6. DISCUSSION

6.1 In Vitro Propagation of Medicinally Important Plant Clitoria ternatea

6.1.1 Surface Sterilization

In this experiment, the pinnacle of sterilization was achieved by the combination treatment of soaking explants in 0.1 % fytolan containing tween 20 for 8 minutes, followed by washing with 0.1 % mercuric chloride containing tween 20 for 3 minutes. It was observed that 80% explants survived after finishing this treatment. Subculturing the cultures after two days of inoculation further reduced the contamination rate. The survivals of cultures are solely dependent upon the period of subculturing and treatment with fungicide. Several researchers have reported the usage of fungicides for controlling the fungal contaminants in medicinal plants like Cinnamomum camphora (Nirmal Babu et al., 2003) and Datura metal (Muthukumar et al., 2004). In the present experiment, along with fytolan, 0.1 % mercuric chloride was used. Mercuric chloride in 0.1 % treatment attributed antimicrobial property inherent to the plant itself (Eilert, 1987). It was observed that when the sterilization time was increased that paved the way to the death of the epidermal tissues, which inhibited the growth. In other words, the low concentration of mercuric chloride is highly acceptable.

6.1.2 Basal Medium

For in vitro propagation of Clitoria ternatea, the best medium identified was Murashige and Skoog Medium. MS medium is in general, reported as the best medium used for tissue culture studies in shrubs, climbers and trees as evidenced by several reports in support of this fact. Vitex negundo (Das et al., 2005; Vadawale et al., 2006) and Desmodium gangeticum (Behera and Thirunauvoukkarasu, 2006) have used the full strength of MS medium.

6.1.3 Effect of Plant Growth Regulators on Shoot Induction

Shoot initiation from the nodal segments was mainly owing to the effect of cytokinins. In the present study, BA in combination with IAA or NAA produced the maximum number of multiple shoots as well as maximum shoot length within 5-10 weeks from culture. The synergistic effect of BA and auxin has been demonstrated in many medicinal plants, for example C. ternatea (Najma Ismail et al., 2012), Bupleurum fruticosum (Fraternale et al., 2002) and turmeric (Salvi et al., 2002). The important observation of these investigators was that low concentration of an auxin in
combination with cytokinins positively modified the frequency of shoot induction and growth. The role of BA in stimulating multiple shoot formation was reported in many plants (Normadiha Mohamed and Rosana mat taha, 2011; Vadawale et al., 2006; Mohapatra and Rout, 2005).

Experiments confirmed that when the concentration of BA increased, the multiple shoot per explant decreased. This result was in accordance with the results of many medicinal plants such as *Plectranthus vetiveroides* (Sivasubramaniam et al., 2002), *Rhinacanthus nasutus* (Johnson et al., 2002) and *Baliospermum montanum* (Johnson and Manickam, 2003). The lower concentration of BA induced shoots. Production of multiple shoots in low concentration of cytokinins was reported in *Plectranthus vetiveroides* (Sivasubramaniam et al., 2002). Perusal of literature suggests that BA, a cytokinin is most active at a concentration of 1.0-2.0 mg/L in many plant systems (Kathiravan and Ignacimuthu, 1999).

KN was found to be less effective than BA in the initiation of the shoots. Similar result was observed in *Salvadora persica* (Mathur et al., 2002) and *Rotula aquatica* (Martin, 2003). In the present study, the combination of IBA and IAA showed inhibitory effect on the shoot initiation. This was reported in *Pelargonium graveolens* (Gupta et al., 2002) and *Geoderum purpureum* (Mohapatra and Rout, 2005).

**6.1.4 Effect of Plant Growth Regulators on Callus Induction**

Callus is an unorganized amorphous tissue formed by vigorously growing cells containing auxin at relatively higher levels with or without cytokinins (George and Sherington, 1984). In the present study, various 2, 4-D and IAA concentrations were used to enhance the callus formation (Murashige, 1974). Yellow calli were obtained on the internodes in the medium supplemented with 2, 4-D (1.5 mg/L). Similar reports were observed in Indian ginseng *Ocimum basilicum* (Anilkumar et al., 2005). Callus proliferation declined at lower concentrations of 2, 4-D and at higher concentrations only a modium amount of hard callus was obtained. Kambaska Kumar Behra and Santilata sahoo (2009) reported similar observation in sugarcane. Calli were also obtained from the internodal segments when the medium was supplemented with IAA at 1.5 mg/L. Similar report has been observed in *Salvia fruiticosa* (Karam et al., 2003).
6.1.5 Effect of Plant Growth Regulators on Root Induction

The *in vitro* shoots were transferred to full strength as well as half strength MS media fortified with IBA, IAA and NAA. Mohammed faheem *et al.*, (2011) in *catharanthus roseus* gave similar reports. Induction of rooting in half strength as well as full strength indicates that IAA was helpful in increasing root induction. Similar results were reported earlier in sugar cane (Kambaska Kumar Behra and Santilata sahoo 2009), *Desmodium ooejinense* (Vasanthakumari and Shivanna, 2005), *Geoderum purpureum* (Mohapatra and Rout, 2005) and *Centella asiatica* (Shashikala *et al.*, 2005). Nair and Seeni (1998) obtained a maximum of 8 roots in quarter strength MS medium supplemented with 2.0 mg/L IAA in *Blepharistemma membranifolia*. At higher percentage of IAA, the percentage of root formation as well as root number decreased. Similar result was observed in *Hoslundia opposite* by Prakash and Staden, (2007). Rooting was also observed in MS medium supplemented with IBA. In most of the studies, IBA was used for the efficient root initiation (Loreti *et al.*, 1988). The effect of IBA in rooting has been reported in the medicinal plants like *Gymnema sylvestris* (Komalavalli and Rao, 2000), *Hemidesmus indicus* (Sreekumar *et al.*, 2000), *Wedelia chinensis* (Kameri *et al.*, 2005) and *Vitex negundo* (Vadawale *et al.*, 2006). These results were similar to those of the present study. MS medium with IAA showed the maximum number of roots and at lower concentration, the percentage of root formation was less. Similar results were reported in *Vitex negundo* (Das *et al.*, (2005), *Entada pursaetha* (Vidya *et al.*, 2005), *Maurua oblongifolia* (Rathore *et al.*, 2005), *Desmodium gangeticum* (Behera and Thirunavoukkarasu, 2006) and *Azadirachta indica* (Reddy *et al.*, 2006). Auxins are widely used for induction of roots on regenerated shoots. Nevertheless, not all auxins are equally effective for the purpose and their response varies significantly with the plant used (Anand *et al.*, 2011).

