INVESTIGATION OF BIOACTIVE CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF THE RHIZOME OF *CURCUMA CAESIA* ROXB.

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Abstract

Curcuma caesia Roxb. commonly known as Sanwin tain pyar or black turmeric, is one of the important and endangered species in the genus of *Curcuma*. The present study aimed to conduct phytochemical screening, isolating some organic compounds and evaluating the biological activities of the Myanmar medicinal plant *C. caesia* rhizome, including antidiabetic and anti-inflammatory properties. The preliminary phytochemical screening of the rhizome of *C. caesia* showed the presence of alkaloids, glycosides, phenolic compounds, polyphenols, saponin and terpenoids. Five pure compounds (HHS-1, 2, 3, 4 and 5) were isolated from the *n*-hexane extract of *C. caesia* by using thin layer and column chromatographic separation methods. The isolated compound (HHS-1) was identified as zederone based on FT IR and UV-vis spectral data and melting point (152-153 ºC). Four extracts and the isolated HHS-1 compound were tested for antidiabetic activity using the Cirillo VP method (Glucose Uptake Capacity by Yeast Cells). The test revealed that the ethyl acetate extract and the HHS-1 compound exhibited the highest antidiabetic activity. Furthermore, the anti-inflammatory effect of the compound HHS-1 was determined by the Croton oil-induced ear oedema method**.** Topical application of the compound HHS-1 showed significant inhibition of the induced ear oedema (39 % for 0.75 mg/ear, 44 % for 1.5 mg/ear and 98 % for 2.25 mg/ear). This study will support further investigations on the pharmacological activity of medicinal plant species in Myanmar.

Keywords*:* *Curcuma caesia* Roxb., phytochemical screening, antidiabetic activity, anti-inflammatory activity

Introduction

Medicinal plants are a rich source of diverse bioactive compounds with many therapeutic properties. Myanmar has abundant plant resources and Myanmar people have used their traditional medicines to maintain their health and treat various ailments, including malaria, diarrhoea and fever over millennia of history (Aye *et al*., 2019). In Kachin State, Northern Myanmar, the people have a long history of the use of traditional plants for medicinal purposes (Aung *et al*., 2016). The use of herbal plants has gained interest from many sectors and in combating diseases because of their safety, availability, and low cost (Ibrahim *et al*., 2023).

Curcuma caesia has a bluish-black rhizome with a bitter taste and pungent smell and is widely used in treating haemorrhoids, leprosy, asthma, cancer, epilepsy, fever, wound, vomiting, menstrual disorder, anthelmintic, aphrodisiac, inflammation, and more. *C. caesia* is a family member of Zingiberaceae. *C. caesia* rhizome has been reported to exhibit various therapeutic activities including antioxidant, antibacterial, antipyretic, larvicidal, insecticidal, antimicrobial, wound healing and anti-hyperglycemic properties (Fatt *et al*., 2021). It is reported to be rich in major constituents of carbohydrates, proteins, amino acids, steroids, glycosides, flavonoids, alkaloids, tannins, and phenols (Ibrahim *et al*., 2023). Owing to its high medical and aromatic properties, it is widely cultivated as a medicinal plant in many Southeast Asian countries (Fatt *et al*., 2021). Recently, significant attention has been directed toward natural and synthetic glycation inhibitors to delay the onset or progression of diabetic complications. The potential for discovering and developing new anti-diabetic therapies from nature's pharmacy is vast and deserves corresponding consideration (Naila *et al.*, 2012). Furthermore, there is increasing interest in medicinal plant-based therapies to mitigate the inflammatory processes associated with

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diseases and tissue damage (Padilla-Camberos *et al.*, 2021). According to the World Health Organization (WHO), over 80 % of the world's population relies on herbal and natural products for medicinal treatment (Ibrahim *et al.*, 2023). The present work aims to investigate bioactive constituents and biological activities of the rhizome of *C. caesia* (Figure 1).

