Minireview Article

Clitoria ternatea - Shifting Paradigms: From Laboratory to Industry.

ABSTRACT

Clitoria ternatea commonly known as Butterfly pea is a standard Ayurvedic medicine in many parts of south Asian countries. *C. ternatea* possesses antibacterial, antiviral, and antifungal properties. However, responsible active compound/s isolation and development still remain in its infancy. Despite its enormous potential health benefits, only a single commercial product managed to reach industrial level production. *C. ternatea* cyclotide studies are also limited despite it is the fastest known natural ligase discovered to date.

In this mini summary we have tried to point out innate specific properties of *C. ternatea* and suggested few future studies, more specifically on *C. ternatea* cyclotides against bacterial ClpB for novel antimicrobial discovery and development.

Keywords: *Clitoria ternatea, Plant cyclotides, Bioactive compounds, Antimicrobial activity, Discovery of novel antibiotics, Bacterial ClpB, ESKAPE pathogens*

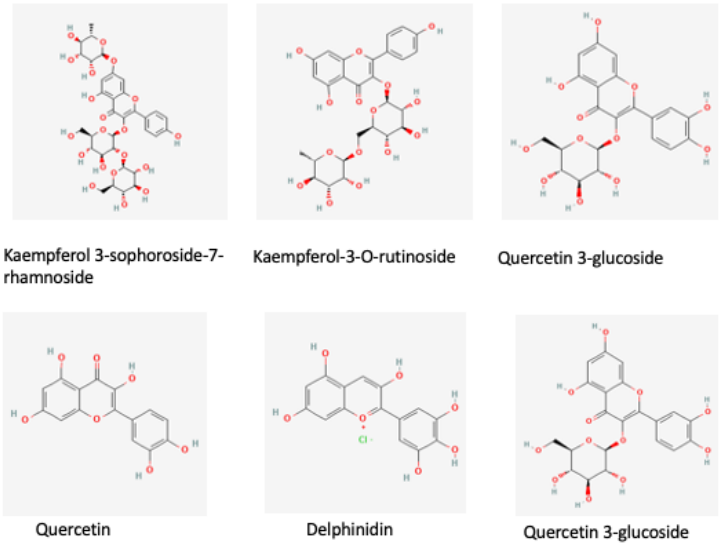
Introduction

Clitoria ternatea (CT), commonly known as 'blue pea' in English is an evergreen perennial plant which grows naturally in several tropical countries. There are two varieties of the plant: Blue flowered and white flowered plant (Figure 1). These flowers resemble to a conch shell and bear five petals. The leaves are compound, alternate, stipulate, and imparipinnate and the root system consists of a stout tap root with few branches and many slender lateral roots. The main root is thick which grows to more than two meters and has one to several glaucous, purplish, nodules. The roots fix nitrogen and have an acrid and bitter taste. In Ayurvedic and traditional medicine, decoctions and extracts made from CT and many other plants have been used extensively to treat many ailments, infections/disorders [1,2,3,4,5,6,7]. CT seeds contain mainly palmitic acid (19%), steric acid (10%), oleic acid (52%), linoleic acid (17%) as fatty major fatty acid compounds [8].

Figure 1. *C. ternatea* Blue, White colour flowers, Pods and plant are shown in clockwise arrangement. Figures were adapted from Royal Botanic Gardens, Kew, Plants of the world Online. http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:486606-1 Accessed on 05th November 2021.

2. MAJOR PHYTOCHEMICAL COMPOUNDS

Clitoria ternatea contains a significant number of phytochemicals (Figure 2) which are believed to be the major contributors for its unique antioxidant, antimicrobial, antidiabetic, anticancer and anti-inflammatory properties [8,17]. Flavanols and Anthocyanins are the major natural products found in *Clitoria ternatea* [8]. It has been reports that blue flowers contain Kaempferol 3-robonoside-7-rhamnoside, Kaempferol 3-rutinoside, Quercetin 3-glucoside and Myricetin 3-neophesperodosie while white flowers contain Kaempferol 3-glucoside, Kaempferol 3-rutinoside and Quercetin. *Clitoria ternatea* leaves mainly contain Kaempferol 3-glucoside and Kaempferol 3-rutinoside. Anthocyanins such as Ternatin A1 to A3, B1 and B2, C1 to C5 and D1 to D3 have been reported in blue flowers while Delphinidin has been reported in mauve flowers [8]. We urge the readers to refer to [8] for a detailed description of major phytochemical compounds.



