IDENTIFICATION OF SOME BIOACTIVE ORGANIC CONSTITUENTS FROM THE AERIAL PARTS OF *Clerodendrum indicum* (L.)KUNTZE(NGA-YANT-PADU) USED IN THE TREATMENT OF ASTHMA

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Abstract

The purpose of this research work was to study bioactive phytochemical constituents from aerial parts of the selected medicinal plant: *C. indicum* (L.) Kuntze (Nga-yant-padu) which are widely used in Myanmar for the treatment of diseases such as asthma, cough, typhoid, vermifuge, diarrhea and urinary complaint. By silica gel column chromatographic separation method, 0.002% of palmitic acid (A) (m.pt 63-64°C), 0.24% of stigmasta-5, 22, 25-triene-3ol (B) (m.pt 151-152°C) was isolated from PE extract of *C. indicum* whereas separation of defatted EtOAc extract of this plant provided hispidulin (C) (0.36%, m.pt 287-288°C), pectolinarigenin (D) (0.064%, m.pt 208-210°C) and stigmasterolglucoside (E) (0.05%, m.pt 283-284°C). The isolated compounds were identified by determination of melting point, some color tests and modern spectroscopic methods. As part of the work on the bioactivity investigation of selected plant, the antiasthmatic action was studied on histamine induced guinea pig trachea *in vitro*. All selected compounds (except palmitic acid) such as stigmasta-5, 22, 25-triene-3ol, hispidulin, pectolinarigenin and stigmasterolglucoside were observed to possess 80.2% ($P<0.01$), 73.0% ($P<0.01$), 70.2% ($P<0.01$) and 63.0% ($P<0.01$) of relaxation response on histamine induced guinea pig trachea. Therefore, it can be inferred that selected plant may be useful as a remedy for the treatment of bronchial asthma.

Keywords : *Clerodendrum indicum* (L.) Kuntze, stigmasta-5,22,25-triene-3ol, hispidulin, pectolinarigenin, stigmasterolglucoside, antiasthmatic activity

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Introduction

Clerodendrum indicum (L.)Kuntze (Nga-yant-padu) belongs to the family *Verbenaceae*, which is distributed in tropical and sub-tropical zones including Hawaii, India, Java, Indo-China and Myanmar. Most *C. indicum* are found in Southern and Eastern India and cultivated for medicinal uses as well as for ornamental purposes. In Myanmar, it is widely cultivated throughout the country and readily available (Hundley and Chit KoKo, 1987).

The roots and leaves of *C. indicum* have been used to treat asthma in Myanmar and Java. In Myanmar, the leaves are also used as a remedy for the treatment of cough, scrofulous affections, vermifuge and bitter tonic. The juice of the leaves and tender branches are employed syphilitic rheumatism, herpetic eruptions and diarrhea. The extract of the whole plant is also used as an anthelmintic, pemphigus and for certain types of mental disorders. Assays realized in India, with alcoholic extract of the whole plant, displayed muscle relaxing effect, anti-inflammatory, confirming its popular use as an antispasmotic. In addition, significant anti-inflammatory activity has been reported to show in the total benzene extract of *C. indicum* against acute, sub-acute and chronic models of inflammation (Aye Than *et al*., 1995).

A hydroquinone diterpenoid – uncinatone, six cleroindicins A – F, stigmasta-5, 22, 25-triene-3ol, β-sitosterol, scutellarein, hispidulin, pectolinarigenin, iridoid glycoside are the chemical constituents of the aerial parts of *C. indicum* (Tian and Zhang, 1997). The aerial parts of that also contain numerous flavonoids and steroidal compounds, possess controllers or anti-inflammatory effects for asthmatic patients (Rang and Moore, 2003).

A diterpene hydroquinone – uncinatone has antitubercular and HIV inhibitory activity. In Dai nationality, there was a report that clerosterolsie., cleroindicins have antimalarial and antirheumatic effects (Tian and Zhang, 1997). One of the major sterol derivative, stigmasta-5,22,25-triene-3ol showed anti-inflammatory and antispasmotic activity (Patrick, 1995). Scutellarein, hispidulin and pectolinarigenin are a class of flavonoids that showed biological activities including antimutagen, antiviral, antioxidant, anti-inflammatory, antihistaminic, cytostatic and antibacterial activity. Airidoid glycoside was reported to exhibit the antimicrobial activity of Streptomyces species (Tian and Zhang , 1997). In the present work, five organic compounds: palmitic acid,

stigmasta-5, 22, 25 triene-3ol, hispidulin, pectolinarigenin and stigmasterolglucoside were isolated from the aerial parts of *C.indicum*. And the anti-asthmatic property of the four isolated compounds (except palmitic acid) were investigated.

Bronchial asthma is a disease of the lungs in which an obstructive ventilation disturbance of the respiratory passages evokes a feeling of shortness of breath. It has also become a major health problem especially in industrialized countries (Aung Htay Oo, 1996). As the number of asthmatics has increased during the last decades, mainly due to air pollution and improper ventilation. Non-allergic bronchial asthma is a genetic predisposition. Nonspecific stimuli such as cigarette smoke, air pollution, medications, emotional factors such as shock, career or family problems, disturbed parent-child relationships and also viral, bacterial or fungal infections can trigger asthma attacks. The pathogenesis of non-allergic asthma causes the release histamine from the mast cell of the bronchial wall. Asthma cannot be cured, but it can be controlled with proper asthma management (Barnes *et al.,*1998).

