

Genistein from *Flemingia vestita* (Fabaceae) enhances NO and its mediator (cGMP) production in a cestode parasite, *Railletina echinobothrida*

B. DAS, V. TANDON* and N. SAHA

Department of Zoology, North Eastern Hill University, Shillong-793022, India

(Received 16 February 2007; revised 13 March 2007; accepted 13 March 2007; first published online 24 April 2007)

SUMMARY

Cyclic GMP (cGMP) is responsible for various cellular functions including signal pathways and it acts as a mediator for nitric oxide (NO). In order to evaluate the anthelmintic efficacy of the plant-derived isoflavones, the crude peel extract of *Flemingia vestita* and pure genistein were tested with respect to the activity of nitric oxide synthase (NOS), NO efflux and the cGMP concentration in *Railletina echinobothrida*, the cestode parasite of domestic fowl. For comparison, the parasites were also treated with genistein (the major isoflavone present in the crude peel extract), sodium nitroprusside (SNP), a known NO donor, and praziquantel (PZQ), the reference drug. At the time of onset of paralysis in the parasite, the activity of NOS showed a significant increase (35–46%) and a 2-fold increase of NO efflux into the incubation medium in the treated worms in comparison to the respective controls. The cGMP concentration in the parasite tissue increased by 46–84% in the treated test worms in comparison to the controls. The results show that the isoflavones, genistein in particular, from the crude peel extract of *F. vestita* influence the cGMP concentration in the parasite tissue, which plays a major role in the downstream signal pathways.

Key words: *Flemingia vestita*, *Railletina echinobothrida*, nitric oxide synthase, nitric oxide, cGMP, genistein.

INTRODUCTION

Isoflavones, genistein in particular, present in the crude peel extract of *Flemingia vestita* (Rao and Reddy, 1991), act as a vermifugal, if not a vermucidal, against several intestinal trematodes and cestodes (Roy and Tandon, 1996; Tandon *et al.* 1997). These isoflavones, as shown from earlier studies, cause flaccid paralysis in trematodes and cestodes, deformity and alterations in the tegumental architecture, and activation of several enzymes that are associated with the tegument (Tandon *et al.* 1997; Pal and Tandon, 1998a, b). The changes in the tegumental architecture are attributed to the permeability changes in the tegument due to an alteration in the Ca²⁺ homeostasis of the parasite (Das *et al.* 2006). The activity of the enzymes associated with the co-ordination system, non-specific esterases and acetylcholine esterase in particular, was also shown to be influenced by these isoflavones (Pal and Tandon, 1998c), as was the activity of nitric oxide synthase (NOS), the free amino acid pool and tissue ammonia (Tandon *et al.* 1998; Kar *et al.* 2002, 2004). The plant-derived isoflavones also affected the

carbohydrate metabolism in *Railletina echinobothrida* (Tandon and Das, 2007).

Recent studies have shown that nitric oxide (NO) – synthesized from L-arginine and molecular oxygen by the enzyme NOS (Nelson and Cox, 2004) – has anti-leishmanial (Holzmuller *et al.* 2005), anti-malarial (Cramer *et al.* 2005) and anthelmintic effects (Mahmoud and Habib, 2003). The biological effects of NO are generally assumed to be attributable to the activation of soluble guanylyl cyclase by nitrosation of its haem moiety, leading to cGMP accumulation (Ignarro, 1990; Lincoln and Cornwell, 1993; Hobbs, 1997). The subsequent increase in cGMP level is involved in many cellular functions by altering mainly three target proteins, the cGMP-regulated ion channels, cGMP-regulated phosphodiesterases and protein kinase G (Schmidt *et al.* 1993; Hofmann, 2005). Besides NO, some hormones, e.g. insulin and oxytocin, as well as acetylcholine and biogenic amines like serotonin and histamine, cause an increase in the cGMP levels (Tremblay *et al.* 1988). Stimulators of guanylate cyclase such as the vasodilators, namely, nitroprusside, nitroglycerin and sodium nitrate also stimulate cGMP levels (Collier and Vallance, 1989). Peptides such as atrial natriuretic factors (ANF) that relax smooth muscle also stimulate cGMP, which acts as secondary messenger for ANF (Sarcevic *et al.* 1989). By cGMP immunostaining, the target cells for NO

* Corresponding author. Tel: +91 364 2722312. Fax: +91 364 2550300/2722301. E-mail: tandonveena@hotmail.com

have been located in adult and larval stages of some platyhelminth parasites (Gustafsson *et al.* 2003; Terenina and Gustafsson, 2003).

In furtherance of our objective to find out the plausible mode of the anthelmintic action of isoflavones from *F. vestita*, we studied the effect of these isoflavones on the accumulation of cGMP. In the present study, the isoflavones from the crude peel extract of *F. vestita* were tested in *R. echinobothrida* with respect to the NOS activity, NO efflux and cGMP concentration.

