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Research Article

DEVELOPMENT OF SINGLE NODE CUTTING PROPAGATION TECHNIQUES AND EVALUATION OF ANTIOXIDANT ACTIVITY OF *CURCUMA AERUGINOSA* ROXBURGH RHIZOME

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ABSTRACT

Objective: The aim of the present study is to evaluate antioxidant activity and estimation of different phytochemicals of rhizome at different stages of maturity (outer and inner cortex) of *Curcuma aeruginosa* Roxburgh as well as increasing the productivity of rhizome through single node cutting propagation technique.

Methods: The extracts were screened for their possible *in vitro* antioxidant potentials by 2,2-diphenyl-1-picrylhydrazyl, reducing power and nitric oxide scavenging assays as well as quantitative phytochemicals were estimated by total phenol, flavonol and curcumine content. Single node cutting method was used for increasing the propagation techniques.

Results: Mature part mainly inner cortex of the rhizome showed excellent performance of antioxidant activity which was increasing towards maturity. Total phenol, flavonoid and curcumin contents were obtained higher in the mature part of rhizome. Free radical scavenging activities were highly correlated with total phenol content. An outstanding result was observed in case of single node cutting propagation technique.

Conclusion: The findings of this study support the view that rhizome of *C. aeruginosa* is promising source of potential antioxidants and may be efficient as preventive agents in many diseases. Single node cutting propagation technique may be the best method for *ex situ* conservation of this critically endangered plant.

Keywords: Propagation, Antioxidant, *Curcuma aeruginosa,* Maturation, Rhizome

INTRODUCTION

Zingiberaceae is a family of advanced monocot plants with numerous ethno-medicinal importance. *Curcuma aeruginosa* Roxburgh (Syn. *C. caesia* Roxburgh) is a rhizomatous herbaceous plant under this family and is commonly known as 'kali haldi'. Fresh rhizomes are aromatic and deep-blue or bluish-black colored cortex with pungent odor. In India it grows in West Bengal, Madhya Pradesh, Orissa, Bihar and Uttar Pradesh and is used by the tribal people to cure their various ailments [1]. Rhizomes of the plant are applied for sprains and bruises and are also employed as cosmetics [2]. In West Bengal it occupies an important place in traditional system of medicine, but according to IUCN the plant is now considered as critically endangered [3].

The rhizome of *C. aeruginosa* is used in treating leucoderma, asthma, tumor, piles, bronchitis etc as well as the paste is applied on contusions and rheumatic pains. Essential oils of *C. aeruginosa* have been known for its antifungal activity [4]. The rhizome is also used in dysentery, diarrhoea and cough. Medicinal uses of the rhizome arise from the bioactive components. Bioactive components like curcuminoids are responsible for anti-inflammatory properties, wound healing, hypoglycemia, anticoagulant and antimicrobial activities [5,6]. Curcuminoids also exhibit free radical scavenging property [7] and antioxidant activity [8].

Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress imposed during depletion of antioxidant enzymes in immune system, change the gene expression pattern and induce abnormal proteins. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Due to depletion of immune system, natural antioxidants may face several difficulties and under this crisis, consuming antioxidants for scavenging free radicals may be necessary [9]. Currently available synthetic antioxidants have been suspected to cause or prompt negative health effects. Recently there has been a growing interest for investigating the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Many plant species have been investigated in search of novel antioxidants [10-13]. Phytochemicals such as essential oils, polyphenols, flavonoids and anthocyanins have been of great interest as source of natural antioxidants, since they are safer and environment friendly than their synthetic counterparts. Numerous natural antioxidants have already been isolated from different varieties of plant material such as leafy vegetables, fruits, seeds, cereals and algae [14]. The medicinal properties of plants belonging to Zingiberaceae family are supposed to be due to the presence of certain bioactive compounds having antioxidant properties. Previous report confirms that DPPH scavenging property of *C. aeruginosa* rhizome extract were significantly correlated with the total phenol content [15]. Due to commercial requirements, the rhizomes are normally harvested in a young (immature) to mature stages randomly, although rhizomes could not achieve their highest biomass at young stage. If the mature rhizomes are proved to be a rich source of antioxidant agent as compared to immature ones, their higher yield in terms of biomass could be advantageous for both farmers and consumers. Based on this concept, the aim of the present work was to determine antioxidant activity and estimation of total phenol, flavonol and curcumin content of rhizome at different stages of maturity of *C. aeruginosa* as well as increasing the productivity of rhizome through single node cutting *ex situ* propagation technique for conservation and management of this endangered plant.

