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Anthelmintic efficacy of *Flemingia vestita*: genistein-induced effect on the activity of nitric oxide synthase and nitric oxide in the trematode parasite, *Fasciolopsis buski*

Pradip K. Kar, Veena Tandon*, Nirmalendu Saha

Department of Zoology, North-Eastern Hill University, Shillong 793 022, India

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Abstract

The root-tuber peel of *Flemingia vestita*, an indigenous leguminous plant of Meghalaya (Northeast India), has usage in local traditional medicine as curative against worm infections. The peel and its active component, genistein, have been shown to cause flaccid paralysis, deformity of tegumental architecture and alterations in the activity of several enzymes in platyhelminth parasites. To investigate further the mode of action and anthelmintic efficacy of the plant-derived components, the crude peel extract of *F. vestita* and genistein were tested, hitherto for the first time, in respect of the unique neuronal messenger nitric oxide (NO) and the enzyme nitric oxide synthase (NOS) in *Fasciolopsis buski*, the large intestinal fluke of swine and human host. NADPH-diaphorase histochemical staining (a selective marker for NOS in neuronal tissues), which was demonstrable in the neuronal cell bodies in the cerebral ganglia, the brain commissure, the main nerve cords and in the innervation of the pharynx, ventral sucker, terminal genitalia and genital parenchyma of the parasite, showed a stronger activity in the treated worms. In biochemical analysis also, the NOS activity showed a significant increase in the parasites treated with the test materials and reference drug, compared to the untreated controls. The increase in NOS activity in the treated parasites can be attributed to an inducing effect of the plant-derived components.

Keywords: Trematode; *Fasciolopsis buski*; Nitric oxide; Nitric oxide synthase; Genistein; *Flemingia vestita*; Anthelmintic

1. Introduction

The edible tuberous roots of *Flemingia vestita* Benth and Hooker (family Fabaceae) have their usage as anthelmintic in traditional medicinal system practiced in Meghalaya (Northeast India). In earlier studies pertaining to efficacy of plant-derived components the crude extract of the root peel of *F. vestita* along with its active component genistein (4′,5,7-hydroxyisoflavone) was shown to induce flaccid paralysis and pronounced tegumental damage and disruption in the soft-bodied platyhelminth parasites in an in vitro exposure [1,2]. The plant-derived components also caused altera-

*Corresponding author. Tel.: +91-364-550105/551508; fax: +91-364-550108/550300.

E-mail address: tandonveena@hotmail.com (V. Tandon).

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tions in the activity of certain tegumental enzymes [3]. A pronounced decline was also recorded in the activity of the non-specific esterases (NSE) and cholinesterases (ChE) in the treated cestodes [4] as well as in the trematodes, *Fasciolopsis buski* and *Artyfechinostomum sufrartyfex* [5]. Esterases, acetylcholinesterase (AChE) in particular, are closely associated with the cholinergic components of the central and peripheral nervous system in trematodes and cestodes, and their role in nervous co-ordination has been established [6,7]. In addition to cholinergic innervation, the presence of nitrergic components in the nervous system of cestodes and flukes has been established [8–10]. Tandon et al. [11] described the occurrence of nitric oxide (NO), the nitrergic transmitter, in *F. buski* by demonstrating the activity of nitric oxide synthase (NOS). NO represents a novel category of neuronal signal substances: a transmitter gas [12,13]. The NO molecule is small, electrically neutral and diffuses freely within the tissue. NO is an unstable compound and is generated when necessary from L-arginine and molecular oxygen by a complex family of NOS enzymes: neuronal (nNOS); inducible (iNOS); and endothelial (eNOS) [14,15]. Under defined fixation conditions, the NADPH-diaphorase (NADPH-d) reaction is a histochemical marker for nNOS [16,17].

Signal transduction with the diatomic NO is involved in a number of important physiological processes. In the central nervous system, NO plays important roles in neurotransmitter release and reuptake, neurodevelopment, synaptic plasticity, smooth muscle relaxation and regulation of gene expression [18,19]. NO can both promote and inhibit lipid peroxidation [20]. Localization of NOS in the sperm of filarial nematodes suggests a role for NO during fertilization as has been described for sea urchin and ascidians [21]. Presence of NO and its plausible role in the neuromuscular system of *Ascaris suum* has been suggested; the profile of the putative NOS characterized in the parasite is suggested to be different from that of rat nNOS [22]. Neuronal NOS has also been found to have adverse neurotoxic effects [23]. Overproduction of NO can cause apoptotic or neurotic cell death through oxidative stress, disrupted energy metabolism, DNA damage, activation of poly (ADP-ribose) polymerase, or disregulation of cytosolic calcium [24]. The role of NO in neurotransmission and neuromodulation has been studied both in human and animal models, while it has also been implicated in neurotoxicity as well as neuroprotection [18,25–27].