Figure 1. Morphological characters of *C. caesia* (A) Habit (B) Rhizome

Materials and Methods

Collection of Plant Material and Preparation of Sample

The rhizome of *C. caesia* used in the present study was collected from Lamone Village, Tanai Township, Kachin State, Myanmar. The rhizome was further identified in the Department of Botany, University of Mandalay.

Phytochemicals Screening

The phytochemical screening of the selected sample was carried out according to the reported method (Harborne, 1998). The presence of different phytochemicals was analysed using different specific reagents.

Extraction from the Rhizome of *C. caesia*

The dried sample (700 g) was percolated with 95 % ethanol (a total of 9.0 L) twice, with each percolation lasting ten days. Then, the solution was filtered and the ethanol solvent was removed using a rotary evaporator. A total of 73.82 g of ethanol crude extract was obtained. A mixture of *n*-hexane and ethanol (1:1 v/v) (1 L) was added into 32.40 g of ethanol crude extract then it was partitioned by using a separating funnel. A total of 11.92 g of *n*-hexane extract, 7.90 g of ethyl acetate crude extract and 12.58 g of ethanol-water extract were obtained.

Isolation of Pure Compounds

The *n*-hexane crude extract 9.87 g was separated by column chromatography on silica gel with various solvent ratios of *n*-hexane: ethyl acetate from non-polar to polar. Totally 169 fractions were obtained. Every fraction was checked on TLC, UV detector and developing reagent. The fractions of the same R_f value were combined and then, 9 combined fractions were obtained. Combined fractions 9, 7 and 2 were rechromatographed by microcolumn. The combined fractions 9 and 7 were rechromatographed by microcolumn with the same adsorbent and eluent mentioned in the previous column. About 202.1 mg of pure compound HHS-1 was obtained from combined fraction 9. 147.3 mg of pure compound HHS-2, 13.4 mg of pure compound HHS-3 and 3.5 mg of pure compound HHS-4 were obtained from the combined fraction 7. The combined fraction 2 was rechromatographed by microcolumn on silica gel with various solvent ratios of *n*-hexane: chloroform from non-polar to polar. Totally 54 fractions were obtained. Every fraction was checked on TLC, UV detector and developing reagent. The fractions of the same R_f values were combined and then, 5 combined fractions (1-5) were obtained. Among them, combined fractions 2 was rechromatographed by microcolumn on silica

gel with various solvent ratios of *n*-hexane: ethyl acetate from nonpolar to polar. About 0.9 mg of pure compound HHS-5 was obtained from the combined fraction 2. The R_f values of these five isolated compounds were determined using *n*-hexane: ethyl acetate = 9:1 v/v solvent system.

Identification of the Isolated Compound HHS-1

Among five isolated compounds, compound HHS-1 was chosen to identify using FT IR and UV-Vis spectroscopic techniques, and comparing its melting point with the reported data.

The functional groups of isolated compound HHS-1 were assigned by FT IR spectra measured using a Fourier Transform Infrared Spectrophotometer (IR Prestige-21. Shimadzu, Japan) at the University Research Center, University of Mandalay. The UV-visible spectrum of pure compound HHS-1 was also measured at Mandalay University Research Center, University of Mandalay. It was taken using a UV-visible spectrophotometer (UV-1800 SHIMASZU) with a slit width of 2 nm, using a 10 mm cell at room temperature.

The melting point of pure isolated compound HHS-1 was measured using the Thiele tube method (Stuart SMP30 melting point apparatus) at Mandalay University Research Center.

