DOI: 10.13140/RG.2.2.29052.13446
http://dx.doi.org/10.13140/RG.2.2.29052.13446
Cultivos Tropicales, 2016, vol. 37, no. 4, pp. 173-181 October-December

ISSN impreso: 0258-5936
ISSN digital: 1819-4087

Ministerio de Educación Superior. Cuba
Instituto Nacional de Ciencias Agrícolas
http://ediciones.inca.edu.cu

ANTIFUNGAL ACTIVITY OF ULTRASOUND-ASSISTED ACETONE EXTRACT AND ALKYLRESORCINOL-ENRICHED FRACTIONS FROM *Hordeum vulgare* L. AGAINST *Fusarium oxysporum*

Actividad antifúngica del extracto en acetona asistido por ultrasonido y fracciones enriquecidas en alquilresorcinoles de *Hordeum vulgare* L. contra *Fusarium oxysporum*

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ABSTRACT. Phenolic lipids are a series of natural origin compounds which are produced, among others, by species of the family Poaceae, such as barley (*Hordeum vulgare* L.). Such compounds have been described with significant antimicrobial properties. Therefore, as part of a program for the search for antifungal compounds, an acetone extract and depurated fractions were obtained from grain flour of barley (andina variety) and tested *in-vitro* against *Furarium oxysporum*. The acetone extract showed high alkylrecorcinols content compared to previous works, whose chromatographic profile led to identify at least eight major alkylresorcinols. The purification of the extract (monitored with specific revealer) led to obtain three alkylrecorcinol-enriched fractions. Both the crude extract and the purified fractions showed antifungal activity at different levels, with a dose-dependent response. Thus the treatments showed ED_{50} values were between 3,3-24,0 μg mL^{-1}. However, enriched fractions showed greater activity to that of crude extract, indicating that the direct effect of alkylresorcinols on the phytopathogen is representative to inhibit their growth. All of the above mentioned facts suggest these compounds as good candidates for controlling this phytopathogen.

Key words: Poaceae, antifungal properties, phenolic compounds, lipids

RESUMEN. Los lípidos fenólicos son una serie de compuestos de origen natural que son producidos, entre otros, por especies de la familia Poaceae, como es el caso de la cebada (*Hordeum vulgare* L.). Tales compuestos se han descrito en algunos trabajos con propiedades antimicrobianas importantes. Por tanto, como parte de un programa de búsqueda de compuestos anti fúngicos, se realizó la evaluación de la actividad *in-vitro* contra *Furarium oxysporum* del extracto en acetona y de tres fracciones depuradas, obtenidas a partir de la harina de granos de la variedad andina de cebada. En comparación con trabajos previos, el extracto en acetona presentó contenidos más altos de alquilresorcinoles, cuyo perfil cromatográfico permitió identificar al menos ocho alquilresorcinoles mayoritarios. La depuración del extracto bajo monitoreo con revelador específico, llevó a la obtención de tres fracciones enriquecidas en alquilresorcinoles. Tanto el extracto crudo como las fracciones depuradas mostraron actividad anti fúngica a diferentes niveles, con respuesta dependiente de la dosis. Así, los valores de ED_{50} de tales tratamientos estuvieron entre 3,3-24,0 μg mL^{-1}. Las fracciones enriquecidas mostraron mayor actividad, indicando que el efecto directo de los alquilresorcinoles sobre el fitopatógeno es representativo para inhibir su crecimiento. Todo lo anterior sugiere a estos compuestos como buenos candidatos para ser estudiados en trabajos futuros en el control de este fitopatógeno.

Palabras clave: Poaceae, propiedades antimicosicas, compuestos fenólicos, lípidos

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INTRODUCTION

The alkylrecorcinols chemically defined as phenolic isoprenoid lipids. Its structure consists of a benzene ring with two hydroxyl groups in positions 1,3 and generally an aliphatic chain at position 5, which can range from C1 to C29 and be saturated and unsaturated. They are amphipathic molecules, which depend on the length of the aliphatic chain, saturation, and the functional groups present (1).

Some authors reported the presence of alkylrecorcinols in 11 plant families, two families of algae, three kinds of mosses, fungi two families, three families of bacteria and a marine sponge species (2). In the case of plants, alkylrecorcinols accumulate in the cuticle of the leaves, floral buttons, fruits and seeds.

