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STUDIES ON APOPTOSIS INDUCTION AND P53 GENE EXPRESSION AT IC50 CONCENTRATION OF A PETROLEUM ETHER EXTRACT OF A POLY HERBAL FORMULATION ON A549 CELL LINES

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Abstract
A polyherbal formulation comprising extracts of seeds of Nigella sativa and fruits of Vitis vinifera, Cucumis sativus, Phoenix dactylifera, Punica granatum, and Ficus carica, using petroleum ether solvent. Using the MTT assay, this formulation's cytotoxic ability against A549 lung cancer cells was assessed, yielding an IC50 value of 87.25 µg/ml.
Remarkably, the petroleum ether extract's IC50 concentration caused A549 cells to undergo apoptosis, indicating a potent anti-cancer action. Additionally, the formulation increased the expression of the P53 gene, a crucial tumour suppressor gene that controls apoptosis and cell cycle progression.
Pursuant to the study's findings, the polyherbal concoction may have chemo-preventive attributes, and regular use of it as part of a regular diet or as a dietary supplement may help prevent cancer. The formulation may also be a useful source for the creation of innovative cancer treatments, which calls for more research and validation through clinical trials and in vivo investigations.

Keywords: Cytotoxic, Prophetic tradition, poly herbal, P53 gene, Apoptosis.

Full forms: PH Aqueous- aqueous poly herbal mixture, PH Methanol- methanol poly herbal mixture, PH PET ether- petroleum ether poly herbal mixture, IC_{50} – half maximal inhibitory concentration.

1. INTRODUCTION

Islamic medicine, also known as Unani or Tibb-e-Nabavi, is a traditional system of healing rooted in the Quran and Hadith. This natural healing approach like other traditional medicinal practices like ayurveda presses on the use of plants and other natural products to prevent and treat various diseases. Prominent Islamic Scholars such as Abdul Malik Bin Habib Undlasi have made major contributions by documenting the medicinal properties of plants mentioned
in Islamic scriptures, including grapes, figs, ginger, garlic, and lentils. The study of the medicinal properties of these plants not only highlight the significance of Unani Tibb in preventive medicine but also is a way forward to the possibility of finding new medicinal substances. The development of complementary and alternative remedies for a variety of illnesses, such as cancer and other infectious diseases, may benefit from further research in this field (Habiba Sajid et al., 2019). Islamic scholars made profound contributions to the field of medicine, particularly in the realm of natural remedies and pharmacology based on the prophetic Saying, "For every disease, there is a cure," (Sahih Bukhari) which, instilled a sense of responsibility and brought curiosity among Islamic physicians to investigate into the medicinal properties of various plants mentioned in Quarn and hadith. Notably, the Quran references 12 plants, while the Hadith provides a more extensive list. The curative effectiveness of several of these plants has been substantiated by scientific investigations. Nonetheless, many are still uncharted, offering chances for further study and exploration (El-Seedi HR et al 2019).
Cancer is a major global health concern, as it is a both metabolic and genetic disorder and antioxidants from plants and other natural sources play vital in prevention and treatment. Chemoprevention using common plants used in daily diet has gained attention, with phytochemicals showing promise in inducing apoptosis and antiproliferative activity in cancer cells. Vitis vinifera (grapes) is a rich source of beneficial compounds like resveratrol, proanthocyanidins and anthocyanins. Extracts of grape seeds have demonstrated anticancer effects in animal models and cell cultures, effectively inhibiting tumour growth. Studies such as these highlight the ability of natural compounds in cancer prevention and treatment (Grace Nirmala J et al., 2018). Nigella sativa contains a substance called thymoquinone (TQ), which has anticancer effects by causing cancer cells to undergo apoptosis. By inducing cell cycle arrest and death, a study on ovarian cancer cells demonstrates TQ's potential as a successful treatment

(Karaosmanoğlu Ö et al., 2024). In India, 25–32% of female malignancies are breast cancer, making it a serious health concern. Current therapies have a detrimental impact on healthy cells and are less effective. Plant-based phytochemicals may aid in the prevention and treatment of breast cancer. Rich in flavonoids and other antioxidants, ajwa dates exhibit antiproliferative properties against cell types that cause breast cancer (Khan, M.A et al., 2021).

