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Research Article

Evaluation of Anti-Atherosclerotic Potential of Herbal Formulation iPulse+ through modulation of PCSK9 Gene Expression in the HepG2 Cell Line: An *In vitro* Study

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Abstract

The main cause of cardiovascular disorders is atherosclerosis, an inflammatory pathology of the vasculature. To reduce the morbidity and mortality of this illness, a wide range of therapeutic and preventative measures are being investigated. However, a few of the treatments were quite expensive and caused a number of negative side effects. The aim of the present study was to assess the anti-atherosclerotic properties of the herbal formulation I-PULSE+ in order to ascertain the modulatory potency of the anti-atherosclerotic effects on the PCSK9 gene expression levels in the HepG2 cell line. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to evaluate the cytotoxicity of I-PULSE+ in the Human Hepatocellular Carcinoma Cell Line (HepG2) at different concentrations (1000 to 62.5µg/ml). The impact of the I-PULSE+ anti-atherosclerotic effect on the PCSK9 gene was determined using a semi-quantitative gene expression method. This method indicated the transcript level of PCSK9 in the human hepatocellular carcinoma cell line (HepG2) in comparison to untreated HepG2 cells. I-PULSE+ exhibits a CTC50 value of >1000 µg/mL on the HepG2 cell line. The results of the gene expression analysis indicate that PCSK9 gene expression was significantly reduced by I-PULSE+ at lower concentrations (62.5 and 125 µg/mL) as compared to the control. The results of this study indicate that I-PULSE+, a herbal formulation, may function as a potential nutraceutical agent to manage atherosclerosis.

Keywords: I-PULSE+; Herbal Formulation; PCSK9 Gene Expression; HepG2 Cell Line; Anti-Atherosclerosis Effect

Introduction

In developed countries such as the USA, Europe, and Asia, atherosclerosis is one of the cardiovascular diseases that has been the leading cause of death [1]. The characteristic of atherosclerosis is a progressive hardening and thickening of the arteries, which leads to plaque accumulation in the end [2]. Calcium, fat, cholesterol, and other chemicals found in the blood make up plaque. Dyslipidemia is a major contributing factor to the advancement of atherosclerosis, among other well-known risk factors. Hyperlipidemia, or hypercholesterolemia, is a kind of dyslipidemia that was thought to be the cause of atherosclerosis. One of the major risk factors for coronary artery disease is atherosclerosis. The development of lesions brought on by fat buildup in the artery walls characterizes this complex, multifactorial inflammatory disease. The development and progression of the atherosclerotic plaque are influenced by several genetic, metabolic, and environmental factors. Hypercholesterolemia, or high total cholesterol (TC) and low-density lipoprotein cholesterol (LDLc), is a well-known risk factor in humans [3]. Inflammation, oxidative stress, and insulin resistance are other significant factors that contribute to this condition [4,5].

The most important pathophysiological mechanism behind cardiovascular diseases (CVD), gangrene, and stroke is atherosclerosis. A complicated illness involving multiple metabolic, immunological, and circulatory system components, atherosclerosis progresses through different stages [6]. Atherosclerosis develops in

areas where blood flow is disrupted in the subendothelial space of the medium and large arteries. The development of lipid-enriched plaque and thickening of the artery wall are two features of atherosclerosis, a chronic inflammatory disease [7,8]. Diabetes affects clinical results and speeds up the course of atherosclerosis. Atherosclerosis is independently associated with a higher incidence of diabetes mellitus [9], and patients with diabetes have a higher risk of atherothrombotic coronary artery disease [10]. Diabetes causes a substantial acceleration of the evolution of atherosclerosis, infiltration of inflammatory cells into the arterial wall, and plaque necrosis [11].

Many ailments, including dermatitis, asthma, menstrual disorders, rheumatoid arthritis pain, headaches, menopausal symptoms, chronic fatigue, contentious internal disorders, and growth, are treated with herbal medication [12]. Many times, scientists are unsure of the exact component in a given herb that is meant to treat a disease or condition. Whole herbs are made up of many different components that work together to produce a beneficial outcome. Herbs are frequently used in combination because the combination is thought to be more potent and to have fewer side effects [13]. The current study aims to determine the thrombolytic action of the herbal anti-atherosclerosis formulation. Consequently, the formulation could be an effective therapeutic source.