Materials and Methods

The experimental works were conducted at the Department of Chemistry, University of Yangon (UY). Aerial parts of Nga-yant-padu (*C. indicum*, family *Verbenaceae*) were collected from Hlaing Township, Yangon Region during November to December in 2003. The selected plant was identified by authorized botanist at Botany Department, Yangon University. After cleaning the sample, they were air-dried at room temperature. Then, they were ground into powder by a motar and pestle and stored in air-tight container to prevent contamination and kept for isolation of organic compounds. The following instruments were used for structure elucidation of isolated compounds: Gallenkamp melting point apparatus (Organic Lab, UY), UV spectrophotometer (URC, Lower Myanmar), FTIR spectrophotometer (URC, Lower Myanmar), ^{1}HNMR ($CDCl_3$, 400MHz, BrukerAvance 400 Spectrometer) (University of Gottingen, Germany), ^{13}C NMR (DMSO, 75MHz, BrukerAvance 400 Spectrometer) (University of Gottingen, Germany), ESI-MS (JEOL JMS-DX 300 Mass Spectrometer) (University of Gottingen, Germany) and EI-MS (URC, Lower Myanmar). Column

chromatography was performed using Silica gel (40-60 μm, Wakogel) and precoated TLC plates (GF_{254}Aluminium plates, Merck) were applied for thin layer chromatographic separation. All of the solvents used were purified by distillation at their boiling point ranges.

Preparation of Crude Extracts

The dried powdered sample (ca. 300 g) were percolated in 95% ethanol (1L) for one week and filtered. This procedure was repeated three times. The combined filtrate was concentrated under vacuum rotatory evaporator to obtain ethanol crude extract. The ethanol crude extract was then successively extracted by partition with pet-ether (60-80°C) and ethyl acetate. The condensed pet-ether and ethyl acetate extracts were kept for isolation of organic constituents.

Isolation of Compounds from Pet-ether Extract

Pet-ether crude extract of *C. indicum* (5 g) was mixed with a little amount of silica gel in a motar. This mixture was separated by silica gel column chromatographic method using solvent system of PE and EtOAc (19:1 to 9:1 v/v). The eluent was collected with 3mL/fraction to provide 25 fractions (f_1- f_{25}) that were monitored by TLC to obtain two main fractions: F – I (f_1 – f_9) and F – II (f_{10} – f_{25}) resulting compound **A** (0.18 mg, 0.002%) and compound **B** (12 mg, 0.24%), respectively. The compound A was purified by recrystallization using PE and EtOAc and compound B was recrystallized from PE and EtOAc giving colourless needle shaped crystal.

Isolation of Compounds from Ethyl Acetate Extract

The ethyl acetate crude extract of *C. indicum*(5 g) was fractionated by using silica gel column chromatography, successively eluting with toluene : ethyl acetate (4 : 1, 1 : 1 v/v) and ethyl acetate and methanol (98 : 2, 95 : 5 v/v) to give 87 fractions (3mL/fraction). After combining the fractions giving the similar appearance on TLC chromatograms, three main fractions were finally collected. From these three main fractions, three compounds (**C**, **D** and **E**) were obtained in the yield of 0.36%, 0.064% and 0.05%, respectively.

Structural Elucidation of Isolated Compounds

The structures of isolated compounds A, B, C, D and E were elucidated by using modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, ESI-MS and EI-MS spectroscopies.

Determination of Antiasthmatic Activity of Isolated Compounds

The antiasthmatic activity of four isolated compounds (B, C, D and E) was studied on normal and histamine induced contraction on isolated guinea pig trachea in *in vitro*. The relaxation response (%) of the four isolated compounds from Nga-yant-padu on histamine induced tracheal chain was determined at different doses : 0.1 mg, 0.2 mg, 0.3 mg, 0.4 mg and 0.5 mg/mL bath concentration.

Results and Discussion

Isolated Compounds from Nga-yant-padu

Column chromatographic separation of pet-ether extract provided two compounds: compound A (Palmitic acid, 0.002% yield) and compound B (Stigmasta- 5, 22, 25 triene – 3ol, 0.24% yield). From active ethyl acetate extract, two flavonoids (C, Hispidulin, 0.36% yield), (D, Pectolinarigenin, 0.064% yield) and one steroidal glycoside (E, stigmasterolglucoside, 0.05% yield) were obtained.

Structural Identification of Isolated compounds

Stigmasta-5,22,25-triene-3ol (B) : $C_{29}H_{46}O$, colourless needle shaped, R_f 0.40, PE:EtOAc 5:1; violet colour by heating with 5% H_2SO_4, m.pt151-152°C ; UV λ_{max} (MeOH) (Figure 1) : 225 nm and 270 nm ; FT IR νcm^{-1}(KBr) (Figure 2): 3451 (ν_{O-H}), 2937, 2891, 2862 (asym&symν_{C-H}), 1641($\nu_{C=C}$),1450(δ_{CH} of CH_3& CH_2), 1380(δ_{CH} of dimethyl gp),1060(asymν_{C-OH}); ^{1}H NMR δ ppm($CDCl_3$, 300 MHz) (Figures 3 and 4): 0.7(s, 3H, H-18), 0.8(t, J=7.5 Hz, 3H, H-29), 1.0(d, J=7.5 Hz, 3H, H-21), 1.02(s, 3H, H-19), 1.2-1.5(m, 15H), 1.64 (s, 3H, H-27), 1.8-2.5(m, 10H), 3.5(m, 1H, H-3), 4.7(m, 2H, H-26), 5.2(m, 2H, H-22,23), 5.35(m, 1H, H-6) (Silverstein and Webster, 1998); EI-MS(m/z): 410$[M]^+$ (Figure 5).