Determination of Antidiabetic Activity of the Extracts and Isolated Compound HHS-1

This assay was performed according to the well-defined method of Cirillo, 1962. About 1 g of commercial Barker's yeast was dissolved in 100 mL of distilled water to prepare 1 % suspension. The suspension was kept overnight at room temperature (25 ℃). Then the yeast cell suspension was centrifuged at 4200 rpm for 5 minutes. The process was repeated by adding the distilled water to the pallet until a clear supernatant was obtained. Exactly 10 parts of the clear supernatant fluids were mixed with 90 parts of distilled water to get a 10 % v/v suspension of the yeast cells. Each of the 5 mg/mL of ethanol, ethanol-water, ethyl acetate and *n*-hexane extracts was mixed with 1 mL of dimethyl sulphoxide (DMSO) until fully dissolved to prepare the extract solutions. The pure compound HHS-1 was also prepared following the same procedure.

Each of the 500 µL of the extract solutions was mixed with 500 µL of various concentrations (5, 10 and 25 mM) of glucose solution and incubated for 10 min at 37 ℃. To initiate the reaction, 100 µL of yeast suspension was poured into the mixture of glucose and extract, vortexed and incubated for another 60 min at 37 ℃. After incubation, the tubes were centrifuged for 5 min at 3800 rpm and glucose was estimated by using UV-vis spectrophotometry at 520 nm. Glucose uptake capacity for pure compound HHS-1 was carried out by using the same procedure. Absorbance for the respective control was also recorded on the same wavelength. The percent increase in glucose uptake for each sample was calculated by the following formula:

$$\% \text{ increase in glucose uptake} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where, A_{sample} = Absorbance of the sample

A_{control} = Absorbance of the control (solution having all reagents except test sample)

Determination of Anti-inflammatory Activity of the Isolated Compound HHS-1 by the Croton Oil-induced Ear Oedema Method

The *in vivo* anti-inflammatory activity of the pure compound HHS-1 was tested via the Croton oil-induced ear oedema method according to Manga *et al.*, 2004. The internal surface of the right ear of five groups of mice with a weight of 25-30 g was treated with 20 µL of 5 % croton oil (in ethanol) as an irritant to induce skin inflammation. The left ear remained untreated. The experimental mice were topically applied 0.75 mg/ear, 1.5 mg/ear and 2.25 mg/ear of pure compound HHS-1 before 30 min Croton oil application, and the positive control group received

0.50 mg/ear aspirin. Ear thickness was evaluated with a calliper 6 h after oedema induction. The mice were randomised into five groups, each of which included three mice. (1) negative control group: treated with 5 % Croton oil only, (2) positive control group: received aspirin (0.50 mg/ear), (3) Groups A, B and C: received 0.75 mg/ear, 1.5 mg/ear and 2.25 mg/ear of pure compound HHS-1. The percentage of oedema inhibition was defined with the control group, which received the Croton oil solution, according to the following formula:

$$\text{Inhibition \%} = \frac{(D_{\text{Control}} - D_{\text{Treated}})}{D_{\text{Control}}}$$

where D_{Control} is the difference in oedema thickness in the control group and $D_{\text{Treatment}}$ is the difference in oedema thickness for the treated groups.

Results and Discussion

Phytochemicals Present in the Rhizome of *C. ceasia*

Preliminary phytochemical screening was carried out to detect the presence of organic constituents in the rhizome of *C. caesia*. It indicated that the rhizome of *C. caesia* consists of alkaloids, glycosides, phenolic compounds, polyphenols, saponin and terpenoids.

Five pure compounds HHS-1, 2, 3, 4, and 5 were isolated from the chromatographic separation. The R_f values of these five compounds: HHS-1, 2, 3, 4 and 5 were observed as 0.35, 0.6, 0.27, 0.15, and 0.77, respectively, on silica gel (602 F_{254}) TLC chromatograms with n-hexane: ethyl acetate 9:1 v/v solvent system.

