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ORIGINAL RESEARCH **INTENSIVE CARE**

Integrating eugenol with intensive care in leukemia patients: exploration of pro-apoptotic potential against HL-60, human leukemia cell line

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ABSTRACT

Objective: The objective of this in-vitro study was to explore the pro-apoptotic potential of eugenol (4-allyl-2-methoxyphenol) on Human Leukemia-60 (HL-60) cell line as a potent phytochemical in intensive care setting to leukemia patients.

Methodology: After formal approval by all of the respective ethical committees, this study was simultaneously conducted at all respective institutional departments. The study included culturing HL-60 cell line and its treatment with serial concentrations of eugenol for calculation of subsequent IC50 values (half-maximal inhibitory concentration) via MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, expression analysis of gene, comparative analysis of relative gene fold (Intrinsic Biomarkers Caspase-3, Caspase-9) as apoptosis mediator markers followed by RT-qPCR. Cellular apoptotic morphology was confirmed via Hoechst 333258 staining.

Results: For HL-60 cell line, the IC_{50} of eugenol (14.1 uM) showed high gene expression of pro-apoptotic biomarkers (Caspase-3 and Caspase-9). Hoechst 333258 staining showed prominent apoptotic bodies leading to nuclear fragmentations.

Conclusion: Eugenol proved to possess robust pro-apoptotic potential leading to diagnostic efficacy against leukemia HL-60 cell line. Further studies would help in identifying key mechanisms by which eugenol exhibits anti-cancer potential against HL-60 cell lines.

Abbreviations: HL-60 - Human Leukemia-60; IC50 - Half-Maximal Inhibitory Concentration

Keywords: Eugenol, Pro-apoptotic Effects, Intensive Care, Anti-Cancer Mechanisms, HL-60, Natural Chemotherapeutics

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1. INTRODUCTION

Leukemia is widely called blood cancer. In context of advanced chemotherapeutic regimens causing poor prognoses, patients tend to face short- and long-term adverse effects of anti-cancer agents. Therefore at large extent natural sources seem to reduce the shortcomings of cancer chemotherapeutics. The last three decades have been very important targeting anti-cancer potential of dietary phytoconstituents.

Eugenol is an inherent extract of clove oil (*Syzygium aromaticum*) with extensive pharmaceutical potential, demonstrates anti-cancer prowess through a myriad of mechanisms and the main phenomenon is induction of apoptosis. [1] Eugenol modulates cellular signaling pathways crucial for cancer development, induces DNA damage, influences DNA repair mechanisms and consequently a disruptor of cancer cells metabolism through molecular warfare. [2] Due to multidirectional potential both compounds have long been used in medicine and pharmacology. [3]

Antitumor activity has been reported for essential oils against several tumors cell lines and these compounds contain a high percentage of phenylpropanoids.[4] HL-60 is leukemia cell lines having adherent growth properties. Eugenol has apoptosis-inducing chemotactic properties in HL-60 cells dependent on ROS and influencing signal pathways and killing cancer cells. [5] Similarly, phytochemicals possess potent bioactive anti-cancer activity against HeLA cell lines showing cell cycle arrest and reduced angiogenesis when combined to Cisplatin. [6,7] Cisplatin is designated with pre-defined MTT assay values and therefore it is used as a standard to check the anti-cancer and anti-growth properties of selected constituent of phytochemical. The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is a sensitive and reliable indicator of the cellular metabolic activity.

Objective: The objective of this in-vitro study was to explore the pro-apoptotic potential of eugenol on HL-60 cell line as a potent phytochemical providing intensive care to leukemia patients.

2. METHODOLOGY

After REC approval (143-2024), this study involved authors' affiliated institutional research laboratories at Liaquat University of Medical & Health Sciences, Jamshoro, Government College University, Faisalabad, and Forman Christian College (A Chartered University), Lahore, from November-2023 to June-2024.

