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Investigation of Phytochemical Constituents and Smooth Muscle Relaxation Activity of Various Herbal Plants in Myanmar

Phyu Phyu Myint[1*], Myint Myint Kyi[2], Saw Hla Myint[3] and Daw Hla Ngwe[3]

[1]Department of Chemistry, Sittway University, Myanmar.
[2]Department of Chemistry, Myin-Gyan Degree College, Myanmar.
[3]Department of Chemistry, University of Yangon, Myanmar.

Authors' contributions

This work was carried out in collaboration between all authors. Author PPM designed the study, wrote the protocol and wrote the first draft of the manuscript. Author SHM managed the literature searches. Author DHN managed the analyses of the study and performed the spectroscopy analysis. Author MMK collaborated the experimental process. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

The leaves from *Ageratum conyzoides* L., *Aegle marmelos* Correa., *Clerodendrum indicum* (L.) Kuntze, and *Mimosa pudica* L. plants, reputed in folk medicine in Myanmar for their value as an anti asthmatic remedy, were selected for this study. These leaves were carried out to investigate the phytoconstituents and smooth muscle relaxation activity in both normal and histamine-induced guinea-pig. The compounds namely stigmasterol, myristic acid, palmitic acid, 9-hydroxy nonan-2-one and kaempferol from *A. conyzoides*; β-sitosterol and N-2-[4-(3′-methyl butoxy) phenyl] ethyl cinnamide from *A. marmelos*; palmitic acid, stigma-5,22,25-trienen-3-ol, hispidulin, pectolinarigenin

**Corresponding author: E-mail: phyuphyumyint2007@gmail.com;*

and stigmasterol glucoside from *C. indicum* and 1-methylene-1H-indene and γ–sitosterol from *M. pudica* leaves could be isolated. *In vitro* screening on the alcoholic and aqueous extracts revealed the smooth muscle relaxation activity in the order of *C.indicum* > *A. conyzoides* > *M. pudica* > *A. marmelos* leaves. Maximum dose for all extracts except *A. marmelos* was found to be 2 mg L^{-1} bath concentration. Isolated compounds exhibited anti-asthmatic effect in the order of stigma-5, 22, 25-trienen-3-ol > hispidulin > pectolinarigenin> stigmasterol glucoside > N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide > stigmasterol> kaempferol. These results might supply the scientific evidence for smooth muscle relaxation activity of the leaves for the treatment of bronchial asthma in traditional herbal medicine.

Keywords: Bronchial asthma; smooth muscle relaxation activity; histamine- induced asthmatic guinea pig; aqueous and ethanolic extracts.

1. INTRODUCTION

The number of asthmatics has increased during the last decades, mainly due to air pollution and improper ventilation. The rates of asthma have enlarged in the developed world since 1960 whereas recent increases primarily in the developing world [1]. According to the latest WHO report on May 2014, Asthma Deaths in Myanmar reached 13,297 or 3.34% of total deaths and the age adjusted Death Rate is 34.30 per 100,000 of population ranks Myanmar in the world [2]. There has been increasing demand for the use of traditional medicine and medicinal plants for the treatment of asthma and chronic bronchitis in our country due to its long and deep based tradition and also due to the trust placed by the people in its therapeutic qualities. Moreover, development of the use of traditional and herbal drugs is also among the items in the national health policy. There are many herbal medicine and traditional medicine plant's application practices for the treatment of Asthma in our country. Commonly used Myanmar medicinal plants comprise *Adhatoda vasica* Nees, *Aegle marmelos* Correa, *Ageratum conyzoides* L., *Clerodendrum indicum* (L.) Kuntze, *Coelus aromaticus* Benth, *Ocimum sactum* L., *Mimosa pudica* L., *Piper betle* L. and *Piper longum* L. They are utilized for treating conditions such as bronchitis, cough, expectorant, tightness of chest and sore throat. With wide consumption of medicinal plants in bronchial asthma treatment, due to their minimum side effect and ecological aspects, extensive experimental and clinical studies are required to prove that some possess smooth muscle relaxation activity. In this study, the comparison between four different medicinal plants which are mostly famous in rural area and their isolated compounds having smooth muscle relaxation activity in guinea pig trachea chain were illustrated.

The wide spread *Ageratum conyzoides*, annual aromatic plant that grows in tropical area, has long been known in herbal or folk medicine as a remedy for various ailments in our country. The several reports are already found that *Ageratum conyzoides* possessed anticancer and antiradical properties [3], wound healing activity [4], and anti-inflammatory activity [5] but not for antiasthmatic action even though still used for the treatment of bronchitics in Myanmar.

