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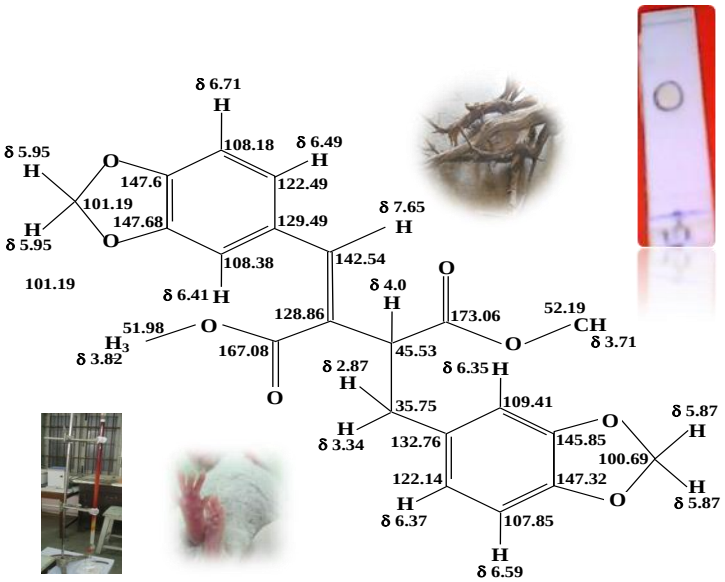
Determination of Anti-inflammatory Activity of the Ethanolic Plant Extract and Structure Elucidation of Isolated Compound from the Root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (Kyauk pan)



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ဒေါက်တာသီတာချို၊ လက်ထောက်သုတေသနအရာရှိ
ဒေါက်တာအံဝင်း၊ တွဲဘက်ပါမောက္ခ
လွင်လွင်အောင်၊ သုတေသနလက်ထောက် (၂)

စာတမ်းအကျဉ်း

ဤသုတေသနပြုချက်တွင် ဆေးဘက်ဝင်အပင်တစ်မျိုးဖြစ်သော *Chamaecyparis pisifera* (Siebold & Zucc.) Endl.(မြန်မာအမည်ကျောက်ပန်းပင်) ကို chemical analysis ပြုလုပ်ရန် ရွေးချယ်ခဲ့ပါသည်။ ကျောက်ပန်းအမြစ်ကို နေပြည်တော်တိုင်းဒေသကြီး၊ သစ်တော သုတေသနဌာနမှ စုယူခဲ့ပါသည်။ ကျောက်ပန်းအမြစ်၏ ကျောက်ပန်းအမြစ်၏ anti-inflammatory activity ရှိ၊ မရှိကို သိရှိနိုင်ရန် carrageenan induced inflammation method ဖြင့် စမ်းသပ်ခဲ့ပါ သည်။ ကျောက်ပန်းအမြစ် extract (high dose -1000 mg/kg) သည် anti-inflammatory activity ရှိမှုကို သိသိသာသာပြပြီး medium (500 mg/kg)နှင့် low dose(500 mg/kg) သည်လည်း ၎င်း activity ကို ပြပါသည်။ ထို့အပြင် ကျောက်ပန်းပင် အမြစ်မှ pure organic compound ကို Thin Layer နှင့် Column Chromatographic နည်းစဉ်များကို အသုံးပြု၍ ခွဲထုတ်ခဲ့ပါသည်။ Pure organic compound (TDC-1) သည် pure pale yellow oily form ရှိပါသည်။ ထို့အပြင် ကျောက်ပန်းအမြစ်မှ spectroscopic နည်းစဉ်ကို အသုံးပြု၍ ခွဲထုတ်ထားသော pure compound ၏ molecular formula မှာ ($C_{22}H_{20}O_8$) ဖြစ်ကြောင်း တွေ့ရှိရပါသည်။ ဖော်ပြပါ pure compound ၏ molecular masse မှာ 412 ဖြစ်ပြီး ၎င်းတို့၏ hydrogen deficiency indexe မှာ 13 ရှိပါသည်။ Isolated pure compound ၏ complete structure ကို DQF-COSY, ^{1}H NMR splitting patterns, coupling constant နှင့် HMBC signals များဖြင့် ခွဲခြားခဲ့ပါသည်။ Isolated pure compound (TDC-1) သည် Phenyl propanoid ဒြပ်ပေါင်းမှ ဆင်းသက်လာပြီး IUPAC အမည်မှာ

(Z) - dimethyl 2 - (benzo [d] [1', 3'] dioxol - 5' - yl methyl) -3 - (benzo [d] [1″, 3″] dioxol - 5″ - yl methylene) succinate ဖြစ်ကြောင်း တွေ့ရှိ ရပါသည်။ ကျောက်ပန်းအမြစ်သည် antibactial, antifungal, antioxidant, anti-inflammatory, antineoplastic, insecticidal, analgesic, aphrodisiac, diuretic, neuralgia and antirheumatic remedia စသည့် ဆေးဘက်ဝင်ဂုဏ်သတ္တိ အများအပြား ပိုင်ဆိုင်ပါသည်။

ဖော်ပြပါ ဂုဏ်သတ္တိများဝိုင်ဆိုင်ခြင်းမှာလည်းPhenyl propanoid ဒြပ်ပေါင်း ပါဝင်နေခြင်း ကြောင့်ဖြစ်ပါသည်။

Determintion of Anti-inflammatory Activity of the Ethanolic Plant Extract and Structure Elucidation of Isolated Compound from the Root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (Kyauk pan)

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Abstract

In this research work, one of Myanmar indigenous medicinal plants, *Chamaecyparis pisifera* (Siebold & Zucc.) Endl., Myanmar name Kyauk pan was selected for chemical analysis. It was collected from Forest Research Institute (FRI) campus, Yezin, Zaeyar Thiri Township, Naypyitaw Region. Moreover, a potent anti-inflammatory activity of the root of Kyauk pan was determined by carrageenan induced inflammation method. This plant showed remarkable inhibitory activity in high dose and also the activity followed by the remaining medium and low dose. High dose was found to possess significant anti-inflammatory activity, which supported the traditional application of the test plant in acute inflammatory activity. Furthermore, the pure organic compound (TDC-1,) was isolated from the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. by using Thin Layer and Column Chromatographic techniques. The pure pale yellow oily form (19.5 mg, 0.4% yield) were obtained. Furthermore, molecular formula of pure compound (TDC-1) isolated from the root of selected plant (Kyauk pan) was determined as ($C_{22}H_{20}O_8$) by using some spectroscopic technique such as FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, and EI Mass spectral data respectively. The molecular mass of that pure compound is 412 and its hydrogen deficiency index(HDI) is 13. The complete structures of isolated pure compounds were elucidated by DQF-COSY, ^{1}H NMR splitting patterns, coupling constant (J values), and HMBC signals. The IUPAC name of isolated pure compound (TDC-3) is (Z) - dimethyl 2 - (benzo [d] [1', 3'] dioxol - 5' - yl methyl) – 3 - (benzo [d] [1″, 3″] dioxol - 5″ - yl methylene) succinate. It is phenyl propanoid derivative compound. Phenyl propanoid compound may be useful in the prevention and treatment of many health conditions. The root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. possess many medicinal properties such as antibactial, antifungal, antioxidant, anti-inflammatory, antineoplastic, insecticidal, analgesic, aphrodisiac, diuretic, neuralgia and antirheumatic remedia. In fact, many of the medicinal effects of the root of Kyauk pan has been directly related to their phenyl propanoid derivative compound content.

Key words: Anti-inflammatory, Thin Layer and Column Chromatographic, IUPAC name phenyl propanoid, HDI

Determination of Anti-inflammatory Activity of the Ethanolic Plant Extract and Structure Elucidation of Isolated Compound from the Root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (Kyauk pan)

1. Introduction

Myanmar is rich in natural resources. Among them, plants are essential for human being. They are important sources for the preparation of natural remedies, food additives, and other ingredients, as they contain many biologically active compounds and other very important phytochemicals. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semisynthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as phytochemicals ('phyto-' from Greek - *phyto* meaning 'plant') or phyto-constituents and are responsible for protecting the plant against microbial infections or infestations by pests (James Hamuel Doughari (2012). At present, not only herbal plants but also woody plants have been predominantly used as crude drugs and resources for the isolation of biologically active compounds.

Nowadays plant-based traditional medicines are still importance sources of medicines, especially in developing countries. It is estimated that 60% of the world population and 80% of the population of developing countries rely on traditional medicine, mostly plant drugs, for their primary health care needs (Gairola sanjay (2010). Therefore, drug discovery from plants should remain an essential component in the search for new medicines and the scientific study of traditional medicines, concerned medicinal plants are thus of great importance.