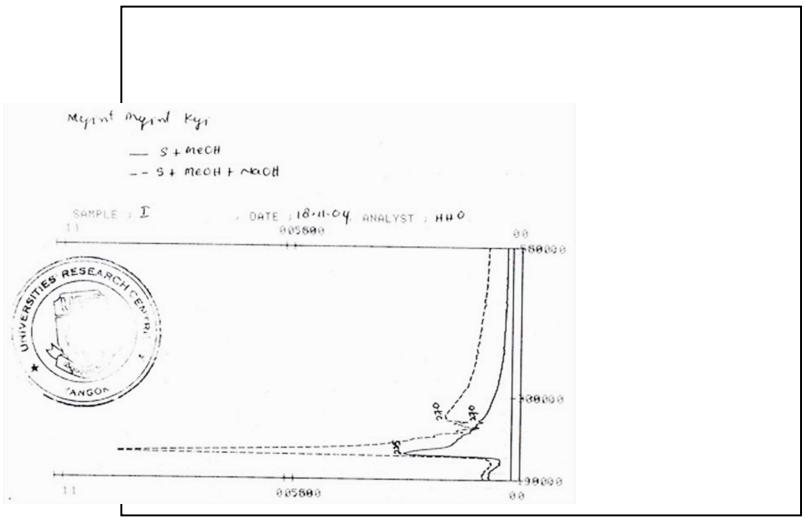


Figure 1 UV spectrum of compound B

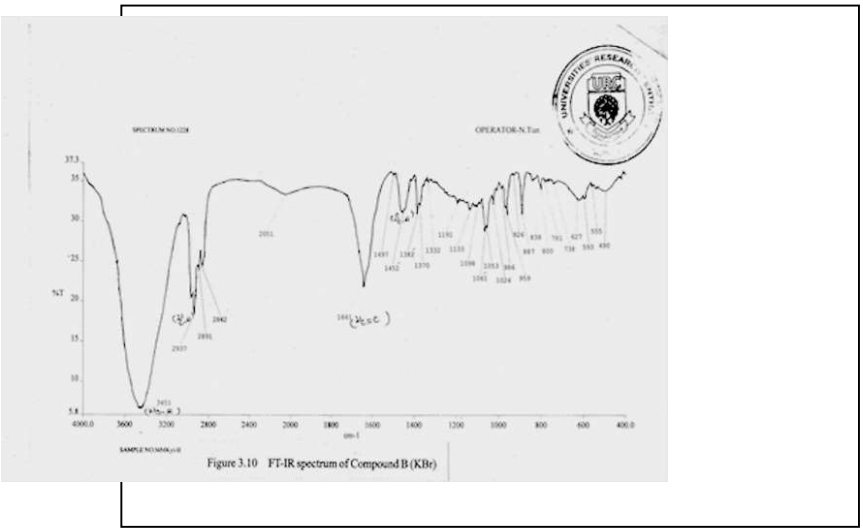


Figure 2 FT IR spectrum of compound B

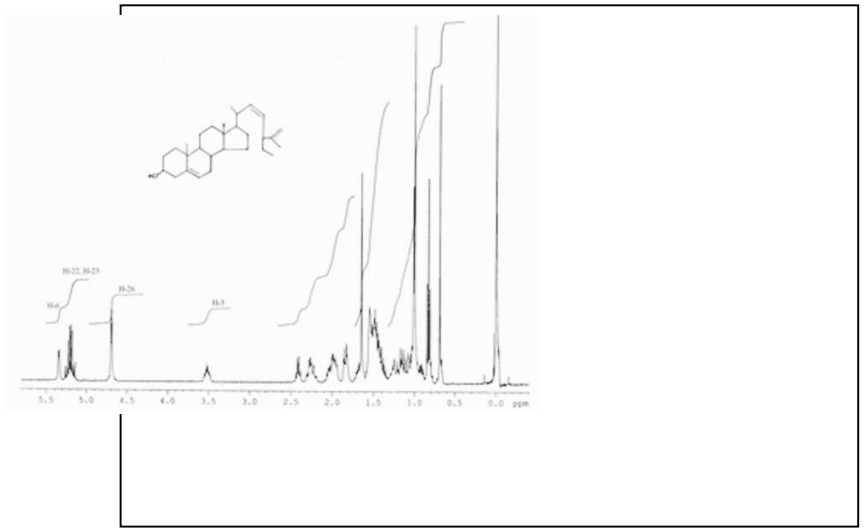


Figure 3 ^{1}HNMR ($CDCl_3$, 300 MHz) spectrum of compound B

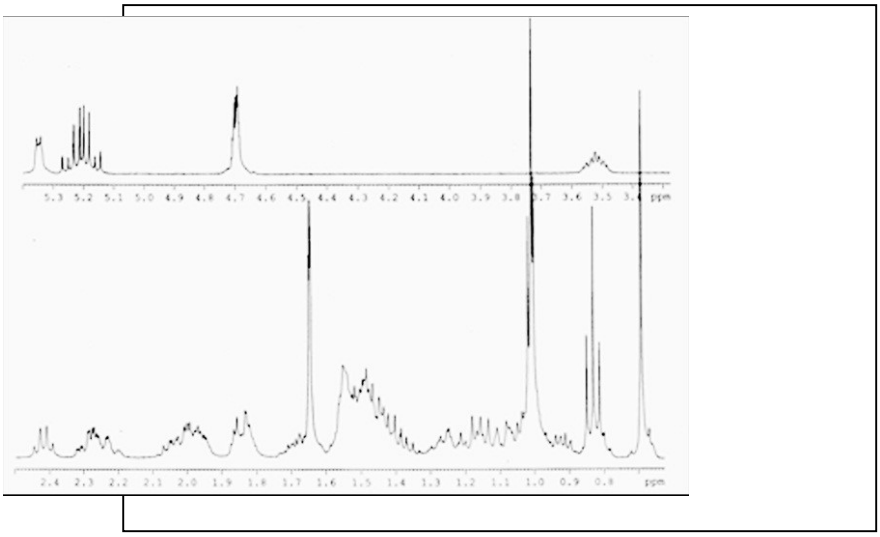


Figure 4 ^{1}HNMR spectra expended of compound B

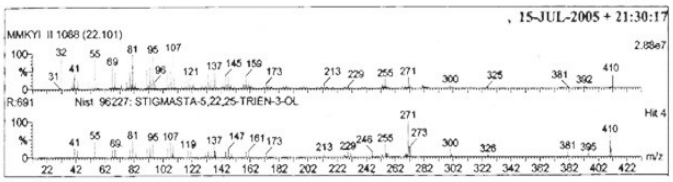
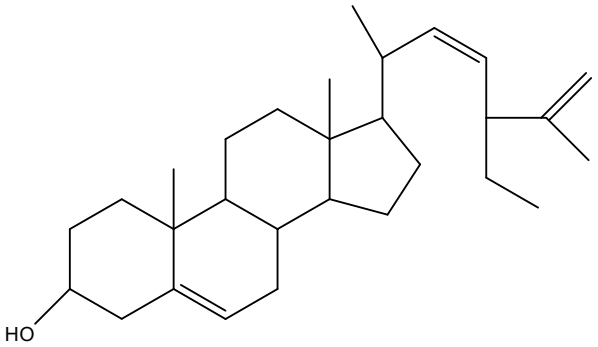


Figure 5 EI-MS spectrum of compound B



Stigmasta-5,22,25-triene-3ol($C_{29}H_{46}O$)

Hispidulin(C): $C_{16}H_{12}O_6$, pale yellow plate, R_f 0.31, Tol : EtOAc 2:1 ; dark yellow colour by heating with 5% H_2SO_4, m.pt 287-288°C ; UV λ_{max}(MeOH and MeOH/NaOH) (Figure 6) : 270, 340 nm and 270(sh), 325, 396 nm ; FT IR νcm^{-1} (KBr) (Figure 7): 3439(ν_{O-H}), 2943(ν_{C-H} of CH_3gp), 