RESEARCH
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THE EFFECT OF *BRASSICA NIGRA* SEED ALCOHOLIC EXTRACT ON ESOPHAGEAL CANCER CELL LINE

Rafal Hussein abdali[1], Aizhar Hamzih Hasan[2], Ali Hmood Al-Saadi[3]
[1] Department of Biology, College of Science, University of Babylon, Iraq. rafalhye04@gmail.com
[2] Department of Biology, College of Science, University of Babylon, Iraq. Sci.azhar.hamza@uobabylon.edu.iq
[3] Department of Biology, College of Science, University of Babylon, Iraq. Alhassanalsaadi9797@yahoo.com

Abstract
Background: The seeds of *Brassica nigra* have important medicinal uses in treatment of rheumatism , joint pains, anticancer agent that lowers the risk of cancer and lowering the growth of cancer cells.
Objectives: The objectives of this work were to extract and analyze the phytochemical components in mustard seed alcoholic extract using GC-MS, as well as investigate the cytotoxicity effect of *Brassica nigra* alcoholic seed extract against esophageal cancer cell line and MDCK normal cell line .
Materials and Methods: Because the seeds of *Brassica nigra* have a range of medicinal properties, Using GC-MS, qualitative analysis was utilized to identify the phytochemicals contained in *Brassica nigra seed* extract. The cytotoxicity test was established using esophageal cancer cell line (KYS-30) and the Madin-Darby Canine Kidney (MDCK) normal cell line was employed to evaluate the crude extract's safety. The cells was cultivated in 96-well microtiter plates. And the plates were subjected to a series of (1000, 500, 250, 125, 62.5, and 31.25 µg\ml) dilution concentrations of alcoholic *Brassica nigra* seed extract alone and in combination with Cisplatin drug. We utilized the crystal violate test to assay the viability of cells.
Results: By using gas chromatography-mass spectrometry (GC-MS), 40 volatile chemicals were found in *Brassica nigra* isopropanolic extract. The extract contained bicyclo[10.10]tridec-1-ene (11.73%) as the most volatile component. The cytotoxicity results show no significant decrease in MDCK normal cell line and a significant ($P \leq 0.05$) decrease in cell viability with *Brassica nigra* alcoholic extract on esophageal cancer cell line (KYS-30) at a concentration of 1000 ug/ml after incubation for 48 hours. When the plant extract was combined with an anticancer drug (cisplatin), the results showed a significant decrease in cell viability at all concentrations on the esophageal cancer cell line (KYS-30).
Conclusion: The crude extract of *Brassica nigra* seed alone or in combination with cisplatin, showed a growth-inhibitory effect as it reduced the viability of the esophageal cancer cell line, and the safety is demonstrated by showing that normal cells are not affected.
Keywords: *Brassica nigra*, Cancer, KYS-30 cell line, Cisplatin , GC-MS.

INTRODUCTION

Medicinal plants have been used as a resource for healing for thousands of years around the world. As well as drug discovery, since 1981 to the present day, about 46% of newly dependable medications are derived from natural sources or natural product mimics(1). The annual plant *Brassica nigra*, also known as black mustard, is grown for its dark brown or black seeds, which belong to the *Brassica* genus (Family: *Brassicaceae*). This weedy annual plant has an enormous of medical benefits (2).
The genus *Brassica* has thirty-seven distinct species. *Brassica* vegetables are abundant in fiber, vitamins, minerals, and fat, along with a number of phytochemicals. It also prevents oxidative stress, stimulates detoxification enzymes, increases immunity, lowers the risk of cancer, and prevents carcinogenic mutations and malignant transformation, in addition to lowering the growth of cancer cells(2). Cancer is a disease that has a significant effect on the human population. Esophageal cancer is sixth globally in terms of cancer-related fatalities in 2020, with 544,000 cases reported worldwide(3). Adenocarcinoma and squamous cell carcinoma are the two primary types of esophageal cancer. While squamous cell carcinomas account for 90% of esophageal carcinomas in Asia, Eastern Europe, and Africa, adenocarcinoma is recognized to be the most frequent type in the West(4).

MATERIALS AND METHODS

Plant collection and identification:

Mustard seed brought from the nearby market in Iraq. The plant were identified by Dr.Neddaa Adnan (Plant Herbarium / Department of Biology / College of Science/ university of Babylon).