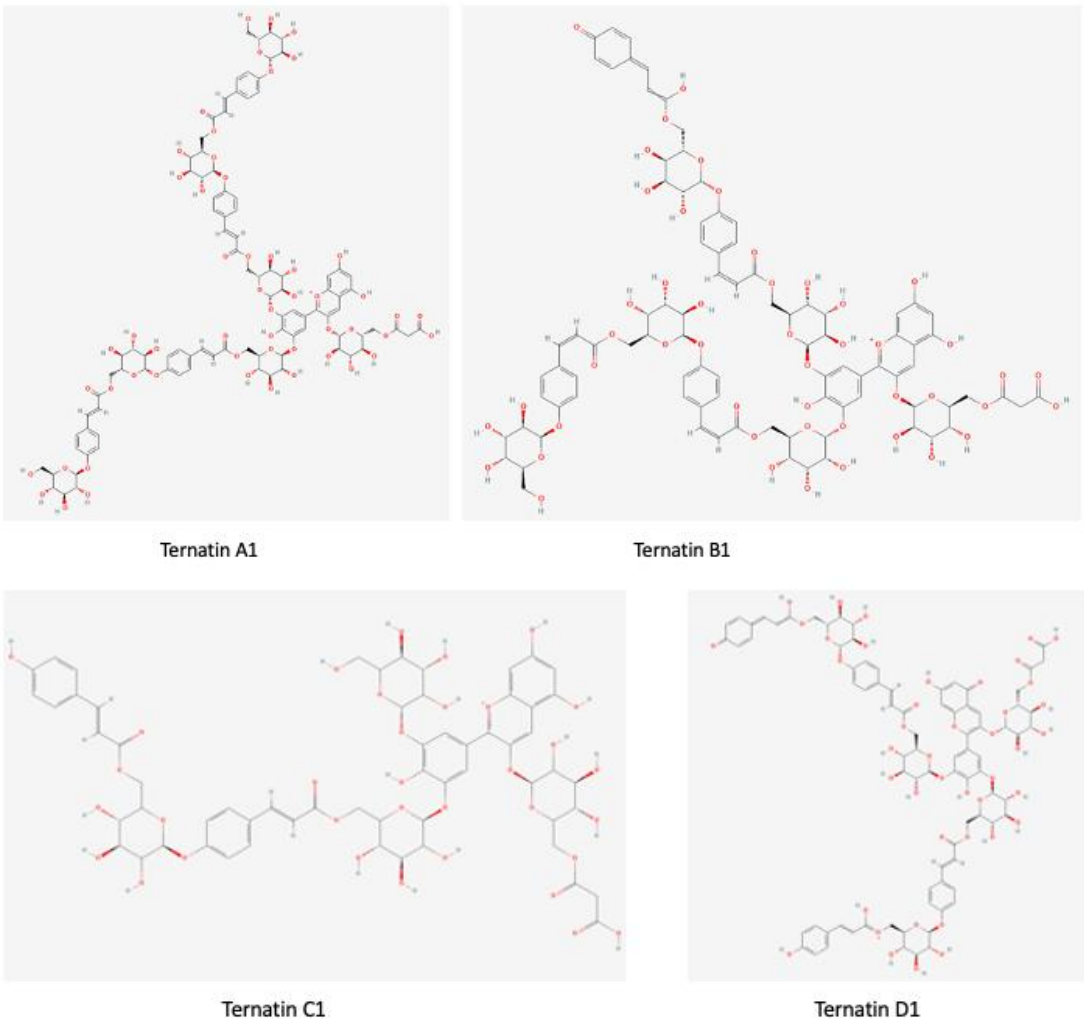


Figure 2: Few reported Phytochemical compounds found in *C. ternatea*. Compound structures were taken from PubChem, https://pubchem.ncbi.nlm.niwetwetwetdryh.gov/

