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Action of polygodial, a sesquiterpene isolated from *Drymis winteri*, in the guinea-pig ileum and trachea 'in vitro'

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Abstract

This study describes the action of the sesquiterpene polygodial, the major constituent isolated from the bark of *Drymis winteri* in the guinea pig ileum and trachea in vitro. Polygodial (5 to 128 μM), added for 20 min, did not affect the resting tone of the preparations, but caused graded inhibition, associated in some cases with rightward displacement of the acetylcholine, histamine (1 nM to 10 μM), bradykinin (0.1 nM to 1 μM) and KCl (1 to 100 mM)-contraction response curves. When assessed in the guinea-pig trachea, polygodial (5 to 342 μM) caused significant inhibition of bradykinin (10 pM to 1 μM), 9,11-dideoxy-9α,11α-methano-epoxy prostaglandin $F_{2\alpha}$ (0.1 to 1000 nM) and KCl (1 to 100 mM)-induced contractions, although the action against bradykinin was not concentration-dependent. Polygodial (5 to 80 μM) caused a small but significant shift to the right of substance P and also the selective agonist of tachykinin NK_2 receptor [β-Ala^8]neurokinin A-(4-10)-induced contractions in guinea pig trachea. This action of polygodial seems to be quite selective towards tachykinin NK_2 receptors since up to 432 μM, polygodial had no effect against contraction caused by tachykinin NK_1 receptor agonist, substance P methyl ester. When tested in the guinea-pig trachea from animals which had been actively sensitised to ovalbumin, polygodial (30 to 40 μM) caused time and concentration-dependent inhibition of ovalbumin-mediated contraction. In addition, polygodial (85 to 342 μM) inhibited contraction induced by compound 48/80 (1 to 1000 μg/ml), in the guinea-pig trachea from non-sensitised animals. These findings and those from our previous study are consistent with the notion that the main sesquiterpene polygodial isolated from the bark of *D. winteri* is responsible for most, if not all, of the relevant pharmacological action reported previously for the extract of this plant. Thus, polygodial could be of potential value in the development of a new drug for the treatment of asthma, allergy and other inflammatory processes. © 1998 Elsevier Science B.V.

Keywords: Polygodial; Ileum, guinea-pig; Trachea, guinea-pig; Contraction; Prostanoid; Bradykinin; Compound 48/80; Ovalbumin; Asthma; Allergy

1. Introduction

In a previous study, we reported that the hydroalcoholic extract obtained from the barks of *Drymis winteri* J.R. et Forster (Winteraceae), a plant largely used in folk medicine in Brazil and other countries for the treatment of respiratory disease such as asthma, allergy and bronchitis, and as an anti-inflammatory remedy (Morton, 1981), inhibited, in a concentration-dependent and reversible fashion, the contractions induced by several mediators associated with the aetiology of asthma, allergy and inflammatory processes (El Sayah et al., 1997). Furthermore, the extract of *D. winteri* antagonised, in a concentration and time-dependent manner, contraction elicited by ovalbumin in guinea pig trachea obtained from animals that had been actively sensitised to this antigen (El Sayah et al., 1997).

In the present work, we examine the effects of the sesquiterpene polygodial, the main principle isolated from the barks of *D. winteri*, on contractions induced by several chemical mediators, such as bradykinin, tachykinins, histamine, acetylcholine and the stable analogue of thromboxane A_2, U 46619, known to participate in the aetiology of respiratory diseases. We additionally investigate whether polygodial interferes with ovalbumin and compound 48/80

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known to mediated contractions in the guinea-pig trachea by the release of histamine obtained from animals actively sensitised to ovalbumin and non-sensitised animals, respectively.

2. Material and methods

2.1. Procedure for isolation of polygodial

Barks of *D. winteri* were collected in Bom Retiro, state of Santa Catarina, and were classified by Dra. Leila da Graça Amaral (Department of Botany, Federal University of Santa Catarina). A voucher specimen was deposited in Herbarium FLOR (UFSC, Florianópolis) under the number 26313.

The methanolic extract obtained from dried barks of *D. winteri* (750 g) was exhaustively partitioned with dicloromethane. After solvent removal, the fraction was chromatographed on a silica gel column eluted with hexane:acetone gradient, giving polygodial (yield of 0.032%) (Fig. 1). This compound was identified by direct comparison with authentic sample and by spectroscopic data Infrared, Proton Nuclear Magnetic Resonance, Carbon-13 Nuclear Magnetic Resonance. Further details regarding the procedure of isolation and elucidation of polygodial structure will be published elsewhere.

