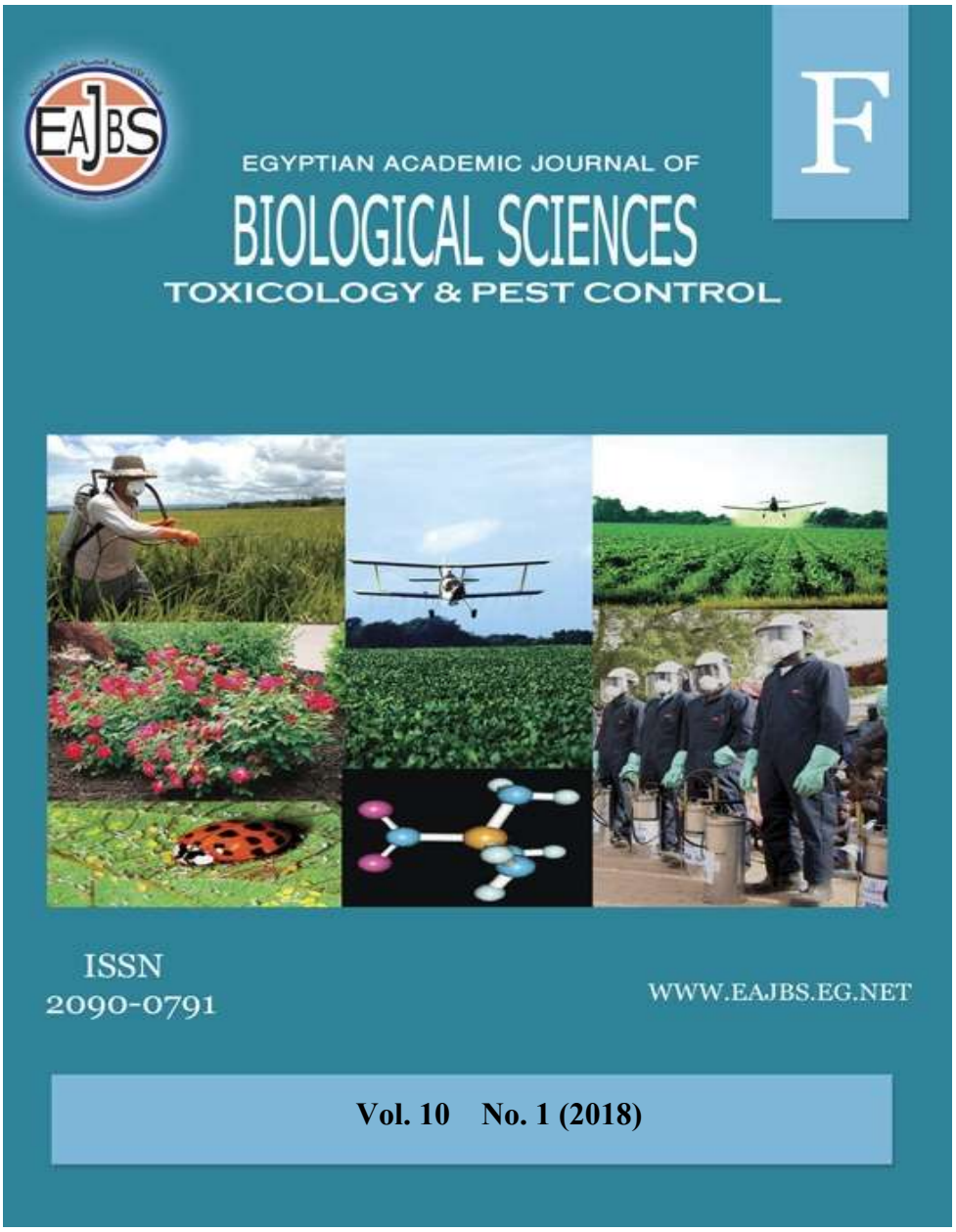

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Nematicidal Potential Impact of Phosphodiesterase (PDE) Inhibitors against Root-Knot Nematode, *Meloidogyne incognita*.

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ABSTRACT

Soon after the discovery that phosphodiesterase (PDEs) are presented in nematodes and regulate the signaling pathway responsible for its motility and reproduction, many of PDE inhibitors have been examined for their nematicidal effects. The present study evaluates the nematicidal activity of seven commercial drugs that act as PDE inhibitors namely diazepam, papaverine, theophylline, caffeine, cilostazol, vinpocetine and sildenafil against root-knot nematodes (*Meloidogyne incognita*) under laboratory and greenhouse conditions. The results showed that all tested PDE inhibitors reduced tomato root galling significantly compared with the untreated check. Diazepam, papaverine and caffeine treatments gave the highest reduction in root galls by 95.5, 93.3 and 91%, respectively. Diazepam exhibited strong nematicidal activity against second-stage juveniles (J2) of *M. incognita* with 48hrs-LC_{50} value of 4.63 ppm. Therefore, using phosphodiesterase inhibitors could serve as the basis for novel nematicides with greater efficacy and reduced environmental impact.

