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RESEARCH ARTICLE

Antibacterial and *in vitro* Anticancer Study of Methanol Extracts of *Clitoria ternatea* Leaves

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Abstract

Clitoria ternatea is often known as butterfly pea plant by the natives and is one of the major plants used in *Ayurveda* and traditional medicine worldwide. It is also used in food and cosmetic industries for various purposes. In this study, methanol leaf extract of *Clitoria ternatea* was studied for its antibacterial and in vitro anticancer study against human promyelocytic leukemia cells (HL60). Secondary metabolites like alkaloids, phlobatannin, triterpenoids, flavonoids, lipids, steroids, terpenoids, tannins and glycosides are identified in the extract by phytochemical screening. Leaf extract showed the presence of ethers and carboxylic acid groups (GCMS analysis). The leaf extract showed resistance against *Salmonella typhi*. Thus, from this study it can be concluded *Clitoria ternatea* leaves methanol extract has bioactive compounds showing anticancer activity against HL60 cells.

Keywords: Antibacterial Activity, Anticancer Activity, *Clitoria ternatea*, GC-MS Analysis, Phytochemical Screening, MTT Assay

1. Introduction

Clitoria ternatea (Fabaceae) is known as Butterfly pea, Asian pigeon wings, blue bellvine, cordofan pea and *Shankpushpi*. It is predominantly found in tropical equatorial area, a perennial twinning leguminous herb. The length of the plant can vary from 0.5 m to 3 m. The plants have 5 to 7 leaflets with pinnated leaves. The length of the petioles ranges from 1.5 cm to 3 cm. They have a persistent stipule which is narrowly triangular with 1mm to 6mm length and are subulate with predominantly 3 nerved. Filiformed stipules are 2mm long and shows characteristic elliptical leaflets. *Clitoria ternatea* a perennial climber with white or blue coloured flowers[11]. The leaves are both elliptic and obtuse in nature. Being a vine or creeper, it naturally grows well in neutral and moist soil. The fruit from these plants are usually 5 to 7 cm long and possess 6 to 10 seeds within each flat pod. The tender nature of the fruit makes it an edible fruit[2]. The flowers are axillary and solitary.

The leaf extract of *Clitoria ternatea* being colourful, after purification is used as a natural colouring agent or natural dye in various food industries. Being a traditional medicinal herb *Clitoria ternatea* is the only species among the Fabaceae family that is bestowed with the presence of cyclotides. Highest concentrations of cyclotides are found to be present in the roots of *Clitoria ternatea*. These cyclotides greatly possess antibacterial and immune stimulating activities[12]. The seeds of *Clitoria ternatea* also yielded lectin that agglutinates trypsin-treated human B erythrocytes. Among the various parts of the plant extracts, the extract of the seed has an effective larvicidal activity.

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Silver Nano particles can also be synthesised from the extract of *Clitoria ternatea*. The seed extract of *Clitoria ternatea* is also used for the isolation of protein named 'finotin' that has enhanced antimicrobial and insecticidal activities[9].

Clitoria ternatea being a medicinal plant has tremendous therapeutic compounds and hence occupies an important place in pharmaceutical industry. The plant is used to cure numerous diseases both in *Ayurveda* and traditional medicines. Various parts of the plant has numerous medicinal functions. The flower extract of the plant act as a good anti-inflammatory, analgesics and also as antidiabetic agent. The leaf extract of the plants helps in preventing neurodegenerative diseases, diabetes mellitus and also controls excessive sweating. The root extract of the plant has good antioxidant activity and its bark is used as a diuretic and laxative. For irritation in bladder and urethra its decoction is used as a demulcent. The seed extract of the plant is used in swollen joints, abdominal viscera enlargement and dropsy. For centuries the extract of the plant has been used as a memory enhancer, antidepressant, stress reducer, anoxiolytic, sedative and tranquillizing agent[2]. The extract of the whole plant is used in treating sexual ailments such as gonorrhoea and infertility.

