Isolation of Phenolic Compound and Antioxidant Activities of *Clerodendrum indicum* L. Leaves

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Abstract

This paper concerns with the phytochemicals, FT-IR spectrum of phenolic compound, total phenol content, antimicrobial and antioxidant activities of *Clerodendrum indicum* leaves which were selected from Sagaing, Sagaing Region, in Myanmar. Firstly, preliminary phytochemicals screening of sample was done by using standard method which showed the presence of alkaloids, glycosides, flavonoids, phenolic compounds, tannins, saponins, terpenes and steroids. Moreover, functional groups of phenolic compound of crude sample were examined by spectroscopic method. After that, total phenol contents of ethanol and water extracts of sample were performed by using the Folin-Ciocalteu method. The total phenol content of ethanol extract was larger than that of water extract of the sample. In addition, antimicrobial activities of five solvent extracts of sample were carried out using agar well diffusion method. Finally, the antioxidant activities of ethanol and water extracts of the sample were evaluated by (2, 2-diphenyl-1-picryl hydrazyl) DPPH Radical Scavenging Assay. The experimental results contributed that the rich phenol fractions of *Clerodendrum indicum* leaves gave a potential source of natural antioxidants.

***Keywords*:** *Clerodendrum indicum*, antimicrobial and antioxidant activities, DPPH

Introduction

Verbenaceae is a large plant family consisting of trees, shrub and herbs (Trease and Evan, 1983). Modern research carried on the Verbenaceous plants revealed that most of the plants belonging to this family are medicinally important as they contain biologically active compounds. *Clerodendrum indicum*, locally known as Nga-yantpatu in Myanmar, belongs to the family Verbenaceae. It has considerable reputation for its medicinal values as traditional medicine. The root is considered useful in asthma, cough and scrofulous affections, the resin is employed in Syphilitic rheumatism and the juice of leaves is used with ghee as an application in hepatic eruptions and pemphigus (Kirtikar and Basu, 1994). The leaves and root are extremely used to treat tumours and certain skin diseases. The dried leaves are smoked like cigarettes to relieve asthma and liver cancer. Hence, leaves are selected for this study.

Materials and Methods

Plant material and preparation

Fresh *Clerodendrum indicum* leaves were collected from Sagaing, Sagaing region and washed thoroughly in distilled water and dried in shade at room temperature. Dried leaves were powdered evenly using an electrical blender. The powder was extracted in ethanol, methanol, and distilled water. The dried extracts were collected in air tight container for experiments. *Clerodendrum indicum* was taxonomically identified by authorized Botanist, Professor Dr. Soe Myint Aye, Botany Department, University of Mandalay.

Phytochemicals Screening

Phytochemicals screening of *Clerodendrum indicum* leaves was performed by using the standard methods for the presence of alkaloids, glycosides, flavonoids, phenolic compounds, tannins, saponins, terpenes, and steroids. (Harbone, 1993).

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Isolation of pure compound

Pure compound was isolated from *Clerodendrum indicum* leaves by using modern separation technique including thin layer and column chromatography.

Study on FT-IR Spectroscopy

FT-IR spectrum of pure isolated compound of *Clerodendrum indicum* leaves was measured in the range 4000-450cm^{-1}using FT-IR Spectroscopic method.

Determination of Total Phenol Content by FCR Method

One of the antioxidant factors, total phenol content (TPC) was measured by spectrophotometer using the Folin-Ciocalteu method.

Preparation of sample solution

The sample solution was prepared by dissolving 1 mg of respective crude extract in 1 mL of distilled water.

Preparation of standard gallic acid solutions

The stock solution of standard gallic acid (1 mg/mL) was prepared by dissolving 1mg of gallic acid in 1 mL of distilled water. This stock solution was two- fold diluted serially with distilled water to get the standard gallic acid solutions with the concentration of 125, 62.5, 31.25, 15.63 and 7.81µg/mL.

1:10 (v/v) FCR (Folin-Ciocalteu Reagent)

Folin-Ciocalteu reagent (1 mL) was mixed with distilled water (10 mL).

1 M Sodium carbonate solution

Sodium carbonate (106 g) was mixed with distilled water (1000 mL).

Construction of gallic acid standard curve

Firstly, 1 mL of different concentration of Gallic acid solution (125, 62.5, 31.25, 15.625, 7.8125 µg /mL was mixed with 5 mL of diluted F-C reagent (FCR: H_2O, 1: 10) and incubated for 15 min. To each tube, 4 mL of 1 M sodium carbonate was added and the tubes were kept at room temperature for 30 minutes and the UV absorbance of reaction mixture was recorded at λ_{max} 765 nm. A standard curve was prepared by plotting the absorbance against concentration of gallic acid. The prepared standard curve is shown in Figure 3.

