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Phytochemicals from *Flemingia vestita* (Fabaceae) and *Stephania glabra* (Menispermeaceae) alter cGMP concentration in the cestode *Raillietina echinobothrida*

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ABSTRACT

Cyclic GMP (cGMP) mediates various physiological functions of nitric oxide (NO) synthesized by nitric oxide synthase (NOS). A crude peel extract and purified fraction of *Flemingia vestita*, as well as a crude rhizome extract of *Stephania glabra* and fractions were tested with respect to the activity of NOS, NO efflux and cGMP concentration in the cestode *Raillietina echinobothrida* in order to find out the possible mode of anthelmintic action of these plant-derived components. For comparison purposes, the parasites were also treated with pure genistein, sodium nitroprusside (SNP–a known NO donor), and the reference drug, praziquantel (PZQ). At the time of onset of paralysis in the parasites, a significant increase (32%–87%) in the NOS activity and a two to three fold increase of NO efflux into the incubation medium were observed in the treated parasites in comparison to their respective controls. The cGMP concentration in the treated parasites' tissue was also increased by 44%–103%. However, in the presence of N^G-nitro-L-arginine methyl ester, a potent inhibitor of NOS, there was no increase in the cGMP concentration in the parasite tissue. This study indicates that the phytochemicals, in particular genistein and tetrahydropalmatine, from *F. vestita* and *S. glabra*, respectively, disturb the downstream signalling pathway of NO, as indicated by the change in cGMP concentration in the parasite tissue.

1. Introduction

Phytochemicals obtained from the crude peel extract of *Flemingia vestita* (Fabaceae) act as vermifugal agents against several intestinal trematodes and cestodes (Tandon et al., 1997), and cause flaccid paralysis as well as various detrimental effects in these parasites (Tandon et al., 1997; Pal and Tandon, 1998a). The activity of nitric oxide synthase (NOS) and the enzymes associated with the co-ordination system, in particular non-specific esterases and acetylcholine esterase, have been shown to be altered by these phytochemicals, as well as the free amino acid pool and tissue ammonia (Pal and Tandon, 1998b; Tandon et al., 1998; Kar et al., 2002, 2004; Das et al., 2007). The phytochemicals from *F. vestita* also affect the carbohydrate metabolism in the cestode, *Raillietina echinobothrida* (Das et al., 2004; Tandon and Das, 2007). Treatment with a crude rhizome extract of *Stephania glabra* (Menispermeaceae), which is also used in traditional medicine (in Meghalaya, Northeast India) as an anthelmintic against gastrointestinal worms, shows a definite effect on the cestode and trematode worms; a dose-dependent gradual decline in physical motility has been observed in *R. echinobothrida* and *Fasciolopsis buski* (Tandon et al., 2004).

The conversion of L-arginine to citrulline and nitric oxide (NO) is catalyzed by nitric oxide synthases (NOS; EC 1.14.13.39; L-arginine, NADPH: oxygen oxidoreductases (nitric-oxide-forming)). The enzyme NOS exists in three isoforms, which are either constitutively expressed (cNOSs) in endothelial cells (eNOS) and neurons (nNOS) or are induced (iNOS) by endotoxin and by inflammatory cytokines. The activity of cNOS depends on the intracellular Ca^{2+} (Malinski and Taha, 1992), whereas that of iNOS depends on immunological and inflammatory stimuli (Nathan, 1992). At a molecular level, NO exerts its most relevant physiological action by activating the soluble form of guanylyl cyclase, leading to the accumulation of cGMP, an important messenger mediating the functions of NO inside the cell. Subsequently, many cellular functions are altered by altering essentially three target proteins of cGMP—Protein kinase G, ion channels regulated by cGMP and cGMP-regulated phosphodiesterases (Hofmann, 2005). The cGMP concentration inside the cell is increased by various means; a) by hormones (such as insulin and oxytocin), acetylcholine and biogenic amines (like serotonin and histamine); b) by vasodilators (nitroprusside, nitroglycerin and sodium nitrate), the stimulators of guanylate cyclase; and c) by peptides such as atrial

