Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Issue 2, 2013 ISSN - 0974-2441

Research Article

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF *IN VITRO* GROWN PLANTS *CLITORIA TERNATEA* L., USING DPPH ASSAY

KUMARI MADHU

Department of Botany, B.R.A.Bihar University, Muzaffarpur,Email: madhu_kumari58@yahoo.com

Received: 6 December 2012, Revised and Accepted: 26 January 2013

ABSTRACT

The present study was aimed to establish a protocol for multiple shoot induction via the culture of nodes of *Clitoria ternatea* L., through callogenesis and organogenesis. Explants were cultured on Murashige and Skoog's medium supplemented with different concentrations and combinations of BAP, 24-D and NAA for shoot and root induction. The sub-cultured of callus on MS basal medium supplemented with BAP (2.5 mg/l) and NAA (0.5mg/l) showed highest rate of shoot multiplication. *In vitro* shoots were rooted on to the MS basal medium supplemented with NAA (0.5mg/l). The preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, steroids and phenol in *in vitro* grown products of *Clitoria ternatea* L. DPPH free radical scavenging assay was studied for evaluation of antioxidant potential. The ethanolic extract of *in vitro* grown *Clitoria ternatea* significantly inhibited the DPPH free radicals at the concentration ranging from 25-600µg mL^{-1}. It showed highest inhibition 67% at 600µg $mL,^{-1}$ equal to *in vivo* grown plant 70% at 600µg $mL,^{-1}$. The *in vitro* multiplication ensures germplasm conservation of rare, endangered, aromatic medicinal plant while intrinsic natural antioxidants level could be harnessed for cure of diseases.

Keywords: *Clitoria ternatea* L, *in vitro* multiplication, ethanolic extracts, antioxidant activity, growth regulator.

INTRODUCTION

Plants are a potential source of natural antioxidands. Natural antioxidants are secondary metabolites of plants such as phenolic acids, carotenoids, flavanoids, tannins, tocopherols, flavones glycosides etc [1]. Easily cultivated, *Clitoria ternatia* with its wide range of antioxidants can be a major source of natural antioxidants or phytochemicals. Free radicals or reactive oxygen species (ROS) are formed in our body as a result of biological oxidation and metabolism. Free radicals are molecules that have lost an electron and try to replace it by reacting with other molecules. This causes the substances to break down. Most of the reactive free radicals are scavenged by endogenous defense systems such as catalase, superoxide dismutase and peroxidase-glutathione system [2]. The over production of free radicals such as hydroxyl radical, super oxide anion radical, hydrogen peroxide can causes damage to the body and contribute to oxidative stress [3,4]. Oxidative damage of proteins, DNA and lipid is associated with chronic degenerative diseases including cancer, coronary artery disease, hypertension, diabetes etc[5]. Antioxidants are substance that significantly prevents or delays the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [6]. Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage and prevent aging and other degenerative diseases. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various human ailments because they contain the components of therapeutic value [7]. The natural antioxidants are not only helping in health promotion but they are safer and environment friendly than synthetic antoxidants. Plants are a potential source of antioxidant compounds like phenolic acids, polyphenols and flavanoids that scavenge free radicals such as peroxide, hydrogen peroxide of lipid hydroxyl and thus inhibit the oxidative damage that lead to the risk of various degenerative diseases associated with oxidative stress [8].

Clitoria ternatea L. is commonly known as "butterfly pea" found all over India, belong to family Caesalpiniaceae. The plant extracts has anthelmintic [9] antipyretic, anti-inflammatory, analgesic [10], anxiolytic, antidepressant, anticonvulsant, sedative , hypoglycemic, anticancer, properties [11,12]. This species has been listed as a rare plant species by the International Union for Conservation of Nature and Natural Resources [IUCNNR] [13]. It is also used to treat neurological disorders and is considered to be wholesome for the intellect. Polyacyl glycosides of delphinidin-type and eight anthocyanin ternatin pigments [14] have been isolated from the plant. The role of medicinal plants in disease prevention or control has been attributed to antioxidant properties of their constituents [15]. The main objective of our study is to establish an efficient, quick and reliable protocol for *in vitro* regeneration of C*. ternatea*. To fulfill the increasing demand of this potent medicinal plant, *in vitro* culture is an alternative method for conservation of this diminishing plant population. Some studies have reported *in vitro* plant regeneration and micropropagation of *C. ternatea* [16,17,18]. The present work describe the procedure for proliferation, of *in vitro* grown plants as well as its preliminary phytochemicals screening and antioxidant property potential of the ethanolic extracts of *Clitoria ternatea* L by determining DPPH method.