3. MEDICINAL PROPERTIES AS REPORTED IN LITERATURE

As mentioned earlier CT has been used in traditional medicine for over centuries. This has stimulated investigators to unravel pharmacological effects of CT, using various plant extracts from CT. It has been well documented that CT extracts from leaves and roots had some significant anti-inflammatory, analgesic, and anti-pyretic activities. Recently in 2014, Ranaweera and colleagues [1] reported that aqueous root extracts of CT possessed a significant *in vitro* inhibitory activity against heat induced albumin denaturation process. This was a significant observation as this demonstrated that aqueous root extracts may content compounds for antirheumatic arthritic activity. Subhash C Mandal and colleagues and Debapriya Garabadu and colleagues had reported that methanolic root extracts showed a significant inhibition in Carrageenin induced paw oedema [9,10]. Interestingly, Subhash C Mandal and colleagues observed that methanolic extracts of CT had a noteworthy anti-pyretic activity in albino rats [9]. This effect was dose dependent, and they stated that ant-pyretic activity observed was comparable to the control drug (paracetamol) as well.

C. Kulkani in 1988 reported about local anaesthetic effect of CT [11]. According. To Manisha Bhatia, Jagbir Chahal and Sumeet Gupta, both petroleum and ether extracts of CT possessed a long-lasting analgesic effect up to 2 hours in albino male wistar rats [12]. S. BKasture and his group conducted an interesting study to investigate to determinine the spectrum of activity of the methanolic extract of *Clitoria ternatea* (CT) on the central nervous system [13]. They used methanolic extracts of CT and studied for its effect on cognitive behaviours, anxiety, depression, stress, and convulsions induced by pentylenetetrazol (PTZ) and maximum electroshock. Their study indicated that methanolic extracts of CT were found to possess nootropic, anxiolytic, antidepressant, anticonvulsant and antistress activity. In 2018, Sirichai Adisakwattana and colleagues conducted a series of elegant experiments to assess the effect of the *Clitoria ternatea* flower extract, on the inhibition of pancreatic α-amylase, in vitro starch hydrolysis, and predicted the glycemic index of different type of flours including potato, cassava, rice, corn, wheat, and glutinous rice flour [14]. According to Sirichai Adisakwattana and

colleagues, *Clitoria ternatea* flower extract could reduce the starch digestibility, predicted glycemic index and the hydrolysis index, of flour through the inhibition of carbohydrate digestive enzymes.

N Kamkaen and J M Wilkinson studied potential antioxidant activity of CT extracts and an extract containing eye gel formulation in 2009 [15]. They reported that aqueous extracts of CT had a stronger antioxidant activity (as measured by DPPH scavenging activity) than ethanol extracts. At the same time, they reported that when aqueous CT extracts were incorporated into the formulation eye gels, these gels were also shown to retain antioxidant properties. In a separate study where protective effect of CT flower extracts with antioxidant activity on male reproductive parameters were conducted by Sitthichai Iamsaard and colleagues in 2014 [16]. They had performed few well planned *in vitro* and *in vivo* experiments and they reported that CT flower extracts possessed antioxidant activity and extracts were not harmful to the male reproductive system. Their findings indicated that CT flower extracts can be used for protection against testicular damage in Ketoconazole induced rats. According to a study on chemical composition and anti-proliferative properties of flowers of *Clitoria ternatea* [18], showed that the water extracted of CT had significant effects against hormone dependent breast cancer cell line MCF-7 with an IC_{50} value of 175.35 µg/ml. Methanol extracts of CT had been used to study *in vivo* to evaluate its anticancer activity in Dalton's lymphoma (DLA) bearing mice [20]. The results from DLA bearing mouse study indicated suggest that methanol extract had a significant antitumour effect in DLA bearing mice

4. ANTIMICROBIAL PROPERTIES

The antimicrobial properties of *C. ternatea* extracts have been well documented. Sreenivasan Sasidharan and colleagues used methanolic extracts of CT of the leaf, stems, flower, seed, and roots to study its antimicrobial properties against 12 bacterial species, 2 yeast species, and 3 filamentous fungal by agar diffusion and broth dilution methods [21]. Their results showed that the leaf and root extracts were the most effective against all the tested organisms. In 2019, Kathirvel Brindhadevi and the group studied antimicrobial activities of crude extracts from *Clitoria ternatea* tested against the urinary tract infection causing pathogen *Proteus mirabilis* [22]. Kathirvel Brindhadevi and colleagues had used clinical samples for the respective study. According to their findings, the highest antibacterial activity was observed for acetone and the lowest antibacterial activities were observed for isopropyl alcohol, and petroleum ether extracts against *Proteus mirabilis*. Interestingly, in a recent review in 2021 stated that *Clitoria ternatea* plant extracts contain antiviral potential and can be used for COVID-19 prophylaxis efforts based on natural antiviral Plant extracts and their compounds [23]. At the same time, it is worth mentioning here that in Srilankan traditional medicine treatment regime *Clitoria ternatea* has been mentioned as a suitable plant to treat rabies [24].

