J. Pharm. Pharmacol. 1995, 47:846–851
Received February 6,1995
Accepted July 10, 1995

Analysis of the Mechanisms Underlying the Contractile Response Induced by the Hydroalcoholic Extract of *Phyllanthus urinaria* in the Guinea-pig Urinary Bladder In-vitro

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Abstract

The hydroalcoholic extract of *Phyllanthus urinaria* (Euphorbiaceae), substance P and substance P methyl ester all caused graded contractions in the guinea-pig urinary bladder. Responses to hydroalcoholic extract and substance P were markedly inhibited in calcium-free Krebs solution, this effect being reversed by reintroduction of calcium in the medium.

The contraction in response to hydroalcoholic extract was unaffected by atropine, propranolol, prazosin, yohimbine, tetrodotoxin, w-conotoxin, nicardipine, HOE 140, guanethidine, staurosporine, phorbol ester or indomethacin, excluding the involvement of nervous mediated responses, or action via cholinergic, adrenergic, kinins, cyclo-oxygenase metabolites, protein kinase C or activation of L or N-type calcium channels. The selective NK_1 tachykinin antagonist (FK 888), but not NK_2 (SR 48968) antagonized substance P-induced contraction, but both drugs failed to effect *Phyllanthus urinaria*-induced contraction. Prolonged desensitization of guinea pig urinary bladder with capsaicin (10 μM) or pre-incubation of guinea-pig urinary bladder with capsazepine did not affect contraction caused by hydroalcoholic extract. Ruthenium red almost completely abolished capsaicin-induced contraction, but had no effect on hydroalcoholic extract-mediated contraction. Substance P and the hydroalcoholic extract caused marked potentiation of the twitch response in the preparations field stimulated. The facilitatory effect of substance P, but not that of hydroalcoholic extract, was prevented by the NK_1 (FK 888), but not by NK_2 (SR 48968) antagonist.

We concluded that contraction induced by hydroalcoholic extract of *Phyllanthus urinaria* in the guinea pig urinary bladder involves direct action on smooth muscle and relies on the mobilization of extracellular calcium influx unrelated to activation of L- and N-type calcium channels or activation of protein kinase C mechanisms. In addition contraction caused by the hydroalcoholic extract of *Phyllanthus urinaria* in guinea-pig urinary bladder does not involve the activation of tachykinin or vanilloid receptors.

The plants belonging to the genus *Phyllanthus* (Euphorbiaceae) are widely distributed throughout the tropical and subtropical countries and are used in folk medicine for the treatment of several diseases, including disturbancies of kidney and bladder calculi, intestinal infections, diabetes and hepatitis B virus (Morton 1981; Oliver-Bever 1983; Unander et al 1990, 1991, 1992). There is now a great amount of evidence based on biochemical, pharmacological and clinical studies that confirm, at least in part, the mentioned medicinal uses of some species of the genus *Phyllanthus* (Calixto et al 1984; Venkateswaran et al 1987; Thyagarajan et al 1988, 1990; Blumberg et al 1989; Ogata et al 1992; Shead et al 1992).

We have previously shown that the hydroalcoholic extract of *Phyllanthus corcovadensis* (Gorski et al 1993), as well as the methanolic extract of callus culture of some species of *Phyllanthus* (Santos et al 1994), the hydroalcoholic extract of *Phyllanthus urinaria, Phyllanthus niruri, Phyllanthus tenellus* and *Phyllanthus sellowianus* (Santos et al 1995a), and some phytosteroids isolated from *Phyllanthus corcovadensis* (Santos et al 1995b), exhibited potent and dose-related systemic antinociceptive effect when tested in several models of nociception in mice, especially against neurogenic pain. However, the mechanism responsible for such effects still remains poorly understood.

The present study was designed to investigate some of the mechanisms underlying the contractile response induced by hydroalcoholic extract of *Phyllanthus urinaria* in the guinea-pig isolated urinary bladder by the use of selective agonist and antagonists and ion-channel blockers.

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Material and Methods

Preparation of the crude extract

Botanical material was collected and classified by Dr Leila da Graça Amaral and Ms Mirian Ulyssea (Department of Botany, Universidade Federal of Santa Catarina). The dried leaves, stems and roots of *Phyllanthus urinaria* were minced and extracted with 50% ethanol-water in the proportion of 1 : 3 (w/v), being stirred and macerated at room temperature (21 ± 3°C) for 15 days. The ethanol was evaporated and the extract was concentrated to the desired concentration and

stored at −20°C. The extracts were dissolved in 0·9% NaCl solution at the desired concentration just before use.

