

Nitric Oxide and Modulatory Effects of the Root extracts of *Phlogacanthus tubiflorus* against Oxidative Stress induced by Hydrogen Peroxide

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ABSTRACT

Introduction: Nitric oxide (NO) mediated signaling is known to influence tumor progression and has been implicated as a novel therapeutic target cancer therapy. **Methods:** In the present study, we have reported the modulatory effects of the root extracts of *Phlogacanthus tubiflorus* on NO levels in the lymphocytes cultured *in vitro* and exposed to oxidative stress induced by H₂O₂. **Results:** Increase in the levels of NO was observed in the lymphocyte treated with increasing concentration of H₂O₂ (0.1, 0.2, 0.5 and 1.0%), but the cell viability declined significantly. Treatment of Lymphocyte with root extract resulted in the decrease of NO level and increase in the Cell Viability. We also observed declined NO levels and increase in cell viability in the lymphocyte pre-treated with the different concentrations of (50, 100, and 200 µg/ml) of root extracts and followed by the 1% H₂O₂ treatment. **Conclusion:** The present data suggests that NO and cell viability are inter related and extract used is rich in phytochemicals with modulatory effect on both NO and cell viability and hence, might find relevance in chemoprevention of oxidative stress related diseased conditions.

Key words: Chemo-modulation, Nitric Oxide, Oxidative stress, *Phlogacanthus tubiflorus*.

INTRODUCTION

Nitric oxide (NO) is highly reactive free radical capable of multitude of the reactions. It acts as an intracellular messenger and known to influence signaling pathways related to regulation of cell growth, differentiation and apoptosis and many physiological action including modulation of blood pressure and synaptic plasticity.^[1] Nitric oxide also regulate hepatic metabolism and plays role in cardioprotection including regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion and prevention of smooth muscle cell proliferation.^[2,3] Besides this NO mediated signaling also influences solid tumor progression resulting in growth, invasion, metastasis and ability to induce angiogenesis.^[4,5] The dual role of NO

depends on its threshold level. In normal tissue, NO is generated from L-arginine by Nitric Oxide Synthase (NOS) and the level exceeded the basal level in certain pathophysiological condition and stress condition. This elevated NO level also cause toxicity to the cell and leads into cell death and apoptosis.^[6]

The elevated level of NO can be reduced to optimum level either by using NOS inhibitors or NO scavenger. Several arginine analogs have been tested for their NO inhibitory action and as these agents exhibit other effects too, so application becomes limited. Quercetin also screened as a NO scavenger, but threshold maintenance with no or least side effects has not been achieved.^[7] Therefore, present study is focused on herbal regime for nitric oxide modulation that might have pharmacological importance.

Phlogacanthus tubiflorus Nees (Family: Acanthaceae) is a traditional medicinal plant used by the tribal population of North Eastern region of India for treating wounds, tumorous growth and also as a blood purifier (Indigenous knowledge). Its bitter

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tested leaves and flowers are used for relieving cough, stomach ache and scabies.^[8-10] Flower is also used for treating intestinal worm and rheumatism.^[11,12] Leaf extract exhibit antioxidative, acaricidal and fecundity reducing activity.^[13,14] Phlogacanthin, a Lacton was isolated from root.^[15] The objective of the present study was to investigate the modulatory effects of hydroalcoholic root extracts of *Phlogacanthus tubiflorus* on the Nitric oxide levels in the cells exposed to oxidative stress induced by H₂O₂ and tried to correlate with cell viability.

MATERIALS AND METHODS

Chemical and reagents

Histopaque 1077 and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma Chemical Co. (St Louis, MO, USA). RPMI 1640, Fetal Bovine Serum (FBS) were purchased from HiMedia Laboratories (Mumbai, India). The rest of the chemicals were of analytical grade and obtained from local firms of India.

Collection and identification of plant material

The roots of *Phlogacanthus tubiflorus* were collected from Tezpur, Assam (India) and authenticated by a competent Botanist, Prof. S K Borthakur, at the Department of Botany, Gauhati University, Gauhati, Assam (India) and a voucher specimen was preserved in our laboratory. The roots were washed with running tap water repeatedly and finally with distilled water to remove impurities and dried at shade. The dried plant materials were finely powdered and stored in air tight container.

