Antimicrobial Activity of *Clitoria ternatea* (L.) Extracts

L. Kamilla,[a] S.M. Mnsor,[a] S. Ramanathan,[a] and S. Sasidharan,[b]

[a]Centre for Drug Research, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.
[b]Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

E-mail: srisasidharan@yahoo.com

Summary

The antimicrobial activities of the methanol extracts of the leaf, stems, flower, seed and roots of *Clitoria ternatea* were studied. The extracts of *C. ternatea* were tested in vitro against 12 bacterial species, 2 yeast species, and 3 filamentous fungal by the agar diffusion and broth dilution methods. The leaf and root extracts were found to be most effective against all of the tested organisms ($p < 0.05$). The MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration) and MFC (minimum fungicidal activity) values of *C. ternatea* extracts ranged from 0.3 mg/ml to 100.00 mg/ml.. The *C. ternatea* extracts were also screened for tannin, phlobatannin, flavonoid, antharaquinone, alkaloid, saponin, cardiac glycosides, volatile oils, steroids and terpenoids. The anthraquinone and saponin were absent in all the plant material investigated. Hence, *C. ternatea* can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals in food preservation as well as natural plant-based medicine.

Key Words: antimicrobial activity, *Clitoria ternatea*, food preservation, phytochemical screening

Introduction

Many extracts from medicinal plant have been known to possess antimicrobial effects and used for the purpose of food preservation and medicinal purposes (1-3). The growth of food spoilage and food-borne pathogens in food can lessen nutritional quality of the food by consuming fat, protein and carbohydrate that are present in the food for their survival, accordingly lead to food discoloration, heating, mustiness, biochemical changes, weight loss and accumulation of toxic substance as by products. Some species of food spoilage and food-borne pathogens are capable to produce highly toxic compounds in food which can adversely affect the health of humans (4, 5). The way of microbial growth inhibition most appropriate to food is the use of food preservatives. A perfect food preservative must be inexpensive, corrosion-free, low in toxicity, and have good antimicrobial activity. The inhibitors of food preservative available for practical use today are mainly chemical preservations. Nevertheless, the safety problems with chemical preservatives are receiving extensive attention world wide, and natural preservatives derived from the natural resources such as medicinal plants have high potential for the food industry as food preservative (4, 6).

Malaysia, being one of the 12 mega-diversity centres of the world, is reach in all three levels of biodiversity (7), as species diversity, genetic diversity and habitat diversity, with many plants used for medicinal and nutritional purpose. In order to fully tap the natural resources of our country, it is very important to put the comprehensive utilization and process of natural resources on this agenda. Therefore, in this study, we focus on the studies on the antimicrobial activities of extracts from *Clitoria ternatea* L., belong to the Fabaceae family against general food spoilage and human pathogens so that new food preservatives can be explored and developed on the basis of the natural resources.

Materials and Methods

Materials

The leaf, stems, flower, seed and roots of *C. ternatea* were collected from the Seberang Jaya, Penang, Malaysia, in January of 2008 and authenticated by the botanist of the School of Biological Sciences, University Science of Malaysia, where a specimen was deposited in the herbarium (Voucher number of 11006).

Test microorganisms and growth media

The following Gram-positive and Gram-negative bacteria, yeasts, and molds were used for antimicrobial activities studies: : Gram-positive bacteria included *Bacillus cereus*, *Bacillus subtilis*, *Bacillus thuringiensis, Staphylococcus aureus*, *Streptococcus faecalis*; Gram-negative bacteria included *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Enterobacter aerogens, Proteus mirabilis, Herbaspirillum* spp.; yeasts included *Candida albicans* and *Saccharomyces cerevisiae*; molds included *Rhizopus* spp., *Aspergilus niger, Penicillium* spp., were used in this study. The bacterial strains were grown in Mueller–Hinton agar (MHA; Difco, USA) plates at 37 °C, whereas the yeasts and molds were grown in sabouraud dextrose agar (SDA; Difco, USA) plates and potato dextrose agar (PDA; Difco, USA) plates media, respectively, at 28 °C. The stock culture was maintained on nutrient agar slants at 4 °C.