MATERIALS AND METHODS

Plant extract and its fractions

The alcoholic crude peel extract of *F. vestita* and its hexane (non-polar), ethyl acetate (semi-polar) and n-butanol (polar) fractions were obtained following the procedure as described earlier (Tandon *et al.* 1997). Crude peel extract and its different fractions were collected and tested against the cestode parasite.

Chemicals and reagents

Genistein (G 6649) and the enzyme immunoassay cGMP kit (CG-201) were obtained from Sigma Chemicals (St Louis, USA). The required enzymes and co-enzymes were from either Sigma or Roche (Germany), whereas the reference drug, praziquantel (PZQ), was from Bayer (India). Other necessary chemicals were of analytical grade and from Sisco Research Laboratory (India). For all chemical preparations deionized double-distilled water was used.

In vitro treatments

Live parasites from the intestine of freshly slaughtered domestic fowl (*Gallus domesticus*) were collected in 0.9% phosphate-buffered saline (PBS, pH 7.2) and immediately exposed to various treatments. Parasites, approximately 0.2 g wet weight, were incubated in 10 ml of PBS at $38 \pm 1^\circ\text{C}$ with defined concentrations of various treatments, i.e. 5 mg/ml each of (i) the crude peel extract, (ii) its hexane-, (iii) ethyl acetate- and (iv) n-butanol-fractions, (v) 0.2 mg/ml genistein, (vi) 1.5 mg/ml sodium nitroprusside (SNP) and (vii) 1 $\mu\text{g}/\text{ml}$ PZQ, dissolved in 1% dimethylsulfoxide (DMSO), with maintenance of respective controls containing only 1% DMSO in PBS. Parasites from a single host were taken for each set of treatments and the treated parasites and their respective controls were retrieved from the incubation media at the time when paralysis started to set in and were processed for assay of NOS activity and estimation of the cGMP concentration in the parasite tissue, and NO release into the culture medium from the incubated parasites.

NOS assay

A 10% (w/v) homogenate of the treated parasites as well as their respective controls was prepared in a homogenizing buffer containing HEPES buffer (20 mM, pH 7.2), mannitol (250 mM), EDTA (1 mM), DTT (1.5 mM) and PMSF (0.1 mM) using a Potter-Elvehjem glass homogenizer. The homogenate was treated with 0.5% (v/v) Triton X-100 at a 1:1 ratio for 30 min and sonicated for 30 sec using a sonicator (Soniprep 150, UK) and centrifuged for 10 min at 10 000 g. The supernatant was used for the measurement of NOS activity. All the steps were carried out at 4°C .

NOS activity was assayed following the method of Salter and Knowles (1998) with certain modifications. The reaction mixture (1 ml) contained potassium phosphate buffer (50 mM, pH 7.2), L-arginine (50 mM), MgCl_2 (1.2 mM), CaCl_2 (0.25 mM), NADPH (0.15 mM), urease (20 U) and enzyme source (0.05 ml). The reaction mixture was incubated at 38°C for 15 min and 1 ml of 10% perchloric acid (PCA) (v/v) was added to stop the reaction. The reaction mixture was centrifuged to precipitate out the protein. The citrulline concentration, formed in the reaction mixture, was estimated spectrophotometrically at 490 nm against a reagent blank, in which 10% PCA (v/v) was added before addition of the enzyme source, following the method of Moore and Kauffman (1970). One unit of enzyme activity is the amount of enzyme catalysing 1 μmole of citrulline formation/h at 38°C .

NO estimation

Cestode parasites were incubated in 10 ml of PBS at $38 \pm 1^\circ\text{C}$ with different concentrations of treatments, with maintenance of respective controls, as described in the section 'In vitro treatments'. At every hour, 1 ml of incubation medium was removed until the paralysis time for estimation of NO released by the parasite was reached, and was then centrifuged at 600 g for 10 min to precipitate out the debris, if any. NO is oxidized mainly to nitrite (NO_2^-) with little or no formation of nitrate in oxygenated aqueous solution in the absence of oxyhaemoglobin (Ignarro *et al.* 1993). NO_2^- concentration in the incubation medium, which is equivalent to NO efflux by the cestode parasite, was estimated spectrophotometrically at 540 nm following the Griess reaction as described by Sessa *et al.* (1994). NO_2^- concentration in the incubation medium was calculated against the standard curve of sodium nitrite.

cGMP estimation

For quantitative determination of the cGMP concentration in the parasite tissue, the enzyme immunoassay cGMP kit (CG-201, Sigma) was used.