Polyphenols, which are plentiful in pomegranates, having anti-inflammatory, antioxidant, and anti-cancer effects. Pomegranate juice in particular shows three times more antioxidant activity than red wine and green tea, emphasizing its potential for therapeutic applications. Through processes including angiogenesis suppression, cell-cycle arrest, and apoptosis induction, the polyphenols in pomegranates have been demonstrated to suppress tumor development and trigger apoptosis in a variety of cancer cell lines (Turrini E et al., 2015).

Cucumber, lodges significant medicinal properties, including anticancer, anti-inflammatory, and cytotoxic effects. Cucurbitacin's, a prominent bioactive compound in cucumbers, have efficiently shown tumour growth inhibition. Recent studies have identified tetracyclic compounds, such as gitoxigenin, in cucumber extracts, which significantly inhibited the growth of MDA-MB-231 breast cancer cells, suggesting cucumber's potential as a therapeutic agent for breast cancer treatment (Kumaraswamy et al., 2025).

Fig latex has emerged as a promising solution for treating cervical cancer, particularly in cases associated with high-risk human papillomaviruses (HPVs). It selectively suppresses growth in HPV-positive cervical cancer cells. Transcriptomic analysis reveals that fig latex regulates genes involved in critical cancer-related pathways, including apoptosis, tumour suppression, and cell cycle control, indicating its potential as a targeted therapy for HPV-related cervical cancer (Cakir MO et al., 2023).

The present study investigates the P53 gene expression and Apoptosis Induction at IC50 concentration of a petroleum ether extract of a poly Herbal formulation on A549 Cells. The study's findings may provide insights into the potential therapeutic applications of a poly herbal formulation and also may be referred that the particular plants be used in our daily diet as precautionary measure against cancer.

2. MATERIALS AND METHODS

2.1 Sample preparation and MTT assay

Based on the Prophetic traditions, mentioned in the Quran and books of Ahadits viz., seeds of Nigella sativa and entire fruits of Vitis vinifera var. Red globe, Cucumis sativus, Phoenix dactylifera L. var. Ajwa, Punica grantum, and Ficus carica var. Kadota were collected (El-Seedi HR et al., 2019)., shade dried and finely powdered using a blender, the powdered material was weighed and was subjected to Maceration (cold extraction) technique wherein 100 grams of each powdered material was immersed in each of 500 ml of Petroleum, after 72 hours the material was filtered using a strainer and then using a Whatman no. 1 filter paper, later the solvent was evaporated using china dish and the crude extract in powdered form was collected. The extracts were mixed in the equal ratio to produce a poly herbal mixture which was further used for MTT assay (tested against A549 cell lines) at different concentration (100, 200, 300, 400 and 500 µg/ml) and the IC50 value was determined to to be ~ 87.25 µg/ml, which is further analysed for apoptosis induction and P53 gene expression.

2.2 Apoptosis Induction

Test sample details:

Table1: Details of samples

Sl. No.	Sample Code	Concentrations	Incubation period	Cell line
1	PH PET ether	IC_{50} (87.25µg/ml)	24hrs	A549

To check the apoptosis inducing nature of test compound (PH PET ether) on A549 cell line by flow cytometry.
Steps followed
standard procedure for detecting apoptosis in cells using Annexin V-FITC and Propidium Iodide (PI) staining, followed by flow cytometry analysis.

