



***In vivo* induction of hepatocellular carcinoma by diethylnitrosoamine and pharmacological intervention in Balb C mice using *Bergenia ciliata* extracts**

K. K. Dar^a, S. Ali^{b*}, M. Ejaz^c, S. Nasreen^c, N. Ashraf^e, S. F. Gillani^c, N. Shafi^c, S. Safeer^c,
M. A. Khan^{c,d}, S. Andleeb^c and T. A. Mughal^c

^aCollege of Life Science and Technology, Beijing University of Chemical Technology, 100029, Beijing, China

^bDepartment of Zoology, Government College University Lahore, Lahore-54000, Pakistan

^cMicrobial Biotechnology and Medical Toxicology Laboratory, Department of Zoology, University of Azad Jammu and Kashmir, Muzaffarabad-13100, Pakistan

^dSchool of Material Science and Engineering – MSE, Nanyang Technological University, Singapore

*e-mail: shaukatali134@yahoo.com

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(With 3 figures)

Abstract

Background: Hepatocellular carcinoma is the most frequent primary malignancy of liver and accounts for as many as one million deaths worldwide in a year. Objectives: The aim of the present study was to evaluate the anti-cancerous efficiency of *Bergenia ciliata* rhizome against diethylnitrosoamine induced hepatocarcinogenesis in Balb C mice. Methods: One percent diethylnitrosoamine was prepared by using 99 ml of normal saline NaCl (0.9 percent) solution to which was added 1 ml of concentrated diethylnitrosoamine (DEN) solution (0.01 µg/µl). Extract of *Bergenia ciliata* was prepared by maceration technique. Mice were classified into four groups as follows: Group 1 a control group (N=7) received saline solution (3.5 µl/mg), group 2 (N=14) received diethylnitrosoamine (3.5 µl/mg) intraperitoneally once in a week for eight consecutive weeks, group 3 (N=7) received plant extract (150 mg/kg (Body weight)) once in a week, while group 4 (N=7) was given combination of diethylnitrosoamine (3.5 µl/mg) and plant extract (150 mg/kg (Body weight)). After eight weeks of DEN induction group 2 mice were divided into two subgroups containing seven mice each, subgroup 1 was sacrificed while subgroup 2 was treated with plant extract (150 mg/kg (Body weight)) once in a week for eight consecutive weeks. Results: The model of DEN injected hepatocellular carcinomic (HCC) mice elicited significant decline in levels of albumin with concomitant significant elevations in tumor markers aspartate aminotransferase, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alpha feto protein (AFP), gamma glutamyl transferase (Y-GT), 5 nucleotidase (5NT), glucose-6-phosphate dehydrogenase (G6PDH) and bilirubin. The intraperitoneal administration of *B. ciliata* as a protective agent, produced significant increase in albumin levels with significant decrease in the levels of tumor markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alpha feto protein (AFP), gamma glutamyl transferase (Y-GT), 5 nucleotidase (5NT), glucose-6-phosphate dehydrogenase (G6PDH) and bilirubin. Conclusion: *Bergenia ciliata* has potent antioxidant activity, radical scavenging capacity and anticancerous properties. *Bergenia ciliata* extracts may provide a basis for development of anti-cancerous drug.

Keywords: hepatocellular carcinoma, diethylnitrosoamine, Balb C mice, anti-cancerous activity, *Bergenia ciliata*, alpha feto protein.

Indução *in vivo* de carcinoma hepatocelular por dietilnitrosoamina e intervenção farmacológica em camundongos balb c usando extratos de *Bergenia ciliata*

Resumo

Antecedentes: O carcinoma hepatocelular é a neoplasia primária mais frequente do fígado e é responsável por até um milhão de mortes em todo o mundo em um ano. Objetivos: O objetivo do presente estudo foi avaliar a eficiência anticancerígena do rizoma de *Bergenia ciliata* contra a hepatocarcinogênese induzida por dietilnitrosoamina em camundongos balb c. Métodos: Um por cento de dietilnitrosoamina foi preparado usando 99 ml de solução salina normal (0,9 por cento) à qual foi adicionado 1 ml de solução concentrada de dietilnitrosoamina (DEN) (0,01 µg / µl). O extrato de *Bergenia ciliata* foi preparado pela técnica de maceração. Os ratos foram classificados em quatro grupos:

