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# Anthelmintic effect of *Psidium guajava* and *Tagetes erecta* on wild-type and Levamisole-resistant *Caenorhabditis elegans* strains

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**ABSTRACT**

***Ethnopharmacological relevance***

*Psidium guajava* and *Tagetes erecta* have been used traditionally to treat gastrointestinal parasites, but their active metabolites and mechanisms of action remain largely unknown.

***Aim of the study***

To evaluate the anthelmintic potential of *Psidium guajava* and *Tagetes erecta* extracts on Levamisole-sensitive and Levamisole-resistant strains of the model nematode *Caenorhabditis elegans.*

***Materials and Methods***

[1] These authors contributed equally to this work.

Aqueous extracts of *Psidium guajava* (PGE) and *Tagetes erecta* (TEE) were assayed on locomotion and egg-laying behaviors of the wild-type (N2) and Levamisole-resistant (CB193) strains of *Caenorhabditis elegans*.

***Results***

Both extracts paralyzed wild-type and Levamisole-resistant nematodes in a dose-dependent manner. In wild-type worms, TEE 25 mg/mL induced a 75% paralysis after 8 h of treatment and PGE 25 mg/mL induced a 100% paralysis after 4 h of treatment. PGE exerted a similar paralyzing effect on N2 wild-type and CB193 Levamisole-resistant worms, while TEE only partially paralyzed CB193 worms. TEE 25 mg/mL decreased N2 egg-laying by 65% with respect to the untreated control, while PGE did it by 40%.

***Conclusions***

*Psidium guajava* leaves and *Tagetes erecta* flower-heads possess hydrosoluble compounds that block the motility of *Caenorhabditis elegans* by a mechanism different to that of the anthelmintic drug Levamisole. Effects are also observable on oviposition, which was diminished in the wild-type worms. The strong anthelmintic effects in crude extracts of these plants warrants future work to identify their active compounds and to elucidate their molecular mechanisms of action.

**Keywords**

Anthelmintic, *Psidium guajava*, *Tagetes erecta*, *Caenorhabditis elegans*, Levamisole-resistance.

## 1. INTRODUCTION

Parasitic nematodes affect the health of humans, plants, livestock and domestic animals, often resulting in significant economic losses. It is estimated that helminth infections affect a third of the world population, mostly in developing countries (Brooker,

2010). Currently, parasite control by commercially-available anthelmintics is insufficient, particularly in light of the acquired resistance to the main families of broad-spectrum anthelmintic drugs by many, often virulent, parasitic helminths (Papadopoulos *et al*., 2012). Moreover, the increasing use of synthetic anthelmintics for livestock has led to residual levels of these compounds in cattle feces that could be environmentally hazardous (Wardhaugh *et al*., 2001; Lumaret and Martínez, 2005; Schmitt and Römbke, 2008). However, in contrast to antibacterials, development of new anthelmintic drugs has been extremely slow (Holden-Dye and Walker, 2007). Therefore, there is a pressing need to find new compounds that counteract helminth activity (Kaplan, 2004; Kaminsky *et al*., 2008).

The search for potential anthelmintic metabolites from natural sources such as plants is one of the alternatives being investigated as a solution to the problem of resistance. Such studies are strongly supported by traditional knowledge on the use of plants (Geary *et al*., 2012; Popović *et al*., 2016) and by recognition of nature as a rich source of bioactive compounds with therapeutic potential (Harvey *et al*., 2015; Newman and Cragg, 2016).

*Psidium guajava* L (Myrtaceae) and *Tagetes erecta* L (Asteraceae) have been largely used to treat gastrointestinal parasites by different traditional medicine systems (Martínez, 1989; http://www.medicinatradicionalmexicana.unam.mx/monografia.php?l=3&t=Guayaba; http://www.medicinatradicionalmexicana.unam.mx/monografia.php?l=3&t=Cempasúchil% ; Tangpu and Yadav, 2006), and some experimental studies have recognized the antiparasitic properties of these plants. Organic crude extracts of *P. guajava* leaves induced a 70% mortality rate in adult specimens of the parasitic trematode *Paraphistomum cervi* (Zahir *et al*., 2009) and a 100% paralysis rate on *Haemonchus contortus* after 8 h of treatment (Molla and Bandyopadhyay, 2014). Aqueous extracts of this plant material have

also shown inhibition of *H. contortus* egg hatching (Pathak *et al*., 2013). Similarly, it was reported that an acetonic extract of *T. erecta* flowers had a nematicidal effect on *H. contortus* larvae (Galicia-Aguilar *et al*., 2008).