Clitoria ternatea has enhanced anxiolytic activities, anti- inflammatory and analgesic activities, antimicrobial activity, anticarcinogenic activity, nephroprotective activities, antistress activities. Its effect on general behaviour includes larvicidal activities, proteolytic activities, antihelmintic activities and antihyperglycemic activities. It has good antioxidant and antihistaminic profile making the plant more important in cancer treatment.

2. Methodology

2.1 Collection of Plant

Clitoria ternatea leaves were obtained from Sathya Mangalam forest. Using plastic bags, the leaves collected were transferred to lab. The powdered and sieved leaves of size 1 mm were finely powered and was stored in the Non-toxic-polyethylene bag.

2.2 Plant Extract Preparation

10gm powder mass was extracted using 200mL of methanol solvent. Dark maceration for 72 hours at 27˚C was carried out and Whatman filter paper was used for filtration. The filtrate obtained was subjected to evaporation at 45˚C and the residue was used for further analysis.

2.3 Phytochemical Screening

2.3.1 Test for Alkaloids

To 3ml of the extract 1 ml of Mayer's reagent was poured and agitated well, presence of Alkaloids was indicated by the white precipitate at the bottom[5].

2.3.2 Test for Phlobatannin

10 ml of aqueous extract of flower was boiled with 1%HCl. Presence of phlobatannin was indicated by the thick red precipitate deposition in the bottom[5].

2.3.3 Test for Triterpenoids

2 ml of extract was added with 5 drops of concentrated sulphuric acid and kept undisturbed. Presence of triterpenoids was indicated by the appearance of greenish blue colour[5].

2.3.4 Test for Flavonoids

The presence of flavonoids was identified by the use of alkaline reagent in the extract. Few drops of 10%NaOH solution was added to 1 ml of the extract and flavonoids was indicated by intense yellow colour, which disappeared on addition of a few drops of dilute acid[5].

2.3.5 Test for Lipids

0.5 N alcoholic potassium hydroxide was added along with a drop of phenolphthalein to 10 ml of the extract. The mixtures were incubated on water bath for 1 hour. The presence of lipids was indicated by the formation of foam or soapy layer[5].

2.3.6 Test for Steroids

2 ml of chloroform was added to extract and few drops of concentrated H_2SO_4. The presence of steroids was

indicated by the appearance of red colour in the upper layer while yellow with greenish fluorescence appears in the H_2SO_4 layer[5].

2.3.7 Test for Terpenoids

To 1 ml of the aqueous extract 1 ml of chloroform was added, mixed well and left for 5 minutes, 1 ml concentrated H_2SO_4 was added after 5 minutes. The presence of terpenoids was indicated by the appearance of greyish layer[5].

2.3.8 Test for Tannin

Braemer's test was used to indicate the presence of tannin. To 1 ml of the extract, 2 ml of water was added, boiled and filtered. Few drops of 5% ferric chloride was added to the filtrate. The presence of tannin was indicated by a dark green, blue or brown colour[5].

2.3.9 Test for Glycosides

To 0.5ml of methanol extract 1ml of glacial acetic acid with a trace amount of ferric chloride was added followed by 1ml of conc. sulphuric acid. The presence of glycoside was indicated by the formation of reddish-brown colour ring at the junction of 2-layers, upper layer turned in to bluish green colour[5].

3. Structural/Compound Estimation of *Clitoria ternatea*

3.1 GC-MS

The sample was subjected to GC-MS evaluation to quantify the number of molecules and its structures. The analysis was performed using GCMS (Perkin Elmer model: Clarus 680) with mass spectrometer (Clarus six hundred(EI) analysed by (TurboMassver5.4.2) software program. The Clarus 680 GC employed a fused silica column, filled with Elite-5MS (5% biphenyl 95 % dimethylpolysiloxane, 30 m × zero.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant drift of 1 ml/min. The injector temperature was set at 260°C throughout the chromatographic run.The 1µL of extract was injected into the device, the oven temperature was set in the following sequence; 60 °C (2 min); followed by 300 °C at 10 °C min−1; and 300 °C for 6 min.The mass detector had transfer line temperature at 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a test time of 0.2 sec and test interval of 0.1 sec. The fragments analysed were ranging from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum in GC-MS NIST (2008) library.

