INVITRO STUDIES ON ANTI ATHROSCLEROTIC EFFECT OF ‘CY-HEART PREMIUM HEALTH TONIC’ USING ANTIATHROSCLEROTIC MARKERS APoE MMP2 & IL6 GENE EXPRESSION ANALYSIS AND GRIESS (NITRIC OXIDE RELEASE) ASSAY

ARVIND PRABHU.T*
PRABHURAMAN.T. V, BABURAMAN.T.V, GANESAN.G, REVETHI.K.S
Department of Bio-Engineering, Vels University, Pallavaram, Chennai 600117

Abstract:
Atherosclerosis remains a major problem in the modern society being a cause of life-threatening cardiovascular diseases. The development of atherosclerosis is characterized by lipid accumulation in the aortic wall and formation of foam cells overloaded with large amounts of lipid inclusions in the cytoplasm. This is one of the main causes of cardiac ischemia, peripheral vascular disease and heart failure is the result of endothelial cell injury and damage. The endothelial damage results in the formation of plaque, narrowing of the arteries and thickening of the arterial wall by cholesterol accumulation. Hence the present study was made to investigate the Cyheart against atherosclerosis. The present study was made with the following objectives: MTT viability assay, Atherosclerotic markers Metalloproteinase 2 (MMP2), ApoE (Apolipoproteins) and Interleukin 6 (IL6) were used for gene expression study of Anti atherosclerotic effect of CYHEART and Quantitative analysis of endogenous nitric oxide (NO) in Human endothelial cells with CYHEART using Griess assay. We observed dose dependent viability of Endothelial cells, implying non-toxic nature of ‘CYHEART’ even at higher doses (100µg/ml) by retaining cellular morphology (disrupted in Standard drug Ecosprin Gold). Further the dose dependent NO (Endogenous nitric oxide) release was observed. It is higher at 100µg/ml concentration demonstrate the anti-atherosclerotic effect of ‘CYHEART ’ compared to control and standard. Significant increase in relative gene expression of specific anti-atherosclerotic marker ApoE exemplify the effective anti-atherosclerotic effect of ‘CYHEART’. The studies Conclude CY HEART PREMIUM HEALTH TONIC has anti-atherosclerotic and Cardioprotective effects which is equal or more effective than standard Ecosprin Gold 10

Keywords: Atherosclerosis, Cardiac ischemia, Cholesterol accumulation, CYHEART, Cardioprotective effects

Introduction:
Atherosclerosis remains a major problem in the modern society being a cause of life-threatening cardiovascular diseases. Subclinical atherosclerosis can be present for years before the symptoms become obvious, and first manifestations of the disease in a form of acute ischemia of organs are often fatal. The development of atherosclerosis is characterized by lipid accumulation in the aortic wall and formation of foam cells overloaded with large amounts of lipid inclusions in the cytoplasm. This is one of the main causes of cardiac ischemia, peripheral vascular disease and heart failure is the result of endothelial cell injury and damage. The endothelial damage results in the formation of plaque, narrowing of the arteries and thickening of the arterial wall by cholesterol accumulation. Effective anti-atherosclerotic drugs based on natural products would be a preferred alternative compared to synthetic drugs because of the limited indications, severe side effects and higher cost of treatment. In this study, we used human endothelial cells to test a natural product-based drug on atherosclerotic parameters and compared it with a synthetic commercial anti-atherosclerotic standard drug Ecosprin. Current therapy of atherosclerosis is aimed mostly at the normalization of the blood lipid profile, and has no direct activity on the atherosclerotic plaque development. It is therefore necessary to continue the search for substances that possess a direct anti-atherosclerotic effect, preventing the cholesterol deposition in the arterial wall cells and reducing the existing plaques. At present no specific therapy is developed so far for direct prevention and treatment of subclinical atherosclerosis, partly because current understanding of the exact mechanisms
and hence relevant therapeutic targets is not sufficient. Statins are currently regarded as most promising therapeutic agents for prevention and treatment of atherosclerosis (Stein and Raal 2014). It is known that regular long-term statin therapy prevents the development of novel and promotes regression of existing atherosclerotic lesions. However, recent opinion is that although statins are effective at reducing cholesterol levels, they fail to substantially improve cardiovascular outcomes. The considerable progress was made during the recent years, our understanding of possible cardio-protective effects of nutraceuticals, such as lowering of blood cholesterol and blood pressure regulation, and their impact on cardiovascular disease risk factors. Hence the present study was made to investigate the Cyheart against atherosclerosis. The present study was made with the following objectives 1.MTT viability assay to determine the cytotoxicity toxicity and assess the optimal concentrations for further investigations.

