Effect of Brassica nigra seeds extract against hepatocellular proliferation induced by phenobarbital as tumor promoter agent

Efecto del extracto de semillas de Brassica nigra contra la proliferación hepatocelular inducida por fenobarbital como agente promotor de tumors

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Abstract

Hepatocellular proliferation is one of the leading causes of hepatocellular carcinoma (HCC), and is a widely distributed type of cancer-related death. There are considerable challenges in treating hepatocellular carcinoma, including ineffectiveness, adverse effects, and drug resistance to available drugs. It has been established that a high intake of Brassica vegetables is linked to a lower risk of many cancers. In the study, Brassica nigra seeds were extracted; phytochemical analysis of the extract was done, including phytochemical screening tests and Gas chromatography-mass spectrometry (GC-MS) analysis. Antiproliferative activity of hydroalcoholic Brassica seeds extract has been studied at 800mg/kg and compared with the control group (given normal saline), phenobarbital group (Phenobarbital 75mg/kg), and combination group (Brassica extract 800mg/kg+ Phenobarbital 75mg/kg). The GC-MS analysis revealed the presence of the isothiocyanate compound. Compared to the phenobarbital group, Combining groups pretreated with Brassica nigra seeds for 14 days and phenobarbital for seven days caused a significant reduction in Ki67 and Bcl2 expression that was obvious in immunohistochemical slides. Also, combination groups showed a substantial decrease in the expression level of genes related to the cell cycle, including minichromosome maintenance genes 2 (Mcm2) and cyclin A2 (Ccna2), and a significant reduction in the expression level of Cytochrome P450, including CYP2B10 and CYP2C29 in comparison with phenobarbital group. Brassica nigra seeds extract showed antiproliferative and apoptotic effects on the liver tissue, suggesting it has a beneficial impact on minimizing the risk of liver cancer.

Keywords: Hepatocellular proliferation, Phenobarbital, Brassica seeds.

Resumen

La proliferación hepatocelular es una de las principales causas de carcinoma hepatocelular (HCC) y es un tipo de muerte relacionada con el cáncer ampliamente distribuido. Existen desafíos considerables en el tratamiento del carcinoma hepatocelular, incluida la ineficacia, los efectos adversos y la resistencia a los medicamentos disponibles. Se ha establecido que un alto consumo de vegetales Brassica está relacionado con un menor riesgo de muchos tipos de cáncer. En el estudio se extrajeron semillas de Brassica nigra; Se realizó un análisis fitoquímico del extracto, incluidas pruebas de selección fitoquímica y análisis de cromatografía de gases-espectrometría de masas (GC-MS). La actividad antiproliferativa del extracto hidroalcohólico de semillas de Brassica se estudió a 800 mg/kg y se comparó con el grupo de control (administrado con solución salina normal), el grupo de fenobarbital (fenobarbital 75 mg/kg) y el grupo combinado (extracto de Brassica 800 mg/kg + fenobarbital 75 mg/kg). El análisis GC-MS reveló la presencia del compuesto de isotiocianato. En comparación con el grupo de fenobarbital, la combinación de grupos pretratados con semillas de Brassica nigra durante 14 días y fenobarbital durante siete días provocó una reducción significativa en la expresión de Ki67 y Bcl2 que fue evidente en los portaobjetos inmunohistoquímicos. Además, los grupos de combinación mostraron una disminución sustancial en el nivel de expresión de genes relacionados con el ciclo celular, incluidos los genes de mantenimiento de minicromosomas 2 (Mcm2) y ciclina A2 (Ccna2), y una reducción significativa en el nivel de expresión de Citocromo P450, incluidos CYP2B10 y CYP2C29 en comparación con el grupo fenobarbital. El extracto de semillas de Brassica nigra mostró efectos antiproliferativos y apoptóticos en el tejido hepático, lo que sugiere que tiene un impacto beneficioso para minimizar el riesgo de cáncer de hígado.

Palabrasclave: Proliferación hepatocelular, Fenobarbital, Semillas de Brassica.

Introduction

Hepatocellular carcinoma (HCC) is a cancer which begins in the liver, it is considered the most widespread form of primary liver cancer, most commonly occurs in patients with chronic liver disorders, like cirrhosis, infection by hepatitis C or B virus[1]. It is widely agreed that hepatocarcinogenesis is very complicated disease and arises during the malignant transformation of normal hepatocytes via a multi-stage biological process in which numerous variables are involved, particularly genetic and epigenetic alterations[2].