2.2. Experimental procedure

2.2.1. Guinea-pig ileum

Ileal strips of about 10 to 20 mm long were obtained from guinea-pigs of either sex (300–500 g) and were taken from the portion situated 10 to 30 cm proximal to the ileo–caecal junction. Preparations were set up for recording of isotonic contractions in 5 ml jacketed organ baths containing Krebs Henseleit solution at 37°C continuously bubbled with air under 1 g of tension, as described previously (Calixto, 1995). The solution had the following composition (mM): NaCl 118.0, KCl 4,7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.1, KH_2PO_4 0.9 and glucose 11.0. After an initial equilibration period of about 60 min, usually 2 to 3 complete cumulative concentration–response curves for each agonist were obtained per preparation, at 30 min intervals between curves. The curves for bradykinin were obtained in the presence of captopril 1 μM, an inhibitor of kininase II. After obtaining two or three complete concentration–response curves and once the responses became reproducible, different concentrations of the polygodial (5–128 μM) were added to the bathing solution 20 min before constructing new curves for the agonists. The mean maximal response obtained from the first two cumulative concentration–response curves was taken as the 100% response value and all other responses were calculated as a function of this value. In separate sets of experiments, in order to correct for spontaneous and/or vehicle-induced desensitisation, control experiments for KCl, acetylcholine, histamine or bradykinin were carried out in presence of the corresponding concentrations of absolute ethanol, the vehicle used to dilute polygodial. Only one agonist was tested in each preparation. In order to correct for any spontaneous and/or agonist-induced changes in the contractile response to the agonists, parallel control experiments were carried out in presence of ethanol.

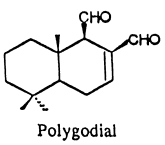

Fig. 1. Molecular structure of polygodial.

2.2.2. Guinea-pig trachea

Guinea-pigs of both sexes (200–350 g) were killed by a blow on the head and were exsanguinated from carotid arteries. The trachea was rapidly removed and after being freed from connecting tissues was cut into six transverse rings (3–4 mm wide), each containing 3 cartilages as described previously (Schelemper and Calixto, 1994). The rings were opened and usually 6 strips of 8 to 10 mm in length obtained from the same animal were suspended in individual 5 ml jacketed organ baths containing Krebs–Henseleit solution maintained at 37°C, pH = 7.4, gassed with 95% of O_2 and 5% of CO_2. The Krebs solution had the following composition (mM): NaCl 118.0; KCl 4.4; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; $MgSO_4$ 1.1; KH_2PO_4 1.2, glucose 11.0. Tissues were allowed to equilibrate for at least 60 min before drug addition, during which the fresh buffer solution was changed every 15 min, under a resting tension of 1 g. Isometric contractions were measured by means of F-60 force displacement transducers and were recorded on a polygraph (Narco Biosystem). In experiments for bradykinin the epithelial layer of the trachea was gently removed with a cotton-tipped applicator. The absence of epithelium was confirmed by assessing the lack of relaxation response caused by bradykinin (100 nM) in preparations under spontaneous tonus (Schelemper and Calixto, 1994). All experiments for substance P and analogues were carried out in the presence of captopril (3 μM) and phosphoramidon (1 μM), incubated 15 min before the addition of drugs to prevent the action of peptidases. Responses for bradykinin were obtained in presence of captopril (3 μM). Only one agonist was tested in each preparation. In order to correct for any spontaneous and/or agonist-induced changes in the contractile response to the agonists, parallel control experiments were carried out in the presence of ethanol.

After the equilibration period of at least 60 min, complete cumulative concentration–response curves were obtained for KCl, bradykinin, prostaglandin E_2, acetylcholine, substance P, histamine, U46619 (a stable throm-