Tissue preparation

Guinea-pigs of both sexes, 200–350 g, were killed by a blow on the head and were exanguinated from carotid arteries. The urinary bladder was isolated, and 3 or 4 strips about 10–12 mm long and 3–4 mm wide were obtained from each animal. Preparations were suspended in 5-mL organ chambers containing Krebs–Henseleit solution (composition, in mM: NaCl 118·0; KCl 4·4; $MgSO_4$ 1·1; $CaCl_2$ 2·5; $NaHCO_3$ 25·0; KH_2PO_4 1·2 and glucose 11·0), maintained at 37°C and pH 7.4, gassed with 95% O_2–5% CO_2. Isometric tension changes were recorded by means of a force transducer (TRI–201–Letica Scientific Instuments), under a basal tension of 1·0 g.

Preparations were allowed to equilibrate for at least 60 min before drug addition, during which the bath solution was changed every 20 min. All experiments with substance P and analogue were carried out in the presence of captopril (3 μM) and phosphoramidon (1 μM), to prevent the action of proteases. The contractile responses are expressed in grams of tension.

Following the equilibration period, complete non-cumulative concentration–response curves were performed for hydroalcoholic extract (0·03–3·0 $\mu g\,mL^{-1}$), substance P (0·1 to 100 nM) or for substance P methyl ester (a selective NK_1-receptor agonist, 0·3 to 1000 nM), at 20-min intervals between doses and 60-min intervals between curves. The contact time of the agonists or the hydroalcoholic extract was 3–5 min. Only one agonist was tested in each preparation. To correct for any spontaneous or agonist-induced changes in the contractile response to the agonists, control experiments were carried out in the presence of saline (0·9% NaCl).

Effects of different classes of drugs on the contractile responses to extract of Phyllanthus urinaria *and substance P*

To assess the possible mechanism of action of the hydroalcoholic extract of *Phyllanthus urinaria*, following 60-min equilibration, in a new series of experiments, single doses of hydroalcoholic extract (2 $mg\,mL^{-1}$) or substance P (20 nM) were added to the bath in the absence or in the presence of one of the following drugs, each added to the bath 10–30 min beforehand: atropine (a muscarinic receptor antagonist, 1·0 μM), prazosin (an α_1-adrenergic receptor blocker, 0·1 μM), yohimbine (an α_2-adrenergic receptor antagonist, 0·1 μM), tetrodotoxin (a Na^+-channel blocker, 0·1 μM), staurosporine (an inhibitor of protein kinase C, 0·1 μM), indomethacin (a cyclo-oxygenase inhibitor, 3 μM), HOE 140 (a β_2-receptor antagonist, 1 μM), guanethidine (a noradrenaline depletor, 3·0 μM), nicardipine (an antagonists of L-type calcium channels, 0·1 μM), w-conotoxin (an antagonist of N-type calcium channels, 0·1 μM), phorbol 12 myristate 13-acetate (an activator or protein kinase C, 1 μM), FK 888 (a selective NK_1 receptor antagonist, 1 μM), SR 48968 (a selective NK_2 receptor antagonist, 1 μM), ruthenium red (a dye which behaves as a functional antagonist of capsaicin, 3 μM) or capsazepine (a selective capsaicin-receptor antagonist, 1 μM).

Influence of extracellular calcium

To investigate the influence of extracellular calcium on hydroalcoholic extract- or substance P-induced contraction in guinea-pig urinary bladder, after obtaining stable contractions to the agonists in normal medium, preparations were transferred to Krebs–Henseleit solution without calcium, containing 1 mM of EGTA, for 20 min, during which the bath solution was renewed every 5 min. After this, the tissues remained 10 min in a calcium-free solution without EGTA, and new responses to hydroalcoholic extract or substance P were obtained.

Field stimulation

In a separate series of experiments, preparations were submitted to field stimulation with trains of rectangular pulses of 0·6-ms duration and supramaximal voltage at 10 Hz for 0·5 s every 10 s, delivered transmurally via platinum electrodes. After complete stabilization of twitch responses, the hydroalcoholic extract (2 $mg\,mL^{-1}$) or substance P (20 nM) was added to the bath for 5 min at 20-min intervals between doses in the absence or in the presence of NK_1 or NK_2 tachykinin antagonists.