Extract preparation

The various plant part extracts were prepared by maceration of dried powdered plant material in methanol solvent (8) for 3 days. 200 grams of powdered leaves, stems, flowers, seeds and roots were macerated in methanol under stirring conditions for 72 hours. The macerated extracts were then filtered through No. 1 Whatman filter paper. The methanolic crude extracts were obtained. The crude extracts were then vaporized to dryness using the rotary evaporator (BUCHI Rotary Evaporator R-110, USA).

Qualitative phytochemical screening

The methanolic crude extracts were subjected to qualitative phytochemical testing for the detection of major chemical groups. Extracts were screened for the presence of tannin using Braemer's test (9, 10), phlobatannin (11), flavonoids using Shinoda test (9), saponin using frothing test, cardiac glycosides using Keller-Kiliani test (10), volatile oil (12). The Liebermann-Burchardt test was used to test the presence of steroids and terpenoids (9). In addition, the salkowski test was used to test the presence of terpenoids (11).

Antimicrobial disk diffusion assay

Antibacterial and antifungal activities of the five plant extracts were investigated by the disk diffusion method (13, 14). The MHA plates, containing an inoculum size of 10^6 colony-forming units (CFU)/mL of bacteria or $2x10^5$ CFU/mL yeast cells or molds spores on SDA and PDA plates, respectively, were spread on the solid plates with an L-shaped glass rod. Then disks (6.0-mm diam.) impregnated with 25 µL of each extract at a concentration of 100.0mg/mL were placed on the inoculated plates. Similarly, each plate carried a blank disk by adding solvent control alone in the centre, and antibiotic disks (6.0-mm diam.) of chloramphenicol, levofloxacin (30 µg/ml, for bacteria) and myconazole (30 µg/ml, for fungal) were also used as a positive control. All of the plates were incubated at 37°C for 18 hours for bacteria and at 28°C for 48 hours for fungi.

The zones of growth inhibition around the disks were measured after 18 hours of in incubation at 37°C for bacteria and 48 hours for fungi at 28°C, respectively. The sensitivity of the microorganism species to the plant extracts was determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks. All of the experiments were performed in triplicate. The results are reported as the average of three experiments. The scale of measurement was as the following (disc diameter included): ≥ 20 mm zone of inhibition is strongly inhibitory; 20 – 12 mm zone of inhibition is moderately / mildly inhibitory; and ≤ 12 mm is no inhibitory (Rota et al., 2008). Strains that were inhibited by more than 12 mm were considered for the MIC, MBC and MFC studies.

Determination of minimum inhibitory concentration (MIC)

MIC was determined by broth dilution methods (15). Two-fold serial dilutions (0.3125 – 100 mg/mlL) of the 5 extracts, with the appropriate antibiotics, were prepared as positive controls in Mueller-Hinton broth for bacteria and Saboraud glucose broth for fungi. For broth dilution tests, 0.1 mL of standardized suspension of bacteria (10^6 CFU/mL) and fungal cell or spores (5×10^5 CFU/mL) was added to each tube (containing fractions of 5 extracts at a final concentration of 0.3125 to 100 mg/mL) and incubated at 37 °C for bacteria for 18 h or at 28 °C for fungi for 48 h. MICs were taken as the average of the lowest concentration showing no growth of the organism and the highest concentration showing visible growth by macroscopic evaluation (16). Each assay was performed in triplicate.

Determination of minimum bactericidal (MBC)

A loop full of microbe culture was taken from each test tube and inoculated onto MHA agar plates. Then, the plates were incubated at 37ºC for 24 hours. The MBC was recorded as the lowest concentration of the extract that gave complete inhibition of colony formation of the test bacteria at the latter cultivation. Each assay was carried out in triplicate.