INTRODUCTION

Plant parasitic nematodes cause severe damage to a wide range of economic crops. Root-knot nematodes (*Meloidogyne spp*.) are among the most economically damaging genera of plant parasitic nematodes on horticultural and field crops, responsible for a large part of the annual 100 billion dollar losses attributed to nematode damage worldwide (Ralmi *et al*., 2016).

Current approaches to nematode control are often unsafe and ineffective. Thus, developing nematicides with an increased selectivity, efficacy, and safety profile is a high priority for safe agriculture.

Phosphodiesterases (PDEs) are a diverse family of enzymes that hydrolyse cyclic nucleotides and thus play a key role in regulating intracellular levels of the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and hence cell function. It was found that PDEs are found in all eukaryotes except higher plants. They differ in their substrate specificity, mode of regulation, pharmacological properties and tissue distribution (Boswell-Smith *et al*., 2006). Nematodes contain only 6 of 11 members of vertebrate PDE families (Schuster *et al*., 2015). PDE inhibitors have been developed by pharmaceutical companies to combat a variety of human health conditions and it was discovered that these PDE inhibitors interact with nematode PDE enzymes (Cote, 2015).

A selective PDE inhibitor may be a PDE inhibitor that significantly reduces PDE activity of members of a PDE family at a concentration that does not significantly reduce PDE activity of members of another PDE family. Thus, PDE inhibitors are classified according to which enzymes they act upon. For example, PDE3 inhibitors for congestive heart failure, PDE4 inhibitors for inflammatory airways disease and most successfully, PDE5 inhibitors for erectile dysfunction (Boswell-Smith *et al*., 2006).

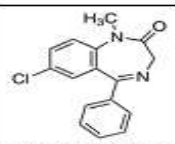
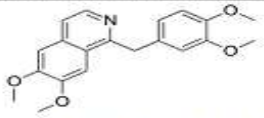
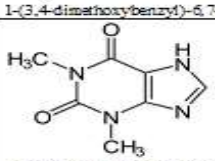
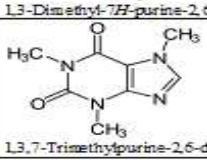
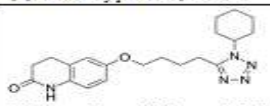
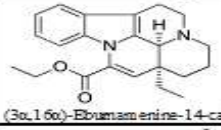
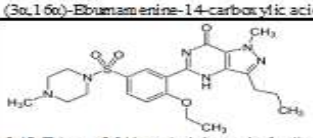
The present study aims to evaluate the potential nematicidal effects of seven commercial drugs that act as PDE inhibitors against second stage juveniles (J2) of root-knot nematodes (*Meloidogyne incognita*) under laboratory conditions as well as their effects in reducing tomato root galling under greenhouse conditions.

MATERIALS AND METHODS

Phosphodiesterase (PDE) Inhibitors Used.

Seven PDE inhibitors were selected from previous publications (Boswell-Smith *et al*., 2006; Schuster *et al*., 2013 & 2015; Cote, 2014) and from "Merck index". They purchased from local pharmacies. Their commercial products, names of active ingredients and chemical structure are listed in Table (1).

Table 1: List of PDE inhibitors used in the present study.

PDE inhibitor	Commercial product	Chemical structure
Diazepam (PDE4 inhibitor)	Neuril (10mg /2ml ampoule)	 7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-one
Papaverine (PDE10 inhibitor)	Vasorin (0.06g /2ml ampoule)	 1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline
Theophline (Non selectivePDE)	Minophilline (300mg tablet)	 1,3-Dimethyl-7*H*-purine-2,6-dione
Caffeine (Non selectivePDE	Caffeinospire (20mg/ml ampoule)	 1,3,7-Trimethylpurine-2,6-dione
Cilostazol (PDE3 inhibitor)	Pletaal (100mg tablet)	 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone
Vinpocetine (PDEI inhibitor)	Vinporal (10mg tablet)	 (3α,16α)-Ebumamenine-14-carboxylic acid ethyl ester
Sildenafil (PDE 5 inhibitor)	Viagra (100mg tablet)	 5-(2-Ethoxy-5-[(4-methylpiperazin-1-yl)sulfonyl]phenyl)-1-methyl-3-propyl-1H, 6H, 7H-pyrazolo[4,3-d]pyrimidin-7-one

Preparation of Nematode Inoculums.