Classically, HCC appears and develops in silent manner, making its detection difficult before the later stage of disease development[3]. Hepatocellular carcinoma is among cancers that have poor prognosis because this malignancy resists the majority of chemotherapeutic treatments[4]. Sorafenib and lenvatinib still considered as single-drug regimens that are highly effective[5,6]. Chemoprevention of cancer with natural phytochemical constituents is a revolutionary technique for stopping, or curing cancer[7]. In several years have been seeing that high consuming of Brassicaceae vegetables minimize the risk of a large number of cancers, like lung[8], gastrointestinal tract[9,10], prostate[11] and bladder cancers[12-14]. The aim of this study was evaluation of the effect of Brassica nigra seeds extract against hepatocellular proliferation induced by phenobarbital, as tumor- promoting agent.

Materials and Methods

Chemicals and reagents: Chloroform where purchased from British drug house, UK. Ethanol 99 from Alpha chemo, India. Formaldehyde PanReac Applichem, USA. Na2HPO4 and NaH2PO4 from Fluca, Germany. Paraffin wax and Xylene from Scharlau, Spain. Phenobarbital ampoule (200mg/ml) supplied from Samarth life sciences PVT.LTD, India. From Elabscience Biotechnology-USA purchased the followings: Bcl2 Monoclonal Antibody, Ki-67Monoclonal Antibody and Step plus Poly-HRP Anti Mouse/Rabbit IgG detection system, while RNA extraction kit, Transcript One-step gDNA Removal and cDNA Synthesis SuperMix kit and Trans Start Tip Green qPCR SuperMix were purchased from TRANS Company in china. Five genes (forward and reverse primers) were employed in this study, supplied by Bioneer, Korea manufacturer with sequences as follows:

MCM2: TCACGGTGCGCCACATCGAG, and CCGGGCAAAAGTCTTGCGCA

CCNA2: TACCCCCCAGAAGTAGCAG, and GGGTCAGCATCTATCAAAC

CYP2B10: 59-CAGGTGATCGGCTCACACC-39, and 59 TGACTGCATCTGAGTATGGCATT-39

CYP2C29: 59-GGGCTCAAAGCCTACTGTCA-39, and 59-AACGCCAAAACCTTTAA-39 Housekeeping gene that used in the current study was GAPDH: CGGGTTCCTATAAATACGGACTG, and CCAATACGGGCCAAATCCGTTC.

The plant: Brassica nigra seeds were collected from Baghdad/Alshorga local market in September 2020, then it had been successfully identified by professional plant taxonomists in the Department of Pharmacognosy/ Collage of Pharmacy/ Mustansiriyah University, and in the Collage of Science/ University of Baghdad, then seeds had stored in dark place few days prior to extraction process.

Animals: Twenty eight, non-previously treated male Swiss albino mice aged (8weeks) their weight (25±5) gm provided from Iraqi Center for Cancer Research were retained in well ventilated place with woodchip bedding with well-marked by their tails for identification and were maintained under standard conditions of relative humidity (70±5%). Temperature (25 ± 2°C), and a 12-hour light-dark cycle with free access to water and food (pellets) in the animal house of the Collage of Pharmacy/ Mustansiriyah University.

Extract preparation: Brassica nigra seeds (2000g) were air dried and finely grinded by electrical mill. These seeds were subjected to extraction by reflux extraction processes. Firstly, the powdered seeds had been immersed with absolute ethanol (99%) and water at 70:30 percent in round reflux apparatus at a temperature range from 50-55^{0}C for 1hr, after that the seeds residues were filtered using filter paper to get clean crude extract. Finally, the filtrate undergo evaporation by a rotary evaporator and further air drying till a semisolid paste was obtained. The extract that obtained at weight 140gm was labelled and preserved in a sealed glass container in refrigerator at 4^{0}C till the time of experiment. The yield of extract was found to be 7% (w/w) according to the following equation[15].

The yield of extract = Weight of material obtained /Weight of starting material × 100.

Phytochemical screening: Phytochemical screening of hydro alcoholic extract of Brassica nigra using standard procedure for detection the presence of glycosides, alkaloids, flavonoids, saponin, tannin and phenols[16-18].

