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Cytotoxic effect of *Brassica oleracea* on liver cancer cell lines

Vanitha V*, Pushpabharathi N, Kripa KG, Bothiraj K.V, Murugan K

Department of Biochemistry, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 600 117 Tamil Nadu, India

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ABSTRACT

The present study was conducted to evaluate the phytochemical and to assess the anti-carcinogenic activity of *Brassica oleracea* on hepatocellular carcinoma. The extraction was done using ethanol solvent and the collected extract was analyzed for its phyto-compounds. The presence of flavonoids, terpenoids, tannins, alkaloids, phenols, carbohydrates, glycosides and amino acid were observed. Then the extract was estimated for its total protein content by Lowry's method. The total protein content of 0.2ml and 0.4ml of b ethanol extract were determined to be 10mg and 11.5mg respectively. To justify the anticancer property, *Brassica oleracea* ethanol extract has been subjected to MTT assay on Hep G2 cell line. The cell viability and cytotoxicity were assessed and IC 50 was calculated to be 40.92ug. To analyze the apoptotic induction CASPASE, 9 activity was measured. It shows the elevated level of caspase 9 on comparing to non-treated Hep G2 cell line. Thus, this present investigation proved that *Brassica oleracea* ethanol extract possesses phytocompounds that can serve as an efficient anticancer drug on hepatocellular carcinoma without any adverse effect.

* Corresponding Author

Name: Dr. V. Vanitha
Phone: +91-994170966
Email: vanitha.sls@velsuniv.ac.in

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INTRODUCTION

Many of our traditional plants have been used over the years to treat many diseases. This property is mainly accredited to the medicinal or healing properties of our traditional plants such as neem, lemon, Tulasi, garlic, ginger, turmeric, pudina etc. Herbal medicines have been universally accepted and used extensively over the past decade for this purpose. Natural products have begun to gain popularity worldwide for promoting healthcare as well as disease prevention and been used as conventional or complementary medicines for both treatable and incurable diseases (Lopenz.D *et al*. 2009). Most of the world's population, health welfare was turned towards the medicinal plants (Akerele 1988). Traditional plants as medicines are extensively used in India. These plants contain many bioactive compounds in them that possess anticancer activity. Also, they are safe, eco-friendly, less expensive than chemotherapy and do not have any cytotoxic effects on normal cells. Many herbal products have been clinically available as potent hepatoprotective agents against commonly occurring hepatic diseases. Herbal remedies for liver cancer involves the use of resources that plants or their derivatives offer. These medicinal plants owe it to various phytochemicals present in their extracts which are biologically active and work for the benefit of not only preventing and treating cancer but various other diseases. The immunomodulatory and antioxidant properties of certain Medicinal plants lead it to be an efficient anticarcinogen. They are known to have versatile immunomodulatory activity by stimulating both non-specific and specific immunity (Govind Pandey *et al*. 2006, Agrawala.S.K *et al.* 2001). Among medicinal plants, tradition use of *Brassica oleracea* (broccoli) was

chosen in order to investigate its anticancer activity against liver hepatocellular carcinoma – 'Hep-G2' cell lines. Broccoli, classified in the cultivar group Italica belongs to the family Brassicaceae (species *Brassica oleracea*). It is mainly grown for its edible flower heads which are used as the vegetable. Naturally, broccoli consists of phytocompound such as vitamin c, glucosinolates, phenolic compounds, minerals and some nutrient that are bioactive against cancer and pathogens. Thus, they were promised to be an effective nutraceutical (Kaur C *et al*. 2007, Ares AM *et al*. 2007). Broccoli in a diet serves as an enriched nutrient that can prevent chronic diseases, such as carcinogenic pathologies, cardiovascular and breast and prostate cancers (Jeffery EH *et al.* 2003, Moreno DA *et al.* 2006).