6.1.6 Hardening and Acclimatization

The rooted plants were transferred to polycups filled with autoclaved garden soil and sand (2:1) and irrigated regularly with 10X diluted liquid MS basal medium. The papercups were covered with polythene sheet to maintain humidity. Hu and Wang (1983) have advocated a period of humidity acclimatization for the newly transferred plantlets to make them adapted to the external environment. This method has been used for the hardening of plants by Das (1992) in *Agave sisalana* and Karwa (2003) in *Citrus reticulata*. Maintenance of humidity was reported to be essential in the hardening phase of *Uteria salicifolia* (Gangaprasad *et al.*, 2003). 70% of *C. terna*tae plants survived in this hardening treatment. After the lapse of four weeks in the net house, the hardened plants
were transferred to pots filled with garden soil, sand and compost (2:1:1). Krishnan and Seeni, (1994) reported that the root plants of *Woodfordia fruticosa* were directly transferred to the potting mixture to deliberately omit the step of hardening usually followed so as to make the system more economical and less cumbersome.

### 6.2 Seed Germination and Dormancy Breaking Techniques

The hard seed coat of many leguminous species meticulously offers marked ecological advantages. This feature obviously favors the accumulation of persistent seed banks in the soil, spreads germination over time and increases the chance that some seeds will germinate, establish and complete the life cycle successfully (Gutterman, 1993). High rapid and uniform seed germination is however pre-adequate for the successful establishment of plant species such as *C. ternatea* under *in vitro* conditions.

This study actually probes into the role of some important factors in the germination of *C. ternatea* seeds. Temperature is one such factor, which seems to play a key role in controlling the germination response of *C. ternatea* seeds. Maximum germination under *in vitro* conditions occurs only at 30°C and decreases with changes in temperature. This optimum temperature ranges are often similar to that found in natural habitat of the species. The fact remains that germination has severely reduced at 35°C. It is, indeed, the outcome of sensitivity of embryo to high temperature. This result was similar to that of other legumes (Tigabu and Oden, 2001; Mackay *et al.*, 2001).

Seeds of *C. ternatea* germinated completely and rapidly in response to concentrated sulphuric acid treatment (5 min) followed by mechanical scarification with blade. Germination of several hard seeded species had been enhanced by mechanical scarification and concentrated sulphuric Acid (Tigabu and Oden, 2001; Sy *et al.*, 2001; Vilela and Ravetta, 2001; Ortega Baes *et al.*, 2002). Damages caused by prolonged immersion or low integument resistance are common in *Tylosema esculentum* and other arid-adapted hard coated legumes (Sy *et al.*, 2001; Vilela and Ravetta, 2001; Ortega Baes *et al.*, 2002). Mechanical scarification disrupts the barriers for the uptake of water by the seed and permits radicle emergence by weakening coat structures. The disintegration of the seed coat as well as the micropylar plug is the reason for increase in imbibition and subsequent germination in seeds treated with concentrated sulphuric acid (Egley, 1989). Efolloit and Thames (1983) expressed that sulphuric acid treatment softened the seed coat by oxidation and increased the permeability of air and water through seed and thereby enhanced the germination percentage. The fact of the matter is, diluted sulphuric acid (10% - 80%) treatment failed to improve upon the level of germination in *C. ternatea*
could be attributed to the degree of thickness of its seed coat, apparently resulting in very high levels of dormancy. Demel teketay (1996) suggested that the degree of seed coat thickness is the cause of differential responses to various pre-sowing treatments. Parameshwari et al., (2001) reported that while treating the seed with acid the duration is critical since over treating the seeds very often causes injury to the seeds and exposed the endosperm. In the present study also 3 min is found to be sub-optimal and 5 minutes is identified as optimal scarification duration for *C. ternatea*. Additional acid scarification decreased germination compared to the 5 min duration. This scarification treatment is less than that of *Tylosema esculentum, Astragalus hamosus and Medicago orbicularis* (Patane and Gresta, 2006), Albizia spp (Tigabu and Oden, 2001), *Tamarindus indica* (Parameswari et al., 2001; Dimophandra Mollis (Hermansen et al., 2001).

The effect of hot water differs from species to species. Hot water treatment has been reported to improve upon the level of germination in *Lupinus arboreus* (Mackey et al., 2001), *Astragalus hamosus* (Patane and Gresta, 2006). Contrasting results of hot water effectiveness in promoting seed germination are reported also for *Albizia Procera, Albizia falcataria* (Kannan et al., 1996), *Medicago* spp and *Trifolium* spp (Uzun and Aydin, 2004), *M. orbicularis* (Patane and Gresta, 2006). Germination response of *Clitoria ternatea* seeds to hot water treatment is generally poor at 60°C, 80°C and 100°C. The limited germination of seeds may have been due to imbibitions as a result of soaking rather than softening of the seed coat (Karam and Salem, 2001). Also the poor performance of *C. ternatea* seeds in hot water treatment could be due to the thickness of the seed coat or soaking injury.