- Cell Preparation: To enable adhesion and proliferation, cells are seeded at a density of 0.5 x 10^6 cells/2 ml in a 6-well plate and incubated for 24 hours. Following the aspiration of the wasted media, cells are treated for a further twenty-four hours with experimental compounds and controls in two millilitres of culture medium.
- Harvesting and Preparation for Staining: after treatment, cells are harvested by trypsinization and washed with PBS to remove any residual medium and trypsin. This step is crucial for ensuring accurate staining and analysis.

- Annexin V-FITC Staining: FITC-conjugated Annexin V is used to stain cells by resuspending them in a binding solution and allowing them to bind to the phosphatidylserine that is visible on the outer leaflet of apoptotic cells.
- Propidium Iodide (PI) Staining: To dye necrotic or late apoptotic cells with impaired membrane integrity, PI is given to the cells. Early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+) can be distinguished using Annexin V-FITC and PI.
- Flow Cytometry Analysis: Using flow cytometry, the labelled cells' fluorescence patterns are used to quantify the populations of viable, early apoptotic, late apoptotic, and necrotic cells (BD Biosciences, Catalog no.556547).

2.3 P53 gene expression

To determine the effect of test compound (PET ether) on P53 gene expressions in A549 cells by RTqPCR method. Test sample details:

Table 2: Details of the experimental samples

Sample Code	Concentrations	Incubation period	Cell line
PET ether	IC50 (87.25µg/ml)	24hrs	A549

PROTOCOL AND METHODOLOGY:

a.) Trizol based RNA Extraction from Cell Pellet

- Cell Lysis and Homogenization: Centrifugation is used to pellet the cells, and the supernatant is disposed of. To lyse the cells and liberate their contents, TRIzol Reagent (1 mL of TRIzol per 0.33 mL of cell suspension) is added to the cell pellet. To guarantee homogeneity, the lysate is pipetted up and down multiple times before being incubated at room temperature for five minutes.
- Phase Separation: The homogenized lysate is mixed with 0.2 mL of chloroform for every 1 mL of TRIzol Reagent, shaken briskly to mix, and then allowed to sit at room temperature for two to three minutes to allow the mixture to separate into different phases. The mixture is separated into three phases by centrifugation at 12,000 × g for 15 minutes at 4°C: a colourless upper aqueous phase containing the RNA, an interphase, and a lower phenol-chloroform phase containing DNA and proteins.
- RNA Recovery: The RNA-containing aqueous phase is cautiously moved to a fresh tube, being careful not to disturb the lower organic phase or the interphase.

b) RNA Isolation

- RNA Precipitation: To improve RNA recovery, 6 µl of carrier RNA is added to the aqueous phase. To precipitate the RNA, isopropanol (0.5 mL per 1 mL of TRIzol
- Reagent) is subsequently added. After 10 minutes of incubation to allow for RNA precipitation, the mixture is centrifuged at 12,000 × g for 10 minutes at 4°C, resulting in a white, gel-like RNA pellet at the tube's bottom.
- RNA Washing and Resuspension: After discarding the supernatant, the RNA pellet is cleaned of contaminants using 1 millilitre of 75% ethanol per millilitre of TRIzol Reagent. After a quick vortex, the sample is centrifuged for five minutes at 4°C at 7500 × g. After discarding the supernatant, the RNA pellet is allowed to air dry for five to ten minutes in order to eliminate any remaining ethanol.
- RNA Resuspension and Storage: To ensure full dissolution, the RNA pellet is pipetted up and down in 45 µL of RNase-free water and then incubated for 10 to 15 minutes at 55 to 60°C in a water bath. After then, the resuspended RNA is kept at -20°C until it is needed again.