The culture of root-knot nematode, *Meloidogyne incognita* was prepared from naturally infected eggplant (*Solanum melongena*) roots collected from the fields. Individual egg-mass with her mature female was removed from root tissues and placed in a small glass capsule containing fresh water. The female was preserved in 4% formaldehyde solution to be identified into species according to the basis cited by Hartman and Sasser (1985).

A pure stock culture of *Meloidogyne incognita* was prepared from tomato seedling planted in a clay pot (25 cm in diameter) filled with previous steam sterilized sandy loam soil was inoculated with egg-mass. Inoculated pots were kept in the greenhouse and irrigated regularly. The inoculum was propagated on tomato seedlings. Infected tomato plants were the source of experimental inoculum.

To obtain second stage juveniles for the experiments, mature egg masses obtained from the source culture were placed onto a paper tissue supported in a basket sitting in shallow water; hatched juveniles passed through the tissue and were collected daily (Whitehead and Hemming, 1965).

Acute Toxicity of PDE Inhibitors to 2nd Stage Juveniles of Root-Knot Nematode *in vitro*.

According to the concentration of active ingredient present in each drug package, a wide range of concentrations (at least 6-7 concentrations) of each drug was prepared using distilled water. One ml of each drug concentration was added to one ml of newly hatched J2 suspension (containing about100 individuals) to get the examined drug concentrations. Each treatment was replicated three times. The estimation of percent mortalities was calculated according to Abbott's formula (1925) after 48 hr. The obtained data were expressed as toxicity lines, thus, LC_{50} values (the concentration in which 50% of the nematodes were killed) for the tested drugs was determined by probit analysis software programme according to Finney (1971).

Greenhouse Experiment to Evaluate the Effect of Tested PDE Inhibitors on Roots Infection with Root-Knot Nematodes.

Pot experiment was conducted under greenhouse conditions at the Department of Plant Protection, the Faculty of Agriculture in Cairo, Al-Azhar University. Clay pots (15cm in diameter) were filled with 1kg steam sterilized soil (2:1) (sand: clay). Three week old Seedlings of tomato (*Solanum lycopersicum* L. cv. Super Strain B) were transplanted in each pot at one seedling / pot. A week later, the plants were inoculated with 1000 freshly hatched second stage juveniles (J2) of *Meloidogyne incognita* per pot by suspended J2 in 10ml of water and pipetted into 4 equidistant 3cm-deep holes surrounding the root zone of each plant. Immediately after inoculation the holes were covered with soil. Each of the tested drugs was applied directly after inoculation as soil drench at the rate of 1000ppm in a total volume of 25 ml distilled water. Inoculated pots were drenched with 25 ml sterilized distilled water left as check treatment. All treated pots including controls (untreated inoculated with *M. incognita*) were arranged in a complete randomized block design and each treatment was replicated three times. Two weeks after inoculation, plants were gently removed from pots, washed free of adhering soil and the number of galls per root system was counted. The reduction of root galls was calculated according to the formula:-

$$\% \text{ reduction of root galls} = \frac{\text{No. of galls in control} - \text{No. of galls in treatment}}{\text{No. of galls in control}} \times 100$$

Statistical Analysis:

Data were subjected to ANOVA by using Costas program (1988) and significant difference among the treatments was portioned by Duncan's multiple range test at probability levels of P = 0.05.

RESULTS AND DISCUSSIONS

The laboratory experiments were conducted to study the potential nematicidal activity of tested PDE inhibitors against second-stage juveniles (J2) of *M. incognita*. A wide range of seven tested drugs concentrations were added to nematode suspension and the percent mortalities were determined after 48 hrs. The toxicity lines are presented in Fig. (1) and the LC_{50} values are listed in Table (2).