1651($\nu_{C=O}$ of α, β unsat'd carbonyl), 1615, 1583, 1492($\nu_{C=C}$ of aromatic),1439(δ_{CH} of CH_3gp), 1372(δ_{O-H}), 1252, 1178(ν_{C-O-C}), 1097(ν_{C-OH}), 827(δ_{oopC-H} of aromatic); ^{1}H NMR δ ppm(DMSO, 300 MHz) (Figure 8): 2.5(s, 1H, 4′-OH), 3.67(s, 3H, O-CH_3), 6.6(s, 1H, H-8), 6.75(s, 1H, H-3), 6.95(d, J=9.52 Hz, 2H, H-3′,5′), 7.9(d, J=9.52 Hz, 2H, H-2′,6′), 10.4(s, 1H, 7-OH), 13.05(s, 1H, 5-OH); ^{13}C NMR δ

ppm (DMSO, 75 MHz) (Figure 9): 163.72, 102.32, 182.01, 152.69, 131.28, 157.13, 94.14, 152.31, 104.00(C-2 to C-10), 121.19, 128.34, 115.85, 161.02(C-1′to C-6′), 59.9(6-OMe) (Takeshi *et al*., 1995) ; ESI-MS(m/z) : 300$[M]^+$ (Figure 10).

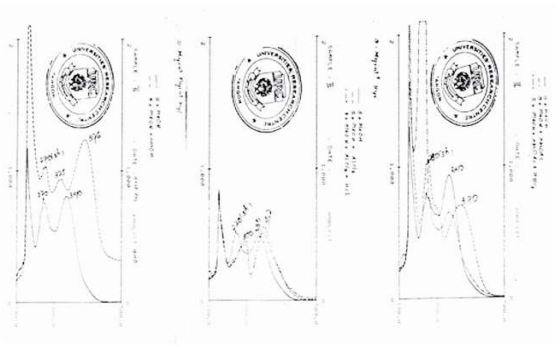


Figure 6 UV-Visible spectra of compound C

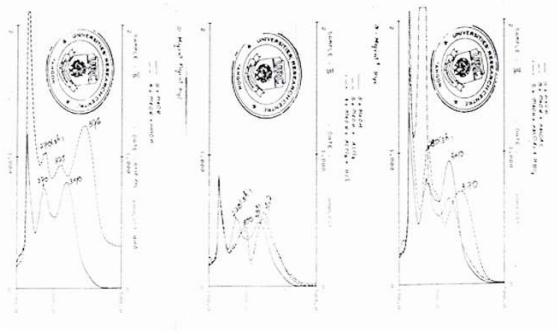


Figure 7 FT IR spectrum of compound C (KBr)