Table -3 RNA yield:

Sample ID	Nucleic Acid concentration (ng/µl)	A260	A280	260/280
UTD	633.28	0.79	0.42	1.88
PET ether	659.44	0.82	0.43	1.9
Standard: Cisplatin - 11µM	777.04	0.97	0.51	1.9

c.) cDNA synthesis

- cDNA first strand reaction mixture (10µl):
- (RNA, Prime Script RT Enzyme Mix, 5X Prime Script Buffer, Oligo dT Primer, Random 6 mers, Nuclease free water up to 10µl)
- After 15 minutes of incubation at 37°C and a 5-second heat inactivation of the room temperature reaction at 85°C, the mixture was snap-chilled on ice.
- The first strand of cDNA reaction mix is ready to be used for PCR amplification

d.) Experimental Conditions
PCR Reaction Mix:
(2X Green Mastermix for RT PCR, Reverse Primer, Forward Primer, cDNA, Nuclease free water) (make up to 20µl) (BD Biosciences, Catalog no.556547).

Table – 4 PCR conditions

	HK	**Test Gene**
Initial denaturation	95°C (30 sec)	95°C (30 sec)
Denaturation	95°C (5sec)	95°C (5sec)
Annealing	55°C (30sec)	60°C (30sec)
Number of cycles	45	45
Channel	FAM	FAM

PCR Standardisation of P53 and GAPDH

Table 5. Primer standardisation details

Target	**Primer Sequence**	**Standardised Temperature**
P53-F	ACCTATGGAAACTACTTCCTGAAA	58
P53-R	CTGGCATTCTGGGAGCTTCA	
GAPDH-F	GTCTCCTCTGACTTCAACAGCG	58
GAPDH-R	ACCACCCTGTTGCTGTAGCCAA	

3. RESULTS AND OBSERVATION

Apoptosis studies results
In this study, IC50 concentration of test compound (PH PET ether) and with two controls (untreated and standard) were used to study their apoptosis and necrosis inducing capabilities on A549 cell line. The used concentrations of the compounds to treat the cells are as follows:

Table 6: Details of the experimental conditions used for the study

SL. No	**Test Compounds**	**Cell lines**	**Concentration treated to cells**
1	Untreated	A549	No treatment
2	Standard (Cisplatin)	A549	11 µM
3	PH PET ether	A549	IC_{50} (87.25µg/ml)

Annexin-V/PI expression study on A549 cell line:

Fig. 1. Quadrangular plots representing the Annexin V/PI expression in A549 cells upon culturing in the presence and absence of test compound. Analysis was done by usingBD FACS Accuri, FCS Express 7. Here, Annexin V-FITC - PrimaryMarker, PI- Propidium Iodide (Secondary fluorescence Marker)