The first step was the extraction of eugenol. Briefly, collected phytocomponents were converted into fine powder by grinding these under hygienic conditions, followed by freeze drying at −58°C using a freeze dryer. Then crushed using a mortar and pestle under extreme care to avoid contamination. Powder formation increases the surface area that facilitates the extraction process in ethanol solvent undergoing maceration (reflux extraction) until maximized yield. For treatment of cells, customized concentrations of 10, 20, 40 and 60 µg/mL were freshly prepared Gibco (Dulbecco's Modified Eagle Medium) DMEM (catalog#11966025) medium supplemented (or enriched) with L-glutamine (2 mM), 10% fetal bovine serum (FBS), sodium pyruvate (1 %), penicillin and streptomycin (100 IU/mL) and vortex for 10-12 min.

HL-60 cells were initially obtained from commercially available sources (Thermosfisher certified) and controls were also extracted from leukemic blood samples and stored in pre-defined Cryo vials preparations in liquid nitrogen at −180°C. After thawing and following routine sub culturing protocols, cells were cultured in DMEM supplemented medium (10% FBS) and treated with pre-formulated concentrations of eugenol (05, 10, 15, 20, 25, and 30 µM) for MTT assays of 24, 48, and 72 h respectively. Briefly, cells (10000 cells/well) were seeded in 96-well plates (100 µL media/well), at pre-optimized cell densities and were treated with serial dilutions of increasing concentrations (10, 20, 40 and 60 µg/ml) of four compounds and their combinations for incubation period of 24, 48, and 72 h respectively to find out 50% inhibitory concentration (IC50) values. 96-well plates were designed in a way that the triplicate of wells undergoing same treatment concentrations and reading of MTT assay at 480 nm were recorded and average of readings were taken to minimize the chances of random and systematic errors.

After the treatment, MTT solution 10 mg/mL in phosphate-buffered saline (PBS) was added at a volume of 10 µL/well and the plate was incubated for 4 h at 37°C and 5% CO_2. After reproducing the experiments, as per greater reproducibility only 24 h MTT was selected for IC50 (the concentration at which approximately 50% cells were found dead). From treated cells, total RNA extraction was performed followed by quality check (ng/µL) and cDNA synthesis as per kit protocols (Reagent Catalog# AM7832, Thermofisher Scientific, USA). Primers sets of Caspase-3 and Caspase-9 are shown in Table 1. Primers were designed on serial cloner by taking the consensus CDS sequence of required genes from NCBI and confirmed by doing In-Silico PCR on UCSC genome browser. Gradient PCR reactions were done multiple times to achieve optimization.

Table 1: Sequences of Caspase-3 and Caspase-9 Primers set	
BIOMARKER GENES	**SEQUENCES OF SELECTED PRIMERS**
Caspase-3	**Forward:** 5'GCGAATCAATGGACTCTGG3'
	Reverse: 5'GACATCTGTACCAGACCGAG3'
Caspase-9	**Forward:** 5'CAGGCCCCATATGATCGAG3'
	Reverse: 5'CTGTGTCCTCTAAGCAGGAGA3'