The people of India have greatly benefited from alternative medical systems, including Ayurveda, Siddha, Unani, and various tribal folklore medicines based on herbal remedies. These days, these systems compete with one another in addition to being complementary in the treatment of different diseases. The wide range of pharmacological activities of herbs and herb-based compounds have drawn a lot of attention in recent years, and they have long been a valuable source of medications for a variety of ailments.

There are currently no medications that can be used to treat or prevent atherosclerosis over the long term without running the risk of experiencing severe adverse effects. Consequently, research on and application of medicinal plants are highly relevant for long-term anti-atherosclerotic therapy. Actually, people have known about the therapeutic benefits of a variety of herbs since antiquity. Certain herbal medicines have the ability to prevent harmful immunological reactions, and some of these have long been used to control the progression of atherosclerosis. Their comparative safety, lack of adverse effects, and apparent increased efficacy provide a justification.

To prevent atherosclerotic diseases, it is crucial to find new targets for nutraceutical drugs. Thus, the objective of this study was to examine the effect of the herbal formulation I-PULSE$^+$ on PCSK9 gene expression levels in the HepG2 cell line, as well as its potential to promote atherosclerosis. Consequently, I-PULSE$^+$ would be a more effective formulation for treating atherosclerosis. These results highlight the significance of medication selection in the management of atherosclerosis. For managing atherosclerosis, I-PULSE$^+$ can acts as the best nutraceutical formulation.

Materials and Methods

Preparation of I-PULSE$^+$

I-PULSE$^+$ is manufactured and registered by Indusviva Health Sciences Pvt. Ltd., Jayamahal Extension, Bangalore, Karnataka, India.

Preparation of the test solution

To prepare a stock solution with a concentration of 10 mg/mL for cytotoxicity investigations, 10 mg (w/v) of the I-PULSE$^+$ was individually dissolved, and the volume was made up with DMEM supplemented with 2% inactivated FBS. The stock solution was then sterilized by 0.22µ syringe filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Cell line and culture medium

The National Center for Cell Sciences, Pune (NCCS) provided the human hepatocellular carcinoma cell line HepG2. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), 100 IU/ml penicillin, 100 µg streptomycin, and 5 µg/ml amphotericin B until confluent. The culture was conducted at 37oC in a humidified atmosphere with 5% CO2. TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) was used to separate the cells. Every experiment was conducted in 96 microtitre plates, and the stock cultures were grown in 25 cm2 culture flasks (Tarsons India Pvt. Ltd., Kolkata, India).

Cytotoxicity studies

A medium containing 10% FBS was used to trypsinized the monolayer cell culture and adjust the cell count to 100,000 cells/mL. 0.1 mL of the diluted cell suspension was added to each well of

the 96-well microtitre plate. Following the formation of a partial monolayer in microtitre plates, a partial monolayer was left for 24 hours. The supernatant was then discarded, the monolayer washed once with medium, and 100 µl of various test concentrations of the I-PULSE⁺ were added. After that, the plates were incubated for 3 days at 37°C in a 5% CO2 atmosphere. During this time, microscopic analysis was done, and observations were recorded every 24 hours. Each well received 50 µl of MTT in PBS after the drug solutions in the wells were discarded after 72 hours. The plates were incubated for three hours at 37°C in a 5% CO2 environment after being gently shaken. After removing the supernatant and adding 100 µl of DMSO, the plates were gently shaken to dissolve the formazan that had formed. A microplate reader operating at 570 nm was used to measure the absorbance. Based on the dose-response curves for each cell line, the percentage growth inhibition was determined and the concentration of I-PULSE⁺ required to inhibit cell growth by 50% (CTC50) values were calculated.