Gas chromatography-mass spectrometry (GC-MS analysis): This technique commonly used for both qualitative and quantitative analysis of a sample containing a variety of organic compounds [18]. The GC-MS technique was used in the current study to recognize the phytoconstituents present in the Brassica nigra extract. This work was done in the Ministry of Science and Technology, Environment and Water Department. The GC-MS was carried out using a Schimadzu (QP2010) PLUS system. The column used in GC-MS system was (optima-5ms, Medium non polar 30m length, 0.25 um thickness). The chemical compounds from the Brassica nigra extract were known by comparing between the retention times of the obtained chromatographic peaks.

Animal grouping: Twenty eight male Swiss albino mice were randomly divided into four groups (n=7 for each): First group (Control group): received normal saline by oral gavage for one week and continue with normal saline intraperitonially for the second week. Second group (Phenobarbital group): received normal saline by oral gavage for one week, then intraperitoneal phenobarbital at 75mg/kg /day for the second week. Third group (Extract alone group): received the extract at dose 800mg/kg/day by oral gavage for one week, then

continue with extract at dose 800mg/kg/day by oral gavage for the second week. Fourth group (Combination group): received the Brassica nigra extract at dose 800mg/kg/day by oral gavage for one week, then continued with Brassica nigra extract at dose 800mg/kg/day by oral gavage with intraperitoneal phenobarbital at 75mg/kg /day for the second week.

Liver harvest: After the ending of treatment period, animals were euthanized by placed in a container that contained the chloroform. To ensure circulation, the chemical was soaked in cotton wool and placed in the container for 5 minutes before to exposure and liver tissues were collected surgically.

Immunohistochemistry analysis: The liver tissues transferred in buffered formalin (100 ml of 40% formalin+900 ml distilled water+4 gm of sodium di-hydrogen phosphate+6.5 gm of sodium phosphate dibasic). After fixation, the tissue samples were immersed in ascending grades of ethanol to avoid excessive tissue hardening to prepare the sample for the next step as a following procedure (Eighty percent ethanol for 15 minutes, Ninety five percent ethanol for 15 minutes), Absolute ethanol (99.9%) for 15 minutes. Chloroform for 15 minutes. Then Paraffin wax was molted at 60˚C for 15 minutes[19,20]. Finally, samples were labeled and inserted in paraffin wax as a blocks that prepared to immunohistochemistry analysis of Ki67 and Bcl2 expression under standard protocol[21,22]. To assess the fraction of immunolabeled cells in liver specimens from each animal included in the current study, the labeling index expressed as the percentage of positive cells out of the total number of cells were counted in five area that randomly selected with 100, 400 magnification per each section from every mouse. The percentage of Ki-67 positive cells was graded as 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The degree of intensity (which indicates the concentration of the stain in positive cells) was graded as (0) for negative staining and +, ++, and +++ for weak, moderate, and strong intensity, respectively. The following scores was used to calculate the percentages of Ki67 positive cells: 1+ (weak) Means less than 10%, 2+ (moderate) = 11 to 50%, and 3+ (strong) = more than 50%[23]. Bcl2 staining intensity was rated on a 5-point scale: 0 represented no staining; 1+ represented weak staining; 2+ represented moderate staining; 3+ represented strong staining; and 4+ represented intense immunostaining. The following scores was used to calculate the percentages of Bcl2 positive cells: 0 (no staining); 1+ mean 1 to 25% cells stained positive; 2+ mean 26 to 50% cells stained positive; 3+ mean 51 to 75% cells stained positive; 4+ mean76 to 100% cells stained positive[24].

Gene expression analysis: Firstly 50- 100 gm of the liver tissue transferred into Eppendorf tubes which contained 1ml of triazole, then stored in deep freeze until assay for gene expression investigations. Using RNA extraction kit (transcript)[25], RNA was obtained then cDNA were synthesized from total RNA using cDNA synthesis supermix (transcript) kit[26] and were amplified using the sets of primers. In this study the q RT-PCR was used to quantify for four genes (MCM2, CCNA2, CYP2B10 and CYP2C29) according to manufacture protocol. The final solution was placed in RT-PCR system for thermal reaction. Table (1) shows the thermal cycling protocol with suitable melting point for each primer according to manufacturer protocol[27].

