ORIGINAL ARTICLES

Antihistamine Effect of a Pure Bioactive Compound Isolated from Slug (*Diplosolenodes occidentalis*) Material

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ABSTRACT

Objective: *Folklore claims of the therapeutic effect of garden slug* (Diplosolenodes occidentalis) *extract used to relieve bronchoconstriction in asthmatic individuals were never validated scientifically. The aim of this study was to isolate the pure bioactive compound from slug extract causing this effect.*
Methods: *The crude ground material was prepared in ethanol and after filtration, separation by flash column chromatography method was done. The structure was elucidated by data from hydrogen and carbon nuclear magnetic resonance (NMR) profiles. The bioactive compound was assessed for dose dependent response effects on guinea pig tracheal smooth muscle pre-contracted with histamine. Receptor specificity studies were done by using HTMT dimaleate (H_1 agonist). The type of antagonism was also identified.*
Results: *The pure component isolated from garden slug material was identified by spectral studies as glyceryl trilinolenate. It caused dose-dependent relaxation in guinea pig tracheal smooth muscle strips pre-contracted with histamine, it acted* via *H_1 type receptors and showed non-competitive antagonism.*
Conclusion: *Glyceryl trilinolenate produced dose-dependent relaxation in tracheal smooth muscle strips in the presence of the agonist histamine. Glyceryl trilinolenate displayed non-competitive antagonism at H_1 receptors in the trachea. This agent was able to alleviate bronchoconstriction in individuals presenting with atopic asthma in rural agricultural areas in Jamaica (verbal communications). It is possible that glyceryl trilinolenate can be used therapeutically to produce tracheal smooth muscle relaxation in individuals presenting with atopic asthma.*

Keywords: Atopic asthma, H_1 antagonist, garden slug, glyceryl trilinolenate, folklore practice, smooth muscle relaxation, trachea

Efecto Antihistamínico de un Compuesto Bioactivo Puro Aislado a Partir de Extractos de Babosas *(Diplosolenodes occidentalis)*

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RESUMEN

Objetivo: *Las afirmaciones de la medicina tradicional respecto al efecto terapéutico del extracto de la babosa de jardín* (Diplosolenodes occidentalis) *usado para aliviar la broncoconstricción en individuos asmáticos, no han sido nunca validadas científicamente. El objetivo del presente estudio fue aislar el compuesto bioactivo puro del extracto babosa que causa este efecto.*
Métodos: *El material molido crudo fue preparado en etanol, y tras la filtración, se efectuó la separación mediante el método de cromatografía en columna ultrarrápida. La estructura fue esclarecida por los datos de los perfiles de resonancia magnética nuclear (RMN) de hidrógeno y carbono. El compuesto bioactivo se evaluó en cobayos, para investigar los efectos de respuesta dependiente de la dosis en el músculo liso traqueal precontraído con histamina. Se realizaron estudios*

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de especificidad del receptor utilizando dimaleato HTMT (agonista H_1). También se identificó el tipo de antagonismo.

Resultados: *El componente puro aislado a partir de la babosa jardín, fue identificado mediante estudios espectrales como trilinolenato de glicerilo. El mismo causó la relajación dependiente de la dosis en tiras del músculo liso traqueal de cobayos, precontraídas con histamina, actuando vía receptores de tipo H_1 y mostraron antagonismo no competitivo.*

Conclusión: *El trilinolenato de glicerilo produjo relajación dependiente de la dosis en tiras del músculo liso traqueal en presencia de la histamina antagonista. El trilinolenato de glicerilo mostró antagonismo no competitivo en los receptores de tipo H_1 en la tráquea. Este agente fue capaz de aliviar la broncoconstricción en individuos con asma atópica en zonas rurales agrícolas de Jamaica (comunicaciones verbal). Es posible que el trilinolenato de glicerilo pueda usarse terapéuticamente para producir la relajación del músculo liso traqueal en individuos con asma atópica.*

Palabras claves: Asma atópica, antagonista H_1, babosa de jardín, trilinolenato de glicerilo, práctica de la medicina tradicional, relajación del músculo liso, tráquea

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INTRODUCTION

This study investigates the validity of the use of slug (*Diplosolenodes occidentalis*) extract in folklore practice to treat breathing difficulty in bronchial asthma. The slug material was either parched or ground and put in meals as a powder or placed in alcoholic drinks and consumed. The leather leaf slug used in Jamaica (1) is from the phylum Mollusca and lives in a moist environment in garden areas.