Determination of minimum fungicidal concentration (MFC)

The hyphal growth inhibition test was used to determine the antifungal activity of the *C. ternatea* extract against *A. niger* and *Rhizopus sp.* The procedure used in the hyphal growth inhibition test has been described previously (17). Briefly, dilutions of the test solutions dissolved in methanol were added to sterile melted Potato Dextrose Agar at 45 ºC to give final concentrations of 100, 10, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/mL. The resultant solution was thoroughly mixed and approximately 15mL was poured onto

the Petri plate. Plugs of 1 mm of fungal mycelium cut from edge of active growing colony were inoculated in the center of the agar plate and incubated in a humid chamber at 25ºC. Control cultures received an equivalent amount of methanol. Three replicates were used for each concentration. Radial growth was measured when the control colonies almost reached 1.5cm. Results were expressed as the percentage of hyphal growth inhibited (18).

Statistical analysis

The triplicate data were subjected to an analysis of variance for a completely random design using Statistical Analysis System (SPSS version 12) programs. Multiple-range test was used to compare the difference among means at the level of 0.05.

Results and Discussion

The results of the qualitative phytochemical screening to test the presence of tannin, phlobatannin, flavonoid, antharaquinone, alkaloid, saponin, cardiac glycosides, volatile oils, steroids and terpenoids in the extracts from various parts of *C. ternatea* are shown in Table 1. The preliminary phytochemical screening study revealed that the leaf of *C. ternatea* contains moderate level of tannin, cardiac glycosides and steroids and mild level of alkaloid. There were no phytochemicals noted in the stem. Both the flowers and seeds of *C. ternatea* contain phlobatannin, flavonoid, terpenoid at moderate levels. In addition, the seeds contain moderate presence of alkaloid and mild presence of volatile oil. The roots contain small amount of flavonoid, volatile oil and terpenoid. These findings had provided a general understanding of the antimicrobial properties of the extracts tested in this study.

Table 1. Phytochemical Screening of Secondary Metabolites from *Clitoria ternatea*.

Secondary metabolites	Name of the test	Leaf	Stem	Flower	Seed	Root
Tannins	Braemer's test	++	-	-	-	-
Phlobatannins	-	-	-	++	++	-
Flavonoids	Shinoda test	-	-	++	++	+
Anthraquinone	KOH test	-	-	-	-	-
Alkaloid	Dragendorff test	+	-	-	++	++
Saponin	Frothing test	-	-	-	-	-
Cardiac glycosides	Keller-Kiliani test	++	-	-	-	-
Volatile oils	-	-	-	-	+	+
Steroids	Liebermann Burchardt test	+	-	-	-	-
	Steroids test	++	-	-	-	
Terpenoids	Liebermann Burchardt test	-	-	++	++	+
	Salkowski test	-	-	++	++	++

'++' Moderate, '+' Present mildly, '-' Absent.

The results of the antimicrobial screening assay of the extracts of all parts of *C. ternatea* are shown in Table 2. All parts of plant included in the present study were found to be active on at least one of the selected microbial strains tested. In general, among the tested microbial strains, bacteria were found to be more sensitive to the test extracts than fungi. The preliminary disk diffusion assay of *C. ternatea* extracts against microbes showed that the leaf and root extracts were more favourably compared to the rest of the extracts (which inhibited 16 out of 17 test microorganism with zone of inhibition 10-25 mm).The antimicrobial screening assay of *C. ternatea* extracts against yeast showed that, all the extracts inhibited *C. albicans* (with zone of inhibition 9-20 mm) tested. As shown in Table 2 no matter what extract was used, of all the fungal strains included in the test found to be virtually sensitive to all extracts except the *A. niger* only sensitive to the leaf extract. The antimicrobial activities of the herbal drugs (leaf, stems, flower, seed and roots) were found to be less effective to the positive controls (chloramphenicol, levofloxacin for bacteria and myconazole for fungus). The negative controls methanol was devoid of any antimicrobial activity. The different extract of *C. ternatea* showed different spectrum of activities, especially by the disk diffusion method where the microorganisms tested produced difference zones of inhibition. The extracts of difference parts of *C. ternatea* showed different efficacy against the tested microorganisms. These differences could be due to the nature and level of the antimicrobial agents present in the extracts and their mode of action on the different test microorganisms (19).