Table 1. Thermal cycling conditions

Step	Temperature	Time	Cycle
Denaturation	94 ºC	5 sec	40
Annealing	50-60 ºC	15 sec	40
Extension	72 ºC	10 sec	40

Calculations

The cycle threshold (CT) of genes of interest was normalized to that of internal control gene. The difference in the cycle threshold (Ct) values between the GAPDH (internal control gene) and the interest genes include: MCM2, CCNA2, CYP2B10 and CYP2C29, was calculated as the following equation[28]:

$$\Delta CT\ (test) = CT\ gene\ of\ interest\ (test) - CT\ internal\ control$$

Calibrator was chosen from the control samples, DCT of calibrator was calculated according to following equation: $\Delta CT\ (calibrator) = CT\ gene\ of\ interest\ (test) - CT\ internal\ control$. The DCT of the test samples was normalized to the DCT of the calibrator:DD CT was calculated according to the following equation: $\Delta\Delta\ CT = \Delta CT\ (test) - \Delta CT\ (calibrator)$ Finally, the expression ratio was calculated according to the formula: 2^{-DDCt} = Normalized expression ratio[28].

Statistical analysis: The statistical analysis of this prospective study was performed with the statistical package for social sciences (SPSS) version 24.0 and Microsoft Excel 2020. Categorical data formulated as count and percentage. Chi-square, Kruskal Wallis and Mann Whitney tests were used to describe the association of these data. Numerical data were described as mean and standard error. Analysis of variance (ANOVA) was used for comparison among more than two groups. P- Value <0.05 considered as a significant difference.

Results

Phytochemical screening tests: In this study, the yield percentage of the Brassica nigra seeds extract was 7%, since 140 gm was obtained from 2000gm of Brassica nigra seeds. Qualitative phytochemical studies were performed on the hydro alcoholic extract of Brassica nigra seeds to determine the presence of various phytochemical constituents. The results showed the presence of glycosides, alkaloids, terpenoids, flavonoids, phenols, tannin, saponin and coumarin, table (2).

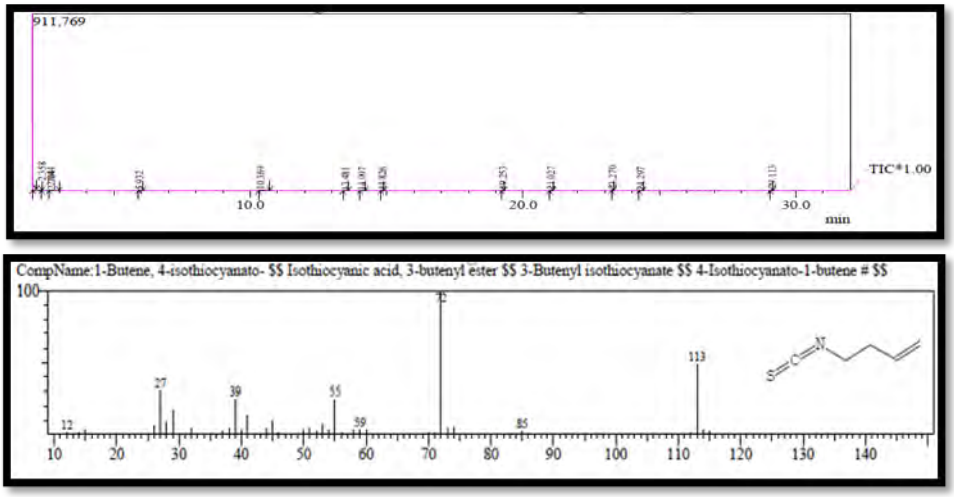
Table 2. Preliminary tests of hydro alcoholic extract of Brassica nigra seeds.

Chemical	Result	Chemical	Result
Alkaloids	+	Terpenoids	+
Flavonoids	+	Glycosides	+
Saponin	+	Cumarin	+
Tannins, Phenols	++		

(+): present, (++): highly present

Gas chromatography-mass spectrometry (GC-MS) analysis: The most important recognized chemicals were isothiocyanates like allyl and butyl isothiocynate. Figures (1) showed the peaks of these chemicals.

Figure 1. (A) GC-MS analysis results of Brassica nigra hydro alcoholic seeds extract. (B) Isothiocynate compound detected by GC-MS.



Effect of hydro alcoholic seeds extract of Brassica nigra on immunohistochemistry markers within mice liver tissues: The effect of hydro alcoholic seeds extract of Brassica nigra was evaluated by immunohistochemical expression of Ki-67 protein in the studied groups of the current study. Figure (2) showed the nuclear and cytoplasmic expression of Ki-67 in control, phenobarbital, extract, and combination group. While figure (3) revealed the cytoplasmic expression of Bcl2 in in control, phenobarbital, extract, and combination group.