MATERIALS AND METHOD

Extraction

The *Brassica oleracea* edible parts were cut into very small pieces and allowed to dry for about 7 days in the shade. Once the cut pieces were thoroughly dried, they were ground into coarse powder using a grinder. The coarse powder obtained was used for extraction purpose. Extraction was performed by the following method:

Ethanolic Extraction

The use of 99.8% ethanol extracted the coarse powder. The coarse powder was first transferred to a 500ml beaker. The powder was then completely submerged in ethanol (400ml). The content was incubated for 72 hours inside an incubator. After completion of the incubation period, the content was filtered using muslin cloth; the filtrate was then transferred to another beaker and the filtrate was kept open in a closed space for the ethanol to evaporate completely which took about 4 days. The remains of the filtrate (paste) which had a dark green colour (figure 1) were then scrapped and put inside an Eppendorf tube (figure 2). The paste was designated as the crude extract of broccoli. This extract was then preserved at 4°C for further use.

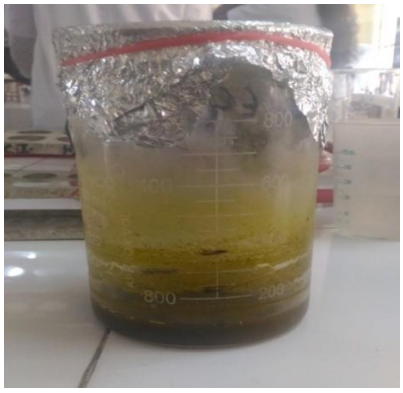

Figure 1: Remaining filtrate after complete evaporation of ethanol

Figure 2: Crude extract of broccoli Phytochemical screening

For phytochemical screening, 100mg of ethanolic extract was dissolved in 10ml of ethanol. Then 10ml of that mixture was diluted to 100ml using distilled water. Phytochemical screening of ethanolic extracts obtained was performed to profile various phytochemicals present in broccoli. The phytochemical screening method was carried out by a standard method (Sofowora A 1993 and VA Doss *et al*., 2016).

Test for Flavonoids

To 1ml of extract, a few drops of 10 % lead acetate solution were added. The colour of the extract was changed to white colour which on adding of acid changed to colourless clear solution depicted the presence of flavonoids.

Test for Steroids

Extracts were treated with chloroform. There was a formation of the ring at the junction and its lower was yellow while adding concentrated sulphuric acid along the sides of the test tube. This shows the presence of sterols.

Test for Terpenoids

Salkowski test: 5 ml of each extract was mixed in 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. The reddish-brown colouration of the interface was formed to show the presence of terpenoids.

Test for Tannins

Ferric chloride test: There was an appearance of brownish green or blue-black colour in 0.5g of the extract on adding few drops of 0.1% ferric chloride.

Test for Saponins

Foam test: if foams were produced on shaking the small amount of extract and lasted for 10 minutes, it indicates the presence of saponins.

Test for alkaloids

Mayer's Test: on treating the extracts with Potassium Mercuric Iodide (Mayer's reagent) a white coloured precipitate was formed, indicates the presence of alkaloids.

Wagner's Test: Few ml of extracts were taken and Wagner's reagent was added. The formation of reddish brown colour shows the presence of alkaloids.

Test for Phenol

Ferric chloride Test: To few ml of extract, few drops of 5% lead acetate was added. The occurrence of bluish green indicates the presence of phenols.

Test for Quinone

The extract was taken and added conc. H_2SO_4 in the sides of the test tube which develops colour formation indicated the presence of quinine.

Test for Carbohydrates

Few ml of extract was boiled on a water bath with 1 ml of Fehling solutions I and II. A red precipitate indicated the presence of sugar.

Test for Glycosides

Keller Killian's Test: The extract was treated with glacial acetic acid, few ml of ferric chloride was added and Conc. H_2SO_4 was added to the sides of the test tube which formation of lower reddish brown and upper bluish green layer indicated the presence of glycosides.

Test for Aminoacids

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to a few ml of the extract. A characteristic purple colour indicated the presence of amino acids.

Quantitative Analysis of Proteins

Determination of proteins by *Lowry's* method:

Reagents: Bovine serum albumin (BSA) working standard. Reagent I: Alkaline copper reagent; Reagent II: Folin-phenol reagent.

