ISOLATION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM BARK OF *HOLOPTELEA INTEGRIFOLIA* R. (PHYAUK-SEIK)

Tha Zin Nwe Oo[1], Mya Thandar Aung[2], Saw Hla Myint[3]

Abstract

One of the sources of bioactive compounds and a potential source of new therapeutic medicines are medicinal plants. Due to its traditional therapeutic benefits for the treatment and prevention of numerous ailments, *Holoptelea integrifolia* Roxb. (Ulmaceae) is a well-known medicinal plant in Myanmar. The purpose of this study is to separate various organic components from the bark of *H. integrifolia* (Phyauk-seik) and assess the principal isolated compound's in vitro anti-arthritic and anti-diabetic effects. Three chemicals, including TZNO-I, TZNO-II, and TZNO-III, were extracted from the plant's bark. An advanced spectroscopic method like Fourier Transform Infrared Spectroscopy (FT IR) was used to determine the structures of the isolated chemicals because these structures were also compared to authentic structures. According to the findings of the protein denaturation method used to assess anti-arthritic activity, TZNO-III exhibited considerable activity against both egg albumin and bovine serum albumin that was concentration-dependent and increased in percentage protection at 1600 □g/mL. Comparing TZNO-III to the two common medications metformin and acarbose revealed that it likewise has mild anti-diabetic activity on the α-amylase and α-glucosidase enzymes with IC_{50} values of 56.72 □g/mL and 40.63 □g/mL.

Keywords: *Holoptelea integrifolia*, anti-arthritic, anti-diabetic, protein denaturation method

Introduction

Holoptelea integrifolia Roxb. is a medicinal and ornamental plant that is widely distributed all over the tropical and temperate regions of the Northern Hemisphere, including Pakistan, India, Nepal, Srilanka, Cambodia, Laos, Myanmar, Vietnam, etc. (Mahmud *et al*., 2010; Kirtikar and Basu, 1999). It is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, hemorrhoids, and rheumatism (Warrier *et al.*, 1995). Bark and leaves are used as astringent, anti-inflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, urinary astringent, and rheumatism (Prajapati *et al*., 2003). Its English name is Indian Elm, which belongs to the family "Ulmaceae". It is a large deciduous tree, growing up to 18 m tall. It contains valuable phytochemicals such as alkaloids, α-amino acids, glycosides, phenolic compounds, steroids, terpenoids, etc.

Different parts of the plant, like stem bark, heartwood, leaves, seeds, and roots, are the major sources of various medicinally important phytochemicals. Two triterpenoid fatty acid esters, Holoptelin-A and B, 2-amino naphthaquinone, friedelin, epifriedelinol, beta-sitosterol, and its beta-D-glucose, beta-amyrin, botulin, and betulinic acid, are derived from stem bark. And, hexacosanol, octacosanol, 1,4-napthalenidone, beta-sitosterol, and alpha-amyrin are derived from leaves (Sharma, 2009; Mondal *et al.*, 2016). The aim of the present study is to isolate some organic constituents and some bioactivities of a major isolated compound from the bark of *H. integrifolia*.

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Materials and Methods

I. Collection and Preparation of Plant Materials

H. integrifolia (Phyauk-seik) bark (Figure 1) was collected from Nyaung Bin Seik Quarter, Mawlamyine Township in Mon State and identified at the Department of Botany, Mawlamyine University.

Plant Barks Leaves

Figure 1 Photographs of plant, barks and leaves of *H. integrifolia*

After being collected, the bark of phyauk-seik was cleaned thoroughly with distilled water, dried under shade, and ground into a coarse powder. The powdered samples were separately stored in airtight containers and kept in a cool, dark, and dry place until analyses were commenced.

II. Preparation of Plant Extracts and Isolation of Organic Compounds

The dried powdered sample of the bark of *H. integrifolia* was maceration in 1000 mL of 70 % ethanol (EtOH) extract for one week and filtered. Then, the filtrate was concentrated with the rotatory evaporator to get 70 % EtOH extract. It was then partitioned with petroleum ether (PE) and successively with ethyl acetate (EtOAc) obtaining PE and EtOAc extracts respectively.

