PRELIMINARY PHYTOCHEMICAL INVESTIGATION AND SCREENING OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES FROM THE STEM BARK OF *CROTON OBLONGIFOLIUS* ROXB. (THAK-RING-KRI)

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Abstract

Croton oblongifolius Roxb., a middle size tree as medicinal plants, was chosen to investigate phytochemical constituents, nutritional values, screening of antimicrobial, total phenolic contents, total flavonoid contents and antioxidant activities. Preliminary phytochemical investigation of Thak-ring-kri bark revealed the presence of flavonoids, phenolic compounds, glycosides, saponin, terpenoids, steroids, carbohydrate, tannin, starch and α-amino acid. Alkaloids and cyanogenic glycoside were not detected in the sample. The AOAC method was used to determine the nutritional values such as moisture, ash, protein, crude fiber, fat, carbohydrate and energy value. The content of these values are 6 %, 10 %, 5.73 %, 11.45 %, 7.43 %, 59.4 % and 327 kcal/100g, respectively. The antimicrobial activity of pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts was determined by agar well diffusion method against six species of microorganisms such as *Bacillus substilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *E. coli*. It was found that all extracts showed antimicrobial activity against all tested microorganisms. Its watery extract showed less activity (12 mm) and pet-ether extract was observed the most pronounced antimicrobial activity (16~35 mm) against all microorganisms tested. Total phenolic contents was determined by spectrophotometric method using Folin-Ciocalteu reagent. Ethanol and watery extracts of the total phenolic contents were found to be (197.50 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg respectively. Total flavonoid contents was studied by spectrophotometric method. The ethanol extract contain flavonoid content (115.34 ± 0.01) µg QE/mg higher than watery extracts (70.00 ± 0.00) µg QE/mg. From the screening of free radical scavenging activity of Thak-ring-kri stem bark by DPPH assay, it was found that ethanol extract (IC_{50} = 5.9 µg/mL) showed the higher radical-scavenging activity than watery extract (IC_{50} = 18.5 µg/mL).

Keywords: *Croton oblongifolius* Roxb., phytochemical investigation, nutritional values, antimicrobial activity, free radical scavenging activity

Introduction

A medicinal plant is plant that has similar properties as conventional pharmaceutical drugs. Humans have used them throughout history to either cure or lessen symptoms from an illness. Nowadays plants are still important sources of medicines, especially in developing countries that still use plant-based traditional medicine for their healthcare. In 1985, it was estimated in the Bulletin of the World Health Organization (WHO) that around 80% of the world's population relied on medicinal plants as their primary healthcare source. *Croton oblongifolius* bark was selected for this research to investigate some biological activities. Scientifically which is known as *C. oblongifolius* and its plant family is Euphprbiaceae. Myanmar name is Thak-ring-kri. *C. oblongifolius* plant is a middle sized tree (about 8 m high) deciduous bark, brownish, branches lapidate. Leaves are alternate and 12.5-25 cm long, crowded towards the end of the branchlets, oblong-lanceolate, subacute and is found in many parts of

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Myanmar and in other Asian countries (Chuakul *et al*., 1997). *C.oblongifolius* plant has been reported to possess antioxidant property, antitumor, antibacterial, antileshmanial and anti-inflammatory activities. Chemical constituents present in Thak-ring-kri stem bark are steroids, monoterpenes, diterpenes, sesquiterpenes, phenyl propanoids, glycosides, mixture of steroid glycosides and flavonoids (Kumar *et al*., 1996). The present study deals with investigation of phytochemicals, antimicrobial activity, total phenolic content and antioxidant activity of the stem bark of *C. oblongifolius*.

Materials and Methods

Sample Collection

The samples was collected from Sittway Township, Rakhine State. After collection, the scientific name of *C.oblongifolius* was identified by authorized botanist at the Department of Botany, Sittway University. The collected sample was clean and dried. The air dried sample was made up to powder in electric grinder and stored in air-tight container to prevent moisture changes and other contamination.

