INVESTIGATION OF PHYTOCHEMICAL CONSTITUENTS AND SOME BIOLOGICAL ACTIVITIES OF THE SPINE OF *ZANTHOXYLUM RHETSA* (ROXB.) DC. (KA-THIT-PHU)

Chan Myae Kyaw[1,2], Khine Zar Wynn Lae[3], Daw Hla Ngwe[4]

Abstract

In the present study, *Zanthoxylum rhetsa* (Roxb.) DC. (Ka-thit-phu) was chosen for the investigation of the phytochemical and some biochemical activities. The spines of *Z. rhetsa* were collected from Dawei Township, Tanintharyi Region. The preliminary phytochemical screening indicated the presence of alkaloids, α-aminoacids, carbohydrate, cardiac glycoside, flavonoids, glycosides, organic acids, phenolic compounds, polyphenol, saponin, steroids, tannins and terpenoids in the spines of *Z. rhetsa.* According to the physicochemical analyses, the dry powdered sample was found to contain 5.46 % of total ash, 1.45 % of water soluble ash, 0.19 % of acid insoluble ash, 8.54 % of moisture content, <100 of foaming index and swelling index of 5 mL/g of the sample. The EDXRF elemental analysis showed some elements such as Si, S, Ca, K, Fe, Mn and Cu present in the sample. The extractable matters (% w/w) were found to be 4.6, 3, 2.6, 2.2, 2.0, 1.0, 0.2 % (w/w) by extracting with different polarities of solvents such as water, methanol, ethanol, acetone, ethyl acetate, chloroform and petroleum ether, respectively. The ethanol extract was observed to have higher total phenol content (51.92 ± 0.54 mg GAE/g) and total flavonoid content (50.61 ± 2.29 mg QE/g) than the watery extract (41.56 ± 0.83 mg GAE/g of TPC and 36.97 ± 1.05 mg QE/g of TFC). Antimicrobial activities (12~20 mm) of seven different extracts of the sample were determined against some fungal and bacterial species by using agar well diffusion method. The antioxidant activities of ethanol extract (IC_{50} = 1.15 μg/mL) and watery extract (IC_{50} = 2.89 μg/mL) were determined by DPPH radical scavenging activity assay. The ethanol extract of the sample was observed to possess the higher α-amylase inhibition activity (IC_{50} = 113.01 μg/mL) and α-glucosidase inhibition activity (IC_{50} = 120.56 μg/mL) than the watery extract (IC_{50} =137.00 μg/mL and IC_{50} = 446.78 μg/mL) and therefore ethanol extract might possess higher antidiabetic potency than watery extract. The crude extracts such as PE, EtOAc, Acetone, MeOH, EtOH, $CHCl_3$ and watery extracts exhibited the inhibition of tumor formation at the dose of 0.3 g/disc up to 7 days determined on tumor produced bacteria by using PCG (Potato Crown Gall) test. *In vitro* antiproliferative activity of ethanol and watery extracts was evaluated against human cancer cell lines: Hela (cervical) (IC_{50} = 3.74 μg/mL and IC_{50} = > 200 μg/mL), MCF-7 (breast) (IC_{50} = 7.71 μg/mL and IC_{50} = > 200 μg/mL), A549 (lung) (IC_{50} = 4.77 μg/mL and IC_{50} = > 200 μg/mL) cancer cell lines by CCK-8 Assay (Cell Counting Kit-8).

Keywords : *Zanthoxylum rhetsa* (Roxb.) DC. (Ka-thit-phu), total phenol content, total flavonoid content, antioxidant activity,antitumor activity, antidiabetic activity, antiproliferative activity

Introduction

At present, natural products have been used for thousands of years for the benefit of mankind, as important sources of food, clothing, cosmetics, building materials, tools, medicines and crop protection agent. Researches in this field are becoming more numerous to the point of getting about half of pharmaceuticals are pesticides from natural sources (Newman and Cragg,

[1] Assistant Lecturer, Department of Chemistry, University of Yangon
[2] PhD Student, Department of Chemistry, University of Yangon
[3] Dr, Lecturer, Department of Chemistry, University of Yangon
[4] Dr, Professor and Head, (Retd.), Department of Chemistry, University of Yangon