Table 1. Efficacy of different test materials on *Raillietina echinobothrida in vitro*(Values are expressed as means \pm S.E.M. ($n=5$.)

| Treatment (mg/ml) | Time (h) taken for paralysis (P) and death (D) | |
|------------------------------|--|-----------------|
| | P | D |
| Control (in 0.9% PBS) | — | 70.5 \pm 0.35 |
| Crude peel extract (5.0) | 6.1 \pm 0.05 | 44.3 \pm 0.44 |
| Hexane fraction (5.0) | 15.5 \pm 0.75 | 30.8 \pm 0.96 |
| n-Butanol fraction (5.0) | 22.8 \pm 0.65 | 41.6 \pm 0.85 |
| Ethyl acetate fraction (5.0) | 2.9 \pm 0.62 | 9.4 \pm 0.52 |
| Genistein (0.2) | 6.4 \pm 0.54 | 48.2 \pm 0.48 |
| SNP (1.5) | 2.8 \pm 0.49 | 4.6 \pm 0.68 |
| PZQ (0.001) | 2.7 \pm 0.65 | 9.8 \pm 0.57 |

Immediately after paralysis, the treated parasites and the controls were frozen. A 10% homogenate was made in 5% cold TCA using a motor-driven Potter-Elvehjem glass homogenizer. The homogenate was centrifuged for 10 min at 600 g and the supernatant was collected in 3 volumes of water-saturated ether. After drying the aqueous extracts, the reconstituted samples were taken for quantitative estimation of cGMP.

Each sample of 100 μ l, in duplicate, was placed into microtitre plate wells coated with goat anti-rabbit IgG and 50 μ l of alkaline phosphatase conjugated with cGMP was added to each well. The plate was incubated on a plate shaker for 2 h at room temp after adding 50 μ l of rabbit IgG to cGMP. The wells were washed 3 times with washing buffer, and then 200 μ l of p-nitrophenyl phosphate were added to each well and the plate was incubated for 1 h at room temperature without shaking. The reaction was stopped by adding 50 μ l of trisodium phosphate to each well and the plate was read immediately at 405 nm using an ELISA plate reader (Multiskan Ex Primary EIA V. 2.1-0). The intensity of the colour is inversely proportional to the concentration of cGMP in the samples.

The concentration of cGMP in the parasite tissue was calculated against the standard curve of cGMP on 5 Cycle Log-Log paper.

Data analysis

Data are presented as the means \pm S.E.M. ($n=4$) and a value of $P \leq 0.05$ was taken to be statistically significant. Using Student's *t*-test, comparisons of the paired mean values were calculated between the treatments and the respective controls.

RESULTS

Table 1 shows the paralysis time in the cestode parasite under different treatment conditions. At

the defined concentrations of various treatments, a flaccid paralysis takes place in the parasite in about 6 h in the case of crude peel extract and genistein and in about 3 h in the case of the ethyl acetate fraction, SNP and PZQ. Hexane and n-butanol fractions of the crude peel extract of *F. vestita* had lesser effects. The control parasites, survived *in vitro* for about 71 h.

The tissue activity of NOS (Tables 2 and 3) was found to be significantly increased in the parasites exposed to various treatments except for the hexane and n-butanol fractions of the crude peel extract. In the control parasites, the tissue activity of NOS was found to be approximately 8–9 units/g wet wt. The activity increased by 37% and 46% after exposure to the crude peel extract and its ethyl acetate fraction, respectively, while there was no significant increase in treatments with the other fractions. Treatments with pure genistein and PZQ resulted in an increase of the NOS activity by 39% and 35%, respectively, in comparison to their respective controls.

The increased NOS activity in the treated parasites was accompanied by a significant increase in the NO efflux into the incubation medium (Tables 2 and 3, Figs 1 and 2). Though there was a continuous NO efflux (about 1.19 nmol/g wet wt/h) into the medium by the control parasites, there was a significant increase in the NO efflux (38–96%) in the treated parasites, excluding hexane and n-butanol fractions of the crude peel extract.

The concentration of cGMP, which is the mediator of the NO action in several cells, was found to be about 22 pmol/g wet wt in the control parasite tissue. At the paralysis time, the cGMP concentration in the parasite tissue increased significantly ($P < 0.001$) by 84% after the SNP treatment, and by 49%, 68%, 52% and 46% after incubation in the crude peel extract, ethyl acetate fraction, genistein and PZQ, respectively.

DISCUSSION

With exposure to the defined concentrations of the crude peel extract and genistein, the onset of paralysis occurred in the parasite, *R. echinobothrida*, after ~6 h of incubation, whilst in the case of the ethyl acetate fraction, SNP and PZQ it occurred in ~3 h. These treatments caused rapid muscular contraction followed by paralysis; however, the mode of action of paralysis is not well understood. Serotonin is reported to also induce a dose-dependent, heterologous flaccid paralysis in *Schistosoma mansoni* (Blair *et al.* 1993).