These and other studies show that *P. guajava* and *T. erecta* effectively possess compounds with anthelmintic potential, although their active metabolites and mechanisms of action are still unknown. Moreover, many of the published studies on the anthelmintic potential of these plants have examined the effect of organic extracts, sidestepping the assessment of aqueous preparations closer to the ethnomedical use of these plants as anthelmintics. Since decoctions of *P. guajava* leaves or *T. erecta* flower-heads are empirically used as vermifuges, we sought to assess the anthelmintic potential of aqueous extracts of these plants by assaying their effects on the locomotion and egg-laying behavior of *Caenorhabditis elegans* strains with different sensitivity to the anthelmintic drug Levamisole. Although *C. elegans* is a free-living nematode, the available knowledge on its genomics and development makes it a useful model system for searching for new anthelmintics and characterizing their mechanisms of action or resistance (Geary and Thompson, 2001; Kaewintajuk *et al*., 2010; Ndjonka *et al*., 2014). Besides, *C. elegans* is a good model system for *in vitro* studies, given its easy maintenance in the laboratory, small size and short generation time, traits that facilitate the evaluation of crude extracts or pure compound effects on worm motility, egg laying and larval development without the need of host infection experiments (Katiki *et al*., 2011).

## 2. MATERIALS AND METHODS

### *2.1. Plant material and preparation of plant extracts*

Leaves of *P. guajava* L. and flower-heads (disk and ray flowers) of *T. erecta* L. were collected in the municipality of Querétaro, México, and taxonomically validated. Voucher specimens of *P. guajava* (Gómez 10063b, QMEX) and *T. erecta* (M. Gómez y Z. Mayoral 10064, QMEX) were preserved at the herbarium of the Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro (QMEX), México. The plant material was dried in darkness at room temperature and then ground in a blender. Aqueous extracts of *P. guajava* leaves (PGE) and *T. erecta* flower-heads (TEE) were obtained by boiling 20 g of dry powdered raw material of each plant in 250 mL of bidistilled water for 2 h with constant stirring. Decoction products were centrifuged at 10,000 rpm for 15 min and the recovered supernatants were first filtered using 11 μm Whatman filters prior to 0.45 μm Millipore membranes. Filtered supernatants were lyophilized in a freeze dry system (Freezone 4.5, Labconco, Kansas City, MO, USA), resulting in 1.66 g for PGE and 1.27 g for TEE. The ratio of the herbal drug to the native herbal drug preparation (DER native) was 12:1 for PGE and 15.7:1 for TEE. Lyophilized extracts were preserved at room temperature and protected from light until used on the nematode cultures.

### *2.2. Nematode strains*

The strains N2 (wild-type) and CB193 (defective in the gene *unc-29* and resistant to Levamisole) (Lewis *et al*., 1980) of *C. elegans* were used for the experiments. Strains were maintained in NGM plates seeded with *Escherichia coli* strain OP50, according to Brenner (1974).

### *2.3. Anthelmintic assays*

Locomotion allows nematodes to perform vital functions such as foraging and

defending against predators (Schafer, 2005). Therefore, paralysis is a main effect to be observed when evaluating potential anthelmintic formulations. For locomotion assays, adult nematodes were exposed to different concentrations of PGE and TEE in M9 buffer (10 individuals per treatment) and paralyzed worms were determined at different times of exposure. M9 buffer was used as a negative control and Levamisole hydrochloride 400 μM (Sigma-Aldrich, Cat. 196142) as a positive control (Gally *et al*., 2004). 12 replicates were performed for each extract and concentration.

*C. elegans* locomotion is closely related to egg-laying behavior (Schafer, 2005). Immediately before an egg-laying event, worms exhibit a transient increase in the forward locomotion and their backward movement is inhibited (Hardaker *et al*., 2001). This would imply that PGE and TEE extracts that inhibit *C. elegans* locomotion might also affect egg-laying behavior of the worms. For egg-laying assays, 15 gravid N2 adult nematodes from synchronized populations were exposed to 25 mg/mL of PGE or TEE for 1 or 2 h, respectively. Next, nematodes were washed twice in M9 buffer and then transferred to NGM plates seeded with *E. coli* OP50. Two hours later the nematodes were removed from plates and the remaining eggs were quantified. Five replicates per condition were performed.