Grupo 1 grupo controle (N = 7) recebeu solução salina (3,5 mL / mg), grupo 2 (N = 14) recebeu dietilnitrosoamina (3,5 mL / mg) por via intraperitoneal uma vez por semana para oito semanas consecutivas, o grupo 3 (N = 7) recebeu extrato vegetal (150 mg / kg (peso corporal)) uma vez por semana, enquanto o grupo 4 (N = 7) recebeu combinação de dietilnitrosoamina (3,5 µl / mg) e extrato (150 mg / kg (peso corporal)). Após oito semanas do grupo de indução DEN 2 ratos foram divididos em dois subgrupos contendo sete ratos cada, subgrupo 1 foi sacrificado enquanto subgrupo 2 foi tratado com extrato vegetal (150 mg / kg) uma vez por semana durante oito semanas consecutivas. Resultados: O modelo de camundongos hepatocelulares carcinômicos (CHC) injetados com DEN provocou declínio significativo nos níveis de albumina com elevações significativas concomitantes nos marcadores tumorais: aspartato aminotransferase, alanina aminotransferase (ALT), lactato desidrogenase (LDH), proteína alfa feto (AFP), gama glutamiltransferase (Y-GT), 5 nucleotidase (5NT), glicose-6-fosfato desidrogenase (G6PDH) e bilirrubina. A administração intraperitoneal de *B. ciliata* como agente protetor produziu um aumento significativo nos níveis de albumina com uma diminuição significativa nos níveis dos marcadores tumorais: aspartato aminotransferase, alanina aminotransferase (ALT), lactato desidrogenase (LDH), proteína alfa feto (AFP), gama glutamiltransferase (Y-GT), 5 nucleotidase (5NT), glicose-6-fosfato desidrogenase (G6PDH) e bilirrubina. Conclusão: *Bergenia ciliata* possui atividade antioxidante potente, capacidade de eliminação de radicais livres e propriedades anticancerígenas. Extratos de *Bergenia ciliata* podem fornecer uma base para o desenvolvimento de drogas anti-cancerígenas.

Palavras-chave: carcinoma hepatocelular, dietilnitrosoamina, camundongos Balb c, atividade anticancerosa, *Bergenia ciliata*, proteína alfa feto.

1. Introduction

Liver comprises about 2.5% of an adult's body weight and is the largest internal organ as well as gland in the body. It performs many important functions in the body such as xenobiotic and drug metabolism, energy metabolism, protein and amino acid metabolism, storage of glucose in the form of glycogen, storage of vitamins and regulation of hormonal functions (Rhoades and Bell, 2013). Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer, it is one of the most common life threatening solid tumors with global annual diagnosis exceeding one million new cases and remains the third leading cause of cancer death (Jemal et al., 2007). Causes of liver cancer include Hepatitis B virus (El-Serag and Rudolph, 2007; McMahon et al., 1990), Hepatitis C virus (Ikeda et al., 1999; Bruno et al., 2001), alcohol (Donato et al., 2002), aflatoxin (Turner et al., 2002), vinyl chloride (Wolk et al., 2001), obesity (Cotrim et al., 2000) and oral contraceptives (Yu and Yuan, 2004).

Diethylnitrosamine (DEN) is amongst the most essential natural carcinogenic agents, which is known to cause changes in the enzymes required in DNA repair replication and is regularly utilized as a cancer-causing agent to prompt liver carcinogenesis in mouse models (Ramakrishnan et al., 2007). Compounds like N-nitroso are well established hepatocarcinogens that are being implicated in the etiology of various human cancers (Bansal et al., 2005). DEN is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agricultural chemicals and pharmaceutical agents and is considered as potential hazard for the development of disease (Bartsch and Montesano, 1984).

Reliable markers for HCC diagnosis are aspartate transaminases (AST) and alanine transaminases (ALT) (Whitby et al., 1984), lactate dehydrogenase (LDH) (King, 1965), bilirubin (Harper, 1961), Gamma-glutamyl

transferase (GGT) (Yao et al., 2004), alpha feto protein (Sell and Becker, 1978), 5-nucleotidase (5NT) (Sadej et al., 2006), Glucose-6-phosphate dehydrogenase (G6PDH) (Molero et al., 1994) and albumin (Vandenberghe, 1996).

