COMPARATIVE STUDIES ON PHYTOCHEMICAL CONSTITUENTS AND SOME BIOLOGICAL ACTIVITIES OF THE SEEDS, FRUITS, LEAVES, AND BARK OF *ZIZIPHUS MAURITIANA* LAM. (ZEE)

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Abstract

The aim of this research is to isolate bioactive compounds and to investigate some biological activities, such as antioxidant, α-amylase and antimicrobial activity, of the fruits, seeds, bark and leaves of *Ziziphus mauritiana* Lam. (Zee). According to the phytochemical tests, Zee bark and leaves have more secondary metabolites than other. By thin layer and silica gel column chromatographic methods, lupeol was isolated from the ethyl acetate extract of the seeds and identified by the nuclear magnetic resonance (NMR) spectroscopic method. The antioxidant activity of bark of ethanol extract (IC_{50} = 2.51 μg/mL) and watery extract (IC_{50} = 3.30 μg/mL) was found, and Zee leaves of ethanol extract (IC_{50} = 3.10 μg/mL) and watery extract (IC_{50} = 2.11 μg/mL) were detected. In α-amylase activity, the percent inhibition of α-amylase activity of ethanol extracts of fruits, seeds, bark and leaves has good activity, but water extracts have mild activity. According to antimicrobial activity, the ethanol extracts of all four portions have good activity.

Keywords: *Ziziphus mauritiana*, Lupeol, Antioxidant, α-Amylase, Antimicrobial activity

Introduction

The medicinal plants have very complex chemical constituents called secondary metabolites, which make them very important in the field of therapeutics. The folkloric system, which is mostly based on phytotherapy, is still used by around 80 % of the world's population. Zee scientifically known as *Ziziphus mauritiana* Lam. is one of the plant family Rhamanacae. Genus is *Ziziphus* and species is *mauritiana.* Myanmar name is Zee Chin. It is grown in dry places and is found in India, Pakistan and China (Perez *et al*., 1990). It can be found in the middle part of Myanmar. The genus Z*iziphus* comprises approximately 170 species and is important in the treatment of various diseases. In Myanmar, Zee seeds were exported to China and Korea for medicinal uses. The fruit is variable shape and size. It can be oval, oblong or round and can be 1-1.5 in long depending on the variety. Its leaves are used in the treatment of liver diabetic, asthma, gonorrhea, and fever. The fruits have been used as anodyne, sedative, tonic, anticancer, potent wound healer. All parts of this plant are extremely effective against various types of diseases (Song, *et al*., 2010).

Hence, *Ziziphus mauritiana* Lam. (Zee) was chosen for this study because it has a variety of biological activities and bioactive chemical constituents, as well as a lack of scientific reports on locally grown Zee plants. In this research work, the isolation of some phyto-constituents and investigation of antioxidant activity, α-amylase activity, and antimicrobial activity of the seeds, fruits, leaves, and bark of Zee were carried out on the respective crude extracts.

Materials and Methods

Plant Materials

The four parts of Zee sample were collected from Kyaukpadaung Township, Mandalay region during April in 2018. After collection the sample was confirmed at Department of Botany,

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University of Yangon. The four parts of Zee sample were cleaned and air dried at room temperature. The dried samples were cut into small pieces and were ground into powder by using a grinding machine. The powdered samples were stored in airtight container to prevent contamination and were kept for the isolation of organic compounds and screening biological activities.

Phytochemical Screening

Preliminary phytochemical tests such as alkaloids, flavonoids, terpenoids, steroids, glycosides, organic acids, starch, phenolic compounds, saponins, tannins, carbohydrates, cyanogenic glycosides, reducing sugars, and α-amino acids tests were carried out according to the appropriate reported methods (Sofowora, 2000).