The ethyl acetate extract (5 g) was used to isolate organic compounds by using a column chromatographic method with gradient solvent systems PE: EtOAc 15:1, 11:1, 9:1, 4:1, and 1:1 v/v, respectively. The isolated compounds TZNO-I and TZNO-II were separately isolated from the PE: EtOAc 11:1 solvent system and the isolated compound TZNO-III was obtained from the PE: EtOAc 9:1 solvent system, respectively.

III. Determination of Melting Points of Isolated Compounds

The isolated compounds (0.5 mg each) were introduced into a capillary tube and their melting points were determined by the melting point apparatus. Each measurement was repeated three times.

IV. Identification of Isolated Compounds by FT IR Spectroscopic Method

The three isolated compounds (TZNO-I, TZNO-II, and TZNO-III) were identified by the Fourier Transform Infrared Spectroscopic method using an IR 8400 spectrophotometer (SHIMADZU, Japan) at the Department of Chemistry, University of Yangon. The FT IR spectra and respective data of the isolated compounds are compared with the authenticated structures.

V. *In Vitro* Anti-arthritic Activity of an Isolated Compound TZNO-III

The *in vitro* anti-arthritic activity of the major isolated compound TZNO-III was determined by protein denaturation methods using fresh hen's egg albumin and bovine serum albumin (Sheelarani *et al*., 2014).

Preparation of sample and standard solutions

The sample and standard solutions were prepared by dissolving 16 mg of an isolated compound TZNO-III and standard diclofenac sodium in 10 mL of distilled water. The stock solution was serially half diluted with distilled water to get the sample and standard solutions with different concentrations of 1600, 800, 400, 200, 100, and 50 µg/mL, respectively.

Procedure for anti-arthritic activity using egg albumin denaturation method

A total of 5 mL of the reaction mixture was formed by mixing 0.2 mL of 100 % fresh hen's egg albumin, 2.8 mL of phosphate-buffered saline (pH 6.8), and 2 mL of test solution or standard drug diclofenac sodium in various concentrations (1600, 800, 400, 200, 100, and 50 µg/mL). An equal volume of double distilled water served as a control solution without test solutions. The test sample and standard solutions were incubated at 37 ± 2 ºC in an incubator for 15 min, followed by heating at 70 ºC for 5 min. The absorbance of these solutions was measured at 660 nm using a UV-Visible spectrophotometer. The percentage inhibition of protein denaturation was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}}}{\text{Abs}_{\text{Control}}} \times 100$$

Procedure for anti-arthritic activity using bovine serum albumin denaturation method

0.05 mL of the different concentrations of the sample or standard solutions were mixed with 0.45 mL of 5 % bovine serum albumin to obtain the test solutions. The test control solution was prepared with 0.05 mL of distilled water and 0.45 mL of 5 % bovine serum albumin, and the product control solution was prepared by mixing 0.05 mL of test solution of various concentrations with 0.45 mL of distilled water. The pH of these solutions was adjusted to 6.3 by using 1 N HCl. All these solutions were incubated at 37 °C for 20 min and then the temperature was increased to 57 °C for 3 min. The solution was allowed to cool for some time, then 2.5 mL of phosphate buffer was added to all the above solutions. The absorbance of the resulting solution is measured at 660 nm using a UV-visible spectrophotometer. The percentage inhibition of protein denaturation was calculated using the given formula.

$$\% \text{ inhibition} = 100 - \frac{\text{Abs}_{\text{test solution}} - \text{Abs}_{\text{product control}}}{\text{Abs}_{\text{test control}}} \times 100$$

VI. *In vitro* Anti-diabetic Activity of an Isolated Compound

In vitro anti-diabetic activity of an isolated compound, TZNO-III was screened by the α-amylase and α-glucosidase bioassays.

α-Amylase inhibition activity of an isolated compound

The α-amylase activity of an isolated compound TZNO-III was measured *in vitro* by the hydrolysis of starch in the presence of the α-amylase enzyme that is described in the Worthing ton Enzyme Manual with slight modification (Kwon *et al*., 2006).

Preparation of sample and standard solutions

The isolated compound (TZNO-III) or standard drugs (5 mg each) were dissolved in a small amount of methanol and the volume was made up to 10 mL of 20 mM sodium phosphate buffer solution (pH 6.8) in a small beaker. A stock solution of 500 µg/mL concentration was obtained. This stock solution was serially half diluted with phosphate buffer solution (pH 6.8) to get the concentrations of 250, 125, 62.5, 31.3, and 15.6 µg/mL, respectively.

