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# *In vitro* propagation and antibacterial activity of *Clitoria ternatea* Linn.

Arumugam M, Panneerselvam R*

*Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu, India – 608 002*

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ABSTRACT

**Objective:** To compare the antibacterial property between tissue cultured and wild plants of *Clitoria ternatea*. **Methods:** Callus formation, shoot multiplication and rooting were carried out in MS medium supplemented with 2,4-D and KN; BA, KN and IAA; NAA and NAA, respectively. The antibacterial activity was evaluated using disc diffusion method against the gram positive bacteria *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus*, the gram negative *Klebsiella pneumonia, Proteus vugaris* and *Salmonella typhi*. Soxhlet apparatus was used for extraction with a series of solvents methonolic, perolium ether and ethyl acetate. **Results:** The best shoot initiation was resulted in KN (1.0 mg/L) and IAA (0.5 mg/L).The maximum shoot multiplication was obtained by BA (0.75 mg/L) and IAA (0.5 mg/L). The maximum callus initiation was observed by adding 2,4-D (1.0 mg/L) and KN (0.5 mg/L) using leaf explants. Callus differentiation was achieved by addition of 2, 4-D (0.3 mg/L) and KN (0.3 mg/L). The combination of plant growth regulators such as 2,4-D and KN induced best callus in all the explants of *Clitoria ternatea* compared to other hormonal combination. Maximum rooting was achieved by adding NAA (1.0 mg/L) on half strength MS medium. The methonolic extract of callus showed higher activity in *Klebsiella pneumonia* and *Staphylococcus aureus* than rest of the organisms. **Conclusions:** From the present investigation, it can be concluded that *in vitro* derived callus and plants of *Clitoria ternatea* exhibits antibacterial activity against certain pathogenic bacteria.

## 1. Introduction

*Clitoria ternatea* Linn. belongs to the family Fabaceae and it seems to be a native of the Caribbean, Central America and Mexico; early after the conquest it was distributed to the Indian Sub continent, in tropical Asia, the Philippine Islands, and Madagascar. It is an attractive perennial climber with blue or white flowers. The pods are 5-10 cm long, flat, nearly straight, sharply beaked and 6-11 seeded. It is a highly palatable forage legume and generally is preferred liver stock over other legumes. Butterfly pea is also used as a cover crop or green manure. The scientific name of the genus is derived from Greek kentron, a spur, prickle, sharp point, the center, a single, referring to the spurred standard petal[1]. Traditionally, the plant has been used in the preparation of more than 40 Ayurvedic formulations. Sankhapushpum is one of the well-established Ayurvedic formulations in India system of medicine, which contains power of roots from 15 different medicinal plant including *Clitoria ternatea*. The preparation is used as a sources of hormone to cure post delivery problems, inflammations, detoxifications of entire body, and an excellent sedative in balancing tri-deoshas (the three imbalances): VATA (regulation of body movement through the nervous system), PITTA (cause of all metabolic processes in the body), and KAPHA (principle of cohesion and function through the body fluids)[2,3].

The drug 'Sankhapushpum' of Ayurveda consists of the roots and seeds of *Clitoria ternatea* and is used as a 'tonic of the nerves', alternative and laxative. The leaves and roots are used in the treatment of a number of ailments including body aches, especially infections, urinogenital disorder, and as an antidote to animal stings. Among the two varieties, the white-flowered one is found to be more therapeutically active, and hence preferred. The blue variety is generally used in the treatment of various diseases, like indigestion, cases of ascetic, enlargement of the abdominal viscera, sore throat and skin diseases. They are also demulcent and given in chronic bronchitis. They are, however, administered with honey and ghee as a tonic to children for improving mental