Furthering the investigations on anthelmintic efficacy of phytochemicals derived from *F. vestita*, it seemed desirable to ascertain whether or not they influence NO, the unique neuronal messenger, and the enzyme NOS. The effect of an anthelmintic drug on the nitrergic nervous system in flat worms has not been hitherto studied. In the present communication, we analyzed the anthelmintic efficacy of *F. vestita* and its active component, genistein, with respect to NO.

2. Materials and methods

2.1. Parasites and drugs

Live specimens of adult *Fasciolopsis buski* were obtained from the intestine of freshly slaughtered pigs at the local abattoirs in Shillong and kept in 0.9% phosphate buffered saline (PBS). An alcoholic extract of the peels of fresh tuberous roots of *F. vestita* was prepared as described earlier [2]. Pure genistein (Sigma code no. G6649) was used as the major active principle of the root peel. Oxyclozanide B.P. (Tolzan, Hoechst India Ltd.) served as the reference drug.

2.2. Treatment and analysis

2.2.1. NADPH-d histochemical localization and NOS activity

The live flukes were incubated at 37 ± 1 °C for treatment with 20 mg/ml peel extract of *F. vestita*, 0.5 mg/ml genistein and 20 mg/ml oxyclozanide, all in PBS with 1% DMSO. The dosages of the test materials were standardized in earlier experiments [1], from which the time taken for onset of paralysis and death following treatment with similar concentrations of the crude peel extract, genistein and oxyclozanide were determined as: 0.6–0.8 h and 1.5–1.8 h; 2.5–3 h and 4–4.5 h; and 0.5–0.6 h and 1.5–2.0 h, respectively. A set of controls in PBS with 1% DMSO was also main-

tained at 37±1 °C, which survived for 18–20 h in the incubation medium.

The paralyzed flukes of various treatments along with the controls were immersion-fixed in freshly prepared 4% paraformaldehyde in 0.1 M PBS at pH 7.4 for overnight at 4 °C. The worms were then preserved in 10% sucrose solution prepared in PBS for few days at 4 °C. The frozen material was sectioned frontally and sagitally at 20 μm on a SLEE HR cryostat at −15 to −20 °C. The sections collected on chrome-alum gelatin/poly-L-lysine coated glass slides were dried for 2 h at room temperature, and directly stained or kept at −70 °C. NADPH-d histochemical staining was performed following the protocol as described by Gustafsson et al. [28] and Lindholm et al. [29]. The concentration of β-NADPH-d in the incubation media was 2 mg ml^{-1}. The sections were incubated at 37±1 °C for 1–4 h, rinsed in 0.01 M PBS with 0.2% Triton-X 100 thrice for 15 min each, rinsed in distilled water and mounted in 50% glycerol in PBS. For controls, β-NADPH-d was substituted with β-NADH in the same concentration and the incubation was performed for 1 h at 37±1 °C.

The NOS activity was assayed separately in the whole worm tissue and also in the preacetabular cut portion of the body both in the treated paralyzed parasites and the untreated controls. A 10% homogenate of the freshly collected worm was prepared in the homogenizing buffer containing 20 mM HEPES buffer (pH 7.2), 300 mM mannitol, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM dithiothreitol (DTT), using a motor driven Potter–Elvehjem glass homogenizer with a Teflon pestle. Phenyl methyl sulfonyl fluoride 10 mg/ml was also added to the homogenate. The homogenate was treated with 0.5% Triton-X 100 in a 1:1 ratio for 30 min, followed by a mild sonication for proper breakage of mitochondria, and centrifuged at 10 000×*g* for 10 min. The supernatant was used for estimation of NOS activity. The enzyme activity was assayed following the method of Salter and Knowles [30] with certain modifications [11]. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.2), 50 mM L-arginine, 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 0.12 mM NADPH and 0.1 ml of tissue homogenate in a final volume of 1 ml. The reaction mixture also contained 20 units of urease, sufficient enough to convert all the urea formed (due to the reaction of arginase) to ammonia and CO_2, so that it would not interfere with the estimation of citrulline. The reaction mixture was incubated at 37 °C for 15 min and the reaction was stopped by adding 1 ml of 10% perchloric acid (PCA), followed by centrifugation to precipitate out the protein. Citrulline so formed as the reaction product was estimated in the supernatant spectrophotometrically (Beckman DU 640) at 490 nm following the method of Moore and Kauffman [31] against a reagent blank, where 10% PCA was added prior to the addition of tissue homogenate. One unit of enzyme activity is defined as the amount that catalyses 1 μmol of citrulline formed per hour at 37 °C.