2007). *Zanthoxylum rhetsa* (Roxb.) DC. is a plant in the family of Rutaceae. In Myanmar, *Z. rhetsa* named Ka-thit-phu is found in the Tanintharyi Region. The plant is also traditionally used as antidiabetes, antispasmodic, diuretic and anti-inflammatory agent and bears significant antinocipective and antidiarrheal activities. The fruits and stem bark of this plant are used traditionally as a stimulant, astringent, stomachic and digestive and prescribed for urinary infection, dyspepsia, heart troubles, tooth ache, asthma and bronchits. Most exotic past research has studied the properties of Z. *rhetsa* extracts from the bark and root, and it was found to reduce the free radicals that cause the death of cancer cells (Sreelekha, 2012). Antimicrobial activities were also reported against some fungal and bacterial species by Poorniam *et al.,* (2018). Oil extracted from seeds can be used for the effective in cholera and is useful as an antiseptic, disinfectant and anti-inflammatory agent (Nalin *et al*., 2018). The water extract of the spine of Z. *rhetsa* was used to treat pain relief and to increase lactation in nursing mothers (Lalitharani *et al.,* 2013). The bark extract of Z. *rhetsa* has great potential for use or a natural active ingredient in anti-ageing cosmetic preparations (Ramesh *et al.,* 2013).

However, there is still lack of reports from the research on locally cultivated *Z. rhetsa*. Hence in this study, the spine of *Z. rhetsa* is chosen for the investigation of some phytochemical composition and some biological activities from its ethanol and watery extracts.

Materials and Methods

The spine samples were collected from Dawei, Tanintharyi Region, Myanmar. The plant was identified and authenticated at the Department of Botany, University of Yangon. After collection, the washed spines were air-dried in shade for about two weeks and ground into the coarse powder with the help of a mechanical grinder. The powders of the samples were used to extract with solvents of various polarities by using ultrasonic effect and to analyze phytochemical composition.

Phytochemical Analysis

The selected spine powders were subjected to qualitative phytochemical tests for the classification of various bioactive constituents present (Harborne, 1973). Phytochemical screenings were carried out by using standard procedures to detect the presence of alkaloids, glycosides, carbohydrates, α-amino acids, phenolic compounds, flavonoids, steroids, terpenoids, saponins, tannins, starch, reducing sugars and organic acids. After addition of specific reagents to the test solution, the observation of colour change or precipitate formation was noted and recorded.

Some Physicochemical Analyses

In this study, the crude plant material was subjected to the evaluation of some physicochemical properties. The various parameters were evaluated such as total ash content, water soluble ash content, acid insoluble matter content, moisture content, foaming index and swelling index. In addition, the relative abundance of elements present in the spine sample was determined by EDXRF spectrometer at the University of Monywa, Monywa.

The crude extracts of the sample were prepared by extracting the sample with different solvents such as petroleum ether, ethyl acetate, ethanol, methanol and water by percolation method. All of these extracts were kept for the determination of total phenol contents, total flavonoid contents, antimicrobial activity, antioxidant activity, antitumor activity, antidiabetic activity and antiproliferative activity.

Determination of Total Phenol Contents

The total phenol contents (TPC) in ethanol and watery extracts were estimated by the Folin-Ciocalteu method according to the procedure described by Saxena *et al.* (2013) and gallic acid was used as a standard. The sample solution (50 ppm) was prepared by dissolving 0.005 g of extract in methanol making up to 100 mL solution. Firstly, 0.5 mL of the prepared sample was mixed with 0.5 mL methanol. Then, 0.5 mL of Folin-Ciocalteu reagent (FCR: H_2O, 1:10) was added to the mixture and incubated for 5 min. 4 mL of 1 M sodium carbonate solution was added to each tube and the tubes were kept at room temperature for 2 h and the UV absorbance of each reaction mixture was recorded at λ_{max} 765 nm. The control solution was prepared as the above procedure by using distilled water instead of sample solution. Total phenolic content was estimated as mg gallic acid equivalents per g of EtOH/water extract.

Determination of Total Flavonoid Contents

The total flavonoids contents of the ethanol and watery extracts were measured by employing the method involving aluminium trichloride ($AlCl_3$) reagent and quercetin was used as standard (Kalita *et al.,* 2011). In the determination of total flavonoid contents, quercetin was used to construct the calibration curve. Quercetin (0.01 g) was dissolved in methanol and then diluted to various concentrations of 6.25, 12.50, 25, 50 and 100 μg/mL. A calibration curve was made by measuring the absorbance of the above different solutions at 415 nm (λ_{max} of quercetin) with a Shimadzu UV-1800 spectrophotometer. Ethanol/watery extract solution in 50 ppm was prepared by dissolving 0.005 g of each extract in 100 mL of methanol solution. Each extract stock solution (0.5 mL), 1.5 mL methanol, 0.1 mL of aluminium chloride, 0.1 mL of potassium acetate solution and 2.8 mL of distilled water were added and mixed well. The blank solution was prepared in similar way by replacing aluminium chloride with distilled water. Their absorbance was measured at 415 nm.