Chemotherapeutic drugs are not recommended for advanced stage HCC treatment because these are chemo-refractory and have adverse events (AEs). The toxicity caused by these chemotherapeutic agents has restricted their usage (Deng et al., 2015). Sorafenib is a multikinase inhibitor that targets the serine-threonine kinase Raf-1 of the Ras/MAPK pathway in addition to having antiangiogenic properties. Besides this Sorafenib also have side effects like, diarrhoea, hand foot syndrome and hypertension. Gemcitabine has a favorable toxicity profile, with bone marrow suppression being the most common side effect (Chung, 2015).

Numerous naturally occurring compounds from plants are being used as anticancerous agents and are currently undergoing medical development. Many plants like *Petasites japonicas* (Kim et al., 2015), *Amorphophallus campanulatus* (Ansil et al., 2012), *Curcuma aromatic* (Li et al., 2014), *Panax ginseng*, *Salvia miltiorrhiza* (Wang et al., 2012), *Tripterygium wilfordii* (Chen et al., 2012), *Astragalus* (Chen et al., 2011) and many other plants are used against HCC as an anticancerous agents with low toxicity. *Bergenia ciliata* (BC) belongs to family Saxifragaceae has various medicinal properties. These properties include antibacterial, antioxidant, anti-inflammatory, anti-diabetics and antiviral activities (Ruby et al., 2012). *Bergenia ciliata* also aids in removing kidney stones (Hafidh et al., 2009).

The aim of current study was to assess the anti-cancerous activity of *Bergenia ciliata* rhizome in Balb C mice against DEN induced hepatocellular carcinoma. The use of natural herbs such as *B. ciliata* will result in decreased toxicity and side effects. Thus, it will increase safety and hence patients associated compliance resulting in overall increase in effectiveness.

2. Materials and Methods

The Minimum Standards of Reporting Checklist contains details of the experimental design, statistics, and resources used in this study.

2.1. Ethical statement

All animal trial techniques were directed as per local and worldwide controls. The nearby direction is the Wet op de dierproeven (Article 9) of Dutch Law (international) and a similar law regulated by the Bureau of Animal Experiment Licensing, local University.

2.2. Chemicals used

All the chemicals used were of analytical grade. Diethylnitrosoamine (Sigma Chemical Co, St Louis, Mo, USA) and normal saline (NaCl 0.9 percent w/v) were used.

2.3. Animals

Balb C mice (35-40 g) of male sex were obtained from the animal house in National Institute of Health (NIH) Islamabad, Pakistan. The animals were maintained in a separate room having temperature 20-23°C and 50%-60% relative humidity with 12:12 h light/dark cycle in polypropylene cages. The animals were fed with standard rodent pellet diet and drinking water. Food was withdrawn 18-24 h before the experiment although water was allowed *ad libitum* and allocated to different experimental groups.

2.4. Preparation of diethylnitrosoamine

One percent diethylnitrosoamine was prepared by using 99 ml of normal saline NaCl (0.9 percent) solution to which was added 1 ml of concentrated diethylnitrosoamine (DEN) solution (0.01 µg/µl).

2.5. Diethylnitrosoamine induced hepatocellular carcinoma

Male Balb C mice (35-40 g) were divided into four groups. Group 1 a control group (N=7) received saline solution (3.5 µl/mg), group 2 (N=14) received diethylnitrosoamine (3.5 µl/mg) intraperitoneally once in a week for eight consecutive weeks, group 3 (N=7) received plant extract (150 mg/kg) once in a week, while group 4 (N=7) was given combination of diethylnitrosoamine (3.5 µl/mg) and plant extract (150 mg/kg).

2.6. Plant material and extract preparation

Rhizome of *Bergenia ciliata* was collected from Jhellum valley Azad Kashmir, Pakistan and washed under running tap water, air dried under shade, coarsely powdered and kept in airtight container for further use. Powder of rhizome was soaked in methanol for 20 days to increase the polarity at room temperature (25°C-30°C). Extract was prepared through maceration extraction method. The crude extract was filtered through Whatmann filter paper I (Sigma Aldrich), evaporated under reduced pressure using rotary evaporator (Temp, 37°C and at speed of 100 rpm) to get the required concentration. The extract was then dissolved in water and methanol in proportion of 23:1 respectively for screening anti-cancer assay.