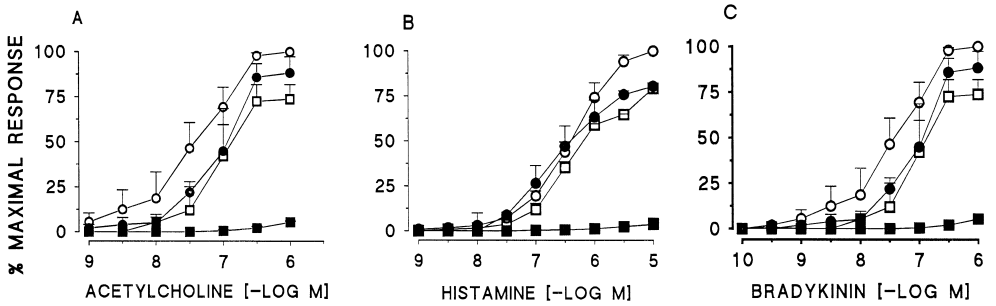

Fig. 2. Mean concentration response curves for acetylcholine (A), histamine (B) and bradykinin (C) in guinea-pig ileum, in the absence (○) or in the presence of polygodial (A and C) 5 μM (●), 10 μM (□) and 20 μM (■); (B) 17 μM (●), 43 μM (□) and 60 μM (■). Values are means ± sem of 4 to 6 experiments.

boxane-like agonist), compound 48/80, [β-Ala^8]neurokinin A-(4–10) (a selective tachykinin NK_2 receptor agonist), and substance P-methyl ester (a selective tachykinin NK_1 receptor agonist), in the absence or in the presence of different concentrations of the compound polygodial which was added to the preparations 20 min beforehand. Usually, 2 to 3 complete concentration–response curves were obtained for each agonist per preparation, at 60 min intervals between curves. Each concentration of the agonist was added when the effect of the preceding addition had reached its maximum. Since contractile response induced for bradykinin was not well reproducible at 1 to 2 h intervals, only one complete cumulative concentration–response curve for this peptide was obtained for each tissue. Therefore, separate control and test tissues were studied simultaneously in adjacent baths.

2.3. Sensitisation procedure and response to ovalbumin

Guinea-pigs of both sexes (200–350 g) were sensitised by intraperitoneal injection of 0.5 ml of 0.9% NaCl solution containing 10 μg of ovalbumin, dispersed with 1 mg of Al $(OH)_3$. The injection was repeated after 14 days and the animals were killed 7–10 days after the second injection (Andersson and Bergstrand, 1981; Pretolani et al., 1989). After removing the trachea from the adjacent tissues, preparations were mounted for isometric recording under a resting tension of 1 g, in a 5 ml organ bath containing Krebs solution, as described before. Following the equilibration period (60 min), preparations were contracted by addition of carbachol (100 μM). After 30 min, preparations were exposed to ovalbumin (1–3 μg/ml) and a contraction of the guinea-pig trachea confirmed that the guinea-pig had been successfully sensitised. The compound polygodial (30–40 μM) was pre-incubated with the preparations 20 min before ovalbumin addition. The contractile responses to ovalbumin, obtained in presence or in absence of polygodial, were expressed as a percentage in relation to the maximal contractile response induced by carbachol (100 μM) or in grams of developed tension.

2.4. Drugs

The drugs used were: acetylcholine iodide, albumin chicken egg (ovalbumin grade V), bradykinin, captopril, carbamylcholine chloride, compound 48/80, phosphoramidon, histamine hydrochloride, substance P, substance P-methyl ester, U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) (all from Sigma Chemical, St Louis, MO), and [β-ala^8]neurokinin A-(4–10) (Peninsula, California).

The stock solutions of most of the these drugs (1–100 mM) were prepared in water or in PBS solution, except polygodial which was dissolved in absolute ethanol, and were maintained at −20°C. The final bath concentration of ethanol did not exceed 0.03% and had no effect 'per se' on

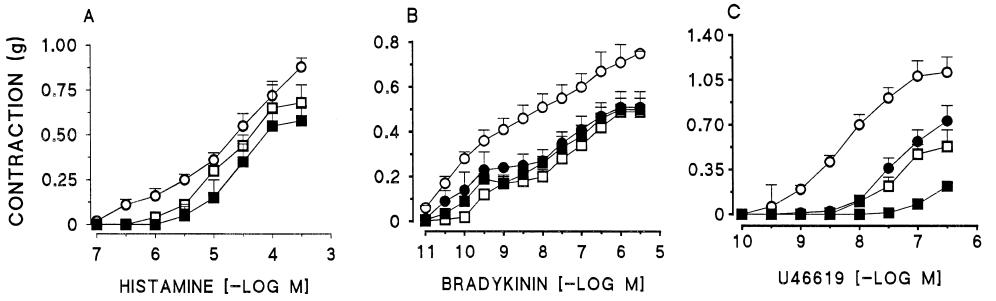

Fig. 3. Mean concentration response curves for histamine (A), bradykinin (B) and U46619 (C) in the guinea-pig trachea without epithelium, obtained in the absence (○) or in the presence of polygodial (A) 171 μM (□) and 342 μM (■); (B) 5 μM (●), 10 μM (□) and 20 μM (■); (C) 10 μM (●), 20 μM (□) and 40 μM (■). Values are means ± sem of 4 to 6 experiments.