There is very little reported on the use of *Chamaecyparis* species in traditional medicine (Turner, 1979). In Myanmar, many people known Kyauk pan as an ornamental plant. But, medicinal uses of Kyauk pan are lesser known. Thus, with the objectives of the promotion of its medicinal uses such as antioxidant, anticancer, antiviral, antifungal and anti-inflammatory, the biologically active organic component in the roots of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. is intended to be investigated.

Moreover, the scientific information regarding effective dose, method of application or administration, efficacy and toxicity of the Kyauk pan should be studied pratically. So,this study is aimed to investigate the anti-inflammatory activity of the Kyauk pan on Carrageena induced paw oedema in albino mice.The mice were inflamed by injecting the carrageenan.Carrageena induced rat paw oedema is a widely used test to determine anti-inflammatory activity and constitutes a simple and routine animal model for evaluation of pain at the site of inflammation without any injury or damage to the inflamed paw (Necas, J et al., 2013). Carrageenan induced paw edema in albino mice of foot- pad have five cardinal signs: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain) and function loss (function laesa). Redness and heat are due to increased blood flow to the inflamed area; swelling is due to accumulation of fluid; pain is due to release of chemicals that stimulate nerve endings; and loss of function is due to a combination of factors (Chandrasoma P *et al*.,1998).Therefore, carrageenan induced acute

inflammation in albino mice is one of the appropriate models for testing anti-inflammatory activity.

In this research, Acetyl Salicylic acid (Aspirin) was used as a standard drug. It is a nonsteroidal anti-inflammatory agent whose pharmacologic activity includes anti-inflammatory, analgesic and antipyretic actions. It inhibits the activity of enzyme cyclooxygenase which lead to the formation of prostaglandins that causes inflammation, swelling, pain and fever (Vane, 2003). Thus, the potent anti-inflammatory activity of 95% ethanol extract of the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. was determined by carrageenan induced paw edema model in which standard drug aspirin was used.

In this study, anti-nflammatory effects of ethanolic plant extract of Kyauk pan was carried out. Moreover, pure organic compound (TDC-1) was isolated from ethyl acetate extract of the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl.The complete structure of pure bioactive organic compound (TDC-1) could be elucidated by using advanced spectroscopic methods such as ^{1}H NMR (500 MH_Z), ^{13}C NMR (125 MH_Z), DEPT, DQF-COSY, HSQC, HMBC and EI-mass spectra respectively.

2. Objectives

The objectives of present research work are as follows:

- To access the potent anti-inflammatory activity of the selected sample on carrageenan induced acute inflammation in albino mice.
- To isolate the biologically active compound from the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. by utilizing solvent extraction method followed by Column and Thin Layer Chromatographic separation methods.
- To assign the molecular formula and elucidate the structure of isolated pure organic compounds by the application of spectroscopic methods.

3. Literature Review

Family	;	Cupressaceae
Genus	;	*Chamaecyparis*
Species	;	*C. pisifera*
Botanical name	;	*Chamaecyparis pisifera* (Siebold & Zucc.) Endl.
Myanmar name	;	Kyauk pan
Part used	;	The whole plant
Medicinal uses	;	antibactial, antifungal, antioxidant, anti-inflammatory, antineoplastic, insecticidal, analgesic, aphrodisiac, diuretic, neuralgia and antirheumatic remedia (Megan Erin Gallagher, 2003).

It is a slow-growing coniferous tree growing to 35–50 m tall with a trunk up to 2 m in diameter. It has thin, horizontal to pendulous branches of a very fine texture which forms a dense, broad pyramid. The bark is red-brown, vertically fissured and with a stringy texture. The foliage is arranged in flat sprays; adult leaves are scale-like, 2 mm long, with pointed tips, green above, green below with a white stomatal band at the base of each scale-leaf. The juvenile leaves, found on young seedlings, are needle-like, bluish-green. The cones are globose, 4–8 mm diameter, with 6–10 scales arranged in opposite pairs, maturing in autumn about 7–8 months after pollination **(***https://en. wikipedia. org/wiki/ Chamaecyparis_pisifera).*

Chamaecyparis pisifera (Siebold & Zucc.) Endl. is mainly distributed in Eastern Asia including Myanmar, western and eastern North America. It is used with folk medicine for aphrodisiac, diuretic and neuralgia in China, Japan and Korea (Min-Gi Kim et al., 2011). The essential oil from *Chamaecyparis* species have been commercially used in soap, toothpaste and cosmetics All species have hard, aromatic wood which is highly prized and has been used by Native American peoples to make bows, canoe paddles and dishes (Turner, 1979).

Myanmar is also rich in biodiversity. Most of the people in Myanmar rely on traditional medicinal plants rather than modern medicines for the treatment of various disorders (Kyaw Sein Win,2003). According to traditional beliefs in Myanmar, there are 96 diseases and afflicted the humans. Myanmar people are believed to be able to cure all of these diseases by using ingredients of medicinal plants such as fresh or dried roots, stems, leaves, buds, and flowers. Only a few of them have been scientifically explored (Swe Swe *et al*., 2015).

In Myanmar, the local communities of different regions have been using medicinal plants as a primary source of their health care system and in fact these medicinal plants are used to cure a large number of diseases. This practice is usually based on experiences, less scientific evidence and therefore, need proper validation on scientific research. (Swe Swe et.al., 2014).

The inflammation reaction is a body defense reaction in order to eliminate or limit the spread of injurious agents (Duta Saryajit et al., 2010). There are two types of inflammation, acute and chronic inflammation. Acute inflammation is of relatively short duration, lasting for minutes, several hours, or a few days. Its main characteristics are odema and the emigration of leucocytes, predominantly leucocytes. There are cardinal signs of acute inflammation.

(i) Redness (rubor): An acute inflamed tissue appears red, for example skin affected by sunburn.
(ii) Heat (color): Increase of temperature on the skin is seen only in peripheral parts of the body. It is due to increased blood flow through the region, resulting in vascular dilation and the delivery of warm blood to the area.
(iii) Swelling (tumor): swelling result from edema, the accumulation of fluid in the extra vascular space as a part of the fluid exudates.
(iv) Pain (dolar): it results partly from the stretching and distortion of tissue due to inflammation edema and in particular, from pus under pressure in an abscess cavity.
(v) Loss of function: It is a well-known consequence of inflammation. Movement of an inflame area is consciously and reflexly inhibited by pain (Macfarlane et al., 2000)

Chronic inflammation is the inflammation of prolong duration in which active inflammation, tissues destruction, and attempts at healing are proceeding simultaneously. Chronic inflammation may follow acute inflammation but it can begin insidiously, as a low-grade, smoldering, often asymptomatic reaction (Robbins et al,1994). Chronic inflammation is characterized by a different set of reactions:

- Infiltration with mononuclear cells, including macrophages, lymphocytes, and plasma cells
- Tissue destruction,largely induced by the products of the inflammatory cells
- Repair, involving new vessel proliferation and fibrosis

Chronic inflammation may arise in the following settings.

(i) Persistent infections by microbes that is difficult to eradicate.
(ii) Immune-mediated inflammatory diseases.
(iii) Prolonged exposure to potentially toxic agents.

(iv) Mild forms of chronic inflammation may be important in the pathogenesis of many diseases that are not conventionally thought of as inflammatory disorders (Robbins et al,2013).

4. Materials and Methods

4.1 Sample Collection

The roots of Kyauk pan were collected from Forest Research Institute (FRI) campus, Yezin, Zaeyar Thiri Township, Naypyitaw Region. Firstly, the root samples were cleaned. Then they were chopped into tiny pieces, and dried in shade at room temperature for about three weeks. These air dried samples were stored in a well stoppered glass bottle and used throughout the experiment.

4.2 Preparation of Ethanol Extract of the Root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl.

About 1000 g of air dried root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. were stored in a stoppered bottle and percolated with 95% ethanol (3 L) at room temperature for about two months. The extract was filtered with filter paper and the filtrate was evaporated at room temperature. Then 95% ethanol (3 L) was added to the residue for about two weeks. It was filtered again and the filtrate was concentrated at room temperature. Totally (43.5 g) of ethanol extract was obtained. The dried extract was stored in a refrigerator for testing the effect of anti-inflammatory by using the experimental animals.