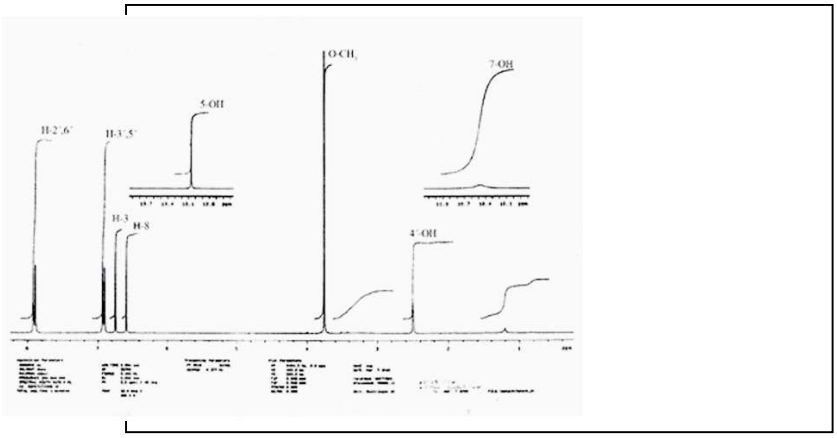


Figure 8 ^{1}HNMR (DMSO, 300 MHz) spectrum of compound C

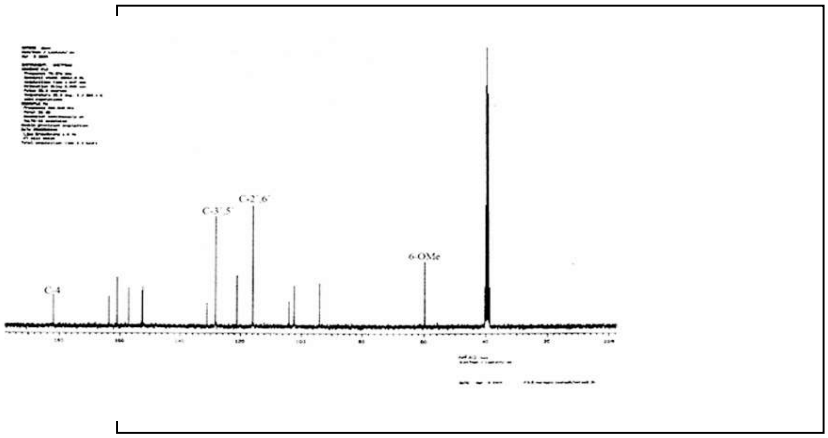


Figure 9 ^{13}CNMR (DMSO, 75 MHz) spectrum of compound C

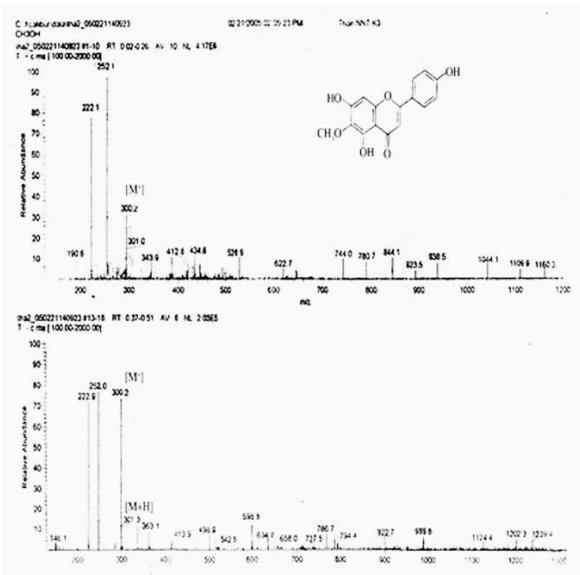
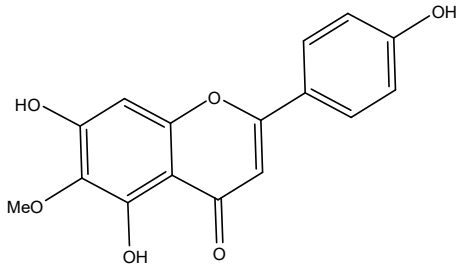


Figure 10 ESI-MS spectrum of compound C



Hispidulin ($C_{16}H_{12}O_6$)

(6-methoxy-4′,5,7-trihydroxy flavones

Pectolinarigenin (D):$C_{17}H_{14}O_6$, pale yellow needles, R_f 0.45, EtOAc : MeOH 98:2 ; yellow colour by heating with 5% H_2SO_4, m.pt 208-210°C ; VU λ_{max}(MeOH and MeOH/NaOH) (Figure 11): 270, 330 nm and 270, 310(sh),

360, 380 nm ; FT IR νcm^{-1} (KBr) (Figure 12): 3420(ν_{O-H}), 1728($\nu_{C=O}$ of carbonyl gp), 1609($\nu_{C=C}$), 1488, 1465(δ_{CH-OH}), 1356(δ_{C-OH} in phenol), 1250, 1177(δ_{CH} of benzene), 1094, 1077(ν_{C-O-C}), 831(δ_{oopC-H} of aromatic) ; ^{1}H NMR δ ppm (Acetone+D_2O, 300 MHz) (Figure 13): 3.62(s, 3H, 4′-OMe), 3.8(s, 3H, 6-OMe), 6.6(s, 1H, H-8), 6.83(s, 1H, H-3), 6.9(d, 2H, H-3′,5′), 7.8(d, 2H, H-2′,6′); ^{13}C NMR δ ppm (Acetone-D_6/D_2O, 75 MHz) (Figure 14): 183.22(C-4), 169.56(C-2), 165.78(C-4′), 161.4s8(C-7), 156.22(C-5), 153.07(C-9), 132.91(C-6), 128.99(C-2′,6′), 121.87(C-1′), 116.48(C-3′,5′), 103.00(C-10), 100.34(C-3), 94.79(C-8), 62.47(6-OMe), 61.23(4′-OMe) (Takeshi *et al*., 1995) ; ESI-MS(m/z)(Figure 15): 314$[M]^+$.