The procedure of α-amylase inhibition activity

A modified dinitrosalicylic acid (DNS) method was used to estimate maltose equivalent. Amylase solution 0.1 mL was mixed with 4 mL of phosphate buffer solution (pH 6.8) containing different concentrations of test samples. After 20 min of incubation at 37 °C, 1mL of a 1% (w/v) starch solution was added, and the mixture was re-incubated for 30 min at 37 °C. Then, 2 mL of the DNS reagent was added and then, the mixture solutions were heated in a boiling water bath for 10 min. The blank solution was prepared without the amylase enzyme and replaced by equal quantities of buffer solution. After that, the absorbance was measured at 540 nm by a UV-visible spectrophotometer. The control solution was prepared as the above procedure by using phosphate buffer without the test sample solution. All experiments were done in triplicate and the % inhibition was calculated by using the following equation.

$$\% \text{ inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}}}{\text{Abs}_{\text{Control}}} \times 100$$

Screening of *in vitro* α-glycosidase inhibition activity

The inhibitory was assessed by the standard method with slight modification. The concentration of the substrate (p-nitrophenyl-α-D-glucopyranoside) by 50 % inhibition concentration (IC_{50}) was determined by monitoring the effect of decreasing concentrations of these samples in the assay on the inhibition values (Kwon *et al*., 2006).

Preparation of stock and standard solution

The isolated compound (TZNO-III) or standard drugs (2 mg each) were dissolved in a small amount of methanol and the volume was made up to 10 mL with 20 mM sodium phosphate buffer solution (pH 6.8) in a small beaker. A stock solution of 200 μg/mL concentration was obtained. This stock solution was serially half diluted with phosphate buffer solution to get the concentrations of 100, 50, 25, 12.5, and 6.26 μg/mL, respectively.

The procedure of α-glycosidase inhibition activity

50 μL of the α-glucosidase solution was mixed with 60 μL of each different concentration of the test samples and incubated at 37 °C for 20 min. After preincubation, 50 μL of 2 μM p-nitrophenyl- α-D-glucopyranoside solution in phosphate buffer (pH 6.8) was added and incubated at 37 °C for 20 min. Then the reaction was stopped by adding 160 μL of 100 mM sodium carbonate and the absorbance reading was recorded at 405 nm by a UV-visible spectrophotometer in the visible region. The control solution was prepared as the above procedure without the test sample solution. The blank solution (without enzyme) and standard solution were also prepared by the above procedure. All experiments were done in triplicate and the % inhibition was calculated by using the following equation.

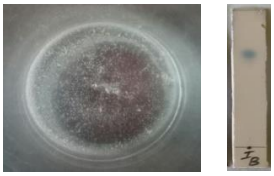
$$\% \text{ inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}}}{\text{Abs}_{\text{Control}}} \times 100 \times 100$$

Results and Discussion

Identification of Isolated Compound TZNO-I

TZNO-I was isolated from the EtOAc extract of the bark of *H. integrifolia* by a column chromatographic method on silica gel as colourless crystals and its R_f value was found to be 0.65 on silica gel TLC with n-hexane: $CHCl_3$ (9:1, v/v) as the developing solvent system (Figure 3). It is UV inactive and gave a blue colour spot with an anisaldehyde solution, suggesting a steroid compound. The melting point of the isolated compound **TZNO-I** is 133.7

°C, which is almost the same as β-sitosterol acetate, 134 °C. In the FT IR spectrum (Figure 4), the absorption band at 3358 cm^{-1} indicated the characteristic of O-H stretching. The asymmetric and symmetric C-H stretching bands of $–CH_2$- and $–CH_3$ groups were observed at 1959, 2924, and 2854 cm^{-1} and their C-H bending appeared at 1464 and 1378 cm^{-1}. The FT IR spectral features of **TZNO-I** are very similar to those of β-sitosterol, except in the addition bands at 1730 and 1272 cm^{-1} for C=O and -O-C-O- stretching of an ester. According to the TLC behavior and the FT IR spectral data, the isolated compound **TZNO-I** may be deduced as β-sitosterol acetate.