Screening of Antimicrobial Activity

Antimicrobial activity of different crude extracts (PE, EA, EtOH, MeOH, $(CH_3)_2CO$, $CHCl_3$ and water) of the sample was screened *in vitro* by agar well diffusion method (Perez *et al*.,1990). Bacterial cultures used in the research involved three strains of gram positive bacteria (*Bacillus subtilis, Staphyloccous aureus* and *Bacillus pumilus*), two strains of gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and one strain of fungi (*Candida albicans*). This experiment was carried out at Pharmaceutical Research Department, Insein Township, Yangon Region, Myanmar.

Determination of Antioxidant Activity

The antioxidant activity of EtOH and H_2O extracts were spectrophotometrically determined by DPPH radical scavenging assay method (Brand-Williams *et al.,* 1995). The

control solution was prepared by mixing 1.5 mL of 60 µM DPPH solution and 1.5 mL of 95 % ethanol with vortex mixer. The sample solution was also prepared by mixing thoroughly the 1.5 mL of 60 µM DPPH solution and 1.5 mL of test sample solution. The solutions were allowed to stand for 30 min at room temperature. After 30 min, absorbance was measured at 517 nm by using a spectrophotometer UV 1601 PC (P\N 206-6750), Shimadzu corporation. Absorbance measurements were done in triplicate for each solution and the mean values obtained were used to calculate % inhibition of oxidation by the following equation,

$$\% \text{ oxidative inhibition} = \frac{A_c - (A - A_b)}{A_c} \times 100\ \%$$

% oxidative inhibition = % oxidative inhibition of test sample

A_c = absorbance of the control (DPPH alone)

A_b = absorbance of the blank (EtOH + Test sample solution)

A = absorbance of test sample solution

Then, IC_{50} (50 % inhibitory concentration) values were also calculated by linear regressive excel program.

Screening of Antidiabetic Activity

The α-amylase inhibitory effects of ethanol and watery crude extracts with five different concentrations (6.25, 125, 250, 500, 1000 µg/mL) were determined by using α-amylase inhibition assay. The tested samples (1 mL) were pre-incubated with 1 mL of phosphate buffer and 2 mL of α-amylase at 37 ºC for 20 min and thereafter 0.4 mL (1 % w/v) starch solution was added. The mixture was further incubated at 37 ºC for 30 min. Then the reaction was stopped by adding 2 mL of DNS reagent and the contents were heated in a boiling water bath for 10 min. A blank was prepared without plant extracts and another without the analyse enzyme, replaced by equal quantities of buffer. The absorbance was measured at 540 nm. The reducing sugar released from starch was estimated as maltose equivalent from a standard graph. Acarbose was used as standard. The anti-diabetic activity was determined through the inhibition of α-amylase which was expressed as a percentage of inhibition and calculated by the following equations.

$$\% \text{ Inhibition} = [A_{control} - (A_{Sample} - A_{Blank}) / A_{control}] \times 100$$

where, % Inhibition = % α-amylase inhibition

$A_{control}$ = absorbance without sample solution

A_{sample} = absorbance of sample

A_{Blank} = absorbance of sample + distilled water solution

Then, the IC_{50} values were calculated by linear regressive excel program.

Determination of α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory effects of ethanol and watery crude extracts with five different concentrations (6.25, 125, 250, 500, 1000 µg/mL) was determined by using a modified assay of that described by McCue *et al*., (2005). The α-glucosidase was assayed using 0.4 mL of sample extracts and 1 mL of 0.1 M phosphate buffer (pH 0.9) containing 2 mL of α-glucosidase solution, which was then uncubated at 25 ºC for 10 min. After the pre-incubatuion period, 0.5 mL

of 0.005M *p*-nitrophenyl-α-D-glucopyranoside solution was added to each well at timed intervals. The reaction mixtures were incubated at 25 ºC for 5 min. After incubation, absorbance readings of the samples were recorded at 405 nm and compared with a control that had 0.4 mL of buffer solution in place of the extract. Acarbose was used as standard. The anti-diabetic activity was determined through the inhibition of α-glucosidase which was expressed as a percentage of inhibition and calculated by the following equations.

$$\% \text{ Inhibition} = [A_{control} - (A_{Sample} - A_{Blank}) / A_{control}] \times 100$$

where, % Inhibition = % α-glucosidase inhibition

$A_{control}$ = absorbance without sample solution

A_{sample} = absorbance of sample

A_{Blank} = absorbance of sample + distilled water solution

Then, the IC_{50} values were calculated by linear regressive excel program.