Different workers have demonstrated the presence of NOS in various helminth groups. The first indication of the occurrence of nitregic mechanisms in the nervous system of parasitic flatworms was provided in *Hymenolepis diminuta*, in which NADPH-d positive neurons were demonstrated (Gustafsson

Table 2. Effects of different test materials on NOS tissue activity (units/g wet wt), NO release (nmol/g wet wt/h) into the culture medium and cGMP concentration (pmol/g wet wt) in *Raillietina echinobothrida* *in vitro* at the time of paralysis

(Values are expressed as means \pm S.E.M. ($n=4$). Percentage increase of NOS tissue activity, NO release and cGMP concentration compared to respective controls are given within parentheses, only if significant.)

| Treatment (mg/ml) | NOS activity* (units/g wet wt) | NO release (nmol/g wet wt/h) | cGMP concentration (pmol/g wet wt) |
|----------------------------------|------------------------------------|-----------------------------------|---------------------------------------|
| 1a. Control (in 0.9% PBS) | 8.59 \pm 0.62 | 1.18 \pm 0.08 | 21.98 \pm 0.62 |
| 1b. Crude peel extract (5.0) | 11.77 \pm 0.72 (37) ^a | 1.68 \pm 0.16 (42) ^a | 32.75 \pm 0.79 (49) ^b |
| 2a. Control | 8.95 \pm 0.65 | 1.18 \pm 0.08 | 22.15 \pm 0.46 |
| 2b. Hexane fraction (5.0) | 9.58 \pm 0.65 | 1.30 \pm 0.10 | 23.81 \pm 0.62 |
| 3a. Control | 8.85 \pm 0.58 | 1.18 \pm 0.08 | 22.05 \pm 0.52 |
| 3b. n-Butanol fraction (5.0) | 9.38 \pm 0.69 | 1.42 \pm 0.07 | 24.81 \pm 0.54 |
| 4a. Control | 8.62 \pm 0.58 | 1.18 \pm 0.18 | 21.95 \pm 0.74 |
| 4b. Ethyl acetate fraction (5.0) | 12.59 \pm 0.86 (46) ^b | 1.83 \pm 0.15 (55) ^b | 36.88 \pm 0.76 (68) ^b |

* One unit of enzyme activity is the amount of enzyme catalysing 1 μ mole of citrulline formation/h at 38 °C.

^a, ^b: *P* values significant at <0.05 and <0.01, respectively.

Table 3. Effects of different test materials on NOS tissue activity (units/g wet wt), NO release (nmol/g wet wt/h) into the culture medium and cGMP concentration (pmol/g wet wt) in *Raillietina echinobothrida* *in vitro* at the time of paralysis

(Values are expressed as means \pm S.E.M. ($n=4$). Percentage increase of NOS tissue activity, NO release and cGMP concentration compared to respective controls is given within parentheses, only if significant.)

| Treatment (mg/ml) | NOS activity* (units/g wet wt) | NO release (nmol/g wet wt/h) | cGMP concentration (pmol/g wet wt) |
|---------------------|------------------------------------|-----------------------------------|---------------------------------------|
| 1a. Control | 8.69 \pm 0.54 | 1.19 \pm 0.08 | 21.84 \pm 0.54 |
| 1b. Genistein (0.2) | 12.08 \pm 0.66 (39) ^a | 1.75 \pm 0.27 (47) ^b | 33.20 \pm 0.46 (52) ^b |
| 2a. Control | 8.95 \pm 0.55 | 1.19 \pm 0.08 | 22.28 \pm 0.69 |
| 2b. SNP (1.5) | 9.93 \pm 0.46 | 2.33 \pm 0.38 (96) ^c | 41.00 \pm 0.85 (84) ^b |
| 3a. Control | 8.75 \pm 0.28 | 1.19 \pm 0.08 | 21.88 \pm 0.75 |
| 3b. PZQ (0.001) | 11.81 \pm 0.60 (35) ^a | 1.64 \pm 0.42 (38) ^a | 31.94 \pm 0.68 (46) ^b |

* One unit of enzyme activity is the amount of enzyme catalysing 1 μ mole of citrulline formation/h at 38 °C.

^a, ^b, ^c: *P* values significant at <0.05, <0.01 and <0.001, respectively.

et al. 1996). Thereafter, neuronal NOS (nNOS) activity was observed in *Fasciolopsis buski*, *H. diminuta*, *Fasciola hepatica* and some free-living flatworms (Gustafsson *et al.* 1998; Tandon *et al.* 2001; Terenina *et al.* 2000, 2003). Recently, NOS activity or immunoreactivity was demonstrated in *S. mansoni* (Kohn *et al.* 2001, 2006). In the present study, the activity of NOS was observed in the whole worm homogenate of *R. echinobothrida*.

