Relaxant and antispasmodic effect in isolated guinea pig ileum treated with extracts of *Xylaria sp* an endophytic fungus of the Mexican yew, *Taxus globosa*

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Summary

Xylaria sp is known as endophytic microorganisms which grown in the Mexican yew, *Taxus globosa* schlec, that is the less known among the four native yews of the western hemishpere. The antispasmodic effect of hexane, chloroform and methanol extracts of *Xylaria* sp were studied *in vitro* on guinea pig ileum against three spasmogens, acetylcholine, histamine and barium chloride. The hexane extract produces a significant antispasmodic effect on the contractions of ileum induced by acetylcholine, histamine and barium chloride. The IC_{50} for each was 7.81, 5.46 and 10.32 mg/mL respectively. These results show that hexane extract of *Xylaria* sp possesses both anticholinergic and antihistaminic properties

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Introduction

Virtually all higher plants are hosts to a diverse community of microorganisms known as endophytic microorganisms. These organisms live in tissues in close association to living plant cells establishing relationships with the plant varying from symbiotic to nearly pathogenic (1). In many cases, establishment of a host–microbe relationship entails the presence of unusual bioactive compounds produced by endophytes which contribute to their host plant providing protection or even being crucial for survival to the plant (2). Novel bioactive compounds such as antibiotics, antimycotics, antioxidants and anticancer compounds are only some examples of what has been found after the isolation and culturing of individual endophytes (3-7). The Mexican yew, *Taxus globosa* schlec, is the less known among the four native yews of the western hemishpere. The tree could be found from the north of Mexico nearby the gulf of Mexico, to Honduras in Central America. In spite of its broad range, populations and individual yews are widely scattered, and located in very specific and relatively small microhabitats. Whereas other species of yew have been studied with considerable detail, primarily by their capacity to produce taxol (paclitaxel), a powerful antileucemic and antitumor agent, studies on *T. globosa* have been very scarce. Also, to our knowledge no endofitic microorganisms associated to this tree have been reported yet, no studied chemistry and pharmacology. The present study reports the antispasmodic activity of hexane, chloroform and methanol extracts of *Xylaria* sp on guinea pig ileum.

Material and Methods

Plant material

The fungus was isolated from a twig of one of the about 20 centennial Mexican yews sited at "El Chico" National Park at the State of Hidalgo, 114 km north of México city. The samples were collected in black bags previously damped

(slightly) with water and transported immediately to the laboratory. Twigs were washed with soap, soaked for 25 minutes in a solution of sodium hypochlorite 3 %, washed with sterilized water and seeded in Petri dishes containing Potato-Dextrose-Agar (PDA) medium. The fungus was isolated after 3 days of incubation at 20 ^{o}C. After 20 days of growth, microscopic analysis revealed small clear rhombic crystals of a substance unknown until now associated to mycelia.

Culture conditions

The static cultures of the fungus were carried out in Erlenmeyer flasks (250 ml) containing 100 ml of culture media with the following composition (g/l): glucose 1,0, fructose 3,0, saccharose 6,0, $MgSO_4.7H_2O$, 0,36, $Ca(NO_3)_2$ 4H2O 0.65, yeast extract 0.5, $ZnSO_4.7H_2O$ 0.0025, $MnCl_2.4H_2O$ 0.0005, $FeCl_2.4H_2O$ 0.002, phenylalanine 0,005, 1,0 sodium acetate and sodium benzoate 0,050, phosphates buffer solution 1 ml/l (pH 6.8). Flasks were inoculated with a small piece of agar of 0.5 x 0.5 cm, containing mycelium of the fungus and incubated for 20 days at 23^{o}C.

Taxonomic identification

After cultivating the fungus in the medium described, standard protocols for DNA extraction and amplification by polymerase chain reactions (PCR) were used (8). The ITS1-5.8S rDNA-ITS2-D1/D2 region of 26S rDNA gen was amplified using the primers 18IT (5'- CTTTGTACACACCGC CCGTC-3') and REPJL (5'-GCTCCGTGTTTCAAGACG-3') described previously by Fell, 1993 (9). PCR were performed using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City) with one denaturing step for 5 min at 92 ^{o}C; 35 cycles of 92^{o} for 1 min, 58^{o}C for 30 s, and 72^{o} for 1 min, with an additional 10-min extension at 72^{o} after cycling. The PCR amplicons were purified using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California, USA) according to manufacturer's protocols. Sequencing PCR utilized ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.) with the primers 18IT y REP described.