Isolation and Identification of Phytochemical Constituent from Ethyl acetate Extract of Seeds of Zee by Column Chromatography

Dried powdered bark sample (1 kg) was percolated in 1 L of 70 % ethanol for one week and filtered. This procedure was repeated three times. Then the filtrate was concentrated by using a vacuum rotatory evaporator to give an ethanol extract (100 g). Then the ethanol extract was defatted by using petroleum ether and the defatted ethanol extract was successively partitioned between ethyl acetate and water. The ethyl acetate layer was concentrated under reduced pressure using a vacuum rotatory evaporator. Ethyl acetate crude extract (45 g) from the seeds of Zee was subjected to column chromatographic separation using silica gel (63-210 µm mesh). Gradient elution was performed successively with the PE: EtOAc system in the ratios of 20:1, 15:1, 9:1, 5:1, 3:1, 1:1, 1:2, and 1:5 v/v followed by ethyl acetate only and methanol only. Successive fractions obtained were combined on the basis of their behavior on TLC. Finally, eight main fractions, F-I to F-VIII, were obtained. When the fraction F-IV was evaporated and washed with petroleum ether, a white powder of the compound A in 65 mg was obtained. The isolated compound was then identified using its physicochemical properties and modern spectroscopic techniques such as ^{1}H NMR and ^{13}C NMR and compared with the reported data. The NMR spectra of the isolated compound were measured at Division of Natural Product Chemistry, Institute of Natural Medicine, and University of Toyama, Japan.

Screening of Antioxidant Activity of Ethanol and Watery Extracts of Seeds, Fruits, Leaves, and Bark of Zee

In this experiment, DPPH (2 mg) was thoroughly dissolved in ethanol (100 mL). This solution was freshly prepared in the brown coloured reagent bottle. Each of the tested samples (2 mg) and 10 mL of ethanol were thoroughly mixed by shaker. The mixture solution was filtered, and the stock solution was obtained. By adding ethanol, the sample solutions in different concentrations of 125, 62.5, 31.25, 15.62, 7.81, 3.91, and 1.95 µg/mL were prepared from the stock solution. The effect on the DPPH radical was determined using the method of Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 50 µM DPPH solution with 1.5 mL of ethanol using a shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 50 µM DPPH solutions and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of each solution was measured at 517 nm by a UV-visible spectrophotometer (GENESYS 10 S UV-VIS, China). Absorbance measurements were done in triplicate for each concentration, and the mean values so obtained were used to calculate the percent inhibition of oxidation. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\%\,\mathrm{RSA} = \frac{A_c - (A - A_b)}{A_c} \times 100$$

Where, %RSA = Radical Scavenging Activity
A_c = absorbance of the control (DPPH only) solution
A_b = absorbance of the blank (ethanol + Test sample solution) solution
A = absorbance of the test sample solution

Determination of *α*-Amylase Inhibition Potency

In *α*-amylase assay, the starch-iodine method was used. First 2 mL of (0.5 %) substrate starch solution and 1 mL of tested solution (Acarbose standard drug, ethanol extract and aqueous extract) of 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91 and 1.95 µg/mL were added in a bottle and these mixtures was incubated for 3 min at room temperature. To start the reaction, 1 mL of *α*-amylase was added in above solution followed by incubated for 15 min at room temperature. To stop the reaction, 4 mL of 0.1 M HCl was added in this mixture and to detect the reaction, 1 mL of Iodine-iodide indicator (1 mM) was added in this mixture. Absorbance was read at 650 nm by UV spectrophotometer in the visible region. The control solution was prepared as above procedure by using phosphate buffer (0.02 M) instead of drug solution. All the experiments were done in triplicate. Percent inhibition of each sample solution was calculated by using the following formula.

$$\%\ \text{inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where,

$A_{control}$ = the absorbance of the control solution
A_{sample} = the absorbance of sample solution

Standard deviation (SD) and 50 % inhibition concentration (IC_{50}) value in µg/mL were calculated by computer excel program.

Screening of Antimicrobial Activity of Various Crude Extracts of the Seeds, Fruits, Leaves, and Bark of Zee by Agar Well Diffusion Method

The screening of antimicrobial activity of various crude extracts such as pet-ether, ethyl acetate, ethanol, and watery extracts of the four parts of *Ziziphus mauritiana* L. were carried out by agar well diffusion method at Department of Botany, Pathein University, Myanmar. Six microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans,* and *Escherichia coli* were used for this test (Perez *et al*., 1990).