Figure 3: Test tubes showing the presence of proteins

0.2-1.0 ml of BSA working standard was pipette out in 5 different test tubes labelled as S_1-S_5 and made up to 2ml using distilled water. The test tube with 2 ml of distilled water alone served as blank. Also, 0.2ml and 0.4ml of ethanolic extracts were added in 2 different test tubes labelled as U_1 and U_2. They were diluted to 2ml using distilled water. To all the test tubes, 4.5 ml of Reagent I was added and incubated for 10 minutes. After incubation, 0.5 ml of reagent II was added and incubated for 30 minutes. Then the absorbance was read at 640 nm and the standard graph was plotted. The amount of protein present in the given sample was estimated from the standard graph (Lowry, O. H. *et al*. 1951). Figure 3 represents the test tubes showing the presence of proteins.

Cell lines and culture

Human hepatocellular carcinoma cell lines, HepG2 cells (American Type Culture Collection [ATCC] CRL 8024) were obtained from National center for cell science, Pune, India was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 50 µg/ml of streptomycin.

MTT assay for cytotoxicity

The Hep G2 cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2X 10^4cells/well and allowed to attach overnight at 37°C. The medium was carefully taken and discarded from wells without disturbing the cells and then cells were incubated with different concentrations of the ethanol extract of broccoli (25, 50, 75, 100 and 125µg) for 24 hours. After the incubation, the medium was discarded and 100µl fresh medium was added with 10µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5mg/ml concentration. The medium was discarded and 100µl of DMSO was added to dissolve the formazan crystals after 4 hours. Then, the absorbance was read at 570nm in a microtitre plate reader. Cyclophosphamide was used as a positive control.

Cell survival was calculated by the following formula,

$$\text{Cell Viability \%} = \frac{\text{Test OD}}{\text{Control OD}} \times 100$$

$$\text{Cytotoxicity \%} = 100 - \text{Viability\%}$$

Determination of CASPASE activity

After the treatment of Hep G 2 cell lines by ethanol extract of broccoli, they were added with cell lysis buffer for 10 minutes on ice. Cell lysates were centrifuged at 10,000×*g* for 1 min and the supernatants (cytosolic extract) were collected. The protein concentration was determined by Bradford's

Table 1: Phytochemical profile of Brassica oleracea

Sl.no.	Tests	Observation	Ethanolic extract
1.	Flavonoids		
	Lead acetate test	A change to a white colour which on the addition of acid changed to the colourless solution.	+
2.	Steroids	Formation of lower yellow with the ring at the junction.	-
3.	Terpenoids		
	Salkowski test	The reddish-brown colouration of the interface.	+
4.	Tannins		
	Ferric chloride test	Brownish green or a blue-black colouration.	+
5.	Saponins		
	Foam test	No foam formation.	-
6.	Alkaloids		
	Mayer's test	Formation of a white coloured precipitate.	+
	Wagner's test	Formation of reddish brown colour.	+
7.	Phenols		
	Ferric chloride test	Bluish green colouration	+
8.	Quinone	No colour change	-
9.	Carbohydrate		
	Fehling's test	The red coloured precipitate formed	+
10.	Glycosides		
	Keller Killian's test	Formation of lower reddish brown and upper bluish green layer	+
11.	Aminoacids		
	Ninhydrin test	Purple colouration	+

method using BSA as a standard. 100-200 µg of cellular extracts were diluted in 50µl cell lysis buffer for assay. The diluted cellular extract was then incubated in 96-well microtitre plates with 5µl of the Ac-LEHD-pNA (CASPASE -9 activities) for 2hr at 37 ◦C. Free pNA formed from the substrate by the action of CASPASE-9 was measured by absorbance at 405nm in a microtitre plate reader. The ratio of the absorbance of treated cells to the untreated cells gives the relative CASPASE-9 activity.