*2.4. Chemical analysis of plant extracts*

HPLC analyses of PGE and TEE were carried on an Agilent 1200 series HPLC (Palo Alto, CA, USA) incorporating a diode detector 230 nm, 20-400 nm, 80 nm, on an analytical column Synergi 4 mm Hydro-RP C18 80 Å (250 mm, 4.6 mm, Phenomenex). The mobile phase consisted in acetonitrile/water using a gradient elution of 40% acetonitrile at 0-15 min, 95% acetonitrile at 15-22 min with a flow rate of 0.80 mL/min. PGE and TEE were dissolved in water (1 mg/mL) and 5 mL were injected into the HPLC system. Gallic acid

and ellagic acid were included as standards.

*2.5. Statistical analyses*

For each assay, the values of the extract were compared to the positive and negative controls per each time and concentration. Statistical significance was determined using a Student's t-test (*$P$<0.05 & **$P$<0.01). Statistical analysis was done in R (version 3.1.0).

## 3. RESULTS

*3.1. Aqueous extracts of P. guajava leaves (PGE) and T. erecta flower-heads (TEE) blocked the locomotion of C. elegans*

PGE and TEE had significant dose-dependent paralyzing effects (*$P$ < 0.05) on the wild type N2 strain of *C. elegans* (Fig. 1). While 95% of nematodes treated with TEE 1 mg/mL retained their typical sinusoidal movement after 8 h, 30% of the worms exposed to TEE 5 mg/mL were paralyzed after 4 h of treatment. In the same time period, TEE 10 mg/mL induced 54% paralysis and TEE 25 mg/mL induced 65% paralysis. After 8 h, exposure to TEE 5 mg/mL or higher induced 65% to 75% paralysis. In these assays, Levamisole 400 μM, the positive control, elicited 100% paralysis in the worms at 2 h, whereas more than 95% of worms maintained in M9 buffer, the negative control, remained motile after 8 h (Fig 1A).

PGE also had a clear inhibitory effect on *C. elegans* locomotion, even stronger than the paralyzing effect observed for TEE. N2 nematodes exposed to PGE 1 mg/mL showed 19% paralysis after 2 h and 50% paralysis after 8 h of treatment. PGE 5 mg/mL induced 68% paralysis after 6 h of exposure and PGE 25 mg/mL induced 75% paralysis after only 2 h of exposure and 100% paralysis after 4 h of treatment. As observed in TEE assays, Levamisole 400 μM induced 100% paralysis at 2 h and M9 allowed normal motility in

>90% of the worms after 8 h (Fig. 1B).

*3.2. PGE and TEE also blocked locomotion of a Levamisole-resistant C. elegans strain*

Locomotion inhibitory effects of PGE and TEE were also assayed on the CB193 strain of *C. elegans*. On these Levamisole-resistant worms, both aqueous extracts exerted paralyzing effects similar to those induced on the wild-type N2 strain, although the magnitude of the response was different. TEE 25 mg/mL paralyzed 58% of CB193 worms after 4 h of treatment and 81% CB193 worms after 6 h, whereas this TEE concentration induced 90% paralysis in N2 worms at 6 h and 100% paralysis in these wild-type worms after 6 h treatment (Fig. 2A). PGE exerted similar paralyzing effects on both wild-type and Levamisole-resistant worm strains: PGE 25 mg/mL paralyzed 84% of N2 worms and 91% CB193 worms after 2 h, and paralyzed 100% of wild-type and Levamisole-resistant worms after 6 h treatment (Fig 2B).

*3.3. PGE and TEE decreased the egg-laying of C. elegans*

Egg-laying of adult N2 nematodes decreased significantly after short-term exposure to TEE or PGE. PGE 25 mg/mL diminished egg-laying by 40%, and TEE diminished egg-laying by 65% after 1 and 2 h of exposition, respectively, as compared to the negative control (**$P < 0.01$; Fig. 3). During these experiments the motility of the nematodes was not affected, thereby ensuring that nematodes were migrating to their food source, which stimulates egg-laying performance.

