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Research Article

ANTIOXIDANT POTENTIAL ASSESSMENT OF HYPOGLYCEMIC POLY HERBAL FORMULATION USING HUMAN PERIPHERAL BLOOD LYMPHOCYTE: AN *IN VITRO* STUDY

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ABSTRACT

Oxidative cell damage due to redox state imbalance is well known to play an important role from cellular aging processes to several pathological conditions including death. The objective of the present study was to evaluate the antioxidant potential of a patented Hypoglycemic Poly Herbal Formulation (HPHF). The formulation comprised of extracts from selected medicinal herbs such as *Azadirachta indica, Momordica charantia, Emblica officinalis, Gymnema sylvestris, Trigonella foenum-graecum, Curcuma longa, Garcinia camboga, Commiphor mukul* and *Ocimum sanctum* mixed with appropriate proportion of active ingredients. Peripheral blood samples collected from healthy female volunteers were used for the study. The lymphocytes were separated using histopaque soon after the collection. After confirming the cell viability of 95%, equal number of cells were divided and incubated with H_2O_2 with various test doses of HPHF (100 to 500µg) for a period of three hours. Cells without any treatment, cells with H_2O_2 alone and cells with H_2O_2 & Vitamin E served as negative, positive and standard controls, respectively. Post treated cells and their medium were subjected to genomic DNA damage and biochemical analysis respectively. HPHF treated cells showed reduction in lipid peroxidation and DNA damage. The activities of antioxidant enzyme and glutathione content were enhanced. The observed results may be due to its direct action in scavenging of free radicals and thereby modulating the antioxidant defense system. Further studies are required to confirm its antioxidant potential.

Keywords: Free radicals, Poly herbal formulation, Antioxidants, Lymphocytes, Hypoglycemic.

INTRODUCTION

In day today life, human cells are constantly exposed to potentially damaging free radical species, which may be intracellular arising from normal cellular metabolism or extracellular due to chemicals, environmental factors and exposures to ultraviolet and ionizing radiations. Reactive oxygen species (ROS) includes the highly reactive hydroxyl radical (OH), superoxide radical (O_2) and non-radical hydrogen peroxide (H_2O_2) which are highly genotoxic and damage cellular macromolecules such as DNA, proteins and lipids[1]. Under normal physiological conditions, there is a balance between endogenous oxidants and various antioxidant defenses[2]. Imbalance in redox state, due to excess generation of ROS compared to poor quenching/scavenging, secondary to biological system incapability, leads to damage of critical cellular components resulting from premature aging to severe pathological conditions such inflammation, metabolic disorders, atherosclerosis, heart disease, stroke, diabetes mellitus, cancer, malaria, rheumatoid arthritis and HIV/AIDS [3]. Hence, identification of natural antioxidants is essential for the prevention of many pathological diseases for the beneficial of society.

The uses of medicinal plants in traditional medicine are widespread and can still serve as lead for the development of novel pharmacological agents[4]. Currently available and practiced anti-diabetic chemical or recombinant drug components possess many adverse effects and hence, attention has been focused towards poly herbal formulations, which are naturally available and are traditionally used as potential therapeutic agents. The Hypoglycemic Poly Herbal Formulation (HPHF) used in the proposed study consists of *Azadirachta indica* (Neem), *Momordica charantia* (Bittergourd), *Emblica officinalis* (Amla), *Gymnema sylvestris* (Madhunashini), *Trigonella foenum-graecum* (Fenugreek), *Curcuma longa* (Turmeric), *Garcinia camboga* (Malabar tamarind), *Commiphor mukul* (Guggul) and *Ocimum sanctum* (Holy basil). Previous ayurvedic literatures have established the roles of HPHF composition in the treatments of diabetes, diabetic related complications, hypercholesterolemia and immunomodulatory activities[5]. Recent animal studies have also supported the medical treatment efficacy of the ingredients. Previous studies had reported that *E. officinalis* and an enriched fraction of Emblica tannoids were found to be effective in delaying the development of diabetic cataract in rats[6]. Exhaustive animal studies documented the beneficiary effects of *Curcuma longa* on hypercholesterolemia and hyperglycemia. Studies have shown that *C. longa* is capable of reversing diabetic nephropathy[7]. There is a direct reference which discusses about treatment of diabetes with neem (*Azadirachta indica*) in Ayurvedic literature. *A. indica* has been reported to possess hypocholesterolemic, hypoglycemic and immunomodulatory activities[8].