Asthma is a respiratory disease which is associated with hyper-responsiveness of bronchial smooth muscle, concomitant with inflammation occurring as a result of eosinophil infiltration. As a result of this, there is obstruction of respiratory flow when tracheal constriction takes place; hence, the hallmark of asthma is obstruction of the airways, inflammation of the mucosa and increased vascular permeability (2).

In Jamaica and the entire Caribbean, the seeds, leaves and roots of plants are used as foods and as home remedies for some medical conditions (3). Plant materials contain many bioactive compounds which may be of potential economic value (4). In Jamaica, 71% of patients use herbal remedies before visiting a physician (5). Bioactive compounds from crude plant extracts and animal species have been used over the centuries for treatment of an array of diseased conditions (6). Animal materials are not popularly used, but bio-organic studies on marine natural products, such as sea slugs, have produced many valuable compounds which can be used in medicine. The spray from the sea slug Aplysia (*Aplysia californica*) is used as a defence tool; further research showed its antimicrobial properties (7). Pharmacologically active compounds have been isolated from soft corals, tunicates and molluscs (8) and the earliest record of animal-based medicine is recorded in the Holy Bible (9).

This study places focus mainly on the ability of the isolated bioactive compound from the slug to produce relaxation of tracheal smooth muscle contraction induced by histamine when the airway is affected by allergens binding to mast cells.

MATERIALS AND METHODS

Source of chemicals

The following chemicals were obtained from Industrial and Technical Supplies, Kingston, Jamaica: sodium chloride, dihydrogen orthophosphate, magnesium sulphate, sodium hydrogen carbonate, potassium chloride (Analar), magnesium chloride (Analar), calcium chloride (dehydrate), dichloromethane, chloroform and ethyl acetate.

The following reagents were obtained from Sigma-Aldrich, St Louis, MO, USA: formaldehyde, hexane, albumin chicken egg (Grade 3), histamine-bisphosphate, diphenhydramine and eosin. Absolute ethanol was obtained from Industrial and Technical Supplies (Kingston, Jamaica) and haematoxylin and eosin from Hopkins and Williams, Ltd, Essex, England.

Physiological solution pH 7.4

Krebs-Henseleit Buffer (KHS) contained the following in mM: 118 sodium chloride (NaCl), 5.9 potassium chloride (KCl), 25 sodium bicarbonate ($NaHCO_3$), 1 sodium dihydrogen bisphosphate (NaH_2PO_4), 2 calcium chloride ($CaCl_2$), 1 magnesium sulphate ($MgSO_4$) and 11 D (+) glucose saturated with 95% O_2–5% CO_2.

Collection of slugs for preparation of extract

Slugs were collected from the garden area of The University of the West Indies, Mona, and were washed in order to remove debris of soil and vegetation. The environment was free of pesticide. Classification was done by the Department of Life Sciences, Zoology section (The University of the West Indies, Mona) as *Diplosolenodes occidentalis* from the phylum Mollusca.

Method of isolation of pure compound from crude extract
Slugs were parched at a temperature of 120 °C. The powdered material was extracted in ethanol (95%) at a temperature of 4 °C for 72 hours. Filtration by gravity was done using Whatman filter paper #2. The crude sample of slug extract was received in ethanol which was evaporated at 60 °C in a rotary evaporator. This crude sample was loaded on the silica flash column (Bond Elute Mega BE-Si, 100GM 600 mL-Silicycle) primed with 200 mL of 100% hexane. The elution continued with 200 mL of 1% ethyl acetate in hexane followed by a percentage gradient increase of ethyl acetate in hexane: 1.5%, 2%, 2.5% to 10%.

Thin layer chromatography (TLC) run was done using the solvent system of 5% methanol in dichloromethane as the mobile phase. The TLC plate was dried and sprayed with developing reagent phosphomolybdic acid solution to identify the number of compounds present. R_f (retardation factor) values were calculated from the TLC profiles and fractions with identical values pooled together and tested for biological activity against histamine-induced tracheal smooth muscle (guinea pig) contraction.