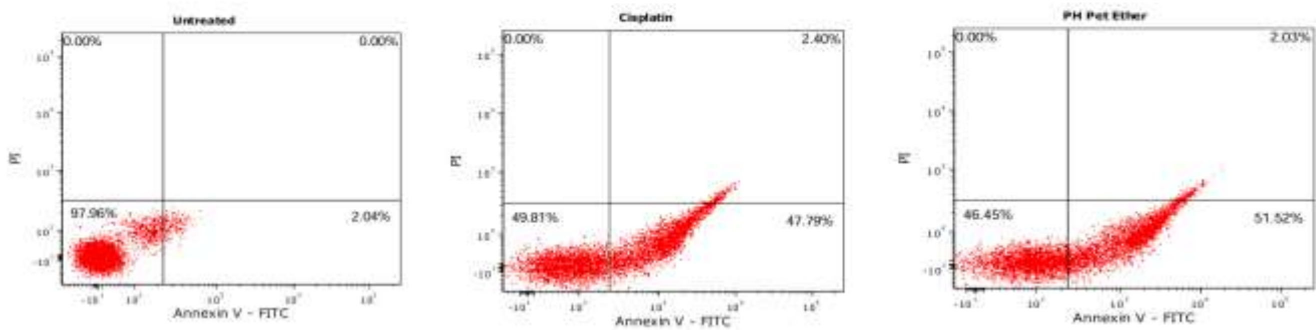


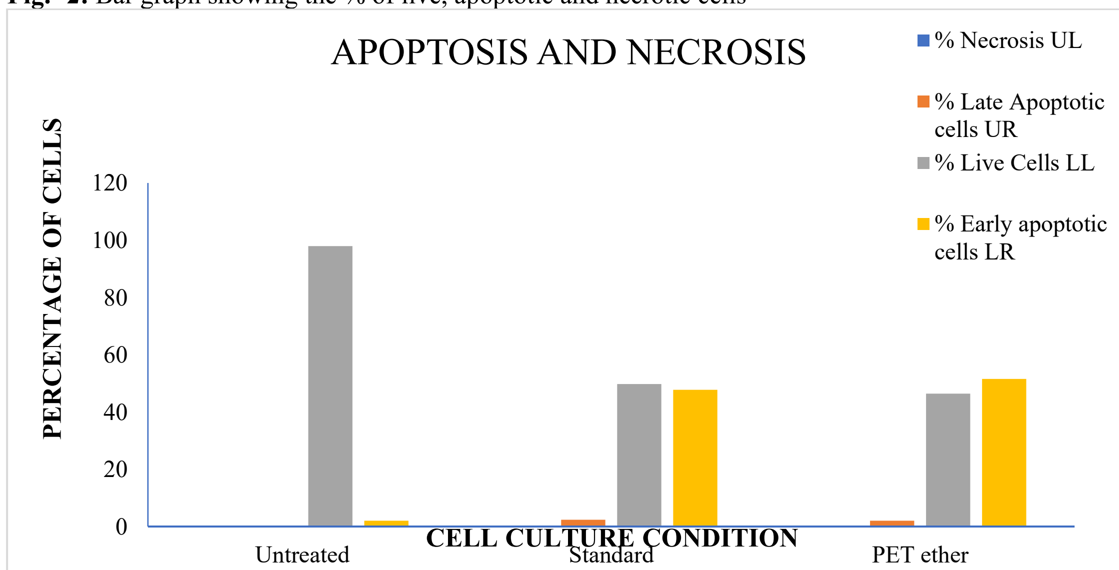
Table 7: Quadrant layout map in apoptosis/necrosis study.

Quadrant	% Necrosis	% Late Apoptotic cells	% Live Cells	% Early apoptotic cells
Label	UL	UR	LL	LR
Untreated	0	0	97.96	2.04
Standard	0	2.4	49.81	47.79
PET ether	0	2.03	46.45	51.52

Table 8: Table showing the % of cells of undergone apoptosis and necrosis, in untreated, standard and sample (PH PET ether) on A549 cells.

Q1 – Upper left: % of Necrotic Cells	Q2 - Upper right: % Late Apoptotic Cells
Q4- Lower left: % Viable Cells	Q3- Lower right: % of Early apoptotic cells

Fig. -2: Bar graph showing the % of live, apoptotic and necrotic cells



P53 Gene expression Results

Table- 9 House Keeping gene – GAPDH

Samples	Ct1	Ct2	Average Ct
Untreated	17.73	16.73	17.23
PET ether	18.85	19.73	19.29
Standard	16.31	15.31	15.81

Table 10 Test Gene- P53:

Samples	Ct1	Ct2	Average Ct	ΔCt	ΔΔCt	2^(ΔΔCt)
Untreated	23.6	22.8	23.2	5.97	0	1.0
PET ether	24.4	23.4	23.9	4.61	-1.36	2.56
Standard	20.2	19.4	19.8	3.99	-1.98	3.94