2.2.2. NO estimation in the culture media

In separate petri dishes, live worms (preweighed) were incubated with various test materials in 15 ml PBS with 1% DMSO and incubated at 37±1 °C for 2 h with simultaneous maintenance of control under similar conditions. The dosages used for the various treatments were 10 mg/ml crude peel extract (with time for onset of paralysis and death being 1.5–2.0 h and 3.7–4.0 h post incubation, respectively), 0.5 mg/ml genistein (time for onset of paralysis and death being 2.5–3 h and 4–4.5 h, respectively) and 10 mg/ml oxyclozanide (with paralysis occurring at 1.2–1.5 h and death, at 3.0–3.5 h). These lower concentrations of the peel extract and oxyclozanide were used so as to prolong the intervening time for onset of paralysis and death following treatment and to facilitate collection of samples at 1 h intervals post incubation for 2 h. The incubation medium was oxygenated at every 30 min interval by administering oxygen to the solution. Incubation medium (0.9 ml) was collected at 1 h intervals for estimation of NO released by the parasite. In the absence of oxyhaemoglobin and in oxygenated aqueous solution NO is oxidized primarily to nitrite (NO_2^-) with little or no formation of NO_3^- [32]. NO_2^- concentration in the incubation medium was determined spectrophotometrically (using Beckman DU 640) at 540 nm following Greiss reaction as described by Sessa et al. [33] and

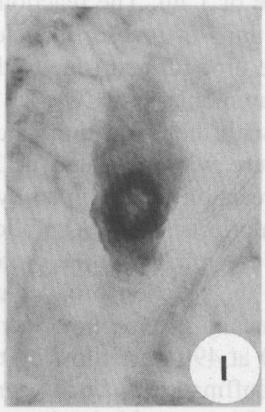

Fig. 1. A NADPH-d positive cell in the course of posterior inner lateral nerve (PILN) lateral to ventral sucker. Control (300×).

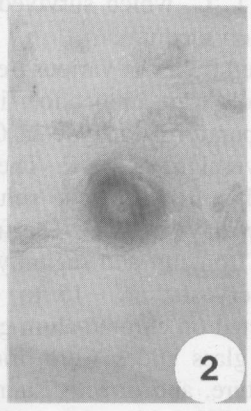

Fig. 2. Another NADPH-d positive cell in subtegumental parenchyma. Control (300×).

detailed in Tandon et al. [11]. A standard curve was prepared with sodium nitrite to calculate the NO concentration in the medium.

Data collected from three replicates were statistically analyzed and presented as mean ± S.E.M. Comparison of the paired mean values was made using Student's *t*-test [34] and $P > 0.05$ was taken as non-significant.

3. Results

NADPH-d staining was demonstrable in the parasites treated with various test materials as well as in their controls. In controls the NOS activity could be localized (Figs. 1 and 2) in the neuropile of the CNS and also in neuronal cell bodies associated with the musculature of the pharynx, ventral sucker, cirrus sac and terminal genital pore region as well as in the subtegumental nerve plexus of the parasite. These observations are in conformity with those made in an earlier study [11]. The controls with β-NADH showed no staining. The intensity of staining was more in almost all the locations in the treated parasites compared to the controls (Table 1, Figs. 3–9). The histochemical studies were also supported with biochemical estimation. As shown in Table 2, the NOS activity

Table 1
Effect of plant-derived test materials and reference drug on NADPH-d activity[a] in the various structures of *F. buski* in vitro: histochemical localization

Location in the parasite	Control (in 0.9% PBS)	Crude extract (20 mg/ml)	Genistein (0.5 mg/ml)	Oxyclozanide (20 mg/ml)
Components of CNS (Cerebral ganglia, cerebral commissure, main nerve cords)	++	++++	++++	+++
Neuronal cell bodies associated with musculature of pharynx and ventral sucker	++	++++	+++	++
Terminals in syncytial tegument and subtegument	++	++++	++++	+++
Cell bodies in musculature of cirrus sac, circum-genital pore Region	+	+++	++	++

[a] + Less activity; ++ medium activity; +++ strong activity; ++++ very strong activity.