Table 1
The mean EC_{50} values for several agonist-induced contractions in the guinea-pig trachea obtained in the absence or in the presence of the compound polygodial

Agonists	Concentration of polygodial (μM)							
	control	5	10	20	40	80	171	342
Histamine (μM)	12.6 (4.3–20.7)[a]	—	—	—	—	—	31.6 (18.0–47.0)	50.1[b] (41.0–59.0)
Bradykinin (nM)	0.4 (0.1–0.8)	50.1 (26.9–85.1)	79.4 (43.0–130.0)	158.5 (82.0–243.0)	—	—	—	—
Substance P (nM)	31.6 (16.4–51.3)	—	—	63.1 (37.3–88.1)	79.4 (41.6–112.4)	316.2[b] (158.6–475.0)	—	—
[β-Ala^8]NKA (4–10) (nM)	31.6 (23.0–40.5)	100.0[b] (90.5–111.0)	501.2[b] (493.0–507.0)	502.3[b] (495.0–509.0)	—	—	—	—
Substance P methyl ester (nM)	39.8 (20.2–51.8)	—	—	—	—	—	—	63.1 (37.2–96.7)

[a] 95% confidence limits. Each group represents the values (means $\pm$ sem) of 4 to 6 experiments.
[b] Significant difference, $P < 0.05$.

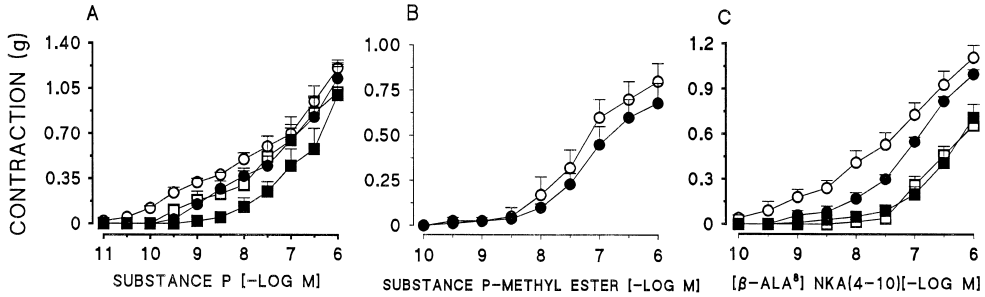

Fig. 4. Mean concentration response curves for substance P (A), substance P-methyl ester (B) and [β-ala^8] neurokinin A-(4–10) (C) in the guinea-pig trachea without epithelium, obtained in the absence (○) or in the presence of polygodial (A) 20 μM (●), 40 μM (□) and 80 μM (■); (B) 342 μM (●); (C) 5 μM (●), 10 μM (□) and 20 μM (■). Values are means ± sem of 4 to 6 experiments.

the tonus of the preparations or on agonist-induced contraction.

2.5. Statistical analysis

Data are presented as the means ± S.E.M. The EC_{50} or IC_{50} values (i.e. the concentration of agonists causing half maximal response or the concentration of the polygodial required to inhibit agonist-mediated contractions to 50% of the control response, respectively) were determined from individual experiments for complete agonists or antagonist concentration–response curves by use of least-square regression analysis. Unpaired Student's '*t*'-test was used for statistical analysis. *P* value less than 0.05 ($P < 0.05$) was considered as indicative of significance.

3. Results

The results presented in Fig. 2 (A, B and C) show that the pre-incubation of the guinea-pig ileum with polygodial (5 to 128 μM) for 20 min, which did not affect the tone of the preparations, resulted in a contraction-dependent inhibition of the acetylcholine, histamine (1 nM to 10 μM) and bradykinin (0.1 nM to 1 μM)-induced contractions, associated in some cases with a rightward displacement of the concentration–response curves. The calculated mean IC_{50} values (and their respective 95% confidence limits) for polygodial were: 87.0 (72.0 to 99.4), 41.6 (30.8 to 52.6) and 13.2 (4.5 to 22.0) μM, respectively. In addition, polygodial (4–12 μM) also caused concentration-dependent inhibition of KCl (1–100 mM)-induced contraction in the guinea pig ileum (results not shown). The calculated mean IC_{50} value was 6.0 (2.5 to 13.0) μM.