Phytochemical Investigation of Thak-ring-kri Stem Bark

A phytochemical is a natural bioactive compound found in plant foods that works with nutrients and dietary fiber to protect against diseases. Phytochemical investigation was carried out to know the types of phytoorganic constituents present in Thak-ring-kri stem bark by test tube method (Trease and Evans, 1980; Robinson, 1983; M-Tin Wa, 1970; Vogel, 1996; Harborne, 1984; Marini-Bettolo, 1981).

Deterimnatoin of Nutritional Values

The nutritional values such as moisture, ash, protein, crude fiber, ether extract (crude fat) and carbohydrate of Thak-ring-kri bark were determined by AOAC methods at Myanmar Food Processors and Exporters Association (MFPEA) in Lanmadaw Township, Yangon, Myanmar (Mark and Stewart, 1975, Pearson, 1981, Joslyn, 1973, AOAC, 2000, Anderson, 1984).

Screening of Antimicrobial Activity

Antimicrobial activity of the Thak-ring-kri stem bark was studied by using agar well diffusion method in various solvents system on six microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon (Finegold, 1978).

Determination of Total Phenolic Contents (TPC)

Total phenolic contents (TPC) was determined spectrophotometrically using Folin-Ciocalteu reagent (Rekha *et al*., 2012). Crude extract (0.01 g) was dissolved in 20 mL methanol to obtain concentration of 500 µg/mL. 5 mL of Folin-Ciocalteu reagent was added to 0.5 mL of the extract and incubated at room temperature for 30 min. Next, 4 mL of 1M Na_2CO_3 was added and kept at room temperature for 15 min and absorbance of reaction mixture was measured at 760 nm by a UV-visible spectrophometer. The samples were prepared in triplicate for each

analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Gallic acid is used as the reference standard compound and total phenolic contents was estimated as microgram gallic acid equivalent per milligram (GAE).

Determination of Total Flavonoid Contents (TFC)

Aluminum chloride colorimetric method was used to determine the total flavonoid contents in the plant extracts. This method is based on the determination of the flavonoid-aluminum complex between flavonoid of the crude extract and aluminum chloride. Briefly, 1 mL of extract in methanol (6.25 - 50 µg/mL) was mixed with 1 mL aluminum chloride in ethanol (20 µg/mL) and a drop of acetic acid. The resulting mixture was then diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min. A blank sample was prepared in similar fashion omitting the extract. The calibration curve of quercetin was plotted using the same procedure and the amount of total flavonoids was calculated from linear regression equation obtained from the curve $y = 0.0023x + 0.0879$, $R^2 = 0.9993$ and expressed as quercetin equivalents (QE) per gram of the plant extract.

Screening of Antioxidant Activity

Antioxidant activity of ethanol and watery extracts of Thak-ring-kri stem bark was carried out by determination of DPPH (1, 1-Diphenyl-2-picryl hydrazyl) free radical scavenging property using UV spectroscopic method (Marinova and Batchvarov, 2011).

2 mg of each test sample and 10 mL of ethanol was thoroughly mixed and the mixture solution was filtered and the filtrate was used as a stock solution. Desired concentrations (5, 2.5, 1.25 and 0.625 µg/mL) of sample solutions were prepared from this stock solution by dilution with appropriate amount of ethanol.

Sample solution was prepared by thoroughly mixing 1.5 mL of 0.002% DPPH solution and 1.5 mL of test sample solution in the brown bottle. The control solution was also prepared by mixing 1.5 mL of 0.002% DPPH solution and 1.5 mL of ethanol. The solutions were then allowed to stand at room temperature for 30 min. Ascorbic acid (Vitamin C) synthetic antioxidnat was used as a standard and ethanol without sample was employed as control. After that, absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Decrease in absorbance indicates increases in radical scavenging activity. Absorbance measurements were done in three times for each sample solution and the mean value so obtained were used to calculate % inhibition of oxidation by using the following equation. Then IC_{50} (50 % inhibition concentration) was determined by using linear regressive excel program.

$$\% \text{ Inhibition of oxidation} = \frac{A_{DPPH} - (A_{sample} - A_{Blank})}{A_{DPPH}} \times 100$$

A_{DPPH} = Absorbance of DPPH solution

A_{sample} = Absorbance of sample + DPPH solution

A_{blank} = Absorbance of solvent

Results and Discussion

Phytochemical Investigation

The phytochemical investigation of Thak-ring-kri stem bark was determined and the results are summarized in Table 1. According to these results, flavonoids, phenolic compounds, glycosides, saponins, terpenoids, steroids, carbohydrate, tannins, starch and α-amino acids were found to be present in the sample. Alkaloids and cyanogenic glycosides were absent in the sample.