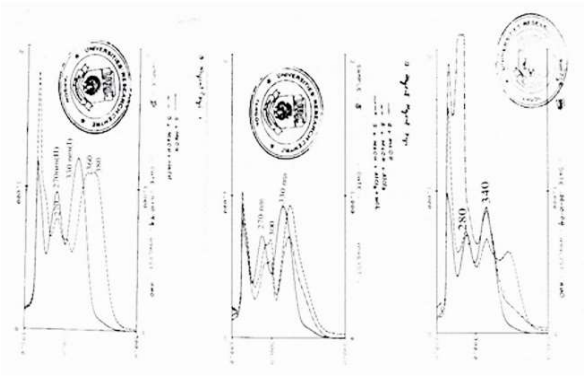


Figure 11 UV-Visible spectra of compound D

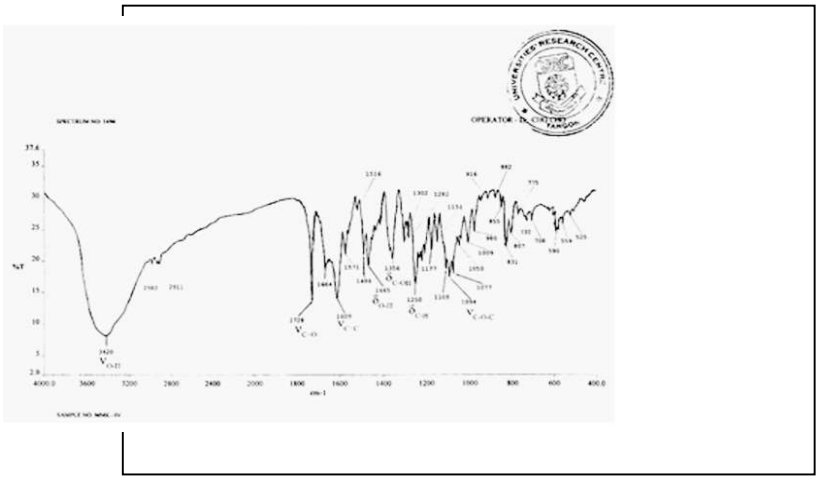


Figure 12 FT IR spectrum of compound D (KBr)

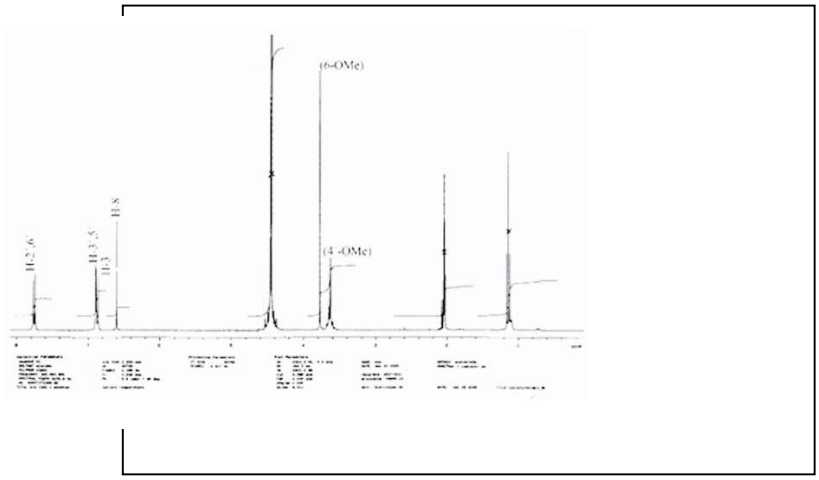


Figure 13 ^{1}HNMR (Acetone+D_2O, 300 MHz)

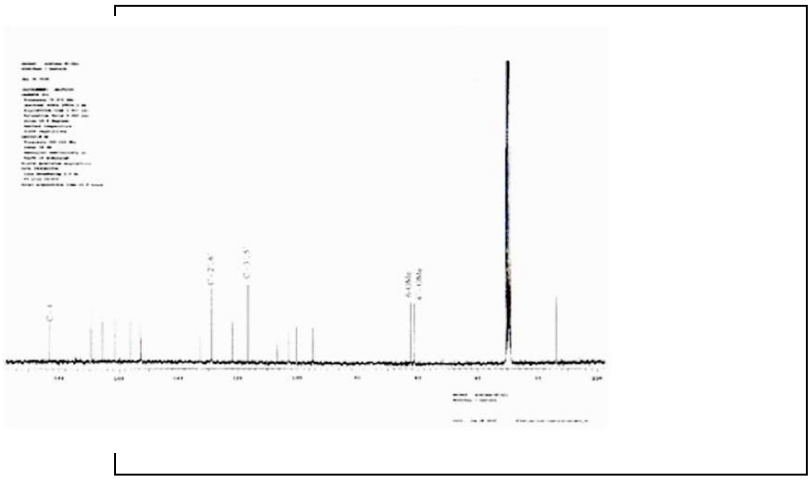


Figure 14 ^{13}CNMR(Acetone-d_6/d_2O, 75 MHz) spectrum of compound D

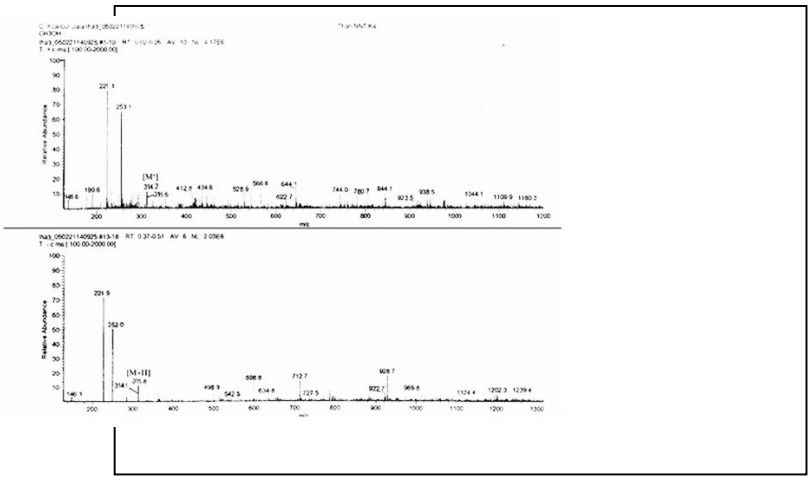
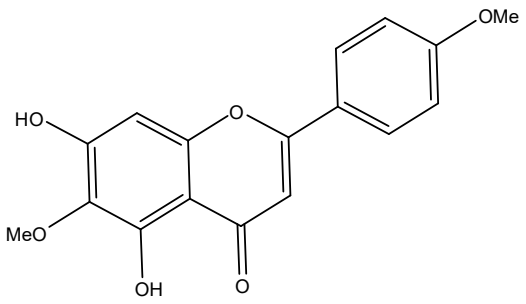