3.2 FT-IR

A pellet was prepared by adding potassium bromide (KBr) with a small quantity of *C. ternatea* methanol leaves extract. Using Thermo Electron Scientific FTIR spectroscope the pellet was subjected to analysis. The characteristics peaks and their functional group was detected[5].

3.3 Antibacterial Assay

3.3.1 Agar Well Diffusion Method

Agar well diffusion is used for analysing the antibacterial activity of the extract. The extract was tested against different bacterial strains such as *Streptococcus agalactiae, Salmonella typhi, Staphylococcus aureus, Enterobacteraerogenes, Escherichia coli* and *Bacillus subtilis.* Nutrient agar medium was used in plating for this assay. These different bacterial cultures were swabbed separately in each different plate. Four wells were made using well injector. Of these four wells, two wells are used as control and the other two wells are used as test. Ampicillin was used as positive control. The test 1 was 50 % of the extract and test 2 was 100% leaf extract. After filling the wells, the plates are incubated at 37°C for 24 hours at optimal conditions. After incubation the zone of inhibition exhibited by the antibiotics and the two test concentrations were compared and analysed.

3.4 Cytotoxic Assay

MTT Assay

The sample was subjected to *in vitro* cytotoxicity test. The used medium from the HL60 cells was replaced with fresh medium. The triplicates of the test were added to the cells, incubated at 37°C for 18 hr. After incubation MTT (1 mg/ml) was introduced in the wells

and incubated for 4 h. DMSO was added in the wells after incubation and the absorbance was read at 570 nm. Cytotoxicity and cell viability were calculated by the below mentioned formula,

Cytotoxicity = [(Control-Treated)/Control]×100

Cell Viability = (Treated/Control)×100

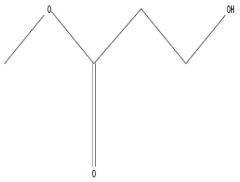
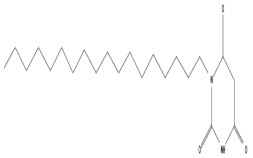
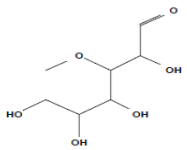
4. Result and Discussion

The chemical components present in the extract was studied using Gas Chromatography Mass Spectrometry analysis. The extract composition was identified based on their retention time. The phytoconstituents were identified as propanoic acid, 3-hydroxy-, methyl ester; pyrimidine-2,4,6(1h,3h,5h)-trione, 1-octadecyl; 1-octanamine, n-methyl-n-nitroso; 3-o-methyl-d-glucose; 2-o-methyl-d-mannopyranosa; undecanoic acid; 4-fluoro-1-methyl-5-carboxylic acid, ethyl(ester); nonadecanoic acid and neopentane-1,1-diol diacetate at various retention time of 14.698, 14.838, 14.998, 19.82, 20.07, 21.446, 22.761, 22.901 and 23.422 respectively. Most of the compounds identified belong to the esters and carboxylic acid family and are tabulated in Table 1.

Well diffusion method was performed to study the antibacterial activity of *Clitoria ternatea* extract. The macerated methanol leaf extract of *Clitoria ternatea* was studied for its antibacterial activity by the determination of zone of inhibition. The methanol leaf extracts were tested against *Streptococcus agalactiae, Salmonella typhi, Staphylococcus aureus, Enterobacteraerogenes, Escherichia coli* and *Bacillus subtilis.* The antibacterial effect of the extract against different bacterial species showed promising results and is tabulated in Table 2. The result of the study suggested that the extract showed greater resistance to *Salmonella typhi* and can be used to treat diseases caused by *Salmonella typhi.*

Phytochemical screening of *Clitoria ternatea* flower extract showed the presence of flavonoids, alkaloids, steroids, lipids, terpenoids, triterpenoids, phlobatannin, tannin and glycosides (Table 3).