Drugs

The drugs used were: substance P, substance P methyl ester, captopril, EGTA (ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*- tetra-acetic acid), indomethacin, yohimbine hydrochloride, ruthenium red (ammoniated ruthenium oxychloride), phosphoramidon, tetrodotoxin, staurosporine, nicardipine, w-conotoxin GVIA, guanethidine, prazosin, phorbol 12-myristate 13-acetate, PBS (phosphate-buffered saline, concentration: NaCl 137 mM, KCl 2·7 mM and phos-

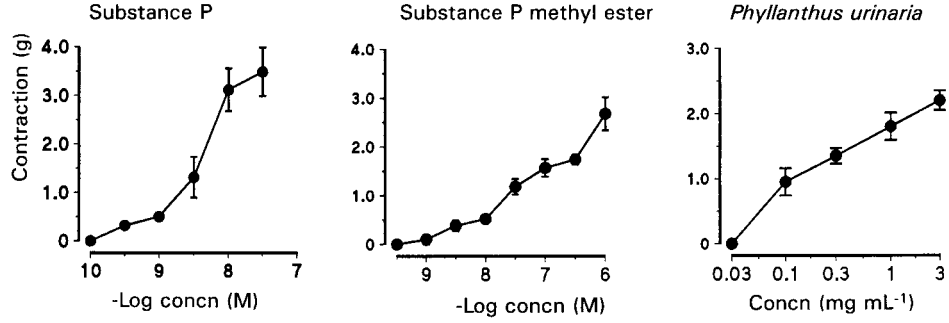

FIG. 1. Mean concentration response curves for substance P, substance P-methyl ester and for hydroalcoholic extract from *Phyllanthus urinaria* in the guinea-pig isolated urinary bladder. Values are mean ± s.e. mean of 4 to 6 experiments.

Table 1. Effect of calcium-free medium plus EGTA on substance P (20 nM)- or extract from *Phyllanthus urinaria* (2 mg mL^{-1})-induced contraction (g) in guinea-pig isolated urinary bladder.

Agonist	Control	Calcium-free	Recovery
Phyllanthus urinaria	1·50 ± 0.22	0·54 ± 0·16**	1·46 ± 0·30
Substance P	1·48 ± 0·14	0·07 ± 0·05**	1·30 ± 0·18

Each group represents the mean ± s.e. mean of 4 to 6 experiments. **$P < 0·01$ compared with control.

phate buffered 10 mM) (all from Sigma Chemical Co., St Louis, MO, USA), HOE 140 (D-Arg-[Hyp^3, Thy^5 D-Tic^7, Oic^8]-BK) (Hoechst, Frankfurt, Germany), atropine sulphate (Merck, Germany), SR 48968 ([*S*])-*N*-methyl-*N*-(4-acetylamino-4-phenylpiperidino-2-(3,4-dichlorophenyl) butyl) benzamide, Sanofi Recherche, Montpellier, France), capsazepine (Sandoz Institute for Medical Research, London, UK), capsaicin (Calbiochem, San Diego, CA, USA), [β-ala^8] neurokinin A-(4–10) (Peninsula Laboratories, Inc., Belmont, CA, USA), FK888 ((4*R*)-4-hydroxy-1-((1-methyl-1H-indol-3-yl) carbonyl)-L-prolyl-*N*-benzyl-*N*-methyl-3-(2-naphthyl)-L-alaninamide, Fujisawa Pharmaceutical Co., Osaka, Japan), dimethyl sulphoxide (Veteq, RJ, Brazil).

Stock solutions of the following drugs (1–100 mM) were prepared and maintained at −20°C in water or PBS solutions: substance P, substance P-methyl ester, ruthenium red, w-conotoxin GVIA, HOE 140, captopril, [β-ala^8] neurokinin A-(4-10), tetrodotoxin, guanethidine, phosphoramidon and atropine. Indomethacin, yohimbine, prasozin, capsaicin, SR 48968, capsazepine, FK 888, and nicardipine were prepared in absolute ethanol. Staurosporine was prepared in dimethylsulphoxide 50%. EGTA was added directly to the Krebs–Henseleit solution. The final bath concentration of ethanol or dimethylsulphoxide did not exceed 0·03% and had no effect on the responses to the agonists or to field stimulation. Solution and experiments with nicardipine were protected from light to avoid degradation.