Among the best known plant species, for its high content of alkylrecorcinols, the Poaceae family stands out, whose most representative examples are barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), triticale (x *Triticum secale*), rice (*Oryza sativa* L.), corn (*Zea mays* L.), millet (*Pennisetum americanum* L.) and oats (*Avena sativa* L.) (3).

Barley is grown in Colombia, in areas with elevations between 1800-3200 meters above sea level and temperatures of 18-11 °C, with an area of 6,372 ha. However, barley is not a primary culture product.

Great efforts are being made to improve supply and demand, whether for human or animal consumption and for the brewing industry. Added to this is the fact that they are good sources of alkylrecorcinols, since in several cases it has been reported that one of the chemical reasons for barley seeds are resistant to fungus is the presence of alkylrecorcinols (4). This opens the possibility of considering these compounds as potential chemical for controlling fungi, including *Fusarium oxysporum* Schlecht is entities, a causative fungus diseases in plants of commercial interest in Colombia and other countries (5).

Several works coincide in explaining that the bioactivity of alkylrecorcinols is determined by their amphipathic nature, which allows them to interact with a large number of molecules, cells and tissue structures (1, 2). Therefore, studies have been focused on the evaluation of the ability of alkylrecorcinols to inhibit *in vitro* growth of microorganisms.

In these studies the antifungal activity stands against *Aspergillus niger* Tiegh., *A. parasticus* Speare, *A. versicolor* (Vuill.) Tirab., *Penicillium chrysogenum* Thom, *P. roqueforti* Thom, *Fusarium culmorum* (W.G. Sm.) Sacc., *Rhizoctonia solani* J.G. Kühn, *R. Cerealis* E.P. Hoeven, *Alternaria alternata* (Fr.) Keissl., *Cladosporium cucumerinum* Ellis & Arthur, *Trichophyton mentagrophytes* (C.P. Robin.) R. Blanch. and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (2, 6).

Other authors conclude that alkylrecorcinols are important and efficient chemical entities with antimicrobial properties, which could be playing a suitable role in protecting against pathogens during the formation of seeds and seedlings biocenosis (6, 7). Therefore, in order to provide related information with the antifungal activity of alkylrecorcinols from known sources, in this paper an experimental design aimed at *in-vitro* against *F. oxysporum* evaluation of the product obtained from barley flour was established by extraction with acetone, assisted by ultrasound, and three alkylrecorcinols enriched fractions.

This work constitutes the first study of the composition in terms of barley alkylrecorcinols grown in Colombia and alkylrecorcinol activity against *F. oxysporum*.

MATERIALS AND METHODS

Biological material

Plant material of Barley -*H. vulgare* L, andean genotype, characterized by six-race spikes, covered grain and mass of 100 grains = 4,83 ± 0,35 g (8) - was grown under organic fertilization without synthetic agricultural products, in a randomized complete block with three repetitions, on the grounds of experimental cultivation of Campus New Granada (clay soil (pH 8,0), 16 °C, 2600 m a. s. l.). The culture was established in accordance with reported in a previous study (8).

Whole grain samples (ca. 2000 grains with pericarp and endosperm), were collected from adult plants in triplicate. The collected samples were lyophilized, and when dried, they were ground in a crusher blades (Brand Waring Model 2.5 Qt) to flour, then held at 20 °C until analysis.

The phytopathogen (*F. oxysporum*) remained cryopreserved in nutrient broth: 70:30 of glycerol at 20 °C and it was reactivated in containing plates of nutrient agar at 25 °C for 48 hours when its use is required.

Extraction

For acetone extraction of barley flour the methodology described in the work done by Magnucka, *et al.* was followed (1). The aforementioned extraction was to take a sample of flour (80 g) and subjecting it to

ultrasound assisted extraction with acetone (120 mL). Extraction consisted of three cycles of sonication of 15 min on a computer Elmasonic S30H. The obtained extract was filtered through filter paper (Whatman Paper No. 4) and then concentrated to dryness under reduced pressure (40 °C) on a rotovap IKA Digital 10.

