EFFECT OF BORNYL-*O*-DECA-*2E,6Z,8E*-TRIENATE ON GROWTH OF SEVERAL PATHOGENS

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Summary

Heliopsis longipes root extract is the source of alkamide, bornyl-*O*-deca-*2E,6Z,8E*-trienate (bornyl decatrienate). This substance exerts an inhibitory effect on fungal growth. In the *in vitro* assay, the fungi affected by the alkamide belong to the Deuteromycete, Ascomycete and Zygomicete classes, but, other pathogens belonging to the Oomycete class were not affected. Furthermore, the inhibitory effect of the bornyl decatrienate against pathogenic fungi was detected *in situ*. The Deuteromycete *Colletotrichum lindemuthianum* the pathogenic agent of anthracnose and the Onygenal *Trichophyton rubrum* the pathogenic agent of tinea, were unable to cause disease in their respective host when the alkamide treatment was present. Interestingly, fungi growth did not occur after the bornyl decatrienate was removed from the growth medium, the rats and the bean plants. These results strongly suggest the biocide effect of bornyl-*O*-deca-*2E,6Z,8E*-trienate against these pathogenic fungi.

Key words: *Heliopsis longipes*, alkamide, bornyl decatrienate, pathogens fungi, anthracnose, tinea

For many centuries, plants have been used as a possible medical treatment due to their specific compounds. At present day, the pharmacological properties are known and indications and dosages have been established for many plants. Usually, these plants go through a strict screening by researchers when looking for a new pharmacological property of the multiple bioactive, chemical, plant components. Some genuses of Heliantheae tribe produce and accumulate different members of alkamides. These alkamides are amides or esters hydrophobic that have an unsaturated fatty acid as part of their chemical structure. It has been demonstrated that alkamides have a biocide action specially against insects; it also has a biological effect against *Schistosoma mansoni* cercaria (1,2,3).

Chilmecatl, chilcuague or peritre are common names of the plant *Heliopsis longipes* (Gray) Blake, a member of the Heliantheae. This plant can be found in the central part of Mexico and has been used by people from the area as part of their traditional medical practice to relieve toothaches; its uses are widely, and it has even been used as a seasoning ingredient in many local recipes. Several reduced amides and *N*-isobuthyl decatrienamide have been isolated and purified from flowers, leaves, but mainly from *H. longipes* roots (4,5,6). They presented different toxic effects against Gram-positive and Gram-negative bacteria and the same effect against the fungus class Ascomycetes and order Endomycetales, *Saccharomyces cerevisiae* (7). Recently, the toxic effect of *N*-isobuthyl decatrienamide and reduced amides against other two fungi belonging to Agonomycetales order and Deuteromicetes class, *Sclerotium rolfsii* and *S. cepivorum* has been reported (7,8). However, the effect of bornyl-*O*-deca-*2E*,*6Z*,*8E*-trienate (bornyl decatrienate) has not been proven in any other taxonomical fungi class, nor the effect of other alkamide, *N*-isobuthyl decatrienamide has been proven *in situ*. Based on this findings, the aim to study was, to evaluate *in vitro* the biocide effect of bornyl-*O*-deca-*2E*,*6Z*,*8E*-trienate against growth of a wide variety of plant and animal pathogens fungi and an oomycete, and *in situ* effect of bornyl decatrienate and *N*-isobuthyl decatrienamide on bean anthracnosis and tinea in experimental models.

Methods

Biological Material. For the *in situ* essay of anthracnose on beans (*Phaseolus vulgaris* L. var. Flor de Mayo), plants were grown in hydroponic system, under regulated and aseptic conditions in groups of 15 plants in each treatment. For the *in vivo* essay on fungi-causing animal infection, 250 g male Wistar rats were used in groups of seven rats in each treatment.

Oomycete and Fungi Strains. The isolates are part of the microbiological collection of Metabolomic Laboratory of Universidad Michoacana de San Nicolas de Hidalgo, see Table 1. The strains were propagated in different types of medium such as Sabouraud, YPG (yeast extract, peptone and glucose) and PDA (potato, dextrose and agar). Dimorphic fungi were growth in conditions and specific medium to induce morphogenesis (9, 10, 11).