Nuclear magnetic resonance spectroscopy
This method was used to give a complete analysis and interpretation of an entire spectrum of the pure compound. Nuclear magnetic resonance (NMR) spectroscopy is an analytical chemistry technique used for determining the content and purity of a sample as well as its molecular structure (10). Nuclear magnetic resonance can quantitatively analyse mixtures containing known compounds. For unknown compounds, NMR can either be used to match against spectral libraries or to infer the basic structure directly.

Animal studies
In vitro studies
The treatment of animals was approved by the University Hospital of the West Indies/University of the West Indies/ Faculty of Medical Sciences Ethics Committee. Adult male guinea pigs (400−450 g) were sacrificed by administration of 75 mg/kg of sodium pentobarbital by the intra-peritoneal route (11). The trachea was excised and placed in Krebs Henseleit solution in a petri dish. The adherent connective tissue was dissected from the outer tracheal rings and the lumen gently flushed with physiological solution. The trachea was cut into sections containing 3−4 cartilage bands (12–14), and each ring was opened to form a strip by cutting through the cartilage on the opposite side of the muscle band (15). This tissue was set up in Krebs Henseleit solution under continuous aeration with 95% oxygen and 5% carbon dioxide in a 20 mL organ bath maintained at a temperature of 37 °C. Each strip was anchored at the bottom of the bath by a loop of thread attached to the lower half section of the cartilage bands. The upper half section of the cartilage bands with the broad band of tracheal smooth muscle in between was attached to a length of thread that was connected to a force displacement transducer which was in turn connected to a polygraph (Grass model 15LT physioamplifier system with 15A12 dual DC amplifier) for recording isometric tension. The tissue was allowed to equilibrate for one hour under a resting tension of 1 g.

Effect of glyceryl trilinolenate on histamine-induced contractions
Histamine-induced control contractions were given *via* cumulative dosing at three-minute intervals or as soon as plateau was attained. The doses of histamine given were 2 µg (3.1 x 10^{-7}) to 32 µg (4.9 x 10^{-6}) as log doses. The doses of pure compound glyceryl trilinolenate (GT) used were 62.6 µg, 125 µg, 250 µg, 500 µg and 1000 µg (n = 6). All doses were added to a 20 mL organ bath. As an oil, GT was dissolved in 95% ethanol before addition to the bath.

Receptor action of glycerol trilinolenate
In further investigations, the effect of the pure compound (GT) was tested in the presence of a selective H_1 agonist, histamine trifluoromethyl toluidide (HTMT) dimaleate. For this investigation, the guinea pig tracheal muscle strips were used. Histamine trifluoromethyl toluidide dimaleate (200 g in 0.3 mL of water as vehicle) was given in the presence of the pure compound (250 µg).

In vivo experiments
Sensitization of animals
Guinea pigs weighing 250−300 g were sensitized with ovalbumin (16). The process involved administration of intraperitoneal injections of ovalbumin (10 mg/kg) on alternate days for three days. Thirty days after the last injection, the animals were tested for cutaneous response produced by intradermal injection of ovalbumin and histamine.

Presentation of the experimental data
Data are given as means ± standard error of the mean (SEM). Test for statistical significance was executed by the Student's *t*-test (paired). Values of $p < 0.05$ were considered to be statistically significant. The graphing programme used is Sigma Plot for Windows, version 11.

RESULTS
Spectral studies
Thin layer chromatography results showed that the bioactive fraction appeared as one spot on the silica plate and the percentage yield was calculated to be 0.05%.

H nuclear magnetic resonance spectral data showed the signal details listed.

The peaks (Fig. 1) were noted and assigned in the following manner:

1) A large singlet at δ 1.29, indicative of terminal methyl groups on the fatty acid of the triglyceride.
2) Multiplets between δ 1.60 and 2.89, representing the methylene portions of the acidic moiety.