MATERIALS AND METHODS

Plant materials

Different parts of rhizome of *C. aeruginosa* Roxb. like outer and inner cortex of different maturity stages were collected from medicinal plant garden of the North Bengal University and surgically separated in the laboratory. The plant material was authenticated from Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material was deposited in the 'NBU Herbarium' and recorded against the accession no 9584 dated 12.03.2010.

Chemicals

Solvents for extraction like methanol, ethanol and chemicals like 2.2-diphenyl-1-picryl hydrazyl (DPPH), potassium ferricyanide

$[K_3Fe(CN)_6]$, trichloroacetic acid (TCA), glacial acetic acid, naphthylethylenediamine dichloride, sulfanilic acid, Folin-Ciocalteu reagent, gallic acid, aluminum chloride, Sodium hydroxide (NaOH), ferric chloride ($FeCl_3$), sodium nitroprusside and sodium carbonate (Na_2CO_3) were either purchased from Himedia or from Merck [India & Germany]. All the reagents used were of analytical grade.

Extraction and determination of methanol extractive values

Fresh parts of *C. aeruginosa* were extracted by standard solvent extraction method [16,17]. Every parts of rhizome were crushed with mortar and pestle and separately extracted with methanol: water :: 4:1, under soxhlet extractor for twenty four hours. The solvents were completely removed by vacuum rotary evaporator at 50°C. These crude extracts were used for further investigation. The extractive value of the plant materials were calculated on dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract} \times 100}{\text{Weight taken for extraction}}$$

Determination of antioxidant activity

DPPH radical scavenging assay

The free radical scavenging capacity of different parts of *C. aeruginosa* was determined by using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) following standard method [18]. 0.1 mM solution of DPPH in methanol was prepared. 1.8 ml of this solution was mixed with 0.2 ml of test solutions at different concentrations [25 mg/ml – 100 mg/ml fresh weight tissues (FWT)] in different test tubes, incubated for thirty minutes at room temperature and after incubation absorbance was measured at 517 nm. Methanol was used as a blank. The percentage of free radical scavenging activity was calculated as follows:

$$\text{Percent inhibition of DPPH radical} = \frac{\text{Abs. of control} - \text{Abs. of sample} \times 100}{\text{Abs. of control}}$$

Reducing Antioxidant Activity

The reducing power of the extract was evaluated according to the method of Oyaizu [19]. The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1%w/v) was added to 1.0 ml of the extract dissolved in distilled water. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

Nitric Oxide scavenging activity

The method of Garratt was adopted to determine the nitric oxide radical scavenging activity of methanolic extract of rhizome of *C. aeruginosa* [20]. Two milliliter of 10 mM sodium nitroprusside, dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (10-100 mg/ml FWT). The mixture was incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [1.0 ml of sulfanilic acid (0.33% in methanol, w/v) in 20% glacial acetic acid incubated at room temperature for 5 min and mixed with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm.

Total phenol estimation

The concentration of total phenolic compounds in the methanol extract of different parts of *C. aeruginosa* were determined spectrophotometrically using the Folin-Ciocalteu reagent which is a combination of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic antioxidants **[**21]. Standard curve was done using different concentrations of gallic acid in methanol. 0.5 ml of 50 % Folin-Ciocalteu reagent was added with (1ml) different extracts of immature to mature parts (inner and outer cortex) of *C. aeruginosa* rhizome, left for 8 min, and then 4 ml of 5% sodium carbonate was added, mixed and allowed to stand for 60 min. protected from light. Their absorbance was measured at 765 nm using methanol as blank and the concentration of the total phenolic content of the extract was calculated. Total phenolic content of methanolic extracts of *C. aeruginosa* in gallic acid equivalents (GAE) was measured by the formula:

$$C = c.V/m$$

Where, C - total content of phenolic compounds, mg/g of extract, in GAE; c - the concentration of gallic acid deduced from the calibration curve (mg/ml); V - the volume of extracts (ml); m - the dry weight of the extract.

