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Mellein, a dihydroisocoumarin with bioherbicidal activity from a new strain of *Lasiodiplodia pseudotheobromae* C1136

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ABSTRACT

The use of synthetic herbicides poses a serious threat to environment, health and food safety. The development of safe and effective bioherbicides for selective control of weeds is thus the primary concern in crop production around the world. Efforts are being made to investigate the use of a safe, ecofriendly and highly selective herbicides from biological origin. Coumarin and its derivatives are natural compounds renowned for their phytotoxic potential.

Mellein, a dihydroisocoumarin designated as (*R*)-8-hydroxy-3-methylisochroman-1-one was isolated as an active metabolite from the broth of a new strain (this strain is one of the best among phytopathogenic strains collected) of *Lasiodiplodia pseudotheobromae*. The structural characterization of the compound was achieved by using combined spectroscopic analysis i.e. 1D and 2D NMR and LC–MS. The host range study was carried-out against plants from *Cucurbitaceae*, *Solanaceae* and *Leguminosae*, *Poaceae and Valerianaceae* families, the isolated phytotoxic metabolite from *Lasiodiplodia pseudotheobromae* at a concentration 10 µg/µl showed selective inhibition at 56%–66% against the latter two families only when inoculated.

Hence, purified compound from *Lasiodiplodia pseudotheobromae* is recommended for large scale production of bioherbicides for the management of weeds in conventional farming to improve yield and enhance food security.

1. Introduction

Tropics and sub-humid tropics dominates many regions in Africa. Humid ecosystem and elevated raise in temperature, make this region a haven for weeds and its sustenance. Weeds make a large building block of the pest network in African farming lands which is a paramount hindrance in harvest and farm outputs. They act at the same tropic level as crops and share or take nutrients and resources useful for plants' development and crops' good yield. Quantitative damages caused by weeds are a result of their competition for water, light and nutrients meant for crops and this ultimately led to reduction in crop yield (Pacanoski, 2006).

Apart from the fact that synthetic herbicides are applied annually or semi-annually, their specificity is low and they destroy organisms. Furthermore, synthetic herbicides are not easily biodegradable (Adetunji et al., 2018). Therefore, they are stored in water and plants leading to severe pollution in the environment and eventually, causing serious health hazards in humans (Adetunji et al., 2017). Approximately one million people reportedly fall ill every year as a result of pesticide pollution (Aneja, 2000). Furthermore, the continuous usage of conventional herbicides has led to the development of resistance to herbicides among weeds (Caamal et al., 1996). This has necessitated rigorous search for novel herbicides which would be new to the weeds and environmentally friendly as well. Specifically, biological (organic) control has been identified as an alternative method to synthetic herbicides (Adetunji and Oloke, 2013). Among the many studies which have been carried out so far on the use of the biological enemies of weeds (bacteria, fungi, insects, nematodes and viruses) to curb weed,

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phytopathogenic fungi have attracted more attention. This is because they can be used easily and its safety is highly guaranteed (Evidente and Abouzied, 2006). Fungal phytotoxins are secondary metabolites that depict an unavoidable role in the initiation of disease symptoms in agricultural practices, forest plants and weeds (Graniti et al., 1989; Ballio and Graniti, 1991; García-Pajón and Collado, 2003; Möbius and Hertweck, 2009). These secondary metabolites belong to different classes of naturally occurring compounds: aromatics, alkaloids, aminoacids, coumarins and its derivatives, cytochalasans, ethanones, furopyrans, flavonoids, quinones, tannins, nonenolides, oxazatricycloalkenones, pyrones, spirophytotoxins, terpenes, trichothecenes, and some others with a intricate and primary carbon skeleton (Charudattan, 2001; Rajcan and Swanton, 2001).

Lasiodiplodia pseudotheobromae emerged from a recent separation of cryptic species originally identified as *L. theobromae* (Alves et al., 2008). The species occur in Africa, Latin America and Europe, where it has been isolated from plants, fruit trees and forest. More and more evidences speak that *Lasiodiplodia pseudotheobromae*, like *Lasiodiplodia theobromae*, has a distribution which is worldwide based and has a large host range (Mehl et al., 2011).

Based on the fore-going facts, we herein report the isolation, structure elucidation, and the herbicidal activity of a fungal metabolite from *Lasiodiplodia pseudotheobromae*.