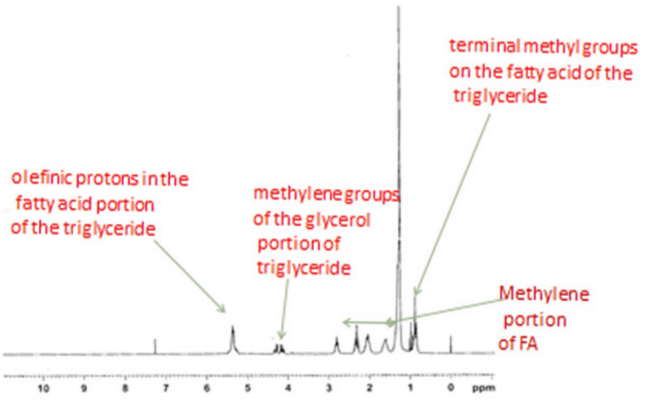

Fig. 1: Record of hydrogen nuclear magnetic resonance (NMR) spectra of pure bioactive compound.

3) Multiplet at δ 4.25 belonging to the methylene groups of the glycerol portion of the triglyceride.
4) Singlet at δ 5.5 representing olefenic protons in the fatty acid portion of the triglyceride.

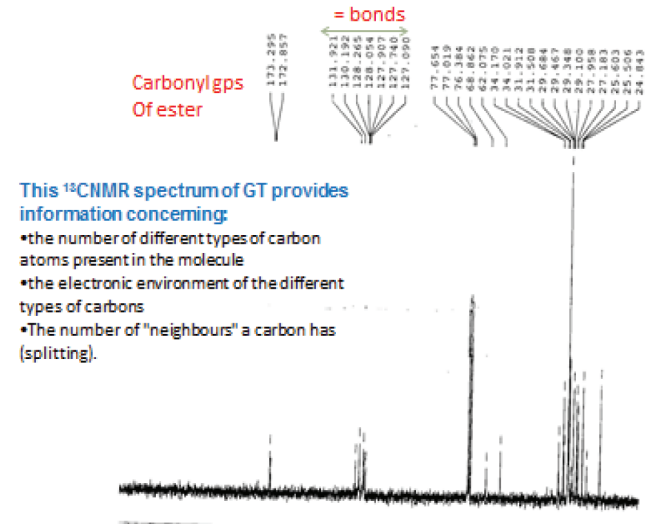

Fig. 2: Distortionless enhancement by polarization transfer data.

This 13CNMR spectrum of glyceryl trilinolenate provides information concerning:

- The number of different types of carbon atoms present in the molecule.
- The electronic environment of the different types of carbons.
- The number of "neighbours" a carbon has (splitting).

1) Signals at 1 δ 73.3 and 172.9 represent carbonyls of ester group in two different environments.
2) Shift at δ 62.1 represents methylene carbons of the glycerol portion of triglyceride.
3) Peak at δ 68.9 represents methane carbon of the glycerol portion of triglyceride.
4) Seven olefinic peaks at 1 δ 27.1, 127.7, 127.9, 128.1, 128.3, 130.2 and 131.9, representing the double bonds in the fatty acid chains.

The compound glyceryl trilinolenate or trilinolenin was identified (Fig. 3). It is a polyunsaturated fat belonging to the

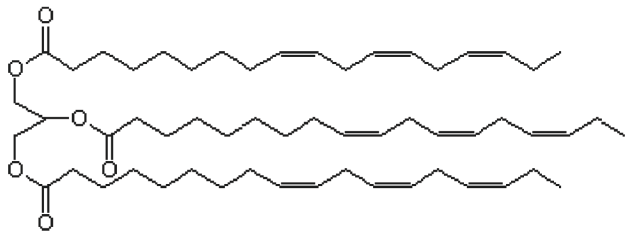

Fig. 3: Chemical structure of glyceryl trilinolenate (molecular weight: 873.33678 [g/mol]; molecular formula: C_{57} H_{92} O_6).

omega-3 category of fats. These omega-3 type fats are associated with beneficial cardiovascular effects. This compound also exhibits antioxidant properties.

Pharmacological studies of the pure compound, glyceryl trilinolenate

Effect of glyceryl trilinolenate on tracheal smooth muscle contraction

In all *in vitro* experiments (n = 6), guinea pig smooth muscle strips were used. The polyview along with a 15LT physio amplifier system (Grass Instruments) connected to a force transducer recorded isometric tension.