Gibberellins have a promotive effect on the germination of many species of angiosperms and gymnosperms (Karam and Salem, 2001; Subodh Airi et al., 1998; Kochankav et al, 1998). In the present study GA3 treatment generally improved germination in *C. ternatea* seeds compared to the control. The promoting effect of GA3 is often attributed to the mobilization of stored reserves and weakening of the mechanical resistance of the endosperm cells around the radical tip (Groot and Karssen, 1987). This weakening of the surrounding structure prior to protrusion is associated with an increased activity of endo-B-1, 4-mannanase (Tigabu and Oden, 2001). It has been also reported that application of GA3 accelerates the disappearance of ABA regulated polypeptides, which are found abundantly in dormant seeds (Nicolas et al., 1997). Sulphuric acid scarification followed by Gibberelic acid treatment resulted to higher germination percentage than single gibberelic acid immersion. Therefore, it is likely that the primary control of germination in *C. ternatea* seeds resides in the seed coats, and that GA3 action is
promoted by a previous seed coat disruption, as reported in *Tylosemia esculentum* and *Capparis spinosa* (Sozzi and Chiesa, 1995).

The study has revealed that dormancy in *C. ternatea* has been attributed to the hard seed coat under *in vitro* conditions. The Sulphuric acid treatment for 5 m followed by mechanical scarification and incubation at 30°C can successfully overcome dormancy in these seeds.

### 6.3 Antibacterial Activity of Different Solvent Extracts of *Clitoria ternatea*

It is a fairly well-known fact that medicinal plants serve as significant therapeutic aids for various many ailments. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century (Zaika, 1975). In India, from ancient times, different parts of medicinal plants had been used to cure specific ailments. Today, there is a widespread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and sound in comparison with synthetic drugs. Natural antimicrobials can be derived from plants, animal tissues, or microorganisms (Gordon and David, 2001). The shortcomings of the drugs are easily available. It propels the discovery of new pharmacotherapeutic agents in medicinal plants (Cordell, 2000). To determine the potential and promote the use of herbal medicine, it is essential to intensify the study of medicinal plants that find place in folklore (Awadh Ali et al., 2001; Nair et al., 2005).

Antimicrobial drugs currently used in medicinal practices for treating various diseases often causes serious side effects such as immunosuppression of the host and development of resistance. Medicinal and aromatic plants and their essences rich in antibacterial compounds could be an alternate way to combat bacterial diseases (Abramowize, 1990; Meera et al., 1999). In order to mitigate the impact of allopathic drugs natural and herbal usage is ideal because it is without any side effects.

Several investigators have reported antimicrobial activity of many medicinal plants like *Argemone mexicana* (Sangameswaran et al., 2004), Sand dune species (Kpemissi et al., 2003), *Dodoneae viscosa, Rumex nervosus, Rumex abyssinicus* (Getie et al., 2003), African combretaceae (Katerere et al., 2003) and *Pergularia daemia* (Rajasekara Pandian et al., 2005).

#### 6.3.1 Antibacterial activity of *Clitoria ternatea* (Blue & White)

In the present investigation, antimicrobial activities of five solvent extracts against four microbial species were recorded. Considerable antimicrobial activity was detected in
ethyl acetate, acetone and ethanol extracts of *C. ternatea* (Blue) and petroleum ether, acetone and ethyl acetate extract of *C. ternatea* (White).

In general, ethyl acetate and acetone extract showed better inhibition for all the four pathogens. The highest degree of inhibition was found against *B. subtilis, K. pneumoniae, E. coli* and *P. vulgaris*. Abiramasundari *et al.*, (2012), have observed that benzene and methanol extracts of *Cocculus chirsutus* leaves have shown strong antimicrobial activity against *B. subtilis, E. coli, S. aureus* and *K. pneumoniae*.

Deshwal Vishal kumar, (2012) found that the ethanol and acetone extract of *Centipeda minima* exhibited antimicrobial activity against *Listeria monocytogenes, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella enteritidis, Yersinia enterocolitica* and *Shigella sonnei*. The greatest inhibition zone was found against *Klebsiella pneumoniae*. This result is in accordance with the present study.

Several workers (Paz *et al*., 1995; Vlientinck *et al*., 1995) have generally reported that water extract of plants does not have much activity against bacteria. Amit kumar *et al*., (2012) reported that methanol was a more effective solvent for plant extraction than water. Similarly, in our study, the ethyl acetate and acetone extracts exhibited higher activity followed by ethanol, petroleum ether and aqueous extracts. Durmaz *et al*., (2006) reported similar observation in *Allium vineale, Chaerophyllum macropodum* and *Prangos ferulacea*.

Brett *et al*., (2012) reported the antibacterial activity of methanol and aqueous extracts of *Morinda citrifolia* against *Escherichia coli* and *Staphylococcus aureus*. In the present study, all the four pathogens showed resistance against petroleum ether, ethyl acetate, methanol and acetone extracts of *C. ternatea*. Aqueous extract showed moderate activity against these pathogens.