Preparation of *Brassica nigra* seed extract:

After the air-dried black mustard seeds were ground into a powder using a mechanical grinder , 36 grams of the powdered seeds were put into a conical flask and 100 milliliters of an 99.7% Isopropanol solution was added into flask and plugged with cotton wool For two days, the beaker's contents were kept at room temperature frequently shaken. alcoholic extract was

filtered many times using medical gauze after that Whitman filter paper was used to filter the separated extract. then, it was centrifuged for five minutes at 1500 rpm in order to remove the solid waste and generate a clear solution, after that we put it in oven at 60 °C. After evaporating the clear solution, the leftover extract was dried, weighed, and the yield was computed and stored at -4°C(5).

Gas chromatography mass spectrometry (GC-MS):
A gas chromatography system and a mass spectrometer (Agilent GC 7890A/MS5975C, Agilent Technologies Inc., Santa Clara, CA, USA) were used to identify the compounds present in the *Brassica nigra* seed extracts. In order to separate the compounds of interest as efficiently as possible and detect them, There was particular temperature programming needed for the GC-MS analysis. For the investigation, we used a positive ionization mode with a detection mode of 70 eV using helium as the carrier gas (1 mL/min). An injection of 1 µL of sample was made while the injector was in split mode and running at 260°C. A GC oven with an Agilent HB-5 MS capillary column was used to perform the separation. After being at 60 °C for four minutes, the temperature was programmed to increase to 100 °C at a rate of 3 °C per minute, and it was then maintained at 250 °C for five minutes(6).

Cancer cell line:
Vials of frozen human esophagus cancer KYS-30 cell line and normal MDCK cell line had been obtained from the Tissue Culture Laboratory at the University of Babylon College of Medicine . to evaluate the cytotoxic effect of *Brassica nigra* seed extract. Cells were grown or maintained upon arrival at 37 °C in incubator with 5% CO2 and 95% atmosphere. All media were supplement with 10% fetal bovine serum (FBS) recommended by American Type Culture Collection (ATTC) (7).

Cytotoxicity assay:
Cytotoxicity was determined by the crystal violate test. MDCK and KYS-30 cells were cultivated in 96-well plates using RPMI-1640 supplemented with 1% gentamicin and 10% FBS, and the temperature was kept at 37˚C. Upon completion of a 24-hour incubation period, the medium was removed, and the cells were subjected to varying doses of of *Brassica nigra* seed alcohiolic extract at (1000, 500, 250, 125, 62.5, and 31.25) µg/mL with three replicates for each concentration of dilution concentrations without control wells .The incubation period was subsequently extended to 48 hours. After incubation period of 48 hours, following the exposure, , the medium was removed from the wells and 200 ul of sterile PBS were used to wash the wells, and 50µl of crystal violet was added to each well. The incubation was continued for 20 minutes. After the removal of the supernatant, the plate was washed with distilled water and allowed to dry at room temperature. The optical density values were determined at 570 nm with Elisa reader .the cellular viability was estimated as % compared to untreated cells (8)
To measure the effect of Cisplatin Drug on KYS-30 cells, KYS-30 cells were cultivated in 96-well plates using RPMI-1640 supplemented with 1% gentamicin and 10% FBS, and the temperature was kept at 37˚C. Upon completion of a 24-hour incubation period, the medium was removed, and the cells were subjected to varying doses of Cisplatin (100, 50, 25, 12.5, 6.25, 3.1) ug/ml in four replicates for each concentration. The incubation period was subsequently extended to 48 hours. After incubation, the crystal violate test was used to measure the cytotoxic effects. The concentration required for a 50% inhibition of viability (IC50) was determined by using an Excel sheet and fitted by blotting graphically of relative cell inhibition percentage in the Y axis versus the concentration of each compound used in the X axis. Calculation of cell viability percentage is done by dividing the absorbance measured for each group by the absorbance of the control group multiplied by 100(9).
Finally the cytotoxicity effect of combination of *Brassica nigra* seed extract and cisplatin Drug on KYS-30 cells Line was evaluate by using 96-well plates seeded with KYS-30 cell lines. 200µl (40µg\ml) of cisplatin were added for each wells without the control wells. the plate were exposed to *Brassica nigra* seed extract in serial dilutions of (1000, 500, 250, 125, 62.5, and 31.25) µg/ ml with three replicates for each concentration. The plate was sealed with a self-closing plastic lid and incubated for a 48-hour period. Following the exposure, 200 ul of sterile PBS were used to wash the wells. By using the crystal violate test, the impact of *Brassica nigra* seed extract and cisplatin drug on KYS-30 cell line growth was evaluated. The optical density values were determined at 570 nm with Elisa reader .the cellular viability was estimated as % compared to untreated cells (8)

Statistical Analysis:
One-way analysis of variance was performed using the SPSS 25 computer program to determine the statistical significance of the variations between the experimental group's and the control group's data. The p-value ($p \leq 0.001$, $p \leq 0.05$) was considered as statistical significance.

