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RESEARCH ARTICLE

Potent Anthelmintic Activity of Chalcones Synthesized by an Effective Green Approach

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Supporting information for this article is given via a link at the end of the document.

Abstract: There is currently an urgent need for new anthelmintic agents due to increasing resistance to the limited available drugs. The chalcone scaffold is a privileged structure for developing new drugs and has been shown to exhibit potential antiparasitic properties. We synthesized a series of chalcones *via* Claisen-Schmidt condensation, introducing a novel recoverable catalyst derived from biochar obtained from the pyrolysis of tree pruning waste. Employing microwave irradiation and a green solvent, this approach demonstrated significantly reduced reaction times and excellent compatibility with various functional groups. The result was the generation of a library of functionalized chalcones, exhibiting exclusive (*E*)-selectivity and high to excellent yields. The chalcone derivatives were evaluated on the free-living nematode *Caenorhabditis elegans*. The chalcone scaffold, along with two derivatives incorporating a methoxy substituent in either ring, caused a concentration-dependent decrease of worm motility, revealing potent anthelmintic activity and spastic paralysis not mediated by the nematode levamisole-sensitive nicotinic receptor. The combination of both methoxy groups in the chalcone scaffold resulted in a less potent compound causing worm hypermotility at the short term, indicating a distinct molecular mechanism. Through the identification of promising drug candidates, this work addresses the demand for new anthelmintic drugs while promoting sustainable chemistry.

1. Introduction

Parasitic nematodes mediate significant problems worldwide leading to substantial morbidity and mortality in humans and animals. Additionally, they negatively impact livestock health and act as major pathogens in plants. The prevalence of resistance to traditional anthelmintic drugs is escalating each year, and resistance to newly developed anthelmintics is appearing at a worrying rate.[1] There is a growing concern related to the limited availability of effective anthelmintic drugs and to the emergence of resistance due to the important threats to human and animal health as well as to livestock and agriculture production. Consequently, it is essential to accelerate the development of new drugs with anthelmintic potential.

The free-living nematode *Caenorhabditis elegans* (*C. elegans*) is widely used as a parasitic nematode model for anthelmintic drug discovery.[2,3] It shares physiological and pharmacological features with parasitic nematodes, including neurotransmitter receptors, and it similarly responds to anthelmintic drugs.[4,5] It is a cost-efficient nematode model that overcomes the disadvantages of working with parasites and is, therefore, highly suitable for the search of novel antiparasitic drugs.

Chalcones are flavonoid and isoflavonoid precursors synthesized by plants as a natural defense mechanism against pathogens.[6,7,8] The chalcone scaffold serves as a privileged structure for the search and development of new drugs, given its bioactive versatility and diverse functionalization possibilities. These characteristics make it an attractive platform for designing molecules with therapeutic potential. Several potential therapeutic effects for chalcones have been reported, including anticancer,[9-11] anti-HIV,[12] antidiabetic,[13] antimalarial,[14,15] anti-

inflammatory,[16,17] antioxidant,[18,19] antituberculosis[20] and antiviral activities.[21] The wide spectrum of activities exhibited by chalcones stems from multifaceted and complex underlying molecular mechanisms. For instance, their anti-inflammatory effects involve inhibition of several enzymes crucial in inflammatory processes, NF-kB (nuclear factor kappa) and microglial cells.[22-24] The antidiabetic effects take place through the modulation of dipeptidyl peptidase-4, peroxisome proliferator-activated receptor gamma, α-glucosidase and tissue sensitivity[25] and some anti-cancer effects take place through downregulation of Bcl-2 and upregulation of Bax expression.[26] Extensive studies are needed to clarify the structure-activity relationships at the molecular and organism levels.

Chalcones have emerged as promising lead molecules in the fight against various parasitic infections,[27-31] including those caused by parasitic nematodes.[32,33] Hybrid benzimidazolyl-chalcones and derivatives have demonstrated *in vitro* nematocidal activity against *Haemonchus contortus*.[34] Furthermore, chalcone derivatives have displayed promising antifilarial activity against *Brugia malayi*.[32,35] For all active chalcones, the IC_{50} values for the antiparasitic effect ranged between 1-100 µM. These findings underscore the potential of chalcones and their derivatives as valuable candidates in the development of novel anthelmintic agents.

A few studies have confirmed the anthelmintic potential of chalcones in *C. elegans*, thereby validating its use as a parasitic model. It was shown that the chalcone scaffold as well as some organic and ferrocenyl derivatives exert long-term paralysis.[36] Also, 4´-dihydroxychalcone (DHC), one of the major constituents present in plant *Zuccagnia punctata*[37,38] kills *C. elegans* after a day of exposure showing an LC_{50} of 17 µg/ml.[39]

The molecular architecture of the chalcone core is based on a chain flavonoid, chemically typified with the 1,3-diaryl-2-propen-1-one framework, in which both aromatic rings (rings A and B) are linked by an unsaturated α,β-carbonyl system. It is believed that the diversity of pharmacological activities can be attributed to the conjugation between the unsaturation and the carbonyl group present in its structure. Their structural diversity is a source of inspiration for drug discovery and the preparation of analogues as simplified, more synthetically accessible and stable models. One of the most traditional reactions to access the chalcone scaffold is the Claisen-Schmidt reaction, which involves the intermolecular condensation between a benzaldehyde and an acetophenone under catalytic conditions.[40,41] During the last decade, significant efforts have been made to develop highly efficient synthetic strategies to prepare chalcone derivatives.[42-48] Unfortunately, the methods generally rely on stoichiometric amounts of strong bases, which should be avoided following by Green Chemistry Principles[49] or toxic transition metal catalysts and suffer from limited functional group compatibility. To overcome these drawbacks, an alternative strategy based on a metal-free catalyst, environmentally benign synthetic systems is very attractive for application in the large-scale pharmaceutical chemical industry. In recent years, microwave-assisted organic synthesis (MAOS) has become an ecological, simple, fast and efficient way.

Biochar (BC) is a carbonaceous material generated from the pyrolysis of waste biomass. Being a low-cost, sustainable material, it has aroused considerable interest in the research community due to its unique characteristics, such as abundant functional groups, high specific surface area, and, pore volume.[50] Besides carbon, BC also contains many other elements such as H, O, S and trace metals that affect and determine its corresponding chemical properties.[51] In addition, BC acts as an electron donor and acceptor, and shows a buffering capacity between acid and base.[52]

We here synthesized a small library of chalcones through a Claisen-Schmidt condensation (CSC), using for the first time a new recoverable BC obtained from the pyrolysis of tree pruning waste, as a catalyst under microwave irradiation and in the presence of a green solvent. It is important to note that the BC was subjected to two different chemical activations by doping with basic or with acidic agents.