RESULTS AND DISCUSSION

Natural plant products have been known as an age-old remedy for cancer in its treatment. However, still use of surgery, chemotherapy and radiotherapy are the main conventional cancer treatment all over the globe. Although these treatments are highly effective, they cause severe side effects which limit their use. Currently, more than half of the widely used anti-cancer drugs in the world are derived from natural sources. In the field of anti-cancer drugs, the research and development of new drugs from natural products show a booming vitality and an attractive prospect. Although drug discovery from medicinal plants continues to provide an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials and their selection.

In this study, we determine the phytochemicals present in broccoli qualitatively which are biologically active and exert hepatoprotective properties essential for the prevention and treatment of liver cancer. Quantitative determination of proteins was also carried out in this study. A person diagnosed with cancer must consume appropriate carbohydrates and proteins for cellular regeneration and recovery which broccoli provides efficiently (Borowski J *et al.* 2008). Also in this study, we report the cytotoxic activity of ethanolic extracts of *Brassica oleracea* against HepG2 liver cancer cells by inducing apoptosis in these cells via activation of CASPASE 9.

Qualitative analysis of phytochemicals: The preliminary phytochemical screening of *Brassica oleracea* extract showed the presence of bioactive components like Terpenoids, Flavonoids, Glycosides, Alkaloids, Phenols, Tannins, Carbohydrates, Glycosides and Proteins was given in table 1.

Flavonoids, terpenoids, tannins, saponins, alkaloids, phenols, proteins etc. are some phytochemicals in broccoli the presence of which are crucial in prevention and treatment of liver cancer. These bioactive compounds have antioxidant effects on free radicals that are known to cause cancer. These compounds inhibit cell proliferation and metastasis of cancer cells via various mechanisms.

Determination of proteins by *Lowry's* method: The optical density of various concentrations of the standard BSA solution and the ethanolic extract of *Brassica oleracea* with the help of which a standard graph was plotted. Concentrations of proteins in the extract were calculated using the standard graph. Figure 4 represents the standard graph of protein estimation of ethanol extracts of *Brassica oleracea* by *Lowry's* method.

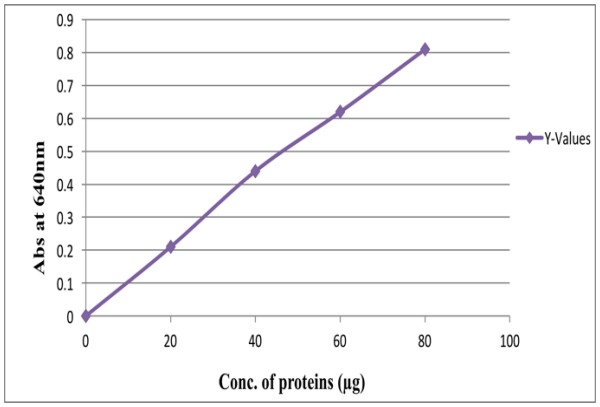

Figure 4: Standard graph for protein estimation of ethanolic extracts of *Brassica oleracea*

0.2 ml and 0.4ml of extract contained 10mg and 11.75mg of proteins respectively. This shows that *Brassica oleracea* contains high levels of proteins. Studies suggest that a diet rich in proteins helps in cellular regeneration and recovery against any cancer which broccoli provides in generous amounts.

MTT assay for cytotoxicity

The MTT assay is based on the reduction of a yellow tetrazolium dye to a purple formazan product by live cells which in turn measurement of purple colour gives the count of live cells (Mossman.T, 1983). The ethanolic extracts of *Brassica oleracea* obtained were subjected to MTT assay against HepG2 cell lines and the cell viability and cytotoxic activity were represented in Table 2 and 3. Observation of morphological changes in cells indicated that the extracts inhibited proliferation of the HepG2 cancer cell lines in a dose-dependent manner. No toxicity was seen in the normal liver cell lines. The IC_{50} values calculated for the ethanolic extracts in the cancer cell lines (HepG2) after treatment for 24 hours.