MATERIALS AND METHODS

Source of explants

In order to obtain *in vitro* plants, a protocol was developed for rapid clonal propagation of *Clitoria ternatea* L through mature nodal explants. The nodal segments were collected from the campus of the department of Botany, B R A Bihar University, Muzaffarpur. These nodal segments were cuts into 1.5cm to 2.0cm length with single node intact. They were washed with 5% (v/v) detergent solution (tepol) for 10 minutes followed rinsing using running tap water for several times. Nodes were further treated with 70% alcohol for one minute followed by 0.1% (w/v) mercuric chloride treatment for 5 minutes, aseptically. It was washed with sterile distilled water for 4 to 5 times and dried using sterile blotting paper which was ultimately used as explants for raising *in vitro* cultures.

Growth medium & culture condition

Murashige and Skoog's (MS, 1962)[19] medium was used as nutrient source with or without growth hormones. The basal constituents supplemented with different concentrations of growth regulators, such as BAP (6-benzylamino purine) , 2-4D (2,4-dichlorophenoxy acetic acid) and NAA (α-napthelene acetic acid) either individually or in combination for culture explants. Sucrose 3% and agar 0.8% (Hi media) were used as carbon source and gelling agent respectively. The pH of the medium was adjusted to 5.8 prior to autoclaving at 15lbs for 20 minutes. The pH of the medium was adjusted by adding 1*N* HCl and NaOH. The cultures were maintained in the culture room at 25°±C 2ºC, under white fluorescent light. All

the experiments were repeated twice and sub-culturing was carried out regularly at 4 weeks interval.

Shoot organogenesis and elongation

For multiple shoot induction from nodal explants were culture on MS medium supplemented with BAP (0.5, 1.0, 1.5, 2.0 & 2.5 mg/l) and 2-4D (0.5, 1.0, 1.5, 2.0 & 2.5 mg/l. Data on percentage of responding explants and number of shoots per explants were recorded after 25 days of initiation culture.

In vitro rooting plant establishment

For multiple roots induction the regenerated shoots (2-3cm) were transferred to MS medium supplemented with NAA (0.5 mg/l to 1mg/l) along with BAP 0.5mg/l. The maximum rooting was obtained on MS with NAA (0.5mg/l) with BAP 0.5mg/l. The well rooting plant lets were transferred to plastic cups containing sterilized soil. The regenerated plantlets were hardened by covering them with a thin perforated transparent polythene bags to maintain humidity. Plantlets were watered with $1/10^{th}$ strength MS salts solution and maintained in the culture condition. Plants started producing fresh shots and roots after one week of transplanting. After two weeks they were transported to the garden while survival rate was (70%) was observed.

Phytochemical screening

The ethanolic extract of *in vitro* and *in vivo* grown plants of *Clitoria ternatea* L was subjected to different tests to identify the nature of chemical constituents present in the plant material. The aqueous extract of the plant samples were prepared by soaking 100gms of *in vitro* grown *Clitoria ternatea* L plant extract in 200ml of ethanol solvent. The crude extracts were screened qualitatively for the phytochemical constituents utilizing standard methods of analysis [20,21].

Determination of total phenolic content

Total phenols were determined by Folin-Ciocalteu reagent [22]. Briefly, a dilute extract of plant extract (0.5 ml of 10 mg/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by calorimetry at 765 nm. The concentration of total phenolic compounds in extract was expressed as milligram of GAE per g dry weight of extract. Total phenolic content was calculated using the formula.

$$\mathbf{TPC = C \times \frac{V}{M}}$$

Where, ´C´ is the concentration of gallic acid mg/ml, ´V´ is volume of plant extract in ml and ´M´ is the weight of pure plant extract in gram.

Estimation of free radical scavenging activity by DPPH method

Most popularly used 1,1 difenyl-2-picryl-hydrazyl (DPPH) is a stable radical that has been used widely to evaluate the antioxidant activity of various natural products [23]. It is a purple colored stable free radical gives a strong absorption band at 517 nm in visible region. The use of DPPH provides an easy and rapid way to evaluate antioxidants. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolorized as the color change from deep violet to light yellow. The degree of reduction in absorbance measured is an indicative of the radical scavenging (antioxidant) power of extract. Sample stock solutions (10mg/ml) were diluted to final concentration of 250, 125, 50 and 25µg/ml in ethanol. One ml of 0.3mM DPPH ethanol solution was added to 2.5ml of sample solution of different concentrations and allowed to react at room temperature. After 30 minutes, the absorbance values were measured at 517 nm and the inhibitory effect of DPPH was calculated according to the following formula [24].