The chalcone derivatives were screened for their rapid effects on *C. elegans* as a model of parasitic nematode. We found that the chalcone scaffold as well as two new derivatives exert potent paralysis in the short term and at low concentrations, thus resulting in the identification of novel, eco-friendly, potent anthelmintic agents.

2. Results and Discussion

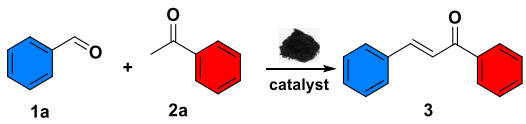
2.1. Chemistry

In the formation of chalcones under homogeneous catalysis, one of the main drawbacks of the CSC reaction is that the speed of the reaction is slow and may take several days to complete. Another problem associated with this transformation is the poor selectivity due to the competitive Cannizzaro reaction of the aldehyde and secondary reactions such as the Michael addition. Despite the exposed limitations, the CSC continues to be used today due to its operational simplicity and high atomic economy.
In order to overcome the drawbacks of CSC and improve its synthetic application from a green perspective, a systematic exploratory study was carried out for the synthesis of chalcones through the synergistic effect of coupling heterogeneous solid catalysis with microwave irradiation to accelerate the reaction rate and improve the selectivity of the process.

Reaction conditions for CSC were optimized using a 1:1 molar ratio of benzaldehyde (**1a**) and acetophenone (**2a**) as model substrates.

Based on our previous experience related to the use of BCs as green support for the catalytic synthesis of relevant heterocyclic structures[53] we started our study by evaluating the catalytic activity of different carbonaceous material (Table 1) under modified standard reaction conditions: equimolar ratio of substrates, catalyst (150 mg), EtOH, 60 °C, 12 h. Taking into account the long reaction times, we used microwave radiation to reduce the reaction times and improve the efficiency of the process in terms of energy and yield.

Table 1. Preliminary studies of some catalysts.[a]



1a 2a 3

Entry	Catalyst	Source	T (°C)	Batch[a] Yield (%)[c]	MW[b] Yield (%)[c]
1	--	--	40	--	--
2	Activated carbon	Commercial	40	--	--
3	BC-1	Commercial[h]	40	trace	10
4	BC-2	Sunflower seed hull	40	--	--
5	BC-3	Peanut hull	40	--	--
6	BC-4	Tree pruning	40	trace	19
7	BC-4	Tree pruning	60	trace	28
8	acidic BC-4	Tree pruning	60	trace	30
9	basic BC-4	Tree pruning	60	15	78
10	basic BC-4	Tree pruning	60	9[d]	44[d]
11	basic BC-4	Tree pruning	60	22[e]	89[e]
12	basic BC-4	Tree pruning	60	18[f,g]	85[f]

[a] **Reaction conditions**: benzaldehyde **1a** (1 mmol), acetophenone **2a** (1 mmol), catalyst (150 mg) in EtOH (2 mL), 12 h at 40 °C. [b] Reaction was conducted at 250 W, 25 min. Time reaction monitored by TLC and GC-MS. [c] Isolated yield after purification by recrystallization. [d] Reaction was conducted using 100 mg of basic BC-4 catalyst. [e] Reaction was conducted using 200 mg of basic BC-4 catalyst. [f] Reaction was conducted using 250 mg of basic BC-4 catalyst. [g] Benzyl alcohol (8%) was detected from crude product. [h] BC-1 was provided by Dinomotive inc.

In the first control experiment, no progress in the reaction was evidenced in the absence of a catalyst (Table 1, entry 1). When the reaction was carried out in the presence of commercial activated carbon as the catalyst, no reaction advance was observed (Table 1, entry 2). Furthermore, in the presence of BC-1 in EtOH, no significant progress towards the formation of the desired product was achieved (Table 1, entry 3). The use of BC-2 as a catalyst also did not lead to the formation of the expected product under both reaction conditions explored (Table 1, entry 4). On the other hand, BC-3 mediated catalytic activity under both reaction conditions (Table 1, entry 5) did not lead to the formation of chalcone **3**. However, when we used our novel BC-4 under microwave irradiation (250 W, 40 °C), progress of reaction was evident even at very short reaction times (25 min) (Table 1, entry 6). On the contrary, when the reaction was performed under batch conditions, a trace of **3** was detected after 12 h. Interestingly, a slight increase in the formation of the chalcone base skeleton was observed when the reaction was subjected to microwave irradiation at 60 °C (Table 1, entry 7).

After these initial investigations, we repeated the CSC reaction but previously chemically activating BC-4 with a basic agent (KOH) and in another case with acid (H_2SO_4), which corresponds to basic BC-4 and acidic BC-4, respectively. When the acidic BC-4 sample was evaluated, no significant improvement in the reaction yield under microwave activation was evidenced (Table 1, entry 8). However, when the condensation was evaluated by the catalytic action of basic BC-4 under microwave irradiation conditions, the desired product was isolated with a very good yield (Table 1, entry 9). Based on this encouraging result, the other BCs were also chemically activated based but in no case was it possible to improve the formation of **3** in terms of yields. The considerable improvement in yield due to the BC-4 catalyst would be related to a synergistic effect of the basic species present in the BC and those introduced in the KOH treatment.

Likewise, when the reaction was carried out under batch conditions, a low yield of **3** (15%) was detected after 12 h. This led us to conclude that both, basic BC-4 and microwave radiation are crucial for CSC.

Next, we optimized the amount of the basic biochar-based catalyst under microwave irradiation at 250 W of power and 60 °C. As illustrated in Table 1, we started our investigation using 150 mg of basic BC-4 thus obtaining the expected product with a 78% yield (Table 1, entry 9). Unfortunately, a lower amount of catalyst (100 mg) was unsatisfactory in terms of performance (Table 1, entry 10), while an amount of 200 mg of catalyst significantly improved the expected chalcone conversion (Table 1, entry 11) when the reaction was carried out under microwave irradiation (89% yield). A further increase in the concentration of basic BC-4 did not significantly improve reaction times and yields (Table 1, entry 12).

It should be noted that under batch conditions benzyl alcohol was detected from the crude product (8%) by GC-MS, presumably due to a concomitant Cannizzaro reaction (Table 1, entry 12).

Once the origin and the charge of the biochar-based catalyst were defined, we proceeded to study the incidence of microwave radiation in the reaction at power values of 100, 200, 250 and 300 W. For this purpose, we used a focused CEM-Discover reactor at 60 °C. The progress of the CSC reactions was monitored by TLC and GC-MS at different times. Complete conversion of the substrates was observed after 25 min at 200 W and 60 °C, isolating chalcone **3** without further purification in 95% yield. Microwave irradiation at 100 and 300 W of power led to lower conversions of starting materials and reaction times of more than 25 min. It should be noted that a shorter reaction time (e.g., 20 min) led to incomplete transformation of the starting materials, while an increase in time up to 50 min or more did not result in an improvement in terms of yield. In this way, we were able to conclude that the best MW power in terms of yields and speed of product formation was 200 W at 60 °C.

As a continuation of our study towards a more eco-friendly protocol, we evaluated other green solvents such as water, *N,N*-dimethylpropyleneurea (DMPU) and 1,3-propanediol but in no case there was an improvement in the course of the reaction towards the formation of the desired chalcone under microwave irradiation. On the other hand, the use of less polar solvents such as 2-methyltetrahydrofuran (MeTHF) or 1,3-dioxolane led to significantly lower product yields. Likewise, all attempts to carry out the CSC reaction under solvent-free conditions were unsuccessful for the synthesis of **3**.

From these studies, we conclude that the optimal reaction conditions for the synthesis of chalcone **3** require the use of benzaldehyde (1 mmol), acetophenone (1 mmol), basic BC-4 (200 mg) as catalyst, and EtOH (2 mL) at 60 °C under microwave

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irradiation (200 W) for 25 min. Surprisingly, under the optimized mild reaction conditions, neither the external presence of stoichiometric amounts of base nor the catalytic action of transition metals were necessary for the successful progress of the reaction. It is also important to note that the formation of by-products such as the *cis*-chalcone isomer, the Michael addition product (1,3,5-triphenylpentan-1-5-dione) and the aldol addition product were not detected from the analysis of the products crude by GC-MS, which denotes the complete selectivity of the present methodology towards the exclusive formation of the (*E*)-chalcone **3** by direct cross-condensation of **1a** with **2a**.

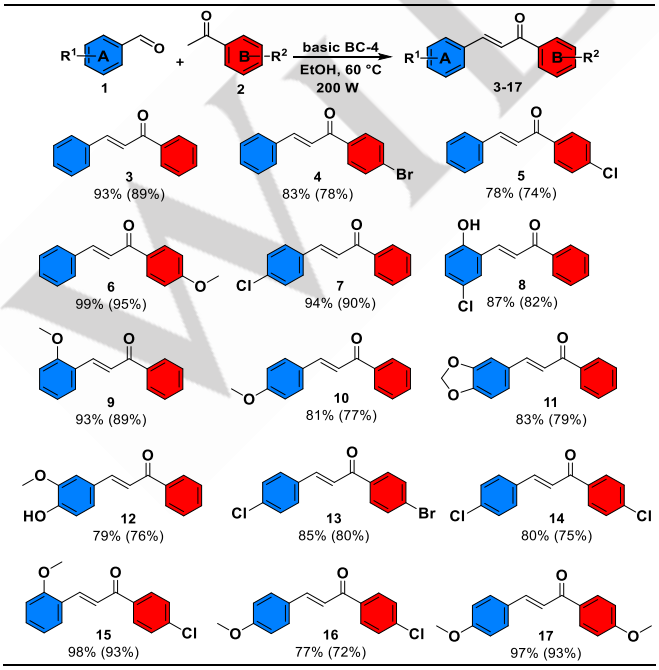
With the optimized conditions in hand, we then explored the scope, limitations, and overall efficiency of CSC by reacting aryl aldehydes with different acetophenones, and the results are summarized in Table 2.

The results described in Table 2 clearly indicate that the new green synthetic methodology is compatible with the presence of a variety of functional groups in the A ring, including chlorine, methoxy, dioxole, and a free phenolic hydroxyl group, providing the desired chalcones in very good to excellent yields (**7**-**12** and **13**-**17**). However, in the presence of a methoxy group in the A ring, the yield of the isolated product decreased slightly to 77% (**10**). The reaction conditions showed a good tolerance to the presence of electron-rich or electron-deficient groups in the B ring, which generally led to the formation of the expected chalcones with very good yields (**4**-**6** and **13**-**17**).

Remarkably, chalcones that contain a hydroxyl and/or halogen in their conjugated structure are useful for the introduction of further functionalization.

The present protocol has also been found to be highly useful for the condensation between 5-chloro-2-hydroxybenzaldehyde and acetophenone, which selectively led to the formation of 2-hydroxychalcone derivative (**8**) without any cyclized side products.

Table 2. Scope for the synthesis of functionalized chalcones via CSC.[a]



[a] **Reaction conditions**: benzaldehydes (1 mmol), acetophenones (1 mmol), basic BC-4 (200 mg) in EtOH (2 mL) at 60 °C under microwave irradiation (200 W) for 25 min. [b] Quantified by GC analysis using internal standard method. Isolated yield after purification (in parentheses).

At this point, it is important to note that the employment of our new heterogeneous catalyst based on a novel biochar from residual biomass allowed the synthesis of a small library of chalcones from benzaldehydes and acetophenones with free hydroxyl groups, thus overcoming the limitations from a large majority of reports of conventional CSC under basic catalysis. Additionally, under these reaction conditions, no by-products such as self-condensation products, the Michael addition product or the aldol addition product were observed. The treatment and isolation of the crude product consisted of adding ethyl acetate to the reaction mixture. The catalyst was recovered by filtration and washed several times with the same solvent. The filtrate was washed twice with saturated NaCl solution and then dried over dry Na_2SO_4. The solvent was removed under reduced pressure and the desired product was purified by recrystallization from ethanol. The structures of all synthesized chalcones (**3**-**17**) were confirmed by comparing their melting points with those reported in literature and on the basis of FT-IR, ^{1}H- and ^{13}C NMR spectral data, as provided in the Supporting Information.

To check the reuse of the basic BC-4 catalyst, we selected the synthesis of chalcone **3** as the test reaction under the optimized conditions. The results are reported in Figure 1. After completion of the reaction (monitored by TLC), the catalyst was easily separated from the reaction mixture by filtration, washed several times with EtOAc, and then dried under vacuum to be used directly for further catalytic reactions. No significant loss of catalytic efficiency was observed up to five cycles. The percentage of yield was 89, 88, 86, 86 and 85% for cycles 1 to 5, respectively (Figure 1). The spent catalyst was characterized by X-Ray Diffraction (XRD) and it was found that there were no changes in the catalyst diffraction pattern before and after the reaction, which shows that the nature of the basic BC-4 sample was not modified in the reaction conditions. XRD pattern for fresh and spent basic BC-4 catalysts are shown in the Supporting Information. In addition, the basic BC-4 and the corresponding spent catalysts were studied by thermal analysis (TGA/DTA), for analyzing the thermal decomposition of the different components of the samples and the amount of ashes. It is observed that the same profile is obtained for both catalysts. Thus, it is concluded that during reaction the catalysts does not leach species towards the reaction media that could carry out a homogenous catalytic process. For further information, see Supporting material regarding thermal studies.