*B. subtilis, K. pneumoniae, E. coli* and *P. vulgaris* showed more susceptibility to the ethyl acetate extract of leaves of *C. ternatea* in the present study. It exhibited activity against every pathogen tested followed by ethanol and aqueous extracts. Burade *et al*., (2005) has reported similar results in *Piper betle*, where methanol as well as ethanol extracts showed stronger and broad-spectrum antibacterial activity against *E. coli, P. aeruginosa, P. mirabilis, S. aureus, S. typhi, S. paratyphi, P. vulgaris* and *Shigella flexneri*. The same researcher has reported antibacterial activity of ethanol and methanol extracts of *Tamarindus indica* leaves against the above said pathogens (Burade *et al*., 2005).
Esath Natheer et al., (2012) reported the antimicrobial activity of ethanol and ethyl acetate extracts of Morinda citrifolia against E. coli, S. aureus, K. pneumoniae and P. mirabilis. In the present study, all the four pathogens showed resistance against ethyl acetate and petroleum ether extracts of C. ternatea and aqueous and methanol extracts showed moderate activity against these pathogens.

6.4 Isoenzyme Analysis of Clitoria ternatea for Genetic Confirmation

Proteins have been used for many years as molecular markers, predominantly in academic laboratories, for plant identification (Kephart, 1990). Proteins are analyzed as isozymes, which are different molecular forms of a protein actively controlling identical biochemical processes of a living cell. Isozymes are separated in an electrical field supported in a poly acrylamide or starch matrix. A characteristic pattern of different isozyme band called an “isozyme fingerprint” is visualized after staining. A disparity between isozyme fingerprints derived from two types of plant is called “polymorphism.” Isozyme analysis has the clear advantage over DNA based markers of relative efficiency and cost effectiveness.

Isozyme electrophoretic characterization has proven to be highly useful as biochemical markers for solving various problems of plant taxonomy in order to distinguish or to confirm the identity of the species. The characterization also allows us to measure divergence between populations at different levels within and between species of related genera (Vander Bank et al., 2001).

Isozymes are the most reliable single gene markers and virtually any plant tissue can be analyzed for identification of cultivars using isozymes (Torres, 1983). Isozymes also have some advantages over other DNA markers such as (i) they are still the most in cost effectiveness, (ii) interpretation of results is easier due to less noise, and (iii) the technique is simpler and still being used effectively (Obara-Okeyo et al., 1998; Kannenberg and Grors, 1999).

The genetic stability of in vitro collection (sources: NBPGR) has been periodically evaluated by using morphological, cytological and biochemical (isozymes and alkaloid) analysis. For example, in sweet potato isozymic analysis was performed on in vitro regenerated plants to study the effect of micropropagation on maintenance of genetic stability of regenerates (Lakhan Paul et al., 1990). Similar studies on genetic stability of tissue culture regenerated Coleus forskohlii by biochemical analysis (Sharma et al., 1991) and Allium tuberosum by cytological analysis (Rao et al., 1992) showed their unaltered characters.
The present study was carried out with the intention of confirming the genetic stability between the mother plant, *in vitro* plantlet and callus of both *C. ternatea* (Blue & White) using four isoenzyme systems namely peroxidase, esterase, alkaline phosphatase and poly phenol oxidase. Several reports were available for the genetic conformity through isozymic analysis. Nair and Seeni, (2001) and Johnson and Manickam, (2003) confirmed genetic stability of *in vitro* plants through isoenzyme analysis. The total soluble proteins were also determined by SDS PAGE analysis.

6.4.1 Peroxidase

Definitive roles of peroxidase in plants have eluded plant scientists so far. There have been numerous reports on the literature with respect to their general involvement in the oxidation of molecules at the expense of hydrogen peroxide. This enzyme exists in larger number of isoforms in plants. However, it is difficult enough to ascribe a particular role to a single isozyme in the physiological events. In addition, plant peroxidases showed specific action with respect to different substrates (Siegel and Siegel, 1970) and the activities changed in response to a wide range of environmental stimuli. Peroxidase is recognized to be one of the most heat stable enzymes in plant and its resistance to heat is reported by numerous workers (Muftugil, 1985; Mc Lellan and Robinson, 1987).

Classical plant peroxidases (donor: \( \text{H}_2\text{O}_2 \) oxido reductase) are heme-containing enzymes that catalyze the oxidation of a diverse group of organic compounds (Dawson, 1988). Peroxidase has been suggested to be involved in various metabolic steps such as auxin catabolism (Haissig *et al*., 1992; Kevers *et al*., 1997), the formation of isodi-Tyr bridges in the cross linking of cell wall proteins (Schnabelranch *et al*., 1996), the cross linking of pectins by diferulic bridges (Amaya *et al*., 1999) and the oxidation of cinnamyl alcohol prior to their polymerization during lignin (Mader and Fussl, 1982; Imberty *et al*., 1985) and suberin formation (Espelie and Kollatukudy, 1985).

Isozymes of peroxidase have been utilized to identify the cultivars and somatic hybrids of basmati rice varieties (Srivastav *et al*., 2002), to confirm the genetic stability in *Celastrus paniculatus* (Maruthi *et al*., 2004), *Ebenus cretica* (Syros *et al*., 2004) and wild diploid wheat (Cheniany *et al*., 2007).

In the present study, the activity staining of the gel for peroxidase revealed identical banding pattern for *in vitro* raised plantlet and mother plant of both *C. ternatea* (Blue & White). The Isozymic profile indicated the genetic conformity among plantlets obtained through *in vitro* propagation and mother plant and thus they were all true to the type. Similar reports have been made by Nair and Seeni (2001) in *Celastrus paniculatus*,

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Maruthi et al., (2004) in Celastrus paniculatus and Reddy et al., (1998) in Gymnema sylvestre. Two different bands were found out in the isozyme electrophoretic pattern of peroxidase with Rf values 0.487 and 0.533. The isoforms with Rf 0.487 was found to be specific to mother and in vitro plants of C. ternatea (Blue).

