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# *In Vitro* Evaluation of Cytotoxic and Antiproliferative Activity of a Polyherbal Extract against H9c2 Cardiac Cells.

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**Abstract:** Recent studies indicate that in addition to necrosis and apoptosis also plays a vital role in the process of tissue damage after myocardial infarction, which has a pathological and therapeutically implications. However, limited effort has been made to correlate these effects to the active ingredients of the polyherbal (PH) extract .The present study was designed to elucidate the cytotoxic and antiproliferative activity against H9c2 cardiac cell line , were analysed the quantitative detection of caspase-3 activity during the early apoptotic process was evaluated by **Fluorometric Immunosorbant Enzyme Assay (FIENA).** Late stage of apoptosis was evaluated by DNA fragmentation. PH extract appear to contain components that inhibit the proliferation of H9c2cardiac cells. Expression of caspase-3 was induced by PH extract at 5g eq/L after 6 hr of treatment increases compared to the control. An increase in DNA fragmentation was also observed in the PH extract treatment.
**Keywords:** Polyherbal (PH) extract, H9c2, apoptosis, DNA fragmentation, Caspase-3, antiproliferation, FIENA.

## Introduction

Myocardial infarction commonly known as heart attack occurs when the hearts supplementary blood vessels are obstruct by an unstable build up of WBC, cholesterol and fat. With no blood flow, the cells die, causing whole portions of cardiac tissues to die. Once these tissues are lost, they cannot be replaced thus causing a permanent damage. Current research indicates, however, that it may be possible to repair damaged cardiac tissues with stem cells [1 ] as human embryonic stem cells can differentiate in to cardiomyocytes under appropriate conditions [2].

Plant based natural constituents can be derived from any part of the plant like bark, leaves, roots, flowers, seeds, fruits and rhizome [3].Plants are the traditional sources for many chemicals used as a pharmaceutical biochemicals, fragrances, food colours and flavours [4].Medicinal plants are at great interest to the researcher in the field of biotechnology, as most of the drug industries depend in part on plants for the production of pharmaceutical compounds. Thus, plants have been used in treating human diseases for thousands of years. The use of medicinal plants is not just a custom of the distant past. Perhaps 90% of the world's population still relies completely on raw herbs and unrefined extracts as medicines [7]. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials [5].Hence, nine new medicinal plants of each different part were taken and prepared a polyherbal formulation. The primary medicinal components such as glycosides, flavonoids, tannins were studied [6]. PH extract is rich in antioxidant phytochemicals including phenolic compounds. The phytochemical profiles of different varieties have been documented , a significant antioxidant activity have been observed from PH extract [8].Biological free radicals inclue ROS as $OH^{-}$,$O^{2-,}$ peroxy radical and reactive nitrogen species such as peroxynitrite, peroxy nitrous acid

and nitric oxide. Free radical inducers include transition metals such as iron III which can oxidize peroxides to prouce $OH^-$ through the fenton reaction. These biological free radicals may damage DNA, protein, lipids [9,10,11], and it is currently believed that they are among the underlying pathophysiological causes of the development of cancer , heart disease, aging and other health affiliations [12,13,14].

The infracted area in the heart consist of necrotic cells as well as so called apoptotic cells which shows characteristics DNA fragmentation [15]. However, the ultra structural features of the apoptotic myocytes subjected to the ischemia-reperfusion insult demonstrated oncosis accompanied by DNA fragmentation [16].

A group of cysteine proteases, caspases play key biological roles in inducing apoptosis. Caspase-1 (Interleukin - 1β converting enzyme –ICE) and caspase-3(CPP32) YAMA/ apopain are detected in the cardiomyocyte [17, 29,30,31].

Caspase 1 and 3 constitute a protease cascade, where caspase-3 is a downstream effector protease leading to DNAse activation [4] followed by DNA fragmentation (Fraser and Evan 1996).In cardiomyocytes, caspase inhibition can reduce incidence of apoptosis induced by metabolic inhibition in the isolated rabbit heart (20). Recently, [21] showed attenuation of myocardial injuiry brought by a caspase inhibitor.

In the current study, we investigated the inhibition of the proliferation of H9c2cardiac cell line. In vitro cytotoxicity and proliferative effects against the cardiac cell line were evaluated using the methylene blue cell counting method. Apoptotic activity was determined by testing for caspase -3 activity and DNA fragmentation. These tests were used to determine a possible mechanism for antiproliferation.