4.3 Anti-inflammatory Activity Study

Anti-inflammatory activity study was carried out on ethanolic extract of root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. using mice as the experimental model. It was assessed by carrageenan induced paw edema method in mice. In this study, mice ICR (Institute of Cancer Research) were taken from Laboratory Animal Services Division, DMR (POLB). The non-fasted mice of both sexes in the weight range 22-40 g were used. Food and water were withheld during the experimental period. The screening was done on 40 albino mice. They were divided into five groups (negative control group, positive control group, three tested groups) and each group contains eight mice. The negative control group was given (vehicle) 10 ml/kg body weight orally; positive control was given Aspirin (Acetyl Salicylic Acid) in a dose of 300 mg/kg. The rest groups were treated with 95% three doses of the ethanolic extract of the root of *Chamaecyparis pisifera* (Sieb. Et Zucc.) Endl.) as low dose (250 mg/kg), medium dose (500 mg/kg) and high dose (1000 mg/kg body weight) respectively. Animals were marked with marker pen at the lateral malleous. Then, basal paw volumes were measured by volume displacement method using Plethysmometer (Model LE 7500) immediately prior to the induction of carrageenan. After one hour of drug administration, freshly prepared suspension of Lambda carrageenan 0.1 mL (1% in 0.9% NaCl) was injected into plantar tissue of the right hind paw. The volumes of paw were again measured by taking baseline volume (0 hour) and then 1, 2, 3, 4 and 5 hour (hr) time interval. (Winter et.al. 1962, Bodakhe et.al. 2008), after carrageenan challenge. The volume of paw was expressed in terms of milliliter (mL). The difference of mean paw volumes between test groups and control group were evaluated for each time interval. The percent inhibition of edema was calculated by using the formula as follow.

$$\% \text{ inhibition} = \left[1 - (Vt/Vc)\right] \times 100$$

Where, Vc = Edema volume of control group

Vt = Edema volume in the drug treated group

4.4 Extraction and Isolation of Pure Compounds from the Root of Kyauk pan

The air dried sample (900 g) was percolated with 95% ethanol (3 L) at room temperature for two months. During percolation, the crude mixture was frequently shaken to achieve the maximum extraction. The extract was filtered with filter paper and evaporated in air. The ethanol extract was then re-extracted with ethyl acetate (300 mL) and evaporated to dryness.

The ethyl acetate crude extract (4.656 g) was chromatographed by silica gel column, using n-hexane and ethyl acetate with various ratios from non polar to polar. Totally (244) fractions were collected. Then each fraction was checked by TLC. The fractions that showed the same R_f values were combined. Eighteen combined fractions were obtained. Each combined fractions were checked by TLC for purity using iodine vapor and UV lamp as developer.

Among eighteen combined fractions, combined fractions (12) and (16) show one spot on TLC plate and also UV active. And then the combined fraction (5) was rechromatographed over silica gel eluted with n-hexane and ethyl acetate to afford pure compound (III). It is labeled as pure compound TDC (1).

4.5 Spectroscopic Studies of Pure Compound

The pure bioactive organic compound, (TDC-1) was subjected to analyze by FT-IR, ^{1}H NMR (500 MHz), ^{13}CNMR (125 MHz), DEPT, HSQC, DQF-COSY and HMBC, EI-MS and NOESY spectroscopic techniques.

FT-IR spectra of pure bioactive organic compound was measured at the Department of Chemistry, University of Mandalay and the remaining spectral data were measured at the Department of Natural Resources Chemistry, Faculty of Pharmacy, Meijo University, Nagoya, Japan.

5. Results and Discussion

5.1 Anti-inflammatory Activity Study

In acute inflammation models, the groups were orally administered with 95% ethanol extract of the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. in the dose of 250 mg/kg, 500 mg/kg and 1000 mg/kg. Paw edema volume was measured before (0 hour) and hourly up to 5 hour after injection of carrageenan by digital Plethysmometer. The paw volumes of treated groups were compared with negative control group (vehical, 10 ml/kg).

In this study, aspirin 300 mg/kg was used as a positive control group. The results of mean paw edema volume in mL after treatment with positive control (Aspirin, 300 mg/kg) were 0.16 ± 0.01 at 1 hour, 0.14 ± 0.007 at 2 hour, 0.13 ± 0.006 at 3 hour, 0.14 ± 0.004 at 4 hour 0.13 ± 0.005 at 5 hour respectively, compared to that of the negative control group (vehicle). Statistically significant decrease in paw edema volumes of mice were found at 2 hour after carrageenan injection ($p < 0.001$) and it has same significant levels from 3 hour and up to 5 hour ($p < 0.001$).

The results of mean paw volumes after treatment with ethanolic extract of 250 mg/kg and 500 mg/kg were 0.18 ± 0.005 and 0.18 ± 0.007 at 1 hour, 0.17 ± 0.008 and 0.17 ± 0.012 at 2 hour, 0.18 ± 0.011 and 0.16 ± 0.014 at 3 hour, 0.19 ± 0.011 and 0.18 ± 0.018 at 4 hour, 0.18 ± 0.006 and 0.17± 0.018 at 5 hour respectively. When compared to the negative control group (vehicle) with 95% ethanol extracts of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl., low dose (test drug, 250 mg/kg) and medium dose (test drug 500 mg/kg), significant anti-inflammatory effects were not showed from 1 hour after carrageenan injection up to 5

hour.The results of mean paw edema volume in mL after treatment with ethanolic extract of 1000 mg/kg were 0.18 ± 0.007 at 1 hour, 0.17 ± 0.009 at 2 hour, 0.15 ± 0.013 at 3 hour, 0.16 ± 0.019 at 4 hour and 0.15 ± 0.015 at 5 hour respectively. When compared to the negative control group (vehicle) with the highest dose (test drug, 1000 mg/ kg), significant anti-inflammatory effects had showed to start at 3 hour ($p < 0.01$), 4 hour and 5 hour ($p < 0.05$) after carrageenan injection.

Thus, it was observed that 95% ethanol extract of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (test drug, 1000 mg/ kg) was found better action from 3 hour after injection of carrageenan up to 5 hour. The results of mean paw volumes of negative control, positive control and the test plant were shown in Table (5.1) and Figure (5.1).

Table (5.1) The Mean Paw Volumes of Negative Control, Positive Control and the Test Plant in Carrageenan Induced Mice

Treatment (Dose)	Mean paw volume (mL)					
	0hr	1hr	2hr	3hr	4hr	5 hr
Vehicle 10 ml/kg	0.11±0.002	0.19±0.008	0.19±0.007	0.19±0.005	0.21±0.01	0.19±0.01
Aspirin 300 mg/kg	0.11±0.006	0.16±0.01	0.14±0.007***	0.13±0.006***	0.14±0.004***	0.13±0.005***
Test plant 250 mg/kg	0.12±0.007	0.18±0.005	0.17±0.008	0.18±0.011	0.19±0.011	0.18±0.006
Test plant 500 mg/kg	0.10±0.008	0.18±0.007	0.17±0.012	0.16±0.014	0.18±0.018	0.17±0.018
Test plant 1000 mg/kg	0.13±0.010	0.18±0.007	0.17±0.009	0.15±0.013**	0.16±0.019*	0.15±0.015*

Results were expressed as Mean ± Standard Error (SE) *P<0.05 **P<0.01 ***P<0.001

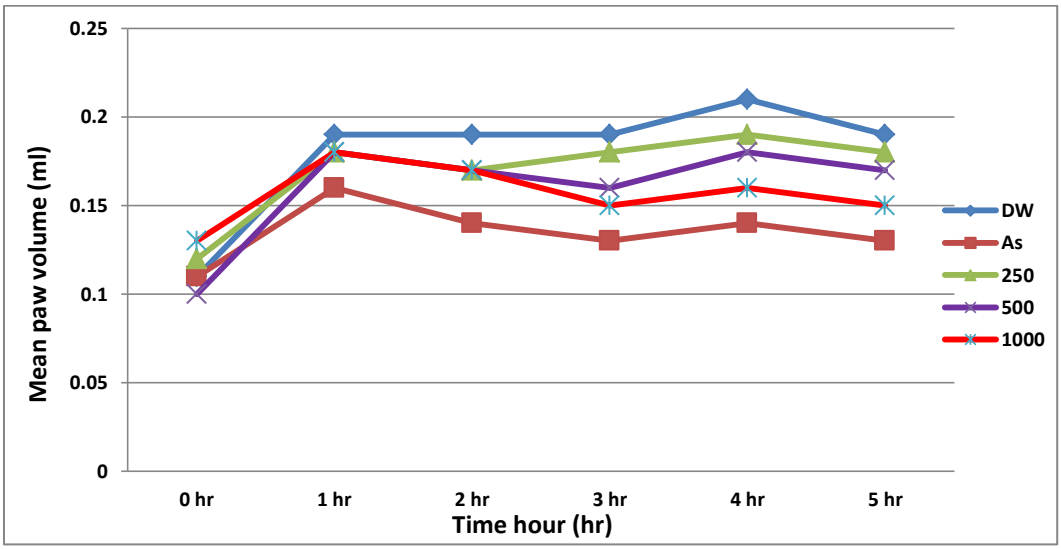


Figure (5.1) Effect of the Root of Kyauk pan, Aspirin and Vehicle on Mean Paw Volume of Mice after Carrageenan Induced Edema

5.2 Percent Inhibition of Carrageenan Induced Paw Edema of 95 % Ethanol Extract of the Root of Kyauk pan and Aspirin

Percent inhibition of paw edema with positive control (Aspirin, 300 mg/kg) were found to be 16%, 26%, 32%, 33% and 29% inhibition for 1 hour, 2 hour, 3 hour, 4 hour and 5 hour respectively. The percent inhibition of right hind paw edema was observed at 1 hour 16% and maximal inhibition was 33 % and it was shown that at 4 hour. The results were shown in Table (5.2.) and Figure (5.2).

