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Phytochemical constituents and cytotoxic effects of *Brassica juncea* extract on different human cancer cell lines *in vitro*

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ABSTRACT

Oxidative stress has been known as the main cause of the development of many diseases. Therefore, supplementation of exogenous antioxidants and elevating of endogenous antioxidant defenses may be the effective way of combating the harmful action of reactive oxygen species, which considered the main reason of oxidative cell damage. The study was conducted to identify the main polyphenolic components of *Brassica juncea* seeds and investigate their cytotoxic effects against different cancer cell lines. Also, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) antioxidant assay and erythrocyte hemolysis were tested against ascorbic acid. The cytotoxic activity of *B. juncea* on all cancer cell lines was significantly higher than the chemotherapeutic drug doxorubicin (Dox). These results confirm that *B. juncea* has a strong cytotoxic effect on most tumor cells; the viability of the six cancer cell lines was significantly decreased when using *B. juncea* extract compared to doxorubicin. *B. juncea* extract showed cytotoxic effect on cancer cells compared to Dox, however, appropriate dose must be determined.

Keywords; *B. juncea*, doxorubicin, cancer cell lines, phenolic compounds.

1. Introduction

The phytochemical and biological studies on the natural crude extracts from plants are considered an important source for discovery of new drugs beneficial to human health. Natural products, especially plant derived constituents are often considered as source of drugs, and a great part of the pharmaceuticals available in modern medicine that are directly or indirectly obtained from natural sources (Ahmed, and Kamel, 2013) .It is important to consume fruits and vegetables as they contain important natural antioxidants such as vitamins C, E, and phenolic compounds, which reduce the possibility of inflammation and chronic diseases. Antioxidants are the first line of defense towards reactive oxygen species (ROS) by preventing oxidative damage of cells and protecting the human body against various diseases as cancer, obesity, diabetes and cardiovascular disorders Ghanad et al., 2019). Cruciferous vegetables, especially those included into the Brassica family such as broccoli and cabbage are an important source of many nutrients and health-improving phytochemicals (Hindson, 2020).

Phytochemicals in Brassicaceae family may induce different effects such as decrease oxidative stresses, induces antioxidant enzymes, activate the immune system, reduces propagation of cancer cells and inhibits malignant transformation and carcinogenic mutations (Horst et al., 2010). They protect our body against ROS that cause DNA damage, modification of gene expression, in addition to lipid and protein oxidation. Most of the Brassicaceae family is known for their antitumor

activity and they play an important role in the protection from many diseases like liver damage, vasospasm, atherosclerosis, heart attack, stroke, and different cancer types (Kapusta-Duch et al., 2012). *B. juncea (L.),* also known by the name of Indian mustard, belongs to the plant family Brassiceae (Cruciferae) or the mustard family which comprises about 375 genera and 3200 species. *B. juncea* is the most common crop than rapeseed-mustard crops in India; where they represent more than 90% of the area (Yadava et al., 2011). *Brassica* is the most famous and important genus in the Brassicaceae (mustard) family and *B. juncea* is a member of this genus. The members of this genus are collectively named cruciferous vegetables such as cauliflower, cabbages, broccoli and brussels sprouts, or mustards.

Cancer is a significant public health problem either in developed or developing countries. Since 1990, there has been a 22% expansion in cancer occurrence and mortality with the four most widespread cancers type being lung, breast, colorectal, and stomach (Balunas et al., 2005). Medicinal plants have played significant roles in the treatment of cancer due to the presence of secondary metabolites (Shabana et al., 2013). Furthermore, hemolytic activities of *B. juncea* extract on human erythrocytes were investigated in this study (Herr and Büchler, 2010; Zohra and Fawzia, 2014). The aim of this study was to identify the main polyphenolic components of *B. juncea* seeds and investigate the cytotoxic effects of *B. juncea* extract against six cancer cell lines included hepatocellular carcinoma (HepG-2); colorectal carcinoma (HCT-116); breast cancer (MCF-7); prostate cancer (PC3); laryngeal carcinoma (HeP2); cervical carcinoma (Hela): In this study *B. juncea* was compared with "Dox" as s standard chemotherapeutic drug. Also, $ABTS^{\cdot+}$ antioxidant assay and erythrocyte hemolysis were tested against ascorbic acid.