*Corresponding author: Panneerselvam R, Professor and Head, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu, India – 608 002.
Tel: 0091 0144 232448, Ext: 354, 0091 9842383346
Fax: 0091 04144 238080
E-mail: rpselvam09@gmail.com
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faculties, muscular strength and complexion tonics and in epilepsy and insanity. The root-juice of the white-flowered variety is blown up the nostrils as a remedy for hemicranias. The decoction or powder of root is given in rheumatism, and as laxative, the action, however, is accompanied by griping in lower abdomen. The seeds are considered for colic, dropsy and enlargement of abdominal viscera; they are also used in swollen joints. The root, stem and flower are recommended for the treatment of snakebite and scorpion sting in India. A bioprospecting study using medicinal plants is important both in terms of adding economic values for biological resources and also creating an economic stake in conservation. Plant tissue culture is an efficient technique for the conservation and large-scale production of various rare, endangered, medicinal, and aromatic plants[4,5]. Although, explants of *Clitoria ternatea* has been reported[6] the lower levels if shoot regeneration are inadequate to meet the pharmaceutical demands. The maintenance *in vitro* culture of micropropagation is known to results in clonal viability[7]. However, in bioprospecting studies, it is an important to use generally stable clones. *In vitro* culture is medicinal plants, which has the ability to synthesize the chemicals de novo under controlled conditions and can be utilized to produce the medicinally important compounds similar to that of the mother plants. The reproducible and efficient propagation protocol is to produce genetically uniform plants. In addition, we also tested the antibacterial activity of *in vitro* raised callus which can be established on an alternative to the wild source of *Clitoria ternatea*.

## 2. Materials and methods

The seeds were collected from naturally growing plants in Annamalai University Campus. The seed coat was removed by scalpel without damaging the embryo because the hard seed coat interrupts the germination and followed by the seeds surface sterilized by sequential washing with 1% sodium hyphochlorite, 70% ethanol for 5 minutes. After the treatments seeds were allowed to sink 0.1% mercuric chloride. The seeds were finally washed with double distilled water and inoculated over the filter paper aseptic condition for germination.

The young and aseptically grown explants were inoculated in MS basal medium supplemented with 2,4-D (0.5-2.0 mg/L) and KN (0.5 mg/L)for callus formation. For the shot multiplication MS medium was supplemented with different concentrations cytokinins 6-BA (0.25-1.25 mg/L), KN (0.5- 2.5 mg/L) and in combination with indole-3-acetic acid (IAA) (0.5 mg/L). Finally root initiation was tested in half strength of MS medium with addition of NAA (0.5-2.0 mg/L) and IAA (0.5-2.0 mg/L). The pH of the media was adjusted 5.8 before adding gelling agent with 0.8% agar, prior to autoclave at 121 ℃ for 15 minutes. The cultures were maintained under controlled conditions such as (25±2) ℃ temperature, (60± 10)% relative humidity, and 16/8 hours photoperiod from cool white fluorescent lamps, with 3000 lux light. Well rooted micropropagules were placed in water along with test tubes for 20 minutes to lose the agar solidification medium, then tubes were inverted to separated the young plants. The individual plants were transferred to pots containing a mixture of soil and sand (1:1), and grown in the green house for acclimatization. Initially, the pots were wrapped with plastic bags having two or three holes for aeration [temperature (25±5) ℃; humidity (80±10)%; maximum light 200 $\mu molm^{-2} S^{-1}$]. After two weeks, the plastic bags were completely removed out, the plants were allowed to transferred to field conditions and watering was made at two days intervals.

### 2.1. Extraction

The tissue culture raised regenerative callus as well as naturally grown plants of *Clitoria ternatea* were collected and dried in shade. The 5 g powder of each sample was soaked separately in 25 mL of methanolic, petroleum ether and ethyl acetate for 24 h at room temperature. Then, the mixture was filtered through Whatman No-1 filter-1 paper. The filtrates obtained were concentrated (to volume of 4 mL) in porcelain dish at 40 ℃. The extracts were preserved at -20 ℃ in screw cap glass vials till further experiments.

### 2.2. Antibacterial assay

The test organisms *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus aureus* and *Salmonella typhi* were collected from Department of Medical Microbiology, Raja Sir Muthaiah Medical college, Annamalai University, Annamalai Nagar, Tamil Nadu, India. These pathogenic organisms were maintained aseptically by using nutrient agar medium. Antibacterial assay were carried out using the disk diffusion method described by Malakzadeh *et al* in 200[8]. 20 μL suspensions containing $10^5$ CFU/mL of bacterial cultures were spread on nutrient agar medium. The disk (6 mm in diameter) impregnated with 10, 20, 30, 40 and 50 mg of extracts were placed on the inoculated nutrient agar petridishes. Negative controls were prepared using the same solvents employed to dissolve the extracts of plant material. All the petridishes were incubated at 37 ℃ for 18 hrs. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms in comparison to negative control and each assay was repeated thrice.