The production of NO from L-arginine by NOS is regulated by various modulators (Nelson and Cox, 2004). In the present study, the NOS activity in the cestode *R. echinobothrida*, was found to be approximately 8–9 units/g wet wt in the control parasites, whilst it was significantly ($P<0.05$) increased by 35–46% when the parasites were exposed to the crude peel extract of *F. vestita* and its ethyl acetate fraction, which is known to contain genistein (Rao and Ready,

1991). The increase in the NOS activity was comparable when the parasites were treated with pure genistein and PZQ. In mammals, constitutive enzymes (eNOS and nNOS) are calcium dependent (Nathan and Xie, 1994) and an increase in the activity of NOS in the PZQ-treated parasites could be explained in the light of PZQ-mediated calcium efflux in the cestode parasite, *R. echinobothrida*. The crude peel extract of *F. vestita*, genistein and PZQ caused a dose-dependent calcium efflux in the parasite (Das *et al.* 2006). The activation of NOS could be perhaps due to the covalent modification of the enzyme, as it seems unlikely to be due to induction of the enzyme in the very short span of time (Stuehr, 1999). The increase in the NOS activity in the parasite was accompanied by an increase in the NO efflux into the incubation medium by the treated parasites in comparison to the respective controls. It is known that

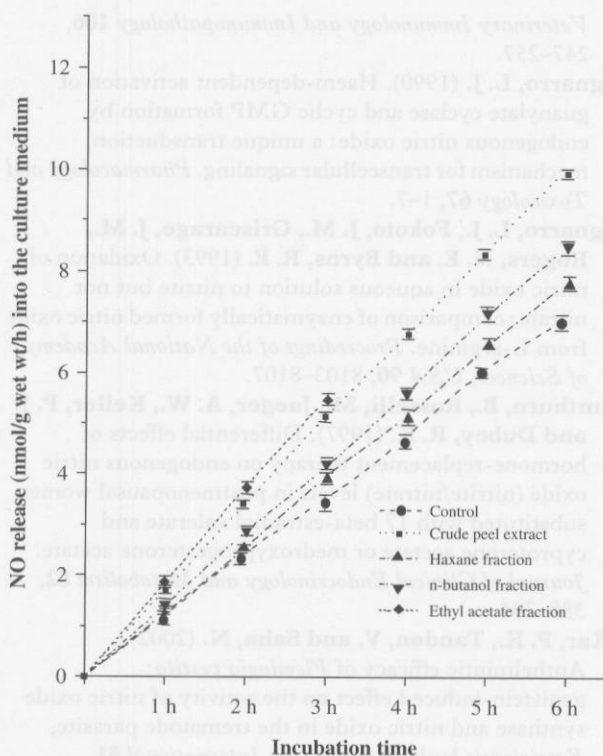


Fig. 1. Effect of crude peel extract of *Flemingia vestita* and its fractions on NO release (nmol/g wet wt) by *Raillietina echinobothrida* into the culture medium at the time of paralysis. NO release, which is equivalent to the NO_2^- formation in the culture medium, was estimated spectrophotometrically at 540 nm following the Griess reaction.

oestrogen induces endothelium-dependent vasodilation, which is likely to be mediated, in part, by augmentation of NO release (Imthurn *et al.* 1997; Best *et al.* 1998). Genistein is a phytoestrogen and in the present study it was shown to increase the NO efflux significantly ($P < 0.01$) by 47% in the treated parasite; the NO efflux in the PZQ-treated parasite was also increased by 38% ($P < 0.05$).

NO, being a sufficiently non-polar molecule, diffuses through cell membranes (Moncada *et al.* 1989) and its cellular functions are mediated by cGMP (Lincoln and Cornwell, 1993; Hobbs, 1997). As expected, the cGMP concentration became accumulated with an increased NO production in the treated parasites. An elevation in the intracellular concentration of cGMP in neutrophils is dependent on increased NO formation (by lipopolysaccharide) and is responsible for downstream signal transmission (Browning *et al.* 1999). However, interestingly NO production caused a decline in the cGMP levels in *H. diminuta* (Onufriev *et al.* 2005). Genistein has been shown to stimulate the osteoblastic proliferation and differentiation by increasing NOS activity, NO formation, and cGMP accumulation in BMSCs cultures (Pan *et al.* 2005). In the present study, genistein increased the NOS activity, NO efflux and cGMP concentration. However, beneficial effects