Nutritional Values

The nutritional values of moisture, fiber, ash, protein, carbohydrate and energy content were found as shown in Table 2 and Figure 1. As a result, the nutritional parameter of carbohydrate was rich and fiber was present as major nutrient in Thak-ring-kri stem bark.

Table 1 Results of Phytochemical Test on Thak-ring-kri Stem Bark

No.	Test	Extract	Test reagent	Observation	Remark
1.	Alkaloids	1 % HCl	Wagner's reagent Dragendorff's	No white ppt No orange ppt	- -
2.	Flavonoids	EtOH	Conc.HCl & Mg turning	Red colour sol^n	+
3.	Glycosides	H_2O	10 % lead acetate	White ppt	+
4.	Phenolic compounds	H_2O	1 %$FeCl_3$ solution & $K_3Fe(CN)_6$ solution	Deep blue colour sol^n	+
5.	Saponins	H_2O	Distilled water	Frothing	+
6.	Terpenoids	$CHCl_3$	Acetic anhydride & conc. H_2SO_4	Pink colour sol^n	+
7.	Steroids	Pet-ether	Acetic anhydride & conc. H_2SO_4	Green colour sol^n	+
8.	Carbohydrate	H_2O	10% α-naphthol and conc. H_2SO_4	Red ring	+
9.	Tannins	H_2O	10% NaCl & 1% gelatin	White ppt	+
10.	Starch	H_2O	I_2 solution	Deep blue sol^n	+
11.	α-Amino acids	H_2O	Ninhydrin	Pink spot on TLC	+
12.	Cyanogenic glycosides	H_2O	Conc. H_2SO_4 & sodium picrate solution	No brick red ppt	–

(-) = absence (+) = presence

Table 2 Results of Nutritional Values from Thak-ring-kri Stem Bark

No.	Types of Nutrients	Observed values
1	Moisture (%)	6
2	Ash (%)	10
3	Protein (%)	5.73
4	Crude fiber (%)	11.45
5	Fat (%)	7.43
6	Carbohydrate (%)	59.4
7	Energy value (kcal/100g)	327