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

The degree of reduction in absorbance measured is an indicative of the radical scavenging (antioxidant) power of extract. The EC50 value was calculated graphically which was the concentration required to inhibit DPPH radical by 50%.

RESULTS AND DISCUSSION

Plants have a great potential for producing new drugs for human benefit. The increased interest in plant derived drugs is mainly because of the wide spread belief that 'herbal medicine' is safer than synthetic drugs having no side effects. The potentially reactive derivatives of oxygen (ROS) such as singlet oxygen, super oxide anion, hydrogen peroxide radical and hydroxyl radical, are continuously generated inside the human body as a consequence of exposure to exogenous chemicals and a number of endogenous metabolic processes involving redox enzymes and bioenergetics electron transfer. The plants have good antioxidant ability can be attributed to the Phenolic acids, flavonoids and tannins found in the plant [25,26]. The antioxidant activity can be attributed to various mechanisms like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity and radical scavenging activity. Hence, there is need to check the antioxidant potential of *in vitro* grown plants for promising biological activity. Micropropagation through culture of explants having pre-existing meristem is powerful options which allow multiplication of genetically stable and type progeny of the species. Plant growth regulators are an important factor of initiation and regeneration of plant. Auxin such as 2, 4-D is commonly used to induce callus growth in rice where as BA have been used for regeneration of plantlets [27]. MS medium supplemented with two different growth hormones BAP and 2, 4-D separately was used to initiate shoot development from nodal explants of *Clitoria ternatia*. (Table 1) Morphogenetic responses demonstrated that both hormones stimulated cell division as well as growth of axillary shoots. BAP was found to be more effective than 2, 4-D in inducing multiple shoots. The nodal explants cultured on MS + 2, 4-D (0.5 – 2.5 mg/L) showed callus formation as well as shoots differentiation. The maximum two to three shoots induction was found in basal medium supplemented with 2, 4-D (2.5mg/L). [Fig-B] On other hand, the explants cultured on medium containing BAP 2.5 mg/L developed maximum number of shoots ranging from (5-6). Repeated sub-culturing of nodal explants isolated from *in vitro* regenerated shoots on medium supplemented with BAP 2.5 mg/L resulted in continuous production of normal and healthy shoots. The elongated shoots were excised from the shoot clumps and inoculated on MS medium containing NAA (0.5 & 1.0 mg/L) alone with BAP (1mg/L) for rooting. The root initiation was observed after two weeks of inoculation. The maximum percentage (80%) of rooting was observed at 0.5mg/L. After 30 days of inoculation in rooting medium, the rooted plantlets were removed from the culture tubes and washed with distilled water. These *in vit*ro derived plantlets were subjected to hardening and finally transferred to clay pots. After 20-28 days, these plantlets were simultaneously exposed to natural environment, where 70% plantlets survived well in the field. Similar rapid and efficient protocol for shoot regeneration was also reported from other medicinally important plants like *Bauhinia variegata* L and *Tinospora cordifolia* (willd) [28,29] (Madhu, 2012a; Madhu, 2012b). Preliminary phytochemical analysis of ethanolic extract revealed the presence of alkaloids, carbohydrates, reducing sugar, glycosides, steroids, terpenes, saponins, phenols and flavonoids *in vitro* grown plant (Table-2). The presence study revealed that the total phenolic compound in the in ethanol extract of *in vivo* and *in vitro* grown *Clitoria ternatea* was 25.5±0.360 mg/g and 19.5±0.458 mg/g GAE units respectively.

The comparative study of anti-oxidant activities was carried out of the ethanolic extracts of field grown *Clitoria ternatea* with that of *in vitro* propagated *Clitoria ternatea*. Scavenging activity of *in vitro* grown plants extracts of *Clitoria ternatea* on DPPH radical has been shown in (Fig 2). The reaction end point is reached when color change stops. The highest inhibition of DPPH radical was 67% and 70% of *in vitro* and *in vivo* grown *Clitoria ternatea* at 600 µg mL^{-1}.