Ethical Approval:
There were no animals or humans involved in this study to get their consent. The study procedures, subject information, and agreement form were reviewed and authorized by a local ethics committee of the University of Babylon, College of Science's Biology Department, according to the document with the number B230601 and the date june 6, 2023.

RESULTS AND DISCUSSION

Analyzing alcoholic extract of *Brassica nigra* seed by Gas Chromatography Mass Spectrometer (GC-MS) :
Brassica nigra extract was subjected to GC–MS analysis, and the profile of the fraction showed that specimen included a distinct number of chemical components with varying retention periods and peak areas, as shown in Table 1.
The GC-MS analysis of the active principles in the isopropanolic extract of *Brassica nigra* seed showed the presence of 40 bioactive compounds (Table 3). The major compounds identified were, 13-Docosenoic acid, methyl ester (8.63%) , (Z)- , Stigmasterol, 22,23-dihydro-(11.01%) and Bicyclo [10.10] tridec-1-ene (11.73%) had the highest area peak (%)

Table 1: Phytochemicals identified in the isopropanolic extract of *Brassica nigra* seed by GC-MS .

No	RT (min)	isopropanolic extract of *Brassica nigra* seed	Area%
1	6.121	1-Butene, 4-isothiocyanato-	1.90
2	19.149	4,5-Dimethoxybenzocyclobutenol	4.74
3	26.009	Hexadecanoic acid, methyl ester	2.06
4	26.144	Methyl glycol phthalate	0.20
5	28.556	Linoleic acid, methyl ester	5.97
6	28.613	Methyl linolenate	3.25
7	28.696	8-Octadecenoic acid, methyl ester	6.57
8	28.779	11-Octadecenoic acid, methyl ester	0.75
9	29.148	Heptadecanoic acid, 16-methyl-, methyl ester	0.68
10	29.345	Cyclododecane	0.31
11	31.4	10-Undecenoyl chloride	1.40
12	31.618	11-Eicosenoic acid, methyl ester	1.48
13	31.711	Methyl linolelaidate	0.82
14	31.825	2-Methyl-Z,Z-3,13-octadecadienol	0.37
15	32.028	Eicosanoic acid, methyl ester	0.37
16	32.137	9-Octadecenoic acid, (E)-	0.33
17	33.013	Cyclododecyne	0.39
18	33.117	2-Methyl-Z,Z-3,13-octadecadienol	0.26
19	33.657	2-Chloroethyl linoleate	2.41
20	33.761	9-Octadecenal, (Z)-	3.45
21	34.316	13-Docosenoic acid, methyl ester, (Z)-	8.63
22	34.596	1,2-Benzenedicarboxylic acid, 3-nitro-	0.72
23	34.679	Docosanoic acid, methyl ester	0.16
24	34.861	Cyclohexene, 1-pentyl-4-(4-propylcyclohexyl)-	0.17
25	35.156	Cyclopentadecanone, 2-hydroxy-	0.62
26	35.54	Erucic acid	0.64
27	36.355	Bicyclo[10.1.0]tridec-1-ene	11.73
28	36.687	trans-.delta.(sup 9)-Octadecenoic acid	0.20
29	36.817	trans-.delta.(sup 9)-Octadecenoic acid	0.32
30	37.393	Erucylamide	3.40
31	38.155	11,13-Eicosadienoic acid, methyl ester	0.57
32	38.747	Erucic acid	7.14
33	40.589	gamma.-Tocopherol	3.54
34	41.128	1,1,1,3,5,5,5-Heptamethyltrisiloxane	0.23
35	41.284	Acetic acid, [4-(1,1-dimethylethyl)phenoxy]-, methyl ester	0.24
36	41.538	D,.alpha.-Tocopherol	0.48
37	41.974	Ergosta-5,22-dien-3-ol, (3.beta.,22E,24S)-	4.53
38	42.83	23 S-METHYLCHOLESTEROL	6.06
39	43.157	Brotizolam	1.91
40	44.2	Stigmasterol, 22,23-dihydro-	11.01