Preparation of plant extract

Dried and coarse powders of *Phlogacanthus tubiflorus* roots (100 g) were macerated with 80% (v/v) ethanol in a shaking condition for one week. The extract thus obtained (PTE) were filtered concentrated and stored at 4 °C. The extract was dissolved in DMSO with final concentration of 2.5 mg/ml.

Preliminary phytochemical screening

The extract was subjected to phytochemical screening for the detection of polyphenols and flavonoids according to the standard procedure.^[16,17]

Isolation and culture of lymphocytes

Lymphocytes were isolated from anticoagulated Chicken blood using Histopaque (1.077 gm/ml), cultured in RPMI supplemented with 10% heat inactivated fetal bovine serum

and were treated with as per experimental requirement. After incubation at 37 °C and 5% CO₂, lymphocytes were centrifuged, washed and homogenized in phosphate buffer saline (PBS). Cell supernatants were used for assaying NO level and protein content.

Determination of nitric oxide levels

NO• levels were determined by the method of Griess in a total volume of 200 µl containing equal volume of Griess reagent and sample and absorbance was read at 550 nm in a microplate reader.^[18] The nitrite content in the sample was calculated in mMole NO/mg protein from the standard curve made with sodium nitrite and finally expressed as percentage change of NO level in comparison to control cells.

Cell viability assay

Cell viability was assayed by the method of Denizot and Lang.^[19] Briefly, after treatments, cells were treated with 10% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chem.) for 2 hours and formazan crystals formed were dissolved in solvent as per manufacturer protocol and absorbance was measured at 570 nm. The background absorbance was measured at 690 nm. The absorbance of control cells was set as 100% viable and the values of treated cells were calculated as percentage of control.

Protein determination

The protein contents were determined according to the method described by Lowry et al., (1951) using bovine serum albumin (BSA) as standard.^[20]

Statistical Analysis

All the data are expressed as means ± SD, n=3. The significance differences between the experimental and the control groups were analyzed by student's t test and three levels of significance were set as p < 0.05, p < 0.01 and p < 0.001.

RESULTS

Preliminary phytochemical screening

Qualitative screening of the plant extract reveal the presence of polyphenols and flavonoids in the root extract used for the study.

Effects of different concentrations of H₂O₂

Exposure of lymphocytes to increasing concentration of H₂O₂ (0.1, 0.2, 0.5 and 1%) for 4 hr, significant increase in

the levels of NO was observed. For similar conditions significant decline in the cell viability in concentration dependent manner was observed (Table 1).

Effects of different concentrations of PTE

Lymphocytes treated with 50, 100, 200 and 300 µg/ml of PTE for 4 hr decreases in the level of NO were observed. The decrease in the level of NO was significant at 200 and 300 µg/ml of PTE treatment. In contrast to the NO, the cell viability was found increased in comparison to untreated cells (Table 2).

PTE as modulator in the cells treated with 1% H₂O₂

Lymphocytes were pre-treated with PTE for 1h followed by the treatment with 1% H₂O₂ for 4 h, decline in the level of NO was observed. The cell viability significantly increased for 50, 100 and 200 µg/ml of PTE as compared to positive control (Table 3).

DISCUSSION

It is well known that Nitric oxide (NO), a highly reactive free radical acts as an intracellular messenger and mediate large numbers of signaling pathways. Beside these, NO is also able to react with other inorganic molecules (oxygen, superoxide or transition metals), structures in DNA, prosthetic groups or with the proteins and indirectly causes