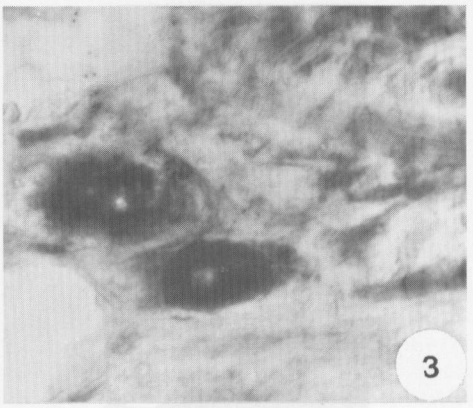

Fig. 3. Strong NADPH-d activity in the cells of the fluke treated with crude peel extract of *Flemingia vestita*. Two cells on the course of PILN (300×).

was significantly stimulated when the worm was treated with the crude root-peel extract, genistein and the reference drug. In the preacetabular part of the parasite body, the NOS activity increased by 92.5, 73.35 and 109.33%, and in the whole worm tissue homogenates it increased by 175.61, 197.5 and 124%, respectively, after the flukes were exposed to the mentioned treatments (Fig. 10).

The increase of NOS activity in the treated worms was also accompanied by a significant increase in the efflux of NO to the incubation medium (Table 3, Fig. 11). The worms kept in 0.9% PBS continuously released some amount of

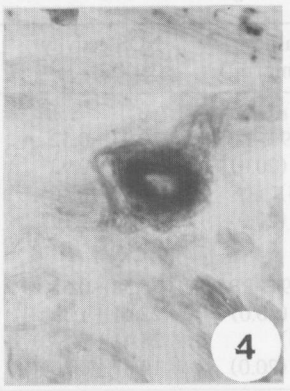

Fig. 4. Strong NADPH-d activity in the cells of the fluke treated with crude peel extract of *Flemingia vestita*. A NOS positive cell by the side of pharynx (300×).

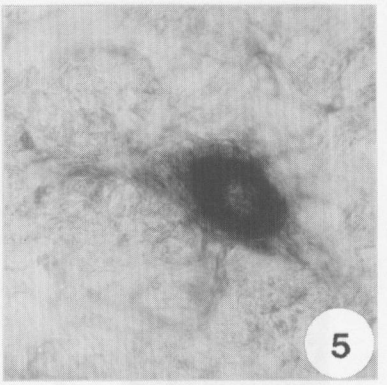

Fig. 5. Strong NADPH-d activity in the cells of the fluke treated with crude peel extract of *Flemingia vestita*. Another cell associated with the PILN (480×).

NO (5.79 ± 0.16 nmol g^{-1} h^{-1}), which further increased by 115.25, 93.16 and 79.9% when they were treated with the root-peel extract, genistein and oxyclozanide, respectively.

4. Discussion

Using NADPH-d staining, the selective marker for nNOS, nitrergic nature of innervation could be revealed in *F. buski*, which also possesses cholinergic neuronal components [5,11]. The demonstration of the release of NO in this parasite also confirmed the presence of NOS in digenetic platyhelminths, as in other groups of helminth parasites [8,10,21,35,36]. Characterization of putative

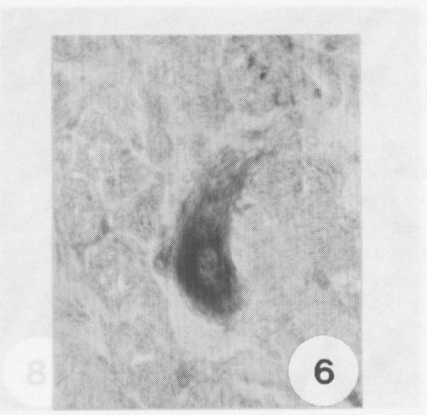

Fig. 6. A NADPH-d positive cell associated with the ventral sucker musculature in genistein-treated parasite (300×).