## Materials & Methods

### 1. Preparation of polyherbal formulation and solvent extraction

Each one gm of a poly herbal (PH) formulation contains equal amount of ***Punica granatum(rind), Catharanthus roseus, Gymnema sylvestre, Cissus quadrangularis, Garcinia cambogia, Tinospora cordifolia, Terminalia Arjuna, Urginea indica, Ficus racemosa***. The plants were authentified in Botanical Survey of India, Coimbatore. 10g of the dried powder of each plant was taken and cold macerated with hydro-ethanolic solvent with occasional stirring for 3 days.

After 3 days, the suspensions was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature ($<40^0$ C) under reduced pressure in a rotary evaporator. The yield of crude extract is called as polyherbal (PH) extract which was found to be -9.64% and were stored in an air-tight desiccator's and used for further analysis.

### 2. Cell Culture

Embryonic rat heart derived cell line H9C2, obtained from the American type culture collection (Manasses, VA) were cultured in Dulbecco's modified Eagles medium (DMEM; invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Atlanta Biologicals,Lawrence ville, GA), L-glutamine (4mM), 1.5g/l sodium bicarbonate, penicillin (100u/ml), and streptomycin (100µg/ml). The cells were maintained at 37° under a water saturated atmosphere of 95% ambient air and 5% $CO_2$. Stock cultures were passaged at 2-3 day intervals. the cells were cultured for 24hr before each experiment in DMEM containg 10% FBS (Merten 2006).

### 3. Cytotoxicity and antiproliferation

PE were dried and then redissolved in 50% DMSO. The 50% DMSO solutions were then examined for cytotoxicity and antiproliferation of theH9c2 cell line. Cytotoxicity and antiproliferation were investigated using the methylene blue assay [ 20, 23, 24]. The test is based on the theory that methylene blue is absorbed into both live and dead cells but is not washed out with water in live cells, whereas, non-viable cells cannot retain methylene blue after washing. Absorbance was read at 570 nm and absorbance readings were directly correlated to live cell number. Briefly, media was removed from wells. The cells were stained with 60 mL methylene blue solution (1.25% glutaraldehyde, 0.6% methylene blue in HBSS without phenol red). Plates were then incubated at 37˚C for 60 min. Next, the wells were washed 4 times in distilled water by emersion. Cells were then destained by adding 100 mL elution solution (50% Ethanol and 1% acetic acid in PBS) per well then shaken at

room temperature for 30 min. A Perkin Elmer Victor$^3$ V 1420 multilabel counter (PerkinElmer, Turku, Finland) was used to measure absorbance. Measurements were taken in triplicate.

For the cytotoxicity tests, $5 \times 10^4$ cells were added to wells in 96 well plates then incubated for 24 hours. Cells were then treated with control or grape pomace extracts in 50% DMSO at 1.25, 2.50, and 5.00 g/ PH extract equivalents per L (g eq/L). All wells contained 0.5% DMSO. Following 24 h of exposure, wells were measured using the methylene blue assay. All tests were conducted in triplicate.

For antiproliferation, $2.5 \times 10^4$ cells were added to wells in 96 well plates and cultured as above. Cells were then treated with 1.31 to 3.50 g eq/L and measured by the methylene blue assay following 72 and 96 h. Triplicate measurements were taken. Effective concentration ($EC_{50}$) was determined after 96 h of treatment. $EC_{50}$ was determined as the concentration of extract equivalents that had 50% of the cells compared to the control.

**4. Apoptosis Assays (Caspase-3 and DNA Fragmentation)**

H9c2 cells were seeded at $2 \times 10^6$ cells per well in 6-well culture plates and incubated at 37˚C for 24 h. The cells were then exposed to control or grape pomace extracts at 5.0 and 10.0 g eq/L for 4 and 12 hours for both apoptosis assays. The caspase-3 analysis was used to determine early apoptosis, and the DNA fragmentation was used to determine late or final stage apoptosis. All treatments contained 0.5% DMSO.

For the caspase-3 assay, cells were washed twice in ice-cold 1 × PBS. Then caspase-3 expression was detected as per the manufacturer's instructions by the fluorometric immunosorbent enzyme assay (FIENA) kit (Roche Applied Science, Mannheim, Germany). The fluorometric determination of proteolytic cleavage of the substrate of the samples was measured with a Victor$^3$ V 1420 multilabel plate reader (PerkinElmer, Turku, Finland) at excitation 425 nm and emission 490 nm.