2. Atherosclerotic markers Metalloproteinase 2 (MMP2), ApoE (Apolipoproteins) and Interleukin 6 (IL6) were used for gene expression study of Anti atherosclerotic effect of CYHEART Premium Health Tonic and Ecosprin Gold 10 was used as standard drug.

3. Quantitative analysis of endogenous nitric oxide (NO) in Human endothelial cells with CYHEART using Griess assay. The pathophysiology and markers of atherosclerosis were given below for the understanding of present experiment. Macrophage APoE secretion and antiatherogenic effects. ApoE secreted after differentiation of monocytes into macrophages is associated with scavenger receptor type A and modulated by positive (sterols) and Negative factors (Cytokines). Secreted APoE forms lipoprotein particles that promote reverse cholesterol transport. Cholesterol thus mobilized from peripheral tissues towards the liver for excretion into the bile. APoE has putative anti-atherosclerotic effect by its antioxidant, antiproliferative, anti-inflammatory antiplatelet and nitric oxide (NO) generating properties. Macrophages promote formation of oxidized LDL favors formation of foam cells whereas apoE inhibits LDL oxidation. (Davignon J.Sang Thrombose et al., Vaisseaux 2002; 14:107-120.)

**APOLIPOPROTEIN E (APoE) AND ATHEROSCLEROSIS:**

In humans, apolipoproteinE (apoE) is a polymorphic multifunctional protein. It is coded by three alleles of a modulator gene (level, variability, and susceptibility gene) at the apoE locus on chromosome 19, determining six apoE genotypes and plasma phenotypes. Its pleiotropic effects are exerted on plasma lipoprotein metabolism, coagulation, oxidative processes, macrophage, glial cell and neuronal cell homeostasis, adrenal function, central nervous system physiology, inflammation, and cell proliferation. ApoE polymorphism modulates susceptibility to many diseases. It is, however, particularly notorious for its role in neurodegenerative disorders and atherosclerotic arterial disease. ApoE can promote atherosclerosis regression independently of lowering plasma cholesterol levels. As a ligand for cell-surface lipoprotein receptors, apolipoprotein E (apoE) prevents atherosclerosis by clearing cholesterol-rich lipoproteins from plasma. However, the mechanisms by which apoE can promote the regression of atherosclerotic lesions, including cholesterol lowering—independent roles.

**Materials & Methods:**


**Table NO: 1 : Ingredients/Composition: CYHEART PREMIUM HEALTH DRINK**

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Quantity % v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garlic (Juice)</td>
<td>Allium sativum</td>
<td>12.5</td>
</tr>
<tr>
<td>Ginger Extract</td>
<td>Zingiber officinale</td>
<td>12.5</td>
</tr>
<tr>
<td>Lemon (Juice)</td>
<td>Citrus limon</td>
<td>12.5</td>
</tr>
<tr>
<td>Apple cedar vinegar</td>
<td>-</td>
<td>12.5</td>
</tr>
<tr>
<td>Honey</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

**Methods:**

**Drug preparation:**

Stock solution of the standard drug (ECOSPRIN Gold 10) was prepared by dissolving 1 mg of the drug in 1 mL of DMSO. For the assays, various concentrations (5, 10, 20, 40, 60, 80, 100 μg/mL) of drug were prepared in DMEM and added directly to the cells.