Screening of Antitumor Activity

The antitumour activity of methanol, ethanol and watery extracts of the sample was examined by Potato Discs Assay Method (Ali *et al*., 2016). This experiment was carried out at Pharmaceutical Research Department, Insein Township, Yangon Region, Myanmar. Tumor producing bacteria, *Agrobacterium tumefaciens*, isolated from *Sandoricum koetjape* Merr. (Thitto) leaves were used in this study. This bacterial strain has been maintained as solid slants under refrigeration. For inoculation on the potato discs, 48 h broth cultures containing 5×10^7~5×10^9 cell / mL were used. Fresh, disease free potato tubers were obtained from local markets and were transferred within 48 h to the laboratory.

Tubers of moderate sizes were surface-sterilized by immersion in 50 % sodium hypochlorite (Clorox) for 20 min. The ends were removed and soaked for 10 min more in Clorox. A core of the tissue was extracted from each tuber by using surface-sterilized (ethanol and flame) 2.5 cm wide cork borer and 2 cm pieces were removed from each end and discarded and the remainder of the cylinder was cut into 1.0 cm thick discs with a surface-sterilized cutter. The discs were then transferred to 1.5 % agar plates (1.5 g of Difco agar was dissolved in 100 mL of distilled water, autoclaved and 20 mL poured into each petri dish). Each plate contained three discs. This procedure was done in the clean bench in the sterile room. The sample (0.1, 0.15 and 0.2 g) was filtered through Millipore filters (0.22 µm) into a sterile tube. A 0.5 mL of this solution was added to 1.5 mL of sterile distilled water and 2 mL of broth culture of *A. tumefaciens* strain (48 h culture containing 5×10^7~ 5×10^9 cells/ mL) were added aseptically.

Controls were made in this way; 0.5 mL of DMSO and 1.5 mL of sterile distilled water were added to the tube containing 2 mL of broth culture of *A. tumefaciens* (from the same 48 h culture). Using a sterile disposable pipette, 1 drop (0.05 mL) from these tubes was used to inoculate each potato disc, spreading it over the disc, surface. The process of cutting the potatoes and incubation must be conducted within 30 min. The plates were sealed with tape to minimize moisture loss and incubated at room temperature and counted with microscope and compared with control. The antitumor activity was examined by observation if tumor is produced or not.

Determination of Antiproliferative Activity

Antiproliferative activity of ethanol and watery extracts were investigated in *vitro* by using cancer cell lines at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan. The cell lines used were Hela (human cervix cancer), A549 (human lung cancer) and MCF 7 (human breast cancer). K562 α-Minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) were used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, sigma) and 1 % antiobiotic antimycotic solution (Sigma). For MCF 7 cell, 1 % 0.1 M non-essential amino acid (NEAA, Gibco) and 1 % 1 mM sodium pyruvate (Gibco) were also supplemented. The *in vitro* antiproliferative activity of the crude extracts was determined by the procedure described by Win *et al.* (2015). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the tested samples were added. After 72 h incubation, the cells were washed with PBS and 100 μL of medium containing 10 % WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance was measured at 450 nm. The concentrations of the crude extracts were 200, 100, 10 μg/ mL and 10, 1, 0.1 mM for positive control were prepared by serial dilution. Cell viability was calculated from the mean values of the data from three wells using the equation below and antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-fluorouracil (5FU) was used as positive control.

$$(\%)\ \text{Cell viability} = 100 \times \frac{\left\{\text{Abs}_{(\text{test samples})} - \text{Abs}_{(\text{blank})}\right\}}{\left\{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}\right\}}$$

Results and Discussion

Phytochemicals Present in the Spine of *Z. rhetsa*

The spine sample of *Z. rhetsa* collected from Dawei Township, Tanintharyi Region was found to contain some of the secondary metabolites such as steroids, glycosides, phenolic compounds and terpenoids (Table 1) according to preliminary phytochemical screening. According to the physicochemical determination of the sample, the spine sample was found to contain (5.46 %) of total ash, (1.45 %) of water soluble ash, (0.19 %) of acid insoluble ash, (8.54 %) of moisture content, (< 100) of foaming index and 5mL/g of swelling index in the sample (Table 2).