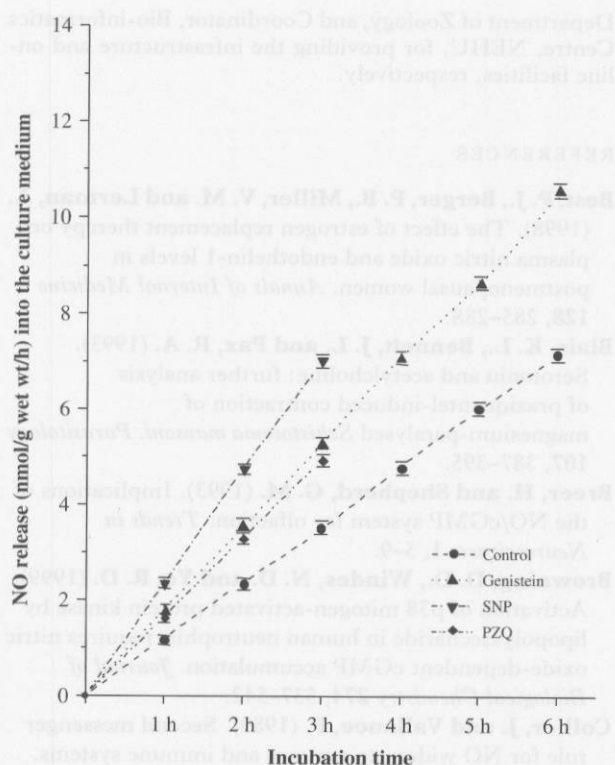


Fig. 2. Effect of genistein, sodium nitroprusside and praziquantel on NO release (nmol/g wet wt) by *Raillietina echinobothrida* into the culture medium at the time of paralysis. NO release, which is equivalent to the NO_2^- formation in the culture medium, was estimated spectrophotometrically at 540 nm following the Griess reaction.

of genistein have also been reported on atherosclerosis and chronic inflammatory diseases by way of inhibiting NO production (Sheu *et al.* 2001). Serotonin, which induces a dose-dependent, heterologous flaccid paralysis in *S. mansoni*, also causes an increase in the cGMP levels (Tremblay *et al.* 1988). Increased cGMP levels by NO mediate signalling pathways by opening Ca^{2+} channels (Breer and Shephard, 1993). One of the important functions of the elevated cGMP by NO is to relax muscle contractions (Toda, 1995). Occurrence of flaccid paralysis due to rapid muscle contractions and changes in the Ca^{2+} homeostasis in *R. echinobothrida*, as observed during the treatments with crude peel extract of *F. vestita* (Das *et al.* 2006), also supplement the action of elevated cGMP concentration in the parasite tissue. These results show that isoflavones, genistein in particular, from *F. vestita*, elevate the cGMP level, which plays a major role in the downstream signal pathways in the parasite, and might also account for the various detrimental effects in the parasite under the resultant anthelmintic stress.

Financial support to B.D. through the DST-SERC Fast Track Scheme (SR/FT/L-107/2004 dated May 04, 2005), GOI, is gratefully acknowledged. We thank the Head,

Department of Zoology, and Coordinator, Bio-informatics Centre, NEHU, for providing the infrastructure and on-line facilities, respectively.