*3.4. Chromatographic profiles of PGE and TEE*

The HPLC analysis of PGE showed an elution profile with three main peaks eluting at 2.245 (1), 2.427 (2) and 3.379 min (3) (Fig. 4). Under the same HPLC conditions, an ellagic acid standard eluted from the column at 1.982 min and a gallic acid standard eluted at 2.082 min, suggesting that neither ellagic acid nor gallic acid were present in PGE. The

HPLC analysis of TEE also showed a simple elution profile with three main peaks eluting at 2.245 (1), 2.427 (2) and 3.379 min (3). As for PGE, peaks with retention times of ellagic acid or gallic acid standards could not be detected in TEE.

## 4. DISCUSSION

Searching for new anthelmintics derived from natural sources for the control of parasitic nematodes is a promising alternative to fight the resistance to current parasiticides and to avoid the ecological costs of their use. Here we show that aqueous extracts of *P. guajava* leaves (PGE) and *T. erecta* flower-heads (TEE) inhibit the normal motility and oviposition in wild-type and Levamisole-resistant strains of the model nematode *C. elegans* in a dose-dependent manner.

Previous works addressed the anthelmintic effects of organic extracts of *P. guajav*a and *T. erecta* on nematodes sensitive to commercial parasiticides (Galicia-Aguilar *et al*., 2008; Ismail *et al*., 2012; Palacios-Landín *et al*., 2015). Our results show that PGE and TEE aqueous preparations strongly suppressed the motility of both Levamisole-sensitive and Levamisole-resistant worms. This implies that PGE and TEE preparations contained water-soluble compounds that could be able to circumvent the resistance to current anthelmintics.

Levamisole is a widely-used commercially-available anthelmintic drug to which some parasites have developed resistance (Becerra-Nava *et al*., 2014; Muñiz-Lagunes *et al*., 2015). *C. elegans* strain CB193 is a Levamisole-resistant strain that carries a mutation in the *unc-29* gene encoding a non-alpha subunit type nicotinic acetylcholine receptor (nAChR), which is the target for Levamisole action (Fleming *et al*., 1997). We used the CB193 strain to initiate characterization of the anthelmintic effects of PGE and TEE. We

found that both extracts exerted paralyzing effects on the Levamisole-resistant CB193 strain, although with different potencies. While the paralyzing effect of PGE on CB193 Levamisole-resistant worms was as strong as on Levamisole-sensitive wild-type worms, TEE exerted a partial but evident paralyzing effect on CB193 strain. These results suggest that the anthelmintic compounds present in PGE are at a higher concentration, or are more active, than those in TEE and that anthelmintic effects of PGE and TEE are mediated by a mechanism of action different from that of Levamisole. In any case, the paralyzing effects shown by both extracts make them two promising sources for finding new anthelmintics able to circumvent the resistance to Levamisole and, potentially, other anthelmintics.

Early studies conducted in mice documented narcotic-like effects that included reduced locomotion after intraperitoneal administration of a non-polar fraction from a methanol extract of *P. guajava* leaves (Lutterodt and Maleque, 1988), and decreased intestinal motility after administration of an aqueous extract of *P. guajava* leaves (Ojewole *et al*., 2008). Whether the paralyzing effects of *P. guajava* formulations on mammalian cells and *C. elegans* are mediated by the same mechanism remains to be elucidated. On the other hand, to the best of our knowledge, a paralyzing effect has not yet been reported for *T. erecta* preparations.

Our study also shows that PGE and TEE had a significant inhibitory effect on the egg-laying behavior of *C. elegans*. *C. elegans* hermaphrodites are self-fertile, and their egg-laying rate and pattern are modulated by various environmental stimuli (Dong *et al*., 2000). Egg-laying takes place when two specific serotonergic neurons stimulate contraction of 16 muscle cells to push the eggs through the uterus and out of the vulva (Desai *et al*., 1988). Furthermore, two opposing pathways involving G protein-coupled muscarinic acetylcholine receptors regulate egg-laying. The Gαq pathway increases the egg-laying frequency, while

the Gα0 pathway reduces it. When nematodes are exposed to an external stimulus such as food, the Gαq signaling pathway is activated; however, the antagonist pathway regulates the frequency of oviposition (Wilkie, 2000). The inhibition of oviposition observed in nematodes treated with PGE or TEE could be the result of blocking the Gαq pathway, stimulating the Gα0 pathway, or from the conjunction of both processes. It was noteworthy that TEE caused lower paralyzing effects than PGE on both wild-type N2 and Levamisole-resistant worms, and exerted a stronger anti-oviposition effect than PGE on the N2 strain. It is possible that mechanisms other than inhibition of locomotion may be involved in the anti-oviposition effect of TEE.