**CYHEART** was directly used for experiment and was taken as v/v. For MTT and Griess assay various concentrations (5, 10, 20, 40, 60, 80, and 100 μL/mL) of the drug were prepared by diluting in DMEM.
MTT assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) \textit{in vitro} basal cytotoxicity assay is based on the ability of viable cells to incorporate and bind MTT, a supra vital dye (Borenfreund and Puerner, 1985). MTT is a weak cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Toxicants can alter the cell surface or the lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become irreversible. Such adverse changes cause cell death and/or inhibition of cell growth, which then decrease the amount of MTT retained by the culture. Since the concentration of MTT dye desorbed from the cultured cells is directly proportional to the number of living cells, cytotoxicity is expressed as a concentration dependent reduction of the uptake of MTT after chemical exposure. The MTT assay uses a 96-well plate format for the production of replicate measurements. In this present study MTT cell viability assay, 10,000 cells/well were seeded in a 96 well plate in triplicates for various concentrations of test and standard drug along with control. Cells were incubated for 24 hours at 37°C and 5% CO2. Once the cells were attached CYHEART (Test Drug) (5, 10, 20, 40, 60, 80, and 100 μL/mL) and standard drug (5, 10, 20, 40, 60, 80, and 100 μg/mL) were added and further incubated for 24 hours. The cell viability at different concentrations of drugs were quantified by the reduction in MTT to produce formazan crystals. MTT was added to each well 24 hours after treatment with drugs at a final concentration of 5mg/mL. After 30 minutes incubation at room temperature in dark, formazan crystals were dissolved by addition of DMSO. The absorbance was measured using microplate reader (PerkinElmer) at 590nm.

Immunocytochemistry of anti-atherosclerotic markers:

For immunofluorescence staining, 30,000 cells/well were seeded in 24 well plate for the effective concentrations of test and standard drug along with control. Cells were incubated for 24 hours at 37°C and 5% CO2. Once the cells were attached test (80 and 100μL/mL) and standard drugs (80μg/mL) were added and further incubated for 24 hours. Cells were fixed using 4% paraformaldehyde (PFA) for 15 minutes at room temperature. PFA was removed and the cells were washed with PBS thoroughly and permeabilized using Triton-X for 15 min and washed with PBS. Blocking was done using 5% BSA in PBS for 30 minutes. Blocking agent was removed and 200-250 µL of primary antibody (cardiac troponin or matrix metalloproteinase 2) prepared in antibody diluent (0.1% BSA in TBST; pH 7.5) was added and incubated overnight at 4°C on a rocker. The primary antibody was removed and was washed two times with PBST. Fluorophore labelled secondary antibody (Alexa 488 anti-rabbit, Invitrogen) was added and incubated for one hour at room temperature. Secondary antibody was removed and cells were washed with PBST twice. The cells were counterstained with DAPI (1:1000 dilution) and washed twice with PBST. 10 μL of DABCO antifade mount was added and coverslip was placed carefully without air bubbles. Cells were observed under 20X in inverted fluorescent microscope (Olympus IX73).

Gene expression analysis of anti-atherosclerotic markers:

For gene expression studies, 1 million cells were plated in a 35mm culture plate for the effective concentrations of test and standard along with control. Cells were incubated for 24 hours at 37°C and 5% CO2. Once the cells were attached the test (80 and 100μL/mL) and standard drugs (80μg/mL) were added and further incubated for 24 hours. Cells were later harvested for gene expression studies. Total RNA was extracted using Trizol method and 500ng of cDNA conversion was made by Prime script 1st strand c-DNA synthesis kit, qPCR was performed using SYBR \textit{Premix Ex-Taq} II mix in QuantStudio 5 for GAPDH, ApoE, IL6, MMP2 primers.
RESULTS & DISCUSSION