lipid peroxidation and formation of several harmful products. NO mediated signaling also influences the regulation of apoptosis and cell viability. High level of NO (as in stress condition, inflammatory response) is known to promote apoptotic death and moderate levels of NO exert protective role through inhibition of caspase processing and activation.^[21,22] Here, in the present study significant increase in the level of NO was observed in the lymphocytes treated with increasing concentration of H₂O₂ (0.1, 0.2, 0.5 and 1%) for 4 hr and at 1.0% of H₂O₂ treatment NO level increased up to 166.65 % (p<0.001) in comparison to untreated cells. This increase in the level of NO might be due to the oxidative stress induced by H₂O₂. For similar conditions significant decline in the cell viability was observed and at 1.0% of H₂O₂ treatment the decline in cell viability was below 50% (Table 1). The results clearly showed the increasing NO level as a result of H₂O₂ treatment induces cell death might be via apoptosis.^[6] Treatment of Lymphocyte with different concentration of PTE significantly lowered the NO level (Table 2). In the same treatment condition cell viability increased significantly in comparison to control suggesting protective role of the extract used against endogenous stress. Lymphocytes pre-treated with PTE for 1h, followed by the treatment with 1% H₂O₂ for 4 h, decline in the level of NO was observed and at 100 and 200 µg/ml of PTE treatment, the decline was significant in comparison to only H₂O₂ treated cells. The cell viability significantly increased for 50, 100 and 200 µg/ml of PTE as compared to positive control (Table 3). The results suggest that low level of NO might delay the triggering of apoptosis and hence contributing to increase cell viability.^[23] The present data concludes that the NO and cell viability are inter related and root extract of *Phlogacanthus tubiflorus* is rich in phytochemicals like polyphenols, flavonoids and have modulatory effects on the NO level and cell viability and hence, might find pharmacological applications in chemoprevention in future.

Table 1: Effects of different concentrations of H₂O₂. Lymphocytes were treated with PTE for 4 hr.

Condition	% change of NO level (mMole/mg protein)	% change of cell viability
Control	100 ± 7.33	100 ± 5.38
0.1% H ₂ O ₂	112.84 ± 7.92	93.38 ± 6.96
0.2% H ₂ O ₂	118.81 ± 0.57 ^c	82.76 ± 7.26 ^c
0.5% H ₂ O ₂	123.55 ± 5.43 ^c	65.66 ± 5.29 ^b
1 % H ₂ O ₂	166.65 ± 10.3 ^a	38.18 ± 9.56 ^a

Values are mean ± SD; n=3; ^ap<0.001 compared to control cells; ^bp<0.01 compared to control cells; ^cp<0.05 compared to control cells.

Table 2: Effects of the root extract of *Phlogacanthus tubiflorus* (PTE). Lymphocytes were treated with PTE for 4 hr.

Treatments	% change of NO level (mMole/mg protein)	% change of cell viability
Control	100 ± 9.12	100 ± 6.05
50 µg/ml PTE	91.27 ± 5.55	104.63 ± 4.96
100 µg/ml PTE	88.70 ± 0.94	109.19 ± 9.21
200 µg/ml PTE	87.32 ± 1.66	119.64 ± 4.89 ^c
300 µg/ml PTE	80.54 ± 2.72 ^c	124.11 ± 7.70 ^c

Values are mean ± SD; n=3; ^ap<0.001 compared to control cells; ^bp<0.01 compared to control cells; ^cp<0.05 compared to control cells.

Table 3: Protective effects of different concentrations of PTE in H₂O₂ treated lymphocytes. Lymphocytes were pre-treated with PTE for 1 h and followed by 1% H₂O₂ treatment for 4hr.

Treatments	% change of NO level (mMole/mg protein)	% change of cell viability
Control	100 ± 1.96	100 ± 6.29
1% H ₂ O ₂	164.48 ± 7.30 ^a	39.45 ± 4.55 ^a
1% H ₂ O ₂ + 50 µg/ml PTE	163.74 ± 10.85 ^a	71.45 ± 8.80 ^{ee}
1% H ₂ O ₂ + 100 µg/ml PTE	136.03 ± 10.3 ^{bf}	86.14 ± 10.70 ^e
1% H ₂ O ₂ + 200 µg/ml PTE	128.36 ± 2.34 ^{ae}	98.33 ± 5.23 ^d

Values are mean ± SD; n=3; ^ap<0.001 compared to control cells; ^bp<0.01 compared to control cells; ^cp<0.05 compared to control cells; ^dp<0.001 compared to cells treated with only H₂O₂; ^ep<0.01 compared to cells treated with only H₂O₂; ^fp<0.05 compared to cells treated with only H₂O₂.

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