2. Materials and methods

Preparation of investigated *B. juncea* extract

Extraction of *B. juncea* was prepared according to the method described by Dent et al. (2013). Accurately 100g of *B. juncea* was extracted using 1.0 L of ethanol (30%), then performed at 60°C for 30 minutes on a horizontal water bath with shaker (Memmert WB14, Germany). The resulting extract was then filtered by using filter paper of the type (Whatman no.1 International Ltd., Kent, UK) and a Büchner funnel, and then the filtrate was adjusted to 100mL in volumetric flasks with appropriate deionized water. The extract was stored at –18°C till use.

Fractionation and identification of phenolic compounds

Phenolic compounds were specified using high performance liquid chromatography (HPLC) Technique at Food Safety and Quality Control (FSQC) Laboratory, Children's Hospital, Mansoura University, Egypt using Agilent 1260 infinity HPLC Series (Agilent, USA), equipped with quaternary pump, aKinetex®5µm EVO C18 (100mm×4.6mm), phenomenex, USA, operated at 30˚C. According to Yang et al. (2014), the separation process was carried out by using a triple linear elution gradient: (A) HPLC grade water 0.2% H_3PO_4 (v/v), (B) methanol, and (C) acetonitrile. The injection volume was 20µL and detection VWD detector set at 284nm. All standard polyphenols were obtained from (Sigma Company) and were dissolved in the mobile phase and injected directly into HPLC instrument. Retention time and peak area were used to calculate the concentrations of phenolic compounds content by analyzing the data of Hewllet packed software.

Cytotoxicity and viability using MTT assay

Six cancer cell lines are used in this study namely; HepG-2, HCT-116, MCF-7, PC3, HeP2, and Hela were obtained from ATCC via holding company for biological products and vaccines (VACSERA), Egypt. The effect of *B. juncea* extract on cell lines would compare with dox. Cytotoxicity was known as the concentration that induced 50% death of cell monolayer (IC_{50}). The colorimetric assay MTT was used for measuring cytotoxicity as an indicator for cell growth according to Bondock et al. (2012). The method depends on the reduction process of the yellow dye MTT which is reduced to purple formazan by the effect of oxidoreductase enzymes related to living cells. RPMI-1640 and 10% fetal bovine serum was used as a medium for culturing our cancer cell lines, also antibiotics were added (100 units/mL penicillin and 100µg/mL streptomycin)

at 37°C in a 5% CO_2 incubator. Then cancer cell lines were seeded in a 96-well plate at a density of 1.0×10^4 cells/well at 37°C for 48 h under 5% CO_2, then the cells were treated with different concentrations of *B. juncea* extract versus doxorubicin and incubated for 24 h. After 24 h of treatment, 20 µl of MTT solution at 5mg/mL was added then incubated for 4 h. Add 100µL of DMSO to each well to dissolve the formed purple formazan. Absorbance of the resulted colour was measured at wavelength 570 nm. IC_{50} was determined through non-linear regression, type sigmoidal, analyzed using Origin 8.0® software (OriginLab Corporation). The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) ×100.

$ABTS^{\bullet+}$ Radical scavenging activity

The appropriate amount of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) El-Refai et al. (2018), transfer diammonium salt and potassium persulfate to 10.0 mL flask then dilute until reach final concentrations of 7.0 and 2.45 mM, respectively. Left the solution in the dark for about 12 to 16 hours to form the $ABTS^{\bullet+}$ radical. Subsequently, the solution of the $ABTS^{\bullet+}$ was diluted with ethanol until the absorbance of the solution was 1.0Au. Determine the radical scavenging activity (RAS) of the extract against $ABTS^{\bullet+}$ after mixing 0.5 mL of the examined sample diluted in 1-butanol with 2.0 mL of the alcoholic solution $ABTS^{\bullet+}$ then read the absorbance at wavelength 734 nm after 15 min. At least five measurements were made for each sample tested, and the percentage of RSA was also estimated by the following equation: Abs (control)- Abs (test)/ Abs (control) X100

Erythrocyte hemolysis assay

Preparation of erythrocytes suspension

Collect 5mL whole blood from healthy rats and transfer immediately into tubes containing EDTA as anticoagulant. The blood was centrifuged at 1500 rpm for 5 min. Plasma was aspirated with micropipette and red blood cells were washed using phosphate buffer saline (pH 7.4). The washing process was repeated three times. The final washed red blood cells were suspended to 0.5% using normal saline (Kumar et al., 2011).