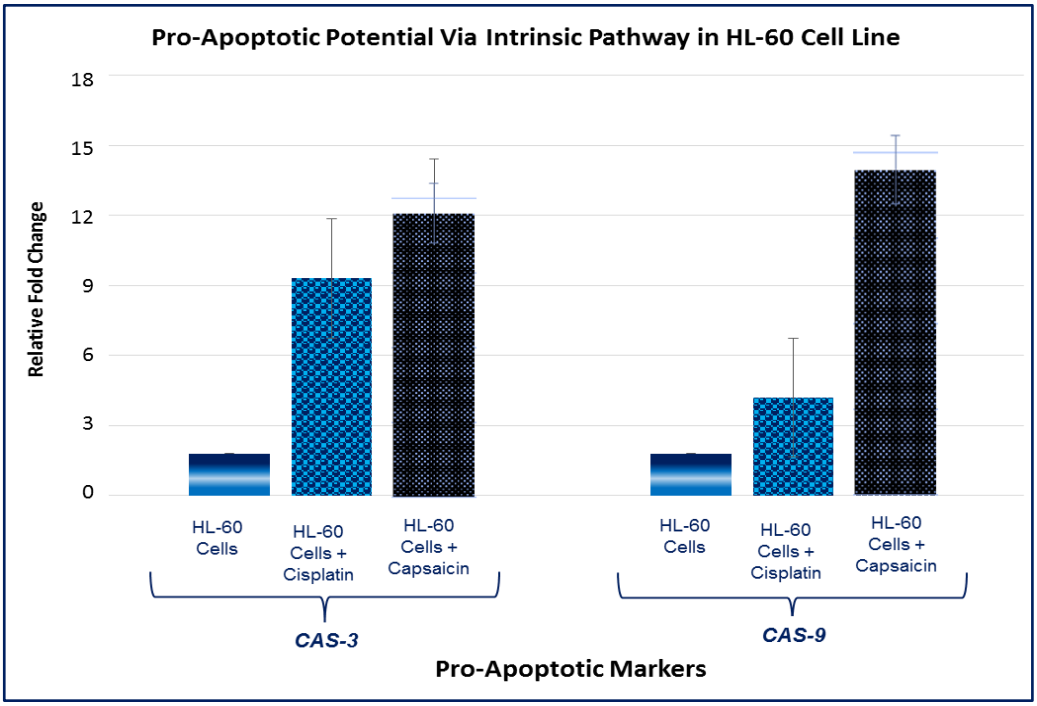

Figure 1: MTT Assay (Expression profiling of selected biomarkers)

To stain the nucleus, Hoechst 33258 was used producing blue florescence. HL-60 cells were incubated after treatment with IC_{50} of eugenol treated cells. After removing culture media, the cell smear (first dried) was immersed in the prepared concentration of formaldehyde upto 4%. Then after soaking with PBS, washed the smear three times and fully covered with Hoechst 33258 (solution). After keeping it at room temperature for 15 min, PBS was used for washing (two to three repetitions). It was sealed in order to observe under microscope.

Statistical Analysis: Data were analyzed using SPSS version 26.

3. RESULTS

HL-60 cells were treated with serial concentrations of eugenol (5, 10, 15, 20, 25 and 30 µM) and MTT assay was performed to calculate the IC_{50} values. Out of 24, 48 and 72 h assays, due to higher experimental reproducibility of MTT assay values, only 24 h MTT by concluding best IC_{50} of assay was selected to proceed further eugenol (IC_{50} of 14.1µM) treatments were performed in comparison with Cisplatin (control). Gene expression profiling (Caspase 3 and Caspase 8) was performed to analyze the pro-apoptotic. Significant associations with the higher relative gene fold depict the efficacy of compounds on HL-60 cells as shown in Figure 1.

Both biomarkers (Caspase-3 and Caspase-9) showed significant expressions as compared to control cells and eugenol treatment. Overall relative gene fold of Caspase-9 (14.1) was found higher as compared to Caspase-3 (12.0).

3.1. Hoechst 33258 staining

Cells after treatments with IC_{50} doses of eugenol showed distinctive morphological features including diversity in

anomalies in different cellular components. Nuclear change was the most prominent feature. Cells with live metabolic machinery were seen as uniformly light blue nuclei. Cell undergoing apoptosis displays the features of karyopyknosis and condensation of chromatin. Control cells did not show any morphological changes as shown in Figure 2.

HL-60 cells exposed to 11.9 µM of eugenol. After treatment, cells showed (brightly) blue fluorescence along with apoptotic morphology. Main changes are rupture of cell membranes and nuclear fragmentations.