Ascorbic acid was used as standard showing highest 87.58% of DPPH radical inhibition. The effect on antioxidants on DPPH is thought to be due to their hydrogen donating ability [30] (Shirwaikar, 2006). The DPPH radical scavenging abilities of extracts were less than those of ascorbic acid. The study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavengers, activity. *in vitro* propagated *Clitoria ternatea* may be considered as good sources of natural antioxidants moreover its bactericidal activity qualify it for medicinal uses[31].

Table 1:Response of nodal explants to different concentrations of BAP and 2, 4-D in *Clitoria ternatea.*

Growth regulator (mg L)		% of Response	Shoot / explant(mean±SE)
2,4-D	0.5	67	1.2±0.04
	0.1	70	1.2±0.09
	1.5	72	1.3±0.08
	2.0	65	1.5±0.18
	2.5	64	2.0±0.09
BAP	0.5	68	2.11±0.16
	0.1	70	2.27±0.17
	1.5	72	3.30±15
	2.0	75	4.32±0.21
	2.5	69	5.94±0.18

Data represents mean ± SE of 3 replicates.

Table 2:Qualitative analysis of the various phyto-constituents on the ethanol, extracts of *Clitoria ternatea L.*

S.no.	Phytochemical test	In vitro *grown plants*	*In vivo* grown plants
1	Test for alkaloids		
	Dragendorff's Test	++	+++
	Mayer's reagent	++	+++
2	Test for saponin	++	++
3	Test for flavonoids	++	++
4	Test for carbohydrates		
	Benedict's Test	++	++
	Molisch's Test	++	++
	Fehling's Test	++	++
5	Test for proteins	++	++
6	Test for steroids	++	+++
7	Test for terpenoids	++	+++
8	Test for tannin	--	++
9	Ferric chloride test for phenol	++	+++

+++ Moderately Present, Present ++, Absent –

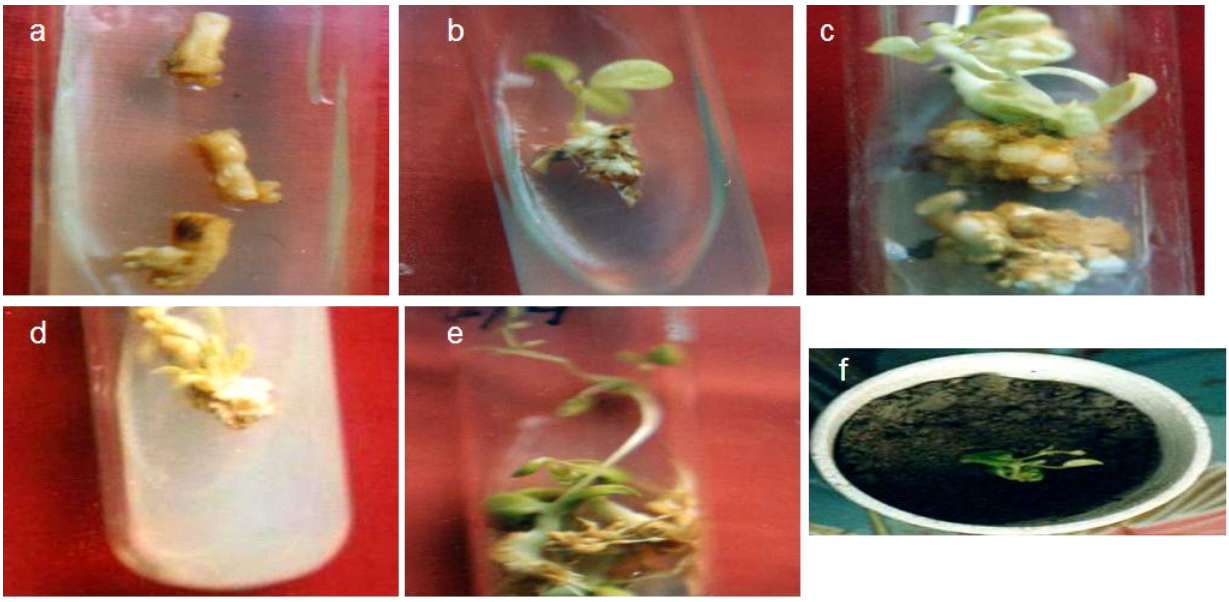

Figure 1: Plant regeneration from nodal segments. (a) The callus emerged from nodal explants on MS +2, 4-D (0.5mg/l). (b) Leafy shoot emerged from callus on MS + 2, 4-D (1mg/l). (c) Two to three to leafy shoot emerged from callus on MS + 2, 4-D (2.5mg/l). (d) Multiple shoot formation from nodal explants on MS + BAP (2.5mg/l).(e) Roots emerged from shoots on MS + BAP (1mg/l) + NAA (0.5mg/l). (f)Hardening of tissue culture raised plantlets.