Clitoria ternatea extract showed a favourable antifungal activity against *Aspergillus niger* [25]. S. Sasidharan and colleagues reported that the extract had an antifungal activity against *A. niger* with a minimum inhibition concentration 0.8 mg/mL and minimum fungicidal concentration 1.6 mg/mL, respectively. In 2014, Ajesh & Sreejith have reported a discovery of a novel antifungal protein with lysozyme-like activity from seeds of *Clitoria ternatea* [26]. They reported that this novel protein had lytic activity against *Micrococcus luteus* and broad-spectrum, fungicidal activity against clinically relevant yeasts, such as *Cryptococcus neoformans*, *Cryptococcus albidus*, *Cryptococcus laurentii*, *Candida albicans* and *Candida parapsilosis*. Sitthichai Iamsaard and colleagues [16] showed that *Clitoria ternatea* flower extracts can be used for protection against testicular damage in Ketoconazole induced rats. David J. Craik and colleagues conducted an extensive RNA-sequencing (RNA-seq) and gene expression profiling on *Clitoria ternatea* plant defence protein known as Cyclotides [27]. Cyclotides are plant proteins which has six conserved cysteines (Figure 3) that form a cystine-knot motif and protect plants from various attacks including pathogens [28, 32]. They had designed few elegant experiments using *C. elegans* to study *in vivo* functions of *C. ternatea* cyclotides. They discovered that cyclotide contained fractions from soil contacting organs were effective at killing nematodes, whereas similar enriched fractions from aerial parts contained cyclotides with stronger interactions with insect like membrane lipids. Based on reported data, generally *C. ternatea* root extracts seem to contain more potent anthelmintic components [29, 30, 31]. It is important to mention here that these cyclic peptides have been used recently to develop eco-friendly pesticide known as Sero-X® [33].

5. CURRENT AND POTENTIAL COMMERCIAL PRODUCTS

C. ternatea plant is used extensively in traditional medicine and reported to have several health benefits in recent findings at the international level. However, to date scientifically validated value-added products from *C. ternatea* is very limited worldwide. Currently, *C. ternatea* plant is being used by an Australian company known as 'Innovate Ag' to commercially produce a bio-insecticide with a trade name Sero-X (https://innovate-ag.com.au/ Accessed on 24/10/2021). According to 'Innovate Ag', Sero-X is a non-toxic, bee-friendly, world first plant extract bio-pesticide, which can be used as an alternative to traditional synthetic pesticides. Sero-X is a natural product that has a high efficacy against a wide range of pests, and no impact on non-target insects (https://innovate-ag.com.au/sero-x/ Accessed on 24/10/2021).
A research group from Sri Lanka attempted to develop a beverage using blue pea flower extract having additional health benefits [34]. Interestingly, they developed a range of blue pea flower incorporated beverages comprising a natural

sweetener (*Stevia* extract) and a flavour (lime). This experimental beverage had a significant antioxidant activity and was shelf stable for a period of 28 days without preservatives [34]. A lemon based mocktail drink with butterfly pea flower extract was used as a market trial [35] and found out that it could be introduced to the consumers as a non-alcoholic drink. Initial test results revealed that the product provides consumers with an appealing and uniquely coloured non-alcoholic drink with a pleasing taste.

A natural enzyme known as Butelase 1 is found in pods of the common medicinal plant *Clitoria ternatea* and it is the fastest known ligase to date [36]. Butelase 1 is a cyclase involved in the biosynthesis of cyclotides, the largest family of plant cyclic peptides [37]. Butelase 1 has the ability to ligase and catalyse peptide cyclization at an extraordinary rate. Butelase 1 cyclization reactions are 20,000 times faster than those of sortase A, a commonly used enzyme for backbone cyclization [36]. A detailed protocol for Butelase 1 purification has been established by James T Pam and his group [36]. According to this protocol Butelase 1 can be purified from pods of *C. ternatea* by a four-step chromatographic procedure to give ~5 mg of enzyme per kg of fresh plant material. Hence, Butelase 1 can be used as a versatile tool in protein engineering and general biological research.