Results and Discussion

Types of Phytochemicals Present in Seeds, Fruits, Leaves, and Bark of Zee

In order to find out the types of phytochemical constituents present in the four portions of *Z. mauritiana* L., preliminary phytochemical tests were carried out according to the reported procedure. From the data findings, it was observed that various secondary metabolites such as alkaloids, terpenoids, steroids, glycosides, organic acids, phenolic compounds, saponins, tannins, and carbohydrates were present, however cyanogenic glycosides, flavonoids, starch, and reducing sugars were not detected in the samples. It can be found as cyanogenic glycosides in leaves sample.

According to these results, it can be seen that the bark and leaves samples might contain potent bioactive secondary metabolites.

Isolation and Identification of Compound from Ethyl acetate Extract of Zee seeds

Compound A was isolated as a white crystal (R_f = 0.6, *n*-Hexane: EtOAc, 4:1 v/v) from the ethyl acetate extract of the seeds by silica gel column chromatographic separation. Compound A is soluble in chloroform and chloroform and methanol mixtures but insoluble in pet-ether, ethanol, and acetone. According to FT IR spectrum data (Figure 1), 3315 cm^{-1} (-OH stretching vibration of hydroxyl group), 3095 cm^{-1} (=C-H stretching), 2923 cm^{-1} and 2852 cm^{-1} (C-H asymmetric and symmetric stretching), 1638 cm^{-1} (=C-C stretching), 1452 cm^{-1} (C-H bending of CH_2), 1379 cm^{-1} (C-H bending of CH_3 group), and 1043 cm^{-1} (C-O stretching vibration) can be found.

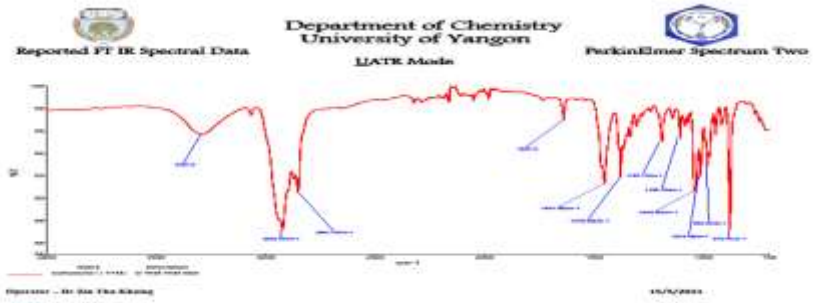

Figure 1. FT IR spectrum of isolated compound A

The structural elucidation of isolated compound A was determined by NMR spectroscopy. The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound A (Figure 2) indicated that the signals at δ_H 4.68, 4.56 (d, *J* = 1.9 Hz, H_2-29), 2.37 (m, H-19), 1.37 (t, *J* = Hz, H-18), 1.02 (d, *J* = Hz, H-15), 3.19 (dd, *J* = Hz, H-3), 0.91 (s, H_3-23), 0.69 (t, *J* = Hz, H-5), and 1.67 (s, H_3-30). The ^{13}C NMR (400 MHz, $CDCl_3$) spectrum of compound A (Figure 3) revealed the presence of 30 carbon signals, which were further classified into 7 methyl carbons at δ_C 27.4 (C-23), 15.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27), 17.9 (C-28), and 19.3 (C-30), 11 methylene carbons at δ_C 38.8 (C-1), 28.4 (C-2), 18.3 (C-6), 34.3 (C-7), 20.9 (C-11), 25.1 (C-12), 27.4 (C-15), 35.6 (C-16), 29.8 (C-21), 39.9 (C-22), and 109.3 (C-29), 6 methine signals at δ_C 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 38.0 (C-13), 48.3 (C-18), and 47.9 (C-19), and 6 quaternary carbon signals at δ_C 38.7 (C-4), 40.8 (C-8), 37.1 (C-10), 42.8 (C-14), 42.9 (C-17), and 150.9 (C-20). The ^{1}H and ^{13}C NMR spectral data of compound A were identical with the reported NMR spectral data of lupeol (Mahato *et al*., 1994). Therefore, the structure of the isolated compound A was assigned as lupeol, and its molecular formula is $C_{30}H_{50}O$ (Figure 4).