### 2.3. Statistical analysis

A completely randomized design was used in all experiments. The experiments were repeated at least thrice. The data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range tests at $P < 5\%$.

## 3. Results

In present study the callus formation was achieved in leaf, node and petiole explants by addition of 2,4-D and KN combinations (Table 1). Among the three explants the leaf explants showed maximum callus in MS basal medium with supplemented with 2,4-D 1.0 mg/L and KN 0.5 mg/

**Table 1.**
Effect of cytokinin and auxin on callus formation of *Clitoria ternatea* from leaf, node and petiole explants.

| Explants | Hormones concentration | | Callus formation | % response |
|---|---|---|---|---|
| | 2,4-D | KN | | |
| Leaf | 0.5 | 0.5 | 5.1±1.1a | 63 |
| | 1.0 | 0.5 | 10.9±1.3b | 86 |
| | 1.5 | 0.5 | 7.0±0.9b | 70 |
| | 2.0 | 0.5 | 6.0±0.4c | 56 |
| Node | 0.5 | 0.5 | 45.1±1.3a | 60 |
| | 1.0 | 0.5 | 85.1±1.1d | 68 |
| | 1.5 | 0.5 | 35.1±1.0c | 64 |
| | 2.0 | 0.5 | 25.1± 0.8c | 50 |
| Petiole | 0.5 | 0.5 | 7.6±0.8a | 46 |
| | 1.0 | 0.5 | 9.0±0.8a | 78 |
| | 1.5 | 0.5 | 6.0±0.4b | 38 |
| | 2.0 | 0.5 | 5.0±0.9c | 20 |

The values represented in the tables are mean ± SE of three independent experiments each containing 21 replicates. Data were recorded after 28 d of culture incubation. Values followed by same letter were not significantly different at $P<0.05$ according to DMRT.

**Table 2.**
Effect of different concentrations of plant growth regulators on *in vitro* shoot multiplication from derived callus of *Clitoria ternatea* L.

| Growth regulators (mg/L) | | No. of shoots per culture | No. of nodes per culture | Length of shoot (cm) |
|---|---|---|---|---|
| BA | IAA | | | |
| 0.25 | -- | 21.2±0.6a | 3.2±0.2a | 4.6±0.2a |
| 0.50 | - | 24.4±0.4b | 3.6±0.4b | 5.8±0.4a |
| 0.75 | - | 31.6±0.5c | 4.4±0.2d | 5.2±0.5c |
| 1.0 | - | 26.2±0.6a | 5.2±0.4c | 5.0±0.6c |
| 1.25 | - | 32.8±0.2a | 4.2±0.5c | 5.4±0.2d |
| 0.25 | 0.5 | 48.6±0.4b | 4.2±0.4d | 6.2±0.4b |
| 0.50 | 0.5 | 58.2±0.5a | 5.8±0.2a | 6.8±0.2b |
| 0.75 | 0.5 | 74.2±0.8a | 7.6±0.7a | 7.9±0.7c |
| 1.0 | 0.5 | 62.0±0.7c | 6.2±0.2b | 8.6±0.4c |
| 1.25 | 0.5 | 49.2±0.5a | 5.9±0.4b | 7.2±0.2b |
| KN 0.25 | - | 18.8±0.4a | 3.4±0.3c | 6.2±0.3c |
| 0.50 | - | 26.4±0.8a | 4.1±0.5d | 6.6±0.6a |
| 0.75 | - | 32.7±0.6b | 3.4±0.4c | 7.2±0.5a |
| 1.0 | - | 28.6±0.7c | 3.6±0.6c | 5.8±0.4b |
| 1.25 | - | 41.6±0.5b | 2.8±0.2a | 5.6±0.2a |
| 0.25 | 0.5 | 46.5±0.4c | 3.8±0.5a | 8.6±0.2c |
| 0.50 | 0.5 | 59.2±0.2a | 4.6±0.3a | 11.7±0.4a |
| 0.75 | 0.5 | 44.8±0.6a | 5.4±0.4b | 10.6±0.2b |
| 1.0 | 0.5 | 39.6±0.6a | 4.6±0.6b | 9.2±0.2c |
| 1.25 | 0.5 | 28.8±0.4c | 4.1±0.6c | 9.6±0.1b |

The values represented in the table are mean ± SE if three independent experiments each containing 21 replicate. Data were recorded often 28 d of culture incubation. Values of followed by same letter were not significantly different at $P<0.05$ according to DMRT.