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natriuretic factors, which relax smooth muscle (Lucas et al., 2000). Besides elevating the cGMP concentration in the cell, NO also modulates mitogen-activated protein kinase cascade, the activity of protein kinase C, and gene expression by modifying the transcription factors, and also causes apoptosis through accumulation of the tumour suppressor p53 (Beck et al., 1999). According to recent studies, NO also has anti-leishmanial (Holzmuller et al., 2005), anti-malarial (Cramer et al., 2005), and anthelmintic effects (Mahmoud and Habib, 2003). In the adult and larval stages of some platyhelminth parasites, the target cells for NO have been located by cGMP immunostaining (Gustafsson et al., 2003; Terenina and Gustafsson, 2003).

In this study, we investigated the effect of the phytochemicals from *F. vestita* and *S. glabra* on the cGMP concentration in the parasite, *R. echinobothrida*, in order to extend the understanding of the possible mode of anthelmintic action of these plant-derived compounds. Therefore, the phytochemicals from *F. vestita* and *S. glabra* were tested *in vitro* with respect to the activity of NOS, NO efflux into the incubation media and cGMP concentration in the parasite tissue. We report here that these phytochemicals possess anthelmintic activities by altering the NOS–NO–cGMP pathway in the cestode *R. echinobothrida*.

2. Materials and methods

2.1. Chemicals

The enzyme immunoassay cGMP kit (CG-201) and pure genistein (G 6649) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). N^G-nitro-L-arginine methyl ester (L-NAME), the enzymes and co-enzymes used in this study were purchased from either Sigma or Roche (Germany), whereas praziquantel (PZQ) and sodium nitroprusside (SNP) were from Bayer (India) and S.D. Fine Chemicals Pvt. Ltd. (India), respectively. Other chemicals were obtained from Sisco Research Laboratory (India). Deionised double-distilled H_2O was used for chemical preparations.

2.2. Isolation of phytochemicals

A crude extract was prepared from *F. vestita* (root peel) and *S. glabra* (rhizome pulp) by soaking the plant material in methanol for two to three weeks, followed by extraction in a Soxhlet apparatus using successive hexane, chloroform and methanol incubations. The solvents were exchanged after 24h of extraction, filtered and concentrated by rotary vacuum-evaporation. The hexane crude extract of *F. vestita* was subjected to fractionation by Flash chromatography (Flash Master Personal, UK) eluted isocratically with 100% hexane and increasing the polarity by increments of 5% until 100% ethyacetate, then 10% methanol in ethylacetate until 100% methanol. The fractions were collected and freeze dried using liquid nitrogen. The elute at a 7:3 benzene:ethylacetate ratio is known to contain genistein (4′,5,7-trihydroxyisoflavone) (Rao and Reddy, 1991). The chloroform crude extract of *S. glabra* was subjected to fractionation by Flash chromatography eluted isocratically with 100% chloroform and increasing the polarity by increments of 5% until 100% ethanol. The elute at 9.5:0.5 chloroform:ethanol contained a pure compound. It was crystallized by slow evaporation from methanol, and the crystals were washed either with acetone or methanol. The structural identity of the purified compound was determined by NMR studies (^{13}C and ^{1}H NMR) and found to be tetrahydropalmatine.

2.3. In vitro treatments

The live cestode parasites were collected in phosphate buffered saline (PBS, pH 7.2) from the intestine of freshly slaughtered domestic fowl and immediately processed. The cestode parasites were taken from a single host for each set of experiments, i.e., treatment and its respective control. For each set of experiments, four to five numbers of freshly collected parasites (~ 0.2g wet mass) were incubated in 10mL PBS at 38 ± 1°C with defined concentrations of test agents; i.e., the crude peel extract and ethyl acetate fraction of *F. vestita* (5, 10, 20mg/mL), the rhizome crude extract of *S. glabra* (5, 10, 20mg/mL) and tetrahydropalmatine (1, 2 and 5mg/mL), genistein (0.2 and 0.5mg/mL), the reference drug, PZQ, (1 and 5μg/mL) and SNP (1.5 and 3mg/mL) dissolved in 1% (v/v) dimethyl sulphoxide (DMSO). In another set of experiments, L-NAME (0.1mM) was used along with the test materials. The respective control of each treatment was also maintained with 1% (v/v) DMSO in PBS.