2. Material and methods

2.1. Materials

Optical rotation $[\alpha]_D^{25}$ of the isolated phytotoxic metabolite from *Lasiodiplodia pseudotheobromae* was measured by using a polarimeter, measured in $CHCl_3$ solution on a JASCOP1010 digital polarimeter; IR and UV spectra were determined as neat and in MeCN solution, respectively, on a Perkin–Elmer Spectrum ONE FT-IR Spectrometer and a Lambda 25 UV–Vis spectrophotometer. ^1H NMR and ^{13}C NMR experiments were carried out on Jeol ECX 300 NMR spectrometer using $CDCl_3$ as solvent, same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Berger and Braun, 2004) DEPT, COSY-90, COSY-135 and HMBC. All evaporation of solvents was done at 40 °C under reduced pressure on BÜCHI Rotavapor-R114, Switzerland. All solvents used for flash chromatography were purchased from Merck, Germany. Aluminum sheets pre-coated with silica-gel 60 F254 of Merck, Germany (Product no. 105554) were used for TLC. Column chromatography was performed with silica gel (60–120 mesh) while flash chromatography was performed using silica gel (200–400 mesh).

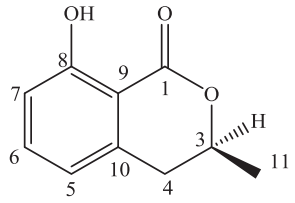
2.2. Media, buffers and solutions used for purification pure compounds

All the media components were prepared in either MilliQ water or double distilled water and sterilized by autoclaving at 15 lb/in^2 for 20 min at 121 °C in liquid cycle. Czapek dox medium was used to culture *Lasiodiplodia pseudotheobromae* at 25 °C. This was used to inoculate 25 × 2 L flasks each containing 400 ml medium and incubated with shaking at 200 rpm for 5 days. Media was isolated by centrifugation. The media cultures were incubated under static conditions at 25 °C in the dark for 4 weeks, then filtered, assayed for phytotoxic activity and lyophilized for the successive purification steps.

2.3. Extraction and isolation of the active metabolite from Lasiodiplodia pseudotheobromae

2.3.1. Purification of phytotoxic compound from Lasiodiplodia pseudotheobromae

For isolation of the phytotoxic compound, the broth was taken after 7 days of fermentation.



(-*R*) -8-hydroxy-3-methylisochroman-1-one

Fig. 1. The structure of (*R*)-8-hydroxy-3-methylisochroman-1-one.

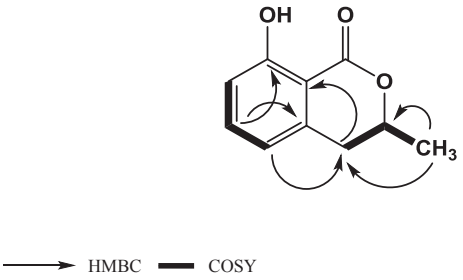


Fig. 2. COSY and HMBC correlation of (*R*)-8-hydroxy-3-methylisochroman-1-one.

2.3.2. Fungal strain and culture condition

The fungal strain *Lasiodiplodia pseudotheobromae* used in this study was isolated from *Tridax procumbus* leave. This strain was grown in Czapek Dox Broth (CDB, 30 g/L) at 30 °C, pH 6.8 at 200 rpm on an orbital shaker. After one week the broth was collected by filtration and used for isolation of phytotoxic compound (Fig. 1).

2.3.3. Isolation and purification of phytotoxic compound

The secondary metabolites were extracted form one week old culture broth by ethylacetate and dried over Na_2SO_4 and evaporated on rotary evaporator under reduced pressure. The crude extract was evaluated for phytotoxic activity by leaf puncture assay. The organic layer retained should have the phytotoxic activity while the aqueous layer should be non-toxic. The crude extract was dissolved in defatting solution which is composed of hexane, methanol and 1 M NaCl in 5:4:1 ratio, respectively. The hexane layer was collected, dried over Na_2SO_4 and evaporated on rotary evaporator under reduced pressure to obtain extract in round-bottom flask. These hexane and aqueous layer extracts were evaluated for their phytotoxic activity. The hexane layer should retain the phytotoxic activity and the nonsignificant phytotoxic activity was shown by the aqueous layer extract. Since, the hexane layer extract has the phytotoxic activity so we attempted to purified the compound responsible for this activity. The hexane layer extract was dissolved in mobile phase and applied on preparative TLC plate. The two major band was separated on TLC plate. Both bands were scratched and extracted by ethylacetate and dried over Na_2SO_4 and evaporated on rotary evaporator under reduced pressure. The phytotoxic activity assay confirmed that upper band with R_f value of 0.51 retained the best activity. This preparative TLC purified sample was further purified by preparative HPLC. The preparative HPLC profile revealed that only one major peak was present which was later isolated. This isolated peak was analyzed for its purity by analytic HPLC (Fig. 3) and HPLC profile determined the purity of the compound.