Total flavonoids determination

Aluminum chloride colorimetric method was used for flavonoids determination [22]. 0.5 ml of sample (400 mg/ml FWT) was mixed with 4 ml of double distilled water. Then the diluted extracts of rhizome were mixed with 5% (0.3 ml) $NaNO_2$. 10% aluminum chloride was then added with reaction mixture. After 6 minute 2 ml (1.0 M) NaOH and 2.4 ml double distilled water was added and mixed well. The absorbance of the reaction mixture was measured at 510 nm with spectrophotometer. The flavonoid content was determined from slope and intercept of calibration curve which was made by preparing quercetin (0-500 mg L^{-1}) in methanol.

Curcumin estimation

Curcumin content from different parts of *C. aeruginosa* was determined through standard method with few modifications [23]. A 100 mg of dried rhizomes were taken in an extraction flask and 20 ml of 95% ethanol was added and refluxed for 3 hour. The refluxed residue was cooled and taken on a filter paper, washed with 80 ml of 95% alcohol. The volume of the filtrate was made up to 100 ml with alcohol and the intensity of the yellow colour in solution was measured at 425 nm in spectrophotometer. Curcumin concentration of the sample was estimated by preparing standard calibration curve of pure curcumin, which showed a linear relationship between A_{425} and curcumin concentration.

Single node cutting method

For single node cutting of *C. aeruginosa*, rhizome propagation technique was followed by the method of Marulanda *et al.* [24] with some modifications. Single node longitudinal cutting, transverse cutting and tip sections of rhizome of *C. aeruginosa* were taken for propagation. Percentages of germination were calculated after one month. Lengths of rhizome were measured after three and six month.

Statistical analysis

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between phytochemicals and antioxidant activity and MS Excel of Microsoft Office, 2007 was used for comparing the antioxidant attributes of different parts of immature to mature rhizome. The data were analyzed by one way ANOVA and different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software ver. 1.022; $p<0.05$ was considered significant in all cases. The software package Statistica was used for analysis of other data. Smith's Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE).

RESULTS AND DISCUSSION

The use of an alcoholic solution provides satisfactory results for extraction of different bioactive phytochemicals [25]. Our result indicates that the methanol extractive (% of yield) of immature tubers of *C. aeruginosa* was highest (7.31%) among all tissues tested but in case of rhizome, methanol extractive was greater (4.50%) at mature stages of inner cortex, when compared with immature and outer ones (Figure 1).

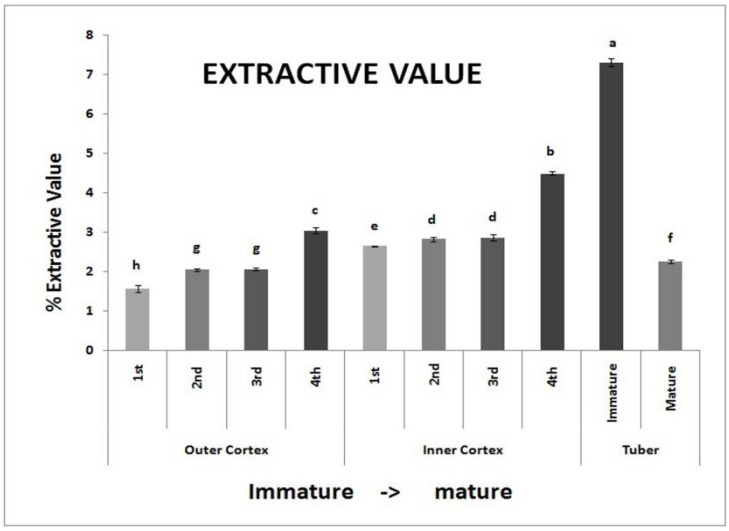

Fig. 1: Methanol extractive values of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SD (n=3). Bars with different letters (a, b, c, d, e, f & g) are significantly ($p<0.05$) different from each other by Duncan's Multiple Range Test (DMRT).