The study of anti-inflammatory effect of 250 mg/ kg and 500 mg/kg of 95% ethanol extract of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. had shown that its action started from 1 hour after carrageenan injection up to 5 hour. Significant anti-inflammatory effects were not shown from 1 hour after carrageenan injection up to 5 hour. Percent inhibition of paw edema with 95% ethanol extracts of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. of low dose and medium dose were found to be 5%, 11%, 5%, 10% , 5% inhibition and 5%, 11%, 16%, 14%, 11% inhibition for 1 hour, 2 hour, 3 hour, 4 hour and 5 hour respectively. According to these results, the maximal inhibitions were 11% for 250 mg/kg at 2 hour and 16% for 500 mg/kg at 3 hour. Hence, low dose and medium dose of plant extract have low inhibition.

On the other hand, percent inhibition of paw edema with 95 % ethanol extract of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (1000 mg/kg) were found to be 5%, 11%, 21%, 24% and 16% inhibition for 1 hour, 2 hour, 3 hour, 4 hour and 5 hour respectively. The maximal % inhibition of high dose of plant extract was 24% at 4 hour. The results were shown in table (5.2) and Fig (5.2).

The percent inhibition on carrageenan induced inflammation in mice were highest in the concentration of the test sample 1000 mg/kg at 4 hour (24%). According to the present study, the test plant has high anti-inflammatory effect at high dose 1000 mg/kg at 3 hour up to 5 hour. Thus, this experiment may provide one of the useful information in the development of Myanmar Traditional Medicine from natural plants.

Table (5.2) Percent Inhibition of Lambda Carrageenan Induced Paw Edema after Treatment with Aspirin and 95 % Ethanol Extract of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl.

Treatment (Dose)	1 hr	2 hr	3 hr	4 hr	5 hr
Aspirin (300 mg/kg)	16%	26%	32%	33%	29%
Test plant 250 mg/kg	5%	11%	5%	10%	5%
Test plant 500 mg/kg	5%	11%	16%	14%	11%
Test plant 1000 mg/kg	5%	11%	21%	24%	16%

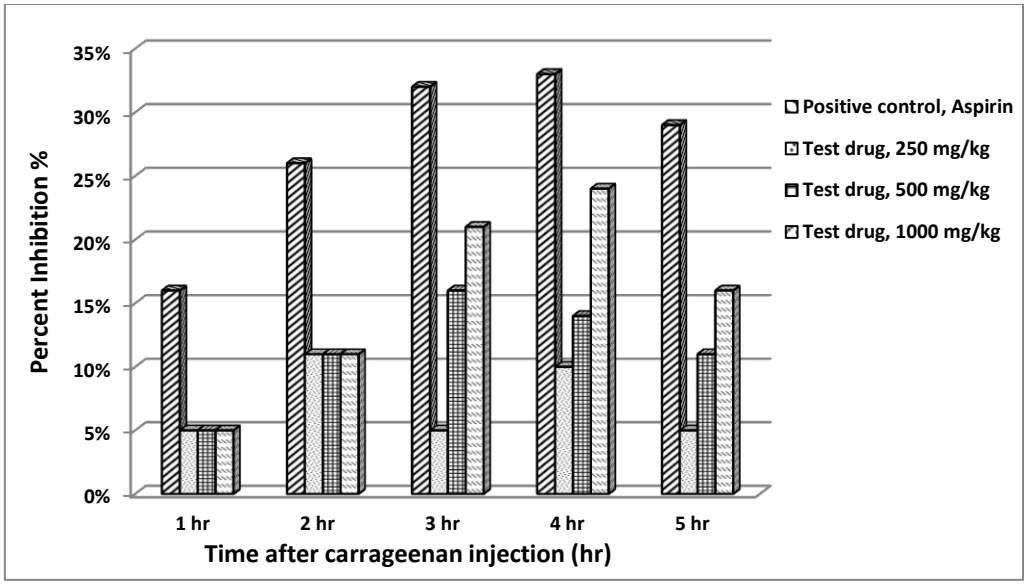


Figure (5.2) Anti-inflammatory Effect of the Root of *Kyauk pan* and Aspirin

5.3 Determination of Molecular Formula of Pure Compound (TDC-1)

The molecular formula of the isolated pure compound (TDC-1) could be determined by some spectroscopic methods such as FT-IR, ^{1}H NMR (500MHz), ^{13}C NMR (125MHz), HSQC and EI-MS spectra respectively.

5.3.1 FT-IR Assignment of Pure Compound (TDC-1)

According to FT-IR assignment, the pure compound (TDC-1) consists of sp^2 hydrocarbon, sp^3 hydrocarbon, carbonyl group, aromatic benzene ring, allyic hydrocarbon, ether group, trans or E alkenic and cis or Z alkenic functional groups respectively. The prominent functional groups containing in this compound are given in Table (5.3).

Table (5.3) FT-IR Assignments of Pure Compound (TDC-1)

No	Absorption peaks (cm^{-1})	Assignments
1.	3044	C- H stretching vibration of sp^2 hydrocarbon
2.	2955, 2911,2874	C- H stretching vibration of sp^3 hydrocarbon
3.	1738,1716	C=O stretching vibration of carbonyl group
4.	1619, 1494	C=C ring skeletal stretching vibration of aromatic benzene ring
5.	1441	C-H in plane bending vibration of allylic hydrocarbon
6.	1246, 1163, 1039	C-O-C stretching vibration of ether group
7.	931.65	C-H out of plane bending vibration of trans or E alkenic group.
8.	862, 813	C-H out of plane bending vibration of cis or Z alkenic group.

5.3.2 ^{1}H NMR Spectrum of Pure Compound (TDC-1)

In accordance with this ^{1}H NMR (500 MHz) spectrum, compound (TDC-1) contains totally (16) protons. The chemical shift values, splitting patterns and J values of protons are shown in Table (5.4).

Table (5.4) ^{1}H NMR Spectral Data of Pure Compound (TDC-1)

No.	Chemical shift (δ/ppm)	No. of protons	Splitting pattern	Coupling constant (J values, Hz)	Proton assignment
1.	2.87 3.34	2H	dd dd	4.86, 14.10 4.86, 14.10	sp^3 methylene protons
2.	4.0	1H	dd	4.86, 9.92	sp^3 methine proton
3.	3.82	3H	s	-	sp^3 methoxy proton
4.	3.71	3H	s	-	sp^3 methoxy proton
5.	5.87	2H	dd	1.56, 10.14	sp^3 methylene proton
6.	5.95	2H	dd	1.44, 2.52	sp^3 methylene proton
7.	6.59	1H	d	7.86	sp^2 methine proton
8.	6.71	1H	d	7.98	sp^2 methine proton
9.	6.41	1H	d	1.60	sp^2 methine proton
10.	6.35	1H	d	1.62	sp^2 methine proton
11.	6.37	1H	dd	7.86, 1.62	sp^2 methine proton
12.	6.49	1H	dd	7.98, 1.60	sp^2 methine proton
13.	7.65	1H	s	-	sp^2 methine proton
	Total number of protons				16

5.3.3 ^{13}C NMR Spectrum of Pure Compound (TDC-1)

^{13}C NMR (125 MHz) spectrum indicates the total number of (22) carbons in this compound. The chemical shift values of carbons and kinds of carbons are described in Table (5.5).