2.3.4. Characterization of phytotoxic compound

The structure elucidation of purified compound was achieved by NMR, IR and MS and was identified as 8-hydroxy-3-methylisochroman-1-one, which is commonly known as mellein. The specific rotation was determined by polarimetry and it was $[\alpha]_D^{25} = -104.6^\circ$ (c 0.56, $CHCl_3$).

The culture media was extracted with ethyl acetate and the resulting

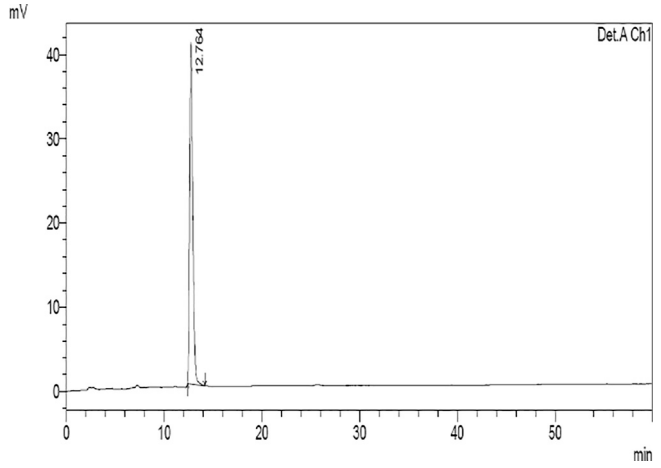


Fig. 3. HPLC chromatogram of (*R*)-8-hydroxy-3-methylisochroman-1-one.

solution was dried over sodium sulphate and evaporated at 40 °C on a rotary evaporator to obtain the crude ethyl acetate extract. This extract which showed necrotic activity was defatted and afterwards subjected to flash chromatography, examining each fraction for necrotic activity. Ninety two (92) tubes of 50 cm^3 each were collected and based on TLC evidence, combined fractions 34–39 which showed significant necrotic activity was further purified by preparative HPLC. The preparatory HPLC was done using C-18 column (250 * 25 mm) and the elution was done with acetonitrile/water (90/10) at a flow rate of 12 ml/min and the detection was done at λ_{254}.

2.3.5. Host range test of the phytotoxic metabolite from Lasiodiplodia pseudotheobromae

In the study, eleven (11) plant species, including weeds, vegetables and field crops were utilized to evaluate and determine the host-range effect of the pure compound from *Lasiodiplodia pseudotheobromae.* The crude extract, the chromatographic fractions and the pure compound were assayed on host plants using the leaf puncture assay. The pure compound, as well as the fractions, were first dissolved in a small amount of methanol and then diluted to the desired final concentration (1 mg/ml concentration) with distilled water. Droplets (15 ml) of assay solution were applied to punctured detached leaves stored in moistened chambers. Symptom appearance was observed 2 or 3 days after application, using the same assay, for selected plants. Throughout the assay, the leaves were kept in a growth chamber at relatively low temperature (23 °C), relative humidity (60%), and illumination (150 $\mu E\, m^{-2}\, s^{-1}$). Distilled water was used as control. Toxicity was then determined with the following scale: − = no symptoms; + = necrosis with diameter around 1–2 mm; ++ = necrosis 2–3 mm; +++ = necrosis 3–5 mm; ++++ = wider necrosis using these as percentage inhibition.