Some Physicochemical Properties of the Spine of *Z. rhetsa*

As shown in Table 3, it can be seen that organic compounds are predominant in the sample, and other elements such as Si, S, Ca are also present in reasonable composition but K and Fe were present in medium amount and Cu was present in very little amounts based on the relative abundance of elements.

The soluble matter contents of the spine powder of *Z. rhetsa* in solvents of different polarities were observed 4.6 % in water, 3.0 % in methanol, 2.6 % in ethanol, 2.2 % in acetone, 2.0 % in ethyl acetate, 1.0 % in chloroform and 0.2 % in Petroleum ether (Table 4). Therefore, it

indicated that the phytochemicals present in spine sample were mostly the polar organic compounds.

Table 1 Results of Phytochemical Screening of the Spine of *Z. rhetsa*

Test	Extract	Test reagents	Observation	Results
Alkaloids	1%HCl	Dragendorff's reagent	Orange ppt.	+
		Mayer's reagent	Cream colour ppt	+
α-Amino acids	H_2O	Ninhydrin	Purple spot	+
Carbohydrates	H_2O	10%α-naphthol, Conc. H_2SO_4	Red ring	+
Cardiac glycoside	Dry powder	Glacial acetic acid, 5% $FeCl_3$	Blue colour	+
Flavonoids	EtOH	Conc. HCl, Mg ribbon	Green colour	+
Glycosides	H_2O	10% lead acetate	White ppt	+
Organic acids	EtOH	Bromocresol green	Blue color	+
Phenolics compounds	EtOH	10% $FeCl_3$	Dark blue colour	+
Polyphenols	EtOH	1% $FeCl_3$, $K_3Fe(CN)_6$	Dark blue colour	+
Protein	NaOH	$CuSO_4$	No colour change	-
Reducing sugar	H_2SO_4	Benedict's solution	No colour change	-
Saponins	H_2O	Shaking	Frothing	+
Starch	H_2O	I_2 solution	No dark blue colour	-
Steroids	PE	Conc. H_2SO_4 & acetic anhydride	Green colour	+
Tannins	H_2O	1% gelatin solution	Green colour	+
Terpenoids	$CHCl_3$	Conc. H_2SO_4 & acetic anhydride	Pink colour	+

(+) = Presence, (−) = absence

Table 2 Approximate Physicochemical Determination of the Spine of *Z. rhetsa*

Parameter	Value
Total ash content (%)	5.46
Water soluble ash content (%)	1.45
Acid insoluble matter content (%)	0.19
Moisture content (%)	8.54
Foaming index	< 100
Swelling index (mL/g)	5

Table 3 Relative Abundance of Elements in the Spine of *Z. rhetsa* by EDXRF

Macro and Micronutrients	Relative abundance (%)
Si	0.131
S	0.061
Ca	0.052
K	0.044
Fe	0.027
Mn	0.002
Cu	0.002
CH	99.681

Table 4 Extractable Matter Contents in the Spine of *Z. rhetsa*

Solvents	Weight of extractable matter (% w/w)
Water	4.6
Methanol	3.0
Ethanol	2.6
Acetone	2.2
Ethylacetate	2.0
Chloroform	1.0
Petroleum Ether	0.2

Total Phenol and Total Flavonoid Contents in the Spine of *Z. rhetsa*

Total phenol and total flavonoid contents in the spine of *Z. rhetsa* are shown in Figure 1 and Table 5. Total phenol contents of the extracts were calculated from the regression equation of calibration curve (Y = 0.0638 x + 0.0169; R^2 = 0.9941) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight. To perform the calculation of the total flavonoid contents in the samples by using Kiranmai *et al.* (2011) method, a standard curve is needed which is obtained from a series of absorbance of different quercetin concentrations (Y= 0.0019x +0.0115; R^2 = 0.9955). It can be seen that the total phenol of the ethanol extract (51.92±0.54 mg GAE/g of extract) was slightly higher than that of watery extract (41.56 ± 0.83 mg GAE/g of extract. In the case of total flavonoid content, total flavonoid content of ethanol extract (50.61 ± 2.29 mg QE/g of extract) is significantly higher than that of water extract (36.97 ± 1.05 mg QE/g of extract). The ethanol extract showed higher total phenol content and total flavonoid content than watery extract, indicating that phenolic and flavonoids compounds were more soluble in organic solvent than water. Generally, extracts with a high amount of phenolic compounds might exhibit high antioxidant activity.