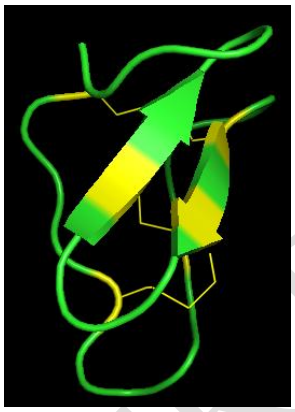


Figure 3: The structure of cyclotide kB1 from *O. affinis* is shown here, PDB code 1NB1. Location of Cysteine residues are shown in yellow and disulfide bonds are shown as yellow sticks. Figure prepared using Pymol molecular visualization system, https://pymol.org/2/ .

5. CONCLUDING REMARKS

Here we have attempted to outline enormous potentials and applications of *C. ternatea* as a medicinal plant. *C. ternatea*, has long been used as a traditional Ayurvedic medicinal plant and various pharmacological activities of *C. ternatea* has been reported in traditional medicine. However, it is worth mentioning here, that only a few biologically active compounds have been isolated from *C. ternatea.* Recently it has been shown that bacterial chaperone ClpB (Caseinolytic Peptidase B) can be used as novel antimicrobial target and development of novel antimicrobials [38,39,40]. Hence it would be interesting to investigate the effects of *C. ternatea* plant cyclotides against bacterial ClpB and ESKAPE pathogens. At the same time, it is important to mention that most of these *in vitro* experiments for *C. ternatea* were done by different research groups under different conditions. Hence, these experiments need to be performed under comparable conditions using valid control experiments. Claims such as *C. ternatea* being used to treat rabies needs to be investigated thoroughly using suitable animal models. Despite there are many exciting research about potential benefits of *C. ternatea*, only a single product which truly managed to find its ways to industrial applications. Therefore, it is important explore ways to translate these initial laboratory findings to next level with the idea of product commercialization.

REFERENCES

1: Ranaweera CB, Pathirana R, Ambalanduwa KC, Jayakody RA, Ratnasooriya WD. In vitro antirheumatoid arthritic activity of aqueous root extract of *Clitoria ternatea*. International research journal of pharmacy. 2014;5(12):926-8.

2: Silva AR, Dissanayake DM, Ranaweera CB, Pathirana R, Ratnasooriya WD. Evaluation of in vitro antibacterial activity of some Sri Lankan medicinal plants. Int J Pharmaceutical Res Allied Sci. 2015;4:54-7.

3: Ranaweera,CB. Abeysekara, WPKM , Pathirana, R., Ratnasooriya WD. In vitro antioxidant activity of methanolic extracts of leaves of *indigofera indica* and stems of *stereospermum suaveolens* grown in Sri Lanka, International Journal of Institutional Pharmacy and Life Sciences 2015 (2), 128-138

4: Ranaweera,C.B. Abeysekara, W.P.K.M , Pathirana, R., Ratnasooriya WD. Lack of in vitro antihyaluronidase activity of methanolic leaf extract of *Indigofera tinctoria* L and methanolic stem bark extract of *Stereospermum suaveolens* DC. Journal of Pharmaceutical Negative Results| Jan-Dec. 2015;6(1):40.

5: Ranaweera, CB, Vidanagamage, AS, Abeysekara, WPK, Silva, ARN, Chandana, AK, Premakumara, S, Pathirana, R, Ratnasooriya, WD. In vitro effects of aqueous extracts of five Sri Lankan medicinal plants on human erythrocyte membrane stabilisation activity. June, 2015,2(6) 0486-0489.

6: Ranaweera CB, Karunathilaka N, Silva AR, Karunarathna S, Pathirana R, Ratnasooriya WD. Antibacterial activity of aqueous root, seed, flower and stem bark extracts of *Acronychia pedunculata* grown in Sri Lanka. International Journal of Pharmaceutical Research & Allied Sciences. 2016 Jan 1;5(2):21-5.