Hemolytic activity

Approximately 0.5mL of different *B. juncea* extract concentrations (125, 250, 500 and 1000 µg/mL in phosphate buffer saline) was mixed with 0.5mL of re-suspended red blood cells. The mixtures were then incubated 30 min for 37°C then centrifuged 10 min at 2000 rpm. Free hemoglobin was measured by reading the supernatant absorbance using spectrophotometer at 540 nm. Negative control was prepared using phosphate buffer saline without extract and positive control using distilled water without extract. Erythrocyte hemolytic activity was calculated for each *B. juncea* concentration from triplicate readings which expressed as the percentage of hemolysis using the following equation (Kumar et al., 2011):

$$\%\ \text{Hemolysis} = \frac{A_t - A_n}{A_c - A_n}\ \text{Hem}$$

Where: A_t is the sample absorbance.
A_n is the negative control absorbance (saline control)
A_c is the positive control absorbance (water control)

Statistical analysis

Statistical analyses of all experimental data were done using the statistical software package CoStat, Version 6.450 (CoStat-Statistics Software, (2017). All comparisons were first subjected to one way completely randomized analysis of variance (ANOVA) and significant differences between treatment means were determined using Duncan's multiple range tests at $p \leq 0.05$ as the level of the significance (Duncan, 1955).

3. Results

Polyphenolic components of *B. juncea* extract

Data in Table (1) showed the quantitative analysis of polyphenols in *B. juncea* extract by HPLC technique. It could be noticed that neringein was the predominant polyphenol in concentration (4431.42 ppm), followed by myricetin, quercitin, benzoic acid, ellagic acid, and rosemarinic acid (1489.55, 868.571, 486.781, 401.242, and 344.456 ppm, respectively). While the rest of polyphenols

ranged from125.121 ppm for syringic acid to 1.476 ppm for rutin.

The cytotoxic effects of *B. juncea* extract on human tumor cells

The findings showed that *B. juncea* had a considerably greater cytotoxic effect on all cancer cell lines than Dox (Table 2). *B. juncea* was found to be a potent active agent against HePG-2, HCT-116, HEP2, and HeLa cell lines (IC_{50} = 10.95±1.1 µg, 13.72±1.2 µg, 19.63±1.5 µg, and 12.53±1.1 µg, respectively). Additionally, *B. juncea*e exhibited moderate activity against MCF-7 and PC3 cell lines (IC_{50} = 25.71±2.0 µg and 32.09±2.5 µg, respectively). These findings support the notion that *B. juncea* is highly cytotoxic to the majority of tumor cells.

Cell viability

The results shown in Table 3, revealed that *B. juncea* was able to decrease the viability of cancer cell lines and inhibit the growth of cancer cell lines under investigation in a dose dependent method

Table 1. Polyphenolic components of *B. juncea* extract

Compound	Conc. (ppm)	Compound	Conc. (ppm)
Pyrogallol	9.259	Ferulic acid	40.958
Quinol	14.76	Benzoic acid	486.781
Gallic acid	68.394	Rutin	1.476
Catechol	2.015	Ellagic acid	401.242
p-Hydroxy benzoic acid	24.572	o-Coumaric acid	15.575
Caffeine	71.056	Salicylic acid	58.541
Chlorgenic acid	3.365	Myricetin	1489.55
Vanillic acid	101.726	Cinnamic acid	2.517
Caffeic acid	89.603	Quercitin	868.571
Syringic acid	125.121	Rosemarinic acid	344.456
Vanillin	2.685	Neringein	4431.42
p-Coumaric acid	18.014	Kampherol	98.146

Table 2. *In vitro* cytotoxic activities of *B. juncea* extract against human cell lines compared with Dox

	In vitro cytotoxicity IC_{50}± SD (µg/ml) •					
	HepG-2	**MCF-7**	**HCT-116**	**PC3**	**HeP2**	**HeLa**
Dox	4.50±0.2	4.17±0.2	5.23±0.3	8.87±0.6	8.54±0.6	5.57±0.4
B. juncea	10.95±1.1	25.71±2.0	13.72±1.2	32.09±2.5	19.63±1.5	12.53±1.1

IC_{50} (µg/ml): 1–10 (very strong), 11–20 (strong), 21–50 (moderate), 51–100 (weak) and above 100 (non-cytotoxic).