At the time when paralysis started to set in, the treated parasites and their respective controls were taken out and processed for NOS activity and estimation of cGMP concentration in the parasite tissue.

2.4. NOS assay, NO efflux and cGMP estimation

For assessment of the NOS activity, the method of Salter and Knowles (1998) with certain modifications as described earlier by Tandon et al. (2001) was followed. In brief, a 10% (w/v) homogenate of the treated parasites and controls was prepared in a homogenizing buffer containing 20mM HEPES buffer (pH 7.2), 250mM mannitol, 1mM EDTA, 1mM DTT and 0.01mM PMSF. The homogenate was mixed (1:1) with 0.5% (v/v) Triton X-100 for 30min and sonicated twice for 30s each using a sonicator (Soniprep 150, UK). The resultant homogenate was centrifuged for 10min at 10,000g and the supernatant was used for assessment of the NOS activity. One mL of reaction mixture contained 50mM potassium phosphate buffer (pH 7.2), 50mM L-arginine, 1.2mM $MgCl_2$, 0.25mM $CaCl_2$, 0.15mM NADPH, 20u urease and 50μL of enzyme source. The reaction mixture was incubated at 38°C for 15min and in order to stop the reaction 1mL of 10% (v/v) perchloric acid was added. The citrulline concentration in the reaction mixture was measured spectrophotometrically at 490nm. One unit (u) of enzyme activity is that amount of enzyme catalyzing 1μmol of citrulline formation per hour at 38°C. The nitrite (NO_2^-) concentration, which is equivalent to NO efflux by the parasite, in the incubation medium was estimated following the Griess reaction (Sessa et al., 1994).

To determine the cGMP concentration in the parasite tissue (treated and controls), the protocol described in the enzyme immunoassay cGMP kit (Sigma) was followed. Briefly, the treated and control parasites were immediately frozen at the time when paralysis began in the treated parasites. A 10% homogenate was made in 5% (v/v) cold trichloroacetic acid and centrifuged at 600g for 10min. The supernatant was collected in three volumes of water-saturated ether. The reconstituted samples, after drying the aqueous extracts, were taken for the estimation of cGMP. To each microtitre plate wells (coated with goat anti-rabbit IgG), 100μL of sample was taken in duplicate. Alkaline phosphatase conjugated with cGMP (50μL) was added into each well and the plate was incubated on a plate shaker for 2h at room temp after adding 50μL of rabbit IgG to cGMP. After washing the wells with washing buffer, 200μL of p-nitrophenyl phosphate was added to each well and the plate was again incubated for 1h at room temp without shaking. Trisodium phosphate (50μL) was added to each well in order to stop the reaction and immediately the plate was read at 405nm using an ELISA plate reader (Multiskan Ex Primary EIA V. 2.1-0). The cGMP concentration in either treated or control parasite tissue was calculated against the standard cGMP curve.

2.5. Statistical analysis

Data are represented as the mean ± SEM ($n = 4$) and probability values less than 0.05 were taken to be statistically significant. Statistical analysis was performed using Student's *t*-test; comparisons of the paired mean values were calculated between the treatments and their respective controls.

3. Results

The phytochemicals from the crude extracts of *F. vestita* and *S. glabra* were tested in order to cause paralysis in the cestode parasite. At the defined concentrations of the test materials (vide the section '*In vitro treatments*'), the onset of paralysis in the cestodes occurred at about 6h, 4h and 2h for the crude peel extract and 6h, 5h and 3h for the ethyl acetate fraction of *F. vestita*, respectively; 18h, 13h and 7h for the crude rhizome extract of *S. glabra* and tetrahydropalmatine; 7h and 5h for genistein, and 3h and 1h for PZQ and SNP, respectively. The control parasites did not show any paralysis and survived for about 72h.