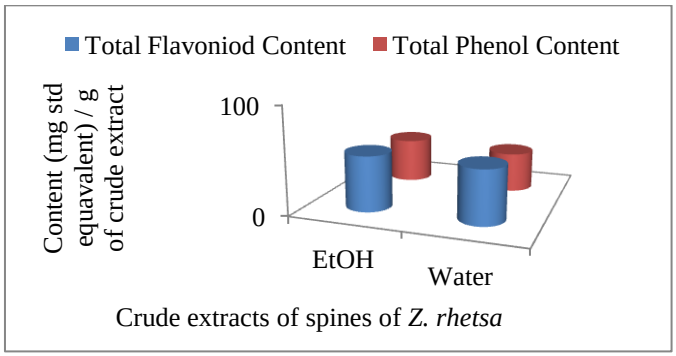

Figure 1 Total phenol and total flavonoid contents of *Z. rhetsa* Spine

Table 5 Total Phenol Content (TPC) and Total Flavonoid Content (TFC) of Crude Extracts

Types of compounds	EtOH extract	Water extract
TPC (mg GAE ± SD)/g of extract	51.92 ± 0.54	41.56 ± 0.83
TFC (mg QE ± SD)/g of extract	50.61 ± 2.29	36.97 ± 1.05

Antimicrobial Activity of the Spine of *Z. rhetsa*

The antimicrobial activity was assessed by agar well diffusion method which is equally suited to the screening of antibiotics or the products of plant evaluation and is highly effective for rapidly growing microorganisms and the activities of the test extracts are expressed by measuring the zones (mm) of inhibition. Generally, the more susceptible the organism, the bigger is the zone of inhibition. Antimicrobial activities of six different extracts (PE, EA, EtOH, MeOH, $(CH_3)_2CO$, $CHCl_3$, H_2O) of the sample were also determined against some fungal and bacterial species. The observed data are tabulated in Table 6. Generally, ethyl acetate and acetone extracts have higher activities (14~24 mm) on gram positive bacteria: *B. subtilis* and *B. pumilus*. Although acetone extract showed higher activity on *S. aureus* and *E. coli* (20 mm), ethyl acetate extract did not inhibit well. Ethyl acetate extract was found to possess higher antimicrobial activity against *P. aeruginosa* (23 mm), however, acetone extract (15 mm), exhibited low activity. Ethanol, methanol, chloroform and watery extracts were observed to exhibit medium antimicrobial activities against all tested microorganisms whereas pet-ether extract did not show any activity against all tested microorganisms.

Table 6 Inhibition Zone Diameters of some Crude Extracts against Six Microorganisms by Agar Well Diffusion Method

Organism	Inhibition Zone Diameters (mm) of Crude Extracts							
	PE	**EA**	**EtOH**	**MeOH**	**$CHCl_3$**	**Water**	**Acetone**	**Control**
Bacillius subtilis	-	24 (+++)	20 (+++)	15 (++)	15 (++)	14 (+)	20 (+++)	-
Staphylococcus aureus	-	14 (+)	17 (++)	15 (++)	18 (++)	13 (+)	20 (+++)	-
Pseudomonus aeruginosa	-	23 (+++)	14 (+)	13 (+)	12 (+)	13 (+)	15 (++)	-
Bacillus pumilus	-	21 (+++)	12 (+)	17 (++)	15 (++)	14 (+)	20 (+++)	-
Candida albicans	-	22 (+++)	15 (++)	15 (++)	15 (++)	13 (+)	18 (++)	-
E. coli	-	16 (++)	15 (++)	17 (++)	15 (++)	-	20 (+++)	-

Agar Well – 10 mm; control - solvent used; 10 mm ~ 14 mm (+) (low activity);
15 mm ~ 19 mm (++) (medium activity); above 20 (+++) (high activity)

Antioxidant Activity of the Spine of *Z. rhetsa*

Most of the medicinal plants possess phytochemicals and antioxidant activity. Flavonoids and tannins are phenolics which are a major group of compound in plants. These compounds act as primary antioxidant or free radical scavengers (Ayoola *et al*., 2008). The antioxidant activity of watery and ethanol extracts of the sample was studied by DPPH free radical scavenging assay (Marinova and Batchvarov, 2011). Gallic acid was used as standard. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay is widely used to investigate the free radical scavenging activities of several natural compounds such as crude extracts of plants. DPPH radical is scavenged by antioxidant through the donation of electron forming the reduced DPPH. Sample's colour change from purple to pale yellow which can be quantified by its decrease of absorbance at wavelength 517 nm (Maw *et al*., 2011). The radical scavenging activity of crude extracts were expressed in term of % RSA and IC_{50} (50 % inhibition concentration). The results are shown in Table 7. From these observations, the larger DPPH radical scavenging activity was observed in ethanol extract, which inhibited 50 % of free radicals at the concentration of 1.15 μg/mL (IC_{50}) than the water extract which inhibited 50% of free radicals at the concentration of 2.89 μg/mL (IC_{50}). It can be inferred that the antioxidant potency of the ethanol extract was found to be stronger than that of the watery extract in antioxidant property.

Table 7 DPPH Free Radical Scavenging Activity (% RSA) and IC_{50} of Crude Extracts of the Spine Sample and Standard Gallic Acid

Samples	% RSA ± SD at Different Concentrations (μg/mL)						IC_{50} (μg/mL)
	0.3125	0.625	1.25	2.5	5	10	
EtOH extract	35.50 ± 1.39	39.63 ± 0.45	52.02 ± 5.58	62.34 ± 3.41	80.63 ± 0.45	88.99 ± 5.31	1.15
Watery extract	28.42 ± 2.51	31.66 ± 1.39	34.41 ± 6.33	47.59 ± 5.21	62.93 ± 4.43	76.30 ± 0.95	2.89
Standard Gallic Acid	43.69 ± 1.28	64.28 ± 1.97	71.45 ± 1.40	86.45 ± 2.22	86.99 ± 1.15	87.12 ± 1.97	0.41

In vitro Screening of Antidiabetic Activity

Diabetes is a clinical syndrome characterization by hyperglycemia due to absolute or relative deficiency of Insulin. Recent decades have experienced a sharp increase the incidence and prevalence of diabetes mellitus. One antidiabetic therapeutic approach is to reduce gastrointestinal glucose production and absorption through the inhibition of carbohydrate digesting enzyme such as α-amylase and α-glucosidase. Inhibition of amylase and glucosidase enzymes involved in digestion of carbohydrates can significantly decreases the past prandial increase of blood glucose after a mixed carbohydrate diet therefore can be an important strategy in management of blood glucose (Narkhede *et al*., 2011).

The ethanol extract of the spine of *Z. rhetsa* (IC_{50} = 113.01 μg/L) possessed the higher α-amylase inhibition activity than the watery extract (IC_{50} = 137.00 μg/L) and also ethanol extract of the spine sample (IC_{50} = 120.56 μg/L) possessed the higher α-glucosidase inhibition property than the watery extract (IC_{50} = 446.78 μg/L) (Table 8 and 9). Both the ethanol and watery extracts possessed lower antidiabetic activity than standard acarbose which showed α-amylase inhibition activity with IC_{50} =0.016 μg/mL and α-glucosidase inhibition activity with IC_{50} = 0.042 μg/mL.

Table 8 α-Amylase Inhibition and IC_{50} of Ethanol and Watery Extracts of Spine of Ka-thit-phu

Samples	% Inhibition in Different Concentrations (μg/mL)					IC_{50} (μg/mL)
	62.5	125	250	500	1000	
EtOH extract	33.85 ± 0.44	53.82 ± 0.78	71.90 ± 0.44	100 ± 0.44	122.8 ± 0.00	113.01
Water extract	31.64 ± 0.22	44.27 ± 0.66	59.19 ± 1.89	89.66 ± 1.22	115.86 ± 4.79	137.00

Table 9 α-Glucosidase Inhibition and IC_{50} of Ethanol and Water Extracts of Spine of Ka- thit-phu