**Table 3.**
Effect of auxins (NAA and IAA) on rooting of *in vitro* regenerated shoots of *Clitoria ternatea* L.

| Growth regulator | Concentration (mg /L) | Rooted shoots (%) | Mean No. of roots/ shoot | Mean root length (mm) | Survival (%) |
|---|---|---|---|---|---|
| NAA | 0.5 | 100[z] | 5.2±0.3a | 12.4±0.1a | 80 |
| | 1.0 | 100[z] | 6.1±0.3a | 13.4±0.2a | 98 |
| | 1.5 | 100[z] | 4.5±0.4b | 13.2±0.6c | 69 |
| | 2.0 | 100[z] | 4.4±0.2a | 10.6±0.2b | 55 |
| IAA | 0.5 | 70 | 2.1±0.2a | 7.3±0.4e | 72 |
| | 1.0 | 72 | 1.9±0.4d | 6.8±0.2a | 76 |
| | 1.5 | 90 | 1.5±0.6b | 5.9±0.2a | 58 |
| | 2.0 | 96 | 1.1±0.9c | 4.6±0.3c | 54 |

The values represented in the table are mean ± SE of the independent experiments each containing 21 replicates/ data were recorded after 28 d of culture incubation. Values followed by same letter were not significantly different at $P<0.05$ according to DMRT.
[z] – *In vitro* regenerated shoots revealed root formation along with callus induction at the cut end of shoot.

**Table. 4.**
Antibacterial activity of Methanolic extracts of plant and callus of *Clitoria ternatea* (measured in terms of diameter of zone of inhibition (mm) around the disk impregnated with extract).

| Bacterial strains | *In vitro* raised plants extracts (mg/disk) | | | | | Callus extract (mg/disk) | | | | | Wild plant extract (mg/disk) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 | 10 | 20 | 30 | 40 | 50 | 10 | 20 | 30 | 40 | 50 |
| *Bacillus subtilis* (+) | – | – | 4 | 6 | 7 | 9 | 10 | 12 | 13 | 15 | – | – | 6 | 8 | 9 |
| *Bacillus cereus* (+) | – | – | 5 | 8 | 9 | 9 | 11 | 12 | 14 | 15 | – | – | 7 | 8 | 10 |
| *Staphylococcus aureus* (+) | – | – | 8 | 11 | 12 | 8 | 10 | 12 | 13 | 14 | – | 6 | 7 | 9 | 10 |
| *Proteus vulcaris* (–) | – | – | – | 8 | 10 | – | 9 | 11 | 13 | 13 | – | – | – | 8 | 10 |
| *Klebsiella pneumonia* (–) | – | 8 | 10 | 12 | 13 | 21 | 22 | 24 | 25 | 26 | – | 7 | 8 | 10 | 11 |
| *Salmonella typhi* (–) | – | – | – | 6 | 8 | – | 6 | 7 | 8 | 10 | – | 6 | 8 | 9 | 11 |

The antibacterial activity was tested based on disk diffusion assay (Malekzadesh *et al* 2001).