The results were shown in Fig. 1a & 1b. The fluorescence micrographs of matrix metallocoproteinase 2 (MMP2) comparing the effect of test drug and standard drug against untreated endothelial cells were shown in Plate 1a & 1b. The results of the present study showed that the dose dependent viability of human endothelial cells especially at 80µg/ml & 100µg/ml higher concentration of CY HEART (SH). At these concentrations, the higher amount of endogenous NO release was observed. The NO release was increasing with increase of SH concentration. Since these concentrations were prominent effect the immuno cytological analysis was made using fluorescence microscope and pictures were observed. The immunofluorescent staining of Matrix metalloproteins (MMP2) were shown the retained cell morphology (at 80 l & 100µg/ml) of SH treated endothelial cells with Cardiac troponins and MMP2 (even at 80 l & 100µg/ml). Even though the protective effects were observed in Ecosprin (std drug) treated cells, cell morphology was disrupted. This exemplified that the Non cytotoxic nature of test drug SH. Furthermore, immunolocalization showed a clear increase of anti-atherosclerotic marker MMP2 in test and standard drug treated samples. Additionally, it was clearly seen that the prominent anti-atherosclerotic marker, ApoE, was significantly increased in test drug, more than even standard drug. The present study investigated the anti-atherosclerotic activity of CYHEART on endothelial cells and compared with Ecosprin Gold 10 based on nitric oxide release, gene expression analysis and immuno localization of different anti-atherosclerotic markers such as cardiac troponin and MMP-2. Standard drug contains clopidogrel, atorvastatin and aspirin as its major ingredients and used being in lipid lowering therapy. The endogenous anti-oxidants balance and inhibit the oxidative stress in living cells. Reactive oxygen species (ROS) induced oxidative stress is a critical and common mechanism in atherosclerosis formation. Endothelium maintains the vascular homeostasis by regulating the vasodilation, vasoconstriction, migration and proliferation of smooth muscle cell, thrombogenesis and fibrinolysis. The disturbance in endothelial cells results in their dysfunction and cause the arterial wall damage then eventually raises the chances for atherosclerosis. The drugs that maintain the integral role of endothelial cells attract potential interest against endothelial dysfunction and lipid lowering therapy in recent years. Nitro vasodilators are of pharmacological importance since nitric oxide (NO) plays a vital role in vasodilation during the heart failure by maintaining the tissue and endothelial homeostasis. Hence, the current study demonstrated the anti-inflammatory potential of SH and also the role of lipolytic activity and also take part in controlling type 2 diabetes.
**Fig. 1b.** Effect of SAVE HEART on viability of endothelial cells

Fig 1a & b: Cytotoxicity study by MTT assay in endothelial cells after exposing to different concentrations of test and standard drug for 24 hours (n=3, and *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA followed by Dunnett’s post test).

**Fig. 2a.** Effect of ECOSPRIN Gold 10 (STD. Drug) on Nitric Oxide release in Endothelial cells
Fig. 3a Effect of CYHEART on VIABILITY OF ENDOTHELIAL CELLS

Fig. 2b. Effect of SAVE HEART on Nitric Oxide release in Endothelial cells

Fig 2a&b: Effect of test and standard drug on nitric oxide release in endothelial cells (n=3, and *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA followed by Dunnett’s post test).
Fig. 3a Effect of CYHEART on VIABILITY OF ENDOTHELIAL CELLS

Fig. 3b. Effect of CYHEART on Nitric Oxide (NO – release)
Fig. 4a. Gene expression analysis for Cardiac troponin I (cTnI), matrix metalloproteinase 2 (MMP2), Apolipoprotein E (ApoE) and Interleukin 6 (IL6) by qRT-PCR in untreated control and in test and standard drug treated cells (n=3, and p<0.05=*, p<0.01=**, p<0.001=*** by one way ANOVA and Tukey's multiple comparison test).

Fig. 4b Analysis of Gene expression for CYHEART on atherosclerosis markers

Plate 1. a. STUDY ON IMMUNOCYTOCHEMISTRY OF CYHEART SYRUP ON MATRIX METALLOPROTEINASE 2
Result and Discussion:

- Dose dependent viability of Endothelial cells was observed, implies non-toxic nature of ‘CYHEART’ even at higher doses (100µg/ml) by retaining cellular morphology (disrupted in Standard drug Ecosprin Gold)

- Dose dependent NO (Endogenous nitric oxide) release was observed. It is higher at 100µg/ml concentration demonstrate the anti-atherosclerotic effect of ‘CYHEART’ compared to control and standard.

- Significant increase in relative gene expression of specific anti-atherosclerotic marker ApoE exemplify the effective anti-atherosclerotic effect of ‘CYHEART’.

- MMP2 and IL6 markers were also significantly enhancing the angiogenesis and anti-inflammatory effect respectively by increased gene expression which exhibit Cardioprotective effect.
Conclusion:

CY HEART PREMIUM HEALTH TONIC has anti-atherosclerotic and Cardioprotective effects which is equal or more effective than standard Ecosprin Gold 10.

Conflict of Interest:
Conflict of interest declared none.

References: