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Investigating the Impact of Phenolic and Terpene Fractions extracted from *Prunus arabica* on p53 Protein Expression in AMJ13 and SK-GT-4 Human Cancer Cell Lines

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ABSTRACT

Breast cancer is the most frequently diagnosed cancer in women, accounting for a quarter of all cases. The burden of cancer in transitional countries is rising. An esophageal cancer diagnosis typically carries a poor prognosis, as well as a high incidence and mortality rate. Apoptosis, angiogenesis, and tumorigenesis are all controlled by the tumor suppressor protein p53. In clinical oncology, many researchers revealed promising effective phytotherapy methods for cancer patients, compared to antitumor xenobiotics. Study objectives were to study the mechanism of cytotoxicity of the phenolic fraction from *Prunus arabica* on breast cancer (AMJ13) and Oesophagus adenocarcinoma cancer (SK-GT-4) cell lines by measuring human tumor protein (p53) expression. Cells were treated with the half maximal inhibitory concentration (IC_{50}) concentrations for each compound, then cells were collected with trypsinization and centrifuged. Cell precipitate was lysed using lysis buffer. The supernatant protein concentration was determined by Bicinchoninic Acid (BCA) procedure, finally p53 expression assayed using an Enzyme-Linked Immunosorbent Assay kit (ELISA). Phenolic fraction showed a significance increase in p53 protein expression on both AMJ13 and SK-GT-4 cancer cell lines (p value <0.05) while terpene fraction did not show any significance in both cell lines in comparison to untreated control group (p value<0.05). Phenolic fractions augment p53 expression giving a mechanistic insight into the fraction's cytotoxic attributes. Consequently, the terpene fraction serves as a potent anticancer agent through an alternate mechanism. The findings of this study offer a novel understanding of the biological functions of the phenolic fraction in relation to cancer therapy.

Keywords: *Prunus arabica*, Tumor protein, phenolic fraction, terpene fraction, breast cancer, esophagus cancer.

Introduction

Globally, breast cancer is the most common non-cutaneous malignancy in women, while esophageal cancer is a major contributor to cancer-related deaths.[1,2] Both cancers have a poor prognosis and high mortality rate, and drug resistance is a major challenge in treatment.[3] Medical practitioners face a formidable challenge when it comes to improving patient survival due to drug resistance, which can be found in various subtypes of breast cancer as well as esophageal cancer.[4] Surgery, radiotherapy, chemotherapy, biologically targeted therapy, and hormonal intervention, as traditional cancer treatment modalities, exhibit inherent limitations.[1]

Natural compounds, such as phenolic compounds and alkaloids, have received significant attention in recent years for their potential cancer chemoprevention and bioactivity.[5,6] These compounds have potent antioxidant and anti-inflammatory properties, and have also been shown to induce apoptosis through the stimulation of p53 expression, inhibition of antiapoptotic activity, and activation of proapoptotic pathways.[7]

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A kind of wild almond called *Prunus arabica* may be found all across the Middle East. Which is a member of the *Prunus* genus and the *Rosacea* family. These species exhibit strong tolerance to drought and high temperature shocks. One species, *P. arabica*, is found in Saudi Arabia, Jordan, Iraq, Syria, and the Southeastern Anatolia Region of Turkey.[8] A wide range of biological and pharmacological effects from different Prunus species show great promise for the treatment of various cancers.[9]

Tumor suppressor protein p53 inhibits the growth of cancer cells.[10] It is estimated that 30% of all breast cancer patients and 40-70% of esophageal cancer patients have a mutation in p53.[11,12] Therefore, the identification of natural compounds that can modulate p53 expression is a promising approach for the development of new and effective anticancer therapies. In our earlier investigation, we observed that extracted fractions (phenolic and terpenoid) from *Prunus arabica* demonstrated cytotoxic, anti-angiogenic, antioxidant, and anti-inflammatory effects.[13] This study investigated the effect of these fractions extracted from Iraqi *Prunus arabica* on p53 expression levels in the breast cancer cell line AMJ13 and the esophageal cancer cell line SK-GT-4. The goal was to determine if p53 regulation is a potential biological mechanism for the anti-tumor effects of phenolic and terpene fractions.

Materials and Methods

Cell lines:

AMJ13 cell line (provided and authenticated by the Iraqi Center for Cancer Research and Medical Genetics)[14], was cultured in Roswell Park Memorial Institute Medium (RPMI-1640) (cat-no. RPMI-A)

supplemented with 10% Fetal Bovine Serum (FBS) (cat-no. FBS-22A) and antibiotics (penicillin and streptomycin) (cat-no. PS-B), all purchased from (Capricorn, Germany). The cells were incubated at 37°C in a humidified incubator with 5% CO_2. (CYANLab CL011, Cypress Diagnostics, Belgium).
SK-GT-4 cell line [15], supplied by European Collection of Authenticated Cell Cultures (ECACC), maintained in Minimum Essential Medium (MEM) (cat-no. MEMA-RXA). Passaging of cells was performed with Trypsin-EDTA (cat-no. TRY-1B), all purchased from (Capricorn, Germany), Incubated at 37 °C (CYANLab CL011, Cypress Diagnostics, Belgium) and replanted at 50% confluence twice a week.

Protein quantification by Bicinchoninic Acid assay
Compared to Lowry method, BCA kit (cat-no. E-BC-K318-M, Elabscience, Houston, TX, USA), provides better protein quantification results. It is fast, sensitive, stable, and reliable, with a small coefficient of variation. The BCA method is also unaffected by most chemicals, making it ideal for a wide range of samples. In this study, Protein quantification of the samples was performed using the same version of the BCA assay as reported by Yangyufan Wang.[16]

Exposure protocol:
In 25T cell culture flasks (Thermo Fisher Scientific, USA), breast and esophageal cell lines were cultured, and after confluency, the IC_{50} concentrations of each compound were applied (obtained from our previous study).[13]
The cells were collected by trypsinization after 24 hours of exposure and centrifuged at 1500rpm/min for 10 minutes (Nest biotechnology, Jiangsu, China). A lysis buffer provided by (ElabScience, Houston, TX, USA cat-no. E-BC-R327) was used to lyse the cell precipitate before measuring protein concertation.

Cell lysis
Cell lysis was conducted according to Liu C. *et. al*,[17] collected cells were washed extensively with chilled PBS (Sigma-Aldrich, Germany cat-no. P5119) before analysis (0.01 M, pH7.4). RIPA Lysate Buffer (Sigma-Aldrich, Germany cat-no. R0278) was added at the proper concentration, and the mixture was lysed on ice for 30 min. Under ice water bath (Memmert, Germany) conditions, the sample was blown through a pipette gun fifty times to ensure the DNA strand was broken and to lessen its viscosity. Then, 10 minutes centrifugation at 12,000 rpm (Nest biotechnology, Jiangsu, China) was conducted at 4°C for 10 minutes finally, the supernatant is taken to measure the protein concentration.

BCA procedure
The BCA assay kit includes Reagent 1 (BCA reagent), Reagent 2 (Copper salt solution), Reagent 3 (Protein BSA standards) and Reagent 4 (Standard diluents)

A. Preparation of standards and samples
According to manufacturer's instruction, Reagent 3 (Protein BSA standards) is mixed with reagent 4 (Standard diluents) to prepare the standards in a concentration of 1 mg/mL. Fresh samples prepared by diluting supernatant from cell lysis step with 0.9 % NaCl.

B. Preparation of BCA working reagent
As directed by the manufacturer (Elabscience, Houston, TX, USA), a fresh batch of BCA working reagent was prepared. Reagent 1 and 2 are mixed in a 50:1 ratio.

C. BCA protein measurement for standard and cell lysate solutions
According to the manufacturer's instruction, in 1.5 mL Eppendorf tubes, 50 µL of each standard or sample was mixed with 1 mL of the BCA working reagent. Then heated in a heating block to 60 °C for 30 min. The final product was cooled to room temperature for 20 minutes prior to measurements of the absorbance at 562 nm (VWR® UV-6300PC, Avantor USA) in disposable plastic cuvettes. [16]

D. Evaluation after BCA analysis
A standard curve was plotted by using optical density values of the standards and corresponding concentration as y-axis and x-axis respectively. A standard curve in GraphPad Prism 7.00 (for Windows) were created to determine unknown sample concentrations.