Table 1. Characteristics of pathogenic fungi and the oomycete used in the study

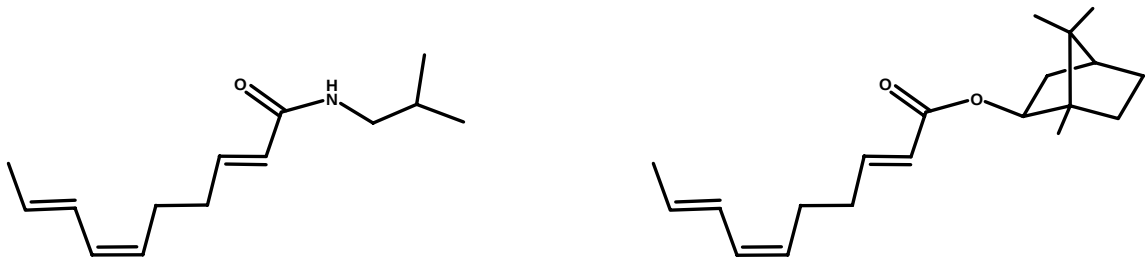
Species	Morphology	Pathogen	Host
Candida albicans	Dimorphic	+	Vertebrate
Colletotrichum gloeosporioides	Mycelial	+	Vegetable
C. lindemuthianum	Mycelial	+	Vegetable
Fusarium moniliformis	Mycelial	+	Vegetable
Monilia sp	Mycelial	+	Vegetable
[1]*Mucor rouxii*	Dimorphic	-/+	Vertebrate
Phytophthora cinnamoni	Mycelial	+	Vegetable
S. cerevisiae	Yeast	-	Unknow
S. cepivorum	Mycelial	+	Vegetable
Sporothrix schenckii	Dimorphic	+	Vertebrate
Trichophyton rubrum	Mycelial	+	Vertebrate
Verticillium albo atrum	Mycelial	+	Vegetable

[1]Opportunist pathogens in the immunocompromised host

Extraction System. Raw extract of *H. longipes* roots were cleaned from soil particles and dried. A portion of 12.5 g was pulverized and put in continuous reflux extraction in a Soxhlet system, using 65 ml ethyl acetate for 3 h at 70 °C. The obtained extract was dried completely in a 45 °C rotary evaporator system; then it was dissolved in 10 ml ethyl alcohol. Finally, the raw extract was storage at a temperature at 4 °C, until being quantify.

H. longipes root metabolites purification (4). The bornyl decatrienate and *N*-isobuthyl decatrienamide went through a purification process. Briefly, the raw extracts obtained from *H. longipes* were purified using column and thin layer chromatography. In the column chromatography, silica gel and *n*-hexane:ethyl acetate (2:1 v/v) were used as support and mobile phase, respectively, over a 118 cm long and 2.5 cm diameter glass column. It was collected a total of 35 fractions each of 10 ml. All fractions were subjected to thin layer chromatography separation using silica gel (G_{60}) over 5 x 5 cm^2 and 6-8 wide glass plaques, and *n*-hexane:ethyl acetate (2:1 v/v) as run phase. The specific metabolites bands were detected by UV light, and following previously the respective standardized R_f of each one (bornyl decatrienate, R_f = 0.8 and *N*-isobuthyl decatrienamide, R_f = 0.5). Purified metabolites quantification was made using a standardized calibration curve system through a spectrophotometric method.

The purity and presence of metabolites was corroborated with gas chromatography coupled with mass spectrometry technical method, see Figure 1. Once the metabolites were quantified, the solutions were standardized with a known concentration. These concentrated metabolites solutions were used as the reference to prepare the doses used in this research.



(***a***) (***b***)

Figure 1. Structural formulas for (**a**) *N*-isobuthyl-*2E,6Z,8E*-decatrienamide and (**b**) bornyl *O*-deca-*2E,6Z,8E*-trienate.

Microbiological Assay. Several concentrations of bornyl decatrienate were dissolved in dimethylsulfoxide (DMSO) and used against the pathogenic fungi and the oomycete. Each concentration was placed in 0.7 cm Wathman paper discs, which were carefully arranged over the growth medium in petri dishes already plated with the fungi. The incubation process took seven days, and then the mark diameter -where the growth inhibition is shown- was measured.

To detect the effect of alkamide as fungicide or as fungistatic compound, an essay was carried out in liquid medium; when the control fungi or oomycete spores germinated (approximately 10 - 20 % rate) it was changed to a new growth medium to bornyl decatrienate. Finally, the total lethal dose was determined. Also, the protein of the medium was determined by Lowry's method and it was considered a growth indicator (9).

Experimental Mycosis. Tinea (fungi skin disease). The method used was the Kishore *et al* (10). Seven male Wistar rats were grouped for each treatment: the group A was treated with a mixture of silica gel and bee honey; group B with the mixture and DMSO; group C with the mixture and *N*-isobuthyl decatrienamide; group D, with mixture and bornyl decatrienate, and group E, with the infection itself (positive control).

Each rat went throughout a depilation process in two zones (4 cm^2 each) of their back. The inoculums were prepared with 5 X 10^{12} spores of *T. rubrum* in 500 mg of silica gel and 2.5 ml of sterile bee honey. The inoculums were applied just after the depilation process and the incubation period lasted for 3 days. The establishment of the disease was detected by the formation of a pink halo in the area of skin treated, and it was confirmed by the pathogen presence isolated from skin flakes. In the animals, the treatments were given seven days after the infection was established. two daily doses of bornyl decatrienate (78 μg) or *N*-isobuthyl decatrienamide (78 μg), then the mycosis and the pathogen presence was quantified.