7: Silva AR, Ranaweera CB, Karunathilaka RN, Pathirana R, Ratnasooriya WD. Antibacterial activity of water extracts of different parts of *Morinda citrifolia* grown in Sri Lanka. Int J Sci Res Publ. 2016;6:124-7.

8: Oguis GK, Gilding EK, Jackson MA, Craik DJ. Butterfly pea (*Clitoria ternatea*), a cyclotide-bearing plant with applications in agriculture and medicine. Frontiers in plant science. 2019 May 28;10:645.

9: Devi BP, Boominathan R, Mandal SC. Anti-inflammatory, analgesic and antipyretic properties of *Clitoria ternatea* root. Fitoterapia. 2003 Jun 1;74(4):345-9.

10: Singh NK, Garabadu D, Sharma P, Shrivastava SK, Mishra P. Anti-allergy and anti-tussive activity of *Clitoria ternatea* L. in experimental animals. Journal of ethnopharmacology. 2018 Oct 5;224:15-26.

11: Kulkarni C, Pattanshetty JR, Amruthraj G. Effect of alcoholic extract of *Clitoria ternatea* Linn. on central nervous system in rodents. Indian Journal of Experimental Biology. 1988 Dec 1;26(12):957-60.

12: Bhatia M, Chahal J, Gupta S. Analgesic and anti-inflammatory activities of *Clitoria ternatea* Linn. leaves extract on rat model. International Journal of Pharmaceutical Sciences and Research (IJPSR). 2014;5(2):600-6.

13: Kasture SB. *Clitoria ternatea* and the CNS. Pharmacol Biochem Behav. 2003;75:529536Jiang.

14: Chusak C, Henry CJ, Chantarasinlapin P, Techasukthavorn V, Adisakwattana S. Influence of Clitoria ternatea flower extract on the in vitro enzymatic digestibility of starch and its application in bread. Foods. 2018 Jul;7(7):102.

15: Kamkaen N, Wilkinson JM. The antioxidant activity of *Clitoria ternatea* flower petal extracts and eye gel. Phytotherapy Research. 2009 Nov;23(11):1624-5.

16: Iamsaard S, Burawat J, Kanla P, Arun S, Sukhorum W, Sripanidkulchai B, Uabun-Dit N, Wattathorn J, Hipkaeo W, Fongmoon D, Kondo H. Antioxidant activity and protective effect of *Clitoria ternatea* flower extract on testicular damage induced by ketoconazole in rats. Journal of Zhejiang University-SCIENCE B. 2014 Jun;15(6):548-55.

17: Jeyaraj EJ, Lim YY, Choo WS. Extraction methods of butterfly pea (*Clitoria ternatea*) flower and biological activities of its phytochemicals. Journal of food science and technology. 2021 Jun;58(6):2054-67.

18: Neda GD, Rabeta MS, Ong MT. Chemical composition and anti-proliferative properties of flowers of *Clitoria ternatea*. International Food Research Journal. 2013 Jul 1;20(3).

19: Shen Y, Du L, Zeng H, Zhang X, Prinyawiwatkul W, Alonso-Marenco JR, Xu Z. Butterfly pea (*Clitoria ternatea*) seed and petal extracts decreased HE p-2 carcinoma cell viability. International Journal of Food Science & Technology. 2016 Aug;51(8):1860-8.

20: Jacob L, Latha MS. Anticancer activity of *Clitoria ternatea* Linn. against Dalton's lymphoma. International Journal of Pharmacognosy and Phytochemical Research. 2012;4(4):207-12.

21: Kamilla L, Mnsor SM, Ramanathan S, Sasidharan S. Antimicrobial activity of *Clitoria ternatea* (L.) extracts. Pharmacologyonline. 2009;1:731-8.

22: Dhanasekaran S, Rajesh A, Mathimani T, Samuel SM, Shanmuganathan R, Brindhadevi K. Efficacy of crude extracts of *Clitoria ternatea* for antibacterial activity against gram negative bacterium (*Proteus mirabilis*). Biocatalysis and Agricultural Biotechnology. 2019 Sep 1;21:101328.

23: Sytar O, Brestic M, Hajihashemi S, Skalicky M, Kubeš J, Lamilla-Tamayo L, Ibrahimova U, Ibadullayeva S, Landi M. COVID-19 prophylaxis efforts based on natural antiviral plant extracts and their compounds. Molecules. 2021 Jan;26(3):727.