The pathways involved in the paralyzing and anti-oviposition effects of PGE and TEE will be more easily characterized when the compounds responsible for these effects are identified. Previous works attributed the anthelmintic effect of diverse plant preparations to gallic acid or ellagic acid (Faizi *et al*., 2011; Ndjonka *et al*., 2014; Akkari *et al*., 2016; Engström *et al*., 2016; Ramsay *et al*., 2016) and gallic acid has been isolated from a polar sub-fraction of guava leaf methanolic extract (Begum *et al*., 2014). Others have demonstrated the nematicidal effects of thiophenes from *T. erecta* roots against plant endoparasites (Uhlenbroek and Bijloo, 1958, 1959), and a recent report showed the presence of these compounds in flower-heads of *T. patula* (Faizi *et al*., 2011). However, the HPLC analyses we conducted did not detect either gallic acid or ellagic acid in the PGE and TEE preparations. Taking into account this result and given the non-polar nature of thiophenes, it is unlikely that these compounds were responsible for the anthelmintic effects of *T. erecta* herein observed. HPLC analyses also showed that the aqueous preparations of PGE and TEE have a simple composition, with less than 10 main components. This will facilitate the future bioassay-guided identification of their anthelmintic compounds.

Taken together, our results show that *Psidium guajava* leaves and *Tagetes erecta* flower-heads possess hydrosoluble compounds that block *C. elegans* motility by a mechanism different than that of the anthelmintic drug Levamisole, and that these compounds also diminish oviposition in this model nematode. The strong effects elicited by the crude aqueous extracts of *Psidium guayaba* and *Tagetes erecta* validate their ethnobotanical use as anthelmintics and warrant future work to identify the active compounds responsible for the effects and to elucidate their molecular mechanisms of action.



The authors have declared that no competing interests exist.

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**Figure 1. Effect of *Psidium guajava* and *Tagetes erecta* aqueous extracts on *Caenorhabditis elegans* locomotion.** Adults of the wild-type N2 strain of *C. elegans* were incubated in M9 buffer and the indicated concentrations of aqueous extracts of *Tagetes erecta* flower-heads (TEE; 1A) or *Psidium guajava* leaves (PGE; 1B) in treatments with 12 replicates. Control worms received M9 or M9 to which Levamisole 400 μM had been

added. At indicated times, the percentage of paralyzed nematodes was recorded. The results were analyzed using a two-tailed t test. ** $P < 0.01$; * $P < 0.05$.

**Figure 2. Effect of *Psidium guajava* and *Tagetes erecta* aqueous extracts on the locomotion of Levamisole-resistant *Caenorhabditis elegans*.** Adults of the wild-type N2 and mutant CB193 strains of *C. elegans* were incubated with 25 mg/mL of PGE (2A) or TEE (2B) in treatments with 12 replicates. Control worms received M9 or M9 to which Levamisole 400 μM had been added. At indicated times, the percentage of paralyzed nematodes was recorded. The results were analyzed using a two-tailed t test. ** $P < 0.01$; * $P < 0.05$.

**Figure 3. Effect of PGE and TEE on *Caenorhabditis elegans* egg-laying.** Gravid N2 adult worms were exposed to 25 mg/mL of PGE or TEE in M9 in treatments with five replicates. The percentage of oviposition in each treatment was recorded. The results were compared using a two-tailed t test, ** $p < 0.01$.

**Figure 4. HPLC profiles of PGE (A) and TEE (B).** PGE and TEE were analyzed on an Agilent 1200 series HPLC with a column Synergi 4 mm Hydro-RP C18 (250 mm x 4.6 mm) using acetonitrile/water as mobile phase.