Statistical analysis

Data are presented as the means ± s.e.m., except for EC50 values (the concentration of agonists or the hydroalcoholic extract causing half-maximal response), which are given as geometric means accompanied by their respective 95% confidence limits. The EC50 values were determined from individual experiments for complete agonist concentration-response curves by using a least-square regression analysis. Statistical analysis of the data was by unpaired Student's *t*-test. A *P* value less than 0·05 was considered significant.

Results

The results (Fig. 1) show that addition of hydroalcoholic extract of *Phyllanthus urinaria* (0·03–3 mg mL^{-1}), like substance P (0·1–100 nM) or substance P-methyl ester (0·3 to 1000 nM), caused a concentration-dependent contraction of the guinea-pig urinary bladder. Response to hydroalcoholic extract was reproducible with no evidence of tachyphilaxis (results not shown). When the preparations were transferred to similar Krebs solution, but free of calcium, the contractile response induced by equi-effective concentrations of substance P (20 nM) and hydroalcoholic extract of *Phyllanthus urinaria* (2 mg mL^{-1}) were markedly reduced (Table 1). The reintroduction of calcium to the Krebs solution completely restored the response to both substance P and hydroalcoholic extract (Table 1). The contraction elicited by the hydroalcoholic extract was not influenced by atropine (1 μM), tetrodotoxin (0·1 μM), HOE 140 (1 μM), indomethacin (3 μM), yohimbine (0·1 μM), prazosin (0·1 μM), guanethidine (3 μM), nicardipine (0·1 μM), w-conotoxin (0·1 μM), phorbol 12-acetate 13-myristate (1 μM) or staurosporine (0·1 μM) (Table 2).

Pretreatment of the guinea-pig urinary bladder with FK 888 (1 μM) caused a significant inhibition of substance P (20 nM)-induced contraction, leaving response to hydroalcoholic extract of *Phyllanthus urinaria* (2 mg mL^{-1}) unaffected (Table 3). In contrast, incubation of the preparations with the selective NK_2 antagonist SR 48968 (1 μM) did not cause any significant inhibition against either substance P or the hydroalcoholic extract-induced contraction (Table 3). However, SR 48968 (1 μM) markedly antagonized the contractile response elicited by the selective NK_2 agonist [β-ala^8] neurokinin A-[4–10] (control response of 1·40 ± 0·08 g and 0·17 ± 0·09 g in the presence of the antagonist, $P < 0·01$, n = 4–8 experiments).

The prolonged desensitization of the preparations with capsaicin (10 μM, for 30 min) did not significantly affect the contractile response induced by the hydroalcoholic extract of *Phyllanthus urinaria* (2 mg mL^{-1}) (Table 3). Preincubation of preparations with ruthenium red (3 μM) or with

Table 2. Effect of various drugs on the contractile responses induced by extract from *Phyllanthus urinaria* (2 mg mL^{-1}) in strips of guinea-pig isolated urinary bladder.

Antagonist	Concentration (μM)	Absence (g)	Presence (g)
Tetrodotoxin	0·1	1·12 ± 0·16	1·27 ± 0·13
Atropine	1·0	1·27 ± 0·13	1·04 ± 0·14
Indomethacin	3·0	1·04 ± 0·14	0·83 ± 0·22
Prazosin	0·1	1·06 ± 0·22	1·52 ± 0·27
Yohimbine	0·1	1·52 ± 0·27	1·36 ± 0·27
Guanethidine	3·0	1·36 ± 0·27	1·39 ± 0·32
HOE 140	1·0	1·17 ± 0·14	1·32 ± 0·20
w-Conotoxin	0·1	1·23 ± 0·06	1·21 ± 0·10
Staurosporine	0·1	1·32 ± 0·20	1·42 ± 0·17
Phorbol ester	1·0	1·57 ± 0·47	1·70 ± 0·35
Nicardipine	0·1	1·03 ± 0·05	1·33 ± 0·30

Each group represents the mean ± s.e. mean of 4 to 6 experiments.

Table 3. Effect of various drugs and capsaicin desensitization upon *Phyllanthus urinaria* ($2\,mg\,mL^{-1}$)-, substance P (20 nM)- and capsaicin (10 μM)- induced contraction in the guinea-pig isolated urinary bladder.

Drug (μM)	*P. urinaria* (g)		Substance P (g)		Capsaicin (g)	
	Absence	Presence	Absence	Presence	Absence	Presence
FK 888 (1)	1·16 ± 0·10	1·20 ± 0·10	1·05 ± 0·05	0·27 ± 0·05**	—	—
SR 48968 (1)	2·07 ± 0·23	2·22 ± 0·23	1·83 ± 0·09	1·66 ± 0·13	—	—
Ruthenium red (3)	1·21 ± 0·09	1·35 ± 0·22	—	—	1·36 ± 0·23	0·15 ± 0·16**
Capsazepine (1)	1·14 ± 0·20	1·47 ± 0·17	—	—	1·12 ± 0·17	0·07 ± 0·05**
Capsaicin (10)	1·46 ± 0·10	1·50 ± 0·17	1·39 ± 0·19	1·43 ± 0·11	1·52 ± 0·22	0·01 ± 0·01**

Each group represents the mean ± s.e. mean of 4 to 6 experiments. $^{**}P < 0.01$ compared with respective control.

Table 4. Effect of the selective NK_1 receptor antagonist FK 888 (1 μM) or NK_2-receptor antagonist SR 48968 (1 μM) on the potentiating effect caused by substance P (20 nM) or extract of *Phyllanthus urinaria* ($2\,mg\,mL^{-1}$) on twitch contractions induced by field stimulation of the guinea-pig urinary bladder.

Agonist	FK 888		SR 48968	
	Absence (g)	Presence (g)	Absence (g)	Presence (g)
P. urinaria	1·17 ± 0·08	1·21 ± 0·14	2·08 ± 0·22	2·22 ± 0·22
Substance P	1·02 ± 0·05	0·25 ± 0·04**	1·81 ± 0·07	1·66 ± 0·11

Each group represents the mean ± s.e. mean of 4 to 6 experiments. $^{**}P < 0.01$ compared with control.

capsazepine (1 μM) almost completely antagonized the contractile response induced by capsaicin (10 μM), but both drugs failed to affect the contraction caused by the hydroalcoholic extract of *Phyllanthus urinaria* ($2\,mg\,mL^{-1}$) (Table 3).

When preparations were electrically stimulated, addition of substance P (20 nM) or the hydroalcoholic extract of *Phyllanthus urinaria* ($2\,mg\,mL^{-1}$) caused a contraction of the smooth muscle followed by a potentiation of electrically driven twitch responses (results not shown). The potentiation caused by substance P (20 nM) but not that of hydroalcoholic extract ($2\,mg\,mL^{-1}$) was inhibited by FK 888 (1 μM) (Table 4). In contrast, SR 48968 (1 μM) affect neither contractions induced by substance P (1 μM) nor those caused by the hydroalcoholic extract of *Phyllanthus urinaria* (Table 4).

Discussion

The present results demonstrate that the hydroalcoholic extract from stems, leaves and roots of *Phyllanthus urinaria*, such as substance P and the selective NK_1 agonist substance P methyl ester, caused a sustained and reproducible concentration-dependent contractile response of the guinea-pig urinary bladder. In addition, both the hydroalcoholic extract of *Phyllanthus urinaria* and substance P caused a significant potentiation of electrically driven twitch tension. Both actions of *Phyllanthus urinaria* on guinea-pig urinary bladder were quite similar to that caused by substance P. Thus, the hydroalcoholic extract and substance P-induced contractions in guinea-pig urinary bladder were almost completely antagonized when preparations were transferred to calcium-free solution containing EGTA (1 mM). Importantly, the addition of calcium (2·5 mM) also completely reverted both hydroalcoholic extract- and substance P-mediated contractions. Preincubation of the preparations with tetrodotoxin, atropine, indomethacin, prazosin, yohimbine, guanethidine, HOE 140, nicardipine, w-conotoxin, phorbol ester or staurosporine, all failed to affect either *Phyllanthus urinaria* or substance P-induced contractions of guinea pig urinary bladder. These results exclude the participation of neural release of neurotransmitters, release of cyclo-oxygenase products from arachidonic acid pathway, activation of muscarinic, release of catecholamine and/or activation of α_1- and α_2-adrenergic receptors or kinin formation. Moreover, these findings also strongly suggest that the contractile responses elicited by hydroalcoholic extract of *Phyllanthus urinaria* and substance P rely largely on calcium influx from extracellular medium being response to the hydroalcoholic extract insensitive to both L- and N-type calcium channel blockers, nicardipine and w-conotoxin, respectively. In addition, both the hydroalcoholic extract- and substance P-mediated contractions seem not to be coupled to activation of a protein kinase C-dependent mechanism.

In spite of these similarities among the responses of hydroalcoholic extract of *Phyllanthus urinaria* and substance P, the selective NK_1-receptor antagonist FK 888 (Hagiwara et al 1993; Murai et al 1993), at concentrations which produced a pronounced inhibition of substance P-induced contraction or potentiation of electrical field-stimulated preparations, had no significant effect on the *Phyllanthus urinaria*-induced responses. In addition, the NK_2-selective receptor antagonist SR 48968 (Advenier et al 1992; Emonds-Alt et al 1992) significantly inhibited the contractile response induced by the selective NK_2 agonist [β-ala^8] neurokinin A-(4-10), leaving responses to hydroalcoholic extract and substance P unaffected, confirming previous

reported data concerning the presence of NK_2 receptors in the guinea-pig urinary bladder. Altogether, these findings provide strong evidence that the contraction as well as the facilitation of the electrically stimulated neurogenic responses in guinea-pig urinary bladder caused by hydroalcoholic extract of *Phyllanthus urinaria* do not involve the activation of either NK_1 or NK_2 receptors. However, our results do not exclude the possibility that the active principles of *Phyllanthus urinaria* might interact wih a novel subtype of receptor insensitive to the classical tachykinin antagonists. Recently, (Meini et al 1994) have provided pharmacological evidence that a NK_1-receptor subtype or a novel type of tachykinin receptor may exist in the rat urinary bladder. Preliminary unpublished results from our laboratory indicate that in contrast to that reported in the guinea-pig urinary bladder, the contraction caused by the hydroalcoholic extract of *Phyllanthus urinaria* of the epithelium-denuded strips of guinea-pig trachea was markedly antagonized by both NK_1- and NK_2-selective receptor antagonists, suggesting the existence of tissue differences in the mechanism of action of the active principle present in this plant.

We next investigated whether the hydroalcoholic extract of *Phyllanthus urinaria* interact with the vanilloid receptors reported to occur in the urinary bladder (Szallasi et al 1993; Szallasi 1994). The prolonged desensitization of the preparations with capsaicin failed to affect the hydroalcoholic extract of *Phyllanthus urinaria*-induced contractions. Furthermore, preincubation of the preparations with ruthenium red, at concentration known to inhibit capsaicin-mediated responses (Amann & Lembeck 1989; Maggi et al 1989; Dray et al 1990; Amann & Maggi 1991; Maggi et al 1993), almost completely abolished capsaicin-mediated contraction in the guinea-pig urinary bladder, but had no significant effect on the hydroalcoholic extract-induced contraction. Additionally, the selective capsaicin receptor antagonist capsazepine (Dickenson & Dray 1991; Bevan et al 1992) significantly prevented capsaicin-mediated contraction in guinea-pig urinary bladder, leaving the contractile response to hydroalcoholic extract of *Phyllanthus urinaria* unaffected. Taken together, these results provide consistent evidence that the contractile response induced by hydroalcoholic extract of *Phyllanthus urinaria* in the guinea-pig urinary bladder does not involve the activation of vanilloid receptor.

Acknowledgements

The authors are indebted to Dr Leila Amaral and Ms Miriam Ulyssea for botanical classification of *Phyllanthus urinaria*, and to Elizabete Ramos Ganzer for help in preparing the manuscript, to pharmaceutical companies for the kind gift of Hoe 140, FK 888, SR 48968 and capsazepine. This study was supported by grants from Financiadora de Estudos e Projetos (PADCT/FINEP), Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and Coordenacao de Aperfeicoamento de Pessoal de Ensino Superior (CAPES), Brazil. Marcos A. Dias and Alexandre H. Campos are undergraduate medical students receiving a grant from CNPq, and Valdir Cechinel Filho is a postgraduate student receiving a grant from CAPES.

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