0.68
NAA (1-Naphthalene acetic acid)	0.5	81.5 ± 0.59
	1.0	76.8 ± 0.77
	1.5	73.5 ± 0.81
	2.0	68.9 ± 0.54
	2.5	65.3 ± 0.63
BAP (6-Benzylaminopurine)	0.5	92.7 ± 0.41
	1.0	87.4 ± 0.62
	1.5	75.3 ± 0.67
	2.0	67.8 ± 0.64
	2.5	64.6 ± 0.56

Note: All treatments had six replicates and were repeated thrice. Each value represents Mean±SE.

Table 2: Preliminary phytochemical analysis of leaf and leaf derived callus extracts of *L. umbellata* (L.) Raf

Phytochemical tests	Leaf					Callus				
	HE	PE	CH	EA	ME	HE	PE	CH	EA	ME
Alkaloids	-	-	+	+	+	+	+	+	+	+
Carbohydrates	+	+	-	+	+	+	+	-	+	+
Flavonoids	-	-	-	-	-	-	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+	+	+
Proteins	+	+	+	+	+	+	+	+	+	+
Resins	-	-	-	-	-	-	-	-	-	-
Phenols	-	-	-	+	+	-	-	-	+	+
Saponins	-	+	-	+	+	-	+	-	+	+
Sterols	-	-	+	+	+	+	-	+	+	+
Tannins	+	+	-	-	+	-	+	-	+	+
Triterpens	-	-	-	-	+	+	-	-	-	+

Note: '+' Present and '–' Absent, HE-Hexane, PE-Petroleum Ether, CH-Chloroform, ME-Methanol, EA-Ethyl Acetate

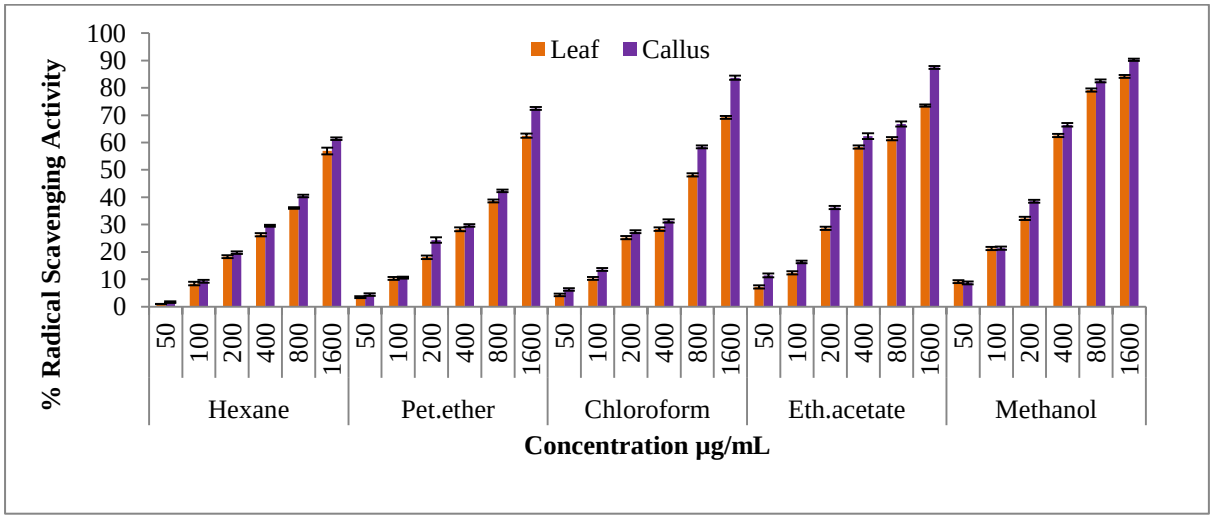

Fig. 2: DPPH radical scavenging activity of leaf and leaf derived callus extracts of *L.umbellata* (L.) Raf