Compound **TZNO-I**
n-hexane:$CHCl_3$ (9:1)
Anisaldehyde, Δ
R_f = 0.65

Figure 3. Photography and thin layer chromatogram of isolated compound TZNO-I

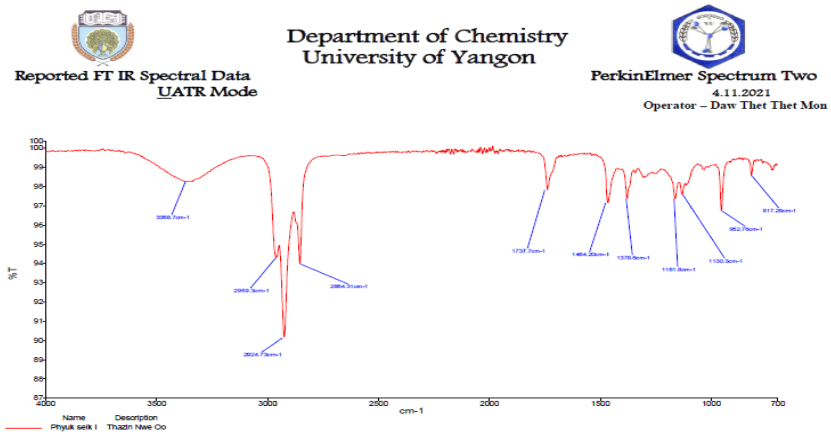


Figure 4 FT IR spectrum of isolated compound TZNO-I

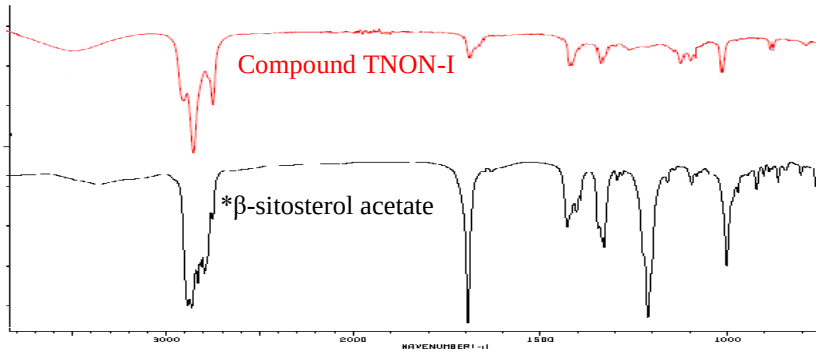


Figure 5 Comparison of the FT IR spectra of the isolated compound TZNO-I (red colour) with the reference standard spectrum*β-sitosterol acetate (black colour)

(* SDBS-Spectral Data Base)

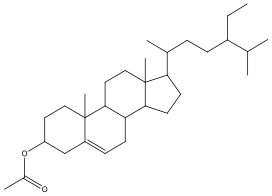
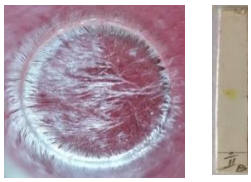


Figure 6 Structure of β-sitosterol acetate ($C_{31}H_{52}O_2$)

Identification of Isolated Compound TZNO-II

TZNO-II was isolated from the EtOAc extract of the bark of *H. integrifolia* by a column chromatographic method on silica gel as colourless crystals and its R_f value was found to be 0.48 on silica gel TLC with n-hexane: $CHCl_3$ (2:1, v/v) as the developing solvent system (Figure 7). It is UV inactive and gives a yellow colour spot with anisaldehyde-sulphuric acid followed by heating. After a few seconds, the yellow colour changed to a pink colour spot. The melting point of the isolated compound **TZNO-II** is 264 °C, which is almost the same as friedelin's 264 °C. In the FT IR spectrum, the absorption bands at 2925 and 2868 cm^{-1} indicated the asymmetric and symmetric C-H stretching bands of $–CH_2$- and $–CH_3$ groups, and their C-H bending appeared at 1461 and 1389 cm^{-1}. The strong absorption band at 1713 cm^{-1} was attributed to C=O stretching of saturated cyclic ketone vibration and 1001, 957 cm^{-1} showed the C-C stretching bands in carbon ring cyclic compound vibration. These absorption frequencies resemble the absorption frequencies observed for friedelin. The FT IR spectrum and observed data of compound **TZNO-II** are described in Figure 8.



Compound **TZNO-II**
n-hexane:$CHCl_3$ (2:1)
Anisaldehyde, Δ
R_f = 0.48

Figure 7 Photograph and thin layer chromatogram of isolated compound TZNO-II

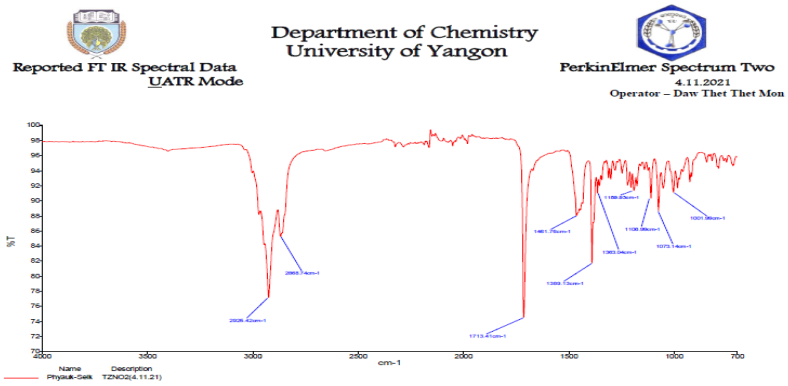


Figure 8 FT IR spectrum of isolated compound TZNO-II

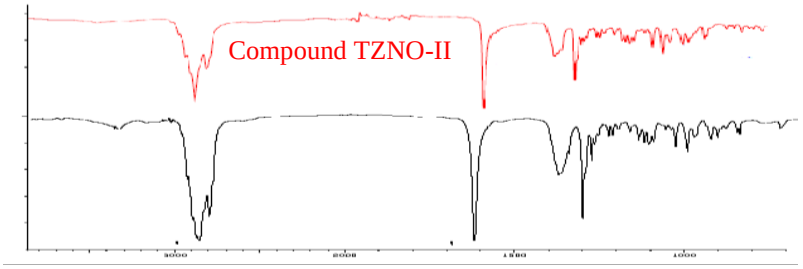


Figure 9 Comparison of the FT IR spectra of the isolated compound TZNO-II (red colour) with the reference standard spectrum *friedelin (black colour)
(* SDBS-Spectral Data Base)

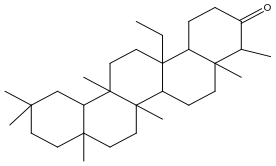
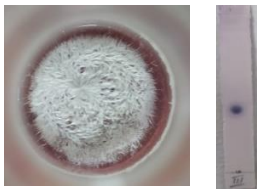


Figure 10 Structure of friedelin ($C_{30}H_{50}O$)

Identification of Isolated Compound TZNO-III

Compound **TZNO-III** was isolated from the EtOAc extract of the bark of *H. integrifolia* by the column chromatographic method on silica gel as colourless crystals and its R_f value was found to be 0.38 on silica gel TLC with PE: EtOAc (4:1, v/v) as the developing solvent system (Figure 11). It is UV inactive and gives a purple colour spot with anisaldehyde-sulphuric acid followed by heating. The melting point of the isolated compound **TZNO-III** is 135.7 °C, which is almost the same as β-sitosterol, 136 °C. On subjection to IR spectroscopic analysis, the broadband that appeared at 3422 cm^{-1} showed the characteristic of OH stretching. The asymmetric and symmetric C-H stretching of $–CH_2-$ and $–CH_3$ groups appeared at 2933 and 2864 cm^{-1} and their C-H bending bands appeared at 1461 and 1375 cm^{-1}. The absorption bands at 1052 and 1023 cm^{-1} attributed to C-OH stretching vibration and 957 cm^{-1} indicated the out-of-plane C-H bending vibration. A weak band at 1644 cm^{-1} corresponded to the C=C stretching of the olefinic group. These absorption frequencies resemble the absorption frequencies observed for β-sitosterol. The FT IR spectrum and observed data of compound **TZNO-III** are described in Figure 12.



TZNO-III
PE : EtOAc (4:1)
Anisaldehyde, Δ
R_f = 0.38

Figure 11 Photograph and thin layer chromatogram of isolated compound TZNO-III

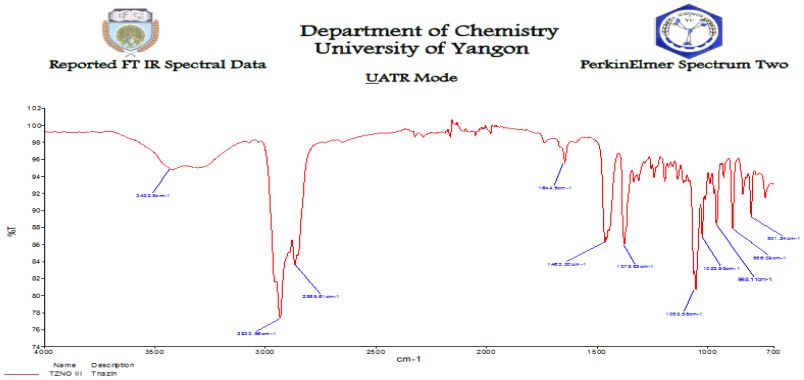


Figure 12 FT IR spectrum of isolated compound TZNO-III

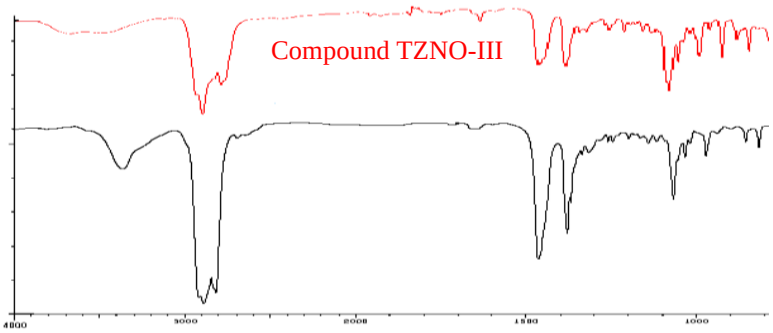


Figure 13. Comparison of the FT IR spectra of isolated compound TZNO-III (red colour) with reference to standard spectrum*β-sitosterol (black colour)
(* SDBS-Spectral Data Base)

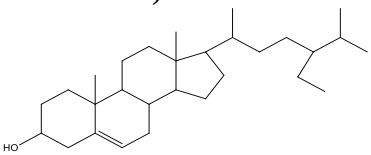


Figure 14 Structure of β-sitosterol ($C_{29}H_{50}O$)

Some Biological Activity of Major Isolated Compound

According to the column chromatographic separation of ethyl acetate extract of the bark of *H. integrifolia*, the three compounds, TZNO-I (colourless crystals, 11 mg, 0.22 % yield), TZNO-II (colourless crystals, 15 mg, 0.30 % yield) and TZNO-III (colourless crystals, 150 mg, 3.00 % yield) were isolated. Among these three isolated compounds, TZNO-III was observed as the major constituent. Therefore, the isolated compound TZNO-III was chosen for the determination of anti-arthritic and anti-diabetic activity.

***In Vitro* Anti-Arthritic Activity of TZNO-III**

The anti-arthritic activity of isolated compound TZNO-III was determined by protein denaturation methods with 100 % fresh hen's egg albumin and 5 % bovine serum albumin. According to the results, the isolated compound TZNO-III at different concentrations (50-1600 µg/mL) provided considerable protection against the denaturation of protein. The isolated compound TZNO-III produced 44.29 % and 85.86 % inhibition of egg albumin and bovine serum albumin at 1600 µg/mL. The standard drug diclofenac sodium exhibited the inhibition of 63.63 % for egg albumin and 83.69 % for bovine serum albumin denaturation at 1600 µg/mL. Both TZNO-III and the reference drug showed concentration-dependent inhibition of heat-induced protein (albumin) denaturation. Therefore, isolated compound III possessed significant activity compared with the standard. These results were tabulated in Tables 1 and 2.

Table 1 Anti-arthritic Activity of TZNO-III by Protein Denaturation Method (Using Egg Albumin)

Activity	Sample	% inhibition (mean ±SD) in different concentrations (µg/mL)						Highest Activity (µg/mL)
		50	100	200	400	800	1600	
Anti-arthritic (Egg lbumin)	TZNO-III	24.01 ± 0.03	26.01 ± 0.05	28.81 ± 0.03	30.00 ± 0.04	41.14 ± 0.03	44.29 ± 0.07	1600
	Diclofenac sodium	42.73 ± 0.03	43.09 ± 0.03	48.61 ± 0.03	53.48 ± 0.03	61.7 ± 0.03	63.63 ± 0.08	1600

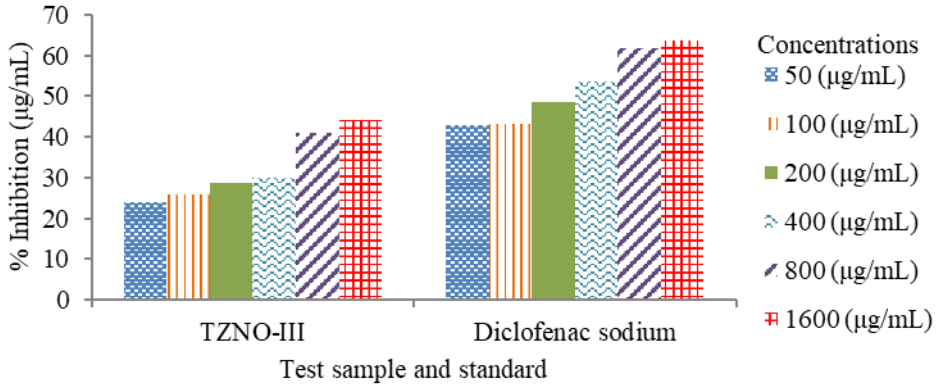


Figure 15 A bar graph of protein denaturation results of TZNO-III and standard drug (diclofenac sodium) by using egg albumin

Table 2 Anti-arthritic Activity of TZNO-III by Protein Denaturation Method (Using Bovine Serum Albumin)

Activity	Sample	% inhibition (mean ± SD) in different concentrations (µg/mL)						Highest Activity (µg/mL)
		50	100	200	400	800	1600	
Anti-arthritic (Bovine Serum Albumin)	TZNO-III	41.39 ± 0.03	52.17 ± 0.03	55.43 ± 0.03	68.44 ± 0.04	82.61 ± 0.05	85.86 ± 0.07	1600
	Diclofenac sodium	38.41 ± 0.03	52.17 ± 0.03	54.35 ± 0.03	56.52 ± 0.03	71.32 ± 0.04	83.69 ± 0.05	1600

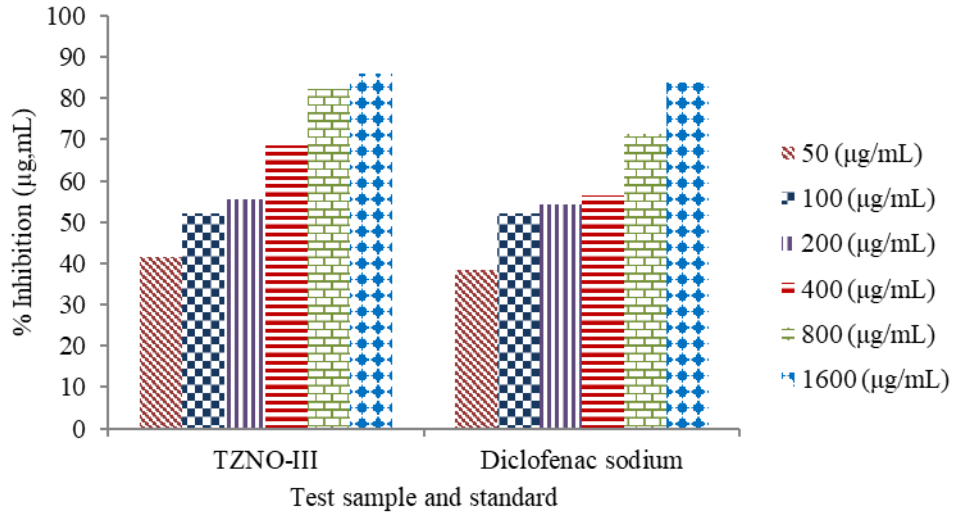


Figure 16 A bar graph of protein denaturation results of TZNO-III and standard drug (diclofenac sodium) by using bovine serum albumin

In Vitro Anti-Diabetic Activity of TZNO-III

The anti-diabetic activity of the isolated compound TZNO-III was determined by α-amylase and α-glucosidase activity. According to the experiment, the IC_{50} values of TZNO-III for α-amylase and α-glucosidase inhibition were found to be 56.72 and 40.63 µg/mL,

respectively. The IC_{50} values for standard metformin and acarbose inhibition of α-amylase activity were 37.15 and 10.69 g/mL, respectively. The IC_{50} values of metformin and acarbose inhibition of α-glucosidase activity were 40.63 and 13.46 g/mL, respectively. As the IC_{50} value of the isolated compound, TZNO-III was slightly higher than that of two standard drugs, the isolated compound TZNO-III possessed mild anti-diabetic activity when compared with the standard drugs. These results were tabulated in Tables 3 and 4.

Table 3 Results of α-Amylase Inhibition and IC_{50} Values of TZNO-III and Standard Drugs

Activity	Sample	% inhibition (mean ± SD) in different concentrations (µg/mL)						IC_{50} (µg/mL)
		15.6	31.3	62.5	125	250	500	
α-amylase	TZNO-III	11.78 ± 0.2	18.38 ± 0.2	57.14 ± 0.5	72.81 ± 0.6	89.79 ± 0.6	93.88 ± 1.4	56.72
	Metformin	24.95 ± 0.2	46.94 ± 0.5	63.27 ± 0.5	77.56 ± 0.5	79.59 ± 0.6	85.71 ± 1.2	37.15
	Acarbose	32.65 ± 0.5	55.10 ± 0.5	71.43 ± 0.5	89.79 ± 0.6	97.96 ± 1.4	98.72 ± 1.4	10.69

Table 4 Results of α-Glucosidase Inhibition and IC_{50} Values of TZNO-III and Standard Drugs

Activity	Sample	% inhibition (mean ± SD) in different concentrations (µg/mL)						IC_{50} (µg/mL)
		6.25	12.5	25	50	100	200	
α-glucosidase	TZNO-III	13.79 ± 0.5	20.69 ± 0.2	32.76 ± 0.2	60.34 ± 0.5	84.48 ± 0.2	94.83 ± 0.5	40.63
	Metformin	32.67 ± 0.2	47.78 ± 0.5	76.67 ± 0.5	78.67 ± 0.8	93.11 ± 0.3	94.44 ± 0.5	13.46
	Acarbose	48.00 ± 0.2	58.44 ± 0.2	84.22 ± 0.6	88.67 ± 0.6	97.78 ± 0.5	98.44 ± 0.2	7.45

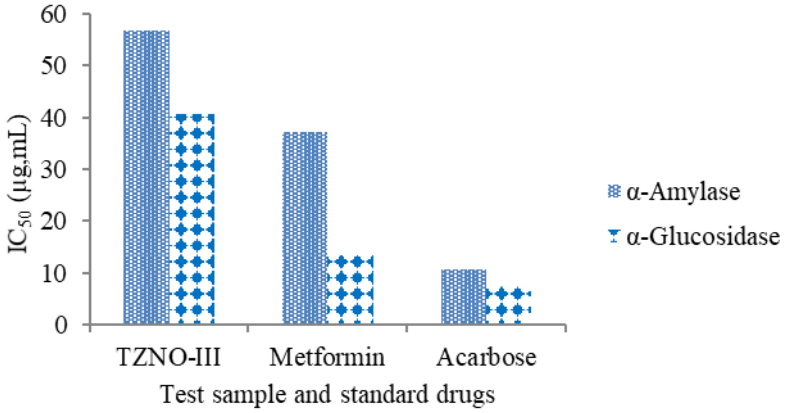


Figure 17 A bar graph of 50 % α-amylase and α-glucosidase inhibitory concentration values of TZNO-III compared with standard drugs (metformin and acarbose)

Conclusion

The overall results of the study indicate that the bark of *Holoptelea integrifolia* R., Phyauk-seik is a good source of biologically active steroids and triterpenoid compounds. It possessed bioactive organic compounds such as β-sitosterol acetate, friedelin, and β-sitosterol. The major constituent is β-sitosterol and it showed significant anti-arthritic activity and mild anti-diabetic activity by comparing with standard drugs.

Acknowledgment

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