The free radical scavenging capacity of these isolated extracts was primarily tested through DPPH assay because the method is rapid, accurate and reproducible. DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule [26]. The reducing purple colour of DPPH to pale yellow hydrazine occurs due to transfer of electron or hydrogen atom to DPPH from the substances which are identified as an antioxidant and free radical scavenger [27]. The degree of discoloration indicates the scavenging activity of the drug [28]. Results designated that the antioxidant potential of different maturation stages of *C. aeruginosa* rhizome were increased towards maturity, when tested with DPPH. It was also observed that DPPH scavenging activity was higher in inner cortex rather than outer one. Figure 2 showed that 4th stage of mature inner cortical part has highest DPPH scavenging activity (IC_{50} 4.72 mg/ml) when compared with all other parts of immature and mature rhizome extracts as well as extracts of root tubers.

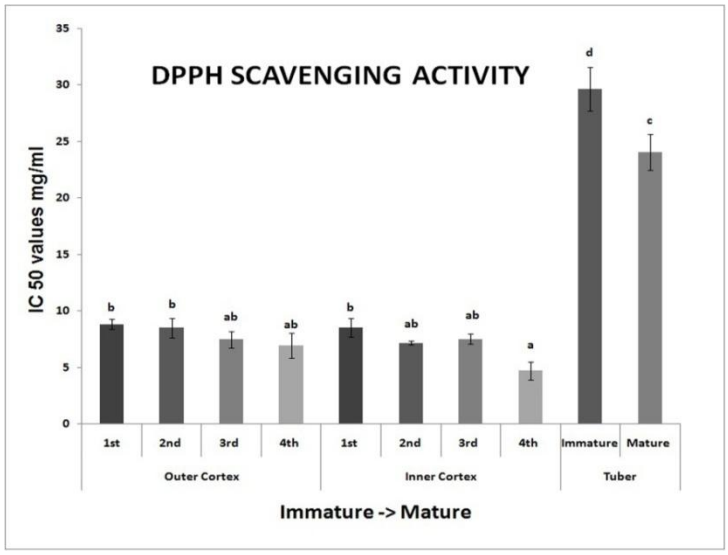

Fig. 2: DPPH radical scavenging (IC_{50}) activity of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b, c & d) are significantly ($p<0.05$) different from each other by Duncan's Multiple Range Test (DMRT).

Figure 3 explained the reducing power of the methanolic extracts of different parts of *C. aeruginosa* rhizome. The reducing capacity of the extracts, another significant indicator of antioxidant ability was also found to be appreciably high. It was observed that there is a great similarity in the result of DPPH scavenging activity with reducing power capability in this plant rhizome. The potent reducing power was observed in the mature inner part of cortex with discrete atactostele (1.687mg/ml half saturation conc.). In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Blue at 700 nm. Enhancement of absorbance with extracts indicates an increase in reductive capacity. The principle of reducing power assay was based on conversion of complex ferric-tripyridyltriazine Fe(III) to a dark blue colored ferrous Fe(II) ion in the presence of antioxidant [29]. The reducing power is expressed when an antioxidant acts as electron donor that reduces the intermediate oxidized substances produced from lipid peroxidation. Therefore, the plant component with optimal reducing power performs as primary and secondary antioxidant [30].

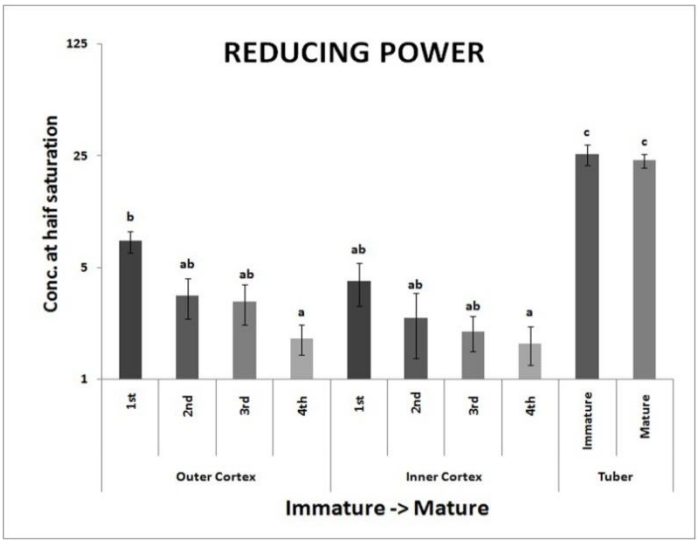

Fig. 3: Reducing power (Conc. at half saturation) of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b & c) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT).