Contractile tension, measured under isometric condition, was induced by histamine 2 µg (3.1 x 10^{-}7 M) to 16 µg (2.48 x 10^{-}6 M] in guinea pig tracheal smooth muscle strips that were maintained under resting tension of 1.0 g in aerated Krebs Heinslet solution pH 7.4. The effect of increasing doses of histamine in the presence of vehicle (2.8% alcohol solution) produced a progressive increase in tension in the tracheal muscle strips; this contractile effect produced a maximum tension of 2.0 ± 0.2 g. In the presence of 62.5 µg of pure compound of GT, there was a slight reduction in tension by 15% (Fig. 4) or 0.3 g ($p > 0.05$, n = 6). This inhibiting effect of GT was not statistically significant. However, in experiments (n = 6) using 125 µg of GT (7.2 x 10^{-}6 M), there was a reduction of contractile tension by 54%. The control maximum rise in tension was recorded as 1.48 g, but in the presence of the 125 µg of GT (Fig. 5), the tension was reduced by 0.8 g ($p < 0.05$, n = 6); this was statistically significant. In experiments (n = 6) using 250 µg of GT (1.43 x 10^{-}5 M), there was a reduction of contractile tension by 70% (Fig. 6).

The control maximum rise in tension was recorded as 1.68 g and in the presence of this concentration of antagonist, tension was reduced to 1.18 g (Fig. 6). Results from data using 500 µg (2.86 x 10^{-}5 M) of GT (Fig. 7) showed a 75%

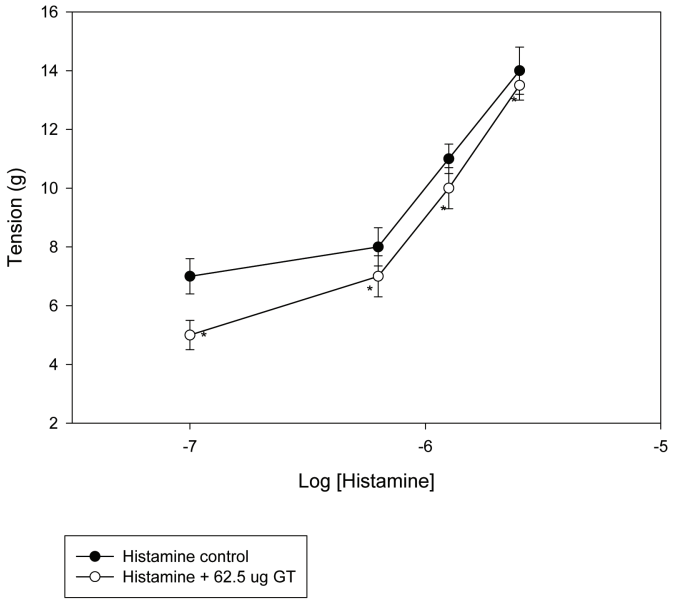

Fig. 4 Graph showing the effect of 62.5 µg of pure bioactive compound glyceryl trilinolenate (GT) on histamine-induced tracheal smooth muscle contraction. Each data point represents the mean ± standard error of the mean (SEM) for n = 6. *$p > 0.05$.

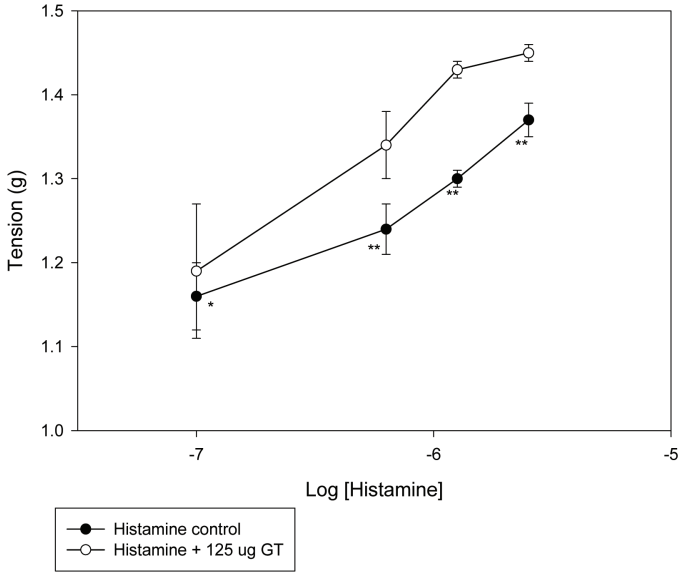

Fig. 5: Graph showing the effect of 125 µg of pure bioactive compound glyceryl trilinolenate (GT) on histamine-induced tracheal smooth muscle contraction. Each data point represents the mean ± standard error of the mean (SEM) for n = 6. *$p < 0.05$, **$p < 0.01$.