24: Anonymous. Osuvisithru Book. Volume 1. Published by Department of Ayurveda Sri Lanka Colombo 1994, pages 265-269.

25: Kamilla L, Mansor SM, Ramanathan S, Sasidharan S. Effects of *Clitoria ternatea* leaf extract on growth and morphogenesis of *Aspergillus niger.* Microscopy and Microanalysis. 2009 Aug;15(4):366-72.

26: Ajesh K, Sreejith K. A novel antifungal protein with lysozyme-like activity from seeds of *Clitoria ternatea*. Applied biochemistry and biotechnology. 2014 Jun;173(3):682-93.

27: Gilding EK, Jackson MA, Poth AG, Henriques ST, Prentis PJ, Mahatmanto T, Craik DJ. Gene coevolution and regulation lock cyclic plant defence peptides to their targets. New Phytologist. 2016 Apr;210(2):717-30.

28: Craik DJ, Daly NL, Bond T, Waine C. Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. Journal of molecular biology. 1999 Dec 17;294(5):1327-36.

29: Bhalke, RD, Nirmal SA, Girme AS, Ghogare PB, Jadhav RS,Chavan MJ. "Anthelmintic activity of *Clitoria ternatea* [Fabaceae]." Portal Regional da BVS, (2008): 19-21.

30: Kumari NV, Devi ML. Effect of some indigenous plant extracts on the inhibition of egg hatching of nematode *Meloidogyne incognita* Chitwood infesting mulberry. HortFlora Research Spectrum. 2013;2(1):35-9.

31: Salhan M, Kumar B, Tiwari P, Sharma P, Sandhar HK, Gautam M. Comparative anthelmintic activity of aqueous and ethanolic leaf extracts of *Clitoria ternatea*. Int J Drug Dev Res. 2011 Jan;3(1):62-9.

32: Poth AG, Colgrave ML, Lyons RE, Daly NL, Craik DJ. Discovery of an unusual biosynthetic origin for circular proteins in legumes. Proceedings of the National Academy of Sciences. 2011 Jun 21;108(25):10127-32.

33: Oguis GK, Gilding EK, Huang YH, Poth AG, Jackson MA, Craik DJ. Insecticidal diversity of butterfly pea (*Clitoria ternatea*) accessions. Industrial Crops and Products. 2020 May 1;147:112214.

34: Lakshan SA, Jayanath NY, Abeysekera WP, Abeysekera WK. A commercial potential blue pea (*Clitoria ternatea* L.) flower extract incorporated beverage having functional properties. Evidence-Based Complementary and Alternative Medicine. 2019 May 20;2019.

35: Bawar SD, Development of mocktail drinks with butterfly pea flower extract. Food & Beverage Services,TESDA Women's Center.
http://twc.tesda.gov.ph/researchanddevelopment/researches/01%20DEVELOPMENT%20OF%20MOCKTAIL%20DRINKS%20WITH%20BUTTERFLY%20PEA%20FLOWER%20EXTRACT.pdf

36: Nguyen GK, Qiu Y, Cao Y, Hemu X, Liu CF, Tam JP. Butelase-mediated cyclization and ligation of peptides and proteins. Nature protocols. 2016 Oct;11(10):1977-88.

37: Serra A, Hemu X, Nguyen GK, Nguyen NT, Sze SK, Tam JP. A high-throughput peptidomic strategy to decipher the molecular diversity of cyclic cysteine-rich peptides. Scientific reports. 2016 Mar 11;6(1):1-3.

38: Glaza P, Ranaweera CB, Shiva S, Roy A, Geisbrecht BV, Schoenen FJ, Zolkiewski M. Repurposing p97 inhibitors for chemical modulation of the bacterial ClpB–DnaK bichaperone system. Journal of Biological Chemistry. 2021 Jan 1;296.

39: Ranaweera CB, Glaza P, Yang T, Zolkiewski M. Interaction of substrate-mimicking peptides with the AAA+ ATPase ClpB from *Escherichia coli*. Archives of biochemistry and biophysics. 2018 Oct 1;655:12-7.

40: Ranaweera CB, Glaza P, Zolkiewski M. Interaction of substrate-mimicking peptides with the AAA+ ATPase ClpB from *Escherichia coli*. poster http://dx.doi.org/10.13140/RG.2.2.19936.79363