Anthracnose (phytomycosis). Bean plants (*Phaseolus vulgaris* var Flor de Mayo) of 7 day old (groups of 15 plants in each treatment) were pathogen infected, after ten days of incubation, an evaluation to determine dead and alive leaves was made. Primary bean plant leaves were cut superficially (wounded leaf) and twice every 16 h, the inoculums of 5 X 10^8 spores of *C. lindemuthianum* -the anthracnose causal agent- were placed with and without 95 μg doses of bornyl decatrienate or *N*-isobuthyl decatrienamide. The observations were closely followed until the infected plant (positive control) died.

Statistic Analysis. In order to show the biocide activity of alkamide, at least ten isolates of each species were tested, because of the different responses and sensitivity to alkamide in fungi and the oomycete. Data were submitted to a randomized complete block analysis of variance, with significance being tested at $p<0.05$. Differences between treatments were additionally tested using the *t*-Student test ($p<0.05$). All statistical analyses were carried out using a computer statistic program, SPSS 7.0.

Results and Discussion

Many extracts and essential oils plants have shown a variety of biological activities, special emphasis has been made toward those whose activity is against pathogens. For example, some members of the plant tribe Heliantheae produce alkamide compounds as secondary metabolites that show biocide action against insects and protozoan. The bornyl decatrienate and *N*-isobuthyl decatrienamide are produced by *H. longipes*, see Figure 1 (4). These compounds are mainly detected in the plant roots. The biological activity of *N*-isobuthyl decatrienamide has been reported as toxic agent against insects, bacteria and some fungi belonging to Endomycetal and Agonomycetal orders (8). But, nothing has been reported about bornyl decatrienate as an inhibitor of fungi growth.

These results suggest that members of other taxonomic class could be susceptible to the toxic effects of *H. longipes's* product. Therefore a different selection of plants and animals pathogenic fungi and an oomycete was made. This selection was tested to determine the possible effect of bornyl decatrienate on them (Table 1). On the other hand, the bornyl decatrienate and *N*-isobuthyl decatrienamide were proven in experimental mycosis.

All the selected pathogenic fungi exhibit a complex morphology, showing mycelial, yeast like and dimorphic growth (yeast-like and mycelial type). An important observation made was that bornyl decatrienate provoked a toxic effect over fungus cell growth; therefore, the lethal dose for each fungus species was determined (Table 2). Their biological activity was a strong toxic effect, a fungicidal effect. The compound inhibited the pathogen's growth, when it was removed from the medium, the fungi were unable to initiate their vegetative growth (Table 3).

Table 2. Bornyl decatrienate minimum lethal concentration estimates over fungi test

Species	[1]Minimum Lethal Concentration (μg/ml)
C. albicans	275
C. gloeosporioides	40
C. lindemuthianum	95
F. moniliformis	172
Monilia sp	125
M. rouxii	350
S. cerevisiae	320
S. cepivorum	29
S. schenckii	235
T. rubrum	78
V. albo atrum	120

[1]Measurement were done on liquid mediums after two days of incubation in bornyl decatrienate presence. The determinations were made independently three times in three replicates. To compare the means it was used a *t*-Student test ($p < 0.05$).

Table 3. Biocide activity of bornyl decatrienate on fungi and an oomicete test.

Species	Growth repeated activation	
	Control	Bornyl decatrienate
C. albicans	15 ± 2.0	5 ± 0.41
C. gloeosporioides	17 ± 2.0	4 ± 1.3
C. lindemuthianum	13± 4.1	3 ± 0.7
F. solani	35 ± 6.8	6 ± 2.9
Monilia sp	21 ± 5.2	6 ± 2.3
M. rouxii	60 ±7.3	3 ± 1.01
P. cinnamomi	35 ± 5.3	35 ± 3.7
S. cerevisiae	65 ± 8.4	8 ± 4.7
S. cepivorum	14 ± 1.7	2 ± 1.1
S. schenckii	40 ± 4.8	4 ± 0.57
T. rubrum	15 ± 2.6	5 ± 0.65
V. albo atrum	23 ± 3.6	5 ± 2.5

Data were collected after two incubation days in liquid growth medium and values are the mean of three independent experiment in three replicates ± standard deviation at $p < 0.05$. When control cultures reached a germination point of 10 al 20%, they were changed to a new medium without inhibitor.