6.4.2 Esterase

Esterase is a monomeric enzyme (Scandalios, 1969) that showed two different zones of enzyme activity. In the present study, a total number of 11 isoforms were observed with their Rf values ranging from 0.105 to 0.466. Among these isoforms, C. ternatea (Blue) exhibited 7 bands with Rf values ranging from 0.105 to 0.462. Isoforms with Rf values 0.107, 0.464 and 0.469 were present only in the mother and in vitro raised plants of C. ternatea (Blue). The isoforms Rf value of 0.462 was common to mother plants, in vitro raised plantlets and callus of C. ternatea (Blue). This showed that these isoforms were specific to C. ternatea (Blue). The in vitro plants showed 91.4 % similarity between the mother plants and callus showed 41.4% similarity. Isoforms with Rf value 0.464 and 0.469 was exhibited by callus of C. ternatea (Blue) and these might be new isoforms that formed during the development of callus. For C. ternatea (White), four isoforms were observed, whose Rf values ranged from 0.105 to 0.432. The in vitro plant of C. ternatea (White) showed 84.8% similarity towards mother plant whereas callus showed 45% similarity. In vitro plant produced new isoform with Rf value 0.107 and it was also found in the callus of C. ternatea (Blue). The callus of C. ternatea (White) does not have first two bands with Rf values 0.105 and 0.111. There were two zones of isozymic activities, the most anodal zone and the cathodal zone. The cathodal zone exhibited five bands with Rf values ranging from 0.105 to 0.432 and anodal zone exhibited six bands with Rf values ranging from 0.107 to 0.469. Similar polymorphism was observed in Prunus by Mowrey et al., (1990) and Prunus persica (L.) by Agarwal et al., (2001). Handa et al., (2000) observed 7 isoesterase bands in the hybrids between Populus ciliata X Maximoviiizii hybrids, and found that male parent had 4 bands and female parent showed 6 bands. None of the hybrid plants had the banding pattern similar to that of the parents. They showed a banding pattern intermediate between the banding pattern identified for male and female plants.

Balen et al., (2003) compared calli, normal and hyperhydric regenerants and tumors in cactus shoots using esterase activity and isozyme pattern. Balen et al., (2004) observed that in Mammillaria gracilll, esterase system was a suitable biochemical marker for characterising different developmental stages. In barley callus Coppens and Dewitte (1990) used esterase zymograms as a biochemical marker for studying embryogenesis and
concluded that esterase system was very sensible for detection of embryogenesis before somatic embryos were formed.

6.4.3 Alkaline Phosphatase

Alkaline phosphatase is an essential enzyme in the cellular process. Phosphatases are vitally important enzymes in tyrosine phosphorylation, which has been shown to be involved in the control of the cell cycle, the cell-cell communication, in cellular responses to growth factor and in embryogenesis (Glenney, 1992). All the studies have focused predominantly on structural polymorphisms, whereas those affecting the amount or the time during which the enzyme is expressed received less attention, largely because their phenotypes and inheritance are generally more complex.

In the present study, three isoforms were observed with Rf values from 0.402 to 0.426. Similar observation was reported in Peach (Agarwal et al., 2001). The mother plant of *C. ternatea* (Blue) exhibited only one isoform (Rf 0.404) whereas *in vitro* plant and callus showed an additional band (Rf 0.426), the pairing affinity was found to be 61.4% for both *in vitro* raised plantlet and callus. In *C. ternatea* (White), both callus and *in vitro* raised plant exhibited identical banding pattern with their mother plant. Hence, the similarity index was found to be 100%.

6.4.4 Polyphenol Oxidase

Polyphenol Oxidase was (EC 1.14.18.1) catalyses enzymatic browning through its action on mono and O-diphenols (Mayer and Harel, 1979; Golbeck and Camarata, 1981). Polyphenol oxidase, a copper-containing enzyme is mainly involved in the synthesis of pigments in plants. Usually, it is formed during the tissue development and bound in the chloroplast thylakoid membranes (Huijiang et al., 2003). Although reports on fruit polyphenol oxidase are available, the studies on leaf polyphenol oxidase are lacking (Meyer and Biehl, 1981; Broothaerts et al., 2000). A polyphenol oxidase from transgenic tobacco leaves (Broothaerts et al., 2000) has been reported. In the present study, leaf samples and callus were taken from the *in vitro* and mother plants of both *C. ternatea* (Blue & White) and polyphenol oxidase isoyme system was used to confirm the genetic stability between them. The similarity and variability in the loci are indicative of diversity and lineage.

Two bands were observed in the present study with Rf values 0.044 and 0.108. Similar results were confirmed in wild diploid wheat (Cheniany et al., 2007). *In vitro* raised plantlets of *C. ternatea* (Blue) showed 100% similarity to the mother plant. In the callus, isoform with Rf value 0.180 was absent. In *Clitoria ternatea* (White), the
micropropagated plants showed variation (pairing affinity 66.7%) to the mother plant, whereas callus showed 100% similarity. An additional band with Rf value 0.108 was observed in the \textit{in vitro} raised plantlet.

Polyphenol oxidase was used to study the variation in the determination of sex in \textit{Actinida}, primary processing of green tea (Hariong \textit{et al}., 1987) and characterization of different organs of the tea shoots. Surprisingly, polyphenol oxidase activity is completely absent in the developing grain of both late and early maturing types of mung bean, which is difficult to explain. However, this enzyme is synthesized in the leaves and stems (Gupta \textit{et al}., 2002).