To find out the effect of the phytochemicals on the NOS activity in the parasite at paralysis time, the NOS activity was assessed after the exposure to various treatments. The NOS activity was measured about 9u/g wet wt. in the control parasites. In the parasites exposed to various treatments, the NOS activity was observed to be increased significantly (32%–87%) ($p < 0.05$), as shown in Fig. 1a and c. Maximal increase in the NOS activity (87%) was observed in PZQ-treated parasites (Fig. 1c). However, there was no obvious change in the NOS activity in the parasites treated with other fractions of *F. vestita* (data not shown) and in SNP-treated parasites (Fig. 1c). In contrast, the NOS activity was found to be decreased by 22% to 47% in the case of

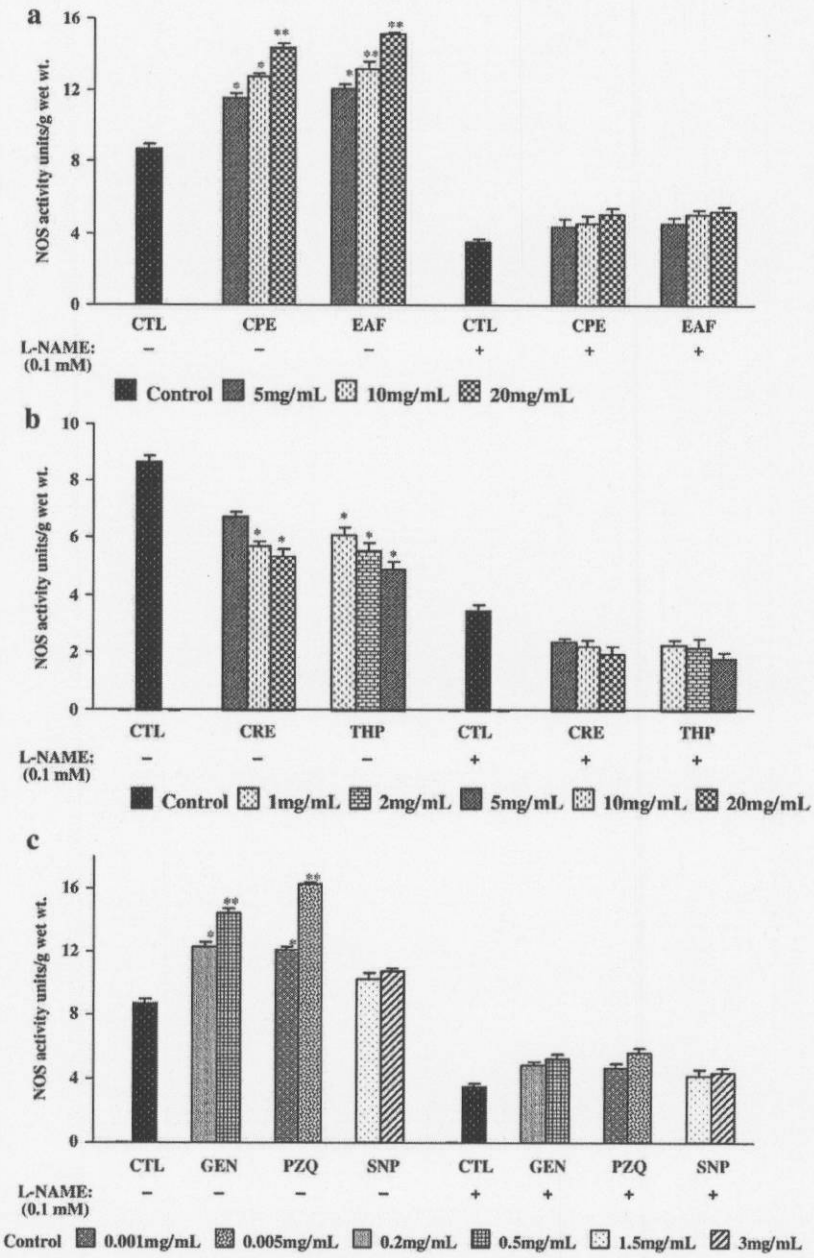

Fig. 1. NOS tissue activity (units/g wet wt) in the cestode *R. echinobothrida* at paralysis time *in vitro* in absence (–)/presence (+) of L-NAME (0.1 mM). Effects of a) crude peel extract (CPE) and ethyl acetate fraction (EAF) of *F. vestita* (5, 10, 20 mg/mL); b) crude rhizome extract of *S. glabra* (CRE; 5, 10, 20 mg/mL) and purified compound, tetrahydropalmatine (THP; 1, 2, 5 mg/mL); and c) genistein (GEN; 0.2, 0.5 mg/mL), praziquantel (PZQ; 1, 5 μg/mL) and sodium nitroprusside (SNP; 1.5, 3 mg/mL). One unit of enzyme activity is that amount of enzyme catalyzing 1 μmol of citrulline formation per hour at 38 °C. Data are expressed as mean±SEM ($n=4$). *$p<0.05$ and **$p<0.01$ vs. control (CTL).

treatments with crude rhizome extract of *S. glabra* and pure compound (Fig. 1b). In the treated parasites, the augmented NOS activity was reduced in the presence of its potent inhibitor, L-NAME. The increased NOS activity was correlated by a two to three fold increase in the NO efflux by the treated parasites into the incubation medium (Fig. 2a and c). There was a significant increase ($p < 0.05$) in the NO efflux by the treated parasites, except in the treatments with *S. glabra* and pure compound, in comparison to that by control parasites (about 1 nmol/g wet wt/h). However, in the presence of L-NAME, there was a significant decline in the NO efflux by the treated parasites.

To examine the effect of the increased NO production in the parasite, the cGMP concentration in the parasite tissue was determined. In the control parasite tissue, the cGMP concentration was found to be about 23 pmol/g wet wt. The cGMP concentration was increased significantly (44%–103%) ($p < 0.05$) in the treated parasites in comparison to their respective controls (Fig. 3a and c). However, insignificant changes were observed in the case of other fractions of *F. vestita* (data not shown). In contrast, the cGMP concentration was decreased in *S. glabra* treatments (Fig. 3b). In the SNP-treated parasites, the cGMP concentration in the parasite tissue was found to be maximal (Fig. 3c). There was a significant