Identification of the Isolated Compound HHS-1

FT IR assignment of the isolated compound HHS-1

The FT IR spectrum of the isolated compound HHS-1 is shown in Figure 2. In this spectrum, the broad band which appears at 3016 cm^{-1} indicates the C-H stretching vibration of sp^2 hydrocarbon. The peaks at 2931 cm^{-1} and 2877 cm^{-1} indicate the asymmetric and symmetric C-H stretching vibration of sp^3 hydrocarbon. The peak at 1658 cm^{-1} gives C=O stretching vibration of the carbonyl group. The peak at 1527 cm^{-1} gives C=C stretching vibration of alkenic group. The peaks at 1458 cm^{-1} and 1234 cm^{-1} indicate C-H bending vibration of sp^3 hydrocarbon. The peaks at 1075 cm^{-1} and 1018 cm^{-1} give C-O-C stretching vibration of the ether group. The peaks at 925 cm^{-1} show C-H out-of-plane bending vibration of the trans or E alkenic group. The functional groups observed in the FT IR spectrum of pure compound (HHS-1) were compared by literature data of zederone (Prapapan *et al*., 2013) as tabulated in Table 1.

Table 1. FT IR Assignments of the Isolated Compound HHS-1

Wavenumber (cm^{-1})		Functional groups
HHS-1	**Literature ***	
3016	3017	C-H stretching vibration of sp^2 hydrocarbon
2931, 2877	2932, 2878	Asymmetric and symmetric C-H stretching vibration of sp^3 hydrocarbon
1658	1662	C=O stretching vibration of the carbonyl group
1527	1523	C=C stretching vibration of alkenic group

Wavenumber (cm^{-1})		Functional groups
HHS-1	**Literature ***	
1458, 1234	1427, 1232	C-H bending vibration of sp^3 hydrocarbon
1075, 1018	1066, 1020	C-O-C stretching vibration of the ether group
925	929	C-H out-of-plane bending vibration of trans or E alkenic group