In the present study, among the four isozymic profiles, esterase showed maximum polymorphism (11 bands with Rf values ranging from 0.105 to 0.466) followed by Alkaline phosphatase (three bands with Rf values from 0.402 to 0.426), Peroxidase (2 bands with Rf value 0.487 and 0.533 and polyphenol oxidase (2 bands with Rf 0.044 and 0.108). Gangopadhyay \textit{et al}., (2004), reported similar observation. The isozymic analysis of tissue cultured \textit{Pandanus amaryllifolius} plantlets was carried out to ascertain their genetic fidelity. The mother and tissue cultured plants of \textit{Pandanus amaryllifolius} revealed identical isozymic profiles. Among the four isozymic profiles, esterase showed maximum polymorphism followed by GOT, acid phosphatase and peroxidase in order.

Petrova \textit{et al}., (2006) studied the isozymic and protein patterns in micropropagated plants of \textit{Gentiana lutea} and observed isozyme polymorphism. Similar observations were made by Diaz \textit{et al}., (1997). They estimated the effect of the \textit{in vitro} regeneration procedure on the isozyme polymorphism in \textit{Brassica napus}, \textit{Brassica rapa}, date palm clones and \textit{Vigna radiata}.

6.4.5 Total Protein Analysis by SDS-PAGE

Electrophoretic analysis of proteins and isoenzymes offers an efficient and cost effective method towards evaluation of geographical and taxonomic distribution of genetic variation for sampling strategies in germplasm conservation (Brown, 1978). In the recent times protein markers are in routine usage and widely accepted as an apt tool for determining the genetic purity and identity of crop varieties (Weir, 1990).

The present study was aimed at confirming the genetic stability of the mother plant and \textit{in vitro} raised plants as well as to probe into the genetic diversity between \textit{C. ternatea} (Blue & White). Analysis of total soluble protein patterns revealed approximately 19 polypeptides. The protein profile of the mother plant, \textit{in vitro} plant and callus of \textit{C. ternatea} (Blue & White) showed an array of proteins with molecular weight ranging from
In *C. ternatea* (Blue), majority of the bands appeared between 60 KD and 15.4 KD. In the mother plant, there was abundance in the expression of high molecular weight proteins whereas in the callus and *in vitro* plant, low molecular weight protein bands were more in number. Some bands observed were unique to *C. ternatea* (Blue) (0.057 and 0.235). The protein band with Rf value 0.933 was observed in the *in vitro* raised plant of *C. ternatea* (Blue).

6.5 Analysis and Identification of Different Bioactive Compounds of Medicinally Important Plant *Clitoria ternatea* L by using HPTLC.

6.5.1 Alkaloids

Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals especially by higher plants - about 10 to 25% of those contain alkaloids and are part of the group of natural products (also called secondary metabolites). They often have pharmacological effects and are used as medications, recreational drugs, or in entheogenic rituals (David, 1979). Depending on the type of plants, the maximum concentration is observed in the leaves (black henbane), fruits or seeds (Strychnine tree), root (*Rauwolfia serpentina*) or bark (cinchona) (David, 1979). Furthermore, different tissues of the same plants may contain different alkaloids. Similar to the previous observations, in the present also, we observed 26 different alkaloids in the different parts of *C. ternatea*. Medical use of alkaloid plants has a long history, and thus when the first alkaloids were synthesized in the 19th century, they immediately found application in clinical practice. Many alkaloids are still used in medicine, usually in the form of salts, including the following: anti-arrhythmic, anti-cholinergic, anti-tumor, vasodilating, anti-hypertensive, cough medicine, anesthetic, on anti/protozoal agent. The results of the present study confirms the folkloric usage and pharmacological studies of the medicinally important plant *C. ternatea* and suggest that some of the plant extracts possess compounds with bioactivity properties that can be used as active principles or agents in new drugs for the therapy of infectious diseases. A recent review shows that the HPTLC techniques can be used to rectify many qualitative and quantitative analytical problems in a wide range of fields including medicine, pharmacy, chemistry, biochemistry and toxicology. In the present study also we identified the alkaloids profile of the medicinally important plant using HPTLC. HPTLC has been recommended for identification of the medicinal plants. In the present study, we also produced the alkaloids profile using HPTLC of the different parts of *C. ternatea*, this profile can be used for the identification of the medicinally important plants from the
adulterant. Further, separation and characterization of the bioactive compounds (principles) from selected plants can be evaluated and reported in near future.

6.5.2 Flavonoids

Flavonoids are ubiquitous in photosynthesising cells and therefore occur widely in the plant kingdom (Deshmukh et al., 2008). They are found in fruits, vegetables, nuts, seeds, stems and flowers and represent a common constituent of the human diet. The results of the present study also confirm the flavonoids presence in the methanolic extract of stem, leaves and seeds of *C. ternatea* there by supplementing the previous observations. Increasingly, flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity (Manokaran et al., 2008; Shirwaikar et al., 2004; Deshmukh et al., 2008; Appia Krishnan et al., 2009). In traditional medicines, medicinal plants have contributed hugely to the traditional and western medicines through providing ingredients for drugs or having played central roles in the drug discovery. The evaluation of a crude drug is an integral part of establishing its correct identity. Before any crude drug can be included in herbal pharmacopoeia, pharmacognostical parameters and standards must be established. Chromatographic finger printing of phyto constituents can be used for the assessment of quality consistency and stability of herbal extracts or products by visible observation and comparison of the standardized fingerprint pattern (Rajkumar et al., 2010). In the present study we established the HPTLC profile for the vegetative and reproductive parts of *C. ternatea* to identify and to differentiate the *C. ternatea* from the other crude drugs and adulterants. HPLTC is useful alternative under circumstances where the other slower and more costly chromatographic methods are not appropriate. It is a suitable method to standardize raw herbs, active constituent enriched extracts and their formulations. The HPTLC method developed for the identification of *Clitoria ternatea* is simple, precise, specific, accurate, rapid and cost effective. This HPTLC procedure may be used effectively for the flavonoids in this plant and its derived products. Developed HPTLC chromatogram of methanol extracts of vegetative and reproductive parts of *C. ternatea* may be treated chromatographic finger prints and could be used efficiently for identification, and quality assessment of the plant.