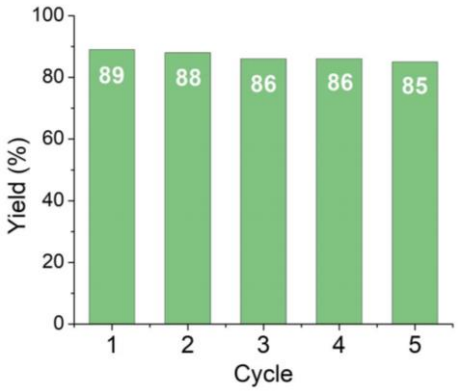


Figure 1. Recyclability of the basic BC-4 catalyst in the synthesis of **3**.

2.2. Biology assays

2.2.1. C. elegans as a nematode model to test anthelmintic actions of chalcones

One reason for the limited number of available anthelmintics may be related to the difficulty in identifying lead compounds by high throughput in parasites. *C. elegans* has been widely used as a parasitic nematode model for anthelmintic drug discovery and for the search of new drug targets and it offers the possibility of performing high throughput screening that is not possible with parasitic species.[2,3,5,54,55] Although some features corresponding to parasites are not present in *C. elegans*, it has been shown that molecules that kill *C. elegans* are fifteen times more likely to kill parasitic nematodes compared with randomly selected molecules, which makes pre-screening with *C. elegans* an attractive option to increase the throughput of future anthelmintic discovery campaigns.[2] Different assays have been developed for testing nematicide drug actions using *C. elegans*. The nematicide action of drugs can be manifested as rapid effects, usually as changes in worm motility, rapid paralysis or impaired pharyngeal pumping, or as long-term effects, evidenced by decreased lifespan or impaired egg hatching. We here used an automatic system that allows continuous and automatic quantification of the movement of worms in liquid medium to evaluate rapid actions of compounds on worm motility.[56,57]

2.2.2. Chalcone 3 leads to a rapid reduction of worm motility

The chalcone **3** has shown activity against parasitic nematodes.[58] Additionally, **3** has been shown to kill *C. elegans* after prolonged exposure of 3 days.[36] We here evaluated its ability to mediate rapid effects on worms by the automatic quantification of animal movements as a function of time of exposure to the drug.

Wild-type young adult hermaphrodite worms were exposed to different concentrations of chalcone **3** (5-80 µM) or DMSO (lower than 0.1% per well) as a vehicle control. As shown in Figure 2, chalcone **3** reduced worm motility as a function of concentration and time. At 5, 10 and 20 µM, the effect of chalcone **3** on worm motility was slight. However, at 50 µM, motility decreased about 55% and 70% after 60 and 120 min of exposure, respectively. At 80 µM, worm motility decreased 80% after 120 min (Figure 2A). The dose-response curve for the 60-min exposure to chalcone **3** yielded an IC_{50} of 52 ± 9 µM (Figure 2B).

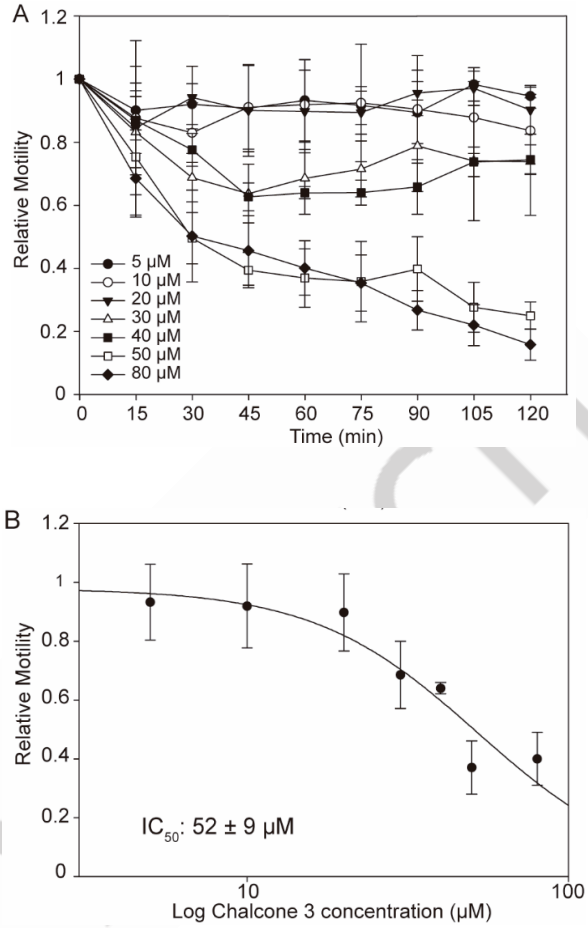


Figure 2. Effects of chalcone **3** on worm motility.

A-Relative motility of wild-type worms as a function of time of exposure to chalcone **3** at different concentrations. The motility was related to the basal measurement before addition of the drug. Each point corresponds to the mean ± SD of at least three independent experiments as described in Methods.

B-Dose-response curves determined for the paralyzing effect of 60 min-exposure to chalcone **3** on wild-type worms. Each point corresponds to the mean ± SD of the relative motility measurement of three independent experiments as described in Methods.

2.2.3. Chalcone 3 induces spastic paralysis not mediated by L-AChRs

Paralyzing drugs affect differently worm body length depending on their mechanisms of action and pharmacological targets. Drugs that induce spastic or flaccid paralysis, such as levamisole or piperazine, decrease or increase worm length, respectively.[54,57] Also, drugs can paralyze worms without affecting their body length.[59]

To evaluate the type of paralysis generated by chalcone **3**, we exposed wild-type young adult worms to the drug in agar plates and measured the body length after 120 min. As a control for spastic paralysis, we exposed worms to the classic anthelmintic drug levamisole. After 120 min exposure to DMSO (0.1%), 1 mM chalcone **3**, or 0.03 mM levamisole, the mean body length was 0.87 ± 0.07 mm (n=101 worms), 0.81 ± 0.07 mm (n=208 worms) and 0.60 ± 0.06 mm (n=155 worms), respectively.

The reduction of body length after treatment with chalcone **3** compared to DMSO was statistically significant ($p<0.001$, Student t-test) (Figure 3), indicating that chalcone **3** exerts spastic

paralysis. As a control drug, we used levamisole, which is an agonist of the levamisole-sensitive nicotinic acetylcholine receptor (L-AChR). L-AChR in *C. elegans* body-wall muscles is a main anthelmintic target and it is composed of three essential, UNC-29, UNC-38 and UNC-63, and two accessory subunits, LEV-1 and LEV-8.[60] Sustained activation of this receptor produces muscle contraction that leads to spastic paralysis.