Fig : 3 Electrophoresis bands for RNA samples extracted from samples

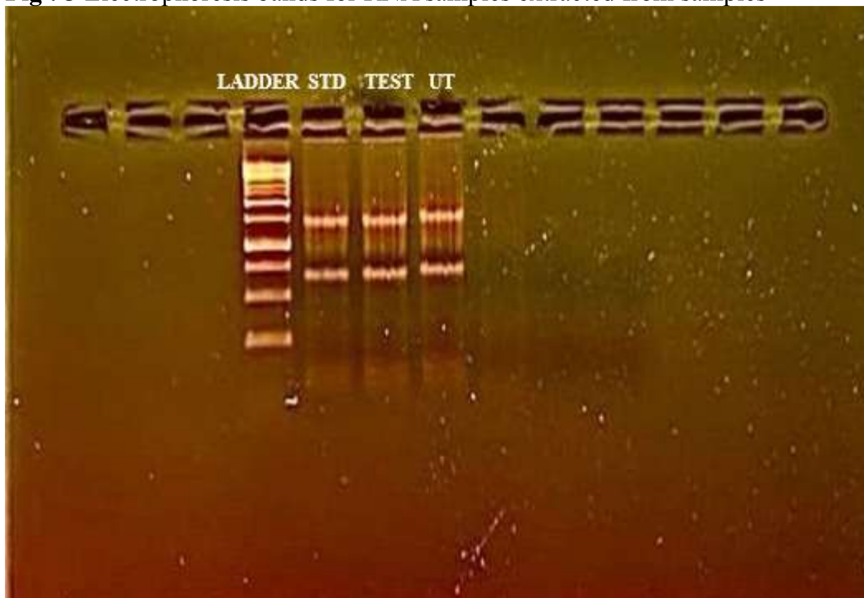


Fig: 4 Amplification and Melt Curve Plot

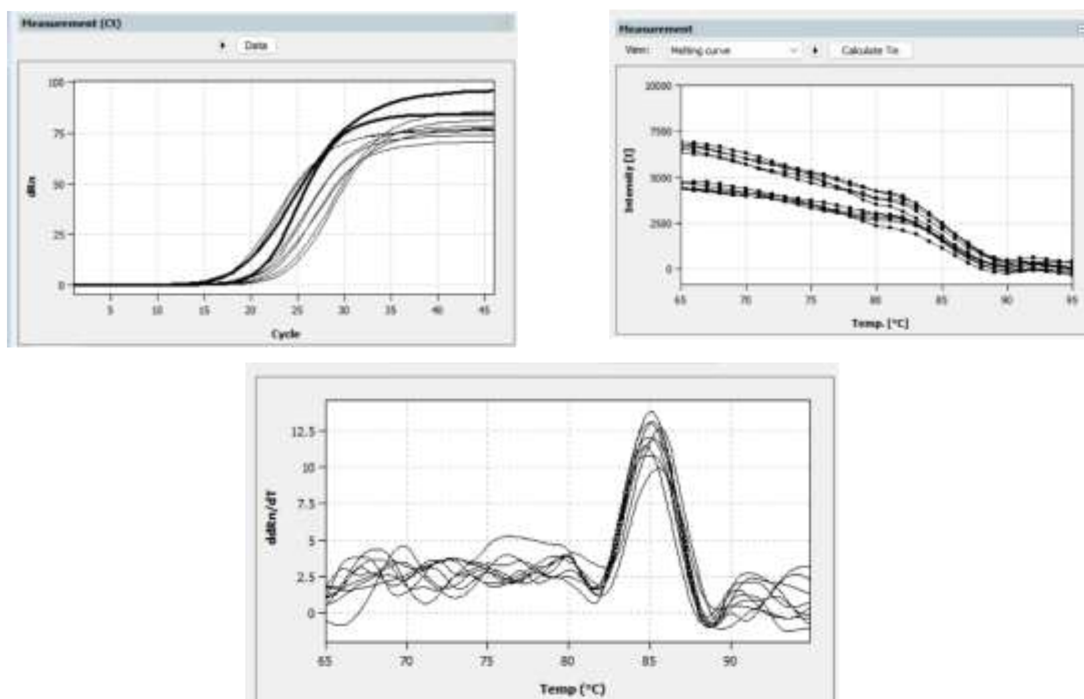
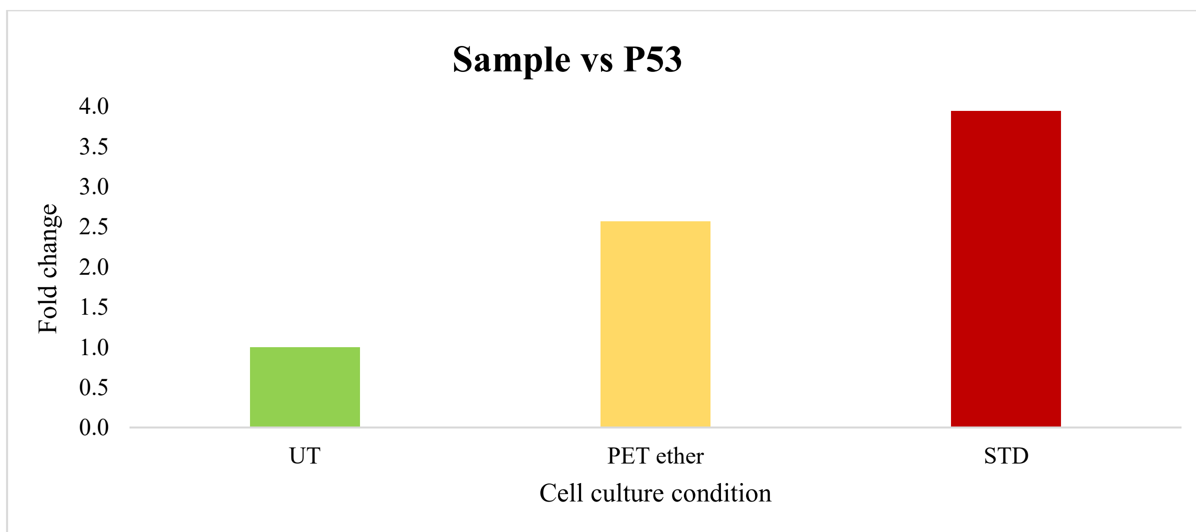