Determination of total phenol content as gallic acid equivalent in sample

The total phenolic content (TPC) in each sample was estimated by Folin-Ciocalteu method. Each extract (1 mg) was mixed with 1mL of distilled water. To this, 5 mL of F-C reagent (1:10) was added and incubated for 15 minutes. To each tube, 4 mL of 1 M sodium carbonate solution was added and the tubes were kept at room temperature for 30 minutes and the UV absorbance of reaction mixture was read at λ_{max} 765 nm. The blank solution was prepared as the above procedure by using distilled water instead of sample solution. Total phenol content was examined as µg gallic acid equivalents per mg of different extract (µg GAE/ mg). The TPC contents of all tested samples are described in Table 3 and Figure 4.

Antimicrobial activity

The petroleum ether, acetone, ethanol, methanol and water extracts of leaves of *Clerodendrum indicum* were tested for antimicrobial activity against the microbial strains *Bacillus pumilus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* using agar well diffusion method at Development Centre for Pharmaceutical and Food Technology (DCPFT), Yangon. The antimicrobial activity was evaluated by measuring zone diameters of inhibition of microorganisms growth surrounding the sample extracts.

DPPH radical scavenging activity

Pure sample (0.004g) was dissolved in (20 mL) ethanol (analar grade). This solution was thoroughly mixed at room temperature for (15) minutes to obtain (200 µg/ mL) of sample solution. The concentrations of standard solution (0.625, 1.25, 2.5, 5, 10, 20 µg/mL) were determined by using parallel dilution method. Sample solution (1ml) and (3) mL of DPPH solutions were thoroughly mixed for about (15) minutes at room temperature. The absorbance of the mixture was measured at (517) nm.

$$\%\,\text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

This formula is the calculation of percent inhibition of (IC_{50}) value. The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function.

Results and Discussion

Phytochemicals screening

Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutically and industrially important compounds. It is imperative to initiate an urgent step for screening of plants for secondary metabolites. The results of major phytochemicals of *Clerodendrum indicum* leaves are described in Table 1.

Table1. Phytochemicals analysis of *Clerodendrum indicum* leaves

No	Phytochemicals	Extract	Reagents used	Observation	Result
1.	Alkaloids	1 % HCl	Dragendroff's reagent	Orange color solution	+ +
2.	Glycosides	Water	10% lead acetate	White ppt	+
3.	Flavonoids	95 % ethanol	Conc: HCl, Mg	Pink color solution	+
4.	Phenolic Compounds	Water	10 % $FeCl_3$	Greenish blue color solution	+
5.	Tannins	Water	10 % $FeCl_3$+ dil HCl	Yellowish brown ppt	+
6.	Saponins	Water	Shaken vigorously	Permant frothing	+
7	Terpenes	95% ethanol	$CHCl_3$,conc: H_2SO_4	Pink color ppt	+
8.	Steroids	Benzene	Acetic anhydride +conc H_2SO_4	greenish yellow color solution	+

(+) indicates presence ppt shows precipitate

As shown in this table, *Clerodendrum indicum* leaves consist of alkaloids, glycosides, flavonboids, phenolic compounds, tannins, saponins, terpenes, and steroids respectively. Presence of phenolic compounds in the plant suggests the potential use of *Clerodendrum indicum* leaves as a source of antioxidant compounds.

Study on FT-IR spectrum

The FT-IR spectrum of pure compound of *Clerodendrum indicum* leaves is represented in Figure 1. Strong and broad bands absorb at 3366, and 3273 cm^{-1} represent O-H group. The symmetric and asymmetric sp^3 C-H stretching bands appeared at 2924, and 2883 cm^{-1} and the C=C stretching bands of aromatic ring observed at 1640 cm^{-1}. There is C-H in plane bending vibration of allylic group at 1457 cm^{-1}. The band at 1252 cm^{-1} shows C-C-O stretching vibration of phenol. The two bands at 1086 cm^{-1} and 945 cm^{-1}suggest C-O-C stretching vibration of ether group and C-H out of plane bending vibration of aromatic OH group respectively. All observed bands in this spectrum showed the presence of phenolic compound in sample.The functional groups of crude extract of *Clerodendrum indicum* leaves in FT-IR are listed in Table 2.