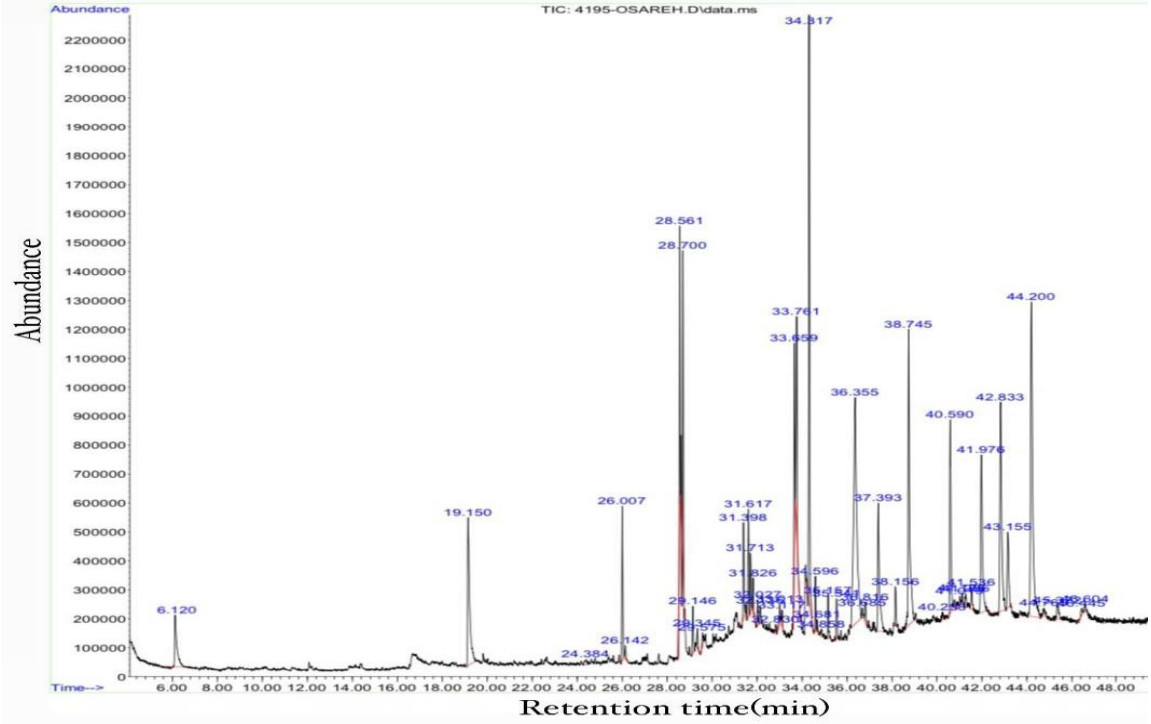

Figure 1: GC-MS chromatogram of isopropanolic extract of *Brassica nigra* seed.

The pharmacological uses of some chemicals found in *Brassica nigra* seed extract are discussed below: Erucic acid is a crucial raw material in the oleochemical sector, utilized in the production of pharmaceuticals, soaps, detergents, cosmetics, plastics, lubricants, rubbers, and coatings. It also has the ability to reduce corrosion and to slide, foam, emulsify, and soften. There was widespread recognition of stigmasterol's antibacterial, anti-cancer, anti-arthritic, anti-asthmatic, diuretic, and anti-inflammatory qualities. Tocopherol, commonly known as vitamin E, is a powerful antioxidant (10).

Vitamin E is a good antioxidant with a wide range of pharmacological activities, including hepatoprotective, analgesic, antidiabatic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, and anticancer properties. Isopropanolic seed extract contains hexadecanoic acid, which has biological activity including nematicide, hypocholesterolemic, and antioxidant properties (11). Like hexadecanoic acid, octadecadienoic acid exhibited anti-arthritic, anti-coronary, hepatoprotective, anti-histaminic, insectifuge, anti-eczemic, and anti-acne properties (10).

It was discovered that eicosanoic acid had anti-inflammatory properties.1-Butene , 4 isothiocyanato, exhibit antioxidant properties. (12). Ergosta-5,22-dien-3- beta-ol It was reported to possess antimicrobial properties. (13).

Cytotoxicity effect of alcoholic extract of *Brassica nigra* seed on MDCK cell Line at different concentrations after incubation for 48 hours:

The MDCK cells were treated with serial concentrations (31.25-1000 µg/ml) of different *Brassica nigra* seed extract, cells were incubated for 48 hours at 37 C, then viability were assessed using crystal violate test.