Analyses

Partial sequences were analyzed using the GenBank BLAST ver. 2.2.3 (10) for sequence similarity search. Multiple alignments were performed with CLUSTAL X and related sequences selected from GeneBank (TaxBrowser). Phylogenetic trees were developed by the distance method using MEGA v. 3.1 software (11). The matrix of nucleotide similitude was calculated with the two parameter Kimura model, the clusters were estimated by neighbor-joining with 1000 Bootstrap type resamplings (12). The nearest sequence was used for taxonomic level assignation to the studied sequence.

Extract preparation

The culture broth of several flasks was poured into the bowl of an Osterizer stirrer and homogenized for 3 minutes, following the homogenate was lyophilized. Microfungus material (50 g) was soxhlet extracted using hexane, chloroform, methanol and water consecutively for 3h. Each extract was concentrated in a rotary evaporator under reduced temperature and pressure. The % yields of hexane, chloroform, methanol and water were 3.2, 1.6, 34.6 and 41.0% respectively. Extracts were sonicated before addition to the organ bath, acetylcholine (Ach), histamine and adrenaline were prepared by adding the substance directly to Tyrode solution.

Biological experimental procedures

Animals

Male guinea pigs (250 to 400 g) were used for all experiments. The animals were housed in a cage under conditions of standard light (light on from 7.0 a.m. to 7.0 p.m.), temperature (22±1˚C) and room humidity (60±10%) conditions for one week before the experimental sessions. The animals were given a commercial feed prepared by Purina and allowed tap water *ad libitum*. The procedures involving animals and their care conformed to the international guidelines Principles of Laboratory Animals Care.

Tissue preparation

Male guinea pigs (250 to 400 g) were sacrificed by a blow to the base of the skull and cervical dislocation and 2 cm pieces of the ileum were dissected from ileum segment 10 to 20 cm proximal to the ileocecal valve. Material was mounted for tension recording and allowed to equilibrate for 1-2 h in 10-ml chambers containing Tyrode solution [composition (mM): 136.0 NaCl, 5.0 KCl, 0.98 $MgCl_2$, 2.0 $CaCl_2$, =.36 NaH_2PO_4, 11.9 $NaHCO_3$, and 5.5 glucose], pH 7.4 maintained at 37 °C and bubbled with air (5% CO_2 and 95% oxygen). In solution with elevated $[K]^+$, $[Na]^+$ was simultaneously decreased to maintain isosmolarity (13). Concentration-effect curves for extracts were performed by cumulative addition to the bath. In experiments examining the relaxation of the basal tonus of the ileum, paired segments of ileum were set up; one piece exposed to the extract and other receiving no treatment. Relaxation was taken to be difference between the tonus of control and test segments for recording the contractions using force traducers connected to a polygraph (Grass D) as previously described (14).

Measurement of contractile activity

After stabilization 30 min the test extracts were added to the bath. The extracts, were dissolved in dimethylsulfoxide (DMSO, Merck). In control preparations of DMSO up to 100 µl was added to the organ bath to determine whether this vehicle alone was able to induced contractions. Next, antispasmodic effect was investigated according to the following experimental schedule:

(a) Hexane, chloroform and methanol extracts at concentrations of 5, 10, 20 and 30 µg/ml organ bath: 15 min contact period.

(b) When stable submaximal responses to standard agonist histamine 3×10^{-7} M, acetylcholine 10^{-8} M, and barium chloride 10^{-4} M were obtained the extracts, were added into the bath (15). Percentage inhibition of histamine, acetylcholine or barium chloride induced contraction, in the presence of extracts, were calculated for each concentration (16).

c) The antispasmodic activity of extract was compared with the standard antispasmodic agent, papaverine (1.0 - 6 µg/ml), (17).