Table 2: Percentage of cell viability of Brassica oleracea ethanolic extract on Hep G2 cell lines

Concentration (µg)	Sample	PC
25	53.39	23.00
50	48.19	21.01
75	43.48	16.52
100	37.62	15.46
125	34.10	13.74

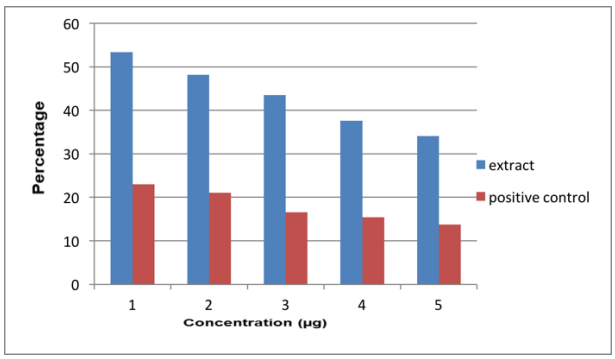

Figure 5: Effect of cell viability of *Brassica oleracea* ethanolic extract on HepG2 cell lines

Table 3: Percentage of cytotoxicity of *Brassica oleracea* ethanolic extract against HepG2 cell line

Concentration (µg)	Sample	PC
25	46.61	77.00
50	51.81	78.99
75	56.52	83.48
100	62.38	84.54
125	65.90	86.26

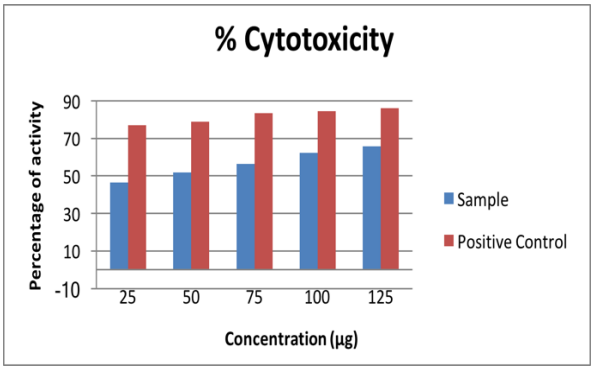

Figure 6: Effect of cytotoxicity of Brassica oleracea ethanolic extract on HepG2 cell lines

Calculation of IC_{50} value

The IC_{50} values were calculated using the graph below shown in figure 7.

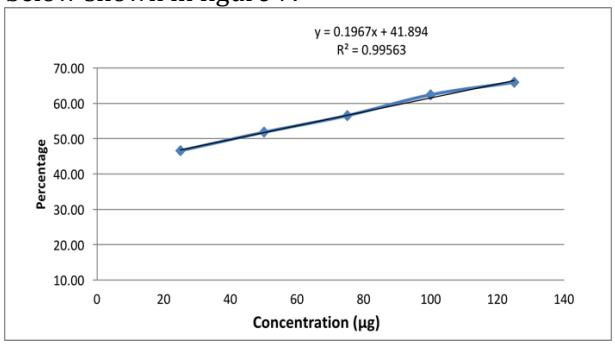

Figure 7: Calculation of IC50 value of ethanolic extracts of *Brassica oleracea*

The IC50 value for the ethanolic extract of broccoli was found to be 40.92µg. This means that only 40.92µg of the extract was needed to inhibit 50% of the HepG2 cancer cells which shows that it is quite potent in its inhibitory effect.

Determination of Caspase 9 Activity

Relative CASPASE-9 activity was calculated as a ratio of the absorbance of treated cells to untreated cells and the values were given in table 4. There is an increase in the concentration of CASPASE-9 as the concentration of ethanol extract of broccoli increased this indicates that there is an apoptotic induction resulted in cell death. This proves that ethanol extract of broccoli induces apoptosis through CASPASE-9.