Nitric oxide (NO) is a free radical produced in mammalian cells and is involved in the regulation of various physiological processes, produced by phagocytes and endothelial cells, to yield more reactive species such as peroxy-nitrite which can be decomposed to form OH radical. However, excess production of NO is a source of several diseases [31,32]. In the present study the nitrile produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25º C was reduced by the methanolic extracts of rhizome of *C. aeruginosa* (Figure 3). This may be due to the antioxidant principles in the extracts which compete with oxygen to react with nitric oxide [33], thereby inhibiting the generation of nitrile. In this study, the level of nitric oxide generation was significantly reduced by the crude extracts of *C. aeruginosa* and the scavenging potency of NO enhanced towards maturity (Figure 4). Since NO plays a crucial role in the pathogenesis of inflammation [34], this may explain the use of rhizome of *C. aeruginosa* for the treatment of inflammation and wound healing.

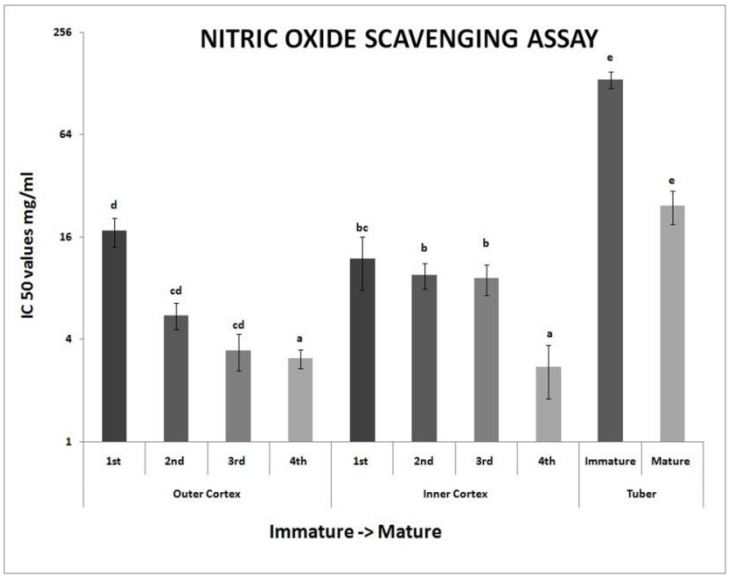

Fig. 4: Nitric oxide scavenging (IC_{50}) activity of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b, c, d & e) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT).

The low IC_{50} value of free radical scavenging activity of mature inner cortex may be due to presence of high polyphenolics and flavonoids. Table 1 demonstrates that DPPH free radicals, NO scavenging as well as reducing power were highly correlated with total phenol content. The table also shows that total flavonoid contents were responsible for DPPH scavenging activity and reducing power. Among the various phenolic compounds, the flavonoids are perhaps the most important group [35]. As the flavonoids have more than one hydroxyl groups, they basically act through scavenging or chelating processes [36,37]. Figure 5 shows that the flavonoid content in the extracts of inner cortical part (0.16 -0.37mg/g FWT) were higher than that in the extracts of outer cortical part of *C. aeruginosa* rhizome (0.05-0.36 mg/g FWT). Phenolic compounds are a class of antioxidant agents which act as free radical terminators [38].

Table 1: Correlation Matrix of antioxidant activity and phytochemicals

	DPPH	NO	RP	EV	TPC	TFC
NO	0.849(**)					
RP	0.986(**)	0.791(**)				
EV	0.567	0.829(**)	0.495			
TPC	-0.863(**)	-0.683(*)	-0.879(**)	-0.205		
TFC	-0.682(*)	-0.561	-0.721(*)	-0.057	0.916(**)	
CUR	-0.601	-0.492	-0.573	0.043	0.797(**)	0.808(**)

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Where, DPPH= 2,2-diphenyl-1-picryl hydrazyl; NO= Nitric oxide; RP= reducing Power; EV= Extractive value; TPC= Total Phenol Content; TFC= Total Flavonol Content; CUR= Curcumin Content.