Fig 5: P53 gene expression after 24hrs of incubation.



4. DISCUSSION

The petroleum ether extract of a poly herbal formulation on MTT Analysis showed ~87.25 µg/ml as IC50 value and this concentration when analysed for its capacity in inducing apoptosis and effect on P53 gene expression by flow cytometry. The experiment results indicated that the test compound induced apoptosis in A549 cells after 24hrs of incubation period as compared to the untreated control, it was observed that the compound (PE PH at IC50 ~87.25 µg/ml) induced 0%, 2.03%, 46.45% and 51.52% necrosis, late apoptotic cells, live cells and early apoptotic cells respectively compared to the standard Cisplatin (11 µM) showing 0%, 2.4%, 49.81% and 47.79% necrotic, late apoptotic cells, live cells and early apoptotic cells respectively(table-7 and graph-2). The effect of test compound (PE PH) on P53 gene expressions in A549 cells was evaluated by RTqPCR method. When compared to the untreated cells, the A549 cells treated with test compound showed increased P53 gene expression. The compound (PE PH at IC50 ~87.25 µg/ml) induces 2.566% P53 gene expression compared to the standard Cisplatin (11 µM) which induces 3.944% P53 gene expression (Graph-4).

5. CONCLUSION

The results indicate that the compound (PE PH at IC50 ~87.25 µg/ml) consisting of Nigella sativa (part- seeds), Vitis vinifera var. Red globe (part- entire fruit), Cucumis sativus (part-entire fruit), Phoenix dactylifera L. var.

Ajwa (part-Entire fruit), Punica grantum (part-entire fruit), and Ficus carica var Kadota (part- entire fruit) in equal ratio induced increased apoptosis and P53 gene expression indicating the above mentioned plants can be included regularly in our diet as a preventive measure against cancer and also hopefully be used in development of drug in cancer treatment.