Values are mean of three independent replicates ± standard error

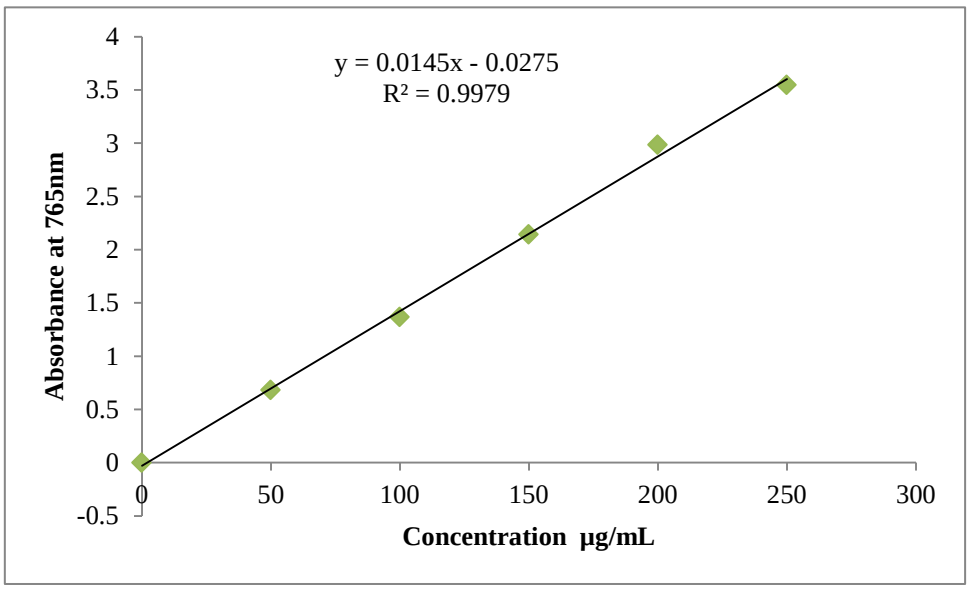

Fig. 3: Total phenolic content (Gallic acid standard curve)

Table 3: Determination of total phenolic content of leaf and leaf derived callus extracts of *L. umbellata* (L.) Raf

	Hexane	Pet.ether	Chloroform	Eth. acetate	Methanol
	% Total Phenol				
Leaf	10.5	13.14	20.07	28.5	32.57
Callus	12.71	17.42	24.92	34.8	39.28

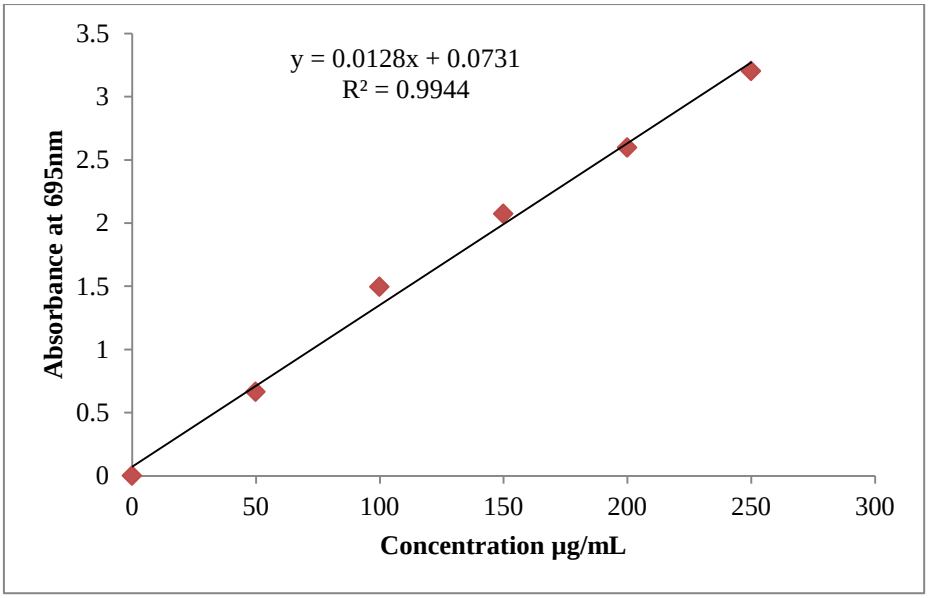

Fig. 4: Total phosphomolybdenum content (Gallic acid standard curve)

Table 4: Determination of total phosphomolybdenum content of leaf and leaf derived callus extracts of *L. umbellata* (L.) Raf