Table (5.5) ^{13}C NMR Spectral Data of Compound (TDC-1)

No.	Chemical shit (δ/ppm)	Carbon assignment
1.	35.75	sp^3 methylene carbon
2.	45.53	sp^3 methine carbon
3.	51.98	sp^3 methoxy methyl carbon
4.	52.19	sp^3 methoxy methyl carbon
5.	100.69	sp^2 methine carbon
6.	101.19	sp^2 methine carbon
7.	107.85	sp^2 methine carbon
8.	108.18	sp^2 methine carbon

9.	108.38	sp^2 methine carbon
10	109.41	sp^2 methine carbon
11.	122.14	sp^2 methine carbon
12.	122.49	sp^2 methine carbon
13.	128.86	sp^2 quaternary carbon
14.	129.49	sp^2 quaternary carbon
15.	132.76	sp^2 quaternary carbon
16.	142.54	sp^2 methine carbon
17.	145.85	sp^2 quaternary carbon
18.	147.32	sp^2 quaternary carbon
19.	147.60	sp^2 quaternary carbon
20.	147.68	sp^2 quaternary carbon
21.	167.08	sp^2 carbonyl carbon
22.	173.06	sp^2 carbonyl carbon
Total number of carbons		22

5.3.4 DEPT Spectrum of Pure Compound (TDC-1)

The DEPT spectrum indicates the numbers and kinds of carbons as well as of protons in this compound. The assignments of DEPT spectrum is shown in the Table (5.6).

Table (5.6) DEPT Spectral Data of Pure Compound (TDC-1)

No.	^{13}C NMR chemical shit (δ/ppm)	Carbon assignment	no. of Carbon	no. of Proton
1.	35.75	sp^3 methylene carbon	1	2
2.	45.53	sp^3 metine carbon	1	1
3.	51.98	methoxy methyl carbon	1	3
4.	52.19	methoxy methyl carbon	1	3
5.	100.69	sp^3 methylene carbon	1	2
6.	101.19	sp^3 methylene carbon	1	2
7.	107.85	sp^2 methine carbon	1	1
8.	108.18	sp^2 methine carbon	1	1
9.	108.38	sp^2 methine carbon	1	1
10	109.41	sp^2 methine carbon	1	1
11.	122.14	sp^2 methine carbon	1	1
12.	122.49	sp^2 methine carbon	1	1
13.	128.86	sp^2 quaternary carbon	1	_
14.	129.49	sp^2 quaternary carbon	1	_
15.	132.76	sp^2 quaternary carbon	1	_
16.	142.54	sp^2 methine carbon	1	1

17.	145.85	sp^2 quaternary carbon	1	_
18.	147.32	sp^2 quaternary carbon	1	_
19.	147.60	sp^2 quaternary carbon	1	_
20.	147.68	sp^2 quaternary carbon	1	_
21.	167.08	sp^2 carbonyl carbon	1	_
22.	173.06	sp^2 carbonyl carbon	1	_
Total			**C_{22}**	**H_{20}**

3.16.5 HSQC Spectrum of Pure Compound (TDC-1)

HSQC spectrum informs the proton-carbon direct correlation. The observations of proton-carbon direct attachment are given in Table (5.7).

Table (5.7) HSQC Spectral Data of Pure Compound (TDC-1)

No.	^{13}C NMR chemical shift (δ/ppm)	^{1}H NMR chemical shift (δ/ppm)	Assignment
1.	35.75	2.87, 3.34	sp^3 methylene carbon
2.	45.53	4.0	sp^3 methine carbon
3.	51.98	3.82	sp^3 methoxy methyl carbon
4.	52.19	3.71	sp^3 methyl carbon
5.	100.69	5.87	sp^3 methylene carbon
6.	101.19	5.95	sp^3 methylene carbon
7.	107.85	6.59	sp^2 methine carbon
8.	108.18	6.71	sp^2 methine carbon
9.	108.38	6.41	sp^2 methine carbon
10.	109.41	6.35	sp^2 methine carbon
11.	122.14	6.37	sp^2 methine carbon
12.	122.49	6.49	sp^2 methine carbon
13.	128.86	_	sp^2 quaternary carbon
14.	129.49	_	sp^2 quaternary carbon
15.	132.76	_	sp^2 quaternary carbon
16.	142.54	_	sp^2 methine carbon
17.	145.85	7.65	sp^2 quaternary carbon
18.	147.32	_	sp^2 quaternary carbon
19.	147.60	_	sp^2 quaternary carbon
20.	147.68	_	sp^2 quaternary carbon
21.	167.08	_	sp^2 carbonyl carbon
22.	173.06	_	sp^2 carbonyl carbon

According to ^{1}H NMR and ^{13}C NMR spectral data, the partial molecular formula of pure compound (TDC-1) could be assigned as $C_{22}H_{20}$ and partial molecular mass is 284.

In the ^{13}C NMR spectral data, the downfield chemical shift quaternary carbons (δ 167.08 ppm and 173.06 ppm) should be two carbonyl groups. It was confirmed by the evidence of C=O stretching band at 1738 and 1716 cm^{-1} in FT-IR spectrum. Therefore the molecular formula could be extended as $C_{22}H_{20}O_2$. Moreover, the two singlet methoxy methyl protons at δ 3.82 ppm and δ 3.71 ppm in ^{1}H NMR spectrum reveals that molecular formula of compound should extended as $C_{22}H_{20}O_4$ and extended partial molecular mass is 348.

In accordance with EI-mass spectrum the molecular ion peak m/z is 412 which indicate the molecular mass of compound. Hence, the remaining partial molecular mass is (412- 348 = 64). It must be four oxygen atoms.

Therefore, the real molecular formula of compound (TDC-1) is $C_{22}H_{20}O_8$.

Hydrogen Deficiency Index = C - H/2 + 1
= 22 - 20/2 +1
= 13

5.4 Confirmation of Molecular Formula of Pure Compound (TDC-1)

Molecular formula of compound (TDC-1) could be confirmed by DEPT spectrum, and FT-IR spectrum as shown in Table (5.8).

Table (5.8) Results Given by DEPT and FT-IR Spectral Data of Pure Compound (TDC-1)

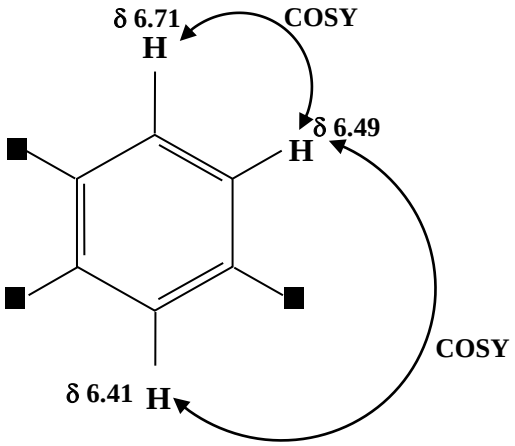
Assignments	no: of carbon	no: of proton	no: of oxygen
From DEPT spectrum			
Two sp^3 methoxyl carbon	2	6	2
Three sp^3 methylene carbon	3	6	-
One sp^3 methine carbon	1	1	
Seven sp^2 methine carbon	7	7	-
Nine sp^2 quaternary carbons	9	-	-
From FT-IR spectrum			
Two C=O group	-	-	2
Partial molecular formula	C_{22}	H_{20}	O_4

Partial molecular formula = $C_{22}H_{20}O_4$
Partial molecular mass = 348
From EI-mass spectrum
Molecular ion peak m/z = 412
Remaining molecular mass = 412- 348 = 64
It must be four hydroxyl groups
Therefore the real molecular formula of compound = $C_{22}H_{20}O_8$

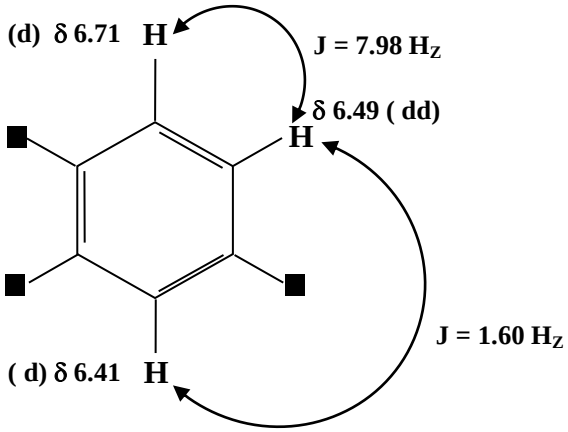
5.5 Structure Elucidation of Pure Compound (TDC-1)

The structure of pure organic compound (TDC-1) could be elucidated by applying DQF-COSY, ^{1}H NMR, HSQC and HMBC spectral respectively.