2.3.6. Bio-herbicidal assay of the purified compound on Echinocholoa crus-galli and cowpea

The effect of different concentration containing 0.1, 1.0, 1.5, 2.0, 2.5 µg/µl of the purified compound was tested on the initial development of *Echinocholoa crus-galli* and cowpea inside a petri dish containing 6.0 ml of the purified compound while control was obtained using distilled water. The treatment were randomised with four repetition of 50 seeds of *Echinocholoa crus-galli* and 10 seeds of cowpea per plate. The seeds were surface-sterilized prior to the experiment for 20 min with 1% NaOCl (4% NaOCl commercial bleach), then rinsed three times with distilled water (SiddiquI et al., 2009). The plates were sealed and incubated in a BOD chamber at 25 °C using a photoperiod of 12 h for 7 days. The effect of the purified metabolite was tested on the hypocotyl, the radicle length, the necrotic area on *Echinocholoa crus-galli* (Brasil, 1992). Moreover, the following characteristics were measured: Leaf mean area (length × breath), germination, seedling root and shoot length (cm) and fresh weight (g). Germination percentage was calculated for each replication using the formula: G = (Germinated seed/Total seed) × 100.

2.4. Statistical analysis

All measurements were done in triplicates. Results were expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA (SPSS software version 21).

3. Results

3.1. Structural elucidation of the isolated bioactive compound from the Lasiodiplodia pseudotheobromae

The HPLC isolated compound (PC) was obtained as a white powder $[\alpha]_D^{22}$ −119° (c 0.43, $CHCl_3$, 25 °C), IR (cm^{-1}): 3310 (OH), 1790 (C=O), 1280 (C–O); Mp: 105 °C; ESI-MS: [M+1], 179. ^{1}H NMR 300 MHz, ($CDCl_3$, δ-TMS) δ_H 4.71 (1H, m), δ_H 2.92 (2H, d, J = 7.23), δ_H 6.67 (1H, d, J = 7.89), δ_H 7.39 (1H, t, J = 8.34, 7.89), δ_H 6.84 (1H, dd, J = 7.6, 1.2), δ_H 10.92 (OH), δ_H 1.5 (3H, d, J = 7.18): ^{13}C NMR, 75 MHz, ($CDCl_3$, δ-TMS): (See Table 1) compared with the literature (Pablo et al., 2013; Vatcharin et al., 2012). The purified compound from the metabolite of *Lasiodiplodia pseudotheobromae* was analyzed by further using ^{1}H NMR, ^{13}C NMR, DEPT-45, DEPT-90, DEPT-135 and COSY (Supplementary data Figs. 1–6).

3.2. Host range test

Host range test was carried out on eleven plants' species which belongs to six families namely *Poaceae*, *Valerianaceae*, *Cucurbitaceae*, *Solanaceae* and *Leguminoceae*. Most of these plant species fall within two major classes of plant i.e. Monocotyledon and Dicotyledon. Tested with the leaf puncture assay on the host plant, the toxin after 2 days produced necrotic circular lesions resembling those caused by the pathogen. The diameter of the necrotic area appeared very wide (up to 0.5 cm) when 10–15 mg/droplets (around 6 and 1.2 × 10^3 M, respectively) were applied to the leaf surface, and was still quite evident at a concentration five times lower. Assayed on several weed and cultivated plants, both monocots and dicots, at 15 mg/droplet, mellin showed interesting selective properties. The results obtained as shown in Table 2, revealed that the phytotoxic metabolite obtained from *Lasiodiplodia pseudotheobromae* did not have bioherbicidal effect on the tested crops except Lamb's lettuce (showed 66.7% inhibition) but it had a bioherbicidal effect on all the tested weeds from the same class (showed selective inhibition). In fact, as shown in Table 2, it was completely ineffective on all the *Solanaceous* species assayed (Red pepper, Potato), *Cucurbitaceae* (Melon, Cucumber), *Leguminosae* (Cowpea) but caused severe (up to 1 cm) necrosis on tested weeds

Table 1
^{1}HNMR and ^{13}CNMR data for PC.

S/N	^{1}H (J in Hz)	^{13}C	DEPT135
1	–	170.05	–
2	–	–	–
3	4.71 (1H, m)	76.21	76.21
4	2.92 (2H, d, J = 7.23)	34.65	34.65
5	6.67 (1H, *d*, J = 7.89)	118.01	118.01
6	7.39 (1H, t, J = 8.34, 7.89)	136.24	136.24
7	6.84 (1H, *dd*, J = 7.6, 1.2)	116.3	116.3
8	– 10.92 (OH)	162.2	–
9	–	108.3	–
10	–	139.5	–
11	1.5 (3H, d, J = 7.18)	21.35	21.35

Table 2
Host range test of the phytotoxic metabolite from *Lasiodiplodia pseudotheobromae* on the leaf punctured assay.

	Common Name	Scientific Name	Family	Class	% Inhibition	Effects on Leaves
1	Fortail millet	*Setaria italic*	Poaceae	M	56.7	+ + + +
2	Perennial ryegrass	*Lolium perenne* L.	Poaceae	M	25	+ +
3	Brome	*Bromus* sp.	Poaceae	M	21.6	+ +
4	Devil grass	*Cyanoclon dactylon*	Poaceae	M	53.3	+ + + +
5	Blanket grass	*Axonopus compressus*	Poaceae	M	58.3	+ + + +
6	Lamb's lettuce	*Valerianaceae*	Valerianaceae	D	66.7	+ + + +
7	Melon	*Cucumis melo*	Cucurbitaceae	D	0	−
8	Cucumber	*Cucumis sativus* L.	Cucurbitaceae	D	0	−
9	Red pepper	*Capsicum annum*	Solanaceae	D	0	−
10	Potato	*Solanum melongena*	Solanaceae	D	0	−
11	Cowpea	*Vigna unguiculata*	Leguminosae	D	0	−

Toxicity determined with the following scale: − = no symptoms; + = necrosis with diameter around 1–2 mm; + + = necrosis 2–3 mm; + + + = necrosis 3–5 mm. + + + + = wider necrosis > 6 mm. M = Monocotyledon; D = Dicotyledon.

which belongs to *Poaceae*, these are monocot. Furthermore plants species from the family of *Poaceae* response differently to the phytotoxic metabolite with *Axonopus compressus* 58.3% with the highest inhibition and *Bromus* sp. (21%) with the least. Table 2 shows that the coumarin derivative exerts selective bioherbicidal activity on monocotyledons than dicotyledons.

3.3. Bioherbicidal assay

There was no significant difference on the germination of cowpea when the different concentration of the purified compound was tested. However an exception of 83.4% was observed at 1 µg/µl when compared with the control ($p < 0.05$) Fig. 5a. The purified extract did not show any inhibitory effect on the development of root and shoot length of the seedlings but their length varied according to the increase in concentration of the pure compound when compared to the control ($P < 0.05$) (Figs. 5b and 4c). The different concentrations of the extract except for of 0.1 µg/µl did not produce any significant difference in the leaf area of the tested weeds when compared to the control (Fig. 6a). The purified compound was able to induce necrotic effect on the tested leaves of *Echinocholoa crus-galli*. Increase in the concentration of the purified compound led to increase in necrotic area in the leaves. The concentration of 2.5 µg/µl had the highest necrotic area of 1.9 mm^2 ($p < 0.05$) on *Echinocholoa crus-galli* leaves when compared to the control that was treated with distilled water (Fig. 6b). Further, when the purified compound was tested on the *Echinocholoa crus-galli* seeds the radicle and the hypocotyl were inhibited as the concentration as the concentration increases (Fig. 6c and d). However, no significant difference was observed in the seedlings of cowpea dry mass treated with different concentrations at the end of the experiment period when compared to the control (Fig. 7).

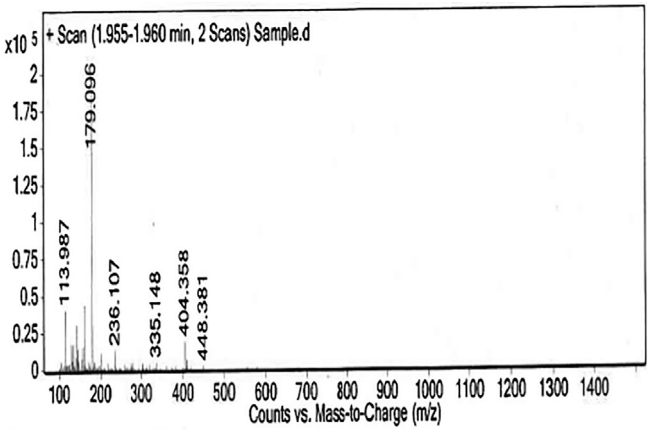


Fig. 4. ESI-MS spectrum of (*R*)-8-hydroxy-3-methylisochroman-1-one.