reduction of contractile tension. The maximum contraction of the control with histamine was 1.57 g and there was reduction to 1.19 g ($p < 0.05$) in the presence of GT; these results were statistically significant. The results obtained with 1000 µg of GT (Fig. 8) was highly statistically significant ($p < 0.00001$, n = 6). In the control experiment, a maximum tension of 1.4 g was recorded, but in the presence of 1000 µg

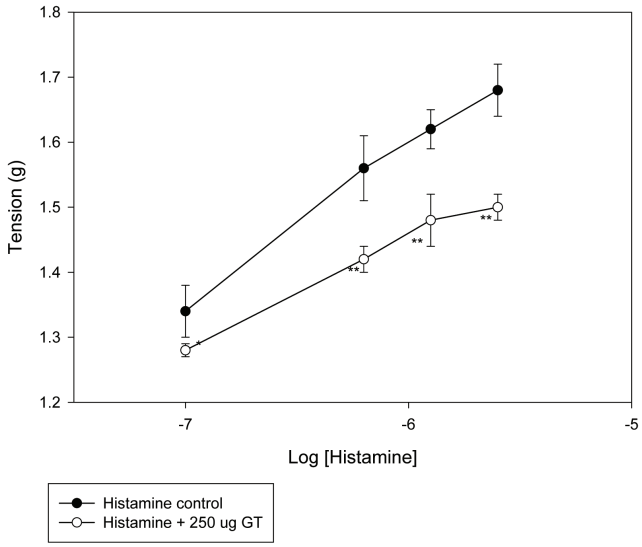

Fig. 6: Graph showing the effect of 250 µg of pure bioactive compound glyceryl trilinolenate (GT) on histamine-induced tracheal smooth muscle contraction. Each data point represents the mean ± standard error of the mean (SEM) for n = 6. *$p < 0.05$, **$p < 0.01$.

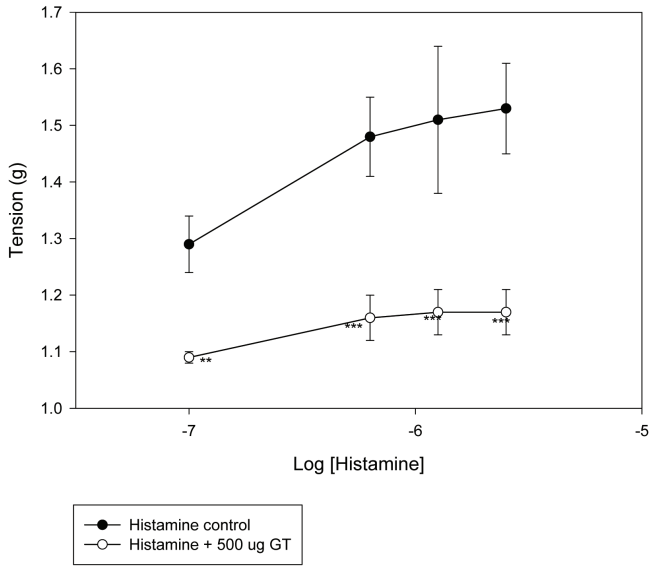

Fig. 7: Graph showing the effect of 500 µg of pure bioactive compound glyceryl trilinolenate (GT) on histamine-induced tracheal smooth muscle contraction. Each data point represents the mean ± standard error of the mean (SEM) for n = 6. **$p < 0.01$, ***$p < 0.001$.

(5.7 x 10^{-5} M) of GT, there was a relaxation of 92% and a reduction in contractile tension to 0.89 g.