Table 1. GC-MS analysis of methanol extract of *Clitoria ternatea* leaves

S. No	Retention Time	Compound Name	Molecular Formula	Molecular Weight	Peak Area	Structure
1	14.698	propanoic acid, 3-hydroxy-, methyl ester	$C_4H_8O_3$	104	7.146	
2	14.838	pyrimidine-2,4,6(1h,3h,5h)-trione, 1-octadecyl	$C_{22}H_{40}O_3N_2$	380	3.133	
3	14.998	1-octanamine, n-methyl-n-nitroson	$C_9H_{20}ON_2$	172	2.545	
4	19.82	3-o-methyl-d-glucose	$C_7H_{14}O_6$	194	25.732	

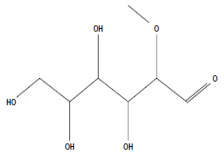
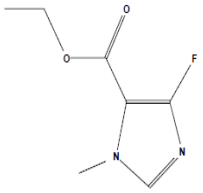
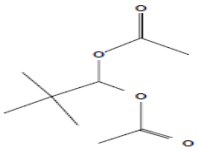
5	20.07	2-o-methyl-d-mannopyranosa	$C_7H_{14}O_6$	194	29.849	
6	21.446	undecanoic acid	$C_{11}H_{22}O_2$	186	21.150	
7	22.761	4-fluoro-1-methyl-5-carboxylic acid, ethyl(ester)	$C_7H_9O_2N_2F$	172	4.577	
8	22.901	nonadecanoic acid	$C_{19}H_{38}O_2$	298	4.320	
9	23.422	neopentane-1,1-diol diacetate	$C_9H_{16}O_4$	188	1.547	

Table 2. Inhibition zone of methanol extract of *Clitoria ternatea*

Microorganism	Inhibition Zone In Extracts (mm)	Inhibition Zone In Ampicillin (mm)
Streptococcus agalactiae	7	8.1
Salmonella typhi	9	13
Staphylococcus aureus	8	9
Enterobacteraerogenes	5.8	7.5
Escherichia coli	6.5	8
Bacillus subtilis	7.5	8.3

The functional groups present in the leaf extract of *Clitoria ternatea* was observed under FTIR Spectroscopy. From the results obtained a graph was plotted between transmittance (%) and wavenumber(1/cm). the functional group corresponding to each peak is tabulated in Table 4 and the graph in Figure 1.

Table 3. Chemical constituents of *Clitoria ternatea* leaf extract

Phytochemical constituents	Present/Absent
Alkaloids	Present
Phlobatannins	Present
Triterpenoids	Present
Flavonoids	Present
Lipids	Present
Steroids	Present
Terpenoids	Present
Tannin	Present
Glycosides	Present

The MTT assay showed slight to severe cytotoxic reactivity to HL60 cells. Triplicates of the samples were done and analysed. The cell death increased with an increase in the concentration of the extract (Table 5). SEM analysis is shown in Figure 2.