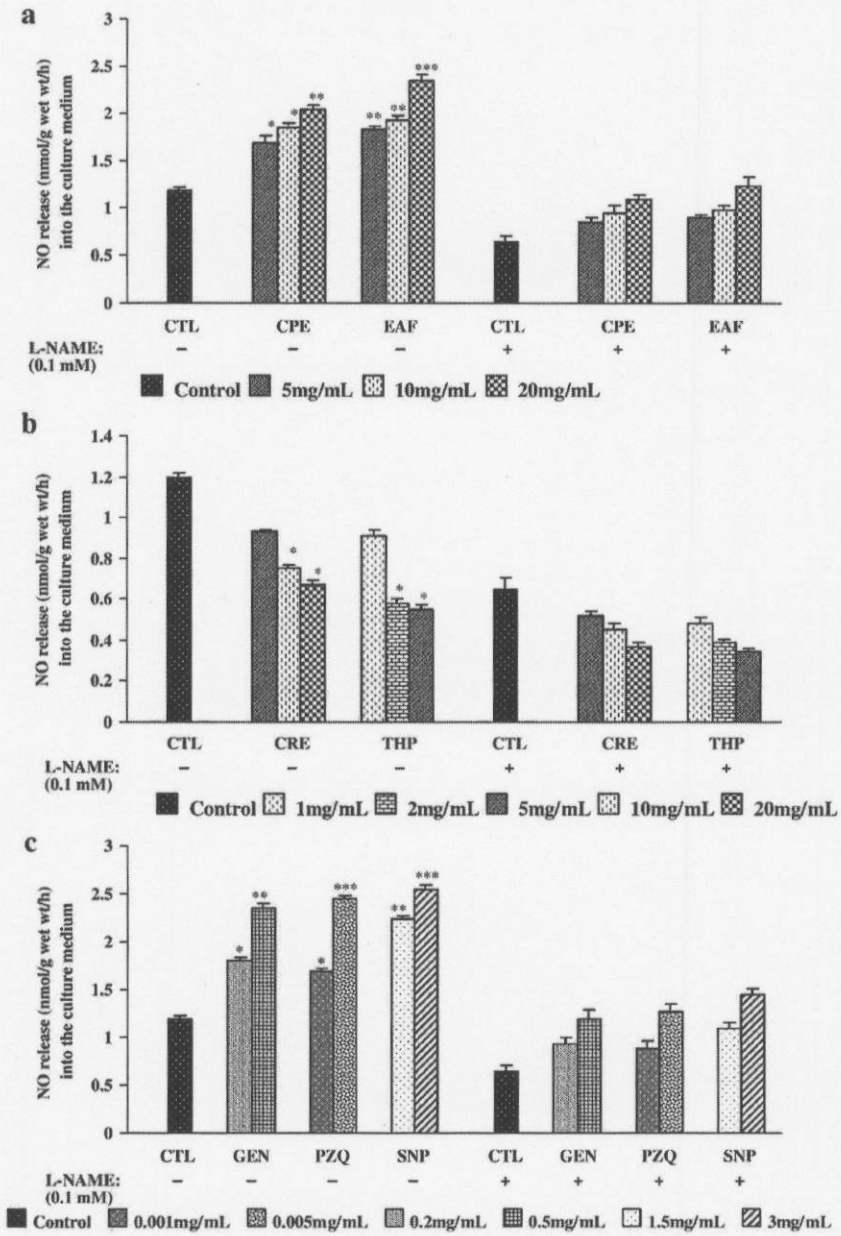

Fig. 2. NO release (nmol/g wet wt/h) by the cestode *R. echinobothrida* into the culture medium at paralysis time in absence (−)/presence (+) of L-NAME (0.1 mM). Effects of a) crude peel extract (CPE) and ethyl acetate fraction (EAF) of *F. vestita* (5, 10, 20 mg/mL); b) crude rhizome extract of *S. glabra* (CRE; 5, 10, 20 mg/mL) and purified compound, tetrahydropalmatine (THP; 1, 2, 5 mg/mL); and c) genistein (GEN; 0.2, 0.5 mg/mL), praziquantel (PZQ; 1, 5 μg/mL) and sodium nitroprusside (SNP; 1.5, 3 mg/mL). Data are expressed as mean±SEM ($n=4$). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control (CTL).