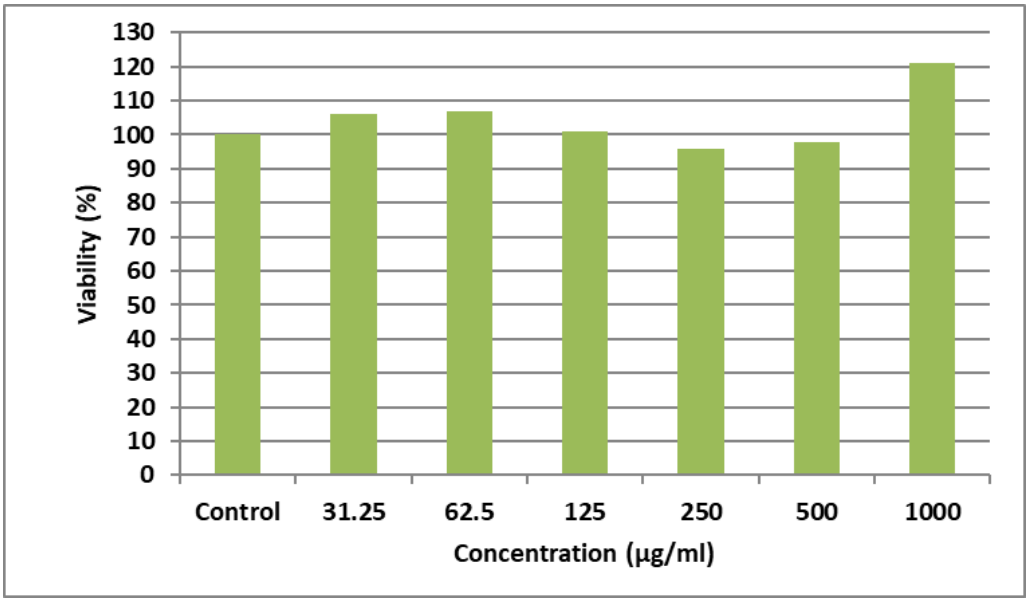

Figure 2: Cells viability percentage of MDCK cell line at different concentrations of *Brassica nigra* extract after incubation for 48 hours by crystal violate test.

Results on the green column doesn't show a significant ($P \leq 0.05$) decrease in cell viability with *Brassica nigra* isopropanolic extract at all concentration .

Our results agree with a study that evaluated the cytotoxicity of *A. paniculata* extracts on MDCK and Vero cells. The results showed that compared to Vero cells, MDCK cells showed higher plant extract resistance (14).

Cytotoxicity effect of alcoholic extract of *Brassica nigra* seed on KYS-30 esophageal cancer cell Line at different concentrations after incubation for 48hours:

Treatment of esophageal cancer with available medicines is quite difficult. So using components that are naturally derived, like plant chemicals, can be considered as a novel approach. *Brassica* vegetables are beneficial to humans because they may help prevent cancer through chemoprevention. Several studies evaluated *B. nigra* potential health benefits as an antioxidant, hypoglycemic, anticonvulsant, anticancer, and antibacterial agent(15).

Our research aimed to determine whether *B. nigra* seed extract had an impact on the proliferation of KYS-30 esophageal cancer cell line. Additionally, we studied the impact of combination of *Brassica nigra* seed extract with Cisplatin drug on KYS-30 esophageal cancer cell line.

The KYS-30 esophageal cancer cell line were treated with serial concentrations (31.25-1000 µg/ml) of both types of alcoholic extract alone and alcoholic extract in combination with drug, cells were incubated for 48 hours at 37 C, then viability were assessed using Crystal violate test . Results are shown in (figure 2) and (figure 5).

Treatment with alcoholic extract resulted in a significant ($P \leq 0.05$) decrease in cell viability at concentrations of 1000 µg/ml when compared to control groups after incubated for 48 hours . while other concentrations do not have a significant decrease in viability of the cell.