Aegle marmelos (L.) Correa, a tree species belonging to the family Rutaceae and one famous herbal plant for Asthma treatment in Myanmar; has been reported to contain several pharmaceutical properties. The leaves, stem, bark and fruits of *A. marmelos* have long been used in traditional medicine for its medicinal value; antidiarrhoel activity [6], antibacterial and antifungal activity [7], anticancer [8], antipyretic activity [9], ulcer healing potential [10,11], antifertility activity [12], antigenotoxic [13], and anti inflammatory activity [14].

Clerodendrum indicum L. which belongs to the family Verbenaceae is widely available in moderate temperature zones Asian countries. Leaves and roots of this plant exert an important part of traditional medicine in many countries like China, India, Japan, Korea, Myanmar and Thailand [15]. Although it is used in the treatment of bronchitis in traditional medicine, there is no scientific research conducted on the smooth muscle relaxation potential of *C. indicum* leaf yet.

Mimosa pudica is well known for its rapid plant movement. All parts of *Mimosa pudica*, induce awareness of the researchers worldwide for its pharmacological activities such as antiulcer [16], antimalarial [17], and antioxidant [18]. Moreover, traditional practitioners in our country have declared that the leaf of *M. pudica* is very useful for the treatment of bronchitic, general weakness and impotence. In the present work, an attempt

has been made to contribute to the health program with the scientific evidence for Myanmar indigenous medicinal plants used for the treatment of bronchial asthma regarding the smooth muscle relaxation action and its responsible chemical constituents.

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

The leaves of *A. conyzoides* L., *A. marmelos* Correa., *C. indicum* (L.) Kuntze and *M. pudica* L. were collected from Yangon Region, Myanmar. These samples were identified by an authorized botanist, lecturer in Department of Botany, University of Yangon. The collected samples were clean, air-dried and pulverized into powder using a grinding mill and then stored in the screw-capped bottles.

2.2 Preparation of Different Extracts

Each dried, purified and powdered sample (50 g) was put into a 1 L conical flask and distilled water was added up to 600 mL. Extractions were done by heating the flask in the water bath. After 6 h of such extraction, it was cooled to room temperature and filtered. The filtrate was then evaporated to dryness over a water bath using evaporator porcelain basin. In this way aqueous extract of samples was obtained. The same amount of each sample was packed in the cotton bag and placed in the Soxhlet extractor, equipped with a round bottomed flask (500 mL) containing 250 mL of petroleum ether (60-80℃). The apparatus was placed in the water bath and heated for 24 hours. The solution containing fatty substances was then discarded. The defatted residue was again extracted in the same way with 250 mL of 70% ethanol for another 40 hours.

2.3 Separation of Phytochemical Constituents of the Samples

The 70% ethanolic extract was partitioned between ethyl acetate and distilled water, resulting in an ethyl acetate fraction and a residual aqueous fraction. The ethyl acetate sub-fraction was evaporated under reduced pressure using a centrifugal evaporator, yielding a dry residue, considered as ethyl acetate extract. The chromatographic column was packed with silica gel (100 g) using petroleum ether: EtOAc solvent system which was chosen depending on TLC behaviour. Five grams of silica gel are mixed thoroughly with ethyl acetate extract (5 g) in a mortar. The resulting free flowing powder was added to the column using a small long necked funnel. The column was eluted consecutively with Petroleum ether:EtOAc:MeOH gradient solvent system. The flow rate was adjusted to about 1 drop per second. Fractions were monitored by TLC. Crystallization of purest fraction was carried out by using a suitable solvent. After washing the purest fractions from the column, some compounds could be isolated.

2.3.1 Structural elucidation and identification

The structures of the isolated compounds were elucidated and identified by the modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, and GC-MS. Some physicochemical properties of these compounds such as melting point, solubility test, phytochemical test and thin layer chromatography with various visualizing reagents were also investigated.

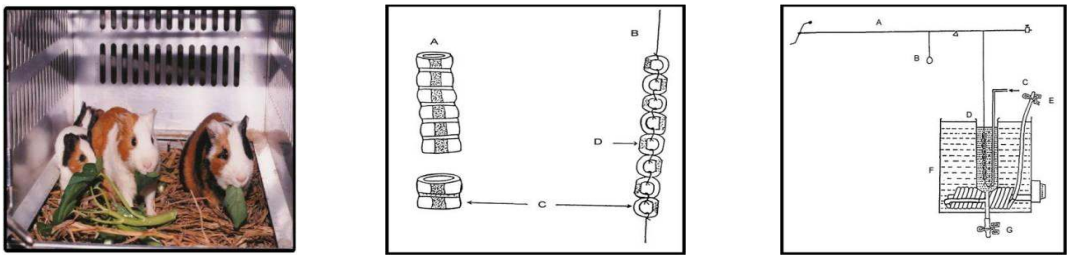
2.4 Effect of Plant Extracts on Tracheal Chain Isolated from Guinea pig

2.4.1 Preparation of isolated tracheal chain from Guinea pig

The isolated trachea chain from guinea pig was done according to the method described by Castillo and De Beer [19].