	Hexane	Pet.ether	Chloroform	Eth. acetate	Methanol
	% Total antioxidant				
Leaf	18.5	21.33	27.83	37.0	41.83
Callus	19.16	23.5	31.5	38.75	49.5

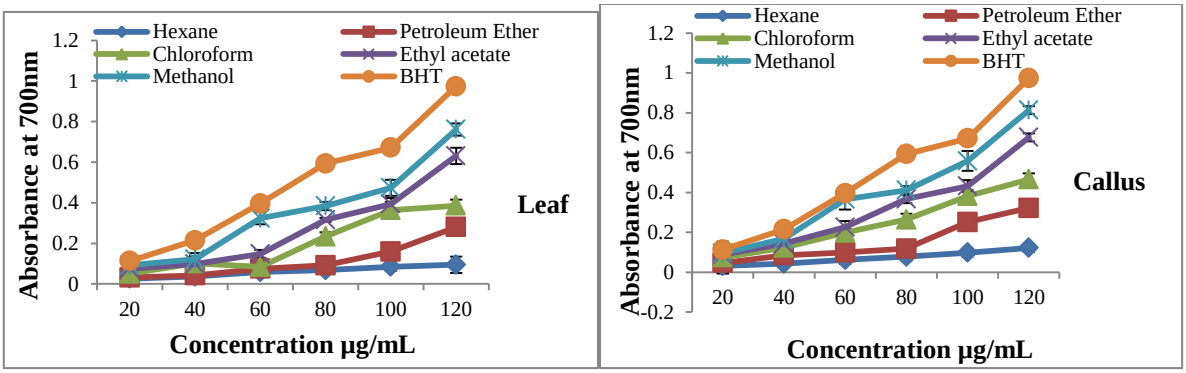

Fig. 5: Reducing power assay of both leaf and leaf derived callus extracts of *L.umbellata* (L.)Raf.

Values are mean of three independent replicates ± standard error

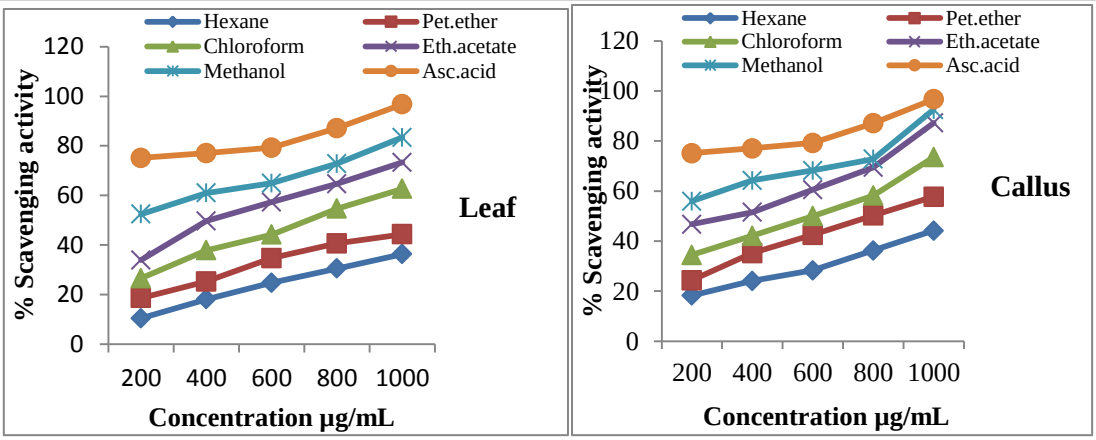

Fig. 6: Nitric oxide scavenging assay of both leaf and leaf derived callus extracts of *L.umbellata* (L.)Raf.