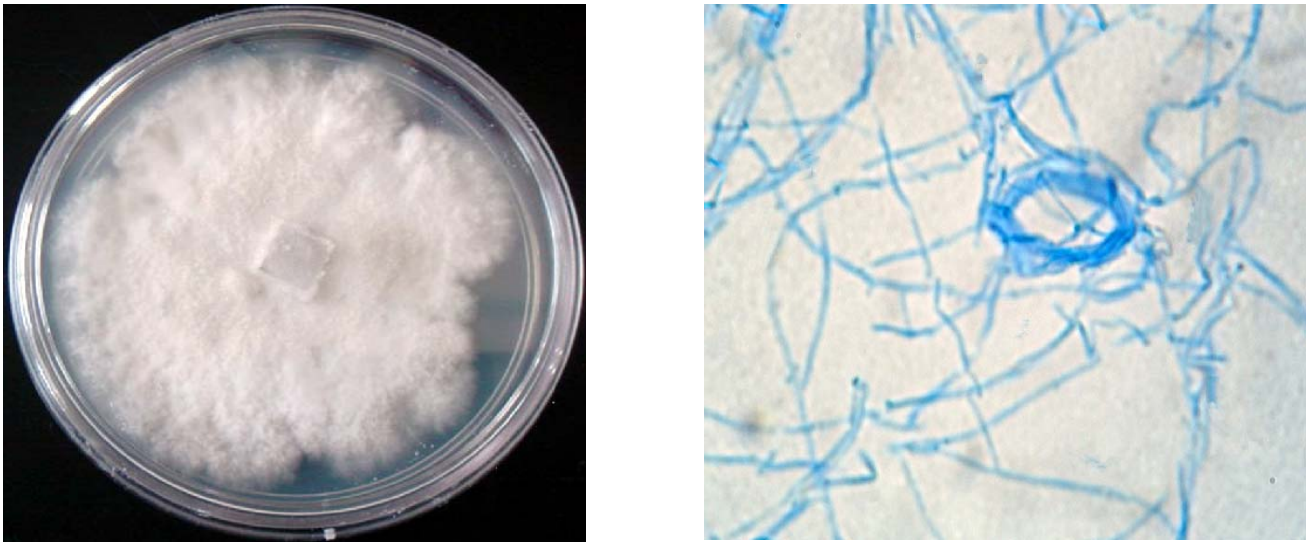
d) The median inhibitory concentration or IC_{50} was determined from the graph plotted of % inhibition versus log dose.

Statistical analysis

The inhibition of ileal contractions by extracts was expressed as the percentage of basal value (mean ± SEM). Regression methods used for statistical analysis and critical significance was set at $p< 0.05$.

Results

Xylaria sp is showed in photography 1.



Photography 1: *Xylaria* sp

The concentration of extracts which inhibited 50% of response (median inhibitory concentration) IC_{50} was determined from the graph plotted of % inhibition versus log dose. Addition of hexane extract of *Xylaria* sp (5-30 µg/mL) elicited a progressively increasing relaxation of the spontaneous tonus of the ileum with IC_{50}

= 11.50 µg/mL (c.l.: 10.1-15.2 µg/mL, n = 6) (Fig. 1). In a preliminary screening the histamine induced contraction in rat ileum with IC_{50} = 22 µg/mL (c.l.: 12-28 µg/mL, n = 6), acetylcholine with IC_{50} = 27 µg/mL (c.l.: 15-30 µg/mL, n = 6), and barium chloride with IC_{50} = 48 µg/mL (c.l.: 25-52 µg/mL, n = 8). IC_{50} for papaverine, used as a reference compounds, were 3.4 µg/mL (c.l.: 1.2-4.5 µg/mL, n = 6), for histamine, 3.8 µg/mL (c.l.: 2.1-5.1 µg/mL, n = 6), for acetylcholine and 3.0 µg/mL (c.l.: 1.8-4.2 µg/mL, n = 6) for barium chloride induced contractions respectively.