* Prapapan *et al*., 2013

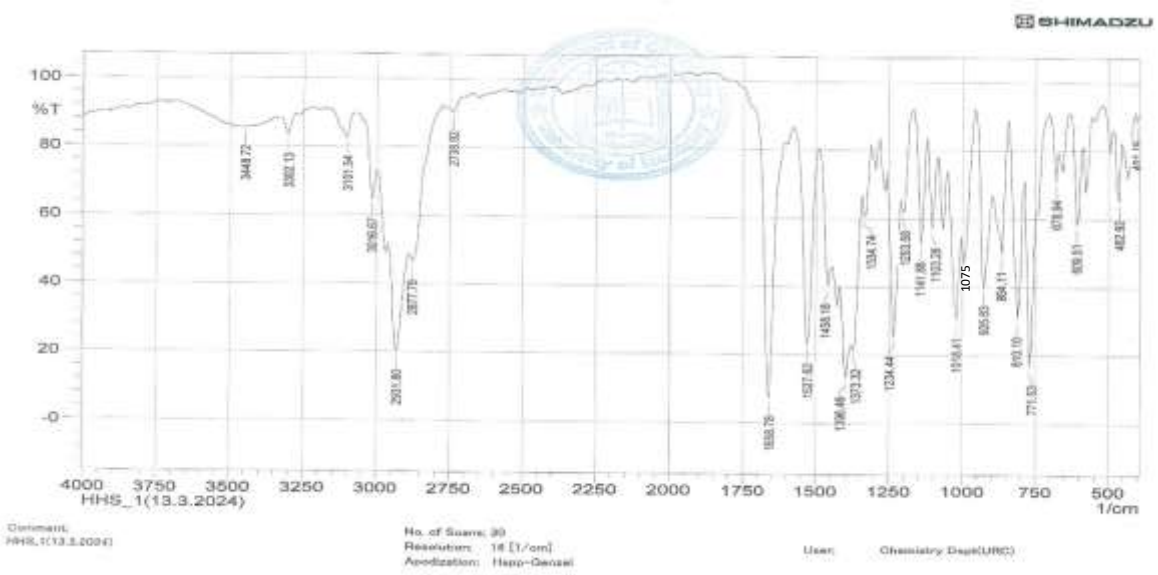

Figure 2. FT IR spectrum of the isolated compound HHS-1

UV-visible Spectroscopic Analysis of the Isolated Compound HHS-1

The UV-visible analysis was performed for the identification of compound HHS-1 containing σ-bonds**,** π-bonds, lone pairs of electrons, chromophores and aromatic rings. The UV-visible spectrum was taken at the wavelength of 200 nm to 700 nm due to the sharpness of the peaks and proper baseline. This profile (Figure 3) showed the peaks at λ_{max}^{MeOH}284 nm with an absorption of 1.242. The peak at 284 nm was characteristic of the carbonyl group corresponding to n $\rightarrow$ π^* transitions (Kania *et al*., 1983). The UV-visible spectral data of the pure compound HHS-1 was compared with literature data of zederone (Prapapan *et al*., 2013) and is shown in Table 2.

Table 2. UV-vis Spectral Data of the Isolated Compound HHS-1

Literature *	HHS-1 compound	Wavelength number (nm)	Absorbance	Remark
281	HHS-1	284	1.242	n $\rightarrow$ π^*

* Kania *et al*., 1983; Prapapan *et al*., 2013

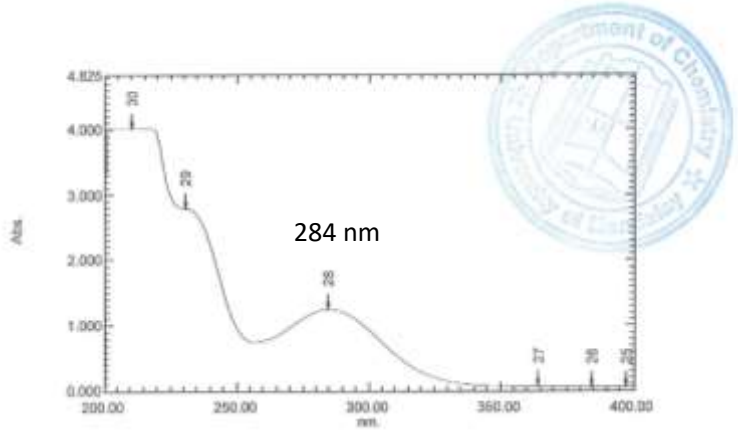

Figure 3. UV-vis spectrum of the isolated compound HHS-1 (in methanol)

Melting Point of the Isolated Compound HHS-1 and Visualization on TLC

The melting point of the isolated compound HHS-1 was found to be 152-153 °C and it is close to the melting point of zederone (153-154 °C) (Prapapan *et al*., 2013). Visualization of the compound HHS-1 on TLC sprayed with developing reagent (*p*-anisaldehyde, H_2SO_4 and EtOH) is identical to the literature (Gerard *et al.*, 2013) as shown in Table 3. Therefore, the isolated compound HHS-1 may be zederone, a sesquiterpene lactone. According to FT IR, UV-vis, melting point measurements and literature data, the isolated compound HHS-1 can be identified as zederone. The structure of zederone is shown in Figure 4.

Table 3. Visualization of the Isolated Compound HHS-1 on TLC Compared with Zederone

Isolated compound HHS-1	Literature *
	

*Gerard *et al.*, 2013; Prapapan *et al*., 2013

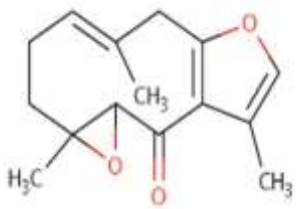

Figure 4. Structure of Zederone

Effects of Four Different Extracts and the Isolated Compound HHS-1 on Glucose Uptake Capacity by Yeast Cells

Four different extracts and the isolated compound HHS-1 showed antidiabetic activity. Among them, ethyl acetate extract showed the highest antidiabetic capacity than other extracts. Pure compound HHS-1 showed the highest antidiabetic capacity at 25 mM glucose concentration (Table 4 and Figure 5). When the percent increases in glucose uptake capacity, the antidiabetic activity shows. The glucose uptake capacity of four different extracts and the isolated compound HHS-1 were dependent on the use of glucose concentrations.