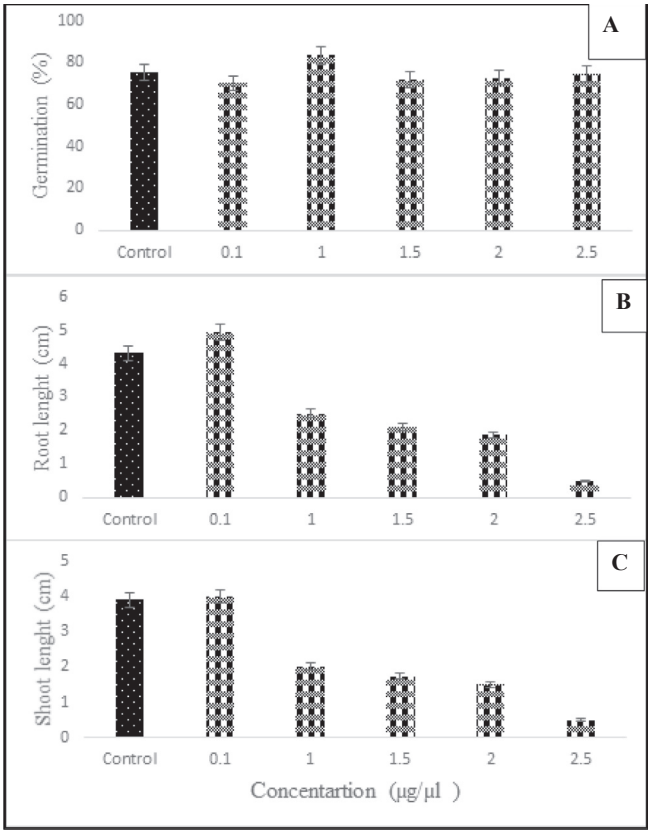


Fig. 5. Germination (A), root length (B) and shoot length (C) of Cowpea seedlings germinated from, (*R*)-8-hydroxy-3-methylisochroman-1-one at five concentrations. The seedlings were evaluated 7 days after germination. Mean followed by the same letter do not differ from one another by the turkey test ($p < 0.05$), mean ± SE. (n = 3).

4. Discussion

The preliminary assays carried out using the crude extract indicated the presence of necrotic compounds in the broth of the fungus culture. The active metabolite was obtained via flash column chromatography and HPLC, as a white compound. IR spectra, thus showed the O–H bond absorbs at 3310 cm^{-1} while the C=O stretching absorbs at the region of 1790 cm^{-1} and at 1280 cm^{-1}, there is a glaring C–O bond. This compound was assigned a molecular formula $C_{10}H_{10}O_3$ according to the $[M+H]^+$ ion at *m/z* 179 in the ESI-MS spectrum. Its ^1H NMR spectrum displayed a methyl doublet at δ 1.51 which indicated the connectivity of the methyl group to a methane proton (H-3). The methane proton was splitted into a multiplet, indicating that it was directly connected to

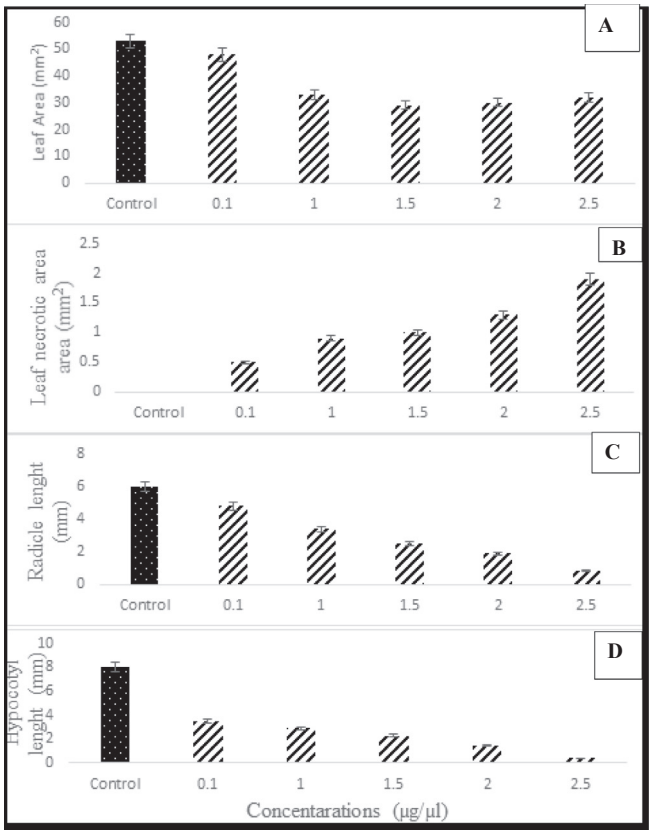


Fig. 6. Mean leaf necrotic area. (A), Mean leaf area values (B), root length (C) and hypocotyl length (D) of *Echinocholoa crus-galli* seedlings germinated, from (*R*)-8-hydroxy-3-methylisochroman-1-one at five concentrations. The seedlings were evaluated 7 days after germination. Mean followed by the same letter do not differ from one another by the turkey test ($p < 0.05$), mean ± SE. (n = 3).