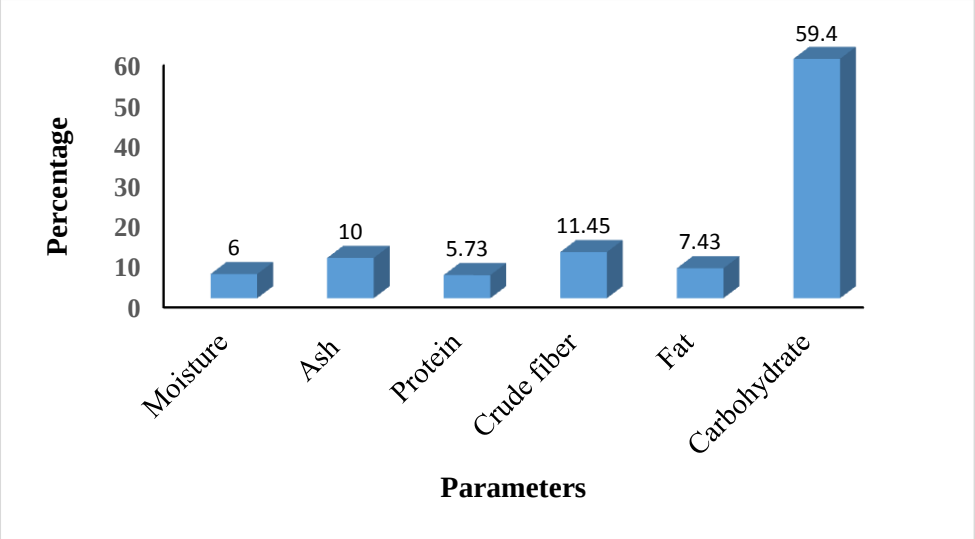

Figure 1 Histogram of nutritional values of Thak-ring-kri stem bark

Antimicrobial Activity

Screening of antimicrobial activity of various crude extracts such as pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from Thak-ring-kri bark was investigated by employing agar well diffusion method. In this study, the samples were tested on six species of microorganisms such as *B. substilis*, *S. aureus*, *P. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli* species. The inhibition zone diameters of all crude extracts against all six microorganisms tested are shown in Table 3 and Figure 2.

From these results, it was found that all extracts exhibit antimicrobial activity against all tested microorganisms. Pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from Thak-ring-kri stem bark exhibited inhibition zone diameters ranged in 16 ~ 35, 15 ~ 20, 14 ~ 16, 16 ~ 18, 17 ~ 19 and 12 mm, respectively, against all microorganisms tested. Thus, its watery extract showed less activty and pet-ether extract was observed to show the most pronounced antimicrobial activity against all microorganisms tested.

Table 3 The Results of Antimicrobial Activity of Various Crude Extracts from Thak-ring-kri Stem Bark

No.	Type of Organisms	Diameter of Inhibition Zone (mm)					
		PE	CH_2Cl_2	MeOH	EtOAc	EtOH	H_2O
1	*B. subtilis*	19	17	18	15	18	12
2	*S. aureus*	20	18	18	16	17	12
3	*P. aeruginosa*	16	15	18	-	17	12
4	*B. pumilus*	35	20	17	14	16	12
5	*C. albicans*	25	18	19	16	18	12
6	*E.coli*	21	18	18	14	17	12

Agar well diameter = 10 mm, 10 mm ~14 mm = (+) (low activity),
15 mm ~ 19 mm = (++) (medium activity),
20 mm ~ above = (+++) (high activity),
Not detected = (-)

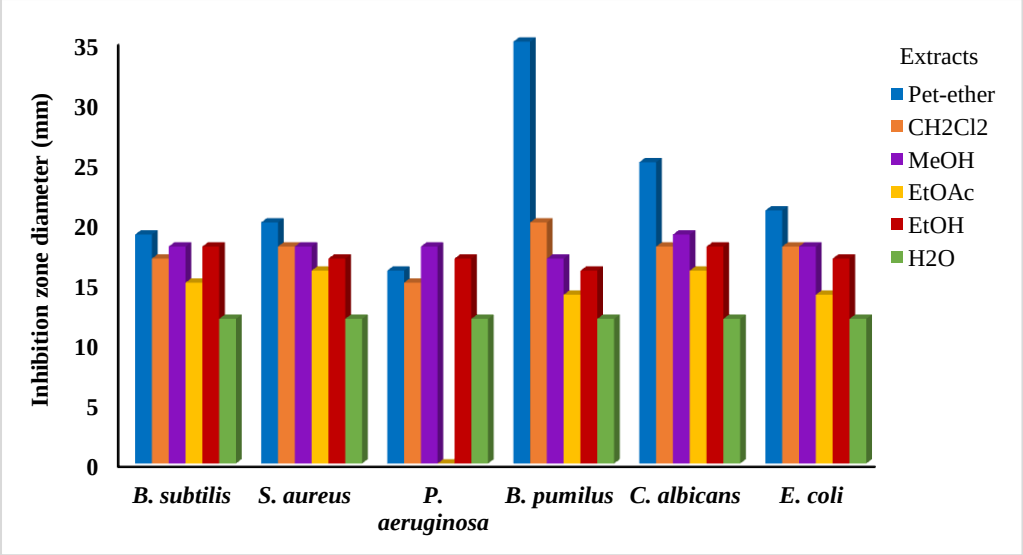

Figure 2 Histogram of antimicrobial activity of different extracts from Thak-ring-kri stem bark on six microorganisms by agar well diffusion method