For the DNA fragmentation tests, DNA was extracted using the Apoptotic DNA Ladder Kit, (Roche Applied Science, Mannheim, Germany) with slightly modification. In brief, cells were washed twice with ice cold 1x PBS, then 200 uL binding/lysis buffer was added and mixed immediately. After holding for10 min at 22˚C, 100 µL of 100% isopropanol was added and the solution was vortexed for 10 sec. The lysate was run through the column then washed twice with washing buffer. DNA was eluted with 200 mL of pre-warmed (70˚C) elution buffer and concentrated with a speedvac. Extracted DNA was subjected to gel electrophoresis, and the image was captured with UVP EC3 bioimaging system (Upland, CA).

**5. Statistical Analysis**

Data was analyzed using SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL). Data were reported as mean ± standard deviation (n = 3). Analysis of variance and Tukey's post hoc analysis was used to determine differences among means. Pearson Correlation Coefficient was used to determine correlations among means. Significance was declared at $P < 0.05$.

## Result and Discussion

**1. Cell cytotoxicity and antiproliferation.**

The crystals of the PH extract was brought in to 50% DMSO solutions for testing. Solutions were tested in theH9c2cardiac cell line for cytotoxicity following 24Hr and antiproliferation following 72 and 96hr treatment. The PH extract showed the highest cytotoxicity which was shown in (figure 1) 30.0%, 46.0%, 68.7% at 2.50,5.00,7.25 eq/l respectively.

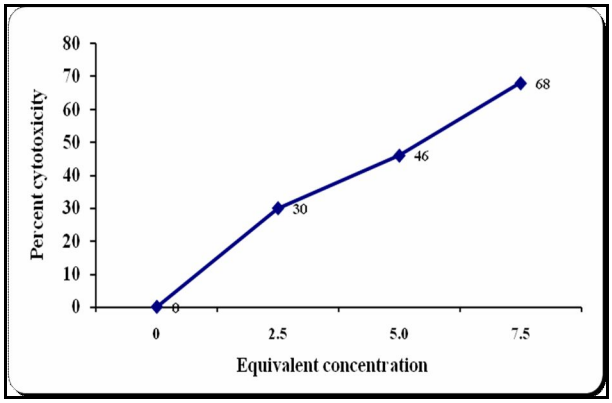


**Figure 1: Cytotoxicity of a Polyherbal Extract.**

Antiproliferation effects of PH extract onH9c2cell line was determined at 4 different concentrations (figure 2).The result showed a dose dependent inhibition of cell proliferation after 72 and 96 hr of treatment compared to control from PH extract samples.

In the H9c2cells , the EC50 of the PH extract were determined. The EC 50 is the concentration of extract in the cell media that causes a 50% lower cell count compared to the control at a specified time period. The proliferation was significantly inhibited at the lowest tested concentration of 1.31 g eq/L after72hr and 96hr of treatment (Figure 2).

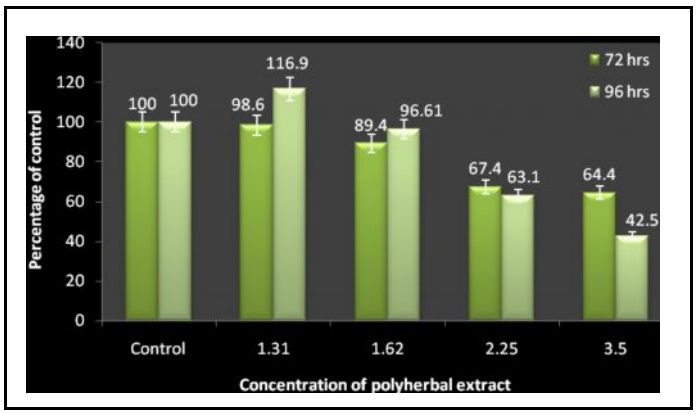


**Figure: 2: Antiprolieration of a Polyherbal Extract**

However, cardiac cells are more antiproliferative compounds and this is also a possible reason for thisH9c2cells were not as adherent to the culture plates and the chemical reactions in the cardiac cells are more favoured to become established on the surface of the plate compared to absorbing outside molecules during the first 24 hr of treatment.