Literature review on the phytochemical constituents of these plants revealed that various secondary metabolites like flavonoids, anthocyanin glycosides, pentacyclic triterpenoids and phytosterols have been isolated from this plant (20). A protein designated as 'finotin' has been isolated from *C. ternatea* seeds and reported to have antifungal, antibacterial and insectidal properties (21). It is possible that this compound was mainly responsible for the observed antimicrobial effects in this study.

Based on the initial antimicrobial screening assay, those extract showed positive results were selected for further studies for the determination of MIC, MBC, and MFC because they were found to be active against bacterial, fungal and yeast strains tested. The MICs, MBCs and MFCs of the extracts are shown in Table 3. The MIC, MBC and MFC values of *C. ternatea* extracts ranged from 0.3 mg/ml to 100.00 mg/ml. The common food borne fungal species such as *A. niger* and *Rhizopus* sp., showed a lower MBC values compared with bacterial species tested in this study with the range of 0.4 mg/mL to 0.8 mg/mL. However the reference antibiotics such as chloramphenicol, levofloxacin and myconazole showed lower MIC, MBC and MFCs values (10 μg/mL to 1000 μg/mL) compared with extracts tested. But, being crude extracts, the overall antimicrobial activity screening results are still indicative of the potential of these herbal drugs for the purpose of food preservation and medicinal purposes against all tested microorganism.

Conclusion

In conclusion, all the extracts investigated possessed activity against at least one strain of bacteria and/or fungi. Further studies aimed at the isolation and identification of active substances from the methanol extracts of *C. ternatea* could also disclose compounds with better value for food preservation as well as natural plant-based medicine.

Strains	Zone of growth inhibition (mm) [a]							
	Clitoria ternatea extracts (100.00 mg/ml)					Reference antibiotic (30 µg/ml)		
	Leaf	Stem	Flower	Seed	Root	Chloramphenicol	Levofloxacin	Myconazole
Gram positive bacteria								-
Bacillus cereus	13.7±1.5	12±2	14±1	12.3±0.6	14.3±1.5	19.7±0.6	23.7±1.1	-
Bacillus subtilis	11.3±1.5	12±1	12.7±1.1	12±2.6	11.3±3.5	21.6±0.6	22	-
Bacillus thuringiensis	10±1	14.3±1.1	15.7±0.6	14±2.6	19±1	24±1	25.33±9.8	-
Staphylococcus aureus	11	12±1	13±1	12.7±1.1	12.3±1.5	19.6±2.5	25.7±3.5	-
Streptococcus faecalis	14.7±1.5	16±1	12±1	12.3±2.5	13.3±0.6	19.33±2.9	26.7±0.6	-
Gram negative bacteria								
Escherichia coli	13.3±1.1	14.3±1.1	13.3±0.6	12.7±1.1	15	17±1.7	26.33±1.5	-
Klebsiella spp	13.3±1.1	11.7±1.1	12.7±0.6	13±1	14.7±1.5	18.33±2.5	16.7±0.6	-
Pseudomonas aeruginosa	13.3±0.6	10.7±2	11.3±1.5	12.3±2	9±2.6	23±2.6	25.33±0.6	-
Salmonella typhi	21±2.3	18.7±2.1	10.3±1.1	11.3±1.7	28.7±4.5	25.6±1.1	25.33±0.6	-
Enterobacter aerogens	13.3±2.1	12.3±0.6	13±1	12.7±1.5	14	14±1	26.33±0.6	-
Proteus mirabilis	18.7±1.5	19.3±0.6	13.7±2.9	15.7±4	23.7±1.5	19±1.7	24.7±2.3	-
Herbaspirillum spp.	14.7±1.1	12.7±0.6	11.3±2.3	14.3±0.6	14.3±3.5	11±1	21.7±2.5	-
Fungi								
Candida albicans	14	-	19	10.7±1.5	25±2.6	-	-	21±1.7
Saccharomyces cerevisiae	-	-	-	-	11.33±0.6	-	-	11
Rhizopus	11.67±0.6	11.67±2.1	11±1	9.67±1.1	11.33±1.5	-	-	14.33±3.8
Aspergillus niger	19.89±0.5	-	-	-	-	-	-	20.33±3.2
Penicillium spp.	11	8.7±0.6	8.33±0.6	9	9.67±0.6	-	-	10.33±2.1