Total Phenolic Contents

Total phenolic contents in the Thak-ring-kri stem bark extracts using the Folin-Ciocalteu's reagent is expressed as microgram of gallic acid equivalent per milligram of crude extract (µg GAE/mg). Standard calibration curve of gallic acid was prepared by dilution of the stock solution (1000 µg/mL) to obtain various concentrations (100, 50, 25, 12.50, 6.25, 3.125) µg/mL. Gallic acid standard curve gave a straight line. The total phenolic contents of ethanol and watery extracts were found to be (197.50 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg, respectively. Thus, ethanol extract was more effective than watery extract. The results are recorded in Tables 4, 5 and Figures 3, 4.

Table 4 Total Phenolic Contents (TPC) of Thak-ring-kri Stem Bark Extracts by Folin-Ciocalteu Method

Sample (500 µg/mL)	TPC (µg GAE/mg ± SD)
Ethanol extract	197.50 ± 0.01
Watery extract	41.42 ± 0.00

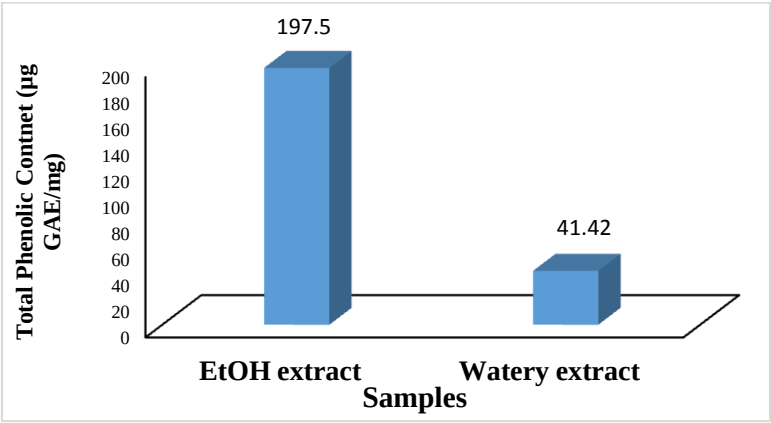

Figure 3 Total phenolic contents of ethanol and watery extracts

Table 5 Absorbance of Various Concentrations of Standard Gallic Acid

Concentration of Gallic Acid (µg/mL)	Absorbance at 760 nm
3.125	0.201
6.25	0.221
12.50	0.237
25.00	0.284
50.00	0.402
100.00	0.598

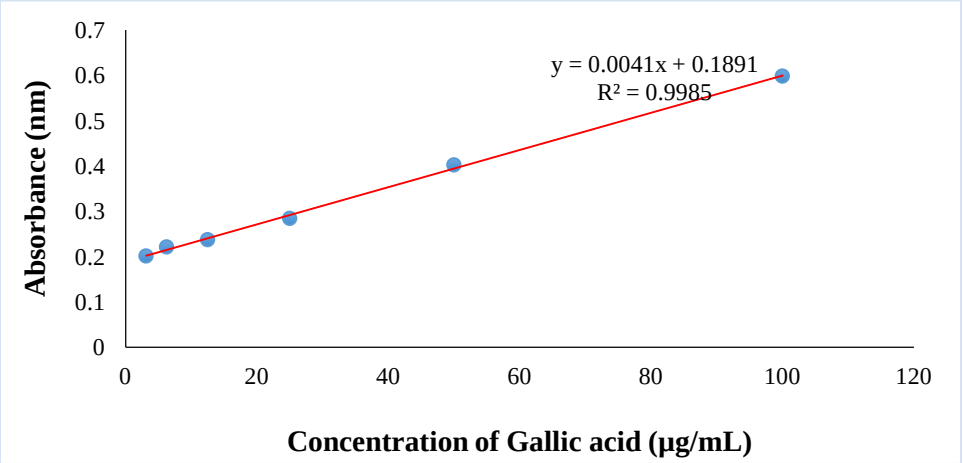

Figure 4 Standard calibration curve of gallic acid concentration vs absorbance at λ_{max} 760 nm