Obtaining enriched fractions in alkylrecorcinols

An amount (5,0g) of acetone extract was fractionated by column chromatography (CC) on silica gel (SiO_2) eluting with a mixture of chloroform: acetone: methanol 90: 10: 1 v/v/v flow 0,5 mL min^{-1}). Fractions were collected in glass vials and were monitored by thin layer chromatography (TLC) (eluted with chloroform: acetone: methanol 82: 15: 3 v/v/v, and developed with Fast Blue RR® 0,5 %). After elution, fractions with TLC-like profile, gathered from the evidence of the presence of alkylrecorcinols.

Colorimetric quantification of alkylrecorcinols in extracts and fractions

For colorimetric quantification of alkylrecorcinols in extracts and fractions of barley flour it was made separately 1 mL of a solution of 1 mg mL^{-1} of the extract or fraction in methanol, were added 100 mL of 10 % Na_2CO_3; then 3 mL of a solution of FBRR 0,01 % were added and 20 minutes after the absorbance was measured at 480 nm (9). Each measurement was performed in triplicate. Absorbance measurements obtained were transformed into concentration values in a previously constructed calibration curve using olivetol (in mg mL^{-1}) as the reference standard (y = 0,1601x; R^2 = 0,9997). The results were expressed as mg equivalents olivetol/g dry matter (DM) (± confidence interval 95 %).

Analysis by HPLC-ESI-MS

The extract solution and the fractions (100 mg mL^{-1}) were analyzed in a chromatograph Shimadzu UFLC with a LC20AD pump and UV SPD20A detector coupled with ESI interface in positive mode on a mass spectrometer of high resolution marks Bruker, Micro ToF-QII model. Chromatographic run for all extracts was performed on a column Phenomenex® mark model Luna C18 of 5 µm of particle size and dimensions 250x100 mm.

The mobile phase used for analysis was a mixture of methanol (phase A) and 0,05 % trifluoroacetic acid (TFA) in water (phase B), gradient elution (phase B: 50 % 0-2 min; 50-100 % of 2-20 min; 100 % 20-24 min, 24-26 min 100-50 %; 50 % of 26-30 min).

The tentative identification was carried out by analyzing mass spectra, recovered from ICT, analysis of the condensed formula through exact mass measurements and compared to data found in literature.

Assessment of antifungal activity

The *in vitro* antifungal activity was evaluated against *F. oxysporum*. The culture medium Potato Dextrose Agar (PDA) was supplemented at different concentrations (100,0 to 0,01 ug mL^{-1}) of the extracts and fractions obtained from *H. vulgare*.

This by a direct (the acetone extract or F1-F3 fractions) mixture between the medium (PDA before solidifying) and treatment was performed, by adding the required amount of medium and treatment, to get the concentration to be evaluated. This mixture was stirred vigorously until a homogenous dispersion before solidifying.

The supplemented media were added in Petri dishes 6 cm in diameter and then a disc of 2 mm, obtained from a colony of the phytopathogen in the center of the box was placed (10). Each trial consisted of a randomized with three replicates (each with three replicates) compared to a control (untreated PDA) design. As a positive control prochloraz® was used.

Assays were terminated once the control mycelial growth reached the edge of the box. When the test was made the data analysis was performed by calculating the percent inhibition (InC).

$$\% InC = [(\text{control mycelial growth area-area mycelial growth treatment})/\text{mycelial growth area control}]*100$$

After the trial the number of conidia (Neubauer chamber) in the different treatments tested are also recorded to calculate the percentage inhibition of conidia production:

$$(InE) (\% InE = [(\text{conidia}/cm^2 \text{of control-conidia}/m^{-2} \text{ treatment})/(\text{conidia}/cm^2 \text{ control})] * 100)$$

where: Spores/cm^2 = # conidia
* Fungal growth area for each treatment or control.