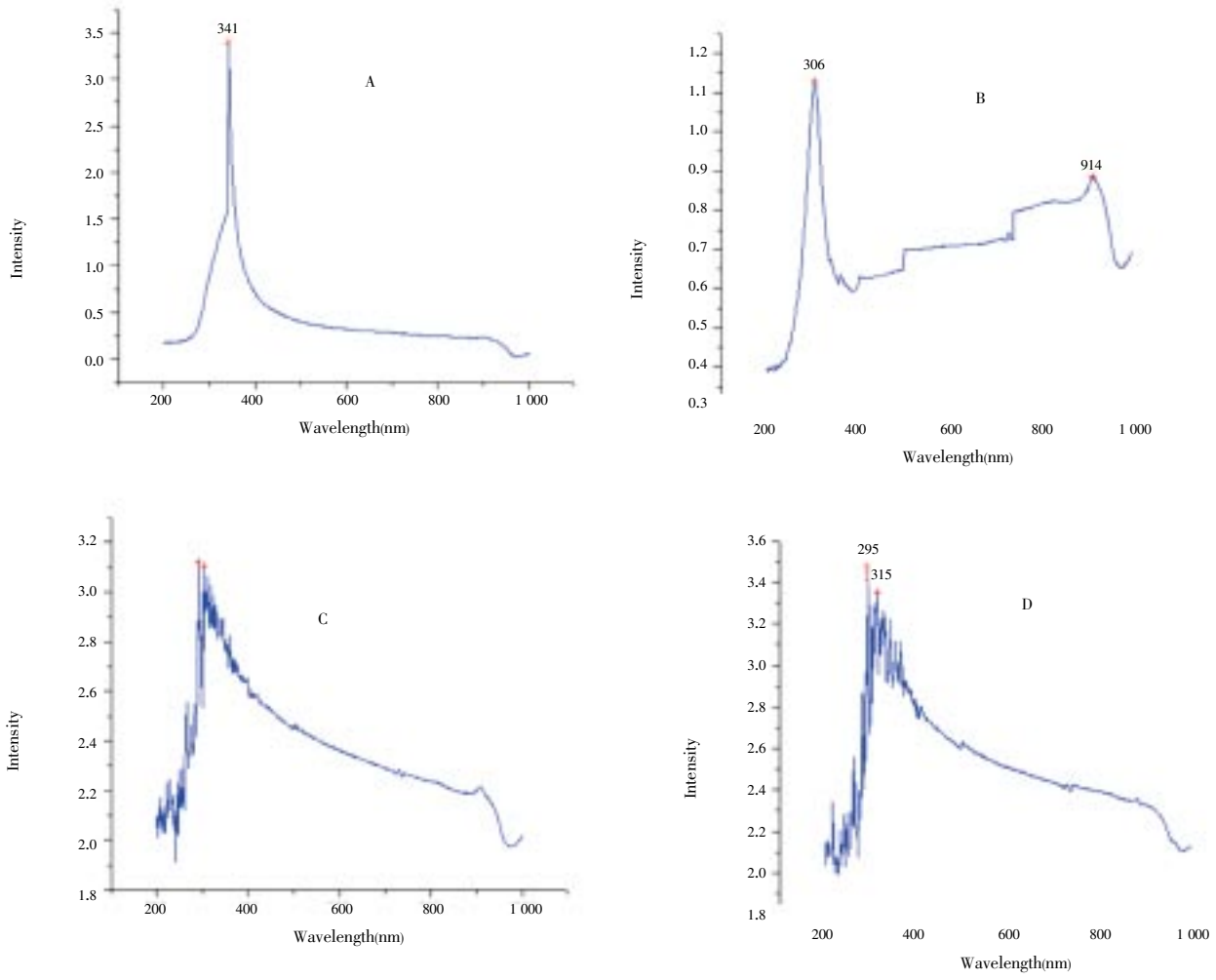


**Figure 1.** Spectral analysis of *M. armatus* mucus extracts (A, B, C and D).

L ( Figure 1b&c). The medium supplemented with 2,4-D 1.0 mg/L and KN 0.5 mg/L also resulted in elongation of shoots (Figure 2 a&b) (Table 2). It showed that MS medium containing 0.75 mg/L BA and 0.5 mg/L IAA induced shoots. Proliferation was 74 per explants within 6 weeks of (Table 2, Figure 2a&b). After 6 weeks of cultured explants produced an average shoot length of 7.9 cm in 0.75 mg/L BA and 0.5 mg/L NAA. Furthermore, the proliferation rate was maintained and shoot regeneration frequency was improved on regulator subculture to the same fresh medium over a period of 6 weeks. After 6 weeks of culture initiation, the highest average shoot length recorded was 11.7 cm, and 10.6 cm on medium containing 0.5 mg/L KN combination with 0.5 mg/L IAA; KN 0.75 mg/L and combination with 0.5 mg/L IAA, respectively (Table 2, Figure 2c). Regenerated shoots about 3 to 4 cm in height were separated and transferred to rooting media. All the treatments tested in the present study induced rooting, including 1/2 strength MS medium with auxins NAA (0.5-2.0 mg/L) and IAA (0.5-2.0 mg/L). The root induction was as high as 98%, with an average of 6.1 roots and 13.4 mm in length by the addition of NAA 1.0 mg/L within 8 weeks (Table 3, Figure 2d). Supplementation of medium with IAA decreased the rooting number of root per shoot, and length of roots when compared to NAA treatments