REFERENCES

- Best, P. J., Berger, P. B., Miller, V. M. and Lerman, A.** (1998). The effect of estrogen replacement therapy on plasma nitric oxide and endothelin-1 levels in postmenopausal women. *Annals of Internal Medicine* **128**, 285–288.
- Blair, K. L., Bennett, J. L. and Pax, R. A.** (1993). Serotonin and acetylcholine: further analysis of praziquantel-induced contraction of magnesium-paralysed *Schistosoma mansoni*. *Parasitology* **107**, 387–395.
- Breer, H. and Shepherd, G. M.** (1993). Implications of the NO/cGMP system for olfaction. *Trends in Neurosciences* **1**, 5–9.
- Browning, D. D., Windes, N. D. and Ye, R. D.** (1999). Activation of p38 mitogen-activated protein kinase by lipopolysaccharide in human neutrophils requires nitric oxide-dependent cGMP accumulation. *Journal of Biological Chemistry* **274**, 537–542.
- Collier, J. and Vallance, P.** (1989). Second messenger role for NO widens to nervous and immune systems. *Trends in Pharmacological Sciences* **10**, 427–431.
- Cramer, J. P., Nussler, A. K., Ehrhardt, S., Burkhardt, J., Otchwemah, R. N., Zanger, P., Dietz, E., Gellert, S., Bienzle, U. and Mockenhaupt, F. P.** (2005). Age-dependent effect of plasma nitric oxide on parasite density in Ghanaian children with severe malaria. *Tropical Medicine and International Health* **10**, 672–680.
- Das, B., Tandon, V. and Saha, N.** (2006). Effect of isoflavone from *Flemingia vestita* (Fabaceae) on the Ca²⁺ homeostasis in *Raillietina echinobothrida*, the cestode of domestic fowl. *Parasitology International* **55**, 17–21.
- Gustafsson, M. K. S., Lindholm, A. M., Mäntylä, K., Reuter, M., Lundström, C. A. and Terenina, N.** (1998). NO news on the flatworm front! Nitric oxide synthase in parasitic and free living flatworms. *Hydrobiologia* **383**, 161–166.
- Gustafsson, M. K. S., Lindholm, A. M., Terenina, N. B. and Reuter, M.** (1996). NO nerves in tapeworm. NADPH-diphorase histochemistry in adult *Hymenolepis diminuta*. *Parasitology* **113**, 559–565.
- Gustafsson, M. K. S., Terenina, N. B., Reuter, M. and Movsessian, S. O.** (2003). NO nerves and their targets in a tapeworm: an immunocytochemical study of cGMP in *Hymenolepis diminuta*. *Parasitology Research* **90**, 148–152.
- Hobbs, A. J.** (1997). Soluble guanylate cyclase: the forgotten sibling. *Trends in Pharmacological Sciences* **18**, 484–491.
- Hofmann, F.** (2005). The biology of cyclic GMP-dependent protein kinases. *Journal of Biological Chemistry* **280**, 1–4.
- Holzmüller, P., Cavaleyra, M., Moreaux, J., Kovacic, R., Vincendeau, P., Papierok, G. and Lemesre, J. L.** (2005). Lymphocytes of dogs immunised with purified excreted-secreted antigens of *Leishmania infantum* co-incubated with *Leishmania* infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. *Veterinary Immunology and Immunopathology* **106**, 247–257.
- Ignarro, L. J.** (1990). Haem-dependent activation of guanylate cyclase and cyclic GMP formation by endogenous nitric oxide: a unique transduction mechanism for transcellular signaling. *Pharmacology and Toxicology* **67**, 1–7.
- Ignarro, L. J., Fokoto, J. M., Griscavage, J. M., Rogers, N. E. and Byrns, R. E.** (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison of enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Sciences, USA* **90**, 8103–8107.
- Imthurn, B., Rosselli, M., Jaeger, A. W., Keller, P. J. and Dubey, R. K.** (1997). Differential effects of hormone-replacement therapy on endogenous nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17 beta-estradiol valerate and cyproterone acetate or medroxyprogesterone acetate. *Journal of Clinical Endocrinology and Metabolism* **82**, 388–394.
- Kar, P. K., Tandon, V. and Saha, N.** (2002). Anthelmintic efficacy of *Flemingia vestita*: genistein-induced effect on the activity of nitric oxide synthase and nitric oxide in the trematode parasite, *Fasciolopsis buski*. *Parasitology International* **51**, 249–257.
- Kar, P. K., Tandon, V. and Saha, N.** (2004). Anthelmintic efficacy of genistein, the active principle of *Flemingia vestita* (Fabaceae): alterations in the free amino acid pool and ammonia levels in the fluke, *Fasciolopsis buski*. *Parasitology International* **53**, 287–291.
- Kohn, A. B., Lea, J. M., Moroz, L. L. and Greenberg, R. M.** (2006). *Schistosoma mansoni*: use of a fluorescent indicator to detect nitric oxide and related species in living parasites. *Experimental Parasitology* **113**, 130–133.
- Kohn, A. B., Moroz, L. L., Lea, J. M. and Greenberg, R. M.** (2001). Distribution of nitric oxide synthase immunoreactivity in the nervous system and peripheral tissues of *Schistosoma mansoni*. *Parasitology* **122**, 87–92.
- Lincoln, T. M. and Cornwell, T. L.** (1993). Intracellular cyclic GMP receptor proteins. *FASEB Journal* **7**, 328–338.
- Mahmoud, M. S. and Habib, F. S.** (2003). Role of nitric oxide in host defence against *Hymenolepis nana* infection. *Journal of the Egyptian Society of Parasitology* **33**, 485–496.
- Moncada, S., Palmer, R. M. and Higgs, E. A.** (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochemical Pharmacology* **38**, 1709–1715.
- Moore, R. B. and Kauffman, N. G.** (1970). Simultaneous determination of citrulline and urea using diacetylmonoxime. *Analytical Biochemistry* **33**, 263–272.
- Nathan, C. and Xie, Q.** (1994). Regulation of biosynthesis of nitric oxide. *Journal of Biological Chemistry* **269**, 13725–13728.
- Nelson, D. L. and Cox, M. M.** (2004). *Lehninger Principles of Biochemistry*. 4th Edn. Freeman, New York.
- Onufriev, M. V., Gulyaeva, N. V., Terenina, N. B., Tolstenkov, O. O. and Gustafsson, M. K.** (2005). The effect of a nitric oxide donor on the synthesis of