Total Flavonoid Contents (TFC)

Spectrophotometric method using Aluminum Chloride Colorimetric (ACC) method was used to determine the total flavonoid contents in the selected plant extracts. This method is based on the determination of the flavonoid-aluminum complex between flavonoid of the crude extract and aluminum chloride. The standard quercetin solution was prepared by dilution with the different concentration of 100, 50, 25, 12.5 and 6.25 µg/mL. The standard quercetin gave a straight line. The amount of total flavonoid contents was calculated from linear regression equation obtained from the curve y = 0.0023x + 0.0879, R^2 = 0.9993 and expressed as quercetin equivalents (QE) per gram of the plant extract. The total flavonoids contents of ethanol and watery extracts were found to be (115.34 ± 0.01) µg QE/mg and (70.00 ± 0.00) µg QE/mg, respectively. Thus, ethanol extract was more effective than watery extract. The results are recorded in Tables 6, 7 and Figures 5, 6.

Table 6 Total Flavonoid Contents (TFC) of Ethanol and Watery Extracts

No.	Extracts (300 µg/mL)	Absorbance	TFC (µgQE/mg ± SD)
1	Ethanol	0.168	115.34 ± 0.01
2	Watery	0.137	70.00 ± 0.00

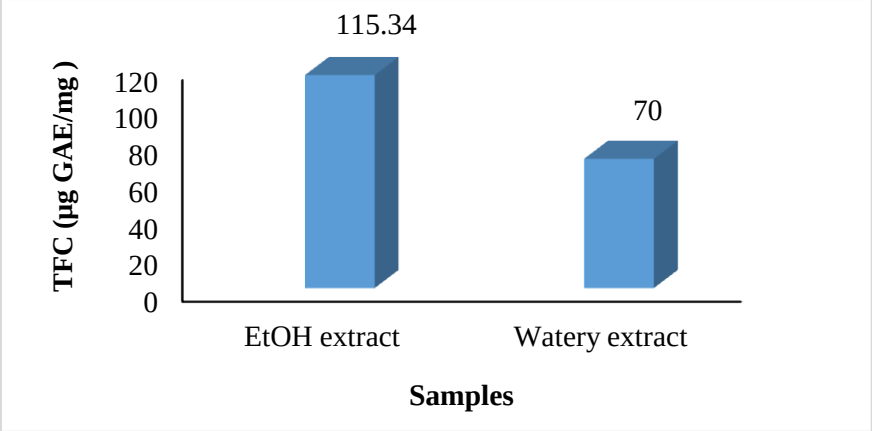

Figure 5 A bar graph of total flavonoid contents of ethanol and watery extracts

Table 7 Absorbance of Standard Compound Quercetin at λ_{max} 415 nm

No.	Concentration of Quercetin (µg/mL)	Absorbance at 415 nm
1	3.125	0.092
2	6.25	0.104
3	12.50	0.118
4	25.00	0.145
5	50.00	0.198
6	100.00	0.314