RNA isolation and cDNA synthesis

A Trizol-extract reagent was used to lyse the HepG2 (Human Liver Carcinoma Cell Line) cells that had been treated with I-PULSE⁺. To extract the whole RNA from the sample, chloroform was added, and centrifugation was then performed. The top layer, out of the three separate layers, was removed and placed in a new tube. An equal volume of isopropanol was then added, and the mixture was incubated for 10 minutes at a temperature of -20°C. An appropriate volume of ethanol was used to resuspend the pellet, followed by centrifugation and incubation. The pellet was air-dried, and the proper volume of TAE buffer was added, followed by incubation and centrifugation. Further cDNA synthesis was done using the extracted total RNA. Reverse transcriptase enzyme treatment was performed in accordance with the manufacturer's protocol (Biorad) after oligo dT primer amplification was completed to create cDNA. The resulting cDNA was used for PCR to amplify the PCSK9 gene and GAPDH (internal control).

RT-PCR procedure

A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to measure the levels of PCSK9 mRNA expression. A PCR was performed on 50 µl of the reaction mixture to amplify the PCSK9 genes. GAPDH, called the housekeeping gene, was co-amplified with each reaction as an internal control when cDNAs were amplified using specially created primers that were purchased from Eurofins, India.

Amplification conditions for the PCSK9 gene

The 35 cycles of denaturation (at 95°C for 30 seconds), annealing Tm (30 seconds), and extension (at 72°C for 45 seconds) were performed after 5 minutes at 95°C. The last extension was performed for 10 minutes at 72°C.

Primers used:

For Ist strand synthesis:

Oligo dT primer.

For IInd strand synthesis

- Forward: 5'- GGGTGAGTGTGAAAGGTGCT -3'
- Reverse: 5'- AGGTGGCTCAGGAAACCAAG -3'

Observation/parameters for evaluation

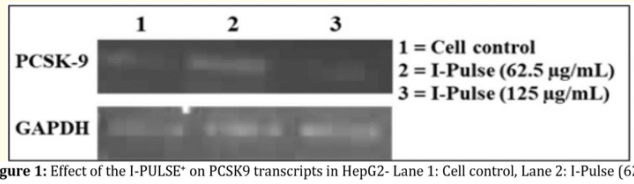

Figure 1: Effect of the I-PULSE⁺ on PCSK9 transcripts in HepG2- Lane 1: Cell control, Lane 2: I-Pulse (62.5 µg/mL) Lane 3: I-Pulse (125 µg/mL)

Figure 1

Results

Cytotoxicity

I-PULSE⁺ was tested for *in vitro* cytotoxicity studies against HepG2 (a human liver carcinoma cell line) by MTT assay, exposing the cells to different concentrations. The CTC50 value of the I-PULSE⁺ on the HepG2 cell line was >1000 µg/mL.

In vitro atherosclerotic effect

The human hepatocellular carcinoma (HepG2) cell line was used to determine the in vitro potency of I-PULSE⁺ to promote gene expression. Gene expression studies were carried out using non-toxic concentrations. The semi-quantitative RT-PCR analysis showed a significant reduction in PCSK9 gene expression when I-PULSE⁺ at lower concentrations (62.5 and 125 µg/mL) was compared to the control in the gene expression analysis.

Table 1: Cytotoxicity of I-PULSE+ against HepG2 cell line.

Sl. No.	Name of Test Substance	Test Conc. (µg/ml)	% of Cytotoxicity	CTC50 (µg/ml)
1	I-PULSE+	1000	46.38 ± 7.27	>1000
		500	38.48 ± 5.54	
		250	35.84 ± 9.94	
		125	34.63 ± 6.01	
		62.5	24.52 ± 0.44	

Table 2: The Quantitative gene expression level of PCSK9 normalized to GAPDH in I-PULSE+.

Test Sample	PCSK9- Regulation in Terms of Folds Against Cell control
Cell Control	1.00
I-PULSE+ (62.5 µg/mL)	1.02
I-PULSE+ (125 µg/mL)	0.92

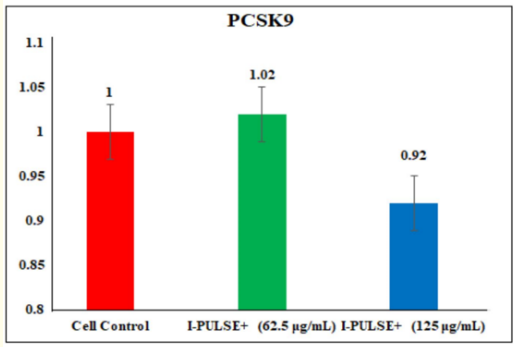

Figure 2: Graph of semi-Quantitative Gene Expression analysis of gene transcripts for fold increase, the relative level of PCSK9 gene expression is normalized to GAPDH. Values shown depict arbitrary units.