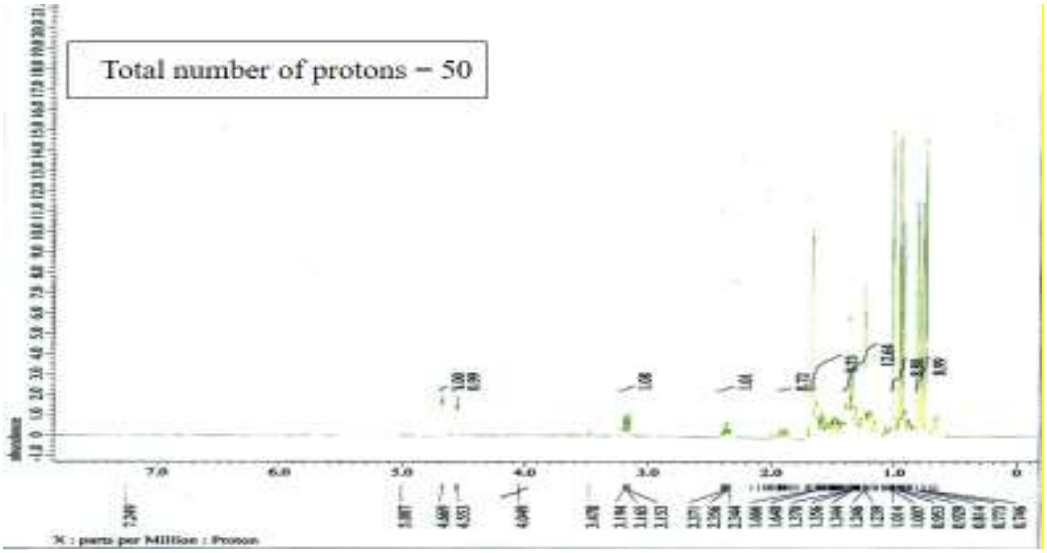

Figure 2. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound A

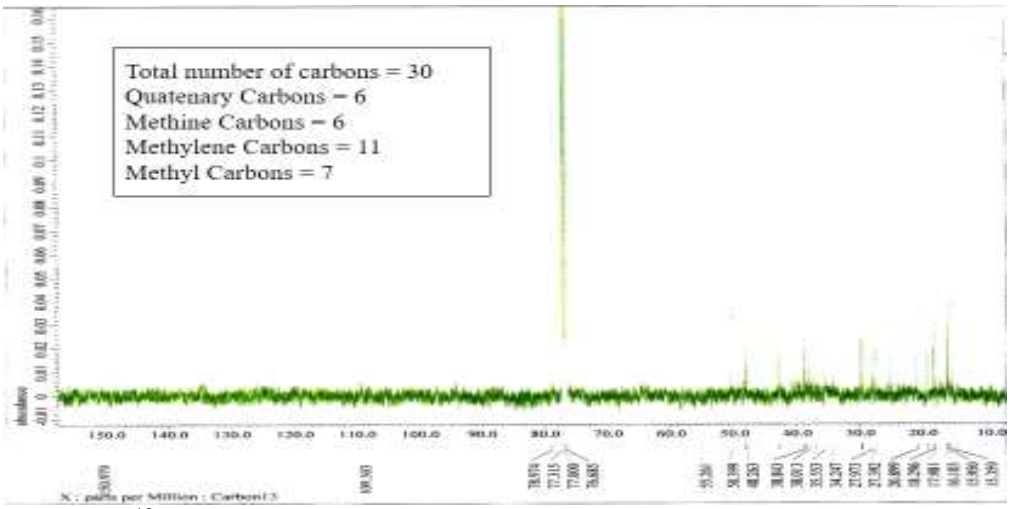

Figure 3. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound A

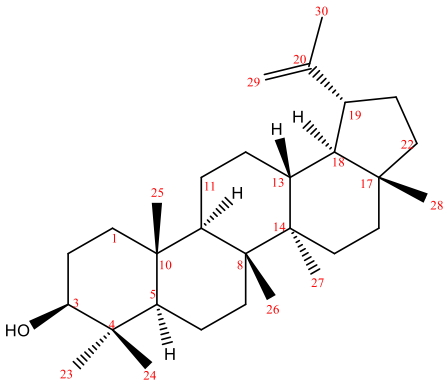

Figure 4. Structure of lupeol ($C_{30}H_{50}O$)

Antioxidant Activity of Crude Extracts of Fruits, Seeds, Bark and Leaves of Zee

The antioxidant activity was measured in terms of hydrogen donation or radical scavenging ability of the ethanol and watery extracts of the samples by using the stable radical DPPH. The radical scavenging activity of standard ascorbic acid and the results are shown in Table 1 and Figures 5 and 6. From these observations, the radical scavenging activity of Zee Bark of ethanol extract (IC_{50} = 2.51 μg/mL) and watery extract (IC_{50} = 2.11μg/mL) was found and Zee leaves of ethanol extract (IC_{50} = 2.51 μg/mL), watery extract (IC_{50} = 3.30 μg/mL) were detected. According to these results, Zee bark and leaves have more potent activities than other portions.

Table 1. % Radical Scavenging Activity and IC_{50} Values of Crude Extracts of Zee Fruits, Seeds, Bark and Leaves by DPPH Radical Scavenging Assay

No.	Samples	Extracts	% Inhibition ± SD at Different Concentration (μg/mL)						IC_{50} (μg/mL)
			1.95	3.91	7.81	15.62	31.25	62.5	
1	Fruits	EtOH	46.18 ± 0.75	48.76 ± 0.23	50.09 ± 0.34	52.06 ± 0.96	53.58 ± 0.27	54.45 ± 0.38	7.54
		Watery	28.05 ± 0.43	34.76 ± 0.16	37.74 ± 0.32	39.99 ± 0.21	45.32 ± 0.48	51.15 ± 0.21	56.34
2	Seeds	EtOH	38.93 ± 0.68	41.41 ± 0.94	45.45 ± 0.63	50.87 ± 0.71	52.62 ± 0.28	54.41 ± 0.55	14.58
		Watery	44.12 ± 0.21	45.41 ± 0.16	46.51 ± 0.16	48.21 ± 0.28	52.11 ± 0.32	55.19 ± 0.48	22.79
3	Bark	EtOH	48.58 ± 0.48	53.54 ± 0.21	55.05 ± 0.62	56.61 ± 0.29	60.56 ± 0.36	67.63 ± 0.41	2.51
		Watery	43.11 ± 0.68	53.08 ± 0.50	56.15 ± 0.50	59.50 ± 0.44	60.65 ± 0.16	64.78 ± 0.25	3.30
4	Leaves	EtOH	47.11 ± 0.89	52.02 ± 0.21	61.62 ± 0.16	64.97 ± 0.14	73.55 ± 0.92	78.65 ± 0.14	3.10
		Watery	49.77 ± 0.60	52.48 ± 0.94	56.75 ± 0.08	60.93 ± 0.48	67.54 ± 0.57	69.05 ± 0.16	2.11
5	Standard	Ascorbic acid	48.39 ± 0.76	54.68 ± 0.95	60.61 ± 0.96	78.42 ± 1.03	85.72 ± 0.08	87.47 ± 0.28	2.45

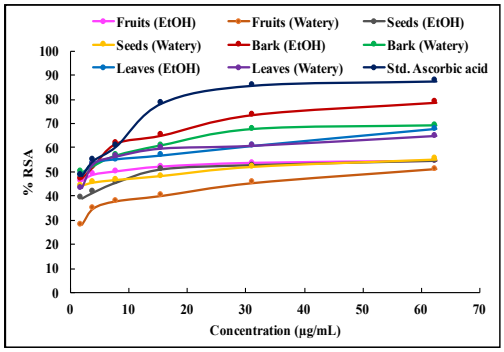

Figure 5. A plot of % DPPH free radical scavenging activity *vs* different concentrations of various crude extracts of Zee