Under the experimental conditions, the body contraction mediated by chalcone **3** was not as profound as that mediated by levamisole, suggesting that chalcone **3** either acts through a different pharmacological target or has reduced efficacy compared to levamisole acting through the L-AChR.

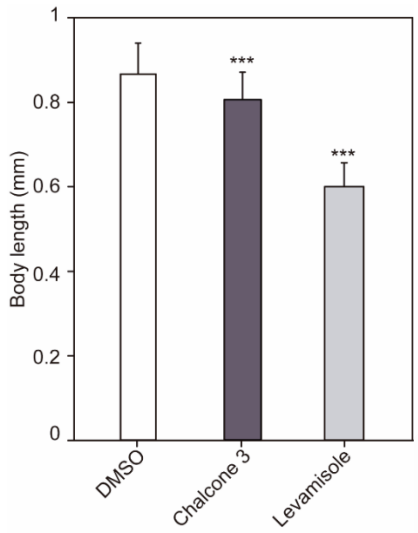


Figure 3. Measurement of body length of wild-type worms in the presence of DMSO (0.1%), chalcone **3** (1 mM) or levamisole (0.03 mM). Each value corresponds to the mean ± SD of the body length of 101, 208 and 155 worms, respectively. Student t-test, ***p<0.001.

To evaluate if L-AChR is the pharmacological target of chalcone **3**, we tested its effects on a mutant strain lacking this receptor. The strain CB904: *unc-38(e264)* lacks the essential subunit UNC-38, hence worms have not functional L-AChRs.[61] Since this strain shows markedly impaired and uncoordinated movement, we used the trashing assay test to evaluate locomotor activity.[62] Wild-type and mutant young adult worms were exposed to 50 µM chalcone **3** or DMSO as a vehicle control, and thrashes per minute were counted for each condition and strain. After 60 min of exposure, the relative reduction of motility in the presence of the chalcone, which was referred to the motility in the presence of DMSO alone for each strain, was 0.75 ± 0.11 for wild-type worms and 0.72 ± 0.15 for mutant worms. No statistically significant differences in the reduction of worm motility between strains were found (Student t-test p=0.754, n=4 experiments).

Therefore, the lack of the L-AChR does not affect the sensitivity of the worm to chalcone **3**, indicating that L-AChR, which is the target of levamisole, is not a main target of chalcone **3**.

2.2.4. Influence of substituents in the B ring on anthelmintic activity

With the aim of determining if the presence of substituents at the B ring increases anthelmintic activity, we synthetized and analyzed a group of synthetic chalcones with different substituents in the B ring: chalcone **4** with a bromine group, chalcone **5** with a chlorine group and chalcone **6** with a methoxy group.

Synchronized young adult wild-type worms were exposed to 50 µM of chalcones **3**, **4**, **5** and **6** or DMSO for 60 and 120 min. We used this concentration of chalcone analogues since it is close to the IC_{50} determined for chalcone **3**. All tested chalcones significantly affected worm motility compared to the control condition in DMSO (lower than 0.1% per well) (Figure 4A). After 60 min exposure to chalcones **3**, **4**, **5** and **6**, the relative locomotor activity was 0.37 ± 0.09 (chalcone **3**), 0.81 ± 0.04 (chalcone **4**), 0.73 ± 0.04 (chalcone **5**) and 0.51 ± 0.14 (chalcone **6**). After 120 min of drug exposure, the relative locomotor activity decreased even more, showing values of 0.25 ± 0.04 (chalcone **3**), 0.73 ± 0.03 (chalcone **4**), 0.67 ± 0.04 (chalcone **5**) and 0.20 ± 0.083 (chalcone **6**) (Figure 4A).

As shown in Figure 4A, chalcones **4** and **5** showed lower efficacy than chalcone **3** and the analogue containing the methoxy group (chalcone **6**). There were no statistically significant differences in the reduction of worm motility between chalcones **3** and **6** (60 min p=0.086 and 120 min p=0.315, Student t-test), indicating that chalcone **6** is as efficacious as chalcone **3**. The IC_{50} value for chalcone **6** obtained from the dose-response curve after 60 min of exposure was 34 ± 15 µM (Figure 4B).

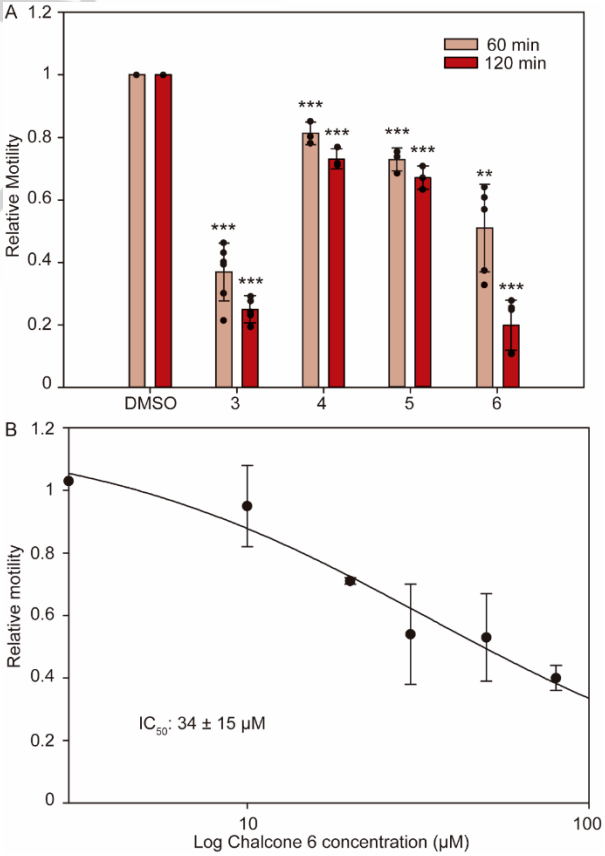


Figure 4. Effects of substituents in the B ring of chalcone **6** on *C. elegans*.

A-Relative motility of wild-type worms exposed to chalcone **3** and chalcones carrying different substituents (**4**, **5** and **6**) in the B ring during 60 and 120 min. The worms were exposed to DMSO (lower than 0.1% per well) and 50 µM chalcones. The values correspond to the mean ± SD of at least three individual

experiments as described in Methods. Statistical comparisons were done against DMSO condition using the Student's t test. **p<0.01; ***p<0.001.

B-Dose-response curve determined for the paralyzing effect after 60 min-exposure to chalcone **6** on worms. Each point corresponds to the mean ± SD of the relative motility of three independent experiments.

2.2.5. Influence of the substituents in the A ring on worm motility

We next explored the anthelmintic properties of the series of synthetic chalcones with different substituents in A ring. In this series of chalcones, the substituents were introduced at different A ring positions.