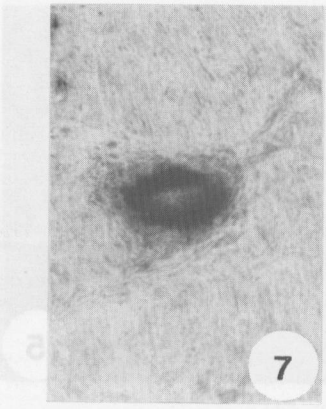

Fig. 7. Another NADPH-d positive cell associated with the ventral sucker musculature in genistein-treated parasite (300×).

NOS in *Ascaris suum* revealed that the parasite's NOS profile is different from that of rat nNOS [22]. This difference in the host/parasite NOS may offer a newer target for plausible drug action. So far no study has been made about the effect of an anthelmintic drug on the nitrergic nervous components in a flatworm.

In control parasites in the present study, strong NADPH-d staining was demonstrable in the neuronal cell bodies in the cerebral ganglia, transverse commissure and along the musculature of the pharynx, ventral sucker, terminal genitalia and circum-genital pore region in *F. buski*. These

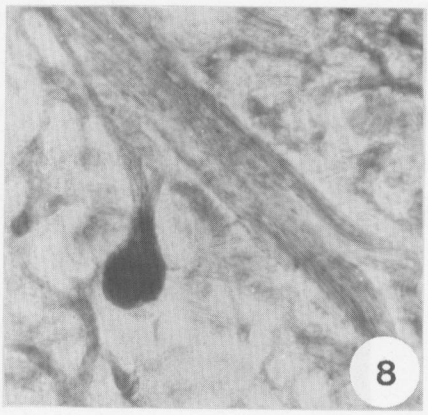

Fig. 8. A NADPH-d positive cell associated with the general parenchyma in genistein-treated parasite (300×).

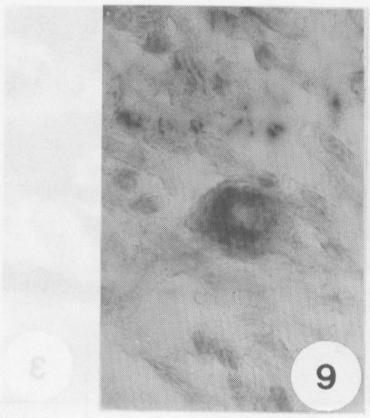

Fig. 9. NADPH-d staining in a cell in the parenchymal tissue by the side of the oral-pharyngeal region. Oxyclozanide treatment (300×).

observations corroborate the distribution pattern of nNOS in *Fasciola hepatica* and other flatworms [10]. However, in comparison to controls, in the parasites treated with the root peel extract of *F. vestita* and genistein, the intensity of NADPH-d histochemical reaction seemed to be considerably higher. Biochemical analysis also revealed an enhanced activity of NOS in the treated flukes. That the test phytochemicals promote NOS activity in the parasite was also supported by the observation of more release of NO in the incubation

Table 2
Effect of plant-derived test materials and reference drug on NOS activity (units g^{-1} wet wt.) in *F. buski* in vitro

Regions of parasite (concentration mg/ml)	NOS activity	% Increase after treatment
Pre-acetabular region		
–Control (in 0.9% PBS)	20.23±0.52	–
–Crude extract (20.0)	38.42±0.93[b]	92.5±4.33
–Genistein (0.5)	36.82±0.46[a]	73.35±3.84
–Oxyclozanide (20.0)	40.55±1.16[b]	109.33±6.74
Whole body		
–Control (in 0.9% PBS)	8.58±0.55	–
–Crude extract (20.0)	22.41±1.39[c]	175.61±21.39
–Genistein (0.5)	28.41±0.23[c]	197.5±11.84
–Oxyclozanide (20.0)	16.81±0.46[b]	124±28.89

Values are expressed as mean±S.E.M. ($n=3$).
[a] P value significant at <0.05.
[b] P value significant at <0.01.
[c] P value significant at <0.001.