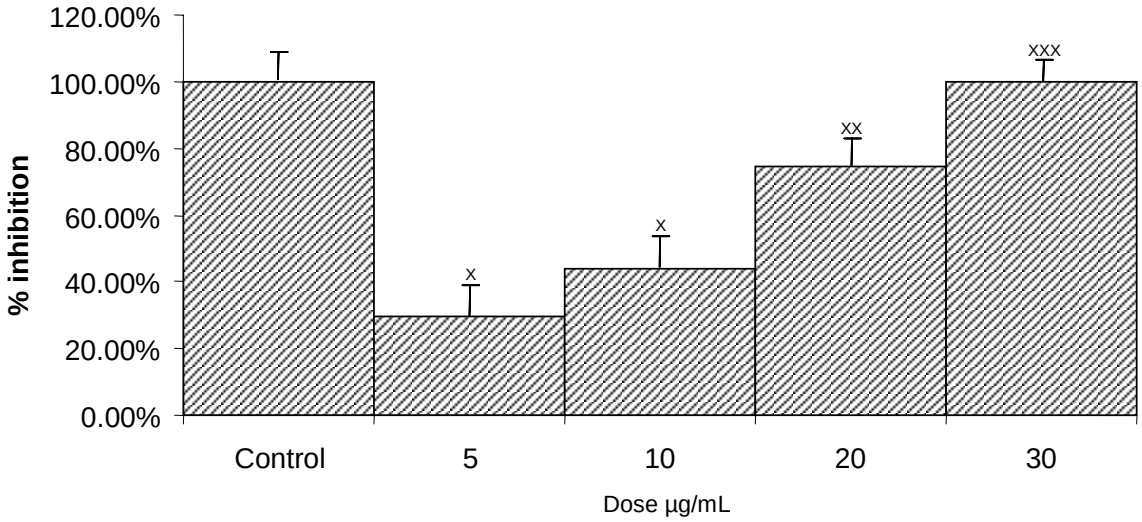


Fig. 1.- The inhibitory effects of the hexane extract of *Xylaria* sp (5, 10, 20 and 30 µg/mL) on spontaneous contraction of Isolated guinea - pig ileum. Results are expressed as mean ± S.E.M. *p<0.05, ** p<0.01 , *** p<0.001 (n=6)

Hexane extract of *Xylaria* sp showed a concentration-dependent inhibition of tone and the amplitude of spontaneous contraction of rat ileum acetylcholine with a IC_{50} = 7.81 µg/mL (c.l.: 5.1-10.1 µg/mL, n = 6), IC_{50} = 5.46 µg/mL (c.l.: 3.26-6.56 µg/mL, n = 6) for histamine, and IC_{50} = 10.32 µg/mL (c.l.: 8.42-11.52 µg/mL, n = 6) for barium chloride. The antispasmodic effect is shown in the Figures 2, 3 and 4. Contrasting, the methanol and $CHCl_3$ extracts did not show any inhibitory effects. Hexane extract was found to antagonize contractions of the rat ileum induced by acetylcholine, histamine and barium chloride in a concentration-dependent way.