Table 2. Functional groups of crude extract of *Clerodendrum indicum* leaves

No.	Wave number (cm^{-1})	Tentative assignment
1	3366,3273	O-H stretching vibration
2	2924, 2883	symmetric and asymmetric sp^3 C-H stretching vibration of hydrocarbon
3	1640	C=C stretching bands of aromatic ring
4	1457	C-H in plane bending vibration of allylic group
5	1252	C-C-O stretching vibration of phenol group
6	1086	C-O-C stretching vibration of ether group
7	945	C-H out of plane bending of aromatic OH group

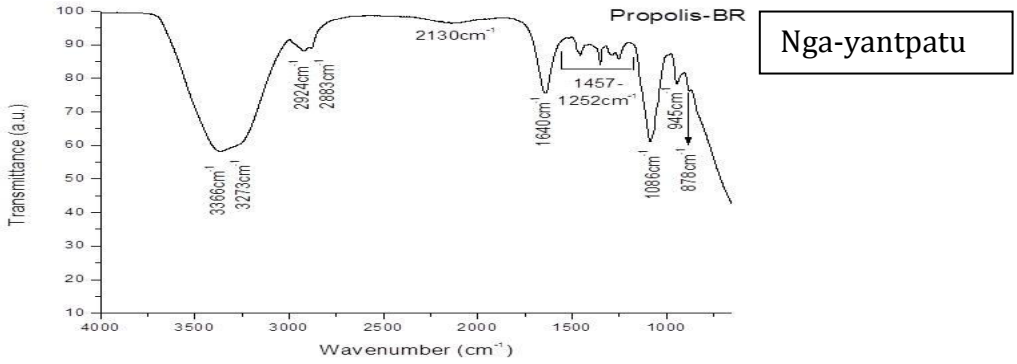

Figure 1. FT-IR spectrum of crude extract of *Clerodendrum indicum* leaves

Total Phenol Content of Crude Extracts of *Clerodendrum indicum* leaves

The phenolic compounds possessing one or more aromatic rings with one or more hydroxyl group are categorized as phenolic acid, flavonoids, coumarins and tannin. Phenolics are the products of secondary metabolism in plants. Phenolic compounds have antioxidant properties of protective against degenerative disease like heart diseases and cancer.

The total phenolic contents of ethanol and water extracts of sample were examined using the diluted Folin-Ciocalteu Reagent (FCR). Gallic acid standard curve is required to evaluate the total phenol content of the selected sample. Because gallic acid is 3, 4, 5- trihydroxy benzoic acid and it is a kind of total phenol. Gallic acid standard curve is shown in Figure 3.

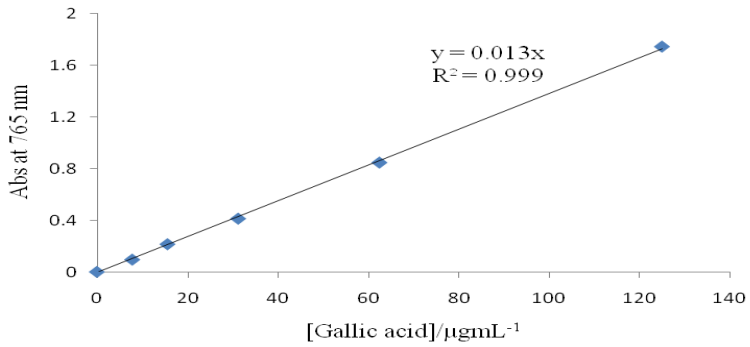

Figure 2. Gallic acid standard graph to determine total phenol content

The results of total phenol contents for ethanol and water extracts of sample are tabulated in Table 3 and Figure 3.

Table 3. Total Phenol Content of Aqueous and Ethanol Extracts of *Clerodendrum indicum* leaves by Folin-Ciocalteu Method

Sample	TPC (µ g GAE ± SD) / mg	
	Aqueous	Ethanol
Leaves	23.39± 0.81	33.91± 2.81

[Data expressed as (µg Gallic Acid Equivalents GAE, (mean SD) in 1 mg of crude extract]

According to experimental data, ethanol extract of selected sample was found to be more total phenol content (33.91µg/mg) than aqueous extract (23.39µg/mg) of sample.