Bornyl decatrienate from *H. longipes* had a toxic effect over various vegetative cycle phases of pathogenic fungi. The compound affected the mycelia and yeast-like growth -mainly the germinative and sporulative processes- of *C. albicans, M. rouxii, S. schenckii, S. cereviseae* and *T. rubrum*, and mycelial growth of *C. gloeosporioides, C. lindemuthianum, F. moniliformis, Monilia* spp, *S. cepivorum* and *V. albo atrum*. On the other hand, it was reported that *N*-isobutyl decatrienamide other alkamide from *H. longipes* has an effect against growth fungi (8). Considering this result, the next step was to determine any therapeutic effect of bornyl decatrienate and *N*-isobutyl decatrienamide.

Two mycotic diseases were induced experimentally, anthracnose and tinea: 1) *Phaseolus vulgaris* plants were infected with *C. lindemuthianum* and, 2) male Wistar rats were infected with *T. rubrum*.

Both are good mycosis experimental models because they can be easily handled in laboratory conditions, and they display distinctive physical characteristics in the presence of disease. In the case of bean phytomycosis, anthracnose, signs of disease can be easy follow with the naked eye, due to characteristic brown-yellow spots present in the death tissue. The rat model, exhibits also a distinctive skin lesion that makes an excellent clinical parameter. The dermatomycosis can be identifying as a round red papule with borders well define, that corresponds to the site where fungi spore were applied on the rat skin.

A unique concentration was tried (95 μg to bean and 78 μg to Wistar male rat), and only for the *N*-isobuthyl decatrienamide twice daily. The development of dermatomycosis in rats was inhibited progressively, and by the end of ten days of treatment, the clinical signs disappeared, and the pathogen could not be detected in flakes taken of rat skin. In primary bean leaves infected and that were treated with the *N*-isobuthyl decatrienamide, the anthracnose disease did not develop for up to fifteen days; the leaves tissue did not show any sign of disease (table 4).

Table 4. *In situ* effect of bornyl decatrienate and *N*-isobuthyl decatrienamide over experimental mycosis

Experimental mycosis	Host	Pathogen	Infection (%)		
			Control	Bornyl decatrienate	*N*-isobuthyl decatrienamide
Tinea	Wistar rat	*T. rubrum*	100	0	0
Anthracnose	*P. vulgaris*	*C. lindemuthianum*	100	19	15

The pathogens used in this study belong to four taxonomic classes, Deuteromycetes, Ascomycetes, Zygomycetes and Oomycetes; and some from their representative taxonomical orders Moniliales, Melanconiales, Endomicetales, Mucorales, Onygenales, and Peronosporales, see table 5.

Pathogens of three taxonomical orders showed susceptibility to bornyl decatrienate, but the oomycete *P. cinnamoni* was not sensitive to the biocide effect, furthermore, this phytopathogen even grew in a relative high compound concentration.

Evidently, bornyl decatrienate had a different effect over pathogens. It is more effective on fungi that posses chitin as main structural polysaccharide in their cell wall, (chitinaceous fungi); while the oomycetes that possess cellulose as a main structural polysaccharide of the cell wall was not

affected. Such is the case of *P. cinnamoni,* a phytopathogen that belongs to Oomycetes class and Peronosporales order.

Table 5. Relationship between biocide effect of bornyl decatrienate and taxonomic class of fungi and an oomycete used in this study

Kingdom	Taxonomic class	Taxonomic order	Fungi Species	Fungicide effect
FUNGI				
	Ascomycetes	Endomycetales	1	Positive
		Onygenales	1	
	Deuteromycetes	Agonomycetales	1	Positive
		Criptococcales	1	
		Melanconiales	2	
		Moniliales	4	
	Zygomycetes	Mucorales	1	Positive
CHROMISTA				
	Oomycetes	Peronosporales	1	Negative

Additionally, in the development of the present work, it was found that *H. longipes* compounds do not have a severe cytotoxic effect over vertebrate host cells, neither on tobacco cells (line TBY2). The result was obtained when the transport (influx and efflux) of the ions were measured; a parameter widely used to study the cell plasmatic membrane integrity because any change in membrane homeostasis is a signal of membrane damage.

It is possible that bornyl decatrienate targets enzymes of cell wall polysaccharides, sterols or fatty acids biosynthesis or all these metabolic routes were affected at same time. For example, vertebrate, plant, oomycete and fungus cells have plasmatic membranes with important differences. These differences are mostly structural, one is in their sterol type: vertebrates have sterol and cholesterol while fungi have ergosterol and plants have stigmasterol and sitosterol. There is a great possibility that this could be the main reason of such different effect obtained with the treatment of *H. longipes* compounds over them (vertebrates and fungi).

Probably, bornyl decatrienate from *H. longipes* has as cell target the ergosterol structure, it's metabolism or fatty acid synthesis in fungi. Other possibility is that the alkamide affecting the permeability of the membrane of fungi. Additional studies will be done to determine the cellular target of alkamides; bornyl decatrienate and *N*-isobuthyl decatrienamide.

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