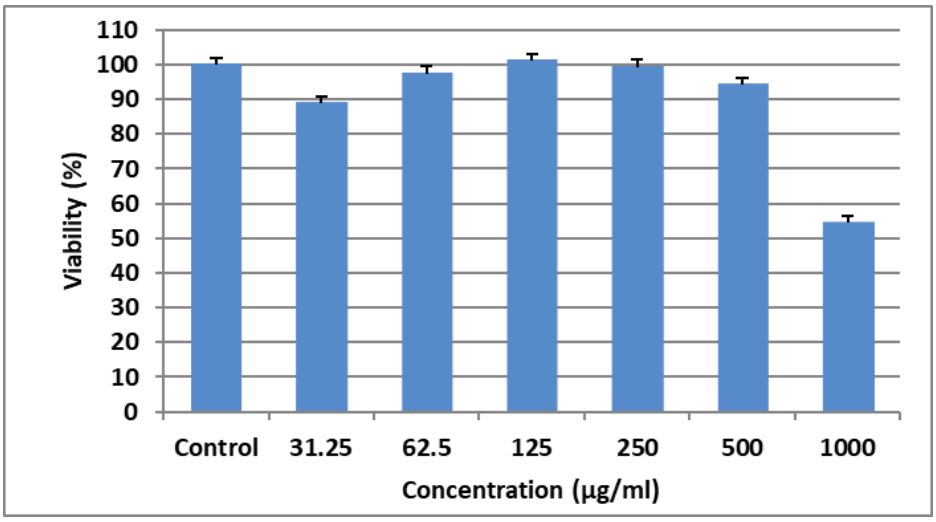

Figure 3: Cells viability percentage of KYS-30 esophageal cancer cell line at different concentrations of *Brassica nigra* alcoholic extract after incubation for 48 hours by crystal violate test.

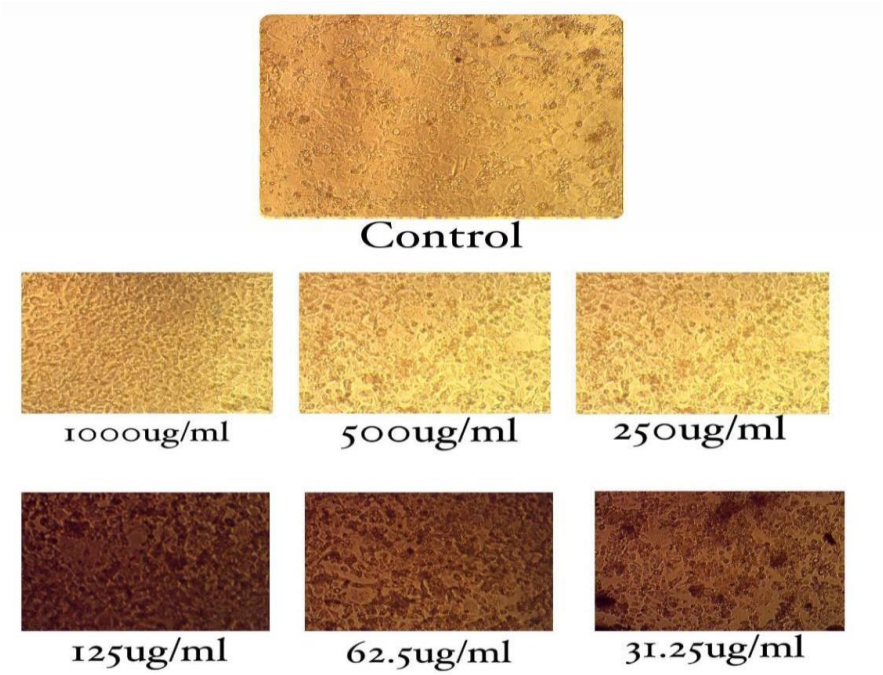

Figure 4: Esophageal cancer cell line after treated with serial concentrations (31.25- 1000 µg/ml) of *Brassica nigra* alcoholic extract.

The antiproliferative properties of *B. nigra* extracts have been shown against human hepatocellular (HepG2), cervical (HeLa), colorectal (HCT), and breast cancer (MCF-7) cells(7).
The results of this study indicate that the high potency of *Brassica nigra* alcoholic seed extract can inhibit the growth of KYS-30 tumor cells in comparison with the control cells, which is dependent on the dose .our result in (figure 2) agree with study the antiproliferative effect *of B. nigra* seed ethanolic extract against Human Lung Cancer which show that *B.nigra* ethanolic extract significantly inhibits the growth of A549 and H1299 human lung cancer cells. This effect may be attributed to the induction of apoptosis and the regulation of the cell cycle through replication stress, which causes DNA lesions known as fork-collapse(15).
An additional study of the cytotoxic effects of *Juniperus excelsa* extract on the KYSE-30 esophageal cancer cell line. The results show that the number of viable cells significantly decreases when the extract concentration is increased(16).
Study by Vahedi Larijani *et al*.,2012 show that the phytochemicals were found in alcoholic extracts of avocado fruit and leaves is considered to be the source of the extract's cytotoxic activity on esophageal cancer cells (KJSE cells), which inhibits cell growth by raising intracellular oxygen radical activity and blocking intratumoral growth signals(17).
Since they are currently unknown, more investigation is required to identify the specific phytocomponents of the mustard seed (*B. nigra*) extract that are causing the anticancer effect. A wide range of bioactive phytochemicals are known to be present in mustard seeds. One of the medicinal components in *B. nigra* that has shown promise in treating cancer is isothiocyanates. Glucosinolates, these compounds' precursors, are hydrolyzed to create them. Two important isothiocyanates that are formed from cruciferous plants are indole-3-carbinol and sulforaphane. Both in vitro and in vivo, sulforaphane has been shown to affect a variety of cell signaling pathways that result in anticancer effects(18),(19). Similar to this, indole-3-carbinol's anticancer action has been linked to its capacity to obstruct several oncogenic signaling pathways that regulate the aggressive behaviors, invasion, and cell cycle progression of cancerous cells(20),(19).
There have been reports of the cytotoxic effects of allyl isothiocyanate, which is found in mustard seeds, against lung cancer(21). Allyl isothiocyanate used proapoptotic and antiangiogenic pathways to suppress the formation of Ehrlich ascites tumors in mice(22).