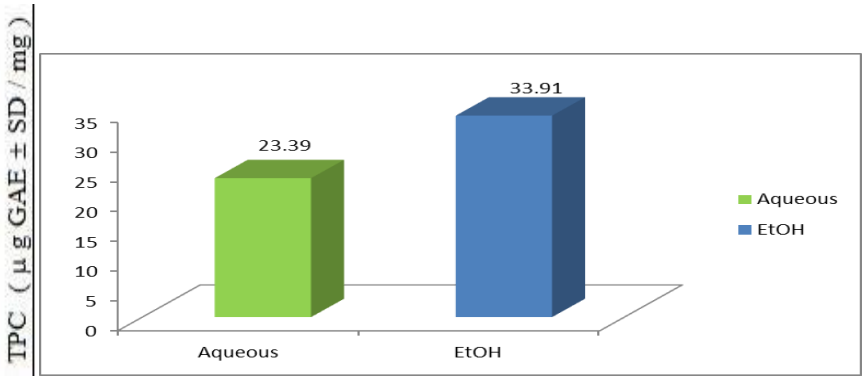

Figure 3. Bar graph for total phenol contents of aqueous and ethanol extracts of *Clerodendrum indicum* leaves

In accordance with Figure 4, total phenol contents of ethanol extract was found to be more than aqueous extract of *Clerodendrum indicum* leaves.

Antimicrobial activity

Antimicrobial activity of petroleum ether, acetone, ethanol, methanol, and aqueous extracts of *Clerodendrum indicum* leaves was screened against *Bacillus pumilus, Bacillus subtilis,*

Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. The results of the zone of inhibition are summarized in Table4 and Figure 4.

Table 4. The results of antimicrobial activities of *Clerodendrum indicum* leaves

Inhibition zone diameters of various solvent extracts against organisms							
Samples	Solvents	I	II	III	IV	V	VI
Leaves	Methanol	-	10mm (+)	-	-	12mm (+)	-
	Acetone	30mm (+++)	31mm (+++)	33mm (+++)	30mm (+++)	32mm (+++)	38mm (+++)
	Ethanol	14mm (+)	14mm (+)	19mm (++)	-	11mm (+)	12mm (+)
	Aqueous	15mm (++)	-	15mm (++)	-	11mm (+)	12mm (+)
	Petroleum ether	-	-	-	-	-	-

Agar-well ~ 10 mm
10 mm ~ 14 mm (+)
15 mm ~ 19 mm (++)
20 mm above (+++)
(+) = low activity
(++) = medium activity
(+++) = high activity
(–) = absent

Organisms
I = *Bacillus subtilis*
II = *Staphylococcus aureus*
III = *Pseudomonas aeruginosa*
IV = *Bacillus pumilus*
V = *Candida albicans*
VI = *Escherichia coli*,

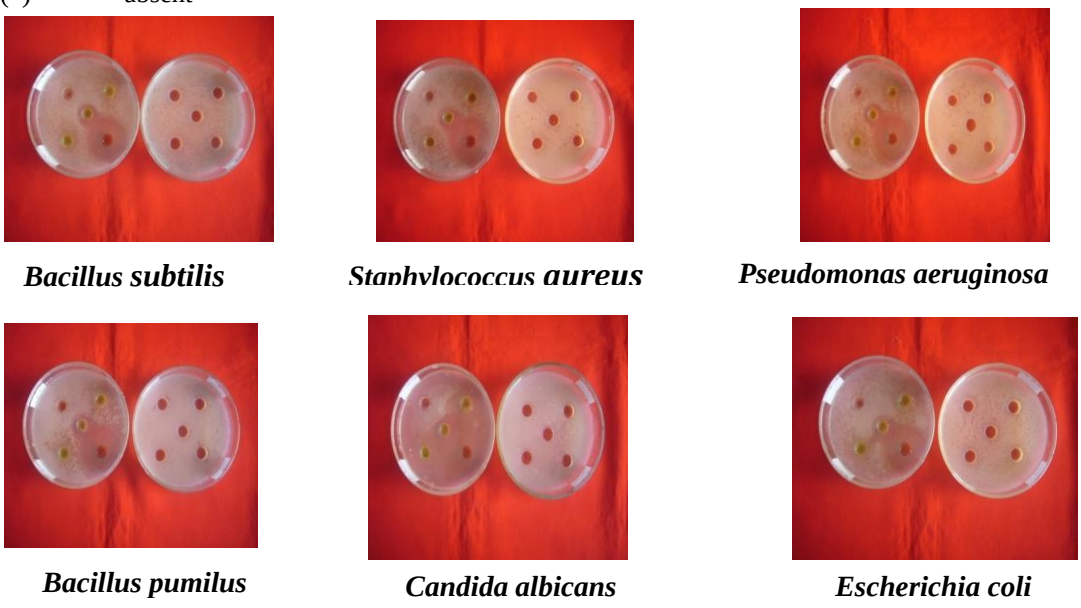

Figure 4. Antimicrobial Activities of extracts of *Clerodendrum indicum* leaves

According to these results, acetone extracts of sample show high activity on all tested organisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans*, and *Escherichia coli*. Petroleum ether extracts sample show no activity on six selected organisms and methanol, ethanol and aqueous extracts exhibit low antimicrobial activity showed the presence of phenolic compound. Hence, *Clerodendrum indicum* leaves consist of bioactive compounds.

