Identification and characterization of phytoconstituents of ethanolic root extract of *Clitoria ternatea* L. utilizing HR-LCMS analysis

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**ABSTRACT**
Medicinal plants act as a vital source in improving health and overcoming the side effects of modern-day medicine. Many evidence-based reports are present in the literature about the benefits of medicinal plants. *Clitoria ternatea* L. belongs to the family Fabaceae and is known to be one of the important Ayurvedic medicinal plant whose uses are specified mainly for the modification of nervous system activities. ‘Medhyarasayana’ is one of the Ayurvedic formulations which is used to promote the intellectual capacity, revive the body and nervous tissue, *Clitoria ternatea* serves as a major constituent of ‘Medhyarasayana.’ Identification and characterization of active metabolites of *C. ternatea* will help to isolate the important phytoconstituents responsible for the central nervous system effects, isolated components can be utilized in future for the formulation of new medicine for various neurodegenerative disorders. In the present study, the phytochemical evaluation of the ethanolic root extract of *C. ternatea* (EECT) was performed using the HR-LCMS technique. Preliminary qualitative phytoconstituents analysis showed the presence of tannins, alkaloids, saponins, steroids, carbohydrate, protein, flavonoids and triterpenoids in the ethanolic root extract. Almost 42 compounds were identified when the EECT subjected to HR-LCMS analysis.

**Materials and Methods**

**Collection and Preparation of *Clitoria ternatea* root extract**
Fresh roots of the white variety of wild *C. ternatea* were collected from Kerala, India. Authentic

**Introduction**
Medicinal plants are considered as amusing resources of ingredients that can be used in drug discovery and development as they are a very vital source to improve health and to overcome adverse effects of allopathic medicine. Many evidence-based reports are present in the literature about the benefits of medicinal plants and their biochemical and molecular effects (1). Worldwide a huge percentage of the population utilize medicinal plants and herbs for their health purpose. Therefore, scientific scrutiny of their phytoconstituents, therapeutic potential, biological properties and safety will be valuable in making wise decisions about their use. (2, 3) Ayurveda is one of the most popular Indian traditional health care systems which labels several herbal preparations which are well-known to uphold health and endurance. 'Rasayana' is the common term representing one of such herbal preparations which is ultimate for the progress of tissue functions in addition to their role as micronutrients (4). ‘Medhyarasayana’ is an Ayurvedic preparation made from the selected plant extracts to revitalize the brain by acting on the nervous system (5).

*Clitoria ternatea* L. belongs to the family Fabaceae, is a perennial twining herb with terete steam. It possess two varieties- white-flower and blue flower varieties (6). The local name is ‘Shankhpushpi’ and this is one of the ‘Medhyarasayana’ ingredients and is reported to promote intellectual capability, revive the body and nervous tissue and because of all these properties it has been widely used as a brain tonic (6). Scientific studies also reported other medicinal properties including antidepressant and anticonvulsant (7), anti-inflammatory, analgesic and antipyretic (8), local anesthetic (9), purgative (10) and anti-diabetic (11) activity. It is also used for the treatment of snakebite and scorpion sting in India (12). In the present study, the phytochemical evaluation of the ethanolic root extract of *C. ternatea* (EECT) was performed using the HR-LCMS technique.
identification was carried by taxonomist Prof.P.Jayaraman, Director, Plant Anatomy and Research Centre, West Tambaram, Chennai. India. A voucher specimen (SES.CLBM.NO. 1458) has been deposited at the Herbarium of Department of Pharmacognosy, C.L.Baid Metha College of Pharmacy, Chennai, India. The collected materials were shade dried at room temperature to remove moisture, then coarsely powdered by using an electric grinder. The powdered materials were stored in an air-tight container and used for further extraction.

**Extraction procedure**

Extraction of roots was carried out using ethanol by continuous hot extraction method using Soxhlet apparatus. The obtained extract was concentrated by gentle heating followed by using rotarat vacuum evaporator. The concentrated extract was then weighed, calculated the percentage yield and stored. The extract was subjected to various preliminary phytochemical tests and HR-LCMS analysis (13). The qualitative phytochemical tests were performed for alkaloids, flavonoids, glycosides, phenolics, terpenoids, saponins, carbohydrate, protein, amino acids and triterpenoids (14, 15).

**High-Resolution Liquid Chromatography and Mass Spectrometry (HR-LCMS) analysis**

The HR-LCMS analysis of the extract was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Mumbai. Methanol was used as the solvent for the preparation of extract and this process was done before subjecting the extract for analysis. Agilent high-resolution liquid chromatography and mass spectrometry model- G6550A (0.01% mass resolution) was used to prepare the chemical fingerprints of the subjected extract. The acquisition method was set to be Mass- minimum range 50 dalton (M/Z) and maximum 1000 Dalton (M/Z). The scanning was done with a rate of each spectrum per second (16).

Hip sampler G4226A-model with ancillary speed 100 µl/min, ejection speed 100 µl/min, flush out factor 5 µl and 8 µl injection volume was used for HR-LCMS. (15) Acquisition time was 30 min with initial 2 min of flow of solvent. The solvent composition used for HR-LCMS was 95: 5-100% water and 100% Acetonitrile. Column details –Hypersil GOLD C18 100 x 2.1mm-3MICRON.

**Identification of components**

Interpretation on mass spectrum HR-LCMS was carried out by comparing the spectrum of unknown components with known components spectrum. For comparison, we have utilized the SAIF -IIT Bombay database, where they have been stored more than 62000 patterns of the spectrum. The name, molecular weight and structure of the components of the trial materials were determined.

**Results and Discussion**

The percentage yield of (EECT) was found to be 10.4%w/w. Preliminary phytochemical evaluation of EECT showed the presence of tannins, alkaloids, saponins, steroids, carbohydrate, protein, flavonoids and triterpenoids (Table 1).

HR-LCMS analysis of EECT showed different major peaks indicating the presence of various phytochemical constituents. The characterization and identification of constituents were done by performing a comparison with the HR-LCMS spectrum of SAIF library compounds. The HR-LCMS study was performed for both positive and negative mode of ionization, the respective chromatogram is represented in Fig. 1 and Fig. 2. The fingerprint obtained was interpreted and mentioned (Table 2, 3). Positive ionization ESI of EECT showed 24 compounds and negative ionization ESI of EECT showed 18 compounds. The MS zoom spectrum of few important compounds identified by both positive and negative ionization ESI are also represented (Fig. 3, Fig. 4).

Neuropsychopharmacologic effects of various crude root extract of *C. ternatea* were reported by different researchers. It was reported that the oral intubation of CT aqueous root extract had shown a significant increase in learning and memory of postnatal and young adult Wistar rats (17). In another study, there are reports the *in vitro* effects of 200 mg/ml of *C. ternatea* aqueous root extract on proliferation, differentiation and growth of anterior subventricular zone neural stem cells derived from prenatal and postnatal rat pups (18). Acetylcholine (ACh) and Acetylcholinesterase (AChE) activity modification in connection with memory and cognitive enhancement of laboratory rodents upon administration of various root extracts of *C. ternatea* was reported by various researchers (19, 20). Anti-depressant and anti-anxiety effects of different root extracts have been studied and reported by different scientists (21, 22).

Even though the preclinical trial on rodents with various crude root extract of *C. ternatea* reported promising results on nervous system, a detailed study on isolated compounds from the root was not done so far. The present study imparts light on various constituents in root ethanolic extract. As per the results of the present study, the identified compounds like Chelidonine, Gibberellin, Elephantopin, Deoxy saponone B 7,3'-dimethoxy ether acetate, 3 hydroxy-3'4'-dimethoxy flavone, Tuberoic acid, Pectolinarin, Isotectorigenin 7-methyl ether, Muconulatol, Biochanin A dimethyl ether and different amino acids may be responsible for the reported effects produced by the root. For the confirmation, a detailed fractionation and constituent's isolation research study have to be performed on its roots. A well-designed constituent isolation and preclinical studies with those isolated compounds will confirm the safety and efficacy of *C. ternatea* against different neurological disorders.

**Conclusion**

The present study investigated and specified the various active metabolites found in the ethanolic root extract of *Clitoria ternatea* by carrying out different qualitative phytochemical screening and HR-LCMS analysis. The results serve as a potential resource to explore the isolation, purification and pharmacological screening of
Table 1. Preliminary phytochemical screening of ethanolic root extract of *Clitoria ternatea* L.

| SL no. | Test for carbohydrates | Test for tannins and phenolics | Test for steroids | Test for triterpenoids | Test for flavones and flavonoids | Test for alkaloids | Test for Glycosides | Test for Proteins | Test for Saponins |
|--------|------------------------|-------------------------------|------------------|------------------------|---------------------------------|-------------------|-------------------|------------------|------------------|
| I      | Test for carbohydrates |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Molisch's test          |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of carbohydrates     |                  |                        |                                 |                   |                   |                  |                  |
| II     | Test for carbohydrates |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Benedicts test          |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of carbohydrates     |                  |                        |                                 |                   |                   |                  |                  |
|        | Fehlings test           |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of carbohydrates     |                  |                        |                                 |                   |                   |                  |                  |
| III    | Test for steroids       |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Salkowski's test        |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of steroids          |                  |                        |                                 |                   |                   |                  |                  |
|        | Libermann Burchard test |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of steroids          |                  |                        |                                 |                   |                   |                  |                  |
| IV     | Test for triterpenoids  |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Isoprenoid test         |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of triterpenoids     |                  |                        |                                 |                   |                   |                  |                  |
| V      | Test for flavones and flavonoids |           |                  |                        |                                 |                   |                   |                  |                  |
|        | Shinoda test            |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of flavanoids        |                  |                        |                                 |                   |                   |                  |                  |
|        | Aqueous sodium hydroxide test |                   |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of flavanoids        |                  |                        |                                 |                   |                   |                  |                  |
| VI     | Test for alkaloids      |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Mayer's test            |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of alkaloids         |                  |                        |                                 |                   |                   |                  |                  |
|        | Hager's test            |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of alkaloids         |                  |                        |                                 |                   |                   |                  |                  |
|        | Dragendorff's test      |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of alkaloids         |                  |                        |                                 |                   |                   |                  |                  |
|        | Wagner's test           |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of alkaloids         |                  |                        |                                 |                   |                   |                  |                  |
| VII    | Test for Glycosides     |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Liebermann's test       |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of glycosides        |                  |                        |                                 |                   |                   |                  |                  |
|        | Borntrager's test       |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of anthroquinone glycosides |            |                  |                        |                                 |                   |                   |                  |                  |
| VIII   | Test for Proteins       |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Millon's test           |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of proteins          |                  |                        |                                 |                   |                   |                  |                  |
|        | Biuret test             |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of proteins          |                  |                        |                                 |                   |                   |                  |                  |
| IX     | Test for Saponins       |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | Foam/Froth test         |                               |                  |                        |                                 |                   |                   |                  |                  |
|        | +                      | Presence of saponins          |                  |                        |                                 |                   |                   |                  |                  |

Fig. 1. HR-LCMS chromatogram (Positive ESI) of ethanolic root extract of *Clitoria ternatea* L.
Fig. 2. HR-LCMS chromatogram (Negative ESI) of ethanolic root extract of *Clitoria ternatea* L.

| Sl. No. | Compound                                                   | Retention time | Mass             | Molecular formula       | DB diff (ppm) | Hits (DB) |
|---------|------------------------------------------------------------|----------------|------------------|-------------------------|---------------|-----------|
| 1       | Chelidonine (+)                                            | 1.245          | 353.1258         | C20 H19 N O5            | 1.49          | 63        |
| 2       | Retusin dimethyl ether                                     | 1.586          | 312.0993         | C18 H16 O5              | 1.46          | 27        |
| 3       | Elephantopin                                               | 1.638          | 360.1199         | C19 H20 O7              | 2.83          | 31        |
| 4       | Sebacic acid                                               | 1.874          | 202.1222         | C10 H18 O4              | -8.33         | 2         |
| 5       | Mycophenolic acid                                          | 1.986          | 320.1271         | C17 H20 O6              | -5.53         | 31        |
| 6       | Deoxysappanone B 7,3'-dimethyl ether acetate               | 6.05           | 356.1247         | C20 H20 O6              | 3.57          | 21        |
| 7       | 7-[2 trifluoromethyl-4-(2-hydroxyphenyl)-1,3-dioxanecis-5-yl]hept-5Z-enolic acid | 6.439          | 374.1351         | C18 H21 F3 O5           | -2.75         | 21        |
| 8       | Deoxysappanone B 7,3'-Dimethyl ether acetate               | 6.713          | 356.1247         | C20 H20 O6              | 3.43          | 21        |
| 9       | 3-hydroxy-3',4'-dimethoxyflavone                           | 8.097          | 298.0831         | C17 H14 O5              | 3.5           | 16        |
| 10      | Isotectogenin, 7-Methyl ether                             | 8.231          | 328.094          | C18 H16 O6              | 1.79          | 7         |
| 11      | Tuberonic acid                                             | 9.675          | 226.1201         | C12 H18 O4              | 1.69          | 20        |
| 12      | Gibberellin A29                                            | 10.232         | 348.1583         | C19 H24 O6              | -3.03         | 29        |
| 13      | Anisodamine                                                | 10.492         | 305.1619         | C17 H23 N4O4            | 2.59          | 27        |
| 14      | 8-(1-Hydroxyethyl)tetodolac                                | 10.621         | 303.1467         | C17 H21 N4              | 1.3           | 47        |
| 15      | Triptolide                                                 | 11.506         | 358.1407         | C20 H22 O6              | 2.55          | 36        |
| 16      | Naloxol                                                    | 12.009         | 329.1622         | C19 H23 N4              | 1.5           | 49        |
| 17      | Butorphanol                                                | 12.846         | 219.2221         | C12 H29 N2O             | -10.41        | 1         |
| 18      | 2-Isoprenyl-3-hydroxy-5-methyl-a-pyrone                    | 13.901         | 194.0939         | C11 H14 O3              | 2.06          | 13        |
| 19      | Lys Ser Lys                                                | 14.17          | 361.224          | C15 H31 N5O5            | 23.0          | 3         |
| 20      | LTB4 ethanol amide                                         | 15.637         | 379.2733         | C22 H37 N4              | -2.86         | 7         |
| 21      | Cer(d18:0/16:0)                                            | 17.631         | 539.5262         | C34 H69 N3              | 2.87          | 1         |
| 22      | Anandamide (20:2, n-6)                                     | 18.23          | 351.3129         | C22 H41 N2O             | 2.45          | 1         |
| 23      | (Z)-N-(2-hydroxyethyl)icos-11-Enamide                     | 19.369         | 353.3286         | C22 H43 N2O             | 2.33          | 1         |
| 24      | Docosanamide                                               | 19.372         | 339.351          | C22 H45 NO              | -4.3          | 1         |
Table 3. HR-LCMS analysis of ethanolic root extract of *Clitoria ternatea* L. at Negative ESI

| Sl. No. | Compound                                      | Retention time | Mass    | Molecular formula | DB diff (ppm) | Hits (DB) |
|---------|------------------------------------------------|----------------|---------|-------------------|---------------|-----------|
| 1       | Pectolinarin                                   | 4.894          | 622.187 | C29H34 O15        | 3.6           | 2         |
| 2       | Glycogen                                       | 5.282          | 666.216 | C24H42 O21        | 8.78          | 3         |
| 3       | 5-Formiminotetrahydrofolic Acid                | 5.95           | 472.181 | C20H24N8 O6       | -0.06         | 14        |
| 4       | Levan                                          | 6.54           | 504.16  | C18 H32 O16       | 11.9          | 9         |
| 5       | Maltotriose                                    | 6.54           | 504.16  | C18 H32 O16       | 11.81         | 6         |
| 6       | Tyr Glu                                        | 6.622          | 438.176 | C19H26N4 O8       | -3.98         | 16        |
| 7       | Sappanone A 7-methyl Ether                     | 6.97           | 298.085 | C17 H14 O5        | -1.3          | 7         |
| 8       | Isotectorigenin, 7-Methyl ether                | 6.982          | 328.095 | C18 H16 O6        | -2.6          | 2         |
| 9       | 6,4-Dimethoxyflavon                            | 7.05           | 282.0894| C17 H14 O4        | -0.76         | 16        |
| 10      | Mucronulatol((+/-))                            | 7.151          | 302.116 | C17 H18 O5        | -2.14         | 12        |
| 11      | Elephantopin                                    | 7.894          | 360.1233| C19 H20 O7        | -6.66         | 33        |
| 12      | Epiafzelechin trimethyl Ether                  | 7.962          | 316.132 | C18 H20 O5        | -4.6          | 13        |
| 13      | Neu5Acalpha2-6Galbeta1-4Glicbeta-Sp            | 7.96           | 702.233 | C25H42N4O19       | 14.96         | 5         |
| 14      | Biochanin A, dimethyl Ether                     | 8.202          | 312.10  | C18 H16 O5        | -7.2          | 7         |
| 15      | 25-O-Deacetylrifabutin N-oxide                 | 9.7            | 820.4247| C4H60N4O11        | 1.43          | 2         |
| 16      | Telmisartan                                    | 10.997         | 514.2466| C33H30N4O2        | -18.91        | 4         |
| 17      | Cys Tyr Arg                                    | 14.815         | 440.185 | C18H28N6O5S       | -3.54         | 48        |
| 18      | DL-8-hydroxy stearic acid                      | 18.598         | 300.2679| C18 H36 O3        | -4.69         | 53        |

Fig. 3. HR-LCMS- MS Zoomed Spectrum of different compounds detected from ethanolic root extract of *Clitoria ternatea* L. at Positive ESI.

Fig. 4. HR-LCMS- MS Zoomed Spectrum of different compounds detected from ethanolic root extract of *Clitoria ternatea* L. at Negative ESI.
various secondary active metabolites from this traditionally well-known medicinal plant.

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Authors’ contributions

PM guided JKN in planning, designing and conducting the research experiment to obtain the data. PM and JKN participated in the manuscript draft and have thoroughly checked and revised the manuscript. The author(s) read and approved the final manuscript.

Conflict of interests

The authors declared that they have no conflict of interest.

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