Chalcone **7** has a chlorine group; chalcone **8** has a hydroxyl group and a chlorine group; chalcone **9** and **10** have a methoxy group in *orto* and *para* position, respectively; chalcone **11** has a dioxole group; and chalcone **12** has a hydroxyl group and a methoxy group.

Synchronized young adult wild-type worms were exposed to 50 µM chalcones **7**, **8**, **9**, **10**, **11** and **12** or to DMSO (lower than 0.1% per well) during 60 and 120 min. Chalcones **7** and **9** reduced motilities very slightly, and the reduction was statistically significant only after 120 min (p<0.001 and p<0.05, respectively).

On the other hand, chalcone **11** did not affect worm motility at any time (p=0.807 and p=0.985 at 60 and 120 min, respectively) (Figure 5A).

In contrast, chalcones **8**, **10** and **12** produced a statistically significant reduction of worm motility compared to the control condition in DMSO (Figure 5A). After 60 min, the relative locomotory activity compared to the control condition was 0.81 ± 0.05 (chalcone **8**, p=0.003), 0.47 ± 0.19 (chalcone **10**, p=0.005) and 0.84 ± 0.05 (chalcone **12**, p=0.006). After 120 min, the relative locomotor activity was 0.82 ± 0.08 for chalcone **8** (p=0.019), 0.35 ± 0.17 for chalcone **10** (p=0.001) and 0.83 ± 0.03 for chalcone **12** (p<0.001) (Figure 5A).

Thus, among this series of compounds, chalcone **10**, which contains the methoxy group as the substituent group in the A ring, generated the greatest reduction of worm motility. The dose-response curve for 60-min exposure yielded an IC_{50} of 41 ± 6 µM (Figure 5B).

The comparison of IC_{50} values of chalcone **3** and chalcones with methoxy groups in B or A rings (**6** and **10**) shows that the three compounds exhibit comparable potency and efficacy in reducing worm motility.

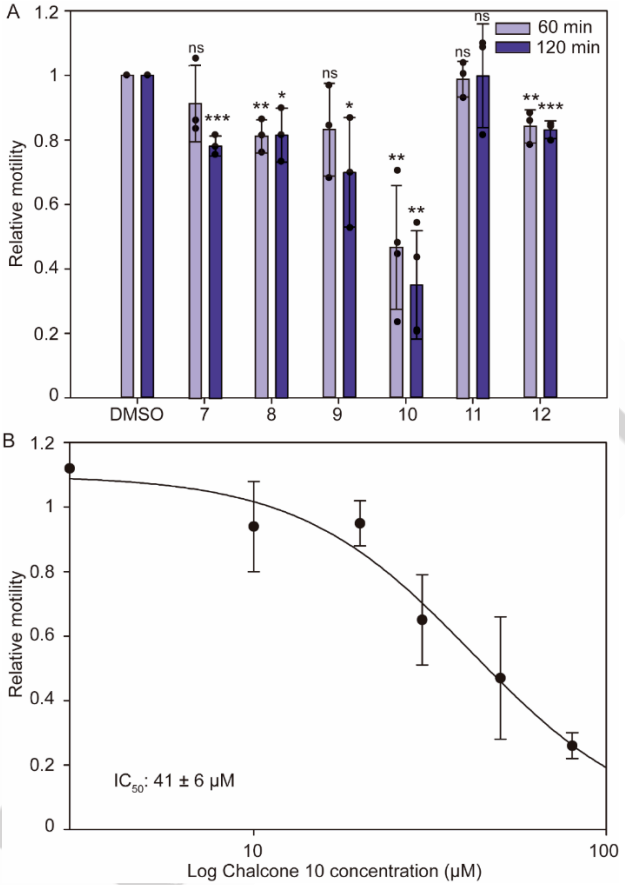


Figure 5. Effects of substituents in the A ring of chalcone **10** on *C. elegans.*

A-Relative motility of worms exposed to chalcones (60 or 120 min) carrying substituents in A ring (**7**, **8**, **9**, **10**, **11** and **12**). The worms were exposed to DMSO (lower than 0.1 % per well) and 50 µM chalcones. The values correspond to the mean ± SD of at least three individual experiments. Statistical comparisons were done using the Student's t-test. *p=<0.05, **p=<0.01; ***p=<0.001.

B-Dose-response curve determined for the paralyzing effect observed after 60 min exposure to chalcone **10** on wild-type worms. Each point corresponds to the mean ± SD of the relative motility measurement of three independent experiments (See Experimental section).

2.2.6. Effects of chalcones with substituents in both rings on worm motility

Since among ring substituents, the methoxy group was the one that conferred the greatest anthelmintic effect (chalcones **6** and **10**), we sought to explore if a chalcone carrying two methoxy groups, one in each ring, showed higher anthelmintic potency than those with a single group.

To this end, we synthesized chalcone **17** carrying a methoxy group in *para* position in each ring. We also explored chalcones with diverse substituents in each ring. Chalcone **13** has a bromine group in *para* position in B ring and a chlorine group in *para* position in A ring; chalcone **14** has a chlorine group in *para* position in each ring; chalcone **15** has a chlorine group in *para* position in B ring and a methoxy group in *orto* position in A ring; and chalcone **16** has a chlorine group in *para* position in B ring and a methoxy group in *para* position in A ring.

Chalcones **13**, **14** and **16** did not affect worm motility after 60 and 120 min of exposure (Figure 6). It is interesting to note that chalcone **16** combines the substituents present in chalcone **5** (chlorine in B ring) and chalcone **10** (methoxy in A ring). Although

the two chalcones are active, the combination results in an analogue that lacks anthelmintic activity. Thus, not only the type and position of substituent groups but also their combination in different rings is important for the pharmacological action of chalcones as anthelmintic drugs.

Chalcones **15** and **17** significatively decreased worm motility. After 60 min, worms exposed to chalcones **15** or **17** showed a 30% decrease of the motility (Figure 6). After 120 min, chalcone **15** did not produce further reduction but chalcone **17** led to a 50% reduction of motility (Figure 6). Increasing the concentration to 80 µM did not enhance the degree of paralysis, indicating no further effect. Thus, the presence of a methoxy group in the two rings slightly decreases the anthelmintic potency compared to that of chalcones showing a single methoxy group in either ring (chalcones **6** and **10**).