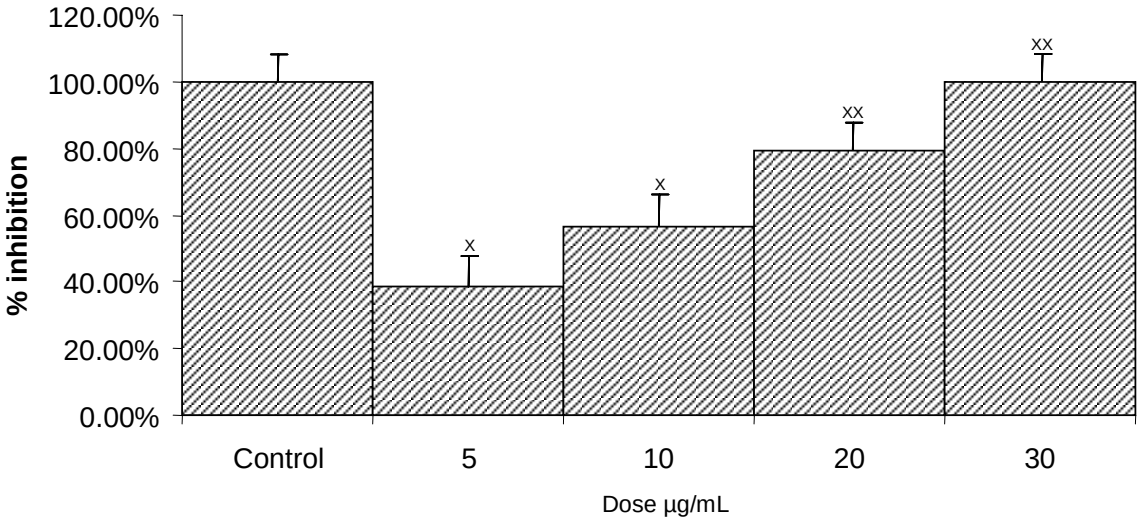


Fig. 2.- Effects of the hexane extract from *Xylaria* sp (5, 10, 20 and 30 µg/mL) on acetylcholine - induced contraction in guinea - pig ileum . Results are expresed as mean ± S.E.M. *p<0.05, ** p<0.01 , ***p<0.001 (n=8)

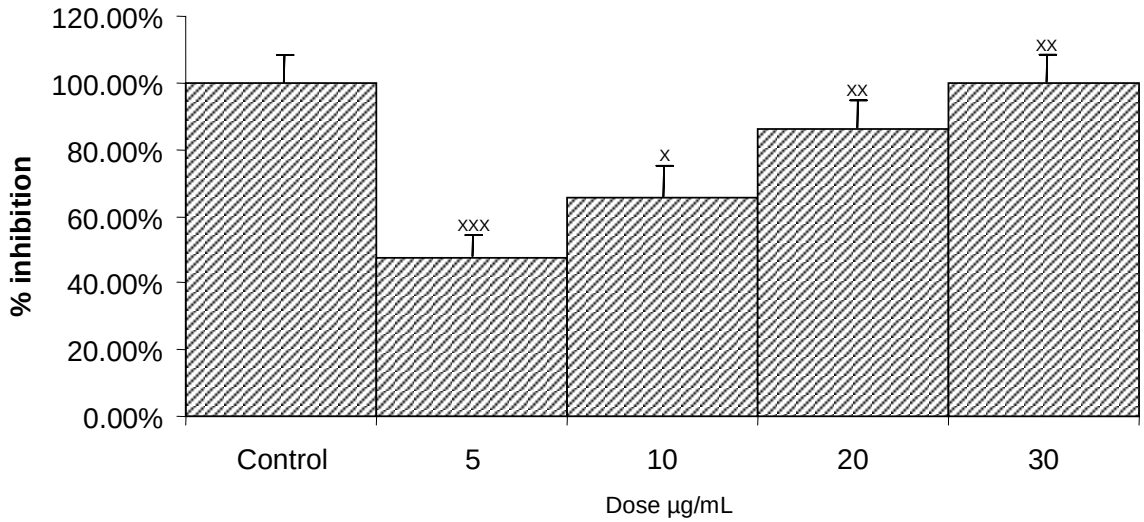


Fig. 3.- Effects of the hexane extract of *Xylaria* sp (5, 10, 20 and 30 µg/mL) on contraction induced by histamine in isolated guinea - pig ileum .Contraction (%) is expressed as a percentage against control contraction induced by histamine in the absence of samples. Each value shows the mean ± S.E.M. of six animals. *p<0.05, ** p<0.01, ***p<0.001 significantly different from the histamine – stimulated group.