Table 4. The Glucose Uptake Capacity Determined by Yeast Cells Assay (Cirillo VP Method)

Sample	% increase in glucose uptake (Mean± SEM)		
	5 mM glucose	10 mM glucose	25 mM glucose
ethanol extract	68.13±0.61	52.73±3.42	42.79±1.82
ethanol-water extract	46.20±3.63	17.41±0.66	22.83±5.74
ethyl acetate extract	62.79±0.35	52.71±0.57	53.66±0.98
n-hexane extract	45.15±0.67	54.32±0.74	39.94±2.39
HHS-1	18.63±13.59	18.95±6.86	36.33±4.14
Metronidazole	52.69±2.08	55.39±1.72	55.53±3.71

5 mg/mL of each sample was used.

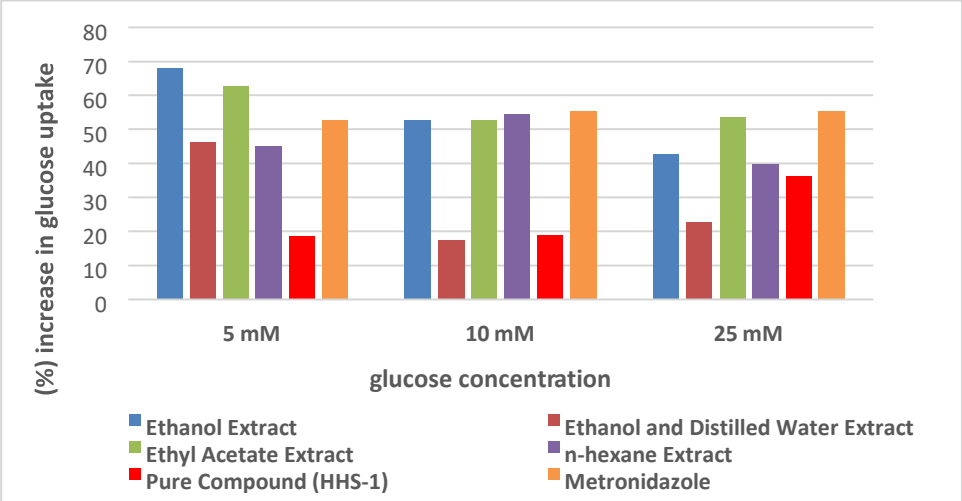

Figure 5. A bar graph of the plot of the concentration of glucose (mM) vs (%) increase in glucose uptake

Effects of the Isolated Compound HHS-1 on Croton Oil-induced Ear Oedema

The anti-inflammatory activity of the pure compound HHS-1 was evaluated with doses of 0.75 (Group A), 1.5 (Group B), and 2.25 mg/ear (Group C) resulting in 39 %, 44 %, and 98 %, respectively (Table 5 and Figure 6). These results indicate significant differences in the efficacy of the groups in reducing inflammation, with an increase in dosage leading to enhanced anti-inflammatory effects. The anti-inflammatory activity of groups A, B and C was compared with negative (treated only with Croton oil) and positive (treated with aspirin) groups how much they can reduce inflammation. Groups A, B and C show higher anti-inflammatory activity than the

negative group. Although groups A and B show less anti-inflammatory activity than the positive group, group C shows higher anti-inflammatory activity than the positive group. Notably, Group C (2.25 mg/ear) demonstrated exceptional efficacy, achieving a very high anti-inflammatory activity of 98 %. This suggests that group C was highly effective in reducing inflammation, almost completely inhibiting the inflammatory process. The significant difference between group C and the other two groups indicates that it could be a promising candidate for further development as an anti-inflammatory agent.