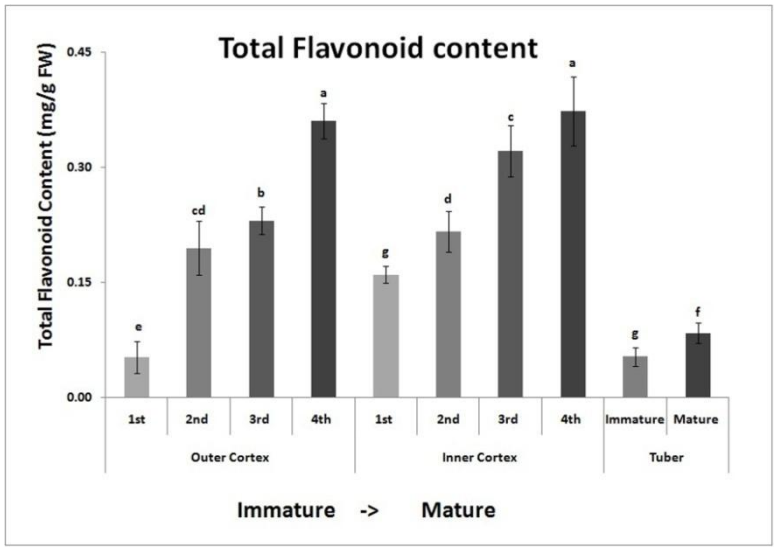

Fig. 5: Flavonol content (mg/g) of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b, c, d, e, f & g) are significantly ($p<0.05$) different from each other by Duncan's Multiple Range Test (DMRT).

In case of inner cortical part of rhizome, the estimated total phenol varied from 2.53 to 3.4 mg/g FWT, which was higher than outer cortex (1.84- 3.25 mg/g FWT) and accumulated basipetally towards mature tissues (Figure 6). From our study it may be concluded that high contents of these phenolic compounds in *C. aeruginosa* rhizome can explain its high radical scavenging activity which increases with the number of hydroxyl groups [39]. Rhizome of *C. aeruginosa* contains high coloring agents i.e. curcumin which acts as antioxidant as well as cytotoxic and tumour reducing properties [40,41]. Figure 7 shows that mature parts of inner cortex contain higher amount (0.5-1.71 mg/100g FWT) of curcumin.

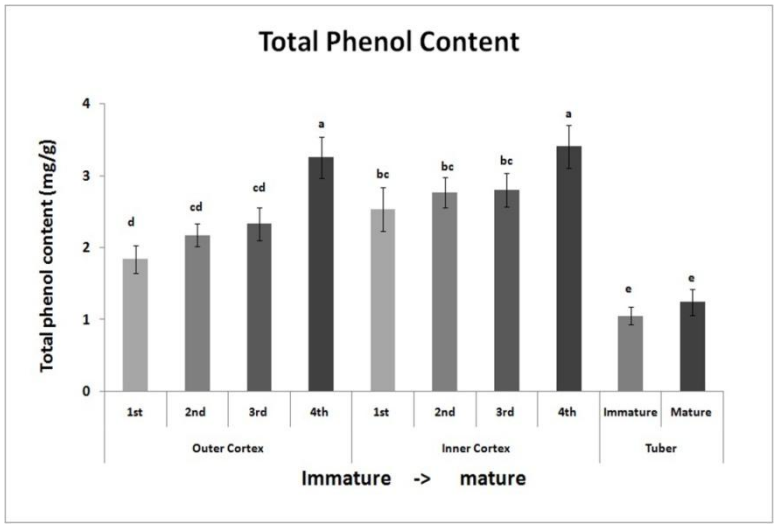

Fig. 6: Phenol content (mg/g) of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b, c, d & e) are significantly ($p<0.05$) different from each other by Duncan's Multiple Range Test (DMRT).