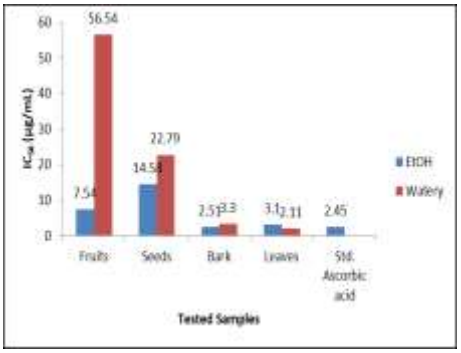

Figure 6. A bar graph of IC_{50} values of Ze Fruits, Seeds, Bark and Leave (DPPH free radical scavenging assay)

α-Amylase Enzyme Inhibition Activity of Seeds, Fruits, Bark and Leaves of *Z. mauritiana* Lam.

The *α*-amylase inhibitory activity of seeds, fruits, bark, and leaves of zee was investigated. The percentage inhibition of the *α*-amylase by ethanol and watery extracts was studied in concentrations of (125, 62.5, 31.25, 15.62, 7.81, 3.92, and 1.95 µg/mL), respectively. The percentage inhibition of the sample on *α*-amylase enzyme activity increased with the increasing concentration. The percentage inhibitions of *α*-amylase activity of ethanol extracts from all four portions have good activity, but water extracts have mild activity. These observations are dedicated in Figures 7 and 8, and Table 2.

Table 2. α-Amylase Inhibition % and IC_{50} Values of the Crude Extracts of Four Tested Samples of Zee and Standard Acarbose

Samples	Extracts	% Inhibition ± SD at Different Concentration (µg/mL)							IC_{50} (µg/mL)
		1.95	3.91	7.81	15.62	31.25	62.5	125	
Fruits	EtOH	39.87 ± 0.61	46.95 ± 0.62	52.27 ± 0.21	53.25 ± 0.41	56.42 ± 0.35	60.1 ± 0.10	64.32 ± 0.27	6.14
	Watery	34.07 ± 0.48	35.71 ± 0.44	43.07 ± 0.51	52.94 ± 0.55	55.67 ± 0.06	62.55 ± 0.99	67.68 ± 0.68	13.29
Seeds	EtOH	46.71 ± 0.71	52.31 ± 0.25	54.71 ± 0.17	58.25 ± 0.39	61.08 ± 0.33	63.08 ± 0.33	67.67 ± 0.22	3.10
	Watery	35.85 ± 0.91	39.74 ± 0.62	45.11 ± 0.24	48.03 ± 0.41	52.19 ± 0.18	58.41 ± 2.27	65.88 ± 0.27	23.02
Bark	EtOH	37.67 ± 0.54	50.74 ± 0.45	53.29 ± 0.48	55.53 ± 0.28	59.03 ± 0.4	64.63 ± 0.14	67.41 ± 0.26	3.79
	Watery	22.64 ± 0.80	33.68 ± 0.23	36.43 ± 0.45	45.16 ± 0.24	48.41 ± 0.43	50.52 ± 0.22	54.53 ± 0.31	54.74
Leaves	EtOH	49.45 ± 0.27	52.93 ± 0.06	56.32 ± 0.15	57.39 ± 0.05	58.52 ± 0.35	59.37 ± 0.1	61.30 ± 0.04	2.26
	Watery	41.67 ± 0.36	48.5 ± 0.37	52.51 ± 0.24	55.18 ± 0.22	56.55 ± 0.40	58.55 ± 0.33	60.32 ± 0.21	5.37
Std.	Acarbose	44.84 ± 0.41	52.27 ± 0.24	58.88 ± 0.42	65.13 ± 0.06	66.65 ± 0.12	71.61 ± 0.12	79.99 ± 0.12	3.31