A guinea pig was killed by a blow on a head and cutting the throat. The neck and upper thorax were opened and the muscles surrounding the trachea were cleared. A length of trachea about 4-6 cm was dissected out from guinea pig and placed in an oxygenated Krebs solution in a petri-dish. By transverse cuts, each tracheal ring containing two cartilage bands were made. Tracheal rings were tied with a fine thread in a series so that the smooth muscle was in a longitudinal plane and consecutive ring shave muscle on opposite sides. About 8-10 tracheal rings were tied to obtain a chain.

The chain was placed in an organ bath, a thread from one end of the chain was fixed to the tissue holder and a thread from the other end connected to a simple lever which was attached to a writing point. The organ bath was filled with Krebs solution and aerated with oxygen and temperature of the bath kept at 37℃. The magnification of the lever for recording was 10-12 folds and a constant tension on tissue was kept between 0.4-0.6 g. An appropriate passive tension was applied and the strip was allowed to equilibrate for half an hour.



(a) (b) (c)

Fig. 1. Pictures of (a) guinea pig (b) guinea pig trachea and tracheal chain (c) set up of apparatus for isolated guinea pig tracheal chain preparation

2.4.2 Action of extracts on tracheal chain isolated from Guinea pig

Following the equilibrating period, the effect of extracts on the isolated tracheal chain was studied at different doses i.e., 0.5, 1, 2, 3, 4 and 5 mg per mL bath. Each dose of extracts was left in contact with tissue for exactly 5 min and the effect was recorded. Then the organ bath was washed 3 times and allowed to rest for 15 min or more until the tissue recovered to the normal baseline.

The same procedure was carried out on the strip which was applied with different concentration of different extracts. Each concentration of different extracts was tested for 3 times.

2.4.3 Action of extracts and isolated compounds on histamine induced tracheal chain

Following the equilibrating period, the strip was allowed to contract by adding histamine (1 µg/mL bath conc.) to the organ bath, left for 5 min, washed 3 times and allowed to rest for 15 min or more until the tissue recovered to the normal baseline. After that histamine (1 µg/mL bath conc.) was added to the organ bath, left for 3 min and followed by an extract (1 mg/mL bath conc.) without washing the bath and left for another 2 min before washing and the effect was recorded. Then the organ bath was washed 3 times and allowed to rest for 15 min or more until the tissue recovered to the normal baseline. The same procedure for each extract and compounds was carried out for 3 times.

3. RESULTS AND DISCUSSION

3.1 Some Chemical Constituents of the Leaf Samples

Stigmasterol, myristic acid, palmitic acid, 9-hydroxy nonan-2-one and Kaempferol were obtained from EtOAc extract of defatted leaves of *A. conyzoides*. In addition, β-sitosterol and N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide were isolated from *A. marmelos* Correa leaves, palmitic acid, stigma-5,22,25-trienen-3-ol, hispidulin, pectolinarigenin and stigmasterol glucoside from *C. indicum* and 1-methylene-1H-indene and γ–sitosterol from *M. pudica* by using column chromatography separation on silica gel, respectively. These compounds were structurally identified by the modern spectroscopic methods such as UV-visible, FT IR, ^{1}HNMR, GC-MS spectroscopy and by joint application of their physicochemical properties, and also by comparing with the reported data.

3.1.1 Stigmasterol

R_f 0.55, Petroleum ether: EtOAc (8:2); m.p. 168-171℃, v cm^{-1} (KBr): 3428 (v_{O-H}), 2960 (asym v_{C-H} for -CH_3), 2936 (asym v_{C-H} for –CH_2-), 1658 ($v_{C=C}$), 1382(sym δ_{-CH_3}), 1061(v_{C-O-H}), 1023 (v_{C-O}), 970(δ_{OOP} C-H trans), 832(δ_{OOP} C-H of trisubstituted d/b); ^{1}H NMR δ_{ppm} (400 MHz, $CDCl_3$), 0.66 (s,3H), 0.82~0.84(m, 10H), 0.91~0.96 (m, 2H), 1.01~1.04 (m, 8H), 1.16~1.22 (m, 4H), 1.24~1.27 (m, 5H), 1.49~1.66 (m, 5H), 1.84~1.86 (m, 1H), 1.07~2.04 (m, 3H), 2.23~2.30 (m, 2H), 3.43~3.53 (m, 1H), 4.09~4.14 (m, 1H, 4.98~5.04 (dd, 1H), 5.12~5.18 (dd, 1H), 5.34~5.35 (m, 1H); ^{13}C NMR δ ppm (75 mHz, $CDCl_3$) : 37.31(C-1), 31.72 (C2/C8), 71.84 (C-3), 42.36 (C-4), 140.8 (C-5), 121.72 (C-6), 31.95 (C7/C25), 50.22 (C-9), 36.56 (C-10), 21.11(C11/C21) , 39.72 (C-12), 42.26 (C-13), 56.9 (C-14), 24.39 (C-15), 28.9 (C-16), 56.02 (C-17), 12.07 (C-18), 19.4 (C-19), 40.47 (C-20), 138.33 (C-22), 129.3 (C-23), 51.27 (C-24), 21.23 (C-26), 19.0 (C-27), 25.41 (C-28), 12.24 (C-29), EI MS (m/z): 412[$M]^+$