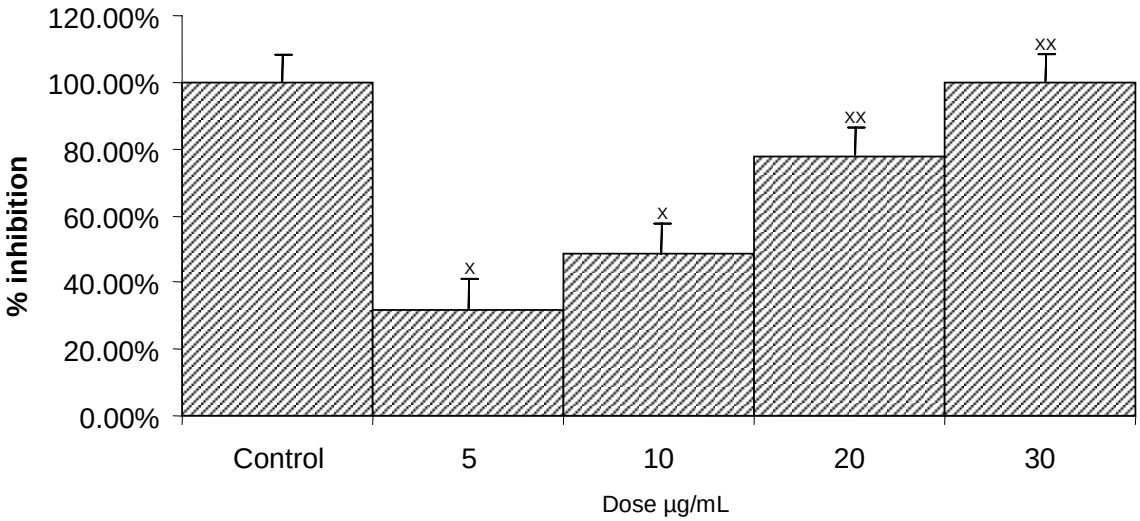


Fig.4.- Effects of increasing concentrations of hexane extract from *Xylaria* sp (5, 10, 20 and 30 µg/mL) on contraction induced by barium chloride in guinea - pig ileum. The contraction are expressed in % of the maximal contraction obtained in the same tissue before the administration of antiespasmodic. Results are expresed as mean mean ± S.E.M. *p<0.05, ** p<0.01, ***p<0.001 (n=6)

Discussion

The present study has shown that hexane extract from *Xylaria* sp exert reversible relaxant and antispasmodic effect on guinea-pig ileum. This is accord with our previous study indicating that both extracts relaxes the basal tone of this muscle with a maximum amplitude similar to that of the well-characterized smooth muscle relaxant papaverine. The antispasmodic activity of hexane extract was less than of papaverine at usual therapeutic dosage of in man. Our current data show that extracts are also capable of inhibiting the response of a wide range of contractile stimuli, such as neurotransmitters acetylcholine and histamine, although showing no obvious selectivity between contractile agents.

The hexane extract of *Xylaria* sp showed a direct action on the smooth muscles of the guinea-pig ileum . Present experiments demonstrated that hexane extract from *Xylaria* sp cultures cause a relaxant effect on ileum but it also antagonized the effect of spasmogens like acetylcholine, histamine and $BaCl_2$. The results confirmed that the crude extract acted as competitive antagonist of contractions induced by acetylcholine, histamine and $BaCl_2$.

The antagonism against these spasmogens which have different modes of action suggests that the hexane extract may act on a common contraction-induced to the contraction mechanism induced of these spasmogens. Acetylcholine, it is well known opens receptor-operated calcium channels and releases calcium from its storage sites, thus inducing phasic and tonic contractions (17). Since the extract inhibited Ach and histamine induced spasms, it could be concluded that the hexane extract inhibited both muscarinic and histaminic receptors. The hexane extract of the *Xylaria* sp possessing both anticholinergic and antihistaminic properties (18). The effect of the extract on transmitter release and receptor functions as evidenced by inhibition of contractions elicited by Ach and histamine suggest a neurotropic mechanism (19). Further, as barium chloride (an agent that releases bound (Ca^{2+}) induced spasms were also inhibited, the extract appears to be also acting by the musculotropic route (Forster et al., 1980, Aquino et al., 2001). and probably inhibit smooth muscle responsiveness, interfering with Ca^{2+} availability to contractile apparatus by inhibiting the release of bound Ca^{2+} (20).

In conclusion, the data obtained have provided evidence to support the antispasmodic activity of microfungus *Xylaria* sp. However, consideration shall be given to the fact that we used a crude extract and that the active ingredient has not been identified yet. Fractionation of the hexane extract is in progress to identify the active fractions, isolate and characterize the actives compounds.

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