Samples	% Inhibition in Different Concentrations (μg/mL)					IC_{50} (μg/mL)
	62.5	125	250	500	1000	
EtOH extract	30.42 ± 3.72	51.49 ± 0.29	80.33 ± 2.05	141.74 ± 1.02	164.44 ± 3.26	120.56
Water extract	35.67 ± 0.34	38.59 ± 1.02	42.47 ± 0.68	52.06 ± 1.88	102.06 ± 5.66	446.78

Antitumor Activity of the Spine of *Z. rhetsa*

The antitumor activity of various crude extracts (MeOH, EtOH, H_2O, Acetone, EtOAc, $CHCl_3$, PE) the spine sample was investigated by using PCG test with the isolated tumor forming bacterium *A. tumefaciens*. The 48 h broth cultures containing 5 x 10^9 cells/mL were used to inoculatethe potato disc. The tested samples were dissolved in DMSO to dilute and the diluted samples were mixed with the bacterial culture for inoculation. After preparing the inoculums, the bacterial suspension was inoculated on the cleaned and sterilized potato discs, and incubated at room temperature for 3 days. Then, the tumors appeared on potato discs and checked by staining the knob with Lugol's (I_2-KI) solution. In the control, the formation of white knob on the blue background indicated tumor formation. The active test samples did not form any tumor on the potato discs and its surface remained blue. This experiment revealed that all of the tested

samples exhibited the inhibition of tumor formation at the dose of 0.3 g/disc after 5 days and 7 days treatment (Table 10).

Table 10 Observation of Tumor Inhibition by Different Concentrations of Crude Extracts of the Spine Sample after 5 Days and 7 Days Treatment

Extracts	Observation of Tumor Formation by Different Concentrations of Crude Extracts		
	0.1 g/disc	**0.2 g/disc**	**0.3 g/disc**
MeOH	+	+	-
EtOH	+	+	-
H_2O	+	+	-
Acetone	+	+	-
EtOAc	+	+	-
$CHCl_3$	+	+	-
PE	+	+	-
Control	++		

(+) Tumor formation (-) No Tumor formation

Antiproliferative Activity of the Spine of *Z. rhetsa*

Cancer is a malignant tumor or malignant neoplasm, is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Many traditional plant treatments for cancer are used throughout the world, and some of these plants have been scrutinized while a good number of them are yet to receive scientific scrutiny. Among them, the spine of *Z. rhetsa* was selected for this study since they are widely distributed in Myanmar. Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. Antiproliferative activity was studied in *vitro* using human cancer cell lines. Screening of antiproliferative activities of ethanol and watery extracts from the spine of *Z. rhetsa* was done by using three human cancer cell lines such as Hela (human cervix cancer) and MCF7 (human breast cancer). Antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-Fluorouracil was used as positive control. The antiproliferative activity of crude extracts are summarized in Table 11. From the results, the IC_{50} values were found to be 3.74 μg/mL against cervix cancer, 7.71 μg/mL against breast cancer and 4.77 μg/mL against lung cancer by ethanol extract and >200 μg/mL against cervix cancer, >200 μg/mL against breast cancer and >200 μg/mL against lung cancer by watery extract, respectively. Since the lower the IC_{50} values, the higher the antiproliferative activity, ethanol extract was more potent than watery extract in antiproliferative activity on the selected cell lines.

Table 11 Antiproliferative Activity of Crude Extracts against Three Types of Cancer Cell Lines

Samples	IC_{50} (μg/mL) of Various Samples against tested cell lines		
	Hela	**MCF 7**	**A 549**
EtOH Extract	3.74	7.71	4.77
Watery Extract	>200	>200	> 200
5FU	14.14	16.04	16.04

Hela - human cervical cancer cell lines MCF 7 - human breast cell lines
A549 - human lung cancer cell lines 5FU* - Fluorouracil (drug for cancer)

Conclusion

From the overall assessment concerning with investigation of phytochemicals and some biological activities on the spine of *Z.rhetsa* (Roxb.) DC. (Ka-thit-phu), ethanol extract was observed to possess higher antimicrobial, antioxidant, antidiabetic and antiproliferative activities than watery extract, it might be due to its higher contents of total phenols and total flavonoids. According to the observations, since the ethanol and watery extract of Ka-thit-pu spine showed these activities, these extracts may be effectively used for the treatment of skin disease, wound infections, diarrhea and also as antioxidant for curing the oxidative stress related diseases, some forms of cancer and some age-related disorders. The quantitative and qualitative phytochemical analytical data are also expected to be applicable to some extent in the medicinal formulation by using the spine of *Z. rhetsa*.

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