3.1.2 Myristic acid

R_f 0.68, Petroleum ether: EtOAc:MeOH (180:20:1); m.p. 56-58℃, v cm^{-1} (KBr): 3500-

3300 (v_{O-H} in acid) , 2909 (asym v_{C-H} for -CH_3), 2847 (asym v_{C-H} for –CH_2-), 1710 ($v_{C=O}$), 1451(δ_{C-O-H}), 1378 (δ_{C-H}), 1051 (δ_{C-OH}), 719 (γ_{C-H}); GC-MS (m/z): 227$[M-H]^+$

3.1.3 Palmitic acid

R_f 0.6, Petroleum ether: EtOAc: MeOH (180:20:1); m.p. 28-30℃, v cm^{-1} (Neat): 3450 (v_{O-H} for alcohol) , 2940 and 2840 (asym- & sym- v_{C-H} for -CH_3 and -CH_2-), 1717 ($v_{C=O}$), 1460 (δ_{C-H} for –CH_2-), 1383 (δ_{C-H} for –CH_3), 1051 (v_{C-OH}), 965 (γ_{O-H}); GC- MS (m/z): 158$[M]^+$

3.1.4 9-hydroxy nonan-2-one

R_f 0.52, Petroleum ether: EtOAc: MeOH (180:20:1); m.p. 62-64℃, v cm^{-1} (KBr): 3500-2661 (v_{O-H} in acid) , 2921 and 2850 (asym- & sym- v_{C-H} for -CH_3 and –CH_2, 1710 ($v_{C=O}$), 1496 (δ_{C-O-H}), 1295 (v_{C-H}); GC- MS (m/z): 265$[M]^+$

3.1.5 Kaempferol

R_f 0.53, Petroleum ether: EtOAc (4:1); m.p. 275-278℃,UV λ_{max} (MeOH): 265 nm and 370 nm; FT IR v cm^{-1} (KBr): 3450 (v_{O-H}) , 1656 ($v_{C=O}$), 1615 ($v_{C=C}$), 1458 (δ_{O-H} for =CH-OH), 1384 (δ_{O-H} for C–OH in phenol), 1176 (v_{C-O} for C-OH in phenol), 847 (δ_{OOP} C-H); ^{1}H NMR δ_{ppm} (400 MHz, DMSO + RD_3OD): 6.17 (d,1H, H-6), 6.4 (d,1H, H-8), 6.9 (d,2H, J=8.5 Hz, H-3, H-4), 8.1 (d,2H, J=8.5 Hz, H-2, H-6), ESI MS (m/z): 286$[M]^+$

3.1.6 N-2-[4-(3'-methylbutoxy) phenyl ethyl cinnamide

R_f 0.7, Pether ether: EtOAc: MeOH (20:20:1); m.p. 108-110℃,UV λ_{max} (MeOH): 270 nm and 320 nm; FT IR v cm^{-1} (KBr): 3418 (v_{N-H}) , 3056 and 3000 (asym- & sym- v_{C-H} for =CH), 1699 ($v_{C=O}$), 1619 ($v_{C=C}$), 1561 (δ_{N-H}), 1452 (δ_{C-H}), 1397 (δ_{C-CH_3}), 1277 (v_{C-N}), 1154 (asym v_{C-O} for C-O-C), 1107 (δ_{C-H}); ESI MS (m/z): 337$[M]^+$

3.1.7 β-sitosterol

R_f 0.5, Petroleum ether: EtOAc (5:1); red with 5% H_2SO_4; m.p. 138-141℃, (same R_f, colour and m.p with authentic β–sitosterol

3.1.8 Stigmasta-5,22,25-triene-3-ol

R_f 0.54, Petroleum ether: EtOAc (5:1); m.p. 151-153℃, UV λ_{max} (MeOH): 225 nm and 270 nm; v cm^{-1} (KBr): 3451 (v_{O-H}), 2937 (asym v_{C-H} for -CH_3), 2891 (asym v_{C-H} for –CH_2-), 1641 ($v_{C=C}$), 1450(sym δ_{C-H}), 1380 (δ_{C-H} for –CH_3), 1060(v_{C-O-H}); ^{1}H NMR δ_{ppm} (400 MHz, $CDCl_3$), 0.80 (t,3H, J=7.5 Hz), 1.00 (d,3H, J=7.5 Hz), 1.02(s, 3H), 1.64 (s, 3H), 3.50 (m, 1H), 4.70 (m, 2H), 5.20~5.35 (m, 3H); GC MS (m/z): 410$[M]^+$