Caspase 3 expression plays a vital role in the early stage of apoptosis. Using an anticaspase 3 specific monoclonal antibody together with a specific caspase substrate, the caspase 3 concentration was determined in theH9c2cardiac cells treated with PHExtract by FIENA (**Fluorometric Immunosorbant Enzyme Assay**). A significant increase in caspase 3 expression was observed after 6hr at 5 and 10g eq/L.H9c2cells were treated with 10eq/L. PH extract showed the highest increase of caspase 3 expression of 406.5% compared to control after 6 hr of treatment (Figure 3).

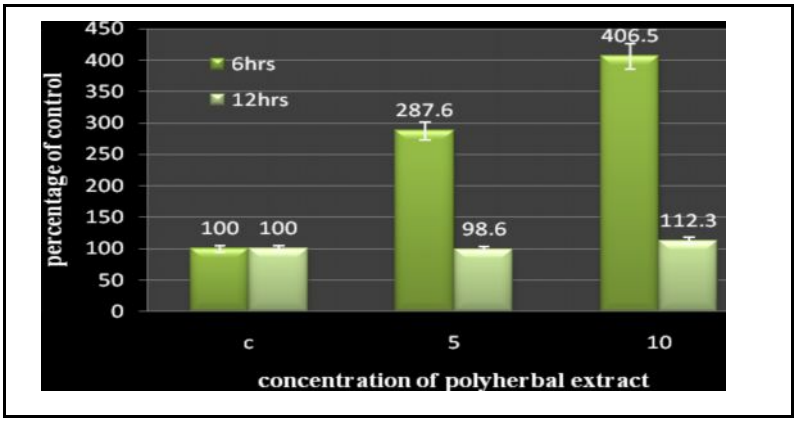


**Figure: 3: Ffiena Assay**

## 2. DNA fragmentation

DNA fragmentation is a marker of late stage apoptosis. It was used to support the caspase-3 expression test to identify apoptosis. Fragmentation was induced inH9c2cardiac cell line after treatment with PH extract using 5 and 10 g eq/L media (**Figure 4**). This Figureure demonstrates internucleosomal DNA degradation from gel electrophoresis of H9C2cardiac cells. A characteristic DNA ladder was observed whenH9c2cells were treated with both 5 and 10 g eq/L of PH extract after 4 h of treatment (**Figure 4**, lanes 4 & 5), and no DNA ladder was shown from the untreated negative control (**Figure 4**, lane 3). Fragmented DNA was also observed in theH9c2cells when treated with 5and 10 g eq/L of PH extract for 12 hours (**Figure 4**, lanes 7 & 8). No DNA ladder was shown from negative control cells (**Figure 4**, lane 6). The highest percentage of the apoptotic cells was observed in theH9c2cell line treated with 5 g eq/L of PH extract for 12 hours.

Caspase 3 expression and DNA fragmentation are indicative of early and late stages of apoptosis, respectively. Recently, the importance of grape seed extract in inhibittion of cancer cell growth has been investigated [26, 27]. In isolated rat hearts subjected to prolonged ischaemia, a significant accumulation of cytochrome- c occurred in the cytosol, which was accompanied by the activation of caspase-3 like proteases [28].

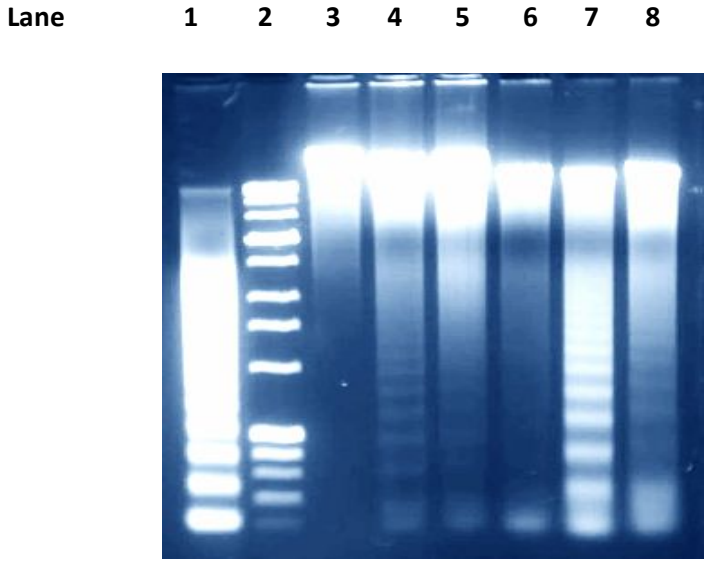


**Figure: 4: DNA fragmentation analysis**

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