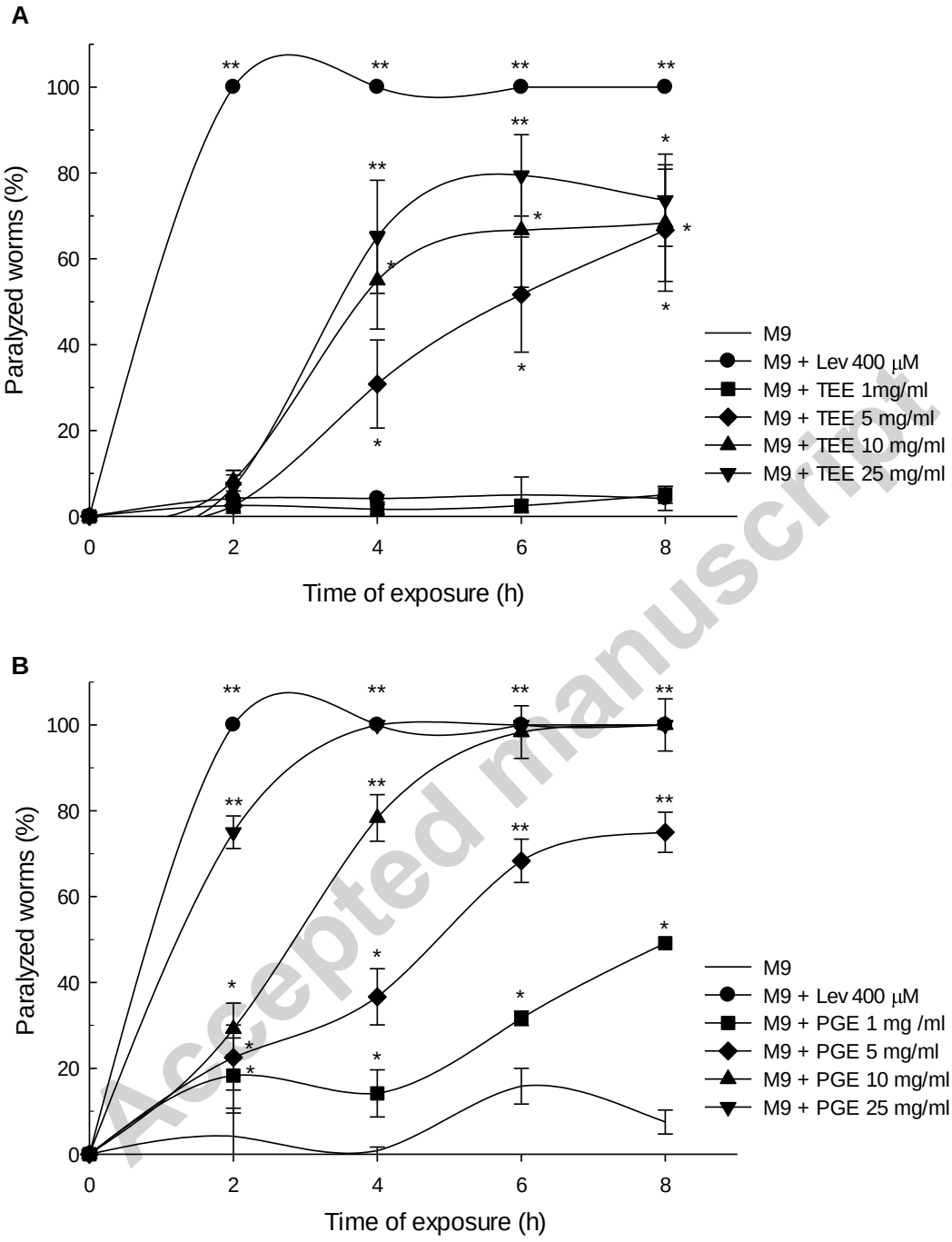


**Figure 1**

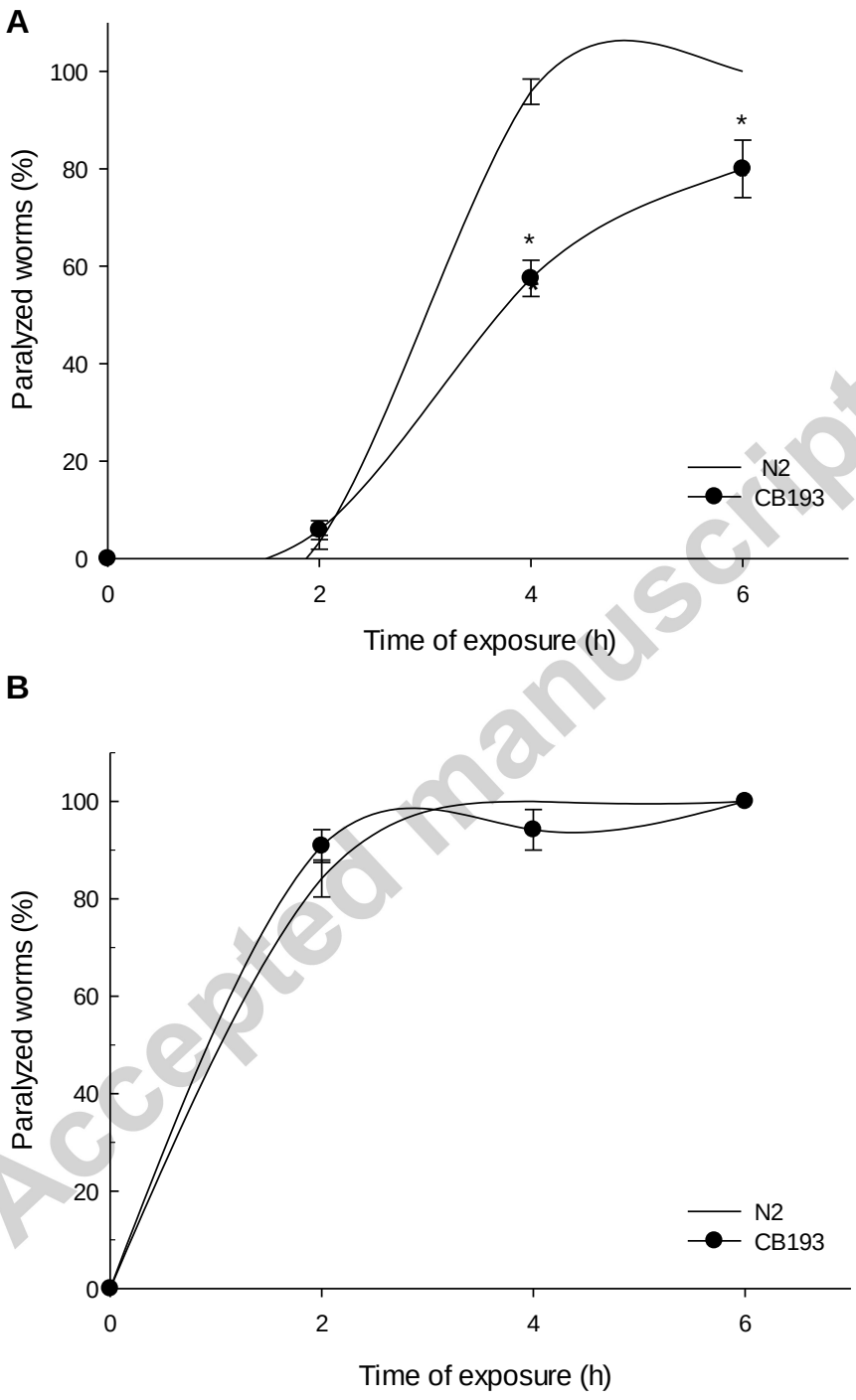


**Figure 2**

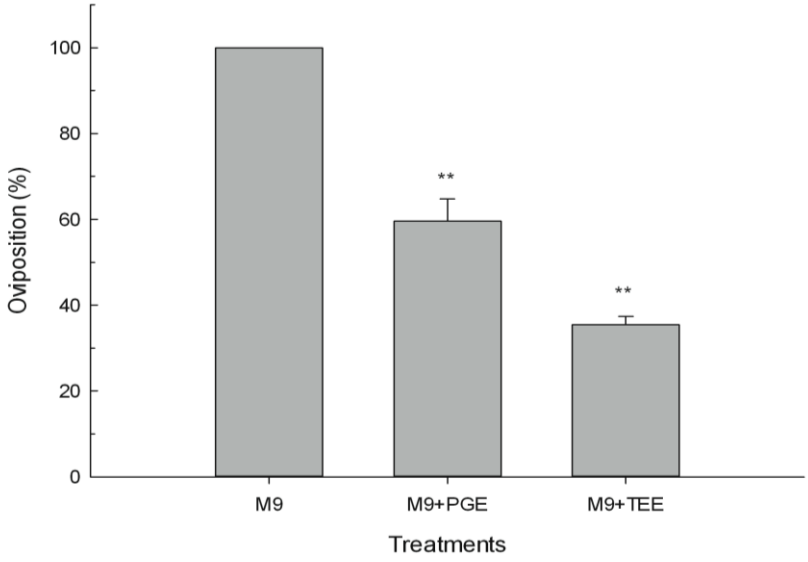