3.1.9 Hispidulin

R_f 0.45, EtOAc: MeOH (98:2); m.p. 208-210℃,UV λ_{max} (MeOH): 270 nm and 340 nm; FT IR v cm^{-1} (KBr): 3439 ($v_{O-H}$for phenolic) , 1651 ($v_{C=O}$), 1615, 1583 ($v_{C=C}$), 1458 (δ_{O-H} for =CH-OH), 1356 (δ_{O-H} for C–OH in phenol), 1077 (v_{C-O} for aliphatic and ring C-O-C), 831 (δ_{OOP} C-H); ^{1}H NMR δ_{ppm} (300 MHz, Acetone-D_6/D_2O), 3.62 (s,3H), 3.80 (s,3H), 6.60 (s, 1H), 6.83 (s, 1H), 6.90 (d,2H), 7.80 (d,2H) ; ^{13}C NMR δ ppm (75 mHz, Acetone-D_6/D_2O) : 183.2 (C-4), 169.6 (C-2), 165.8 (C-4'), 161.5 (C-7), 156.2 (C-5), 153.1 (C-9), 132.9 (C-6), 128.9 (C-2′/6′), 121.9 (C-1′), 116.5 (C-3′/5′), 103.0 (C-10), 100.3 (C-3), 94.8 (C-8), 62.5 (C-6), 61.2 (C-4′)

3.1.10 Pectolinarigenin

R_f 0.45, EtOAc: MeOH (98:2); m.p. 208-210℃,UV λ_{max} (MeOH): 270 nm and 330 nm; FT IR v cm^{-1} (KBr): 3420 ($v_{O-H}$for phenolic) , 1664 ($v_{C=O}$), 1609 ($v_{C=C}$), 1458 (δ_{O-H} for =CH-OH), 1356 (δ_{O-H} for C–OH in phenol), 1077 (v_{C-O} for aliphatic and ring C-O-C), 831 (δ_{OOP} C-H); ^{1}H NMR δ_{ppm} (300 MHz, Acetone-D_6/D_2O), 3.62 (s,3H), 3.80 (s,3H), 6.60 (s, 1H), 6.83 (s, 1H), 6.90 (d,2H), 7.80 (d,2H) ; ^{13}C NMR δ ppm (75 mHz, Acetone-D_6/D_2O) : 183.2 (C-4), 169.6 (C-2), 165.8 (C-4′), 161.5 (C-7), 156.2 (C-5), 153.1 (C-9), 132.9 (C-6), 128.9 (C-2′/6′), 121.9 (C-1′), 116.5 (C-3′/5′), 103.0 (C-10), 100.3 (C-3), 94.8 (C-8), 62.5 (C-6), 61.2 (C-4′)

3.1.11 Stigmasterol glucoside

R_f 0.36, EtOAc: MeOH (95:5); m.p. 284℃, v cm^{-1} (KBr): 3450 (v_{O-H}), 2961 and 2869 (asym- & sym- v_{C-H} for -CH_3 and –CH_2), 1638 ($v_{C=C}$), 1463 (δ_{C-H}), 1450(sym δ_{C-H}), 1383, 1367 (δ_{C-H} for -CH_3), 1165 (v_{C-O-H}), 965 (γ_{O-H}); ^{1}H NMR δ_{ppm} (300 MHz, DMSO), 0.65 (s,3H), 0.85 (d,3H), 1.00(s, 3H), 1.20 (d, 3H), 1.60 (m,1H), 2.00 (m,1H), 2.50 (s,1H), 3.40 (m,1H), 3.70 (t,1H), 3.85 (m,1H), 4.12 (m,1H), 4.23 (d,1H), 4.40 (t,1H), 4.80 (d,1H), 5.10 (d,1H),5.20 (t,1H), 5.39 (t,1H) ; ^{13}C NMR δ ppm (75 mHz, DMSO) : 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)

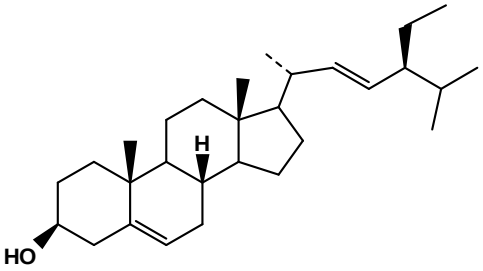
3.1.12 1-methylene-1H-indene

R_f 0.32, Petroleum ether: EtOAc (9:1); m.p. 71-74℃,UV λ_{max} (MeOH): 223 nm, 235 nm and 245 nm; FT IR v cm^{-1} (KBr): 2925 and 2850 (asym- & sym- v_{C-H} for $-CH_3$ and $-CH_2$), 1638 ($v_{C=C}$), 1384 (δ_{C-H} for $-CH_3$); GC MS (m/z): 128$[M]^+$

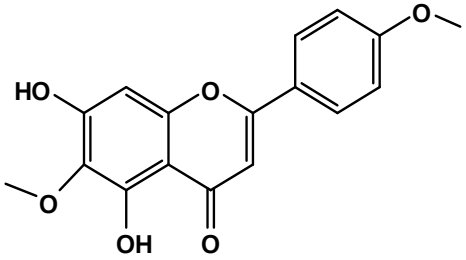
3.1.13 γ-sitosterol

R_f 0.45, Petroleum ether: EtOAc (4:1); m.p. 146-148℃ FT IR v cm^{-1} (KBr): 3430 (v_{O-H}), 2959 and 2868 (asym- & sym- v_{C-H} for $-CH_3$ and $-CH_2$), 1627 ($v_{C=C}$), 1382 and 1370 (δ_{C-H} for $-CH(CH_3)_2$; GC MS (m/z): 414$[M]^+$

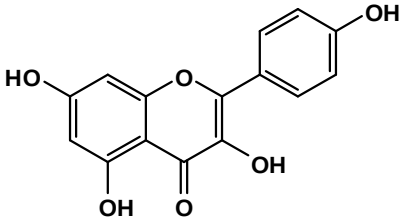
The structures of some isolated compounds are as shown below.



Stigamasterol



Pectolinarigenin



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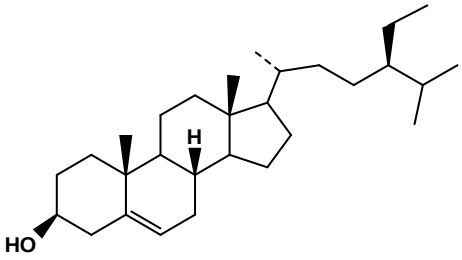
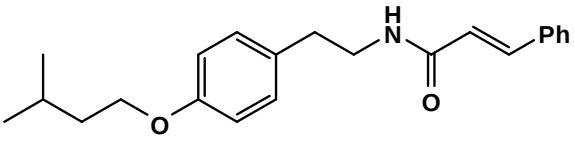
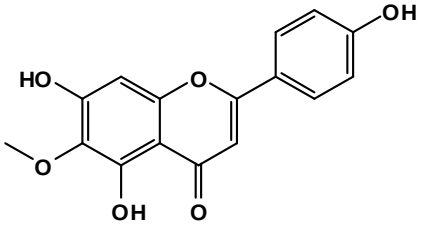


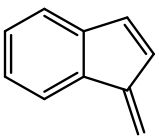
β-sitosterol



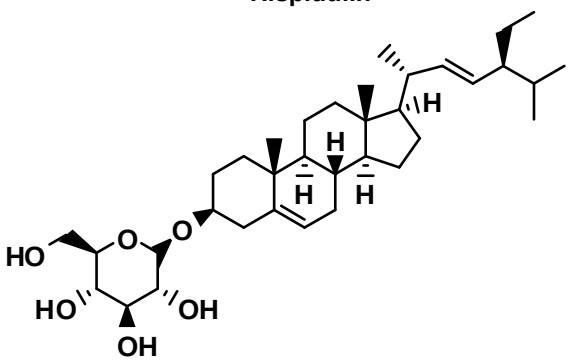
N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide



Hispidulin



1-methylene- 1-H-indene



Stigmasterol glucoside

3.2 Effect of Extracts on Normal Contraction of Isolated Tracheal Chain

Table 1 shows the relaxation effect of the plant sample extracts with different doses (0.5, 1, 2, 3, 4 and 5 mg per mL bath) on normal tracheal chain. The maximum dose for all aqueous and alcoholic extracts except *A. marmelos* was found to be 2 mg mL^{-1}. From the data of these two extracts, it was observed that the alcoholic extract of *A. conyzoides* (75.3%) was slightly more potent than the aqueous extract (72%). While that alcoholic extracts of *C. indicum* (77.8%) and *M. pudica* (39.0 %) was found to more potent than aqueous extracts of these two plants (68.0 % and 38.2 %).

The two extracts of *A. marmelos* leaves had bronchodilation activity. The tissue model treated with these two extracts showed maximum relaxation effect at 1 mg mL^{-1}. The aqueous extract of *A. marmelos* (39%) was more potent than alcoholic extract (31%).

From the result of the experiment carried out on the normal contraction of tracheal chain isolated from guinea pig, it had been observed that the extracts of *C. indicum* and *A. conyzoides* had more bronchodilating activity than that of others. All of these plants were reported to possess muscle relaxation activities and those are popularly used as an anti spasmodic [20-23]. However, from these scientific verifying, it was observed that *C.indicum* and *A. conyzoides* are more feasible and more applicable for therapeutic practices than *A. marmelos* and *M. pudica*.

3.3 Effect of Extracts on Histamine Induced Tracheal Chain

When different extracts were added to the organ bath after stimulating the trachealis muscle with histamine (1 µg mL^{-1}), the extracts were found to inhibit the contraction. The relaxation effect of aqueous and alcoholic extracts from these medicinal plants on the isolated tracheal chain is shown in Table 2. The results of the present study have demonstrated that all these extracts possessed anti-histaminic activity. Aqueous extract and 70% EtOH extract of *A. conyzoides* were found to have 59.6 and 74.2% relaxation response, and 77.8 and 68.0% for that of *C. indicum*, 54.2 and 50.6% for that of *M. pudica* respectively, in histaminc induced tracheal chain. *A. marmelos* aqueous extract and 70% EtOH extract also exhibited 52.6 and 45.6% relaxation response. In these extracts, *C.indicum* 70% EtOH extract was found to posses the most pronounced activity in trachealis muscle relaxation than the others.

Thus the beneficial effect of these plants in bronchial asthma and cough expectorant appears to be due to its anti-histaminic activity. So, it could be concluded that both aqueous and ethanolic extracts of these plants studied might possess some active principles with bronchodilating activity on the trachealis muscle.

Table 1. Relaxation effect of extracts from four medicinal plants on tracheal chain isolated from Guinea Pig (n=3)

Extracts	Relaxation response (%)					
	0.5 mg mL^{-1} (Bath conc:)	1 mg mL^{-1} (Bath conc:)	2 mg mL^{-1} (Bath conc:)	3 mg mL^{-1} (Bath conc:)	4 mg mL^{-1} (Bath conc:)	5 mg mL^{-1} (Bath conc:)
A. conyzoides (aqueous)	40.7 ± 1.7	62.7 ± 3.7	72.0 ± 6.9	68.7 ± 6.9	69.3 ± 5.2	70.0 ± 5.8
A. conyzoides (70% EtOH)	38.7 ± 3.5	56.0 ± 8.3	75.3 ± 2.4	74.7 ± 2.9	74.0 ± 2.3	74.0 ± 3.0
A. marmelos (aqueous)	24.0 ± 1.2	39.3 ± 4.0	21.3 ± 0.7	23.3 ± 1.8	24.7 ± 1.3	26.0 ± 3.7
A. marmelos (70% EtOH)	24.0 ± 1.7	31.3 ± 2.9	22.0±2.03	24.7 ± 0.7	22.7 ± 0.7	22.7 ± 0 .7
C.indicum (aqueous)	35.0±1.7	62.4±1.2	68.0±2.4	66.1±2.7	65.3±2.3	66.0±2 .3
C.indicum (70% EtOH)	40.3±1.2	73.6±1.3	77.8±1.3	77.0±1.7	76.8±1.7	76.0±0 .7
M. pudica (aqueous)	24.2±2.3	34.5±2.9	38.2±2.3	36.8±2.3	36.2±2.7	36.5± 2.7
M. pudica (70% EtOH)	27.0±1.8	29.8±2.4	39.0±1.7	38.2±1.3	38.0±2.3	38.5±1 .7

For *A. conyzoides*, *C.indicum* and *M. pudica* leaves, direct relaxant activity of ethanolic extract was observed to be higher than that of aqueous extract. Therefore the constituents present in ethanolic extract may be more active than that in aqueous extract.

Table 2. Relaxation effect of aqueous and ethanolic extracts of four medicinal plants on histamine induced tracheal chain isolated from Guinea Pig (n=3)

Extracts (1 mg mL^{-1}bath conc.)	Relaxation response (%)
A. conyzoides (aqueous)	59.6 ± 7.1
A. conyzoides (70% EtOH)	74.2 ± 1.8
A. marmelos (aqueous)	45.6 ± 2.7
A. marmelos (70% EtOH)	52.6 ± 4.0
C.indicum (aqueous)	68.0±1.4
C.indicum (70% EtOH)	77.8±1.3
M. pudica (aqueous)	50.6± 1.35
M. pudica (70% EtOH)	54.2 ±1.3

3.4 Effect of Isolated Compounds on Histamine Induced Tracheal Chain

It was observed that many compounds were isolated from the extracts of these plants. Among the isolated compounds, stigma-5, 22, 25-trienen-3-ol, hispidulin, pectolinarigenin, stigmasterol glucoside are the main constituents of the extracts of *C. indicum*. The yield percent of stigmasterol and Kaempferol from *A. conyzoides* and N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide from *A. marmelos* were high and then these compounds are major constituents of the leaves. So, further investigation could be continued and direct relaxant activity of these compounds was studied on histamine induced contraction in tracheal chain isolated from guinea pig.