Figure 15 ESI-MS spectrum of compound D



Pectolinarigenin ($C_{17}H_{14}O_6$)

(5,7-dihydroxy-6,4′-dimethoxy flavone

Stigmasterolglucoside(E) :$C_{35}H_{58}O_6$, fine colourless crystals, R_f 0.36, EtOAc : MeOH 95:5 ; purple colour by heating with 5% H_2SO_4, m.pt 283-284°C ; FT IR νcm^{-1}(KBr) is shown in (Figure 16): 3450(ν_{O-H}), 2961, 2933(asymν_{C-H}), 2869(symν_{C-H}), 1638($\nu_{C=C}$), 1463(δ_{CH}), 1383, 1367(δ_{OH}), 1165(ν_{C-O-C}), 1073-1024(asymν_{C-OH} in sugar), 891(δ_{CH} of sugar), 621, 600(δ_{oopOH}) ; ^{1}H NMR δ ppm (DMSO, 300 MHz) is shown in (Figure 17) : 5.39(t, 1H, H-6), 5.20(t, 1H, H-22), 5.10(d, 1H, H-23), 4.80(d, 1H, H-1′), 4.40(t, 1H, H-6′a), 4.23(d, 1H, H-6′b), 4.12(m, 1H, H-3′), 3.85(m, 1H, H-4′), 3.70(t, 1H, H-2′), 3.4(m, 1H, H-5′), 2.5(s, 1H, H-7a), 2.0(m, 1H, H-8), 1.6(m, 1H, H-7b), 1.2(d, 3H, Me-21), 1.0(s, 3H, Me-19), 0.85(d, 3H, Me-26), 0.65(s, 3H, Me-18) ; ^{13}C NMR δ ppm (DMSO, 75 MHz) is shown in (Figure 18): 139.7(C-5), 136.9(C-22), 127.8(C-23), 121.9(C-6), 102.3(C-1′), 78.5(C-3′), 78.1(C-3), 77.9(C-5′), 74.6(C-2′), 71.0(C-4′), 62.7(C-6′), 58.1(C-14), 56.9(C-17), 51.1(C-9), 46.0(C-24), 43.2(C-13), 39.9(C-12), 38.5(C-4), 37.4(C-1), 36.9(C-10), 35.1(C-20), 32.9(C-7), 32.0(C-8), 30.1(C-28), 29.4(C-16),28.5(C-2), 26.5(C-25), 23.8(C-15), 21.2(C-11), 20.0(C-26), 19.5(C-19), 19.2(C-27), 19.0(C-21), 13.1(C-29), 11.7(C-18)(El -Askary, 2005).

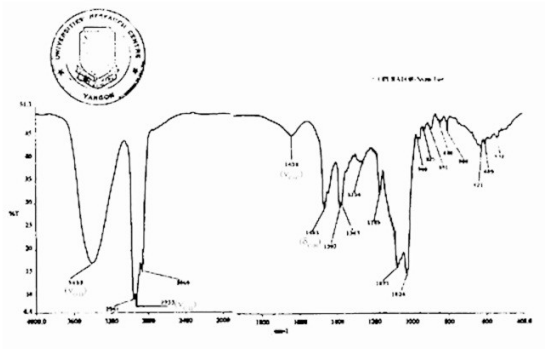


Figure 16 FT IR spectrum of compound E (KBr)

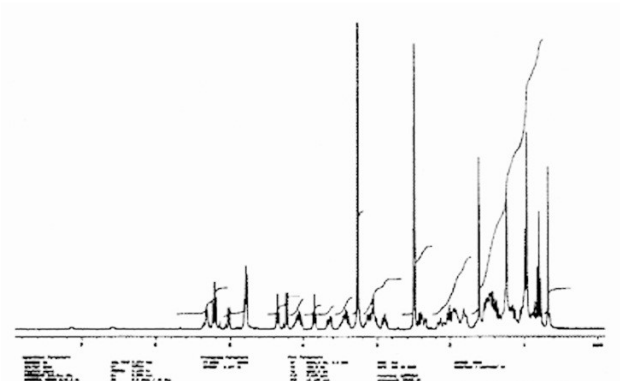


Figure 17 ^{1}HNMR (DMSO, 300 MHz) spectrum of compound E

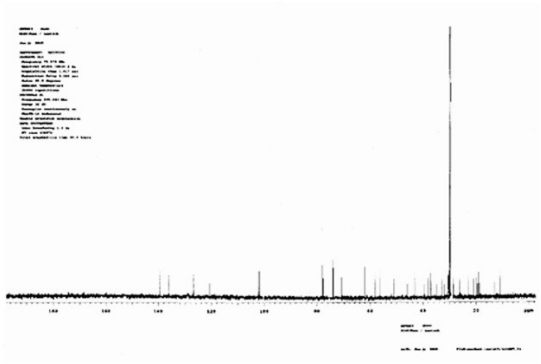
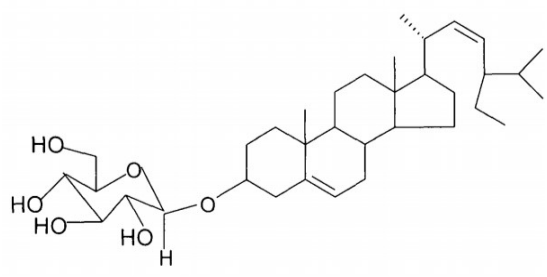