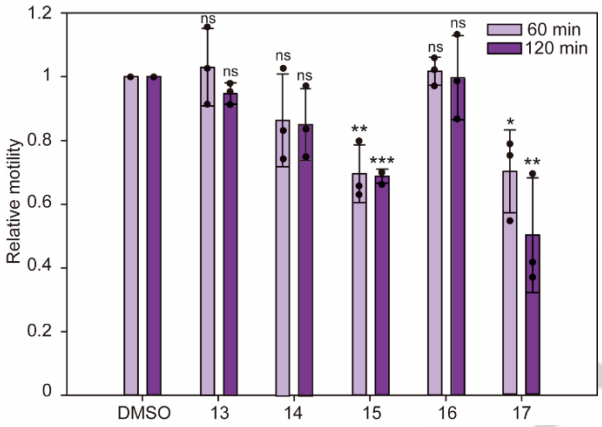


Figure 6. Relative motility of wild-type worms exposed to chalcones (60 or 120 min) carrying substituents in A and B rings (**13**, **14**, **15**, **16** and **17**). The worms were exposed to DMSO (lower that 0.1% per well) and 50 µM chalcones. The values correspond to the mean ± SD of at least three individual experiments. Statistical comparisons were done against the DMSO condition using the Student's t test. *p=<0.05, **p=<0.01, ***p=<0.001.

2.2.7. Changes in worm motility as a function of time during the 120-min exposure to chalcones

We next analyzed in detail how the most active chalcones affect worm motility at 15-min intervals during the 120 min-exposure. At 50 µM, chalcones **3**, **6** and **10** rapidly reduced worm motility after 15 min in liquid medium, and worm motility continued decreasing during the 120 min (Figure 7). When measured at 60 and 120 min-exposure, chalcone **17**, carrying the methoxy group in both rings, was less potent than chalcones **3**, **6** and **10**. Interestingly, chalcone **17** exerted an acceleration of worm motility during the first 30 min of exposure. As shown in Figure 7, chalcone **17** markedly increased worm locomotion at 15 min of exposure and the difference was statistically significative with respect to the chalcone **3** (p=0.002 Student's t-test). This transient acceleration, which revealed a novel mechanism mediated by chalcone **17**, was followed by a sustained reduction of motility (Figure 7). Thus, the detailed analysis demonstrated that, although after 60 min chalcone **17** produces worm paralysis similar to chalcones **3**, **6** and **10**, at shorter times it induces hypermotility, an effect unique to this chalcone. This observation may indicate that chalcone **17** acts through different mechanisms or additional drug targets with respect to the rest of the active chalcones.

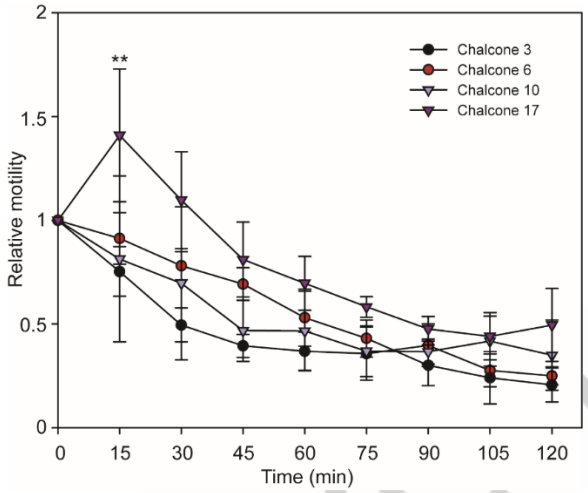


Figure 7. Analysis of motility at 15-min intervals of worms exposed to chalcones during 120 min.
Relative motility of wild-type worms exposed to 50 µM chalcones **3**, **6**, **10** and **17** as a function of time (each 15 min). The values correspond to the mean ± SD of at least three individual experiments. Statistical comparisons were done using the Student´s t-test, **p=<0.01.

The cuticle of *C. elegans* is an extracellular matrix that confers environmental protection.[63,64] and it is an essential barrier for the absorption of many compounds.[4] It has been shown that hydrophobic compounds are more permeable through the cuticle than hydrophilic compounds. In our series of analogues, chalcones with methoxy substituents, such as the active chalcone **6** (log Po/w: 3.27) is less hydrophobic than those carrying a bromine or chlorine substituent in the same position, such as chalcone **4** (log Po/w: 3.92) and chalcone **5** (log Po/w: 3.83), respectively, which are both inactive. Therefore, we suggest that impaired absorption through the cuticle is not the cause of the lack of effect observed in several analogues. Overall, our results indicate that combination of different substituents at both rings govern the selectivity for specific pharmacological targets involved in worm motility. Future studies employing a battery of mutant worms may be undertaken to identify the pharmacological targets of these active chalcones, which, in turn, may help to decipher structure-activity relationships for chalcones.

Chalcones are compounds of natural origin and have a long history of human consumption. Also, they have been administered in large doses to animals showing little or no toxicity.[65-67] There are some toxicity reports indicating the safety of natural and synthetic chalcones in mice and plants.[33,68] *In vitro* assays have demonstrated no or limited cytotoxic effects at medium-high micromolar concentrations depending on the type of substituent of the chalcone and the tested cell line.[26,69,70] Particularly noteworthy are *in vitro* cytotoxicity assays conducted on a series of methoxylated chalcones, which confirmed their lack of significant toxicity.[71] Evaluation of the antimalarial activity of a series of methoxylated chalcones with IC_{50} values against *Plasmodium falciparum* ranging from 1 to > 200 µM, showed that the active compounds were relatively non-toxic for mammalian cells with selectivity indices between 3 and >50.[71] We conducted the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay with human embryonic kidney cells

(HEK-293) to assess cell viability in the presence of the chalcone (**3**) and the three active methoxylated chalcones (**6**, **10** and **17**) at 50 µM. In line with previous studies,[71] no toxicity was detected in the presence of the methoxylated chalcones, whereas a reduction in cell viability was detected with the chalcone **3** after a 4-hour exposure compared to the control exposed to DMSO (0.13%) (Figure S1). Since the permeability properties of the *C. elegans* cuticle may be more restrictive for drugs than those of adult parasites, it is possible to infer that the IC_{50} concentrations for rapid paralysis may be even lower in some parasites.[72] In general, the reduced significant toxicity displayed by chalcones has rendered them attractive scaffolds for drug discovery. However, to validate the therapeutic potential of the active chalcones, further *in vivo* assays are essential including evaluations of toxicity, administration routes, and formulations.

The SwissADME tool[73] was used to predict the physicochemical properties, pharmacokinetics, and drug-likeness of the active chalcones **3**, **6**, **10** and **17** (Table S1). The analysis revealed that these chalcones do not show any violations to Lipinski's rules, which refer to the drug-likeness of the compounds,[74] and do not show PAINS (Pan-Assay INterference compoundS) alert, which refer to molecules that show potent biological outputs that can interfere with biological testing. Additionally, all chalcones exhibit gastrointestinal absorption and blood–brain barrier penetration as determined by the Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) included in SwissADME. These properties are shared by the classical anthelmintic levamisole, which was included in the study for comparison (Table S1). Although experimental *in vivo* studies are necessary to validate these predictions, the analysis supports the drug-likeness of the tested chalcones.