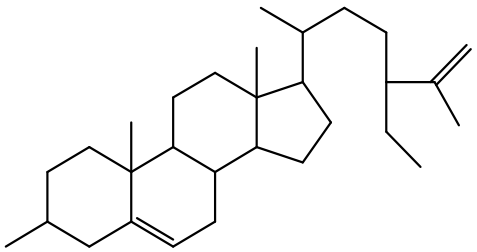
The results of the present study have demonstrated that these compounds possess anti-histaminic activity with relaxation response of 80.2, 73.0, 70.2, 63.0, 56.5, 49.3 and 60.6% by treating with stigma-5, 22, 25-trienen-3-ol, hispidulin, pectolinarigenin, stigmasterol glucoside, stigmasterol, kaempferol and N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide respectively.

Table 3. Relaxation effect of isolated compounds on histamine induced tracheal chain isolated from Guinea Pig (n=3)

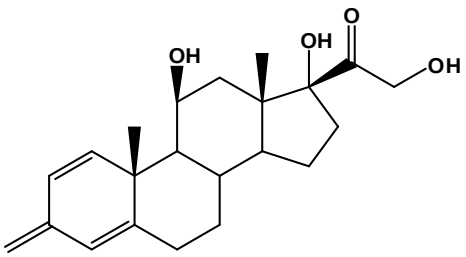
Isolated Compound (0.1 mg mL^{-1} bath conc.)	Relaxation response (%)
Stigmasterol	56.5 ± 1.7
Kaempferol	49.3± 3.55
N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide	60.6 ±0.82
stigma-5,22,25-trienen-3-ol	80.2 ± 0.84
hispidulin	73.0 ± 1.6
pectolinarigenin	70.2 ± 1.52
stigmasterol glucoside	63.0 ± 3.2

Most steroidal compounds are used as controllers or anti-inflammatory agents for asthmatic patients. Steroids are the most effective way of controlling inflammation in the lungs. The steroids used in asthma are corticosteroids [24]. They have been widely and safely used to treat asthma for many years. Some steroids occur naturally in the human body. The most commonly prescribed steroid tablet is prednisolone for people with rheumatic conditions. Stigma-5, 22, 25-trienen-3-ol, stigmasterol glucoside, and stigmasterol, are steroidal compounds. The structure of stigma-5, 22, 25-trienen-3-ol was found to be similar to that of antiasthmatic drug, prednisolone, it may be used for the treatment of smooth muscle contraction problem.

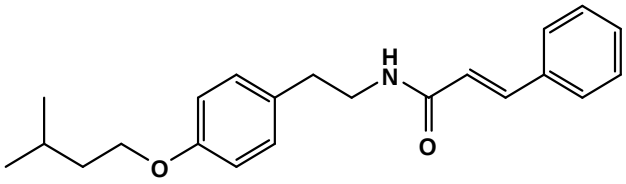
Hispidulin, pectolinarigenin, and kaempferol are flavonoids. According to literature review, flavonoids may contribute to the anti-histamine activity and it can be used as lipoxygenase inhibitor [25]. Flavonoids have been shown that H_2 receptor blocking drugs can reduce histamine to have an antiasthmatic effect [26].



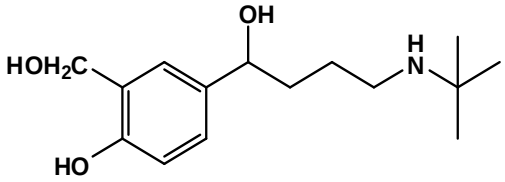
stigma-5, 22, 25-trienen-3-ol



prednisolone



N-2-[4-(3´-methyl butoxy)phenyl] ethyl cinnamide



salbutamol

N-2-[4-(3´-methyl butoxy) phenyl] ethyl cinnamide is an alkaloidal compound. Its structure is nearly equal to the anti-asthmatic drug, salbutamol. Salbutamol is a kind of bronchodilator medicines. It is used to relieve breathing difficulties that come with asthma to widen the airways [27].

If the molecular modification of this steroid (stigma-5, 22, 25-trienen-3-ol) and alkaloid (N-2-[4-(3´-methyl butoxy)phenyl] ethyl cinnamide) could be carried out, the studying should be continued testing on guinea pig trachea in order to obtain new smooth muscle relaxing drugs which could be beneficial for asthmatic patients.

4. CONCLUSION

This study inferred that direct relaxant effect of the extracts (aqueous and 70% EtOH) of *Ageratum conyzoides* and *Aegle marmelos*, *Clerodendrum indicum* (L.) Kuntze, and *Mimosa pudica* L. leaves on both normal and histamine induced tracheal chain preparation using guinea pig. The maximum dose of all extracts was found to be 2 mg mL^{-1} while that of *A. marmelos* extracts to be 1 mg mL^{-1}. And then 45-80 % of relaxation response after treating with extracts as well as some isolated compounds. Therefore, from these results, it is suggested that these plants should be used as a remedy for the treatment of bronchial asthma and structure modification should be accomplished to obtain the new smooth muscle relaxing drugs.

CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the authors.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee”

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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