Tumor Protein p53 (Human TP53) ELISA kit assay
P53 protein levels were determined using commercial ELISA assays (cat-no. E-EL-H0910, Elabscience, Houston, TX, USA) according to Silong Zhang *et. al*,[18] about 100 µL of the standard and sample were added to the wells and incubated 1 hour and 30 min at 37°C. Then the liquid discarded and immediately 100 µL of detection antibody (biotinylated) added to the working solution to each well, incubated for 60 min. at 37°C. Plates are aspirated and washed 3 times. Followed by addition of 100 µL of Horseradish peroxidase (HRP) conjugate to the working solution. Incubated for 30 min at 37°C. Aspirated and rinsed 5 times. Followed by 90 µL substrate reagent addition. Incubated for 15 min at 37°C. finally 50µL stop solution were added and the absorbance immediately read at 450 nm. (MR-100 Microplate Reader, Genex laboratories, Florida, USA)

Statistical analysis
The statistical analysis was conducted using GraphPad Prism version 7. A mean and standard error of the mean (Mean ± SEM) were used as descriptive statistics for the numerical data. The analysis of variance (ANOVA) test was used for statistical analysis, p-values less than 0.05 were considered significant

Results and Discussion

Cancer's etiology can be traced back to malfunctioning, mutated, or inactive P53 genes. Researchers believe that mutations or changes to the P53 gene are responsible for up to 50% of all malignancies.[19]
The protein content in cell lines was assessed using the BCA protein assay kit. The assay uses the principle of the biuret reaction, where the copper ions in the reagent complex with protein peptide bonds to form a purple-colored complex. The amount of protein in the sample is directly correlated with the color's intensity. Bovine serum albumin (BSA) standards were used to calibrate the assay and create a standard curve. The protein concentration of the samples was then determined by measuring the absorbance of the purple complex and comparing it to the standard curve. This method provided a reliable and accurate measurement of protein concentration in the cell lines.
The absorbance values of the BSA standards were plotted against their known concentrations to generate a standard curve. After measuring the absorbance of the samples and comparing it with the standard curve, the protein concentration of the samples was determined as shown in **Error! Reference source not found.Error! Reference source not found.** and **Error! Reference source not found.**. The proteins in all samples were unified by dilution to make 0.5 mg/mL for each sample before measuring the p53 protein in the samples.
The ELISA p53 protein assay was employed to measure the effect of each treatment on p53 protein expression in SK-GT-4 and AMJ13 cancer cell lines. The assay uses an antibody that specifically recognizes and binds to p53 protein to quantify its levels in the samples. Each cell line was treated with phenolic and terpene fractions, and the levels of p53 protein expression were measured using the ELISA assay. The results of this study on AMJ13 cell line provides compelling evidence that the phenolic fraction is a potent regulator of protein expression. Through rigorous experimentation, it was observed that the phenolic fraction exhibited a remarkable ability to stimulate protein expression, displaying a significant increase in p53 protein expression on both SK-GT-4 and AMJ13 cancer cell lines compared to the terpene treatment and untreated control group (p value <0.05) which promotes the cell apoptosis process in these cells. The results of this study support previous findings on the potent effects of the phenolic fraction on protein expression. Consistent with the research by Vakili *et al*. and Salam *et al*. that indicated the crucial effect of phenolic substances is due to their ability to impede the progression of cancer [20,21] by enhancing the expression of p53, which

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activates pro-apoptotic factors and inhibits anti-apoptotic activity, as well as by preventing the activity of telomerase enzymes.

However, the terpene fraction showed a surprising effect on p53 expression, displaying a decrease in p53 expression in AMJ13 cell line and no significant changes in SK-GT-4 cells compared to the untreated control group (p value > 0.5). This result contradicted to the research conducted by Muhseen et al., which reported that terpenes exhibited upregulated p53 expression level.[22] Also in contrast to previous research, which has shown that terpenes positively impact p53 expression Hepatocellular carcinoma, this study found that terpenes had a negative impact on p53 expression.[23] This difference in findings may be due to a number of factors, including the different types of terpenes used in the studies, the different cell lines used, and the different methods used to measure p53 expression.

Our findings suggest that the relationship between terpenes and p53 expression is more complex than previously thought. These intricate and disparate responses emphasize the need for further mechanistic elucidation and contextual exploration to decipher the underlying molecular interplay. **Error! Reference source not found.** and **Error! Reference source not found.** illustrates the effect of different treatments on p53 protein expression in SK-GT-4 and AMJ13 cancer cell line

Table 1: Illustrates Protein concentration in cell lines with different treatments in BCA protein assay

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Cell line	Treatment	concentration mg
AMJ13	Control	0.5892775
AMJ13	Terpene	0.9997497
AMJ13	Phenolic	1.240407
SK-GT-4	Control	0.9143215
SK-GT-4	Terpene	1.447727
SK-GT-4	Phenolic	1.338337

The table provides information on the protein concentration in different cell lines with various treatments. Each row of the table represents a different cell line, and the columns indicate the treatment and corresponding protein concentration. The protein concentration values were determined using a BCA protein assay kit and calibrated against BSA standards.