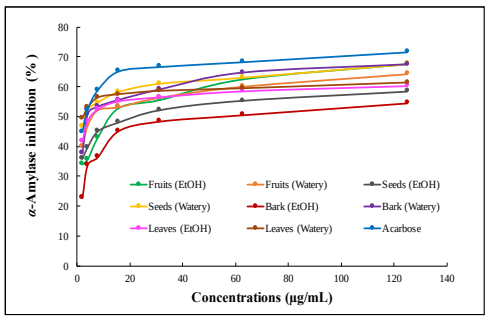

Figure 7. α-Amylase inhibition percent of tested samples of Zee

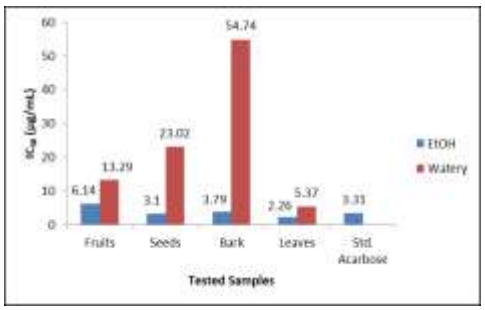

Figure 8. A bar graph of IC_{50} values of α-amylase activity of tested samples of Zee

Antimicrobial Activity of Crude Extracts of the Fruits, Seeds, Bark, and Leaves of Zee

Four crude extracts such as pelidem ether, ethyl acetate, ethanol and watery extracts, from four parts of the samples were subjected to screening for antimicrobial activity against six different pathogenic microbes using the agar well diffusion method. This method is based on the zone diameter, including the well diameter, in millimeter (mm). The larger the zone diameter, the higher the activity. According to the results, the ethanol extracts of all four portions have good activity, but watery extracts have mild activity. The resultant data are shown in Table 3.

Table 3. Inhibition Zone Diameters of Various Extracts of the Seeds, Fruits, Leaves and Bark of Zee against Six Microorganisms by Agar Well Diffusion Method

No.	Microorganisms	Samples	Diameter of inhibition zone (mm) in various crude extracts			
			PE	EtOAc	EtOH	H_2O
1	*B. subtilis*	Fruits	13	32	35	18
		Seeds	-	11	15	-
		Bark	15	15	15	19
		Leaves	13	23	15	18
2	*S. aureus*	Fruits	13	30	33	17
		Seeds	-	12	16	-
		Bark	18	17	16	19
		Leaves	18	24	16	17
3	*P. aeruginosa*	Fruits	17	33	35	26
		Seeds	-	12	14	-
		Bark	17	19	18	20
		Leaves	18	23	18	14
4	*B. pumilus*	Fruits	15	25	35	24
		Seeds	-	12	16	-
		Bark	19	19	17	19
		Leaves	16	23	14	16
5	*C. albicans*	Fruits	18	27	32	24
		Seeds	-	11	18	-
		Bark	19	17	17	20
		Leaves	15	22	16	20
6	*E. coli*	Fruits	13	28	34	20
		Seeds	-	12	17	-
		Bark	15	18	18	20
		Leaves	13	23	15	13

Diameter of agar well = 8 mm
10 mm - 14 mm = weak activity
15 mm - 19 mm = moderate activity
20 mm - above = potent activity

Conclusion

From the overall assessment concerning the investigation of phytochemicals and biological activities on the seeds, fruits, bark, and leaves of *Z mauritiana* Lam. the following inferences could be deduced. Lupeol was isolated from the ethyl acetate extract of the seeds by using silica gel column chromatographic separation technique. The bark and leaves of both extracts possessed greater antioxidant activity than those of other portions. According to the α-amylase activity, ethanol extracts of seeds, bark, and leaves have good α-amylase activity; however, other extracts

showed mild activity. For antimicrobial activity, pet-ether, ethanol, ethyl acetate and watery extracts of fruits, bark and leaves showed good antimicrobial activity against all tested microorganisms whereas seeds of ethanol extract and ethyl acetate extract have mild activity and then pet- ether extract and watery extract did not show antimicrobial activity. In conclusion, the four portions of Zee were found to have rich chemical constituents. According to the experimental result, the present study will contribute that four portions of *Ziziphus mauritiana* Lam. can be used in the traditional medicinal formulation for the treatment of many diseases.

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