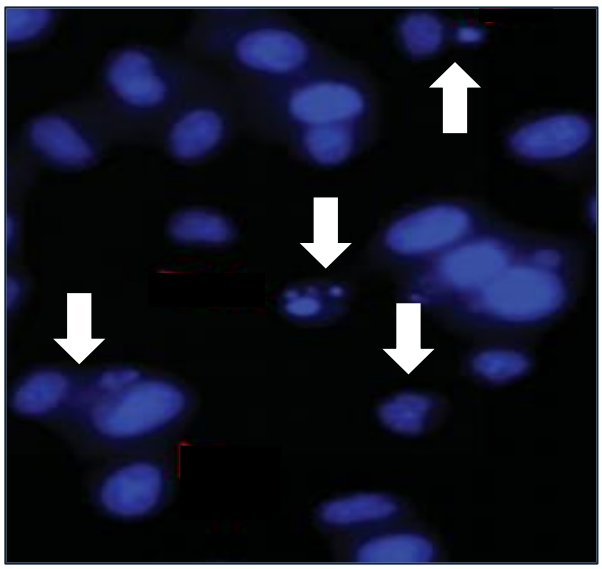

Figure 2: Apoptotic morphology of HL-60 cell line on Hoechst 33258 staining

4. DISCUSSION

Eugenol has the ability to interfere with the initiation and promotion of cancer, has demonstrated anti-oxidative, anti-inflammatory, pro-apoptotic, anti-proliferative, and anti-tumorigenic activities in multiple cancer cell lines.[8,11] Supported by many previous studies, results obtained from our study show that treatment of HL-60 cells with eugenol led to a greater induction of expression of Caspase-3 and Caspase-9 confirming pro-apoptotic (intrinsic) potential of compounds as a major contributing factor to the decrease in cell proliferation.[9,10] Similar results were obtained where piperine induced the expression and subsequent activity of both Caspase-3 and Caspase-8 leading to the apoptosis of the HeLa, human cervical cancer, cells.[11] A group of researchers suggested that eugenol with very low doses (08 µM) interfered with the process of carcinogenesis by inhibiting breast cancer cells percentage and inhibiting the activity of MMP (matrix metalloproteinase). [12,13]

Flow cytometrical analysis on leukemia cell lines after treating with IC_{50} of cinnamaldehyde and eugenol showed similar congestions in cell size and altered granularity.[14] Another experiment on $HepG_2$ cell lines showed decrease in size and varied granularity as indicated by many studies on the effect of eugenol on cancer cell lines.[15] Our findings supported that eugenol possesses strong membrane permeability potential to rupture cell membranes and destroy the cellular metabolic machinery in experiments of *in vitro* analysis.[16,19]

A study suggested that after phytochemical extract treatments, many apoptotic bodies along with disrupted cells showing nuclear fragmentations were seen under florescence microscope with Hoechst 33258 Stain.[17] The addition of eugenol is believed to enhance the inhibition of breast cancer stem cells by Cisplatin by inhibiting the activity of aldehyde dehydrogenases (ALDH) and ALDH positive tumor initiating cells and enhancing NF-κB signaling pathway inhibition.[18,20] On the whole both compounds showed significant pro-apoptotic potential. In the future, the synergistic effect of both eugenol with other natural phytochemicals should be studied on multiple cell lines to understand how these compounds work and if their affectivity is altered.

5. CONCLUSION

Our findings support the evidence of pro-apoptotic potential of eugenol on the HL-60 cells. It was also noted that the eugenol showed strong chemoprotective potential. Furthermore, the observed disruption of cellular metabolic machinery and induction of apoptosis in various cancer cell lines underscore eugenol's promise as an adjunct therapy, warranting further clinical trials to explore its efficacy and safety in cancer patients.

6. Data availability

The numerical data generated during this research is available with the authors.

7. Conflict of interest

The study utilized the Institutional Internal resources only, and no external or industry funding was involved.

8. Authors' contribution

HMHK, RB, PN: Conception and Design of work, Acquisitions and Analysis, Agreement to be accountable for all aspects of work

RB, PN: Resulting and Interpretation Agreement to be accountable for all aspects of work

OA: Interpretation and Drafting, Final Approval Agreement to be accountable for all aspects of work

WJ: Final Approval and Agreement to be accountable for all aspects of work

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