Table 5. The Effect of Isolated Compound HHS-1 on Croton Oil-induced Ear Oedema

Treatment	Dose	Thickness of ear oedema (mm)	Inhibition %
Negative control (Croton oil only)	20 µL/ear	4	-
Aspirin (Positive control)	0.5 mg/ear	2.03	49
Group A	0.75 mg/ear	2.45	39
Group B	1.5 mg/ear	2.25	44
Group C	2.25 mg/ear	0.07	98

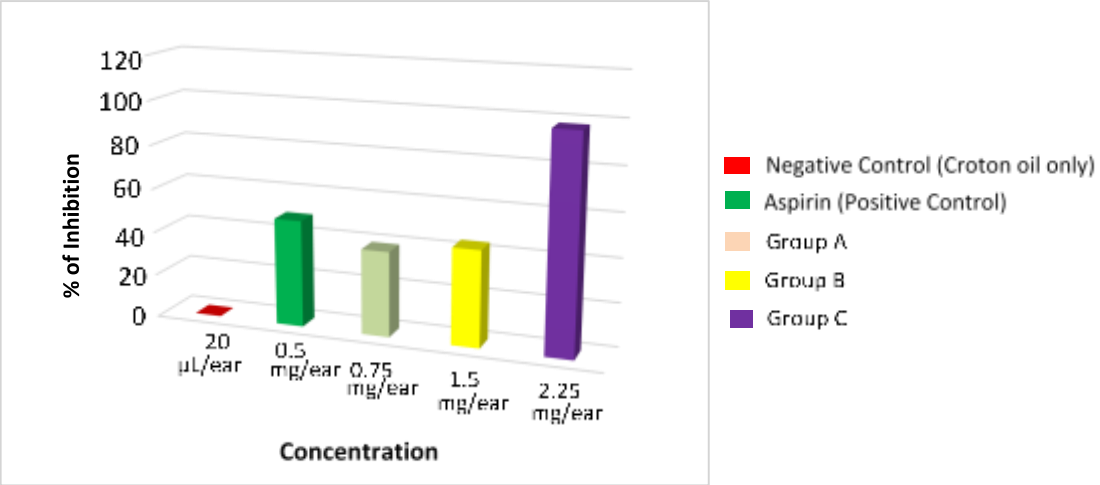

Figure 6. A bar graph of the plot of concentrations vs inhibition (%)

Conclusion

The rhizome of *C. caesia* was collected from Lamone Village, Tanai Township, Myanmar. According to the phytochemical screening, the rhizome of *C. caesia* contains alkaloids, glycosides, phenolic compounds, polyphenols, saponin and terpenoids. Five pure compounds (HHS-1, 2, 3, 4 and 5) were isolated from the *n*-hexane extract of *C. caesia* by using a thin layer and column chromatographic separation methods. Based on FT IR and UV-Vis spectroscopic analyses, as well as melting point determination and TLC visualization compared with reported data, the isolated pure compound HHS-1 (melting point: 152–153 °C) was identified as zederone (reported melting point: 153–154 °C).

Ethanol, ethanol-water, ethyl acetate and *n*-hexane extracts and the isolated zederone (HHS-1) demonstrated antidiabetic activity. Among these, the ethyl acetate extract exhibited the highest antidiabetic capacity. Notably, the isolated zederone (HHS-1) showed the strongest antidiabetic activity at a 25 mM glucose concentration. The comparative analysis of the anti-inflammatory activities at different doses on ear oedema mice demonstrated that a 2.25 mg/ear dose of zederone (HHS-1) was the most effective, achieving an activity rate of 98 %. This highlights its potential as a strong therapeutic candidate for treating diabetic and inflammatory conditions. However, *C. caesia* is a critically endangered species with remarkable medicinal properties, underscoring the urgent need for its conservation. Efforts must be made to value, protect, and sustainably maintain this plant for future generations.

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