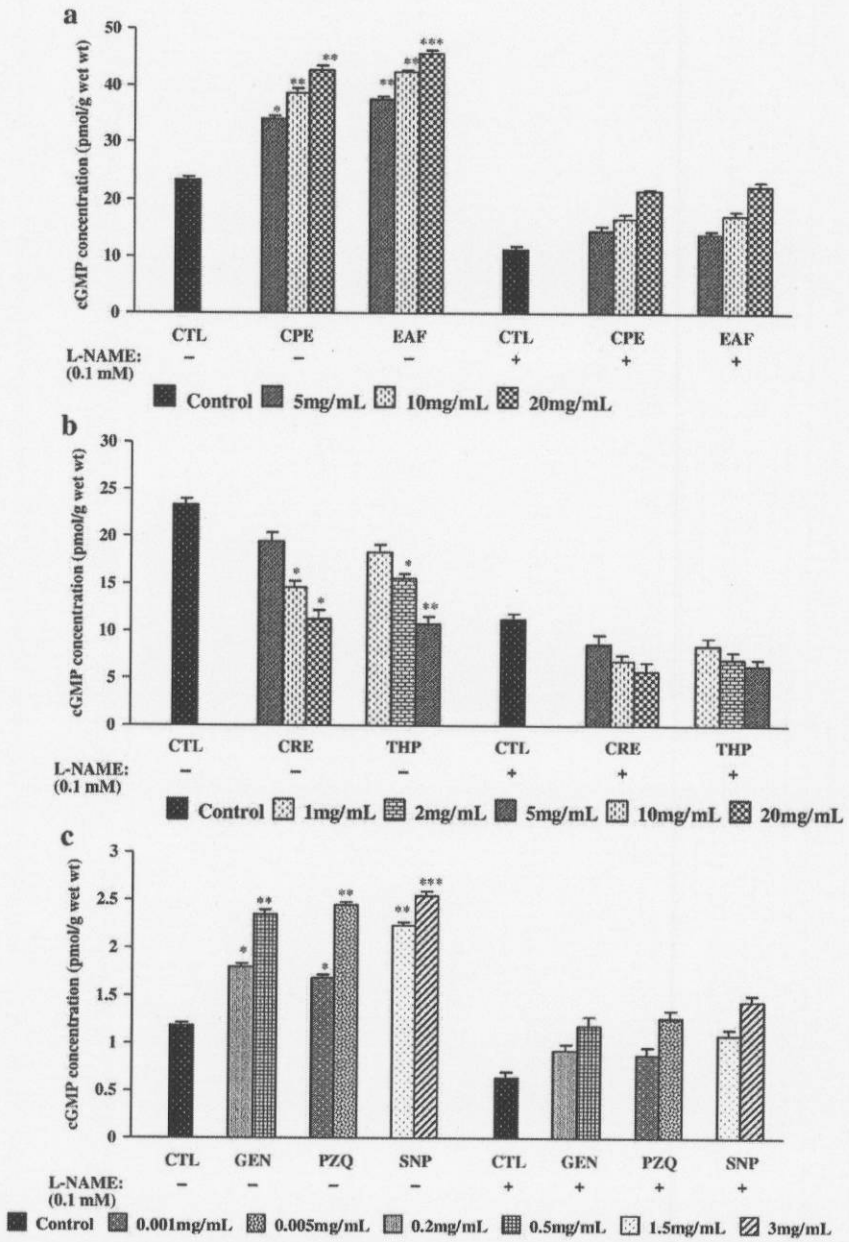

Fig. 3. Alteration in the cGMP concentration (pmol/g wet wt) in the cestode *R. echinobothrida* tissue at paralysis time *in vitro* in absence (−)/presence (+) of L-NAME (0.1 mM). Effects of a) crude peel extract (CPE) and ethyl acetate fraction (EAF) of *F. vestita* (5, 10, 20 mg/mL); b) crude rhizome extract of *S. glabra* (CRE; 5, 10, 20 mg/mL) and purified compound, tetrahydropalmatine (THP; 1, 2, 5 mg/mL); and c) genistein (GEN; 0.2, 0.5 mg/mL), praziquantel (PZQ; 1, 5 μg/mL) and sodium nitroprusside (SNP; 1.5, 3 mg/mL). Data are expressed as mean±SEM ($n=4$). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control (CTL).

reduction in the cGMP concentration in the parasite tissue treated with different test materials along with L-NAME.

4. Discussion

The phytochemicals from *F. vestita* and *S. glabra* caused a dose-dependent gradual decline in physical motility or paralysis in *R. echinobothrida*. The onset of paralysis, as a result of rapid muscular contraction, in the treated parasites is not fully uncovered. In *Schistosoma mansoni*, serotonin (the biogenic amine that alters the cGMP level) also caused a similar dose-dependent, heterologous flaccid paralysis (Blair et al., 1993).

The presence of NOS in various helminths has been demonstrated by various authors. For the first time, NADPH-d positive neurons, an indication of the occurrence of nitregic mechanisms, were demonstrated in the nervous system of parasitic flatworms—the cestode, *Hymenolepis diminuta* (Gustafsson et al., 