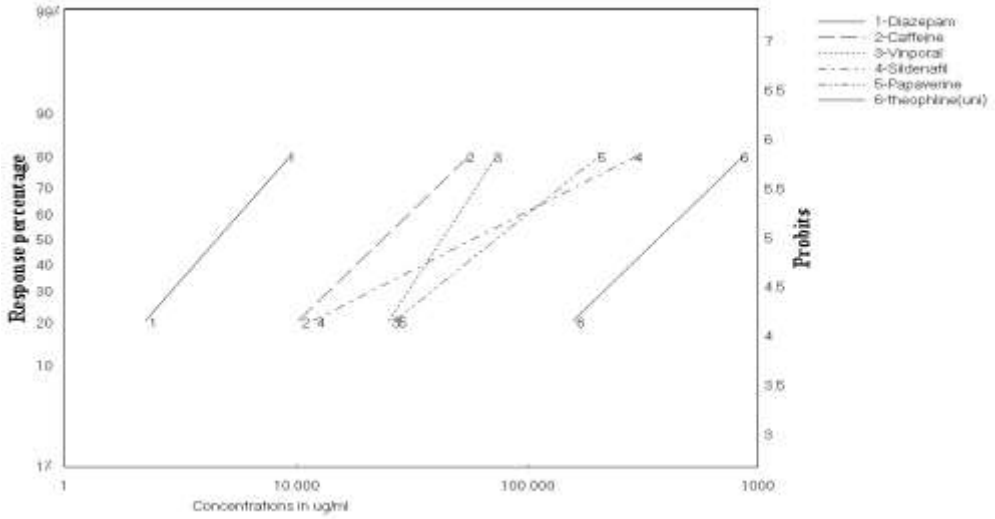


Fig. 1: Toxicity regression lines of tested PDE inhibitors on J2 of root-knot nematode under laboratory conditions

Table 2: LC_{50} and slope values of certain Phosphodiesterase (PDE) Inhibitors to J2 of root-knot nematode (*M. incognita*) after 48 hrs.

PDE inhibitors	LC_{50} values (ppm) (lower-upper limits)	Slope values
Diazepam	**4.63** (4.07-5.30)	2.68±0.23
Caffeine	**24.10** (16.45-33.24)	2.3±0.1
Vinopectine	**43.42** (40.24-47.10)	3.607 ±0.3
Sildenafil	**60.03** 34.87-122.46)(	1.2 ±0.1
Papaverine	**74.85** (52.53-112.27)	1.9 ±0.14
Theophylline	**370.83** (251.84-588.58)	2.284 ±018

As shown, diazepam exhibited the highest nematicidal activity with LC_{50} value of 4.63 ppm, while theophylline had the least nematicidal activity with LC_{50} value of 370.83ppm. Cilostazol did not give any mortality at the concentration of 1000 ppm. The remaining drugs showed intermediate activity.

Wang and Pong (1982) believed that diazepam (as benzodiazepine compound)

immobilized nematodes by blocking the transmittance of electrical activity in nerves and muscle cells by stimulating the release and binding of gamma-amino butyric acid (GABA) at nerve endings. Stewart *et al.* (1994) added that GABA was found also in the second stage juveniles of nematodes. Boswell-Smith *et al.* (2006) reported that theophylline and papaverine are known to be weak inhibitors of PDEs.

Greenhouse experiment was conducted to study the effect of tested PDE inhibitors in reducing the invasion of nematodes to plant roots.

Results listed in Table (3) showed that, all treatments reduced the invasion of nematode to plant roots as indicated by the reduction of the number of root galls compared to untreated check. Diazepam was the most effective compound in reducing the number of root galling (95.5%) followed by papaverine (93.3 %), caffeine (91 %) and theophylline (82%).

Table 3: Effect of certain Phosphodiesterase (PDE) Inhibitors on the number of tomato root galls caused by *Meloidogyne incognita* under greenhouse conditions.

PDE inhibitors	No. of galls/root	% Reduction
Diazepam	4.0^{f}	95.5
Papaverine	6.0^{f}	93.3
Caffeine	8.0^{f}	91.0
Theophylline	16.0^{e}	82.0
Sildenafil	39.0^{d}	57.0
cilostazol	52.0^{c}	42.0

Means in a column followed by the same small letter are no significant at $P<0.05$ according to Duncan's multiple range test.

It was surprising that although theophylline and cilostazol did not show a clear nematicidal effect against 2nd juvenile of nematodes, they reduced the number of root galling which reflect their ability to reduce nematode invasion to plant roots.

Schuster *et al.* (2015) found that the treatment of *C. elegance* and *M. hapla* nematodes with PDE inhibitors such as cilostazol and papaverine decreased their locomotion velocity by 30-50%. They concluded could affect the nematodes by many ways such as disrupt their locomotion, their chemosensation, and/or processes required to invade plant roots.

The impact of the present work is in demonstrating " proof principle" that nematode PDEs are excellent candidates as molecular targets for future development of "next-generation" nematicides capable of disrupting the nematode life cycle with greatly reduced adverse effects on host plants and vertebrates(including humans).

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