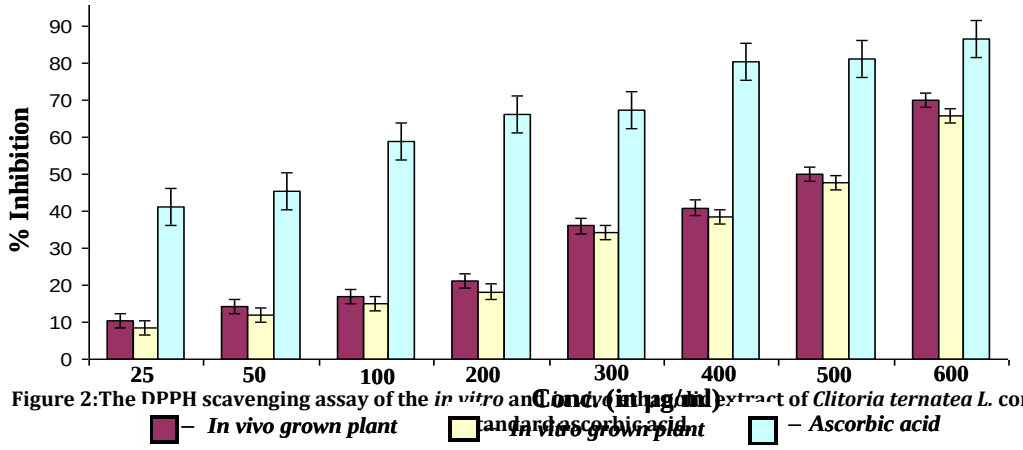

Figure 2:The DPPH scavenging assay of the *in vitro* and *in vivo* grown plant extract of *Clitoria ternatea L.* compared with standard ascorbic acid

CONCLUSION

In conclusion, an efficient protocol was developed for successful micropropagation and multiple plant regeneration of an important medicinal plant *C. ternatea* L It is a widely used in ayurvedic medicine because of its multipotent bioactive molecules. The phytochemical tests revealed the presence of flavonoids, saponins, alkaloids carbohydrate, protein, steroids and terpenoids. DPPH free radical scavenging assay can be helful for primary screening of antioxidant sources. The finding suggested that the ethanolic extract of *in vitro* grown plants could be a potential source of natural antioxidant, that can prevent or slowing the oxidative stress related degenerative diseases.

However, we do not know what components in the plant extracts show these activities.

Therefore, further work is necessary to isolate and characterize these constituents present in *in vitro* grown *C. ternatea.*

ACKNOWLEDGEMENTS

The author would like to express her gratitude to the Head, Department of Botany, B.R.A. Bihar University, Muzaffarpur for providing the necessary laboratory facilities.