Cytotoxicity effect of alcoholic extract of *Brassica nigra* seed combined with cisplatin on KYS-30 esophageal cancer cell Line at different concentrations after incubation for 48hours:
Following treatment of the KYS-30 cancer cell line with a concentration of (100-3,5) µg/ml of cisplatin, we found that the cisplatin drug's IC50 value on the KYS-30 cell line was 40 µg/ml.

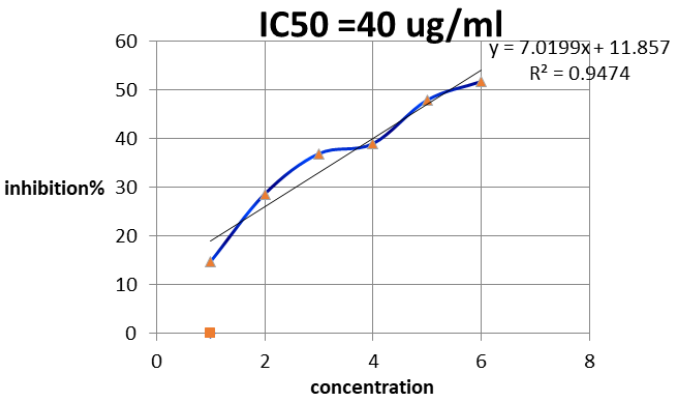

Figure 5: Cisplatin IC 50 value on KYS-30 esophageal cancer cells.

Treatment with combination of alcoholic extract with Cisplatin drug show a significant ($P \leq 0.05$) decrease in cell viability with concentration of (500,1000) ug /ml and significant ($P \leq 0.001$) decrease in cell viability with concentrations of (31.25,62.5,125,250) ug /ml after incubated for 48 hours.

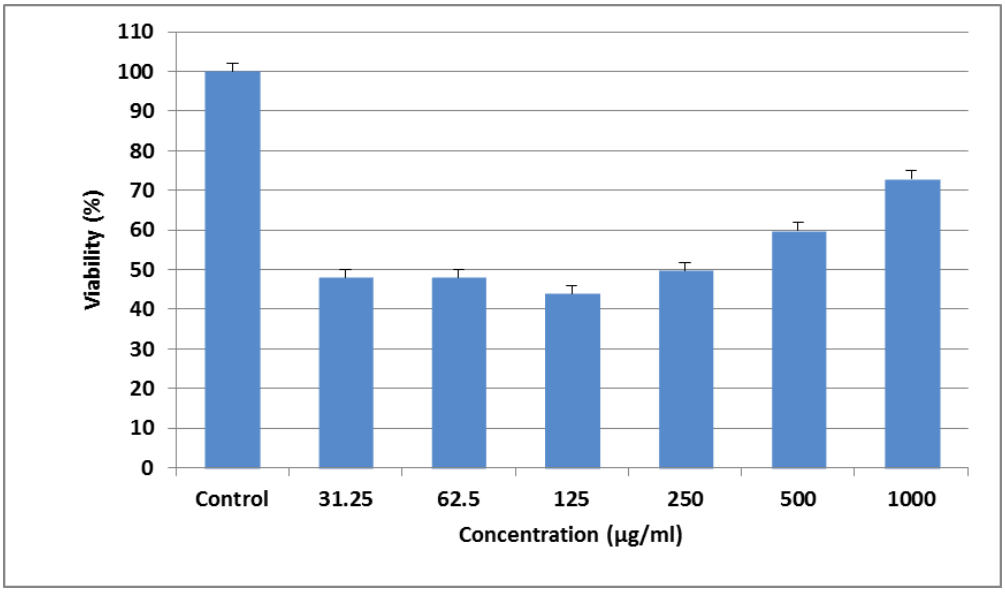

Figure 6: Cells viability percentage of KYS-30 cell line at different concentrations of *Brassica nigra* isopropanolic extract and cisplatin after incubation for 48 hours by crystal violate test.