Table 4: The relative CASPASE-9 activities with different concentrations of the extract

Control	25µg	50µg	100µg
0.5267	0.5905	0.649667	0.6854

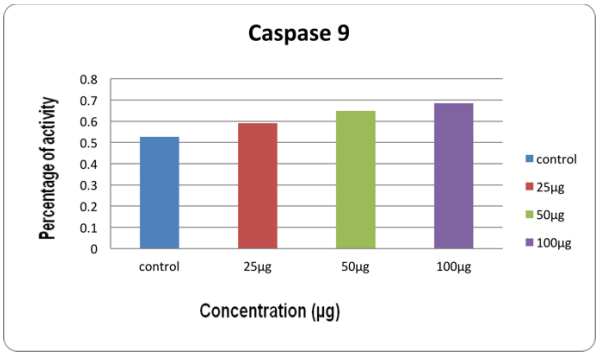

Figure 8: Effect of relative CASPASE-9 activities with different concentrations of the extract

The figure 8 shows that caspase 9 activity of HepG2 cell line, which was elevated with higher concentrations of the extract this proves the initiation of apoptosis of the cancer cells (Shalini, S *et al*. 2015).

CONCLUSION

The cytotoxic activity of the ethanolic extracts of varying concentrations was significantly enhanced with their higher concentrations; the extracts have shown anti-proliferative effects on liver cancer cells. The extract also markedly elevated activation of CASPASE 9 activity respectively with increasing concentrations of the extract which induced cell death or apoptosis of the HepG2 liver cancer cells. All these findings proved that the *Brassica oleracea* is cytotoxic against liver cancer cells without disrupting the normal homeostasis of healthy cells and can be used as a potent anticancer drug against liver cancer cells as it successfully induces apoptosis in them through activation of caspase activity.

REFERENCES

Agrawala, S. K., Chatterjee, S. and Misra, S. K., Immune potentiation activity of a polyherbal formulation 'Immu-21' (research name). Phytomedica, 2001, 2, 1–22.

Akerele, OF., Medicinal plants and primary health care: an agenda for action. Fitoterapia, 1988; 59: 355–363.

Ares AM, Nozal MJ, Bernal J. Extraction, chemical characterization and biological activity determination of broccoli health-promoting compounds. J Chromatogr A. 2013;13(13):78–95.

Borowski J, Szajdek A, Borowska EJ, Ciska E, Zieliński H. Content of selected bioactive components and antioxidant properties of broccoli (Brassica oleracea L.) Eur Food Res Technol. 2008; 226:459–465.

Govind Pandey and Madhuri.S. Medicinal plants: better remedy for neoplasm. Indian Drugs, 2006, 43, 869–874.

Jeffery EH, Brown AF, Kurilich AC, Keck AS, Matusheski N, Klein BP, Juvik JA. Variation in content of bioactive components in broccoli.J Food Compos Anal. 2003; 16:323–330.

Kaur C, Kumar K, Anil D, Kapoor HC. Variations in antioxidant activity in broccoli (Brassica oleracea L.) cultivars.J Food Biochem. 2007; 31:621–638.

López D1, Fischbach MA, Chu F, Losick R, Kolter R.Structurally diverse natural products that cause potassium leakage trigger multicellularity in Bacillus subtilis. Proc Natl Acad Sci U S A. 2009; 106(1):280-5.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. "Protein measurement with the Folin phenol reagent". Journal of Biological Chemistry. 1951; 193 (1): 265–75.

Moreno DA, Carvajal M, López-Berenguer C, García-Viguera C. Chemical and biological characterisation of nutraceutical compounds of broccoli. J Pharm Biomed Anal. 2006; 41:1508-1522.

Mossman, T. Rapid colourimetric assay for cellular growth and survival – application to proliferation and cytotoxicity assays. J.Immunol.Methods 1983; 65: 55-63

Shalini, S.; Dorstyn, L.; Dawar, S.; Kumar, S "Old, new and emerging functions of caspases". Cell Death & Differentiation. 2015; 22 (4): 526–539.

Sofowora A. Medicinal plants and traditional medicines in Africa. Spectrum Books Ltd., Ibadan, Nigeria, 1993; pp. 191–289.

Victor Arokia Doss, Mohanasundaram.S, Prasad Maddisetty. Analysis of hydroethanolic extract of Senna alata (L.) to screen bioactive compounds with inhibitory activity on lipid peroxidation, in vitro antibacterial and antidiabetic efficacy. Int J Pharm Sci., 2016; 6(1): 1360-1366.