6.5.3 Terpenoids

Plants can produce many different types of secondary metabolites, which have been subsequently utilized by humans for their valuable characters in a diverse array of
application (Zwenger and Basu, 2008). Currently, there is an increased interest in natural substances with valuable medicinal properties, such as Terpenoids (hydrocarbon composition) and multiple C$_3$H$_8$. Plant Terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic and other pharmaceutical functions. Terpenoids contribute to the scent of eucalyptus, the flavors of cinnamon, cloves, ginger and the color of yellow flowers. Well-known Terpenoids include Citral, menthol, camphor, salvinorin A in the plant *salvia divinorum* and the cannabinoids found in cannabis. The steroids and sterols in animals are biologically produced from terpenoid precursors. Sometimes Terpenoids are added to protein, e.g., to enhance their attachment to the cell membrane; this is known as isoprenylation. These compounds and their derivatives also belong to other drugs such as validol, bromkamfora, menovasin, turpentine, etc. Turpentine is widely used as external drugs, and it is the main raw material for other products on the basis of Terpenoids. Terpenoids are defined as secondary metabolites with molecular structures containing carbon backbones made up of isoprene (2-methylbuta-1, 3-diene) units. More than 36000 terpenoid compounds have been identified, making Terpenoids the largest class of plant metabolites. Most of the thousands of Terpenoids produced by plants have no discernible role in growth and development and are, therefore, often classified as secondary metabolites. In addition to universal physiological, metabolic and structural functions, many specific terpenoids function in various situations, including communication and defence. Members of the isoprenoid group also include industrially useful polymers (e.g., rubber and chicle) and agrochemicals (e.g., pyrethrins and azadirachtin). It is known that several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. In particular, terpenoids are contained in many herbal plants and several terpenoids have been shown to be available for pharmaceutical applications. For example, artemisinin and taxol are used as malaria and cancer medicines, respectively. Terpenoids are a large and diverse class of naturally occurring organic chemicals found in all classes of living organisms. Plant terpenoids are used extensively for their aromatic qualities and play a role in traditional herbal remedies. They are currently under investigation by numerous groups for antitumor, antibiotic, anticancer, antineoplastic, antibacterial, anti-inflammatory and other therapeutic properties (Heras et al., 2010). Considering the wide therapeutic applications and importance of *C. ternatea*, a HPTLC method was developed to ensure the identity and quality of commercial samples. This will help to obtain monograph of the future medicinally active plant. The method was validated by determining linearity, peak purity and limit of detection and repeatability of terpenoids from the aerial part extract of *C. ternatea*. The developed HPTLC method for terpenoid
profile is simple, precise and accurate and can be used for the identification and commercial application. For developing analytical method, pure active chemical constituents should be isolated in further study and identification on the basis of reference standard shall be made. This profile helps in setting in house standards of the medicinal plants used extensively by herbal manufactures.

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show the immense potential of medicinal plants used in various traditional systems. Plants are rich sources used in flavouring, fragrances, insecticides; sweeteners and natural dyes (Kaufman et al., 1999). Carbohydrates are one such group of carbon compounds which are essential to life. Almost all organisms use carbohydrates as a matter of fact, exploit their rich supply of potential energy to maintain life. The highest amount of carbohydrate was observed in stem of *C. ternatea* 36.24 mg/100g and the highest amount of total sugar was observed in stem of *C. ternatea* 40.92 mg/100g. Total level of protein was found to be higher in seed of *C. ternatea* 13.96 mg/100g. Proteins are the beginners and builders of biochemical reactions. These are the integral Parts of protoplasm varying in their contents from plant to plant.

The total levels of lipids were found to be higher in seed of *C. ternatea* 12.3 mg/100g. Lipids are the supporters and storage molecules of cells. These are greasy materials which play important cellular structures. Lipids are being used by industry as highly stable lubricant and as a renewable source of fuel (Harwood et al., 2000) and the Total Ash 9.95 mg/100g leaf of *C. ternatea*.

### 6.6 Identification of Bioactive Components of *Clitoria ternatea* (Linn.) by GC – MS Analysis

GC- MS Chromatogram of ethanol leaf extract of *C. ternatea* (Blue) has shown the presence of twenty eight phyto compounds. The first compound present in ethanol leaves of *C. ternatea* (Blue) identified with less retention time (7.25) was Quercetin 7,3',4'-Trimethoxy (0.29) whereas 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-,(E)]]- (CAS) (1.97%) was the last compound identified which took longest retention time (44.03) to identify.

GC- MS analysis of ethanol leaf extract of *C. ternatea* (white) has shown the presence of twenty four phyto compounds. The first compound present in ethanol leaf extract of *C. ternatea* (white) identified with less retention time (11.66) was Neophytadiene (1.93%) whereas á-Amyrin (2.41%) was the last compound identified which took longest retention time (37.40) to identify.
The identified compounds have many biological properties. For instance, Hexadecanoic acid, methyl ester which is a palmitic acid compound found to be an antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha is a reductase inhibitors. This supports the findings of Sermakkani and Thangapandian, (2012) who observed the presence of this compound in methanol extract of Cassia italica leaf.