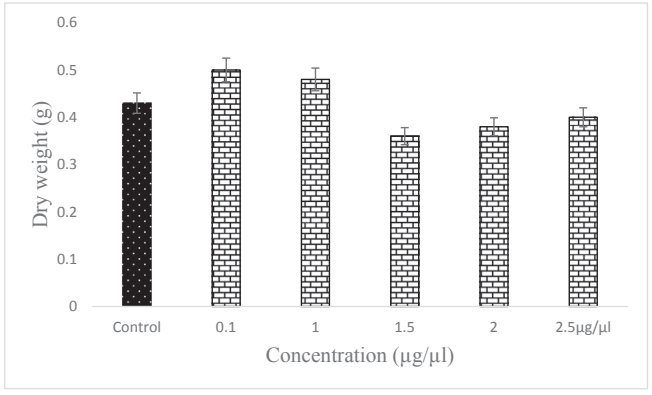


Fig. 7. Dry weight of cowpea seedlings germinated of from, (*R*)-8-hydroxy-3-methylisochroman-1-one at five concentrations. The seedlings were evaluated 7 days after germination. Mean followed by the same letter do not differ from one another by the turkey test ($p < 0.05$), mean ± SE .(n = 3).

methyl and methylene protons. These linkages revealed by the coupling constants were further authenticated by the COSY spectrum which showed the correlation between H-6 and H-7, H-6 and H-5, H-3 and H-4, H-3 and H-9 protons. The signal of the hydroxyl proton was a singlet at δ 11.0. The ^{13}C NMR spectrum of PC indicated the presence of nine carbon atoms which have been assigned as shown in Table 1 with the aid of distortionless enhancement by polarization transfer (DEPT) experiments. The HMBC (Fig. 2) correlations from H-5 [δ_H 6.67 (1H, *d*, J = 7. 89 Hz)] to C-4 (δ_C 34.65), H-11 [δ_H 1.15 (3H, *d*, J = 7.18 Hz)] to C- 4 (δ_C 34.65) and from H-4 [δ_H 2.22 (2H, *d*, J = 7.89 Hz)] to C-9 (δ_C 108.3). The 1H, 1H correlation spectroscopy (COSY) and other HMBC correlations also supported the assignment of the mellein. The configuration of the stereocenter at C-3 in compound 3 was determined to be R based on a comparison of similar NMR data and negative optical rotation (−119.4) with those in the literature (Pablo et al., 2013; Vatcharin et al., 2012; Ningning et al., 2015). Based on the foregoing discussion and in the light of literature (Chacon-Morales et al., 2013) the structure was identified as (R)-8-hydroxy-3-methylisochroman-1-one. Mellin has a molecular formula of $C_{10}H_{10}O_3$.

Coumarins and its derivatives are considered as selective inhibitors and mostly monocotyledons (Sergio et al., 1995). Though further assessments are needed, this semi-selective toxin could have practical applications as a herbicidal compound. At low concentration, it is interesting to note that the toxin is still very active. Sergio et al. (1995) reports earlier that 4-(2,6-disubstituted-phenoxy) coumarin and its derivatives control mono-cotyledenous weed species in the presence of crops and particularly in the presence of cereal crops.

5. Conclusion

In this work, we described the isolation, structural and biological characterization of purified active compound produced by pathogenic fungi. Bioassay-guided and purification of the organic extract from the submerged culture allowed the isolation of a new metabolite with phytotoxic activity on the tested weeds but does not exhibit any adverse effect on cowpea. The phytotoxic metabolites named (*R*)-8-hydroxy-3-methylisochroman-1-one, was characterized by extensive use of spectroscopic (essentially NMR and MS techniques) and chemical methods, as new phytotoxic coumarin. The results suggested that the phytopathogenic coumarin could be promising phytotoxic compound employable as new natural herbicide against moncotyledon weeds.

Acknowledgements

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