3. Conclusion

In this study, we have developed an attractive, rapid and convenient green synthetic protocol for the synthesis of a small collection of chalcone derivatives by using an innovative recoverable biochar-based catalyst via Claisen-Schmidt condensation. The novel biochar obtained from waste biomass was chemically activated to improve its catalytic action. Unlike most classical dissolution procedures, our catalytic system could be recycled up to at least five times without a significant loss of activity. Our new approach offers very good functional compatibility with very good to excellent yields of the desired condensation products, under metal-free conditions and in the presence of a benign organic solvent.

In response to the need for novel antiparasitic drugs, the library of synthesized chalcones was screened for their rapid effects on *C. elegans* as a model of parasitic nematode. Our findings revealed that the chalcone **3**, along with two new derivatives, exhibit potent paralysis at low concentrations over short timeframes. By using a mutant strain, we discarded the muscle levamisole-sensitive nicotinic receptor as the main target involved in chalcone effects. Additionally, we discovered a hypermotility effect associated with another derivative. As a result, we identified novel anthelmintic agents based on chalcone framework. This work opens doors for identifying the target receptors involved in the effects of chalcones on nematode motility.

4. Experimental Section

4.1. Materials and Reagents

Reagents were obtained commercially and used without further purification. Solvents were dried and distilled in accordance with standard procedure.[75] Reactions were monitored by thin-layer chromatography on silica gel plates (60F-254) visualized under UV light and/or using 5% phosphomolybdic acid in ethanol. Chalcone stock solutions were prepared in dimethyl sulfoxide (DMSO).

4.2. Chemistry

Full experimental description including synthetic procedures, characterization data and copies of ^{1}H and ^{13}C NMR spectra for all chalcones, as well as the preparation and characterization of the BC-4 catalyst are provided in the Supporting Information (Figures S2 and S3).

4.3. Biology assays

***Caenorhabditis elegans* Culture**: Nematode strains were obtained from the *Caenorhabditis* Genetics Center, which is supported by the National Institutes of Health - Office of Research Infrastructure Programs (P40 OD010440). The nematode strain used was PD4251: ccIs4251;dpy-20(e1282) that corresponds to wild-type worms and CB904: unc-38(e264) that corresponds to worms lacking the levamisole-sensitive nicotinic receptor (L-AChR). Nematodes were maintained at 18–25 °C using freshly prepared Nematode Growth Medium (NGM) petri dishes that have been spread with *Escherichia coli* (OP50) as a source of food.[59] For all *C. elegans* assays, the final concentration of DMSO was lower than 0.1% and chalcones were dissolved in DMSO just before starting each assay.

Locomotion assays: Motility assays were performed with wMicroTracker (Phylumtech S.A, Argentina), which is a LED-based assay system that allows nematode motility to be recorded over time.[76,77] All assays were done at room temperature (21–23 °C) with young adult hermaphrodite worms from synchronized plates. Prior to the experiment, hermaphrodite young worms were transferred from NGM agar plates into a 15 ml conical tube containing water, allowed to sink to the bottom and washed three times with water. Worms were then transferred to flat bottomed 96-well microplates containing buffer M9. An average of 30-50 worms were used per well and basal motility of worms within the wells was assessed for 30 min. After basal measurement, drugs (prepared in M9 from DMSO stocks) were added to the wells containing worms, and the locomotor activity was tracked continuously for 120 min. At least 16 replicate wells were used in parallel for each experiment, and each condition was repeated in three to five independent experiments and worm batches.

Body Length Measurement: Synchronized young adult worms were picked to fresh agar plates containing 0.03 mM levamisole, 1 mM chalcone **3** or the vehicle DMSO (lower than 1%) (n>100 worms per condition). Worms were incubated 120 min at 22 °C and after that photographs were taken with a digital camera (Toup Tek Photonics) mounted on a zoom stereo microscope. The body length was measure using the FIJI-ImageJ Software.

Thrashing assays: Synchronized young adult worms were placed in 96-well microplates containing 100 µl M9 buffer with drug or the vehicle DMSO in each well at room temperature. After 60 min, the number of thrashes (bends of the body from one side to the other) were counted during 1 minute. Experiments were carried out four independent times (n>15 worms were analyzed per condition in each experiment).

Cytotoxicity assay with MTT: HEK-293 cells (2×10^4 cells/well) were seeded in 96-well plates and grown at 37 ºC. Cells were exposed 4 h to the chalcones at 50 µM or DMSO (0.13%) before performing the MTT assay. MTT is a water-soluble tetrazolium salt which is reduced by mitochondrial dehydrogenases of metabolically viable cells to a colored, water-insoluble formazan salt. Briefly, MTT (2 mg/ ml) was prepared in sterile phosphate buffer saline (PBS) and added to the cell culture medium to reach a final concentration of 0.2 mg/ml. Cells were subsequently incubated for 2 h at 37 °C and lysed with 100 µl/ well of a buffer containing 20% SDS (sodium dodecyl sulfate).

The extent of MTT reduction was measured spectrophotometrically (570 nm absorbance-650 nm absorbance) using a Multiskan™ microplate spectrophotometer (Thermo Fisher Scientific). Results are expressed as percentage with respect to the control condition. 24 samples for each condition were analyzed. Data represent the mean value ± SD of three independent experiments.

Data and statistical analysis: Experimental data are shown as mean ± S.D. Statistical comparisons were done using the Student's test. A level of $p<0.05$ was considered statistically significant. All the tests were performed with SigmaPlot 12.0 (Systat Software, Inc). Dose-response curves were fitted by a logistic function using Sigmaplot 12, from which IC50 (half maximal inhibitory concentration) values were obtained and expressed as mean ± SE.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available.

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Keywords: Chalcones • Claisen-Schmidt condensation • Biochar • Caenorhabditis elegans • Anthelmintic.

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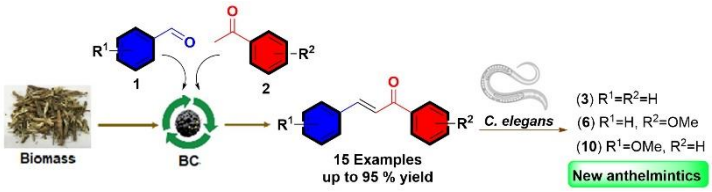

The first heterogeneous catalysis employing a novel biochar from biomass for the synthesis of a chalcone library has been successfully developed. This new approach proved to be fast, eco-friendly and selective. The chalcones obtained were evaluated on the free-living nematode *C. elegans*. The present study led to the identification of novel and potent anthelmintic agents based on chalcone scaffold.