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A Study on The Nitric Oxide (NO) Enhancing Ability of A Natural Phytochemical Formulation

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ABSTRACT

Nitric oxide (NO) has basically dual action in biological systems. Nitric oxide (NO) has basically dual action in biological systems. Nitric oxide plays a crucial role in the vasodilation process. As a result of the vasodilation, the blood flow to the body tissues will be increased, there by influencing the biological actions of the human body. There are many naturally derived phytochemicals which are helpful in the synthesis of nitric oxide. This study report explains the Nitric oxide stimulation activity of a combination of naturally derived phytochemicals.

Key-words: Nitric oxide, Phytochemical Formulation, NO enhancing.

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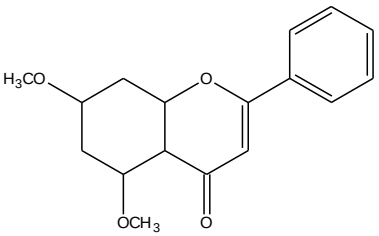
INTRODUCTION:

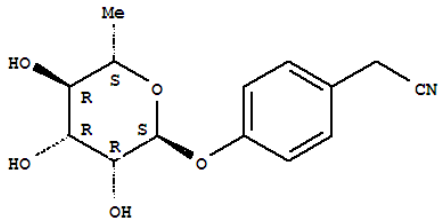
Nitric oxide plays a vital role in many biological activities. Nitric oxide plays a crucial role in the vasodilation process. There are many naturally derived phytochemicals responsible for the stimulation of nitric oxide. L arginine is the precursor of NO production.[1] There are two endothelial forms of NOS: constitutive NOS (**cNOS**; type III) and inducible NOS (**iNOS**; type II). Co-factors for NOS include oxygen, NADPH, tetrahydrobiopterin and flavin adenine nucleotides. Studies reveal that in addition to endothelial NOS, there is a neutral NOS (**nNOS**; type I) that serves as a transmitter in the brain and in different nerves of the peripheral nervous system, such as non-adrenergic, non-cholinergic (NANC) autonomic nerves that innervate penile erectile tissues and other specialized tissues in the body to help in vasodilation.

Many naturally derived phytochemicals possess the potential ability of vasodilation. The extract of *Kaempferia parviflora* is found to promote the Nitric oxide levels of the human body. Endothelial NOS facilitate vascular function by generating NO production in the blood vessels, inhibit smooth muscle contraction and platelet aggregation. Black Ginger Extract enhances NO production, therefore, improve blood circulation and regulate blood pressure. The active components of the extract include the so called methoxy flavones. There are 10 methoxy flavones present in the compound.[2] 5, 7 dimethoxy flavones is the most promising active component. The structure of which is depicted below:



5, 7 dimethoxy flavone

The Moringa Oleifera leaf extract is also found to possess nitric oxide stimulatory effects. The elevation of cGMP induced by *M. Oleifera* extract and the suppression effect of the extract on PDE-5 may enhance the cGMP-dependent vasodilation effect of nitric oxide.[3] Saponins contribute to the health benefits of Moringa leaf.



Niazirin

The pomegranate polyphenols is found to possess the potential ability to protect the nitric oxide levels of the human body.[4] The active components of the pomegranate include hydrolysable tannins. Ellagi tannins and ellagic acid are the active components of the extract.

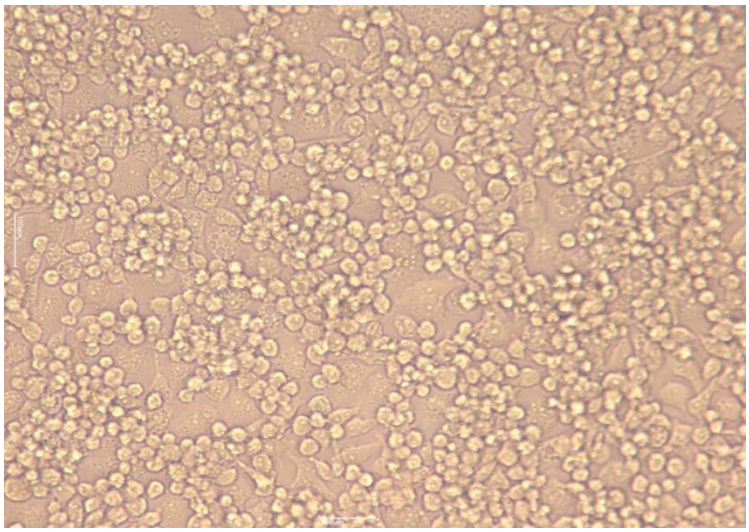
EXPERIMENTS

Cell lines and Culture medium:

Raw 264.7 (Mouse, Macrophages) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated by scrapping and the stock cultures were grown in 25 cm^2 culture flasks

and all experiments were carried out in 96 microtitre plates. Fig 1 represents the Mouse macrophages (RAW 264.7) in culture.

Figure 1: Mouse macrophages (RAW 264.7) in culture



Preparation of Test Doses:

For studies, each weighed test substances were separately dissolved in 1% tween 80 with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay:

The monolayer cell culture was distrusted and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm. The percentage growth inhibition was determined and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curve.

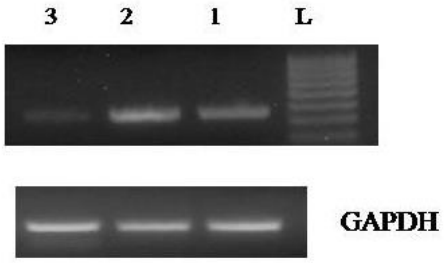
Nitric Oxide modulatory assay:

The assay was carried out in 24h RAW 264.7 cell suspension with cell number of 15 to 20 x10^5cells/ml. The cells were treated with non-toxic concentrations of test substance and incubated at 37^0C with 5% CO_2 for 24h. After incubation, the cell supernatants were collected and centrifuged. Supernatants were stored at -20^0 C till needed for further analysis. The nitrite levels in the samples were estimated by modified Griess method using a micro plate reader. The amounts of nitrite in samples were determined by interpolating from standard curve plotted with known concentrations of nitrite.

iNOS Gene expression study:

Twenty four hours after plating, RAW 264.7 cells were treated with non-toxic concentration of test substances of non toxic concentration test substance i.e. 200 and 100 µg/ml for 24 hr. Along with these positive and negative controls (untreated) were maintained in the experiment. After incubation, the supernatant solution from the cultures was discarded and cultures were subjected for total RNA extraction using Tri Reagent. cDNA was synthesized from total isolated RNA by reverse transcriptase kit. Then 20µl of the reaction mixture was subjected to PCR for amplification of cDNAs using specifically designed primers and as an internal control, the house keeping gene GAPDH was co-amplified with each reaction. PCR was carried out in MJ Mini Thermo cycler (Bio Rad, U.S.A) and PCR conditions for genes were initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 95 °C for 1 minute, annealing of primers (refer table for temperature) for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes. Fig. 2 depicts the iNOS gene expression in RAW cells treated by test substance

Fig. 2: iNOS gene expression in RAW cells treated by test substance



L – Ladder
1 – RR 2721 (Lower Conc.)
2 – RR 2721 (Higher Conc.)
3 – Control

Details of the primers

Primer type	Oligonucleotide bases	Annealing temperature	Product size (base pairs)
INOS	For 5' TGGGAATGGAGACTGTCCCAG 3' Rev 5' GGGATCTGAATGTGAGATGTTTG 3'	58	300
GAPDH	For 5'ACC ACA GTC CAT GCC ATC AC 3' Rev 5'CAC CAC CCT GTT GCT GTA GCC 3'	60	500

RESULTS & DISCUSSION:

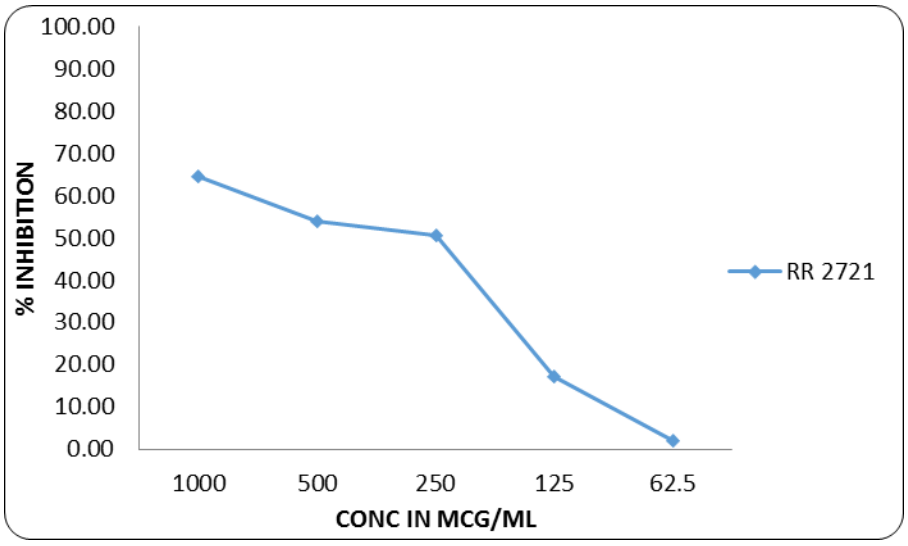
The test substance was evaluated for its modulatory effect in Nitric oxide generation and its role in iNOS gene expression in macrophages. In Nitric oxide assay, test substance exhibited dose dependent increase in NO levels. Table 1 represents the cytotoxic effect of test substance against RAW cell line.

Table 1: Cytotoxic effect of test substance against RAW cell line

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	**RR 2721**	1000 500 250 125 62.5	64.64±6.4 54.07±5.3 50.61±2.2 17.16±1.8 2.02±1.2	249.33±1.15

Figure 3 gives a clear graphical introduction on the cytotoxic effects of test substance against RAW cell line

Fig.3: Cytotoxic effect of the test substance on Raw Cell line



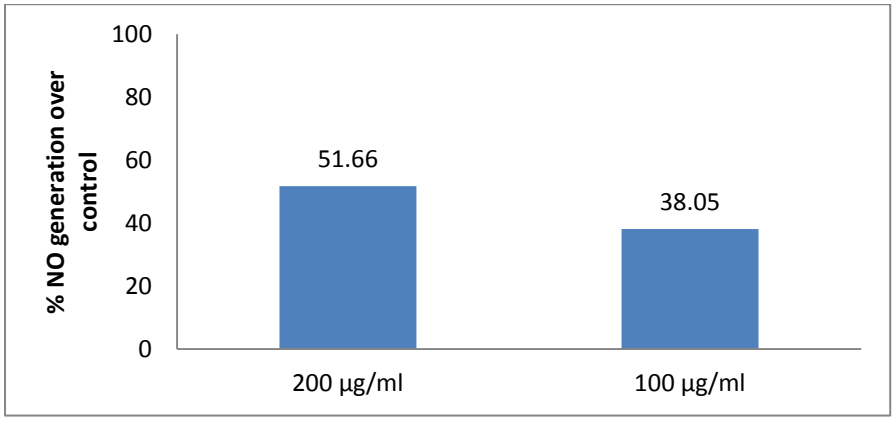
Treatment with test substance increased the NO levels by 51.66 ± 1.13 and 38.05 ± 0.81 % over control. Table 2 represents the Nitric oxide modulatory effect of test substance.

Table 2: Nitric oxide modulatory effect of test substance

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% NO generation over control
1	**RR 2721**	200 100	51.66 ± 1.13 38.05 ± 0.81

Figure 4 represents the modulatory effect of test substance on NO generation in macrophages

Fig 4: Modulatory effect of test substance on NO generation in macrophages



Further, gene expression studies confirmed the same. In gene expression study, results indicated that test substance up regulated iNOS gene expression by 0.27 and 0.23 fold at 200 and 100 µg/ml, respectively as compared to control. Table 3 represents the densitometric analysis of iNOS gene transcripts of sample at different concentrations.

Table 3: Densitometric analysis of iNOS gene transcripts of sample at different concentrations.

Test sample	Control	Higher Concn. (200 µg/ml)	Lower Concn. (100 µg/ml)
Regulation in terms of Folds	1.00	1.27	1.23

CONCLUSION:

Nitric oxide plays a vital role in maintaining the physical endurance of the human body. The use of naturally derived phytochemicals for stimulating the nitric oxide has gained much importance. The study focused on the use of a natural formulation for NO production gave a promising result. The study concluded that the unique formulation containing black ginger extract, Moringa Oleifera leaf extract and pomegranate peel extract gave 50% nitric oxide stimulating property and can be used successfully for maintaining the physical endurance.

REFERENCES:

1. L-Arginine Supplementation and Nitric Oxide Production: No Additional Effect When Associated to Exercise Diego dos Santos Baião1, Carlos Adam Conte Jr.2, Joab Trajano Silva1, Vânia Margaret Flosi Paschoalin1, Thiago Silveira Alvares1, 3.

2. S.K. Wattanapitayakul et al. Journal of Ethno pharmacology 110, 559–562 (2007).

3. Aversa A, Bruzziches R, Francomano D, et al. Testosterone and phosphodiesterase type-5 inhibitors: new strategy for preventing endothelial damage in internal and sexual medicine. Ther Adv Urol. 2009;1(4):179–197. doi: 10.1177/1756287209344992.

4. K. M. Azadzoi, R. N. Schulman, M. Aviram, and M. B. Siroky, "Oxidative stress in arteriogenic erectile dysfunction: prophylactic role of antioxidants.

5. Francis D and Rita L. Rapid "colorometric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability". Journal of Immunological Methods, 1986; 89: 271-277.

6.Sandeep Varma R, Ashok G, Vidyashankar S, Nandakumar KS, Patki PS. Bresol inhibits phosphodiesterase 4 gene expression and modulates the levels of select mediators of inflammation in human monocytic cells. J. Immunotoxicol, 8(4): 315-23, 2011.