Antioxidant Activity of Ethanol and Aqueous Extracts of *Clerodendrum indicum* leaves by DPPH Radical Scavenging Assay

Antioxidant compounds in plants play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk.

The extracts or their constituents when mixed with DPPH decolorized due to hydrogen donating ability. The radical scavenging activity of crude extracts was described by % RSA and IC_{50} (50% inhibitory concentration). These results are described in Table 5 and Figure 5.

Table 5. Radical Scavenging Activity (IC_{50}) of EtOH and Aqueous Crude Extracts *Clerodendrum indicum* leaves and Ascorbic Acid

Extracts of sample	% RSA (mean ±SD) in different concentration (µg/mL)						IC_{50} (µg/mL)
	0.625	1.25	2.5	5	10	20	
Aqueous extract	46.19	55.89	66.45	75.82	84.12	92.01	2.85
	±1.06	± 0.40	± 0.60	± 0.65	± 0.53	± 0.31	
Ethanol extract	19.98	29.52	41.3	57.76	71.62	75.81	1.73
	± 0.88	± 0.6	± 1.97	± 1.37	± 0.46	± 0.4	
Ascorbic acid	25.2	53.58	65.53	74.82	83.32	91.21	1.17
	± 1.4	± 0.88	± 1.13	± 0.59	± 0.78	± 0.48	

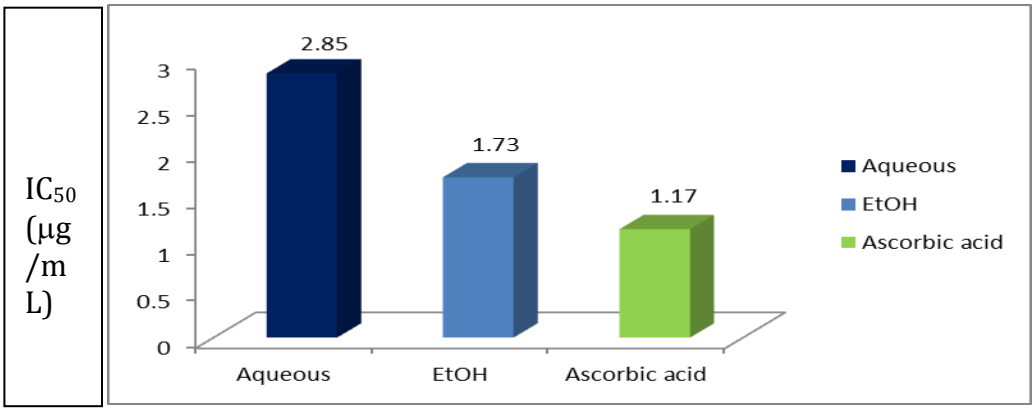

Figure 5. A bar graph of IC_{50}(µg/mL) of aqueous and ethanol extracts of *Clerodendrum indicum* leaves and ascorbic acid.

In this study, IC_{50} values of ethanol extract showed the strong antioxidant activity by compaired with aqueous extract and ascorbic acid. Hence, *Clerodendrum indicum* leaves are good antioxydant activity and leaves are suitable to eat for human health.

Conclusion

Clerodendrum indicum leaves gave alkaloids, flavonoids, glycosides, phenolic compounds, tannins, saponins, terpenes, and steroids. The main constituents found in the extract were flavanoids, phenolic compounds, and terpenes. Secondary metabolites such as alkaloids, and phenolic compounds present in extract can show anticancer potential. The presence of phenols suggests the antioxidant activity of the extract. Tannins are a group of phenolic compounds that are known antimutagentic property and can act against cancer cells.

FT-IR spectrum of pure compound of sample shows the presence of phenolic compound. Ethanol extract of sample for the total phenol content was found to be higher than water extract of sample.

Acetone extract of sample indicates high antimicrobial activities on all tested organisms and petroleum ether extract showed no antimicrobial activity. The strong IC_{50} values of aqueous and ethanol extracts of sample were found to be (2.85 μ g/mL) and (1.73 μg/mL). Antioxidant activity can reduce the risk of cardiovascular disease, some type of cancer and anti-aging.

Clerodendrum indicum leaves were contribued bioactive and good source of antioxidant activities leading to better nutrition for human health. Therefore, bioactive new and noble compounds from *Clerodendrum indicum* leaves would be isolated and characterized for health benefits.

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