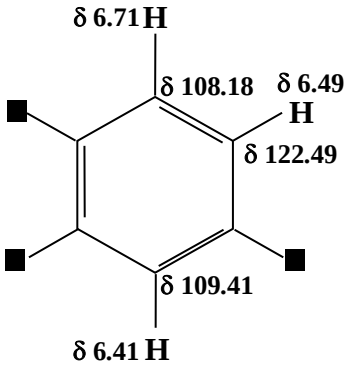
In DQF-COSY spectrum, the occurrence of medium square metrix area of the two aromatic protons (δ 6.71 ppm and 6.49 ppm) and also the observation of small graphic area (or) long range (W coupling) between the two aromatic protons (δ 6.41 ppm and 6.49 ppm) produce the following tri-substituted benzene ring fragment.



Moreover, in the enlarged ^{1}H NMR spectrum, the splitting patterns and the coupling constants of the two aromatic protons (δ 6.49 ppm, dd, J= 7.86 Hz and 1.60 Hz) and (δ 6.71 ppm, d, J= 7.98 Hz) reveals that each of these two protons are oriented at ortho position. And also, another aromatic proton (δ 6.41 ppm, d, J= 1.60 Hz) indicates the meta orientation with its relative proton (δ 6.49 ppm), which confirms the above fragment.

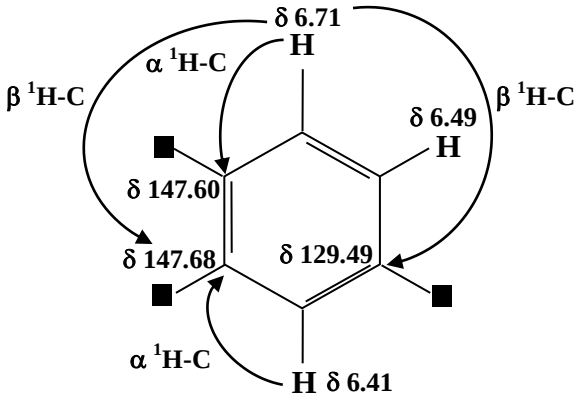


In addition, HSQC spectrum represents the proton carbon direct correlation, in which these three aromatic protons (δ 6.71 ppm, δ 6.49 ppm and δ 6.41 ppm) are directly attached to the aromatic methinecarbons (δ 108.18 ppm, δ 109.41 ppm and δ 122.49 ppm) respectively.



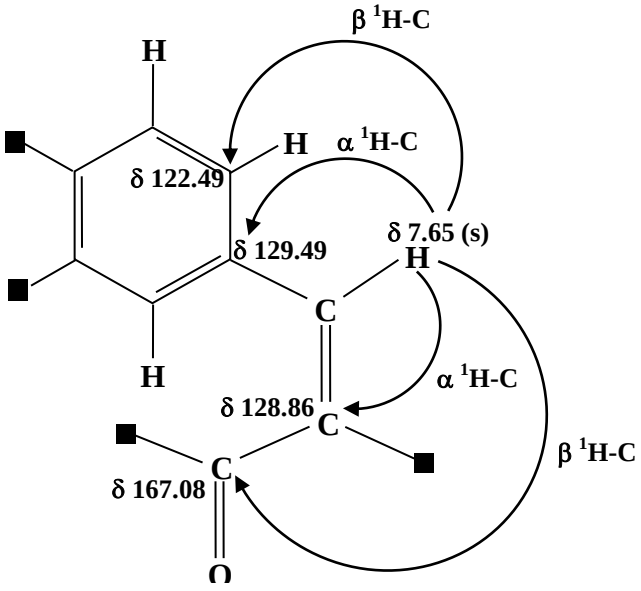
In HMBC spectrum, the aromatic proton (δ 6.71 ppm) responds α and β^{1} H-C long range signal with aromatic quaternary carbons (δ 147.60 ppm and δ 147.68 ppm) and also

the β ^{1}H-C long range coupling with another aromatic quaternary carbon (δ 129.49 ppm). Similarly, the other aromatic proton (δ 6.41 ppm) also responds α ^{1}H-C long range signal with aromatic quaternary carbons (δ 147.69 ppm) which represents the following fragment.



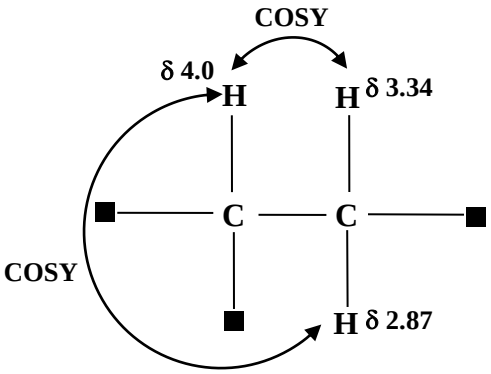
Fragment a

Moreover, the sp^{2} methine proton (δ 7.65 ppm) has α and β ^{1}H-C long range coupling with sp^{2} methine carbon (δ 128.86 ppm) and carbonyl carbon (δ 167.08 ppm) and also the α and β ^{1}H-C long range coupling with two aromatic quaternary carbons (δ 122.49 ppm and δ 122.49 ppm) from fragment a which reveals the following larger fragment b

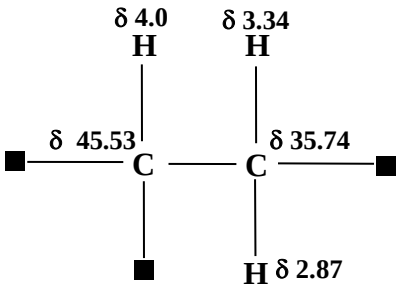


Fragment

In another front, in DQF-COSY spectrum, the observation of medium square metrix area of the sp^{3} methine proton (δ 4.0 ppm) with adjacent two sp^{3} methylene protons (δ 3.34 ppm and 2.87 ppm) shows the following fragment.

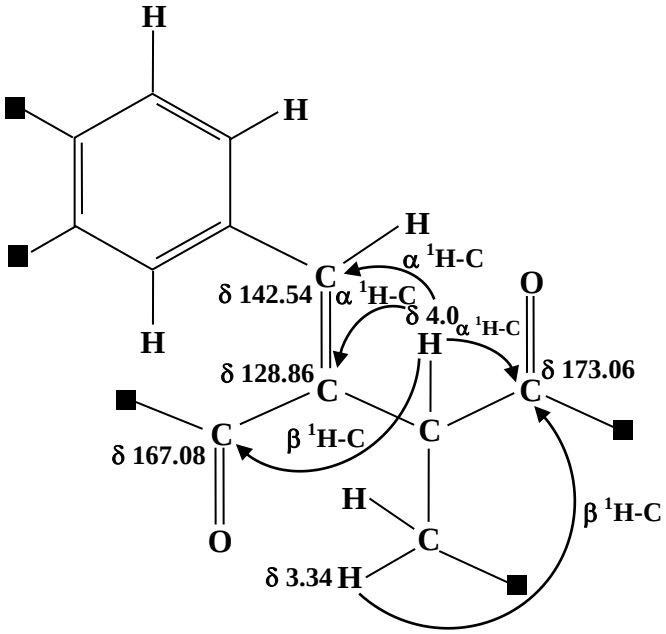


In HSQC spectrum, three protons (δ 4.0 ppm, δ 3.34 ppm and δ 2.87 ppm) are directly attached to the sp^3 methine carbon (δ 45.53 ppm, and sp^3 methylene carbon (δ 35.74 ppm) respectively.



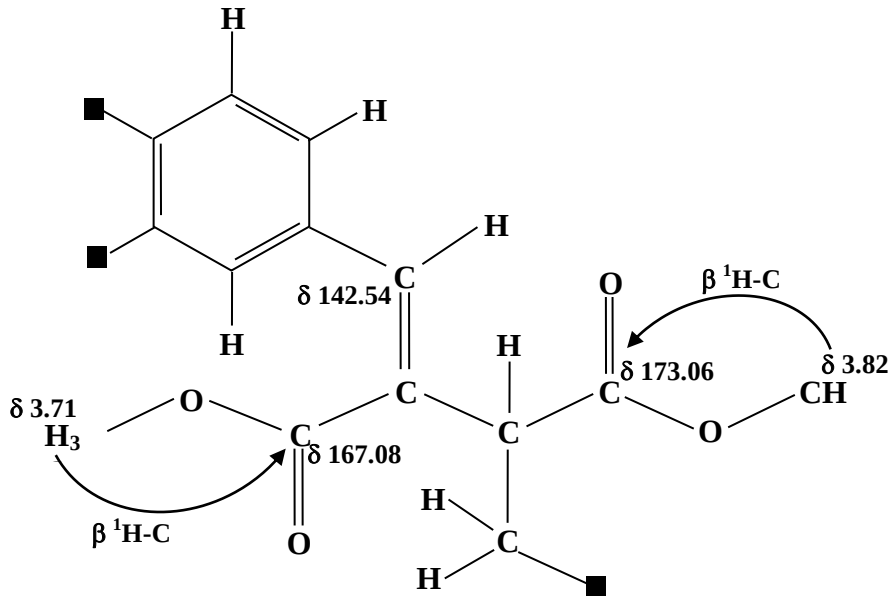
Fragment c

Fragment b and fragment c could be connected by HMBC spectrum. In this spectrum, the sp^3 methine proton (δ 4.0 ppm) has α ^{1}H-C long range coupling with two sp^2 methine carbons (δ 142.54 ppm and δ 128.86 ppm) and one carbonyl carbon (δ 173.06 ppm) and β ^{1}H-C long range coupling with another carbonyl carbon (δ 167.08 ppm). Moreover, singlet sp^3 methylene proton (δ 3.34 ppm) has β ^{1}H-C long range coupling with carbonyl carbon (δ 173.06 pm) which leads to the following extended fragment d .