Receptor actions of glyceryl trilinolenate

Histamine trifluoromethyl toluidide was used as the selective H_1 agonist to produce contractions in the absence and presence of GT. Figure 9 shows that GT inhibited the contractions of the tracheal muscle strips produced by HTMT (200 µg). However, for effects on H_2 receptors, the selective

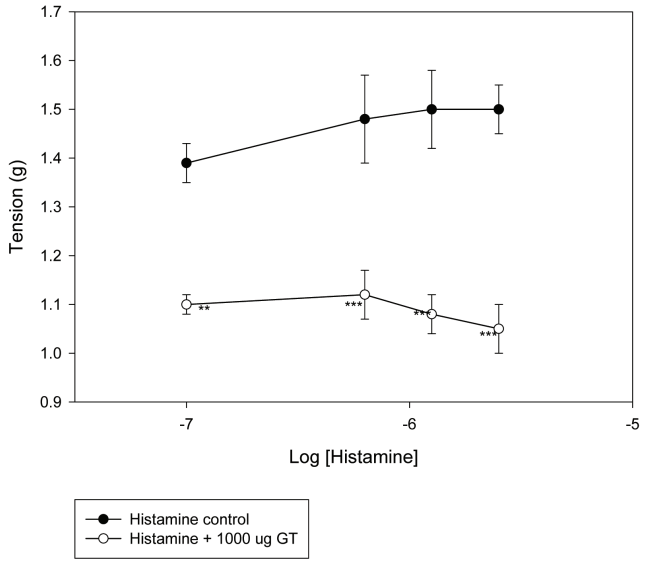

Fig. 8: Graph showing the effect of 1000 µg of pure bioactive compound glyceryl trilinolenate (GT) on histamine-induced tracheal smooth muscle contraction. Each data point represents the mean ± standard error of the mean (SEM) for n = 6. $^{**}p < 0.01$, $^{***}p < 0.00001$.

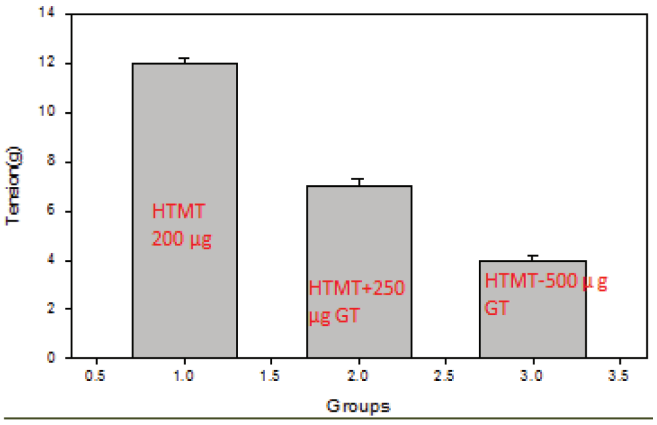

Fig. 9: The graph represents the effect of two doses of glyceryl trilinolenate (GT) on 200 µg of histamine trifluoromethyl toluidide (HTMT) dimaleate.

Group 1 represents the control tracheal strips (HTMT-200 µg).
Group 2 (strips) represents the effect of HTMT in the presence of 250 µg of GT on strips.
Group 3 (strips) represents the effect of 500 µg GT on HTMT dimaleate (200 µg).

H_2 agonist, dimaprit dihydrochloride, did not contract the tracheal muscle strips when various doses (60−180 µg) were used. Therefore, these results suggest that H_2 receptors did not contribute to the contractile tension that was inhibited by GT. These results also suggest that the inhibitory effect of GT against HTMT-induced contractions was due to interaction with H_1 receptors in the tracheal muscle strips.

Type of antagonism produced by glyceryl trilinolenate

Increasing doses of histamine did not surmount the inhibitory action of the antagonist GT. Also, the maximum contractile tension obtained in the presence of GT was depressed in all experiments (Figs. 5−8) and based on these results, competitive antagonism was ruled out. Dose ratios for histamine in the presence of GT could not be established, because the inhibition was not surmounted by increasing doses of histamine. Therefore, the Schild's Plot was not generated and there was no evidence of competitive antagonism by GT. Instead, the evidence suggests that GT may have produced its effects by non-competitive antagonism. Additionally, to support this, dose response curves (Figs. 5−8) showed the characteristic depression of the maximum tension and non-parallel dextral shift to the right in all graphs and this represents the profile of non-competitive antagonism.