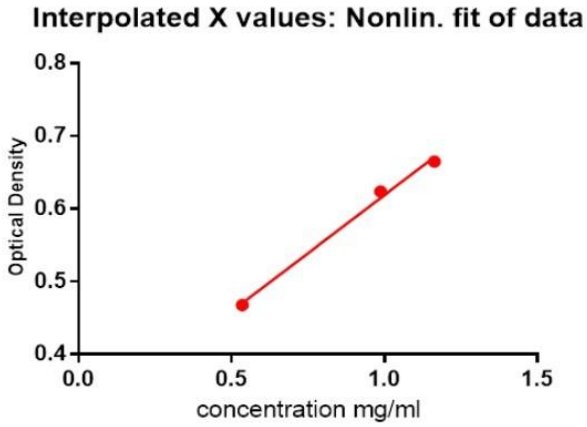

Figure 1: Represents the quantification of protein concentration in cell lines using a BCA protein assay kit. The x-axis of the graph represents the concentration of BSA standards, while the y-axis shows the corresponding absorbance values.

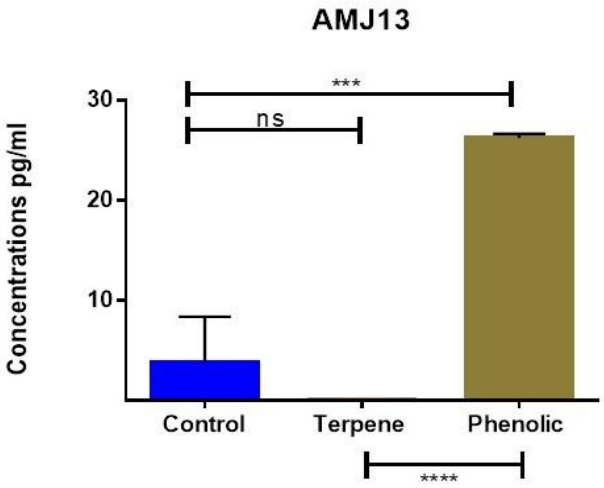

Figure 2: illustrates effect of different treatments on p53 protein expression on AMJ13 cancer cell line. Phenolic fraction showed a significant increase in protein expression in comparison to control and terpene fraction. Terpene fraction showed no significance in comparison to control group.
(*) denotes significant; (*** &****) highly significant; (ns) non-significant. Data are expressed as Mean and SEM.

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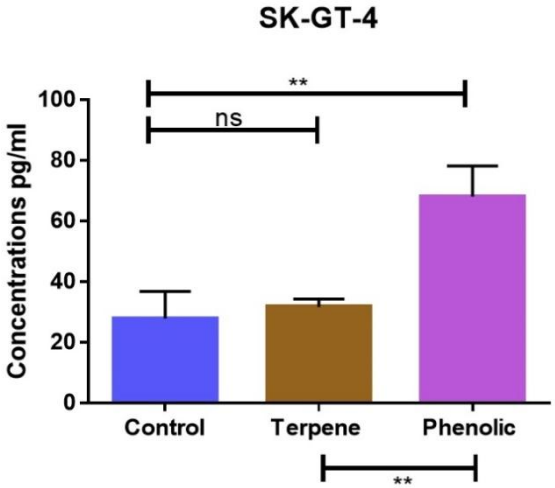

Figure 3: illustrates effect of different treatments on p53 protein expression on SK-GT-4 cancer cell line. Phenolic fraction showed a significant increase in protein expression in comparison to control and terpene fraction. Terpene fraction showed no significance in comparison to control group.
(*) denotes significant; (**) highly significant; (ns) non-significant. Data are expressed as Mean and SEM

Conclusion

The observed augmentation in p53 expression by phenolic fraction lends mechanistic insight into the fraction's cytotoxic attributes, propounding its potential for integration into chemotherapy protocols, either as a stand-alone or adjunctive therapeutic measure. Conversely, the observed unanticipated downregulation of p53 expression induced by the terpene fraction, presents an intriguing and potentially context-dependent modulation of this critical cellular pathway.

In totality, these findings propel the comprehension of intricate pathways governing the bioactivity of plant-derived fractions, substantiating their prospective utility in refined therapeutic paradigms and fostering novel avenues for therapeutic innovation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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