Figure 2. Immunohistochemical expression of Ki-67 in mice liver tissue, black letter indicates groups: (A) control group, (B) Phenobarbital group, (C) Extract group, (D) combination group. Black arrow indicate Ki-67 protein expression, left: (10X) & right: (40X)

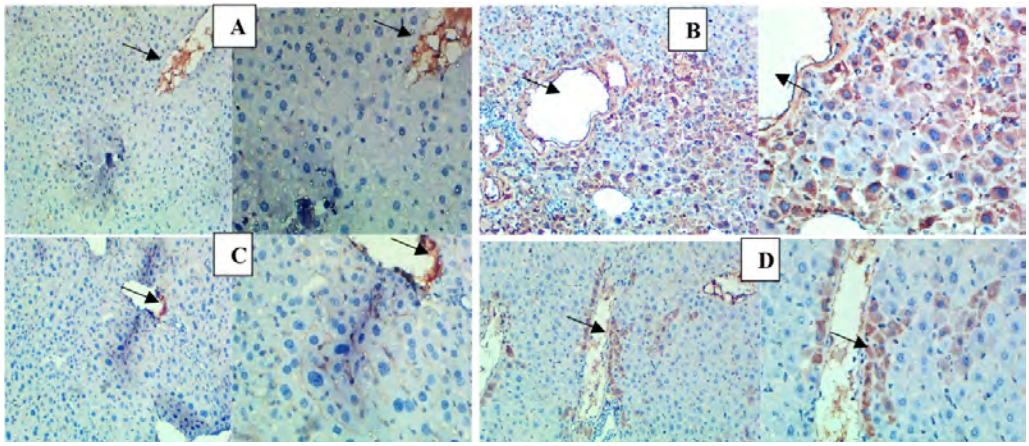
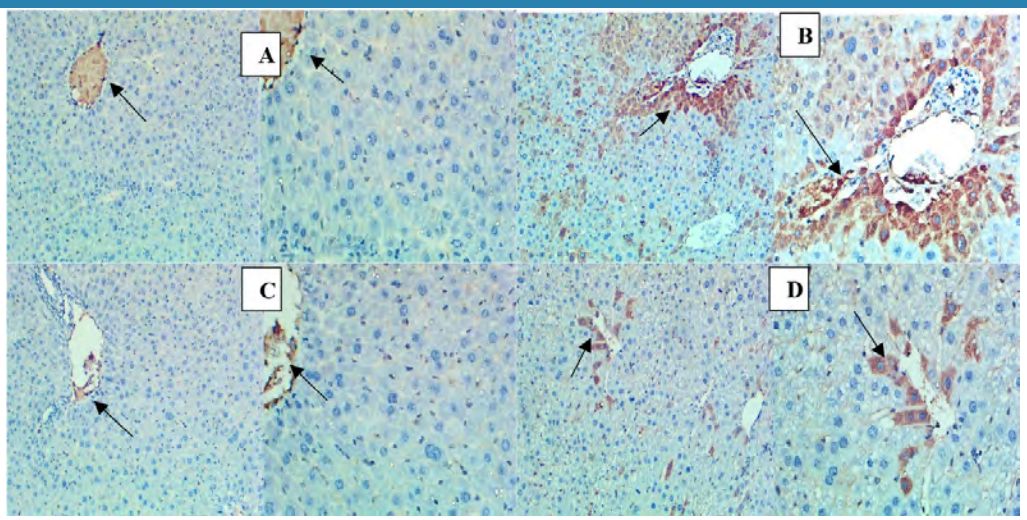


Figure 3. Immunohistochemical expression of Bcl2 in mice liver tissue, black letter indicate groups: (A) control group, (B) Phenobarbital group, (C) Extract group, (D) combination group. Black arrow indicate Bcl2 protein expression, left: (10X) & right: (40X).



Regarding the immunohistochemical expression of Ki-67 and Bcl2 in mice hepatic tissues, the results of the present study showed that there was a significant difference (P value= 0.001) in the staining intensity between control group and both phenobarbital and combination group, while there was no significant difference in staining intensity between control group and extract group for Ki-67 (P value=0.317) table (3) and for Bcl2 (P value=0.606), table (4).

Table 3. Frequency distribution of immunohistochemical expression of Ki-67 in studied groups.