DISCUSSION

In this study, a pure bioactive compound was extracted from crude garden slug (*Diplosolenodes occidentalis*) extract. In Jamaica, parched and ground slug materials are used in folklore practice in some agricultural communities for treatment of bronchial asthma. Since the practitioners who used the slug material are from agricultural areas, it was reasonable to assume that the claimed therapeutic effectiveness of the slug material may be associated with asthma triggered by atopic allergy due to exposure to allergens in the community.

The dark brown crude viscous material was prepared and absorbed onto silica for flash column chromatographic separation. Spots from TLC were identified by spraying with the developing reagent consisting of phosphomolybdic acid and ceric sulphate. This developing reagent permitted identification of a broad range of chemicals such as sphingophospholipids (15) and lipid compounds, steroids, phenols and fatty acids (16) and thus maximized the potential for inclusion of the bioactive compound in one of the separated TLC spots.

Spectral studies identified the bioactive compound GT, a triglyceride of linolenic acid, consisting of a glycerol backbone with substituent of three linolenic acid molecules (Fig. 3).

When GT was investigated for antihistamine properties, it produced a dose-dependent inhibition of histamine-induced contraction of guinea pig isolated tracheal smooth muscle strip. The inhibition was not surmountable with increasing addition of histamine. Glyceryl trilinolenate inhibited H_1 receptor-induced contraction produced by HTMT dimaleate, a selective H_1 agonist (17) on guinea pig tracheal tissue. The selective H_2 agonist, dimaprit hydrochloride, had no effect on isolated tracheal muscle strip (18). This ruled out H_2 receptor mediation of GT inhibition of histamine-induced contraction of the guinea pig isolated tracheal muscle. Therefore, GT was present in the crude material

used by the claimants and was the agent producing the antagonism to histamine. The pure compound GT produced highly significant relaxation in tracheal smooth muscle due to histamine-induced contractions. Histamine-induced contraction is mediated mainly by H_1 receptors in the isolated tracheal smooth muscle (19) and GT is acting on H_1 receptors in this study (Fig. 9).

The type of antagonism produced by GT on H_1 receptors was most likely non-competitive since increasing the doses of histamine did not surmount the inhibition. Also, there was depression of the maximum response in all graphs and a non-parallel rightward shift of the curve in the presence of the antagonist. Despite the fact that most antihistamines show competitive antagonism, GT is probably exhibiting non-competitive antagonism in a similar manner to the newer antihistamines which show this type of antagonism at a "higher than clinical dose" (20).

The chemical structure of GT shows the acyl tails of the linolenic acid substituents which can induce changes in the lipid layer of cell membranes and disrupt binding of agonist to membrane-bound receptors (21). It is possible that GT produced its inhibitory effects *via* this mechanism, by disrupting the binding of histamine to H_1 receptors in the cell membrane. It is also known that H_1 receptors of histamine are located in cell membranes of smooth muscles (22). Glyceryl trilinolenate may have non-competitively inhibited histamine binding to H_1 receptors by induction of changes in the lipid layer of tracheal smooth muscle cell membrane.

Non-competitive antagonists usually target the transduction processes such as G proteins, second messengers and ion channels. Perhaps the mechanism of action of GT is also associated with a second messenger system which produces smooth muscle relaxation involving inhibition of calcium mobilization (23). Inositol 4,5-triphosphate (IP3) diffuses to the endoplasmic reticulum and initiates the release of calcium ions into the cytosol. This action causes the release of calcium and 1,2-diacylglycerol (intracellular messengers) which activate protein kinase C (24). Calcium reacts with troponin and causes a response by actin and myosin which causes muscle contraction. During this activity, there is concomitant decrease in the levels of cyclic adenosine monophosphate [cAMP] (25). Glyceryl trilinolenate may act by inhibiting calcium and diacylglycerol release and increasing the levels of cAMP in cells and hence inducing relaxation.

These investigations provide the first scientific documentation of the effectiveness of slug material used in Jamaican folklore practice for treatment of the symptoms of bronchial asthma, which is most likely the extrinsic type. Glyceryl trilinolenate may have exhibited both antihistamine and anti-inflammatory effects concomitantly, hence its ability to alleviate difficulty in breathing as reported in folklore practice.

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