When analysed in the guinea-pig trachea, polygodial (171 to 342 μM) caused partial inhibition of histamine (100 nM to 100 μM)-mediated contraction (Fig. 3A and Table 1). On the other hand, at much lesser concentrations, polygodial (5 and 10 μM) caused marked inhibition of the contractile concentration–response curves induced by bradykinin (10 pM to 1 μM) or U 46619 (0.1 to 1000 nM) (Fig. 3B and C and Table 1). However, the action of polygodial against bradykinin-mediated contraction was not concentration dependent. The calculated mean IC_{50} value for polygodial in antagonising U 46619-mediated contraction was 30.0 (17.0 to 39.0) μM. Polygodial (20 to 80 μM) caused a discrete, but significant displacement to the right of substance P-induced contraction without interfering with the maximal response for this peptide in the guinea-pig trachea (Fig. 4A and Table 1). A much lower concentration of polygodial (5 to 20 μM) provoked a displacement to the right of the contractile concentration–response curve caused by the selective agonist of tachykinin NK_2 receptor [β-Ala^8]neurokinin A-(4–10) (Fig. 4C and Table 1). At a concentration up to 432 μM, polygodial had no significant effect against contraction caused by the selective tachykinin NK_1 receptor agonist, substance P methyl ester, in the guinea-pig trachea (Fig. 4B and Table 1). Also polygodial (85–342 μM) inhibited, in a concentration-dependent manner, KCl (1–100)-induced contraction in guinea pig trachea (results not shown). The calculated mean IC_{50} value was 258.3 (70.5 to 360.1) μM.

When tested in the guinea-pig trachea from animals which had been actively sensitised to ovalbumin, polygodial (30 to 40 μM) caused time and concentration-dependent inhibition of ovalbumin-mediated contraction (maximal inhibition of 96 ± 7%) (Fig. 5A). In addition, polygo-

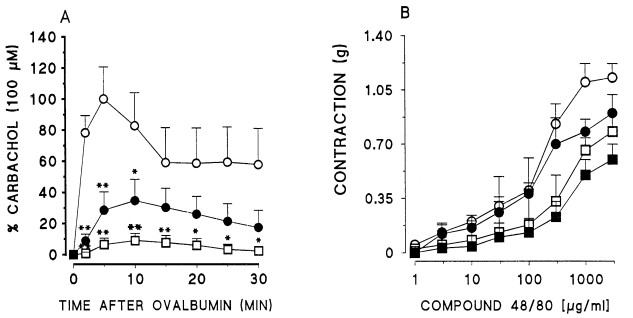

Fig. 5. (A) Mean time-dependent contractile response induced by ovalbumin in the guinea-pig trachea with epithelium obtained from animals actively sensitised to ovalbumin. (B) Mean concentration response curve for compound 48/80 in the guinea-pig trachea from non-sensitized animals without epithelium, obtained in the absence (○) or in the presence of polygodial (A) 30 μM (●) and 40 μM (□); (B) 85 μM (●); 171 μM (□) and 342 (■). Values are means ± sem of 4 to 6 experiments. The maximal developed tensions for ovalbumin and carbachol were 2.10 ± 0.41 g and 1.93 ± 0.16 g, respectively.

dial (85 to 342 μM) caused significant inhibition of the maximal response followed by displacement to the right of the contractile concentration–response curve induced by the compound 48/80 (1 to 1000 μg/ml) in guinea-pig trachea from non-sensitised animals (Fig. 5B). The antagonistic effects caused by polygodial in both preparations were easily reversed following the renewal of the nutrient solution over a period of 30 to 60 min (results not shown).

4. Discussion

Chemical studies carried out with *D. winteri* and related species have revealed the presence of many constituents, mainly sesquiteponoid compounds and flavonoids (Cruz and Silva, 1973; Cortés and Oyarzun, 1981; Torres et al., 1992; Brown, 1994).

The results presented in this study have revealed, for the first time, that the sesquiterpenoid polygodial, a major constituent isolated from the barks of *D. winteri*, exhibits an interesting pharmacological profile when assessed in the guinea-pig ileum and trachea 'in vitro'. Confirming and extending our previous observations carried out with the hydroalcoholic extract of *D. winteri* (El Sayah et al., 1997), the pre-incubation of the preparations with micromolar concentrations of polygodial results in marked, concentration-dependent, and reversible antagonistic effect of the contractions induced by several mediators known to participate in the aetiology of asthma, allergy and other inflammatory processes (see for review: Emanuel and Howarth, 1995; Frossard and Fajac, 1995; Goldie and Pedersen, 1995; Bertrand and Geppetti, 1996).

Consistent with our previous data reported for the extract of *D. winteri*, polygodial interacts, through different mechanism of action, with several inflammatory mediator-mediated contractions in guinea-pig ileum and trachea, namely bradykinin, U46619, and tachykinins by activation of NK_2 but not NK_1 receptors. In relation to the contractile response elicited by acetylcholine, histamine and bradykinin in the guinea-pig ileum the action of polygodial occurs by a typical non-competitive mechanism. However, polygodial produced a marked displacement to the right together with inhibition of the maximal responses of contractions induced by bradykinin and U46619 in the guinea-pig trachea. As demonstrated for the hydroalcoholic extract of *D. winteri*, polygodial caused a rightward displacement of the concentration response curves for tachykinins in the guinea-pig trachea. The antagonistic effect of polygodial against tachykinin-mediated contractions in guinea-pig trachea was quite selective toward tachykinin NK_2 receptor-mediated responses, since at much greater concentration polygodial had no significant effect against contraction induced by the selective tachykinin NK_1 receptor agonist, substance P methyl ester. There is an extensive literature which demonstrates that tachykinin, acting essentially through NK_1 and NK_2 receptors, determines contraction, plasma extravasation, mucus secretion, vasodilatation, and increase in microvascular permeability with accompanying plasma exudation (Barnes et al., 1990; Maggi et al., 1995). In guinea-pig upper airways (trachea and main bronchi) suggestion has been made that the neurogenic plasma extravasation is mediated via tachykinin NK_1 receptors. In contrast, bronchoconstriction in guinea-pig and human airways induced by tachykinins is mediated principally by NK_2 receptors (Ellis et al., 1993; Tousignant et al., 1993; Rodger et al., 1995; Boichot et al., 1996).

Also relevant is the antagonistic action exhibited by polygodial against contractions elicited by bradykinin and the stable analogue of thromboxane A_2, U46619 in the guinea-pig trachea. In contrast to the partial displacement to the right reported for polygodial against responses mediated by tachykinin NK_2 selective agonist, this action against both bradykinin and U46619 involves an apparent non-competitive mechanism of action. Similar properties have been demonstrated previously by the hydroalcoholic extract of *D. winteri* (El Sayah et al., 1997). As both bradykinin and thromboxane A_2 also have a role in asthma and allergy (Barnes, 1992; Kawikova et al., 1993; Bertrand and Geppetti, 1996), such a results may also account for the reported beneficial action of infusion of *D. winteri* in folk-medicinal treatment of asthma and allergy (Morton, 1981). On the other hand, contraction elicited by histamine in guinea-pig trachea was only slightly affected by polygodial, thus confirming our previous findings for the crude extract of this plant (El Sayah et al., 1997). The fact that polygodial, at a concentration similar to that which caused inhibition of agonist-mediated contractions in both guinea-pig ileum and trachea, also concentration-dependently attenuated contraction caused by KCl in both preparations, strongly suggests a non-specific mechanism of action, most certainly by inhibiting calcium influx across the cell membrane. These results may explain, at least in part, the non-competitive action of this sesquiterpene in antagonising contractions elicited by some of the inflammatory mediators in guinea-pig ileum and trachea smooth muscles.

The demonstration that polygodial, at a similar concentration where it antagonises contraction induced by several mediators of asthma, allergy and inflammation, inhibited in a time and concentration-dependent and reversible manner the contraction elicited by ovalbumin in guinea-pig trachea from actively sensitised guinea pigs, seems to be of interest. A very similar effect has been demonstrated previously by using the crude extract of this plant (El Sayah et al., 1997). Also consistent with our previous observations reported for the extract of *D. winteri*, polygodial at similar concentration caused a concentration-dependent reduction of maximal response associated with displacement to the right of the concentration–response curve induced by compound 48/80. As reported for the crude extract, although polygodial has little effect against histamine-mediated contraction, at high concentrations possesses the ability to

inhibit the histamine release caused by compound 48/80, an effect which may also account, at least in part, for the reported beneficial anti-allergic and anti-asthmatic actions of this plant in folk medicine.

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