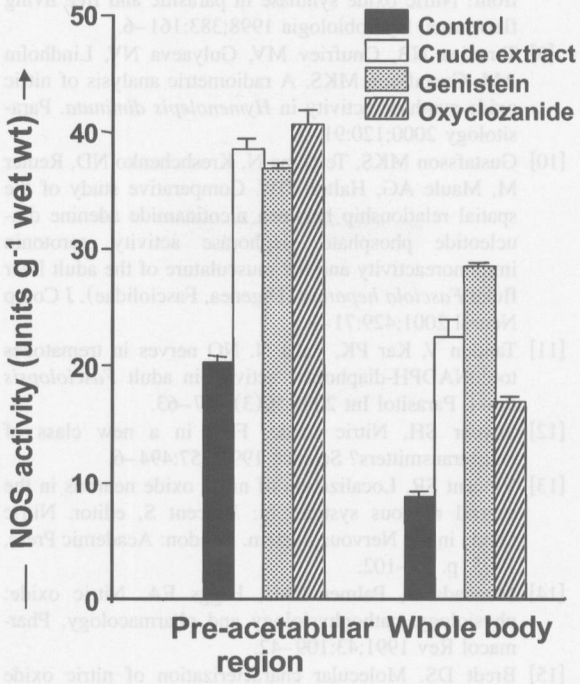

Fig. 10. NOS activity in the parasite tissue homogenates of the parasite following treatment with the test materials.

medium by the treated worms. With the available data, it may be difficult to explain the mechanism (s) of stimulation of NOS activity causing, thereby, more production of NO, in the root peel extract- and genistein-treated flukes. The stimulation of NOS activity in the fluke took place within an hour or less post incubation. Therefore, the increase of enzyme activity would not be expected due to new protein synthesis so rapidly. However, the stimulation of NOS activity perhaps could be due to activation of pre-existing enzymes by changing the phosphorylation status of the enzyme induced by the test plant extract and genistein possibly with the involvement of certain cell signaling molecules [37,38].

In central nervous system, NO has been postulated to have many physiological roles as a neurotransmitter and neuromodulator and implicated in neurotoxicity as well as in neuroprotection [26]. Under conditions of pathologic insult excessive formation of NO can be an important mediator of neurotoxicity and tissue dysfunction [18,27,39,40].

Table 3
Rate of NO release by *F. buski* in the incubation medium under various treatment conditions

Treatment (mg/ml)	NO release (nmol g^{-1} body wt. h^{-1})	% Increase after treatment
Control (0.9% PBS)	5.79±0.16	–
Crude peel extract (10.0)	12.43±0.07[b]	115.25±6.99
Genistein (0.5)	11.14±0.24[b]	93.16±9.39
Oxyclozanide (10.0)	10.38±0.11[a]	79.9±6.69

Values are expressed as mean±S.E.M. ($n=3$).
[a] P value significant at <0.05.
[b] P value significant at <0.01.

In helminth parasites NO has been suggested to have multiple roles: as a neurotransmitter at neuromuscular junction [35], myoinhibitory [10], in fertilization and embryogenesis [21]. After exposure to the test materials the *F. buski* flukes did get paralyzed within varying periods of time in a dose-dependent manner before actual death occurred [1]. Excess production of NO is known

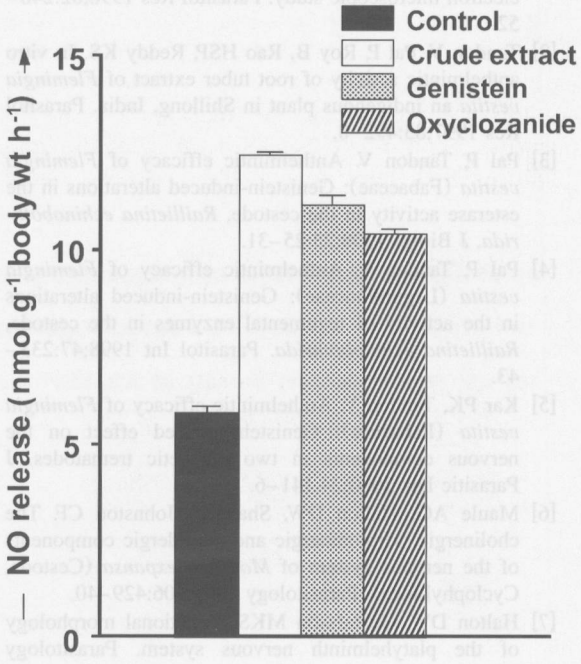

Fig. 11. Efflux of NO in the incubation medium following exposure of the parasite to the various test materials.

to cause oxidative stress, DNA damage and disruption of energy metabolism, calcium homeostasis and mitochondrial function [24]. Enhanced nNOS activity and increased NO release in *F. buski* under the influence of the root-tuber peel extract and genistein in vitro suggest that *F. vestita*-derived phytochemicals cause more NO production in the flukes, which might also account for, among other factors, onset of paralysis—a manifestation of neurotoxicity [1,2]. It may thus be hypothesized that NO is also implicated in putative vermifugal activity of genistein.

Acknowledgments

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