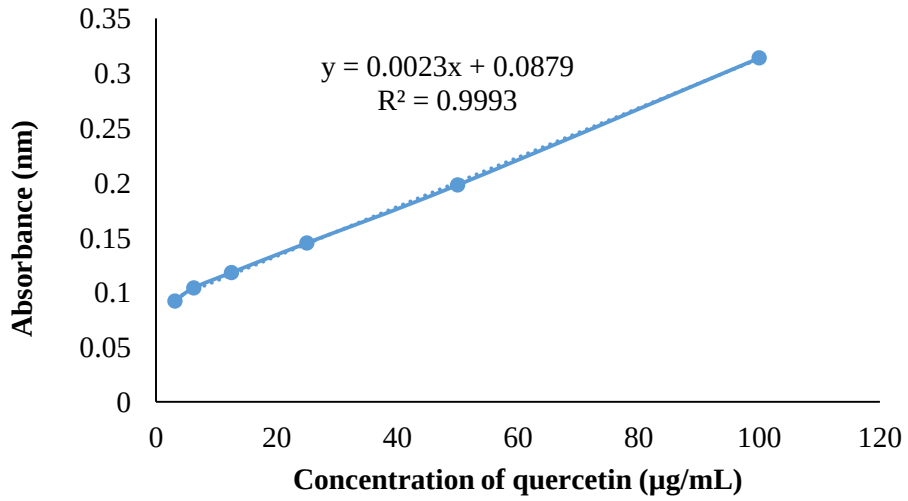

Figure 6 Standard calibration curve of quercetin concentration vs absorbance at λ_{max} 415 nm

Antioxdant Activity

The antioxidant activity of ethanol and watery extracts of Thak-ring-kri stem bark were studied by DPPH free radical scavenging assay. The resultant average % inhibition property values in different concentrations (50, 25, 12.5, 6.25 and 3.125 µg/mL) for all samples is

tabulated in Table 8 and Figures 7 and 8. From these figures, it can be seen that as the concentration of the samples increased, the respective % inhibition also increased.

The antioxidative potential of sample can be determined by IC_{50} (50% inhibition concentration). These IC_{50} value for each extract was determined by linear regressive excel program and can also be obtained from the plot of % inhibition *vs* concentration of the samples. The IC_{50} values were found to be 5.94 µg/mL for ethanol extract and 18.5 µg/mL for watery extract. Since the lower the IC_{50} values, the higher the free radical scavenging activity, i.e., the higher the antioxidative property. Watery extract has the higher IC_{50} than that of ethanol extract. Therefore, the antioxidant potential of ethanol extract was found to be higher than that of watery extract. In addition, it was found that all of these extracts have the lower antioxidant activity than standard ascorbic acid (IC_{50} = 1.9 µg/mL).

Table 8 Average % Inhibition of Oxidation and Values of Watery and Ethanol Extracts and Standard Ascorbic Acid

Samples	Average % inhibition in various concentrations (µg/mL)					IC_{50} (µg/mL)
	3.125	6.25	12.5	25	50	
watery extract	28.23	28.45	42.96	57.60	68.80	18.5
ethanol extract	28.42	52.37	71.22	73.09	76.41	5.9

Samples	Average % inhibition in various concentrations (µg/mL)					IC_{50} (µg/mL)
	0.16	0.8	4.0	20.0	100	
Ascorbic acid	16.34	39.20	70.52	88.09	95.95	1.9

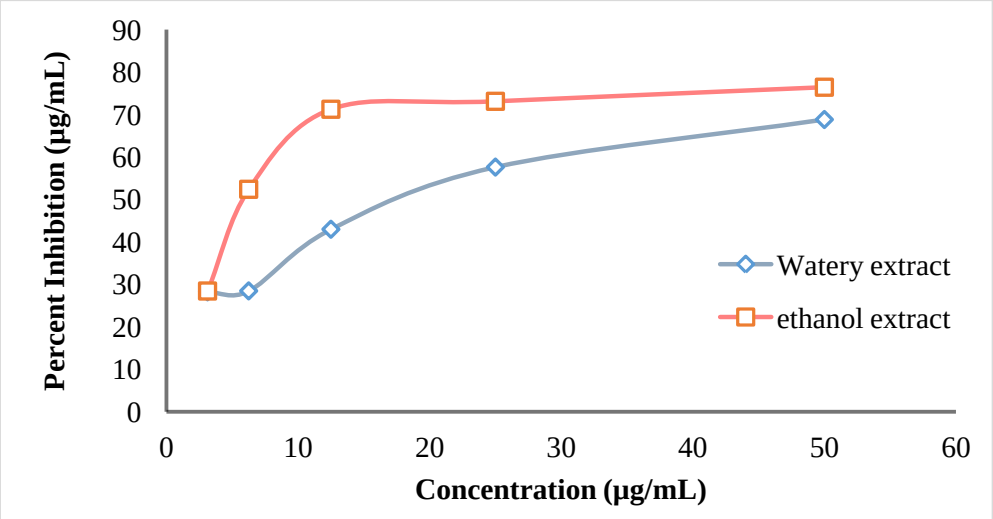

Figure 7 Percent inhibition of oxidation *vs* concentration (µg/mL) of ethanol and watery extracts from Thak-ring-kri stem bark