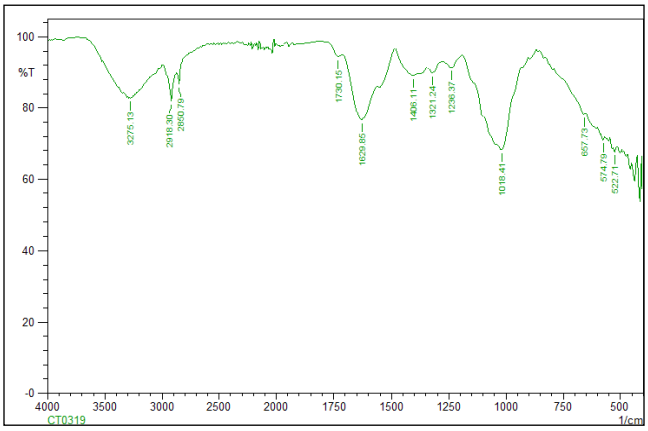


Figure 1. Transmittance % vs Wavenumber (1/cm) for the extract of *Clitoria ternatea*.

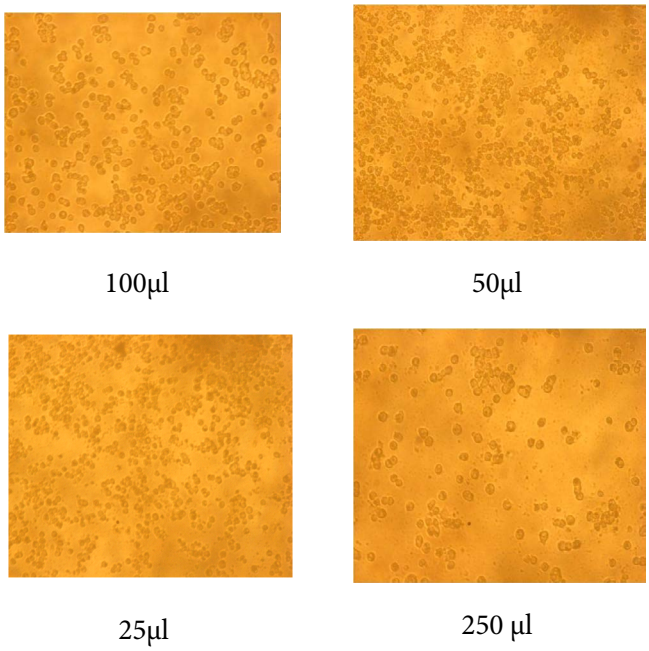


Figure 2. HL60 cells reactions at different sample concentrations.

5. Conclusion

This study gives us evidence about the bioactive compounds present in *Clitoria ternatea*. Extract was analysed and found to be rich in esters and carboxylic acid. The phytochemical secondary metabolites identified in this extract are flavonoids, alkaloids, steroids, lipids, terpenoids, triterpenoids, phlobatannin, tannin and glycosides. The antibacterial assay proved that the extract showed an accountable resistance to *Salmonella typhi*. Also, the extract showed anticancer activity against HL60 cells. Thus, from this study it can be concluded *Clitoria ternatea* leaves methanol extract has bioactive compounds showing anticancer activity against HL60 cells.

Table 4. FTIR peak value corresponding functional group in methanol leaf extract

Wave number (1/cm)	Functional Group/Assignment
3275.13	Normal polymeric OH stretch
2918.30	Methylene C-H asymmetric/ symmetric C-H
2850.79	Methylene C-H asymmetric/ symmetric C-H
1730.15	Aldehyde
1629.85	Organic nitrates
1406.11	Organic sulfates
1321.24	Aromatic nitro compounds
1236.37	Aromatic ethers, aryl O stretch
1018.41	Cyclohexane ring vibrations
657.73	Thioesters/CH_2-S-/C-S stretch
574.79	Aliphatic iodo compounds, C-I stretch
522.71	Aliphatic iodo compounds, C-I stretch

Table 5. Cytotoxic activity of *Clitoria ternatea* leaf extract

Concentration (µg/ ml)-CT0319-HL60	Cytotoxicity (%)	Cell Viability (%)
250	79.93	20.07
100	69.48	30.52
50	54.36	45.64
25	43.91	56.09
12.5	39.03	61.97
6.25	12.11	87.89
3.125	5.81	94.18
1.562	6.58	93.42
0.781	5.49	94.50

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