DECLARATIONS

Author contributions

Khadir Ulla Shariff: collection and processing of plant material, methodology, formal analysis and experimenting, writing — original draft preparation. Prof. C. Maya: conceptualization, methodology, review, editing, and supervision.

Conflicts of interest

The authors declare that they have no personal or financial interests in the present study or there is no funding body that can potentially influence the results.
The authors were not involved in the editorial review or the decision-making body to publish the article.

Ethics approval

Not applicable

Availability of data and material

Not applicable

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REFERENCES

1. BD Biosciences FITC Annexin V Apoptosis Detection Kit I (Technical Data Sheet, Catalog no.556547)
2. Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. J Biol Chem. 1990; 265(9):4923-4928. View reference
3. Casciola-Rosen L, Rosen A, Petri M, Schlissel M. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc Natl Acad Sci U S A. 1996; 93(4):1624-1629. View reference
4. Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingsperger CP, Roos D. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. Blood. 1995; 85(2):532-540. View reference
5. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994; 84(5):1415-1420. View reference
6. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995; 182(5):1545-1556. View reference
7. O'Brien MC, Bolton WE. Comparison of cell viability probes compatible with fixation and permeabilization for combined surface and intracellular staining in flow cytometry. Cytometry. 1995; 19(3):243-255. View reference
8. Raynal P, Pollard HB. Annexins: the problem of assessing the biological role for a genefamily of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta. 1994; 1197(1):63-93. View reference
9. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry. 1992; 13(2):204-208. View reference
10. Karaosmanoğlu Ö, Kamalak Z, Özdemir İ, Öztürk Ş, Tuncer MC. Apoptotic effect of thymoquinone on OVCAR3 cells via the P53 and CASP3 activation. Acta Cir Bras. 2024 Nov 8;39:e399224. doi: 10.1590/acb399224. PMID: 39536185; PMCID: PMC11548134.

11. Grace Nirmala J, Evangeline Celsia S, Swaminathan A, Narendhirakannan RT, Chatterjee S. Cytotoxicity and apoptotic cell death induced by Vitis vinifera peel and seed extracts in A431 skin cancer cells. Cytotechnology. 2018 Apr;70(2):537-554. doi: 10.1007/s10616-017-0125-0. Epub 2017 Oct 5. PMID: 28983752; PMCID: PMC5851950.
12. Khan, M.A., Siddiqui, S., Ahmad, I. et al. Phytochemicals from Ajwa dates pulp extract induce apoptosis in human triple-negative breast cancer by inhibiting AKT/mTOR pathway and modulating Bcl-2 family proteins. Sci Rep 11, 10322 (2021). https://doi.org/10.1038/s41598-021-89420-z.
13. Turrini E, Ferruzzi L, Fimognari C. Potential Effects of Pomegranate Polyphenols in Cancer Prevention and Therapy. Oxid Med Cell Longev. 2015;2015:938475. doi: 10.1155/2015/938475. Epub 2015 Jun 9. PMID: 26180600; PMCID: PMC4477247.
14. Kumaraswamy, Latha & Tallur, Preethi. (2025). Cucumis Sativus L. Foamy Extract Induces Apoptosis In Mda-Mb-231 Cells: Cucumber Extract Induces Apoptosis in Cancer Cells. SAARC Journal of Agriculture. 22. 55-66. 10.3329/sja.v22i2.74341.
15. Cakir MO, Bilge U, Ghanbari A, Ashrafi GH. Regulatory Effect of Ficus carica Latex on Cell Cycle Progression in Human Papillomavirus-Positive Cervical Cancer Cell Lines: Insights from Gene Expression Analysis. Pharmaceuticals (Basel). 2023 Dec 12;16(12):1723. doi: 10.3390/ph16121723. PMID: 38139849; PMCID: PMC10747314.