Ki-67		Study groups			
		Control	Phenobarbital	Extract	Combination
IHC score	0	7 (100%)	0 (0.00%)	7 (100%)	0 (0.00%)
	+	0 (0.00%)	0 (0.00%)	0 (0.00%)	5 (71.44%)
	++	0 (0.00%)	1 (14.28%)	0 (0.00%)	1 (14.28%)
	+++	0 (0.00%)	6 (85.72%)	0 (0.00%)	1 (14.28%)
Total		7 (100%)	7 (100%)	7 (100%)	7 (100%)
P-value		-	0.001	0.317	0.001
			-	0.001	0.001
				-	0.001

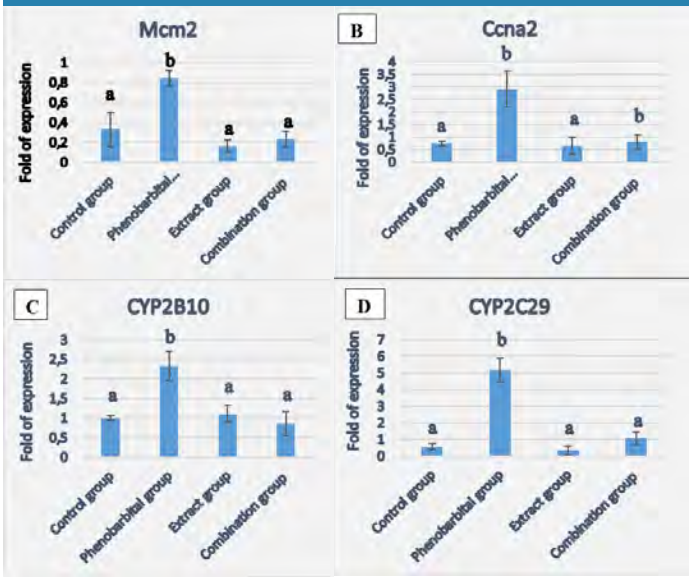
Table 4. Frequency distribution of immunohistochemical expression of Bcl2 in studied groups.

Bcl2		Study groups			
		Control	Phenobarbital	Extract	Combination
IHC score	0	7 (100%)	0 (0.00%)	6 (85.72%)	4 (57.15%)
	+	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
	++	0 (0.00%)	1 (14.28%)	1 (14.28%)	1 (14.28%)
	+++	0 (0.00%)	1 (14.28%)	0 (0.00%)	1 (14.28%)
	++++	0 (0.00%)	5 (71.44%)	0 (0.00%)	1 (14.28%)
Total		7 (100%)	7 (100%)	7 (100%)	7 (100%)
P-value		-	0.001	0.606	0.001
			-	0.001	0.001
				-	0.001

Effect of hydro alcoholic seeds extract of Brassica nigra on gene expression depending on real time reverse transcriptase poly chain reaction (qRT-PCR): The results of the present study clarified that mean expression levels of Mcm2 gene showed a significant elevation in phenobarbital group ($p<0.05$) when compared with control groups, and also with both extract and combination groups (figure (4-A), the last three groups did not showed any significant difference among each ($p>0.05$).Also, the mean expression levels of Ccna2 gene showed a markedly elevation within phenobarbital group ($p<0.05$) when compared with other studied groups, where the mean expression levels of Ccna2 gene were lowered significantly ($p<0.05$) in the extract and combination group when compared with phenobarbital group. However no statistically significant differences ($p>0.05$) in mean expression levels of Ccna2 were detected upon treating with Brassica nigra seeds extract compared with control group or combination protocol, figure(4-B).Regarding the mean expression levels of CYP2B10 gene, there was a significant elevation within phenobarbital group ($p<0.05$) when compared with control, extract and combination group, where there was no statistically significant ($p>0.05$) differences in mean expression levels of CYP2B10 gene among the last three groups (figure 4-C).Considering CYP2C29 gene, mean expression levels were significantly ($p<0.05$) elevated in phenobarbital group when compared other studied groups. Also, there was no statistically significant ($p>0.05$) differences in mean expression levels of CYP2C29 gene among control, extract, and combination group, figure (4-D).

Figure 4. Effect of hydro alcoholic Brassica nigra seeds extract on gene expression. (A): Mcm2 expression, (B): Ccna2 expression, (C): CYP2B10 expression, (D): CYP2C29. Data were expressed as means ± SEM. Different small letters (a, b) represent significant differences among the examined groups. P- Value <0.05 indicate a significant difference.