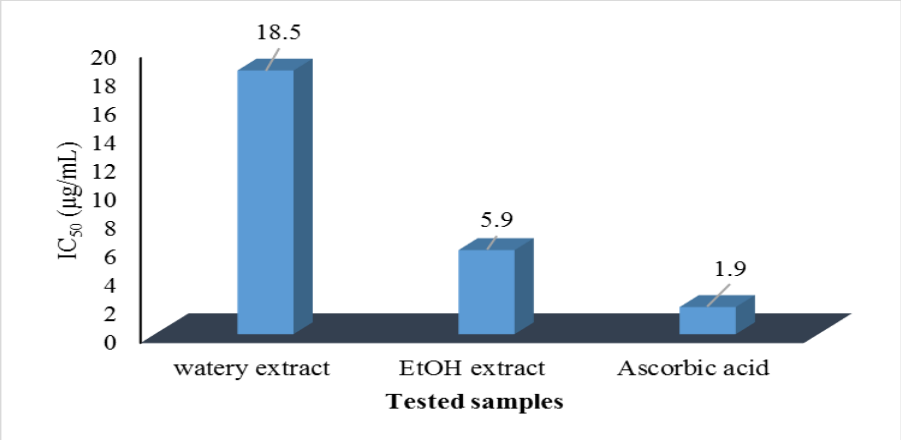

Figure 8 Bar graph of IC_{50} values of ethanol and watery extracts from Thak-ring-kri stem bark compared with standard ascorbic acid

Conclusion

Thak-ring-kri stem bark revealed the presence of flavonoids, phenolic compounds, glycosides, saponin, terpenoids, steroids, carbohydrate, tannin, starch and α-amino acid, and absence of alkaloids and cyanogenic glycosides. Nutritional values were found to be 6% of moisture, 10 % of ash, 5.73 % of protein, 11.45 % of crude fiber, 7.43 % of fat, 59.4 % of carbohydrate and 327 kcal/100 g of energy, based of dried sample.

Antimicrobial activity of six crude extracts such as pet-ether, dichloro methane, ethyl acetate, ethanol, methanol and watery extracts from selected sample was investigated by employing agar well diffusion method aganist *B. substilis*, *S. aeruginosa*, *B. pumilus*, *C. albicans* and *E. coli* species. According to the results obtained, all extracts showed the antimicrobial activity against all tested microorganisms. Pet-ether extract was observed the most pronounced antimicrobial activity and watery extract was the lowest activity agaisnt all tested microorganisms. The total phenolic contents of ethanol and watery extracts were found to be (197.5 ± 0.01) µg GAE/mg and (41.42 ± 0.00) µg GAE/mg respectively. Thus, ethanol extract was more effective than watery extract. The total flavonoids contents of ethanol and watery extracts were observed to be (115.34 ± 0.01) µg QE/mg and (70.00 ± 0.00) µg QE/mg respectively. Thus, ethanol extract was more effective than watery extract. From the screening of free radical scavenging activity by DPPH assay, it was found that ethanol extract (IC_{50} = 5.9 µg/mL) showed the higher antioxidant activity than watery extract (IC_{50} = 18.5 µg/mL). These two extracts showed less activity by comparing with the standard ascorbic acid (IC_{50} = 1.9 µg/mL). The present research is therefore, ethanol and watery extracts of Thak-ring-kri stem bark may be useful for the cure of bacterial infections and oxidative stress related diseases.

Acknowledgements

The authors would like to thank the Department of Higher Education, Ministry of Education, Nay Pyi Taw, Myanmar, for allowing to carrying out this research Programme. Thanks are also extended to Professor Dr. Mya Thu Zar, Professor and Head, Department of Chemistry, Sittway University, for her permission to carry out this research programme.

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