Figure 2

Discussion

The causes of coronary artery disease have been strongly associated with hypercholesterolemia and atherogenic dyslipidemia. Body weight fluctuations are used to track the progression of an illness, monitor a patient's reaction to treatment, and reveal side effects from medication. High serum concentrations of TC (total cholesterol), TG (triglyceride), LDLc (low-density lipoprotein cholesterol), and HDLc (high-density lipoprotein cholesterol) are related to atherosclerosis. Since HDLc and total body cholesterol are inversely correlated, a decrease in HDLc concentration in the plasma can accelerate the onset of atherosclerosis, the precursor to ischemic heart disease, by reducing the arterial wall's ability to remove cholesterol [14]. The mechanism by which extra tissue cholesterol is absorbed and then processed by HDLc particles for potential transportation to the liver for metabolism is primarily responsible for HDLc. As a result, a higher HDLc level reduces the chance of atherosclerosis [15]. Numerous research studies highlight the fact that developing cardiovascular problems is mostly dependent on the interplay between multiple risk factors, including hypertension and dyslipidemia. The development of cardiovascular disease as a whole is ultimately caused by the same factors that contribute to the initiation of atherosclerosis [16].

Inflammation and its mediators significantly decrease the stability and development of a plaque that has already developed [17]. Extensive research has demonstrated that infection and inflammation both significantly enhance PCSK9 expression. It has been demonstrated that pro-inflammatory gene expression is reduced by

PCSK9 inhibition [18,19]. However, it is unclear from clinical trials how PCSK9 inhibition affects inflammatory alterations.

Cardiovascular disease is associated with elevated LDLc [20]. Lipid metabolism is now being studied in relation to novel methods of inhibiting PCSK9 action. PCSK9 was identified as a novel therapeutic target for lowering LDLc levels recently. PCSK9 monoclonal antibodies that inhibit PCSK9 interaction with LDLR have already been approved on the market and demonstrated to reduce circulating LDLc levels with good efficacy and tolerance. There are a number of non-antibody treatments being developed at various phases of preclinical or clinical research, such as antisense oligonucleotides, small interfering RNAs (siRNAs), mimetic peptides, adnectins, and vaccination. However, there is currently insufficient data to determine an effective treatment for atherosclerosis.

Thus, the anti-atherosclerosis efficacy of the herbal formulation I-PULSE$^+$ was the main focus of our in vitro research. Here, the cells were exposed to various combinations of I-PULSE$^+$ in vitro cytotoxicity tests against HepG2 (a human liver cancer cell line) using the MTT assay. The results showed that the expression of PCSK9 mRNA was lower than the control value when the Semi-Quantitative RT-PCR experiment was carried out using PCSK9 specific primers. To prevent different atherosclerotic diseases, it is crucial to find new targets for nutraceutical agents.

Conclusion

The mechanisms of action and potential methods for preparing and utilizing therapeutic preparations from various sources of medicinal plants are hidden from view by both traditional herbal medicine and contemporary methods of researching the effects of medicinal plants. Numerous medicinal plants offer potential for the prevention or treatment of atherosclerosis. Medicinal plants aid in the treatment of atherosclerosis through diverse pathways and mechanisms of action. One important factor in the development of atherosclerosis is oxidative stress. Consequently, it is imperative to create drugs or dietary supplements that target atherosclerosis by improving the induction of antioxidant enzymes, preventing the production of reactive oxygen species (ROS), or obstructing further reactions, like the inhibition of the inflammatory process; all of these contribute to the vicious cycles of oxidative stress. Reliable evidence for the use of this medication will come from further investigation into the therapeutic effect of the herbal formulation I-PULSE$^+$ on atherosclerosis from the perspective of oxidative stress and clarification of the mechanisms and targets. These findings suggest that I-PULSE$^+$ is a promising nutraceutical agent for the treatment of different forms of atherosclerosis.

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Conflicts of Interest

The authors have no conflict of interest regarding the publication of this paper.

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