It has been reported that local traditional healers use *Gymnema sylvestre* to treat diabetes in south India[9]. Anti-obesity[10] and hypolipidemic properties[11] of *Garcinia cambogia* have been well documented. Studies in streptozotocin diabetic rats revealed the hypoglycemic effect *Ocimum sanctum*[12]. HPHF is primarily indicated for treating hyperglycemia while the other beneficiary effects of this synergistic compound have not been extensively explored. Hence, efforts had been made to evaluate the antioxidant potential of HPHF using human blood lymphocytes by *in vitro* assay.

MATERIALS AND METHODS

Composition of HPHF

HPHF consists of *Momordica charantia* L., *Emblica officinalis* Gaertn., *Gymnea sylvestris* Retz., *Trigonella foenum-graecum* L., *Curcuma longa* L., *Garcinia cambogia* Gaertn., *Commiphora mukul* and *Ocimum sanctum* L. The HPHF was formulated without using an organic solvent and the extraction procedure has been patented[13] by Lanson Biotech, Chennai.

Preparation of the aqueous extract

One gram of poly herbal formulation was macerated with 100 ml of sterile distilled water in a blender for 10 min. The macerate was initially filtered through double layered muslin cloth and then centrifuged at 4000 g for 30 min. The supernatant was filtered through Whatman No.1 filter paper and the extract was stored aseptically in a brown bottle at 4° C until further use.

Isolation and Treatment of Lymphocytes

Lymphocytes were separated from the venous blood obtained through venipuncture from a young female healthy donor using Histopaque-1077 according to manufacturer's guidelines.

Lymphocytes were isolated and resuspended in serum free RPMI-1640 media at a concentration of ~1 x 10^6 cells/ml.

The isolated lymphocytes were distributed to various tube (5 x 10^4 cells per tube) and were subjected to H_2O_2 (100µM) treatment to induce oxidative stress. Selected concentrations of HPHF such as 100, 200, 300, 400 and 500µg were added to the tubes to evaluate its antioxidative potential. The final volume of the treatment medium was adjusted to 1ml using serum free media. The medium was treated for 2 hr at 37°C[14]. Lymphocytes treated without 100 µM of H_2O_2, with 100 µM of H_2O_2 and with 100 µM of H_2O_2 + 100 µg Vitamin E served as negative, positive and standard controls respectively. The experiment was performed using triplicate tubes for each treatment and control. After the treatment period, cells were centrifuged at 500g for 3 min. The supernatant and cell pellets were utilized for biochemical analysis and genomic DNA isolation step, respectively. Details of various treatment concentrations and controls employed are given below.

A. Group 1: Negative Control - Untreated lymphocytes
B. Group 2: Positive Control - H_2O_2 (100µM) treated lymphocytes:
1. Group 3: Treatment I - H_2O_2 (100µM) and 100µg HPHF treated lymphocytes
2. Group 4: Treatment II- H_2O_2 (100µM) and 200µg HPHF treated lymphocytes
3. Group 5: Treatment III- H_2O_2 (100µM) and 300µg HPHF treated lymphocytes
4. Group 6: Treatment IV- H_2O_2 (100µM) and 400µg HPHF treated lymphocytes
5. Group 7: Treatment V- H_2O_2 (100µM) and 500µg HPHF treated lymphocytes

C. Group 8: Standard Control - H_2O_2 (100µM) and Vitamin E (400µg) treated lymphocytes

Cell Viability

The cells were analyzed for viability using trypan blue dye exclusion[15] before the start and after the completion of the treatment.

Genomic DNA isolation and Biochemical assay

The cell pellets were subjected to white blood cell lysis for genomic DNA isolation using Lahiri *et al* method[16]. DNA damage was assessed by using 3% agarose gel electrophoresis.

Total flavonoid content present in the extract was determined by aluminium chloride method using catechins as a standard[17]. The total phenolics content in the extract was determined with the Folin-Ciocalteu's reagent[18]. Lipid peroxidation in terms of malondialdehyde (MDA) was determined by Thiobarbituric Acid Reaction (TBARS)[19], the reduced glutathione (GSH),[20] superoxide dismutase (SOD)[21] and catalase (CAT)[22] were estimated by standard methods. Total protein content was determined spectrophotometrically at 595 nm and concentrations were calculated compared to a calibration curve of bovine serum albumin[23].

Data are presented as means ± SD. Initially, the data were analyzed for normality and homogeneity. The analysis indicated a normal distribution and homogeneity for all parameters that were evaluated. The data were then analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. $P < 0.05$ was considered significant.

RESULTS

The 3% agarose gel showed maximal DNA damage in H_2O_2 alone treated cells with minimal damage, noted at 100 and 200µg concentrations of HPHF treated cells. However, subminimal DNA damage was noted at 300 and 400µg concentrations of HPHF treated cells. Cells treated at the highest concentration of 500µg HPHF showed normal DNA bands (Fig 1).

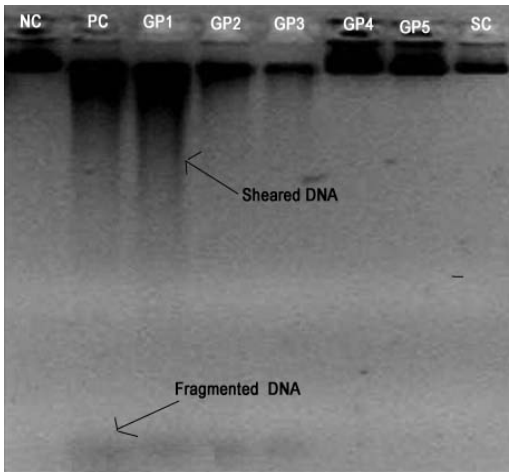

Fig. 1: Effect of HPHF against H_2O_2 induced fragmentation of DNA in human Lymphocytes. Reduction in fragmentation and shearing were observed in GP1, GP2 and GP3 (HPHF treated cells) compared against PC (H_2O_2 treated cells). No shearing and fragmentation were observed NC, GP4, GP5 and SC. (NC: Negative Control, PC: Positive control: GP: Group and SC: Standard Control).

Table 1: *In vitro* effect of HPHF on the levels of lipid peroxides and reduced glutathione in human lymphocytes untreated and treated cells.

Groups	Treatment	Lipid peroxides (n moles MDA/mg protein)	Reduced Glutathione (µ moles/mg protein)
Group 1	Negative Control	0.87±0.02	5.58±0.13
Group 2	Positive Control	4.2±0.16	2.35±0.19
Group 3	H_2O_2 +100µg	3.73±0.09	2.98±0.20
Group 4	H_2O_2 + 200µg	3.13±0.09	3.28±0.14
Group 5	H_2O_2 + 300µg	2.73±0.09	3.7±0.08
Group 6	H_2O_2 + 400µg	2.18±0.17	4.37±021
Group 7	H_2O_2 + 500µg	1.88±0.25	4.80±0.08
Group 8	H_2O_2 + 400µg vitamin E	1.2±0.16	5.11±0.08

The data were then analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. $P < 0.05$ was considered significant.

Total polyphenols and flavonoids were expressed as gallic acid (103 mg) and catechins (41.6 mg) equivalents per gram of formulation, respectively. TBARS was significantly higher ($P < 0.05$) in H_2O_2 treated lymphocytes relative to controls. However, its level recovered significantly ($P < 0.05$) with an increase in concentrations of HPHF (100 to 500µg) in lymphocytes (Table 1).

The concentration of GSH decreased significantly ($P<0.05$) in H_2O_2 treated cells as compared to controls. But, the HPHF + H_2O_2 treated cells showed progressive increment in GSH levels ($P < 0.05$) with an increase in concentrations of HPHF relative to H_2O_2 treated cells (Table 1).

The SOD and CAT activities were decreased significantly ($P< 0.05$) in H_2O_2 treated cells relative to controls. These enzyme activities were restored ($P< 0.05$) in HPHF treated lymphocytes in a concentration-dependent manner (Table 2).

Table 2: *In vitro* effect of HPHF on the activities of superoxide dismutase (SOD) and catalase (CAT) in treated and untreated human lymphocytes

Groups	Parameters	Superoxide dismutase (units/mg protein)	Catalase (µmoles of H_2O_2 consumed / min / mg protein)
Group 1	Negative Control	3.64±0.03	5.2±0.16
Group 2	Positive Control	1.84±0.01	3.16±0.19
Group 3	H_2O_2 +100µg	2.23±0.03	3.38±0.20
Group 4	H_2O_2 + 200µg	2.55±0.09	3.52±0.22
Group 5	H_2O_2 + 300µg	2.86±0.05	4.11±0.09
Group 6	H_2O_2 + 400µg	2.95±0.11	4.63±0.16
Group 7	H_2O_2 + 500µg	2.98±0.11	4.72±0.14
Group 8	H_2O_2 + 400µg Standard Control	3.23±0.13	5.14±0.16

The data were then analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test. $P < 0.05$ was considered significant.

DISCUSSION

Lymphocytes contain a diversified redox and free radical scavenging system[24], which are well known and extensively used for screening of variety of toxicants in human population. In the present study, H_2O_2 was used to induce oxidative stress in lymphocyte as it can freely cross the cell membrane and stimulate hydroxyl radical formation[25]. Any natural or synthetic compound with antioxidant properties is purported to alleviate cell damage either partial or total. In this study, the ameliorating antioxidative effect of HPHF in stressed human lymphocytes was monitored *in vitro.*

The mechanism for antioxidants to scavenge free radicals involves donating hydrogen to a free radical and thereby reducing it to an unreactive species through removing the odd electron feature, which is responsible for radical reactivity[26]. Plants are the sources of wide variety of phytochemicals such as flavonoids and polyphenols. These compounds are responsible for the antioxidant nature of HPHF. Presence of polyphenolic compounds in the plants has been shown to ameliorate cell injury and protect DNA from lesions induced by various toxic chemicals. This is attributed to their ability to scavenge free radicals and thereby reducing the oxidative damage[27].

In the present study, we observed increased levels of TBARS in H_2O_2 treated lymphocytes due to activation of lipid peroxidation. This process leads to excessive free radical formation that attacks the fatty acid components of membrane lipids resulting in membrane rigidity and receptor realignment[28]. Our study also confirms the finding that increased free radical formation causes a decrease in GSH observed in H_2O_2 treated lymphocyte as reported by Demir *et al*[29], which has the potential to impair the cellular defenses and render the cells more vulnerable to oxidative stress. The intracellular glutathione status appears to be a sensitive indicator of the cell's overall condition and its ability to counteract a toxic challenge[30].

The antioxidant enzyme system plays an important role in the defense of cells against oxidative insults. ROS affects the antioxidant defense mechanisms, reduces the intracellular concentration of GSH and other marker enzymes like SOD, and CAT[31]. Decreased activities of SOD and CAT in H_2O_2 treated lymphocytes may be due to the enhanced lipid peroxidation or inactivation of the anti-oxidative enzymes. This would cause an increased accumulation of superoxide radicals, which could further stimulate lipid peroxidation. Lymphocytes treated with different concentrations of HPHF (100-500 µg) following H_2O_2 exposure showed ameliorative effect. The effects were more evident at higher doses of HPHF.

HPHF ingredients such as *Momordica charantia* L.[32], *Emblica officinalis*[33, 34], Gaertn, *Trigonella foenum-graecum* L.[35], *Curcuma longa* L[36, 37] have been reported to exhibit antioxidant properties. *Emblica officinalis* has been established as a rich source of vitamin C, which plays an important role in scavenging free radicals. The individual constituents present in the formulation owing to their phytochemical constituents may either induce antioxidant enzymes or directly contribute to free radical scavenging activity.

The phytochemicals present in the HPHF formulation should possess the ability to cross the membrane *in vitro,* and neutralize the free radicals produced, and check the lipid peroxidation leading to a decrease in TBARS of H_2O_2 stressed cells. Phytochemical constituents present in the herbal formulation may be responsible for scavenging ROS and protecting the biomolecules from ROS induced damage. The concept of synergy is central to the holistic approach. The popular modern concept of isolating pure active compounds may not achieve the desired result as observed in natural version. When the active principles are isolated from the formulation it might lose its activity without its synergistic colleagues.

CONCLUSION

Our present study indicated that HPHF is devoid of pro-oxidant property, which is observed by its protective role against H_2O_2 induced oxidative stress. The antioxidant activity could be correlated to the presence of polyphenolic components in the extract. This protective effect might be due to presence of various phytochemicals present in the formulation. However, further studies are required to be performed on this promising formulation to understand its role in the prevention of oxidative damage in the living system.

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