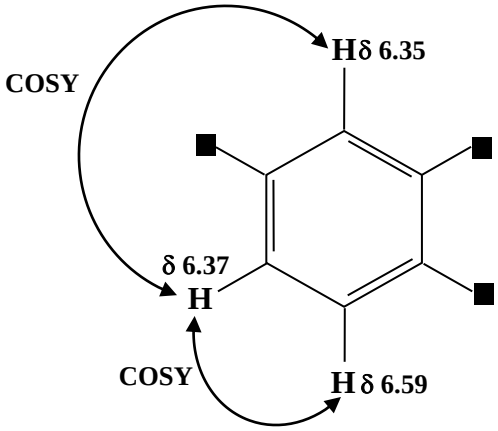
Fragment

On the other hand, in HMBC spectrum, the occurrence of β ^{1}H-C long range coupling of two methoxy methyl protons (δ 3.71 ppm, δ 3.82 ppm) with two carbonyl carbons (δ 167.08 ppm, and δ 173.06 ppm) show the following extended fragment ę .



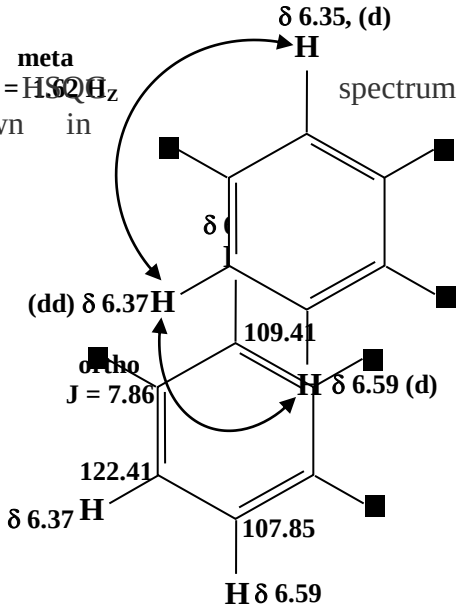
Fragment ę

Furthermore, in DQF-COSY spectrum, the occurrence of medium graphic area of two aromatic protons at δ 6.59 ppm and 6.37 ppm and small graphic area (or) long range (W coupling) of another aromatic proton at δ 6.35 ppm giving rise to the following tri-substituted benzene ring fragment .

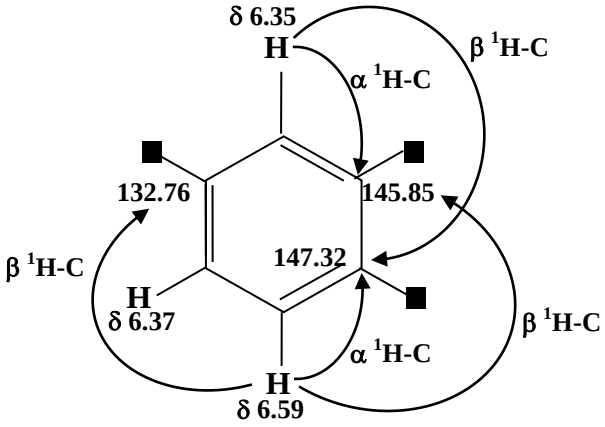


The above fragment could be confirmed by ^{1}H NMR spectrum. The splitting patterns and the coupling constants (J-values) of two aromatic protons (δ 6.37 ppm, dd, J= 7.86 Hz and 1.62 Hz) and (δ 6.59 ppm, d, J = 7.86 Hz) which indicate that these two protons are oriented at ortho position and another aromatic proton (δ 6.35 ppm, d, J= 1.62 Hz) is oriented at meta position with its related proton (δ 6.37 ppm) as described below.

Moreover, HSQC spectrum displays the proton carbon direct correlation as shown in following fragment.



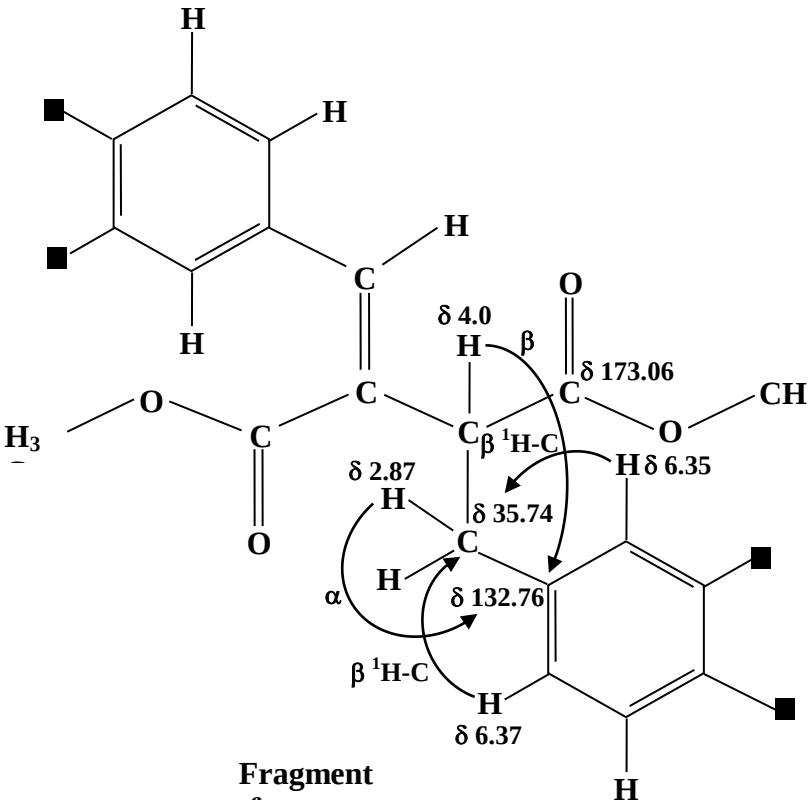
In addition, in HMBC spectrum, the aromatic proton (δ 6.59 ppm) has α and β ^{1}H-C long range coupling with two aromatic quaternary carbons (δ 147.32 ppm, δ 132.76 ppm and δ 145.25 ppm). Similarly, the another aromatic proton (δ 6.35 ppm) also responds α and β ^{1}H-C long range signal with aromatic quaternary carbons (δ 145.85ppm and δ 147.32ppm) which represent the following fragment .