9-Octadecenoic acid which is a linoleic acid compound reported to have an anti inflammatory, nematicide, insectifuge, hypocholesterolemic, anticancer, hepatoprotective, antihistaminic, antiacne, antiarthritic and antieczem properties. Similarly, the presence of 9-Octadecenoic acid was observed in the ethanolic root of Plumbago zeylanica by Ajayi et al., 2011. 2-Hexadecen-1-ol, 3, 7, 11, 15-tetramethyl-[R-[R*, R*-,(E)]] - phytol is a diterpene compound which is reported to possess antimicrobial, anticancer, anti-inflammatory and diuretic agent (Praveen kumar et al., 2010). Similarly Maria Jancy Rani et al., (2011) observed the presence of phytol in the leaves of Lantana camara and Sridharan et al., (2011) in Mimosa pudica leaves. Similar result was also observed in the leaves of Lantana camara (Sathish kumar and Manimegalai, 2008). Phytol was observed to have antibacterial activities against Staphylococcus aureus by causing damage to cell membranes as a result there is a leakage of potassium ions from bacterial cells. Phytol is a key acyclic diterpene alcohol that is a precursor for vitamins E and K₁. It is used along with simple sugar or corn syrup as a hardener in candies (Inoue et al., 2005).

n- Docosane, n- Heptacosane, n- Octacosane and n-Nonacosane are hydrocarbons which are found to be reported in the acetone leaf and ethyl acetate stem extracts. Similarly, Memon et al., (2012) observed these compounds from stem and leaf extracts of Aerva javanica. Hexadecane and dodecane are the fatty acid esters which are found in all the three extracts and are reported to have antioxidant property.

6.7 Polymerase Chain Reaction

According to WHO general guidelines for methodologies on research and evaluation of traditional medicines, first step is assuring quality, safety and efficacy of traditional medicines for correct identification. In the present article, C. ternatea was chosen for the identification through RAPD technique. For accuracy of the results, the high quality and purity of genomic DNA free from secondary metabolites was isolated from these species by modified CTAB method (Khan et al., 2007). For RAPD reaction, it was mandatory to standardize the following variables for successful amplification with PCR: RAPD amplification is not reproducible below a certain concentration of genomic
DNA and produces ‘smears’ or results in poor resolution, if DNA concentration is high, series of dilutions were made to check good amplification. PCR trials were undertaken with different concentrations of MgCl₂ (0.5 mM, 1 mM and 1.5 mM) keeping all other parameters constant. MgCl₂ of 1.5 mM concentration was proved best in 25 ll reaction volume. For most amplification reactions 0.5-1.5 units of the enzyme were used. Initially amplification reactions were carried out with 0.9 units of Taq polymerase, however, this was not found to work to generate better results. Therefore the quantity was reduced to 0.5 units of Taq polymerase per 25 ll reaction volume, which gave better amplification. In all PCR trials the annealing temperature 36°C was used which was determined with gradient PCR. DNA denaturation is a critical step in DNA amplification reactions. For most DNA amplification reactions incubation time for DNA denaturation is 1 min at 94°C. In the present investigation, 4 RAPD primers produced 76 polymorphic bands that unambiguously discriminated C. ternatea respectively. This RAPD marker exhibited 48.10% polymorphism among these species with 4 decamer primers. Our results indicated the presence of wide genetic variability among different C. ternatea. Variations in the DNA sequences lead to the polymorphism and greater polymorphism is indicative of greater genetic diversity. All four species of C. ternatea were differentiated from each other based on unique bands obtained in PCR amplification.

A small number of pairwise differences (high genetic similarity values) among some genotypes are likely due to their genetic relatedness. On the other hand, a large number of pairwise differences (low genetic similarity values) should be observed among those cultivars developed from genetically distant parental lines. If the same statistical procedures are applied to analyze RAPD data, similarity values between cultivars may change, depending upon certain considerations (Kosman and Leonard, 2005). An important consideration is the choice of coefficient for measuring genetic similarity, i.e. common band, shared band or simple matching band. The problem with RAPD dominant markers is to distinguish bands that represent two alleles at a homozygous locus from bands that represent only a single allele at a heterozygous locus. Consequently, it is impossible to determine exact genetic similarity between two cultivars that share a band at the same position. Therefore, the similarity between cultivars of diploid longan distinguished with RAPD markers may be incomplete information. In general, no suitable method for measuring genetic similarity between diploids with RAPD markers can be proposed (Kosman and Leonard, 2005).

High polymorphism obtained in this study clearly indicates the presence of genetic variation among the C. ternatea populations collected from different states in India. This
variation is attributed to the differences in the number of alleles per locus/or loci and their distribution within the population. The presence of a large range of similarity values (0.842) between the tested samples revealed that the differences in the ecosystems of *C. ternatea* might be reflected on RAPD-PCR. Similar results were obtained by Morsy (2007) when fingerprinting four populations of *C. ternatea* using RAPD-PCR. This result indicates that a genetic diversity is found among the studied populations. RAPD analysis has a potential for studying the genetic diversity among and within *C. ternatea* populations. However, the application of biochemical and genetic systems play a vital role in studying the status of wild plant species and in investigating their evolution and migration from their centers of distribution.

The advantages of this technique are its rapidity, simplicity and avoidance of any need such as genetic information about the plant prior to the commencement of the experiment. These characters are especially advantageous for the identification of any herbal drugs mainly because of little DNA existing in the dried material. The significance of the present work lies in the fact that a single primer can differentiate genuine as well as adulterant samples as reported in our study. More reproducible DNA markers such as sequence characterized amplified regions (SCAR) can be developed in further studies, which would provide an alternative tool to monitor the quality of these herbal drugs. Thus, our study has proved that RAPD markers can be used for the identification of commercial *C. ternatea* species and could be a useful tool to supplement the distinctness, uniformity and stability analysis for plant samples to maintain their identity for the protection in the future.

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