In the present study, antibacterial activity of *in vitro* was compared between raised plants and wild plants. Among the

various types of solvent extract, only methanolic extract showed zone of inhibition against the selected test organisms. The methanolic extracts of leaf-derived callus revealed strong antibacterial activity against all the test micro-organisms as compared to *in vitro* raised and wild plants (Table 4). Negative controls which used the same solvents to dissolve the extracts of plant material showed no response of zone of inhibition (data not presented). In

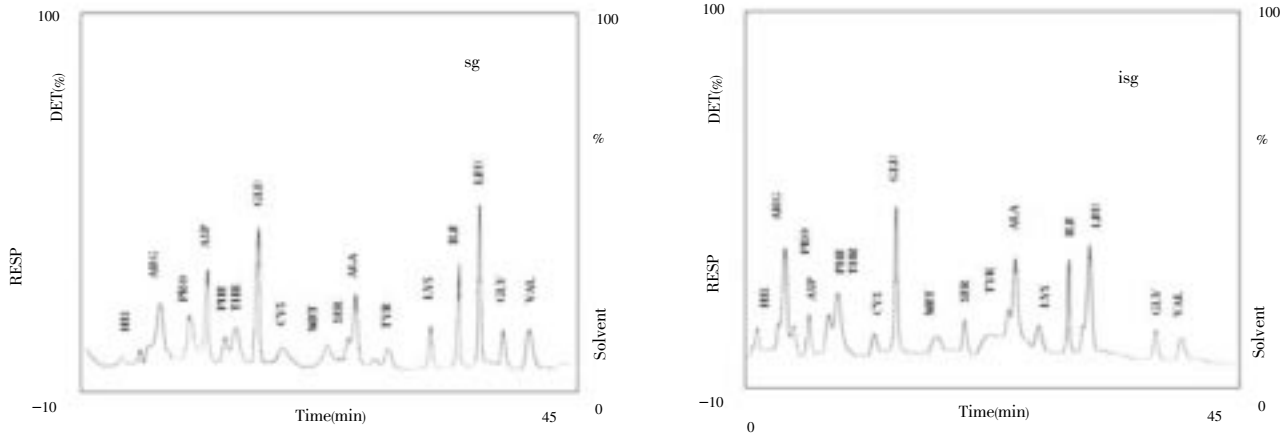


**Figure 2.**The chromatograms showing amino acid profiles of *M . armatus* mucus . sg- Soluble portion isg- Insoluble portion.

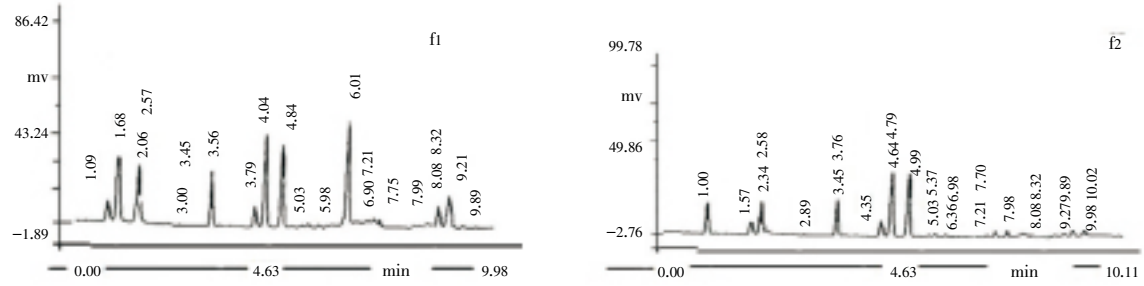


**Figure 3.** The Gas Chromatogram showing the fatty acid profiles of *M. armatus*. f1-Soluble Gel and f2- In Soluble Gel.

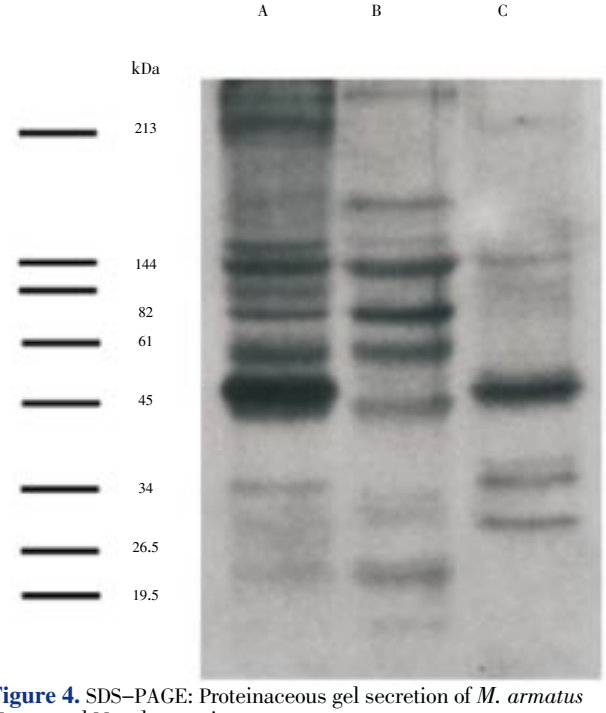


**Figure 4.** SDS-PAGE: Proteinaceous gel secretion of *M. armatus* Mucus and Muscle protein.
A = Molecular weight marker, B=Muscle protein, C= Mucus protein.

order to test the presence of superior chemical constituents of methanolic callus extract as compared to other explant sources, isoflavanoids content were estimated. The higher antibacterial activity of methanolic callus extract revealed significantly the highest content of isoflavanoids [(0.17±0.04) mg/L DW] in comparison with wild plant [(0.15±0.02) mg/L DW]. These results indicate that the leaf derived callus with superior chemical constituents (preferably isoflavanoid) may be used as a substitute to root biomass of naturally grown *Clitoria ternatea* in the preparation of pharmaceutical preparation.

## 4. Discussion

This study showed shoot regeneration response of explants cultured on MS basal media supplemented with various concentration of BA, KN individually and combined with IAA. The inclusion of cytokonins and auxin caused a swelling the base of explants after 6-10 d of culture, and the addition of a cytokonins, auxin to medium was essential to induce axillary shoot proliferation. Of the three different growth regulators tested (BA, KN individually and IAA combined, BA at 0.75 mg/L and IAA 0.5 mg/L was the most effective to shoot multiplication. The concentration and type of cytokinin along with auxin used significantly affected

the number of shoot, number of nodes and length of shoots regeneration. Previous studies showed high frequency of adventitious bud induced from nodal explants (7-32 buds per explants) on MS medium containing BA[9] or in combination with IAA and adenine sulphate[10]. Exogenous auxins are often used in a number of plants species to promote *in vitro* rooting of *in vitro* produced microshoots. There are other reports of NAA being effective in stimulation adventitious such as Zhang[11], Divya Goyal and Seema Bhadauria[12]. The present study also demonstrated that the superiority of NAA over IAA in the induced development of adventitious root formation. Auxin plays a major role in the rooting process and their efficacy depends on several factors and their depends on several factors protein involved that reaches target component cells, the amount of endogenous auxins and the metabolic stability. However, in our study NAA was almost effective in inducing adventitious root theory proposed by Focaga and Fett-Neto[13]. Secondary metabolites are usually not distributed uniformly within the whole plant. Some are restricted to specific organ and may be synthesized in small quantities. Accumulation also depends very much on the seasons, on the developmental stage of the plants, and on surrounding biotic and abiotic factors. Besides, the overexploitation and anthropogenic factors also result in the depletion of natural plant resources[14].

The current study denoted the production of the maximum number of shoots (almost 74 shoots) per culture which revealed a definite advantage over the previous reports for *in vitro* multiplication of *Clitoria ternatea*. The high shoot regeneration and transplant survival rates suggest that the protocol can be employed for the medicinal plant species. The presence of strong antibacterial activity with higher isoflavanoid content in the *in vitro* raised cell biomass offers the possibility for establishment of an alternative system to wild plant biomass, which should help in bioprospecting of *Clitoria ternatea*.

## Conflict of interest statement

We declare that we have no conflict of interest.

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