One of the most promising and commonly used medications for the treatment of a variety of solid tumors, including testicular, ovarian, head and neck, bladder, lung, cervical, melanoma, lymphomas, and several others, is cisplatin, also known as (SP-4-2)-diamminedichloridoplatinum(II). Although cisplatin has anticancer properties through a variety of mechanisms, the most

plausible one is the creation of DNA lesions by interaction with purine bases on DNA, which is followed by the activation of various signal transduction pathways and ultimately apoptosis. However, the two main problems with cisplatin that restrict its use and efficacy are side effects and drug resistance(23).

The field of drug discovery has directed its focus towards combination anticancer treatments that utilize plant extracts' active ingredients. Rich in flavonoids, phenolic compounds, and other phytochemicals, natural plant extracts have a lower potential for side effects than chemotherapy drugs, making them effective anticancer treatments. Several studies show how reducing host cell cytotoxicity and maximizing anticancer treatment can be achieved through combination drug-herb interactions(24).

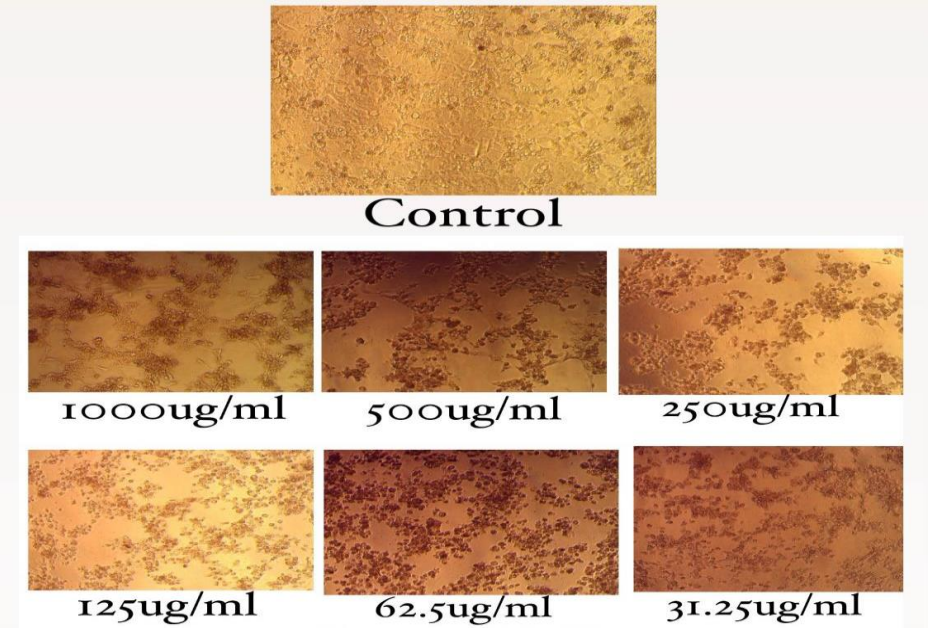

Figure 7: Esophageal cancer cell line after treated with serial concentrations (31.25- 1000 µg/ml) of *Brassica nigra* isopropanol extract combined with cisplatin drug.

This study aimed to identify novel natural compounds that could lower cancer cell proliferation. Related research using a combination of cisplatin and plant extract produced corroborated results, demonstrating that extracts from *Terminalia bellerica* and *Phyllanthus emblica* were particularly toxic to two cancer cell lines and that this combination, along with cisplatin and dovorubicin, increased the extracts' ability to inhibit tumor growth in both A549 and HepG2 cells(25).

Another study show that combination of sulforaphane and cisplatin reduces the stemness and metastatic potential of triple-negative breast cancer TNBCs, By downregulating the sirtuin-mediated Epithelial-to-mesenchymal transition EMT signaling axis(26)

Relate study show that when combined phenethyl isothiocyanate (PEITC), which is predominantly generated from watercress, a member of the *Brassica* family , with cisplatin drug , can enhance cisplatin-induced apoptosis in cervical cancer cells and its mechanisms of action(27).

CONCLUSION

We conclude that an isopropanol extract of *B. nigra* seeds, both alone and in combination with cisplatin medication, has significant antiproliferative action in KYS-30 human esophageal cancer cells based on the data obtained in this study overall, the results of this investigation point to the possibility of developing phytoconstituents derived from *B. nigra* as a chemopreventive and therapeutic agent for esophageal cancer. To fully understand the importance of the findings presented here, however, more research including in vivo experiments is required.

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