Figure 4



Discussion

Hepatocellular carcinoma (HCC) still the most widespread form of primary liver cancer and a major cause of cancer-correlated death around the world[29]. By 2030, the global burden of HCC mortality is expected to reach one million deaths per year[30]. It has been established that a high intake of Brassica vegetables is linked to a lower risk of cancer, this link clearly appears in lung, stomach, colon, rectal and prostate cancer[31-33]. In the present study, the percent of yield from Brassica nigra seed was 7%, using water- ethanol solvent system, according to the reflux extraction method[34,35]. This result was higher than others which were conducted by Nitin Kumar (2011)[36] who used methanol as solvent (4.3% w/w) and Anand P. (2014)[37] that was 5.18 % (w/w) using DW as a solvent and Basha S. (2015)[38] that was 1.4% (w/w) using DW and 6.52 % (w/w) using methanol.

This finding largely related to the extraction process, since the isolation of bioactive chemicals from plant materials using different temperatures during extraction technique is very important, where the findings of previous research revealed that increasing temperature can increase the yield of extrac-

tion for Brassica nigra and this support the current results[39]. The present study aimed to investigate the phytochemicals of Brassica nigra seeds extract. The results showed that the plant contains flavonoids, alkaloids, terpenes, saponins, tannins and glycosides, these classes of chemical compounds were same to those of previous studies conducted by Uddin MS. (2020)[40], Danlami U. (2016)[41], and Basha S. (2015)[42]. In the current study, GC-MS analysis confirmed the presence of isothiocyanates represented by 3-butenylisothiocyanate and methyl isocyanate. The present study come in accordance with sharma et al. (2018) who detect the same isothiocyanate compounds from Brassica juncea (a plant with same family of Brassica nigra), since isothiocyanate present in abundance within Brassicaceae family[43].

In the present study, ki-67 expression was measured due to its intense association with mitotic phase of cell cycle compared to other proliferating proteins, where Ki-67 expression reach its maximum level at G2/M phases[44]. The finding of the current research showed a significant elevation in the nuclear expression of Ki-67 after treatment with intraperitoneal PB compared with control group. Phenobarbital is known as CAR activator agent, so the activation of these receptors in liver caused hepatocyte hypertrophy and hyperplasia. The nuclear protein "Ki-67" is largely accompanying with tumor cell growth and proliferation[44].

This study was in accordance with a study done by Shizu R. (2013) who reported a significant increase in Ki-67 levels within mice hepatic tissue following phenobarbital administration depending on IHC technique[44]. Treatment with Brassica nigra seeds extract in the present study interestingly reduced the percentage of Ki-67 positive cells, suggesting inhibition of hepatocyte proliferation. The suspected mechanism of Brassica nigra in reduced hepatocyte proliferation is related to isothiocyanate compounds which are produced by hydrolysis of their precursor molecules glucosinolates, one of the active substances in Brassica nigra that has shown potential antitumor activity represented by sulforaphane, allyl isothiocyanates (AITC) and indole-3-carbinol[45]. Also, Vrca et al. study proved that Brassica nigra extract have a potent antiproliferative action against different human cancer cell lines[46].

Regarding Bcl-2, overexpression and phosphorylation of this protein, which is the most significant anti-apoptotic marker. Considering the results of the current study, PB alone group exhibited a significant reduction in the apoptosis process, represented by Bcl-2 overexpression. Supporting of this result comes from previous research done by Sanders S. (1999) who indicated that mice taken phenobarbital in the food have high expression level of Bcl-2, as anti-apoptotic protein[47]. A high rate of apoptosis in the liver tissue has been linked to a lower risk of hepatocyte proliferation and hepatic carcinoma. In this study, the hydroalcoholic Brassica nigra seeds extract stimulated apoptosis in liver tissue within combination group (Brassica nigra plus phenobarbital) compared to phenobarbital alone group, represented by a significant decline in Bcl-2 protein expression according to the IHC technique. De Felippe also showed that sulforaphane, a compound found in Brassica nigra, have an ability of increasing mitochondrial apoptosis through suppression of Bcl-2 protein[48]. The seeds of Brassica nigra contain sinigrin in high amounts[49], a previous study done by Jie et al. (2014) observed that sinigrin have ability to cause Bcl-2 down regulation, which support the finding of the present study[49].