Figure 18 ^{13}CNMR (DMSO, 75MHz) spectrum of compound E



Sigmasterolglucoside ($C_{35}H_{58}O_6$)

Anti-asthmatic Activity of Isolated Compounds on Guinea pig Trachea

Among five compounds isolated from pet-ether and ethyl acetate extract of Nga-yant-padu, the anti-asthmatic activity of compounds B, C, D and E were subjected to study on histamine induced contraction in tracheal chain isolated from guinea pig. The relaxation response (%) of isolated compounds: B, C, D and E as well as 70 % EtOH and watery extracts of Nga-yant-padu on histamine induced tracheal chain are shown in Figure 19 and given in Table 1. When the isolated compounds were added to the organ bath,

the compounds (B, C, D and E) were found to exhibit direct relaxation effects of 80.2% (P<0.01), 73.0% (P<0.01), 70.2% (P<0.01) and 63.0% (P<0.01) of smooth muscle relaxation effect. Out of four isolated compounds tested, compound B is the most effective than the others. Compound B is a steroidal compound, stigmasta- 5, 22, 25 triene-3ol and compound E is also a steroidal glycoside, stigmasterolglucoside. Most steroidal compounds are used as controller or anti-inflammatory agents for asthmatic patients. Steroids are the most effective compounds of controlling inflammation in lungs. Compound C and D are flavonoids: hispidulin and pectolinarigenin. Flavonoids has been reported as H_2 – receptor blocking drug that can reduce histamine to have an antihistaminic effect. The anti-asthmatic activity of isolated compounds (at 0.1 mg/mL bath conc.) on histamine induced guinea pig trachea were found in the order of B > C > D > E.

Table 1 Relaxation Effect of Four Isolated Compounds and Different Extracts on Histamine Induced Tracheal Chain

Isolated Compounds and Extracts (n = 5)	Relaxation Response (%)	P – value
Stigmasta-5,22,25-triene-3ol (0.1mg/mL)	80.2 ± 0.84	< 0.01
Hispidulin (0.1mg/ mL)	73.0 ± 1.6	< 0.01
Pectolinarigenin (0.1mg/ mL)	70.2 ± 1.52	< 0.01
Stigmasterolglucoside (0.1mg/ mL)	63.0 ± 3.2	< 0.01
70% EtOH (NYP) (1.0mg/ mL)	77.8 ± 1.3	< 0.01
Watery (NYP) (1.0mg/ mL)	68.0 ± 1.4	< 0.01

Student's t – test NYP = Nga-yant-padu

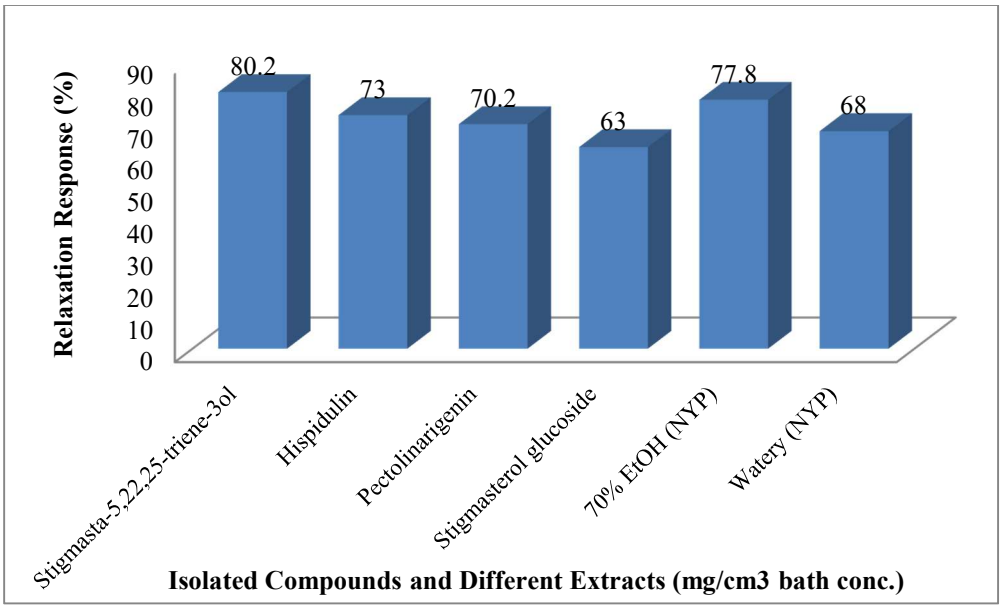


Figure 19 Relaxation effect of isolated compounds and different extracts on histamine induced tracheal chain

Conclusion

From the overall assessment of the present work, the following inferences could be deduced. By silica gel column chromatographic separation, palmitic acid (A, 0.002%), stigmasta-5, 22, 25-triene-3ol (B, 0.24%), hispidulin (C, 0.36%), pectolinarigenin (D, 0.064%) and stigmasterolglucoside (E, 0.05%) were isolated from Nga-yant-padu. *In vitro* screening of anti-asthmatic activity showed that all isolated compounds (except palmitic acid) from Nga-yant-padu possessed the anti-asthmatic property and the muscle relaxation effects of these compounds were found to be in the order of B (80.2%, $P<0.01$) > C (73.0%, $P<0.01$) > D (70.2%, $P<0.01$) > E (63.0%, $P<0.01$) on histamine induced guinea pig trachea. Therefore, it could be suggested that Nga-yant-padu might be used as a remedy for the treatment of asthma.

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