[a] Values for zone of growth inhibition are presented as mean±SD from the experiments in triplicate; '-' inhibition zone was not noted.

Table 2. Antibacterial Activities of *Clitoria ternatea*.Extracts

Strains	Broth dilution method (mg/ml)															
	Leaf		Stem		Flower		Seed		Root		Reference antibiotic (ug/ml)					
	MIC	MBC/ MFC	MIC	MBC / MFC	MIC	MBC / MFC	MIC	MBC / MFC	MIC	MBC / MFC	Chloramphenicol		Levofloxacin		Myconazole	
Gram positive bacteria											MIC	MBC	MIC	MBC	MIC	MFC
Bacillus cereus	3.125	6.25	>100	>100	>100	>100	25	50	50	25	>1000	>1000	<31.25	<31.25	-	-
Bacillus subtilis	12.5	25	50	100	>100	>100	50	100	<0.312	<0.312	>1000	>1000	<31.25	<31.25	-	-
Bacillus thuringiensis	50	100	>100	>100	12.5	25	3.125	6.25	<0.312	<0.312	500	1000	<31.25	<31.25	-	-
Staphylococcus aureus	50	100	>100	>100	>100	>100	50	100	25	50	>1000	>1000	31.25	62.5	-	-
Streptococcus faecalis	50	100	>100	>100	>100	>100	50	100	25	50	>1000	>1000	<31.25	<31.25	-	-
Gram negative bacteria																
Escherichia coli	50	100	>100	>100	>100	>100	50	100	0.125	0.25	>1000	>1000	<31.25	<31.25	-	-
Klebsiella spp	>100	>100	>100	>100	>100	>100	50	100	50	100	125	250	<31.25	<31.25	-	-
Pseudomonas aeruginosa	50	100	>100	>100	50	100	25	50	50	100	>1000	>1000	<31.25	<31.25	-	-
Salmonella typhi	12.5	25	>100	>100	>100	>100	50	100	25	50	>1000	>1000	<31.25	<31.25	-	-
Enterobacter aerogens	50	100	>100	>100	>100	>100	50	100	1.56	3.125	250	500	31.25	62.5	-	-
Proteus mirabilis	12.5	25	>100	>100	50	100	50	100	25	50	250	500	31.25	62.5	-	-
Herbaspirillum spp.	50	100	50	100	>100	>100	50	100	50	100	>1000	>1000	<31.25	<31.25	-	-
Fungi																
Candida albicans	25	50	>100	>100	>100	>100	>100	>100	25	50	-	-	-	-	500	1000
Saccharomyces cerevisiae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	ND	ND
Rhizopus	0.8	1.6	0.8	1.6	0.8	1.6	0.8	1.6	0.8	1.6	-	-	-	-	ND	ND
Aspergillus niger	0.4	0.8	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	<10	<10
Penicillium spp.	0.8	1.6	0.8	1.6	0.8	1.6	0.8	1.6	0.8	1.6	-	-	-	-	ND	ND

[a] Result was the average of records determined by both agar and broth dilution methods; ND= not determined.

Table 3. Minimum Inhibition Concentration, Minimum Bactericidal Concentration and Minimum Fungicidal Concentration of The *Clitoria Ternatea* Extracts.

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