Values are mean of three independent replicates ± standard error

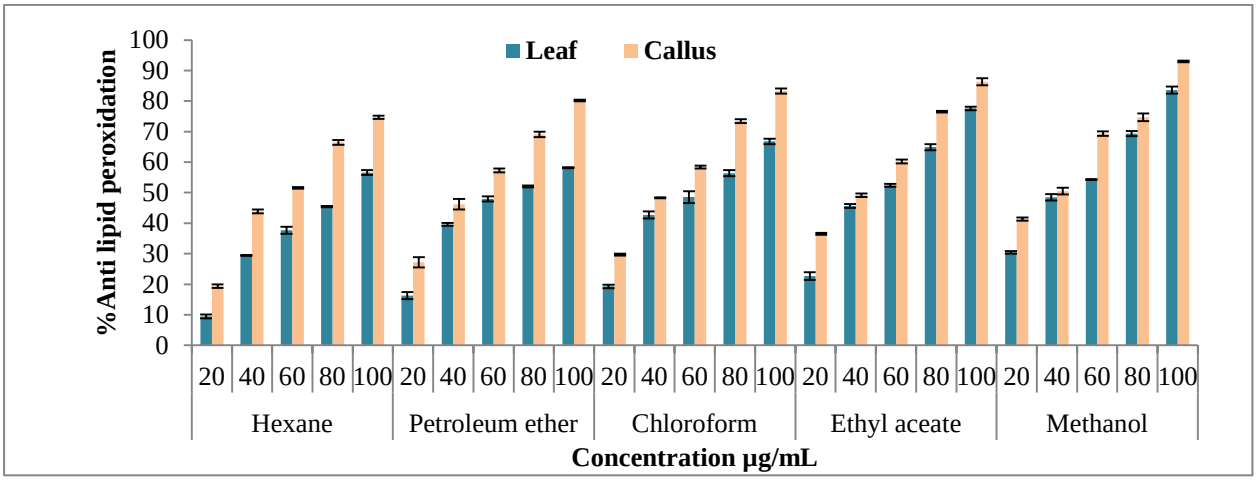

Fig. 7: Anti lipid peroxidation assay of both leaf and leaf derived callus extracts of *L.umbellata* (L.)Raf.

Values are mean of three independent replicates ± standard error

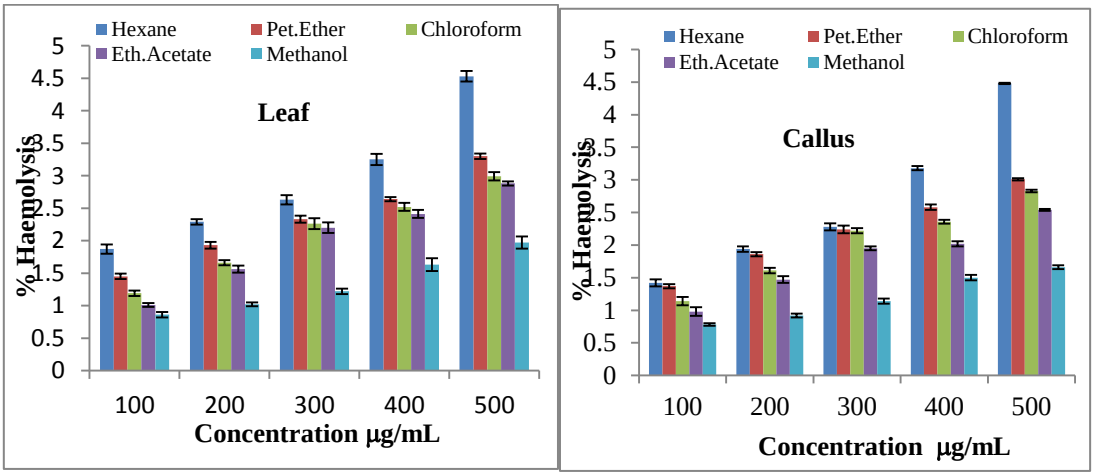

Fig. 8: Hemolytic activity of both leaf and leaf derived callus extracts of *L.umbellata* (L.)Raf.

Values are mean of three independent replicates ± standard error

CONCLUSION

Medicinal plants are the potent source of human health due to the presence of phytochemical constituents that are responsible for various pharmacological activities. On the basis of the results obtained the present work concluded that leaf derived callus of *L. umbellata* (L.) Raf. are rich in phytochemical constituents. The results obtained also represents a worthwhile expressive contribution of antioxidant assays which varied depending upon the type of solvents being used. With these results methanolic extract considered to be the major contributor for the antioxidant activities. Hence it can be concluded that it could be used to invent more potent drugs of natural origin. Further studies are required for identification and to understand the mechanism of action of this plant and callus extracts.

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