- cGMP in *Hymenolepis diminuta*: a radiometric study. *Parasitology Research* **95**, 22–24.
- Pal, P. and Tandon, V.** (1998a). Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): genistein-induced alterations in the ultrastructure of the tegument in the cestode, *Raillietina echinobothrida*. *Journal of Parasitic Diseases* **22**, 104–109.
- Pal, P. and Tandon, V.** (1998b). Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): genistein-induced alterations in the activity of tegumental enzymes in the cestode, *Raillietina echinobothrida*. *Parasitology International* **47**, 233–243.
- Pal, P. and Tandon, V.** (1998c). Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): genistein-induced alterations in the esterase activity in the cestode, *Raillietina echinobothrida*. *Journal of Bioscience* **23**, 25–31.
- Pan, W., Quarles, L. D., Song, L. H., Yu, Y. H., Jiao, C., Tang, H. B., Jiang, C. H., Deng, H. W., Li, Y. J., Zhou, H. H. and Xiao, Z. S.** (2005). Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. *Journal of Cell Biochemistry* **94**, 307–316.
- Rao, H. S. P. and Reddy, K. S.** (1991). Isoflavones from *Flemingia vestita*. *Fitoterapia* **63**, 458.
- Roy, B. and Tandon, V.** (1996). Effect of root-tuber extract of *Flemingia vestita* a leguminous plant, on *Artyfechinostomum sufrartyfex* and *Fasciolopsis buski*: an electron microscopy study. *Parasitology Research* **82**, 248–252.
- Salter, M. and Knowles, R. G.** (1998). Measurement of NOS activity by conversion of radiolabelled arginine to citrulline using ion-exchange separation. In *Nitric Oxide Protocols* (ed. Titheradge, M. A.), pp. 67–74. Humana Press, New Jersey.
- Sarcevic, B., Brookes, V., Martin, T. J., Kemp, B. E. and Robinson, P. J.** (1989). Atrial natriuretic peptide-dependent phosphorylation of smooth muscle cell particulate fraction proteins is mediated by cGMP-dependent protein kinase. *Journal of Biological Chemistry* **264**, 20648–20654.
- Schmidt, H. H. H. W., Lohmann, S. M. and Walter, U.** (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochimica et Biophysica Acta* **1178**, 153–175.
- Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J. and Hintz, T. H.** (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide gene expression. *Circulation Research* **74**, 349–353.
- Sheu, F., Lai, H. H. and Yen, G. C.** (2001). Suppression effect of soy isoflavons on nitric oxide production in RAW 264.7 macrophages. *Journal of Agricultural Food and Chemistry* **49**, 1767–1772.
- Stuehr, D. J.** (1999). Mammalian nitric oxide synthases. *Biochimica et Biophysica Acta* **1411**, 217–230.
- Tandon, V. and Das, B.** (2007). *In vitro* testing of anthelmintic efficacy of *Flemingia vestita* (Fabaceae) on carbohydrate metabolism in *Raillietina echinobothrida*. *Methods* (in the Press) doi:10.1016/j.jymeth.2007.01.005.
- Tandon, V., Kar, P. K. and Saha, N.** (2001). NO nerves in trematodes too! NADPH-diaphorase activity in adult *Fasciolopsis buski*. *Parasitology International* **50**, 157–163.
- Tandon, V., Pal, P. and Saha, N.** (1998). Anthelmintic efficacy of *Flemingia vestita* (Fabaceae): Genistein-induced alterations in the free amino acid pool of the cestode, *Raillietina echinobothrida*. *Journal of Parasitic Diseases* **22**, 110–115.
- Tandon, V., Pal, P., Roy, B., Rao, H. S. P. and Reddy, K. S.** (1997). *In vitro* anthelmintic activity of root tuber extract of *Flemingia vestita*, an indigenous plant in Shillong, India. *Parasitology Research* **83**, 492–498.
- Terenina, N. B. and Gustafsson, M. K. S.** (2003). Nitric oxide and its target cells in cercaria of *Diplostomum chromatophorum*: a histochemical and immunocytochemical study. *Parasitology Research* **89**, 199–206.
- Terenina, N. B., Onufriev, M. V., Gulyaeva, N. V., Lindholm, A. M. and Gustafsson, M. K. S.** (2000). A radiometric analysis of Nitric oxide synthase activity in *Hymenolepis diminuta*. *Parasitology* **120**, 91–95.
- Terenina, N. B., Onufriev, M. V., Gulyaeva, N. V., Moiseeva, Y. V. and Gustafsson, M. K. S.** (2003). Nitric oxide synthase activity in *Fasciola hepatica*: A radiometric study. *Parasitology* **126**, 585–590.
- Toda, N.** (1995). Nitric oxide and the regulation of central arterial tone. In *Nitric Oxide in the Nervous System* (ed. Vincent, S. R.), pp. 207–225. Academic Press, London.
- Tremblay, J., Gerzer, R. and Hamet, P.** (1988). Cyclic GMP in cell function. *Advances in Second Messenger Phosphoprotein Research* **22**, 319–383.