In this study, the proliferation was successively induced by phenobarbital that confirmed by a significant hepatic up-regulation of cell cycle-related genes named minichromosome maintenance gene 2 (Mcm2) and Cyclin A2 (Ccna2) in phenobarbital group compared with control. These findings were closely agreed with a study done by Shizu et al. (2013) who successively proved that mice treated with PB (1000 ppm) in diet for one week accelerate cell cycle through initiation of DNA synthesis as a consequence of elevated levels of cell proliferation markers, including Mcm2 and Ccna2 that were up-regulated in S-phase and m-phase, respectively[44].

The findings of the present study showed a down regulation of Ccna2 gene in combination group significantly compared with phenobarbital group, so the Ccna2 protein cannot contributes with driving the entry into cell cycle, cannot binds to CDK2 in S-phase and cannot binds to CDK1 in G2/M phase of cell cycle. Similarly, significant down regulation of Mcm2 gene was observed in this group that suggest a cell cycle arrest. Therefore, and according to the present findings, down regulation of Ccna2 and Mcm2 proteins after Brassica nigra seeds extract treatment led to cell cycle arresting at S-phase and G2/M phase with inhibition of DNA replication that will lead to block their entrance into mitotic phases. Since combination group showed decreasing in Ccna2 and Mcm2 expression compared to phenobarbital group, this result can confirm the antiproliferative action of Brassica nigra seeds extract on hepatocytes which induced by phenobarbital.

The current finding agreed with cell line study done by Ahmed et al. (2020) which stated that the Brassica nigra seeds extract has significant proliferation inhibitory effect in A549 and H1299 lung cancer cells, so these cell lines were stopped at S and G2/M phase by Brassica nigra seeds extract in comparison with control cells[50], supporting the results of the current study about Brassica nigra anti-proliferative activity. In a previous research, allyl isothiocyanate, and as one of Brassica nigra seeds components, was shown to have growth-inhibitory action against lung cells proliferation and carcinoma[51]. The precise phytocomponents in Brassica nigra seeds extract that responsible for the reported anti-proliferative effect are unknown at this time, so further researches are essential since seeds are reported to contain number of bioactive chemicals. The results of the current study were supported by a study done by Jie et al. (2014) who stated the antiproliferative activity of sinigrin against liver damage induced by carcinogenic substances in rats via cell cycle arrest[49]. Previous studies indicated that allyl isothiocyanate, a major component of Brassica nigra seed, has been found to have antiproliferative properties in a variety of cancer types[52,53].

Cytochrome P4502B (CYP2B) and 2C (CYP2C) protein levels were measured in this study to evaluate CYP expression, as expected, the fold of expression of CYP2B10 gene was

higher in phenobarbital group than others, since phenobarbital is CYP2B inducer and CYP2B10 is highly induced by PB, this finding was in line with Cartea et al. results[54]. In this study, CYP2B10 expression declined significantly in combination group, this finding is in line with a prior research, which found that exposure to glucosinolates, the major metabolite of Brassica nigra inhibited phase I enzymes[55]. Cytochrome P2C29 is one of CAR target genes[56], as reported previously by Hecht [57] and Fahey et al.[58], where CYP2C29 mRNA level was also elevated after PB exposure, this finding was in accordance with the result of the present study. In vitro and in vivo researches have stated that glucosinolate influence many stages of cancer development, comprising the inhibition of enzymes activation, including phase I enzymes[58-62].

As a future work, the following points can be suggested: The precise active phytochemicals of Brassica nigra that responsible for the observed effects are unknown, so fractionation of the extract, isolation and purification of active constituents is essential. Evaluate the in vitro and in vivo anticancer effect of the Brassica nigra extract on different cells and tissues using tumor-initiator agents, and try to understand the molecular mechanism and exact signaling pathway. Further researches may require to ensure the safety and tolerability of Brassica nigra extracts.

Conclusions

In this study, reflux extraction method using water: ethanol solvent system gave acceptable percent of yield, and the major active phytochemicals may be represented by isothiocyanate, depending on GC-MS analysis. Brassica nigra extract showed a remarkable antiproliferative and apoptotic effect confirmed by immunohistochemistry results (ki67, Bcl2). Real time qRT-PCR results further indicated the proposed mechanism of the antiproliferative effect of extract represented by its down regulation on the critical cell cycle genes (Mcm2 and Ccna2) within hepatocyte cells. The extract has inhibitory action on CYP2B10 and CYP2C29 expression which may be useful in protection against carcinogens activated by these enzymes.

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