Fragment e

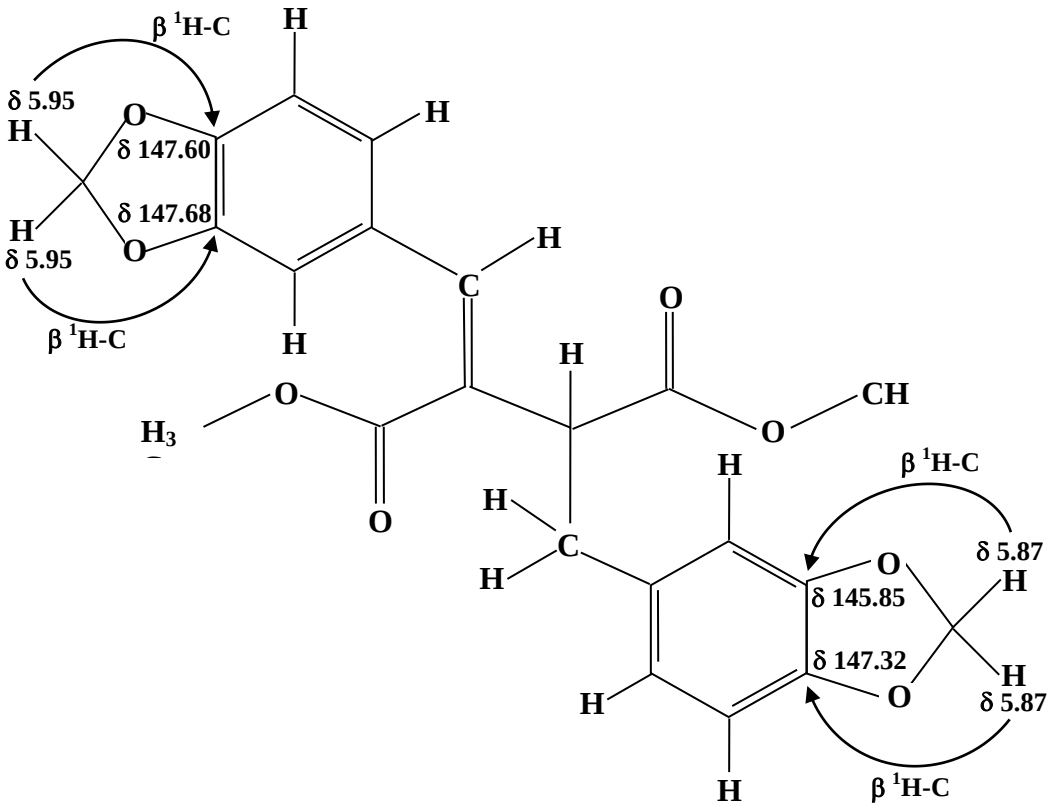
Moreover, the elucidated partial fragments d and e could be connected by HMBC spectrum. In this spectrum, the observation of α and β ^{1}H-C long range coupling of sp^3 methylene proton (δ 2.87 ppm) and another sp^3 methine proton (δ 4.0 ppm) with aromatic quaternary carbon (δ132.76 ppm) reveal the following extended fragment f .

Inversely, the two aromatic protons (δ 6.35 ppm and δ 6.37 ppm) display β ^{1}H-C long range signal with sp^3 methylene carbon (δ 35.74 ppm) which confirms the following fragment f .



Fragment

In addition, the observation of β ^{1}H-C long range coupling of two same chemical shift acetal methylene protons (δ 5.95 ppm) with two aromatic quaternary carbons (δ 147.60 ppm and δ 147.08 ppm) and β ^{1}H-C long range signal of another acetal methylene protons (δ 5.87 ppm) with two aromatic quaternary carbons (δ 147.32 ppm and δ 145.85 ppm) in HMBC spectrum establish the complete structure of pure compound (TDC-1).



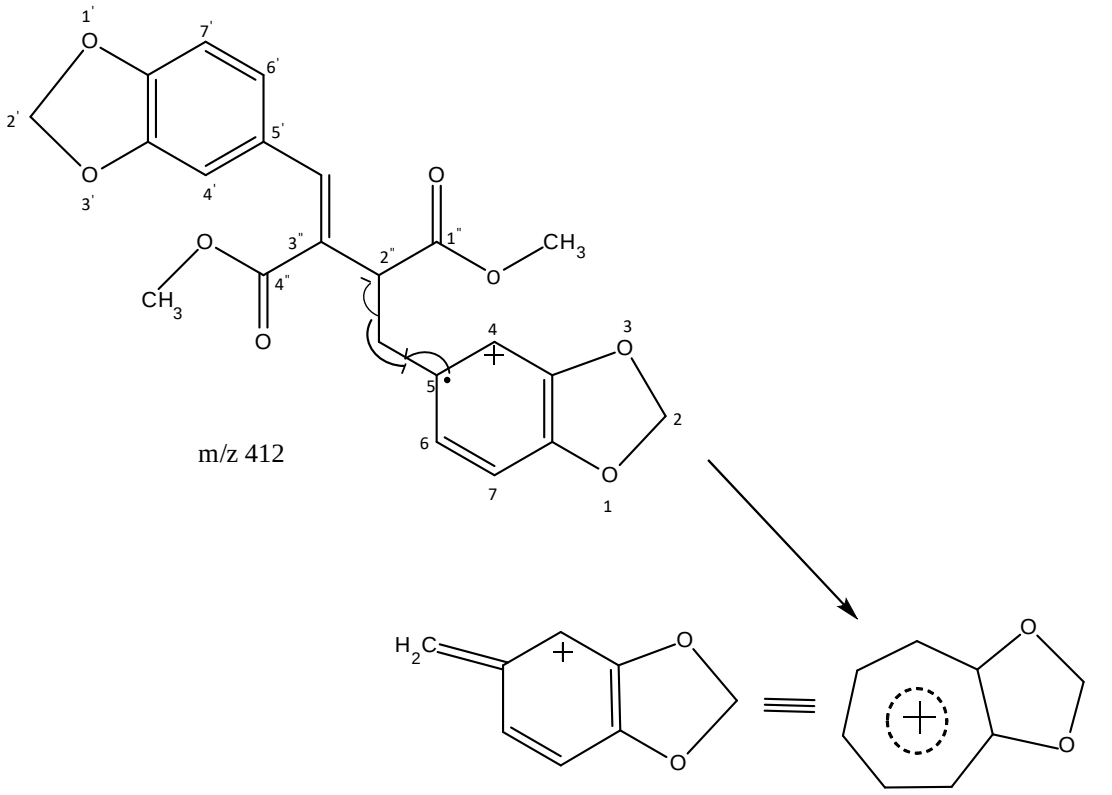
Complete Structure of Pure Compound

The IUPAC name of isolated pure compound (TDC-1) is (Z) - dimethyl 2^{II} - (benzo [d] [1, 3] dioxol - 5 - yl methyl) - 3^{II}- (benzo [d] [1^{I}, 3^{I}] dioxol - 5^{I} -yl methyl) - 3 - (benzo [d] [1, 3] dioxol - 5 - yl methylene) succinate.

5.6 EI Mass Fragmentation Behavior of Pure Compound (TDC-1)

The elucidated structure of pure compound (TDC-1) could be confirmed by EI mass fragmentation behavior. The proposed mechanisms for the fragmentation pattern in EI mass spectrum are shown as follows.

The disconnection of C_2'- methyl bond from the molecular ion (m/z 412) produces the most stable base peak, tropylium ion fragment a (m/z 135).



a m/z 135 (base peak)

In addition, fragment b (m/z 277) could be observed by the rearrangement of hydrogen radical from the molecular ion (m/z 412). Moreover, the cleavage of C_2'- methyl bond and the loss of methoxyl radical from the molecular ion (m/z 412) produce the fragment c m/z 245).

6. Conclusion

In this research work, *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. (Myanmar name Kyauk pan) was chosen for chemical analysis. A potent anti-inflammatory activity of the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. was determined by carrageenan induced inflammation method. In this study, the percent inhibition on carrageenan induced inflammation in mice was highest in the concentration of the present test extract sample 1000 mg/kg at 4 hr (24%) though it was less than aspirin (33%). A potent anti-inflammatory activity was determined against carrageenan induced inflammation for the root extract sample of the test plant showed remarkable inhibitory activity in high dose and also the activity followed by the remaining medium and low dose. High dose was found to possess significant anti-inflammatory activity, which supported the traditional application of the test plant in acute inflammatory activity.

Furthermore, the pure organic compound (TDC-1) was isolated from the root of *Chamaecyparis pisifera* (Siebold & Zucc.) Endl. by using Thin Layer and Column Chromatographic techniques. The pure yellow oily form compound, (19.5 mg, 0.41% yield) was obtained. Molecular formula of pure compound (TDC-1), isolated from the root of Kyauk pan was determined as $C_{22}H_{20}O_8$ by using some spectroscopic technique such as FT-IR, ^{1}H NMR (500 MH_Z), ^{13}C NMR (125 MH_Z), DEPT, HSQC, and EI Mass spectral data respectively. The structure of the isolated compound was elucidated by DQF-COSY, ^{1}H NMR splitting patterns, coupling constant (J values), and HMBC spectral data.

Finally, the pure pale yellow oily form compound isolated from the selected plant which is assigned as ($C_{22}H_{10}O_8$) based on the modern spectroscopic data calculating (13) HDI was elucidated as phenyl propanoid derivative compound as follows. Phenyl propanoid compound may be useful in the prevention and treatment of many health conditions. The root of Kyauk pan possess many medicinal properties such as antibactial, antifungal, antioxidant, anti-inflammatory, antineoplastic, insecticidal, analgesic, aphrodisiac, diuretic, neuralgia and antirheumatic remedia. In fact, many of the medicinal effects of the root of Kyauk pan has been directly related to their phenyl propanoid derivative compound content.

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