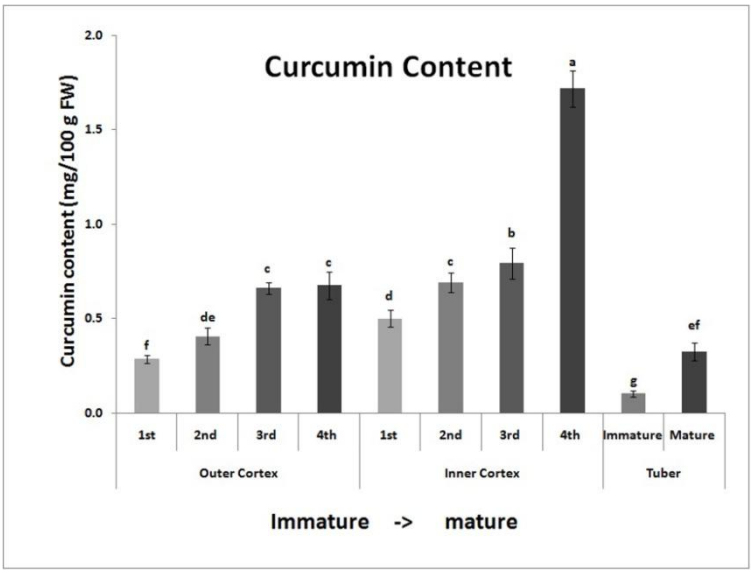

Fig. 7: Curcumine content (mg/100g) of different maturation stages of *C. aeruginosa* rhizome.

Values are expressed as Means ± SEE (n=3). Bars with different letters (a, b, c, d, e, f & g) are significantly ($p<0.05$) different from each other by Duncan's Multiple Range Test (DMRT).

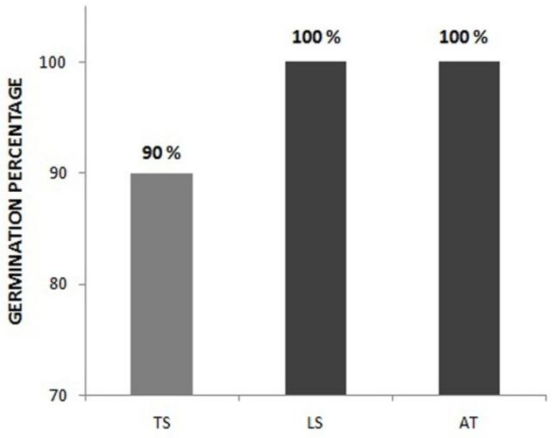

Fig. 8: Percentage of germination from single node cutting of rhizome by TS, LS and AP.

Where, TS: Transverse section; LS: Longitudinal section; AT: Apical tips.

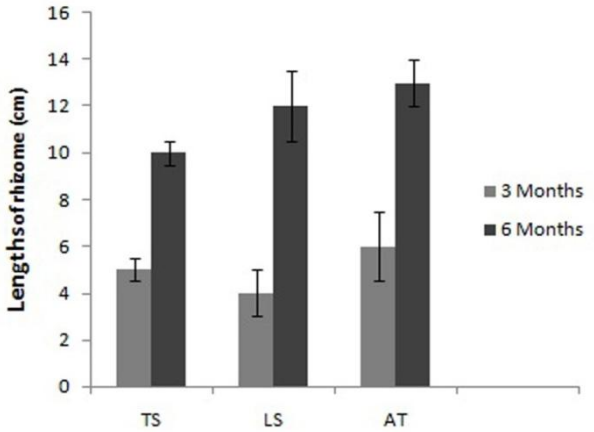

Fig. 9: Rhizome yield (length) from single node cutting of rhizome by TS, LS and AP.

Where, TS: Transverse section; LS: Longitudinal section; AT: Apical tips

One rapid multiplication technique used to produce many plants is single node cutting [42]. Dormant plant part i.e. rhizome was taken and a small piece of the tuber was surgically isolated, where an apical bud was also present [43]. *C. aeruginosa* rhizome can be successfully propagated through single nodal cutting and apical tip culture. 100% germination was observed from longitudinal single nodal cutting as well as apical tip culture and 90% germination was found from transverse single node cutting (Figure 8).

The productivity in terms of length of rhizome was significantly increased through apical tip based propagation. Although after 6 months from transverse cutting, longitudinal cutting, and tip sections respectively 8-10 cm, 9-12 cm and 9-14 cm long rhizome with many fingers was originated (Figure 9).

CONCLUSION

From our results it may be concluded that single node cutting from apical tips are very useful for successful propagation of *C. aeruginosa,* which is critically endangered with high ethno medicinal values. Presence of significant quantity of antioxidant as reflected from our studies may prove the pharmacological importance of this plant. These antioxidant activities enhanced gradually towards mature tissues in basipetal direction. Significant correlations between antioxidant and phenol/flavonol content further indicate that the antioxidant activity is due to polyphenolic component present in this plant.

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