Table 3. Average relative viability of cells (%) using different concentrations of *B. juncea* and Dox.

	Conc.(µg)	HePG-2	MCF-7	HCT-116	PC3	HeP2	Hela
Dox	100	6.3^{v}	6.2^{v}	7.1^{uv}	8.8^{uv}	8.4^{uv}	7.3^{uv}
	50	11.2^{tuv}	10.9^{tuv}	13.9^{stuv}	16.3^{rstuv}	15.3^{stuv}	12.1^{stuv}
	25	14.1^{stuv}	14.3^{stuv}	18.7^{qrst}	21.7^{pqrs}	21.7^{pqrs}	18.9^{qrst}
	12.5	28.3^{nopq}	26.9^{opq}	31.4^{mno}	38.9^{jklm}	37.9^{klm}	30.8^{mnop}
	6.25	45.8^{ijkl}	41.5^{jkl}	47.9^{ij}	59.2^{gh}	58.2^{gh}	51.7^{hi}
	3.125	57.6^{gh}	58.4^{gh}	60.5^{gh}	73.6^{cde}	72.6^{cde}	62.4^{fg}
	1.56	71.2^{de}	69.1^{ef}	73.8^{cde}	95.3^{a}	94.3^{a}	74.0^{cde}
	Conc.(µg)	**HePG-2**	**MCF-7**	**HCT-116**	**PC3**	**HeP2**	**Hela**
B. juncea	100	13.7^{stuv}	26.7^{opq}	19.8^{qrst}	30.6^{mnop}	20.3^{qrst}	16.9^{rstu}
	50	21.5^{pqrs}	38.2^{klm}	27.1^{opq}	41.7^{jkl}	31.4^{mno}	25.3^{opqr}
	25	27.9^{nopq}	47.0^{ijk}	36.4^{lmn}	52.3^{lmn}	43.7^{jkl}	32.2^{mno}
	12.5	42.3^{jkl}	60.4^{fg}	45.2^{ijkl}	64.4^{fg}	52.9^{hi}	43.1^{jkl}
	6.25	63.4^{fg}	75.3^{cde}	60.7^{gh}	$78.9b^{cd}$	71.8^{cde}	65.0^{fg}
	3.125	76.1^{cde}	93.9^{a}	83.3^{b}	97.5^{a}	95.5^{a}	80.4^{bc}
	1.56	98.8^{a}	100^{a}	100^{a}	100^{a}	100^{a}	97.5^{a}

ABTS$^{\bullet+}$radical scavenging activity of *B. juncea* against ascorbic acid

As shown in (Table 4) the radical scavenging activity of *B. juncea* extract was (46.0%), compared with that of vitamin C (88.1%), indicating that the *B. juncea* extract has a moderate antioxidant activity related to vitamin C.

Table 4. ABTS•+ radical scavenging assay (RSA) of *B. juncea* against ascorbic acid

Compounds	ABTS$^{\bullet+}$ Radical Scavenging	
	% RSA*	Absorbance of samples
Control of ABTS	0	0.520
Ascorbic acid	88.1	0.062
B. juncea	46.0	0.281

*****RSA% = Abs (control) - Abs(test) /Abs(control)×100

Erythrocyte hemolytic activities of *B. juncea* extract

In our study we examined the hemolytic activity of different *B. juncea* concentrations and as shown in Table (5) the hemolytic activity was expressed as the percentage of hemolysis and reported as mean ± SD. From the results *B. juncea* showed a dose dependent increase in hemolysis.