REFERENCES

1. Ghasemzadeh A, Jaafar HZE, Rahmat A. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (Zingiber officinale Roscoe). Molecules 2010; 15: 4324-4333.
2. Rice-Evans C. Flavonoids and isoflavones: absorption, metabolism and bioactivity. Free Radic Biol Med 2004 ; 36: 827-828.
3. Diplock AT. Free radical damage and control. In: Rice Evans C.A. and Burden R.H., ed. Antioxidant and free radical scavengers. Elsevier, New York, USA., 1994;113-130.
4. Thomson MJ. The role of free radicals and antioxidants; How do we know that they are working? Crit Rev Food Sci Nutr 1995; 35: 21-29.
5. Lee KG, Mitchell AE, Shibamoto T. Determination of antioxidant properties of aroma extracts from various beans. J Agric Food Chem 2000; 48: 4817-4820.
6. Naik GH, Priyadarsini KI, Mohan H. Free radical scavenging reactions and phytochemical analysis of triphala, an ayurvedic formulation. Curr Sci 2006; 90: 1100-1105.
7. Narayanaswamy N, Balakrishnan KP. Evaluation of some Medicinal Plants for their Antioxidant Properties. International J Pharm Tech Research 2011; 3: 381-385.
8. Vinary RP, Prakash RP, Sushil SK. Antioxidant activity of some selected medicinal plants in Western Region of India. Adv Biol Res 2010; 4: 23-26.
9. Khadatkar SN, Manwar JV, Bhajipale NS. *In vitro* anthelmintic activity of root of *Clitoria ternatea* Linn. Pharmacogn Mag 2008; 4: 148-150.
10. Devi BP, Boominathan R, Mandal SC. Anti-inflammatory, analgesic and antipyretic properties of *Clitoria ternatea* root. Fitoterapia 2003; 74: 345-349.
11. Jain NN, Ohal CC, Shroff SK, Bhutada RH, Somani RS, Kasture VS, *et al. Clitoria ternatea* and the CNS. Pharmacol Biochem Behav 2003; 75: 529-536.
12. Sharma AK, Majumdar M. Some observations on the effect of *Clitorea ternatea* Linn. on changes in serum sugar level and small intestinal mucosal carbohydrase activities in alloxan diabetes. Calcut Med J 1990; 87: 168-171.
13. Harish MC, kumar RS, kumar SR. Efficient *in vitro* callus induction and regeneration of different tomato cultivars of India. Asian J Biotech 2010; 2: 178-184.
14. Bhaskar CR, Kiran Y, Madhavi PR, Koteswara, Raghava TR. Evaluation of antioxidant potential of *Clitoria ternate* L. and Eclipta prostrate L. Indian J Biochem Biophys 2009 ; 46: 247-252.
15. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant activity of extracts from Acacia confusa bark and heartwood. J Agricul Food Chem 2001; 49: 3420-3424.
16. Raageeva B, Madhu K, Shahnawaz Md, Kiran N. *et al*. In vitro plant regeneration in Butterfly pea, *Clitoria ternatea* L. Columban J Life Sci 2011; 12: 23-25.
17. Malabadi RB, Nataraja K. Shoot regeneration in leaf explants of *Clitoria ternatea* L., cultured *in vitro*. Phytomorphol 1990; 51: 169-171.
18. Rout GR. Micropropagation of *Clitoria ternatea* L. (Fabaceae)-A importan medicinal plant. *In vitro* cell dev Bio Plant 2005 ; 41: 516-519.
19. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15: 473-497.
20. Sofowora A. Medicinal Plants and Traditional Medicinal in Africa. 2nd Ed. Sunshine House, Ibadan, Nigeria: Spectrum Books Ltd. Screening Plants for Bioactive Agents. 1993; 134–156.
21. Trease GE,.Evans WC. Pharmacognosy. 15th Ed. London: Saunders Publishers 2002; 331–393.
22. Pourmorad F, Hosseinimehr SJ, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr J Biotechnol 2006; 5: 1142-1145.
23. Wojdylo A, Oszmianski J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem 2007; 105: 940- 949.

24. Yokozawa T, Chen CP, Dong E, Tanaka T, Nonaka GI, Nishioka I. Study on the inhibitory effect of tannins and flavonoids against the 1,1 diphenyl-2-picrylhydrazyl radical. Biochem Pharmacol 1998; 56: 213-222.
25. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem 2003; 51: 609-614.
26. Naik GH, Priyadarsini KI, Mohan H. Free radical scavenging reactions and phytochemical analysis of triphala, an ayurvedic formulation. Curr Sci 2006; 90: 1100-1105.
27. Rattana K, Theerakulpisut P, Bunnag S. The effect of plant growth regulators and organic supplements on Rice (*Oryza sativa* L.). Asian J Plant Sci 2012; 11: 182-189.
28. Madhu K. Evaluation of ethanolic extracts of *in vitro* grown *Bauhinia variegata* L. for antibacterial activities. Int J Pharm BioSci 2012a; 3: 43–50.
29. Madhu K. Evaluation of methanolic extracts of *in vitro* grown *Tinospora cordifolia* (willd) for antibacterial activities. Asian J Pharm Clin Res 2012b; 5: 172-175.
30. Shirwaikar A, Prabhu KS, Punitha IS. *In vitro* Antioxidant studies of Sphaeranthus indicus (Linn). Indian J Exp Biol 2006; 44: 993-996.
31. Madhu K. *In vitro* embryo culture and antimicrobial activity of *Clitoria ternatea* L. Int J Green Pharm 2012; 6: 310-314.