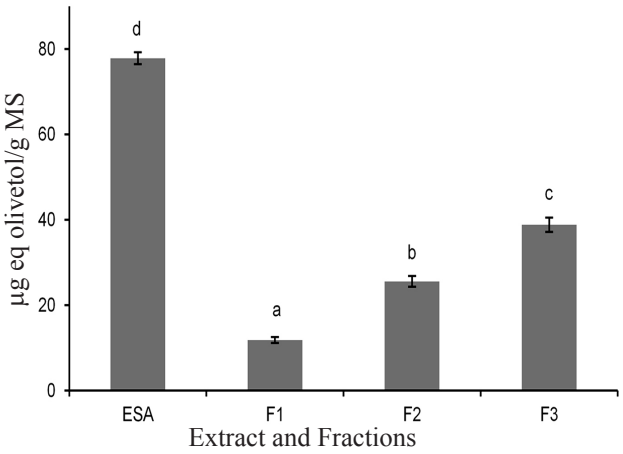
Statistical analysis

In order to determine whether there were differences ($p < 0,05$) among the response variables evaluated, the results of total content of alkylrecorcinols (TCA) and antifungal activity compared to their replicas using ANOVA and Tukey tests (11) . Statistical software R version 3.2.5 (12) was used for this purpose. In addition, inhibition data with the median effective dose (ED_{50}) was determined by nonlinear regression with Graph Pad Prism 5.0 (13) Program.

RESULTS AND DISCUSSION

Alkylrecorcinols in Andean barley

Extraction with acetone gave a yield of 9,1 mg extract per g dry material. Figure 1 shows that the TCA for *H. vulgare*, Andean genotype grown in Colombia, has a value of 77,8±1,4 mg g^{-1} dry material. This result is comparable with that found in previous studies, which describes the TCA value for barley may be between 41-210 mg g^{-1} dry bases (4). However, the result found was higher than the content identified in accessions of Polish origin (14), but lower than those found in other grasses such as wheat (1).



Values expressed as the average of three replicates ± confidence interval. Values with different letter indicate that are significantly different ($p<0,05$) according to Tukey test (11).

Figure 1. Total content of alkylrecorcinols (TCA) for acetone soluble extract (ASE) and the fractions obtained by chromatography in column

From analysis by HPLC-ESI-HRMS the total ion chromatogram (TIC) shown in Figure 2a was obtained. In this ICT 22 chromatographic signals are observed. However, it was found that not all detected compounds correspond to alkylrecorcinols among those considered some phenolic and triglycerides (data not shown).

Under a detailed analysis of mass spectra and condensed formula, deduced by determining its exact mass, eight compounds of alkylresorcinol type, indicated by numbers highlighted in the chromatographic profile of the extract were identified (Figure 2a).

In Table I the eight alkylrecorcinols identified with exact mass values, deduced molecular formula and the name of the identified alkylresorcinol shown.

In previous work the present alkylrecorcinols barley were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). This type of analysis requires prior derivatization to obtain the trimethylsilylates products of alkylrecorcinols so they can be analyzed by GC. In such works, found homologous series of chain saturated of $C_{15:0}$ to $C_{25:0}$, being the majority $C_{21:0}$ and $C_{21:0}$ and $C_{25:0}$ (14). However, in the ESA of the Andean variety, three known alkylrecorcinols were found, but not previously identified in barley samples: $C_{12:1}$ (1), $C_{14:1}$ (2) and $C_{18:0}$ (5). This may be due to the use of sonication to aid in extraction, allowing the removal of such compounds from the plant matrix.

Of all the compounds detected in the ESA, those with the largest area corresponded to $C_{15:0}$ (3), $C_{17:0}$ (4), $C_{19:0}$ (6) and $C_{21:0}$ (7), which could be considered as majority.

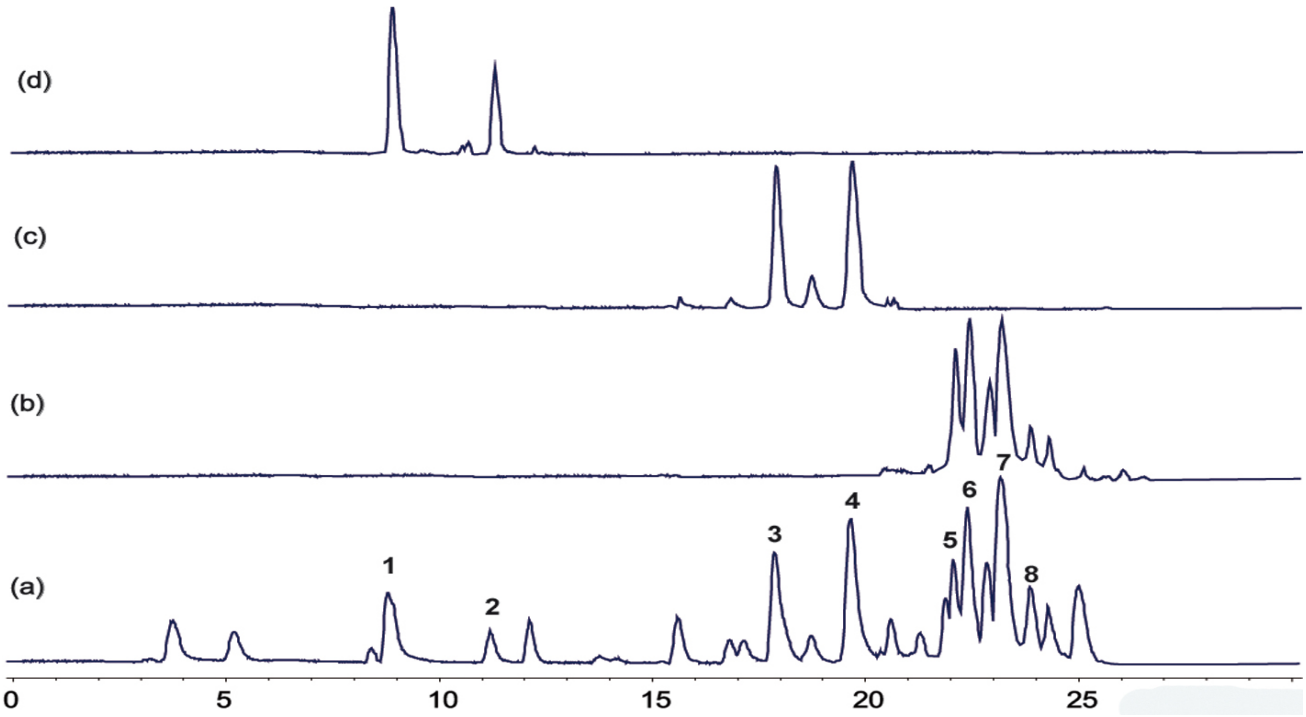
Obtaining enriched fractions in alkylrecorcinols

From chromatography in column three alkylrecorcinols enriched fractions (F1-F3) with Rf values in CCD of 0,81, 0,62 and 0,44 respectively were obtained.

Quantification showed that TCA F3 has the highest content (38.8 mg eq olivetol g^{-1} DM) compared to the other fractions (Figure 1), indicating that the ESA is abundant in long chain saturated compounds. In addition, these three fractions are also analyzed by HPLC-ESI-HRMS whose TIC shown in Figure 2b-d, respectively. In these profiles the good separation achieved in fractions shown, obtained for each fraction alkylrecorcinols three groups, which is indicative of the purification of the extracts.

Fraction 3, due to its complexity grouped as many compounds, including the alkylrecorcinols 5-8 in mixture with some triglycerides. Fractions 2 and 3 consisted of mixtures of two equivalents to 1-2 and 3-4, respectively alkylrecorcinols.

Other signals present in the ESA ICT are not presented in sections since extract purification was based on monitoring the specific developer for alkylrecorcinols.



(a) acetone-soluble extract (ESA) (b) Fraction 3 (c) Fraction 2 (d) Fraction 1

Figure 2. Total ion chromatogram (TIC) obtained by HPLC-ESI-HRMS for extracts and fractions obtained from *H. vulgare*

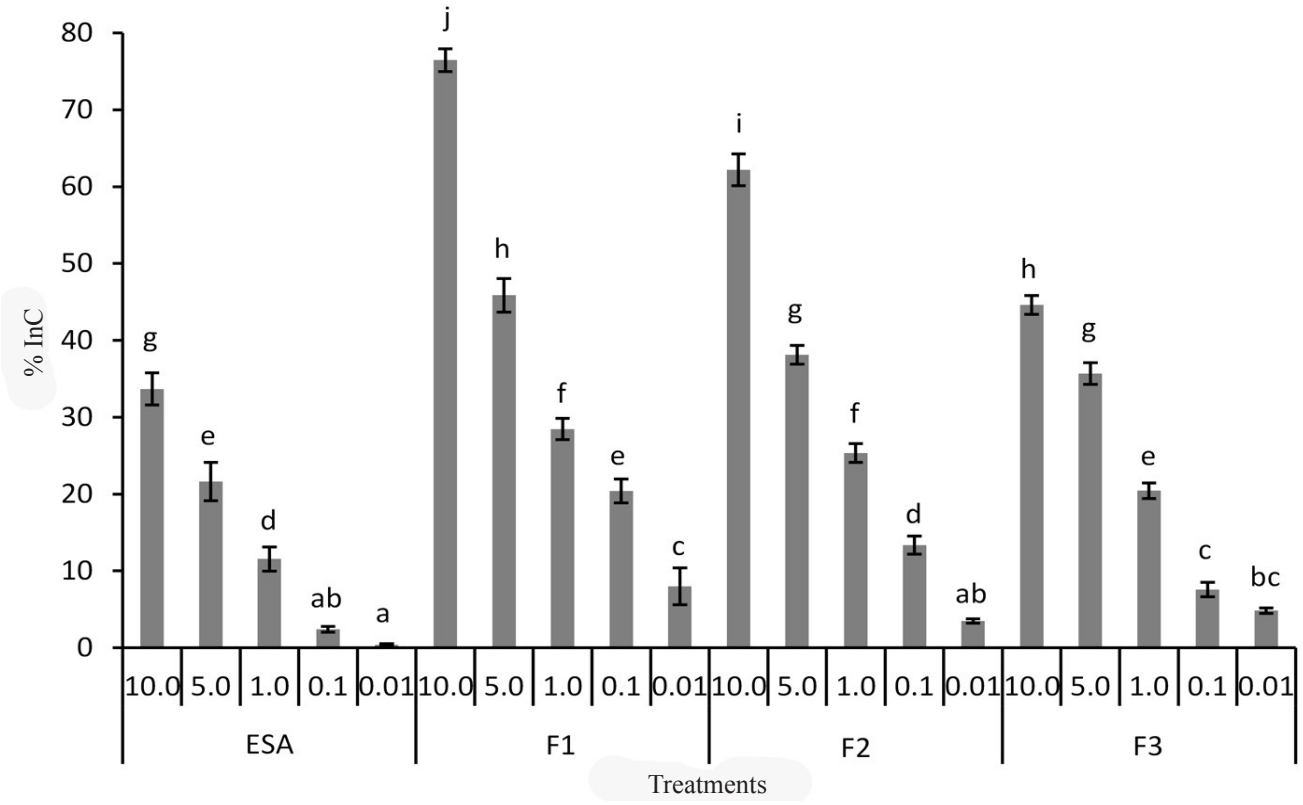
Table I. Alkylrecorcinols identified in the acetone-soluble extract and fractions of *H. vulgare* L.

No	$[M+H]^+$ (m/z)	Molecular formula	Lateral chain (homologous series)	Name
1	277,2163	$C_{18}H_{28}O_2$	$C_{12:1}$	dodecenylresorcinol
2	305,2485	$C_{20}H_{32}O_2$	$C_{14:1}$	tetradecenylresorcinol
3	321,2789	$C_{21}H_{36}O_2$	$C_{15:0}$	pentadecylresorcinol
4	349,3112	$C_{23}H_{40}O_2$	$C_{17:0}$	heptadecylresorcinol
5	363,3260	$C_{24}H_{42}O_2$	$C_{18:0}$	octadecylresorcinol
6	377,3426	$C_{25}H_{44}O_2$	$C_{19:0}$	nonadecylresorcinol
7	405,3741	$C_{27}H_{48}O_2$	$C_{21:0}$	henicosylresorcinol
8	433,4038	$C_{29}H_{52}O_2$	$C_{23:0}$	tricosylresorcinol

Antifungal activity

In Figure 3 the results of InC percent for each of the treatments are shown. It is observed that the ESA dependent behavior presents the dose used, with values between 0,5 to 34,0 % inhibition at doses between 0,01 and 10,0 μg mL^{-1}, and significantly different ($p < 0,05$) with each other. These results are consistent with findings by other authors (7), who evaluated alkylrecorcinols a mixture of C_{13} to C_{27} saturated against three pathogens (*F. culmorum*, *R. solani*, *R. Cerealis*) and found that the mixture inhibition exercised at different levels.

In that study, phytopathogens presented inhibition from 10-20 μg mL^{-1}, being *F. culmorum* the most resistant to treatment. However, when compared with the present results, the soluble crude extract in acetone (ESA) of barley was more active, then at 90 μg mL^{-1} inhibited the growth completely (data not shown), while in other work it was observed the complete inhibition was achieved from 160 μg mL^{-1} (7).



Values expressed as the mean ± nine replicas confidence interval
Values with different letter indicate that are significantly different (p <0.05) according to Tukey (11)

Figure 3. Percentage of mycelial growth inhibition (% InC.) treatment outcomes (extracts and fractions) of *H. vulgare* against *F. oxysporum*

The fractions F1-F3 meanwhile, showed better activity than the ESA, with higher percentages of inhibition and significantly different (p<0,05) the crude extract. Among them, Fraction 1 showed the best inhibition values (77 % to 10 µg mL^{-1}), which contains the unsaturated alkylrecorcinols and C_{12} and C_{14} chain. The other two fractions (F1 and F2) were also more active than ESA, which contain alkylrecorcinols saturated (C_{15}-C_{23}), but less active compared with Fraction 1 (eg, the maximum shown dose, 10 µg mL^{-1}).

On the other hand, ED_{50} results are shown in Table II. It can be seen that the ESA is the least active (24,1 ± 1,3 mg mL^{-1}) and the fraction 1 has the highest activity (two-four times) in terms of mycelial growth inhibition of *F. oxysporum* (3,31 ± 0,54 mg mL^{-1}) and almost comparable to the positive control used (prochloraz®, ED50 = 1,68 ± 0,37 mg mL^{-1}).

In the case of inhibition in the production of conidia (% InE), both the extract and the fractions they showed significant reduction in sporulation, and with a similar distribution (Figure 4).

Fraction 1 showed again the highest values of % InE, but was comparable and not significantly different Fractions 1 and 2. However, the fractions showed slightly higher values than the ESA.

The alkylrecorcinols and phenolic lipids generally are described as compounds with different biological activities (15), especially those obtained from cereals (16). However, the antifungal activity against some phytopathogenic has not been widely studied, being phytopathogenic activity studies alkylrecorcinols obtained rye (7, 17-19), durum wheat (6) and handle shell (20, 21).

To alkylrecorcinols obtained from *H. vulgare* only the study of extracts obtained from different cultivars grain barley Uruguayan origin against *A. niger* and *P. crysogenum* (5) was found, but no previous results evaluating alkylrecorcinols activity against *F. oxysporum*, which is also one of the main innovations of this paper.

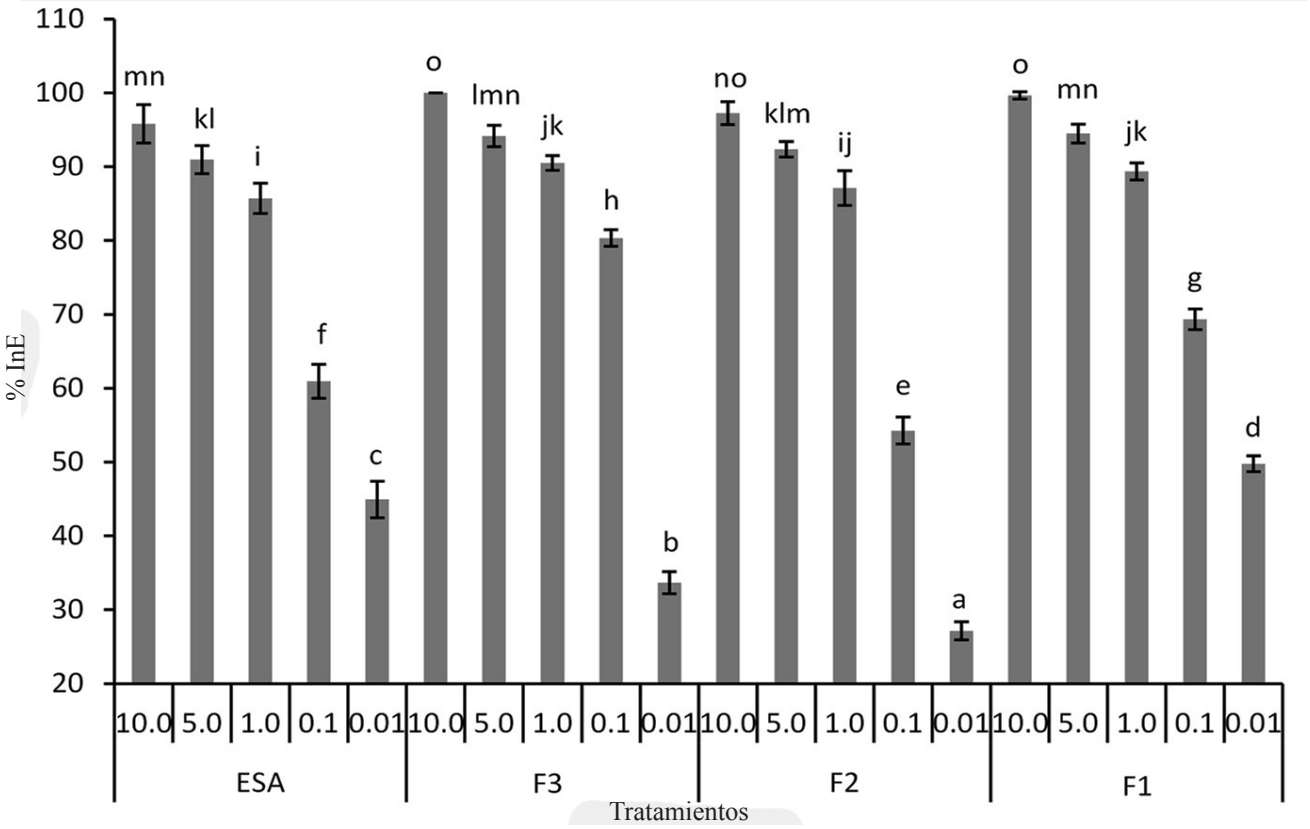


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Figura 4. Percentage of inhibition of production of conidia (% InE) treatment outcomes (extracts and fractions) of *H. vulgare* against *F. oxysporum*

Table II. Median effective dose (ED_{50}) of acetone-soluble extract and fractions of *H. vulgare* L. evaluated against *F. oxysporum*

Tratamiento	ESA	F1	F2	F3
ED_{50} (µg mL)	24,1±1,3	3,31±0,54	6,79±0,65	16,6±0,8

The results show that the alkylrecorcinols have antifungal activity, which is consistent with the provisions of Zarnowski in his study (7).

This also indicates that alkylrecorcinols could be studied in advanced evaluation processes (*in-vivo* and field) on the way to be used as an alternative control of *F. oxysporum*.

Given the chemical characteristics of the alkylrecorcinols present in the fractions, phenolic lipids with unsaturated chains or smaller, have higher antifungal than those with longer chains saturated and activity. This is consistent with a previous study where it was found that the longer saturated and chain alkylrecorcinols may not retain antifungal activity longer, since some plant pathogenic fungi have the ability to metabolize alkylrecorcinols relatively larger chain, transforming them into less toxic compounds (17).

CONCLUSIONS

- This study is the first work in Colombia aimed at establishing the composition in terms of alkylrecorcinols of barley grown in the Colombian Central Andean territory.
- The chromatographic profile of the acetone-soluble extract of the Andean variety of barley identified at least eight major alkylrecorcinols. This extract also presented total content higher than in other studies alkylrecorcinols.
- The three scavenged fractions enriched alkylrecorcinols and showed higher antifungal activity (percentage inhibition of both growth and production of conidia) that crude extract, indicating that alkylrecorcinols could be responsible for the reported activity.

- It was demonstrated that the fractions containing alkylrecorcinols with unsaturated chain with fewer carbons are between two to four times more active than those containing more alkylrecorcinols saturated carbons.
- The results indicate that these compounds are abundant in barley and other grasses, are important and valuable chemical entities that eventually could be subject to further studies, leading to the inclusion of alkylrecorcinols in plant pathogen control programs as *F. oxysporum.*

ACNOWLEDGEMENT

The authors thank the New Granada Military University for funding. CIAS derived product-INV-1471 project, funded by the Vice-Rector of Research UMNG Effectiveness 2014.

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Received: April 9th, 2015
Accepted: February 29th, 2016

Revista Cultivos Tropicales
Instituto Nacional de Ciencias Agrícolas