1996) and the trematode, *Fasciolopsis buski* (Tandon et al., 2001) followed by *Fasciola hepatica* (Terenina et al., 2003) and some free-living flatworms (Gustafsson et al., 1998). Recently, NOS immunoreactivity was also demonstrated in *S. mansoni* (Kohn et al., 2001, 2006), *S. japonicum* and *S. mansoni* (Long et al., 2004), the nematode *Trichinella britovi* (Masetti et al., 2004) and the metacestode of *Taenia solium* (Kim et al., 2007).

When the parasites were treated with pure genistein and PZQ, the increase in the activity of NOS was found at par with that of *F. vestita* treatments; however, in the case of *S. glabra* and its purified compound, significant decrease in the NOS activity was observed. The cNOS activity is known to depend on the intracellular Ca^{2+} (Malinski and Taha, 1992) and an increase in the NOS activity in the treated parasites may be explained in the light of Ca^{2+} efflux. Recently, we reported that the crude peel extract of *F. vestita*, genistein and PZQ caused a dose-dependent Ca^{2+} efflux in the parasite (Das et al., 2006). The NOS activity is also regulated through phosphorylation and protein–protein interactions (Fulton et al., 1999). Logistically, it can be predicted that the activation of cNOS could be due to the change in the phosphorylation status of the enzyme, as it seems unlikely that it would happen due to transcriptional regulation within a short time (Stuehr, 1999). In the presence of L-NAME, the dose-dependent increase in the NOS activity by the various treatments was significantly reduced.