**Figure 3**

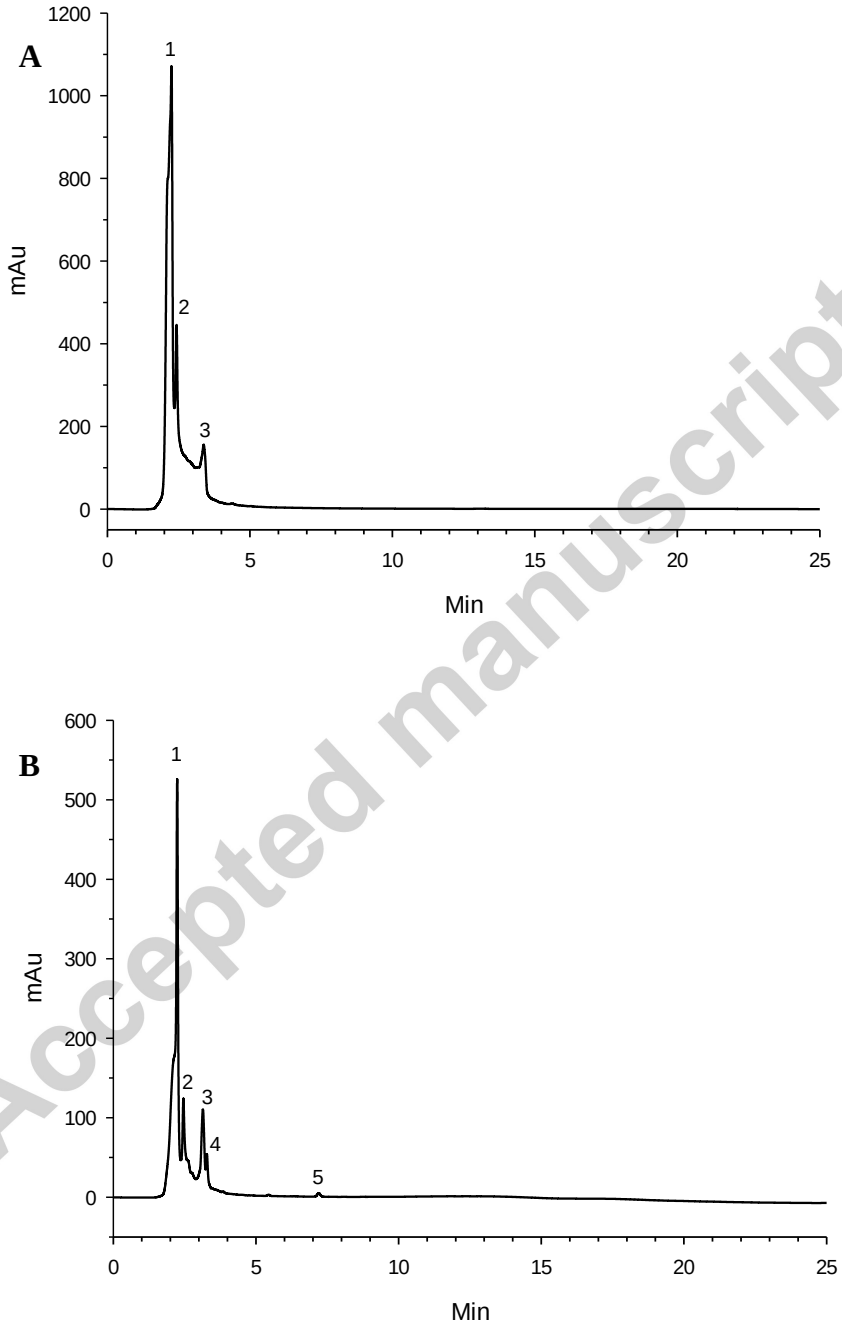




**Figure 4**