Table 5. Erythrocyte hemolytic activity of B. juncea

B. juncea concentrations (µg/mL)	% Hemolysis
125	0.8 ± 0.07
250	2.1 ± 0.05
500	3.7 ± 0.08
1000	5.4 ± 0.07

4. Discussion

The current study aimed to elucidate the antioxidant effect of *B. juncea* on different cancer cell lines *in vitro*. The main reasons for cancer cells proliferation are the unlimited and uncontrolled growth of cancer cells in addition to inactivated apoptosis. Therefore,by growth inhibition and apoptosis restoration in cancer cells would be an effective pathway either in prevention or treatment of cancer (Kwaket al., 2016). In this study the results revealed that the cytotoxic activity of *B. juncea* was significantly higher than the chemotherapeutic drug doxorubicin, but this activity was strong on HepG-2, HCT-116, HEP2, and HeLa. this strong cytotoxic activity of *B. juncea* may be due to the high absorption capacity of these cell lines, which enhancing the cytotoxic action of the extract, but it has a moderate activity on MCF-7 and PC3 cell lines and this may be due to the high resistance of these cell lines (Miceli et al., 2020; Mandrich et al., 2020). The World Cancer

Research Fund points out that diet rich in *Brassica* vegetables protect the human body especially from colon, rectum, and thyroid cancers (Gupta, 2011). Moreover, when consumed in high amounts as a part of the diet beside other vegetables, *Brassica* vegetables generally have protective effects against cancer (Glade, 1999). This anticancer activity is also attributed to the compounds resulting from hydrolysis (Holst, and Williamson, 2004). The capability of the components of brassica vegetables to alter biotransformation enzyme expression also, its activities play an important role in cancer prevention.

The current study found that the viability of the six cancer cell lines was significantly decreased when using *B. juncea* extract compared with doxorubicin, and this decrease in viability is dose dependent where it is inversely proportional with the concentration used either of *Brassica* extract or doxorubicin chemotherapy. The decrease of viability means that there is a decrease in cell growth, which may be due to the impact of some compounds found in *B. juncea* extract (Mohammed et al., 2013). The current study showed the ability of different concentrations of *B. juncea* to decrease the viability of under investigated cancer cell lines compared with the same concentrations of doxorubicin as a chemotherapeutic cancer therapy. These results confirm that *B. juncea* has strong growth-inhibitory activities that may acquire partially by its apoptosis inducing activity.

Oxidative stress may start molecular events in the carcinogenesis, and reducing oxidative stress protects against the occurrence of cancer (Hwang and Bowen, 2007). The Cruciferae family contains many antioxidant substances that may induce oxidant enzymes that protect against carcinogenesis (Williamson et al., 1996). The *B. juncea* extract was screened for its antioxidant activity using the ABTS method compared with vitamin C as positive control. These findings support the antioxidant activity of the volatile constituents obtained from *B. juncea*. Our results were in the same line with many studies (Nawaz et al., 2018), which stated that *Brassica* plants are rich source of phytochemical compounds, which have strong antioxidant potential. Antioxidants such as polyphenols, vitamin C, vitamin E, and carotenoids present in many medicinal plants and natural herbs in large amounts and they have several roles in eliminating free radicals by work as antioxidants, by neutralizing, quenching or reducing peroxides (Eloff, 1998). These natural compounds that have antioxidant potential can be used as a remedy against oxidative damage and its related diseases (Middleton et al., 2000). Most recent studies are interested in substitution of synthetic antioxidants with natural antioxidants to prevent the potential toxicity of synthetic ones (Gulcin et al., 2008).

The results of our study showed that *B. juncea* extract induced hemolysis in a dose dependent manner, only high concentrations of the extract may induce high hemolysis; some components of the extract may induce hemolysis by permeability and destroying erythrocyte membrane (Mouffouk et al., 2020). Hemolysins are compounds capable of lysing the cytoplasmic membrane, inducing cell lysis and death. The activity of these toxic substances is most easily observed with any process causing the lysis of red erythrocytes (Zohra, and Fawzia, 2014). As we support the utilization of *B. juncea* in therapy for its antioxidant properties it was important to study its effect in the process of erythrocyte hemolysis. The results of this investigation showed that *B. juncea* extract, particularly on hepatocellular, colorectal, epidermoid larynx, and epithelioid cervical carcinomas, has a good cytotoxic impact on cancer cells; however, the right dose needs to be found. The ability of different concentrations of *B. juncea* can decrease the viability of investigated cancer cell lines compared with the same concentrations of Dox. These results confirm that *B. juncea* has strong growth-inhibitory activities that may be acquired partially by its apoptosis inducing activity.

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