In the present study, it was observed that there was an increase in the NO efflux into the incubation medium by the treated parasites, which corresponds to the increased NOS activity in their tissue. In the genistein-treated parasites, the NO efflux was significantly increased ($p<0.05$). Genistein is a phytoestrogen and present in the crude peel extract of *F. vestita* (Rao and Reddy, 1991). Induction of endothelium-dependent vasodilation by estrogen is known to be mediated, in part, by augmentation of NO release (Best et al., 1998). In contrast, beneficial effects of genistein, by inhibiting NO production, have been reported in atherosclerosis and chronic inflammatory diseases (Sheu et al., 2001). In the same line, genistein and other non-specific tyrosine kinase inhibitors abolish iNOS expression in vascular cells (Papapetropoulos et al., 2001). Recently, Hatziieremia et al. (2006) reported that cardamonin, a known chalcone, possesses anti inflammatory activities by inhibiting the synthesis of NO through interfering with the binding of NFκB to DNA, while other reports suggest that it induces endothelium-dependent relaxation mediated through endothelial NO (Huang et al., 2000). Being a non polar molecule, the released NO diffuses through cell membranes (Moncada et al., 1989) and its physiological functions are mostly mediated by cGMP (Hofmann, 2005). The dose-dependent NO efflux by the various treatments was significantly inhibited in the presence of the potent inhibitor of NOS, i.e., L-NAME.

The cGMP concentration in the treated parasite tissue also got accumulated, as predicted, with a simultaneously increased NO production. To compare, a known NO donor (SNP) was used and an elevated cGMP concentration was observed in the parasite tissue. However, in another cestode, *H. diminuta*, the cGMP levels declined by NO production (Onufriev et al., 2005). In neutrophils, the elevated cGMP concentration is also dependent on increased NO formation caused by lipopolysaccharides, a known activator of NOS (Browning et al., 1999). It has been shown that genistein stimulates the osteoblastic proliferation and differentiation by altering NOS–NO–cGMP signal pathway in BMSCs cultures (Pan et al., 2005). In our study, genistein significantly increased the cGMP concentration, which corresponds to the increased NOS activity and NO efflux in the genistein-treated parasites. Elevated cGMP level in the cell is responsible for the opening of Ca^{2+} channels (Breer and Shepherd, 1993) and relaxation of muscle contractions (Toda, 1995). Serotonin, the biogenic amine responsible for dose-dependent heterologous flaccid paralysis in *S. mansoni* (Blair et al., 1993), is known to increase the cGMP concentration in the cell (Lucas et al., 2000). The dose-dependent increase in the cGMP concentration, which is a consequence of increased NOS activity, indicates that the phytochemicals from *F. vestita* and *S. glabra*, genistein and PZQ bring about an alteration in the cGMP concentration in the parasite. The elevated cGMP concentration in the parasite tissue due to various treatments might relate to the occurrence of flaccid paralysis and alteration in the Ca^{2+} homeostasis in *R. echinobothrida*, as observed in our previous study (Das et al., 2006). The inhibitory effects of phytochemicals from *S. glabra* on NOS activity, NO efflux and cGMP concentration may be due to the presence of tetrahydropalmatine, which is reported to inhibit NOS expression (Guangtian et al., 2003) and NO production (Yang et al., 1999).

These findings indicate that phytochemicals, in particular genistein and tetrahydropalmatine, from *F. vestita* and *S. glabra*, respectively, alter the cGMP concentration in the parasite tissue, which plays a major role in transmitting NO signals. The elevated cGMP level might also be held responsible for other detrimental effects in the cestode parasite, *R. echinobothrida*, under the consequential anthelmintic stress. These initial research findings provide an insight into the potential development of new anthelmintic drugs of plant origin.

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