2.7. Toxicity studies

Assessment of toxicity studies were conducted by utilizing the acute toxic classic method (Hussain et al., 2012). For each step, three male Balb C mice were used in this study. The mice were kept fasting with water overnight and extract was administered intraperitoneally at a single dose of 150 mg/kg. Mice were regularly observed for 4 h after initial administration and then once in a day for consecutive two weeks. If the mortality occurred in two out of three mice than the dose was toxic, if not than this dose was used as standard. Mice survived at the dose of 150 mg/kg and this dose was used as a standard in the whole experiment.

2.8. In vivo pharmacological interventions against hepatocellular carcinoma

After eight weeks of DEN induction group 2 mice were divided into two subgroups containing seven mice each, subgroup 1 was sacrificed while subgroup 2 was treated with plant extract (150 mg/kg) once in a week for eight consecutive weeks.

2.9. Biochemical analysis

After the last treatment, the mice were fastened overnight, and all of the mice were anesthetized with chloroform presented on a cotton ball and euthanized by cervical decapitation. Ethylene diamine tetra acetic acid (EDTA) tubes (size 13*75, containing ~1.8 mg K2EDTA per ml blood) were used for collection of blood samples. Blood samples were centrifuged for a minimum of 10 minutes at 1000-2000 RCF (Relative Centrifugal Force). Centrifuged plasma was used for various biochemical measurements. The livers were extracted instantly from the mice, washed with ice-chilled physiological saline and refrigerated until examination.

The activities of biochemical parameters like aspartate transaminase (AST) and alanine transaminase (ALT) were estimated by the method of (Reitman and Frankel, 1957), while Gamma glutamyl transferase (Y-GT) was estimated by methods of (Szasz, 1976; King, 1965). Total bilirubin level (TBL) was determined by modified dimethyl sulfoxide (DMSO) method (Dangerfield and Finlayson, 1953) on the basis of sulfanilic acid reaction with sodium nitrite to produce deoxidized sulfanilic acid. Activity of lactate dehydrogenase (LDH) was assessed by the method described by (King, 1965), while total albumin levels were measured by procedure demonstrated by (Savory et al., 1976). Activity of 5 nucleotidase (5NT) was measured by the process explained by (Rieder and Otero, 1969). G6DPH was assessed by the method explained by (Minucci et al., 2009). Quantitative estimation of tumor marker alpha fetoprotein (AFP) antigen (CEA) was based on solid phase enzyme linked immunosorbent assay using the UBI MAGIWELL (USA) enzyme immunoassay kit (Macnab et al., 1978; Sell and Becker, 1978). Activity of G6PDH was assessed by the method explained by (Tepperman and Tepperman, 1962)

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs with error bars of standard errors of the means (SEM). To analyse the impact of different treatments on levels of biomarkers in mice, one-way analysis of variance and a Dunnett's multiple comparison test with probability level of five percent as the minimal criterion of significance was used.

3. Results

3.1. Effect of BC extract on body weight, liver weight and relative liver weight

Table 1 depicts initial and final average body weight, absolute and relative liver weights of different treatment groups. Final body weight of the control group I mice was (244.85±0.98) g which on administration of DEN was significantly decreased to (172.57±0.68) g in group II mice. In mice group IV treated with BC extract (150mg/kg) after DEN induction, the weight of mice showed significant increase in weight (210.28±1.84) g when compared to group II DEN treated mice. Further, the group II intraperitoneally DEN administered mice resulted in significant increase of relative liver weight/100 g body weight to (5.47±0.06) g as compared to control, (2.94±0.01) g. The treatment with BC extract in group V DEN administered mice significantly decreased the relative liver weight (3.52±0.008). While concomitant administration of DEN and BC extract resulted in maintaining the body weight (240.57±1.2) g and relative liver weight (2.63 ± 0.01) g as compared to DEN administered mice. The administration of BC extract alone did not show any significant effect on body weight and relative liver weight Table 1.

3.2. Effect of BC extract on HCC tumour markers

3.2.1. Effect on ALT

Intraperitoneal administration of DEN (3.5 µl/mg body weight once in a week for eight consecutive weeks in mice) caused highly significant increase in levels of ALT (control: 29.0 ± 1.8 U/L; DEN: 141.5 ± 2.2 U/L; BC: 32.7 ± 3.2 U/L). When extract of BC was given in combination with DEN no significant increase in levels of ALT was observed (DEN+BC1: 49.1 ± 3.6 U/L). When extract of *B. ciliata* (BC) was injected intraperitoneally (150 mg/kg

body weight once in a week for eight consecutive weeks) in DEN treated mice, significant decrease in level of ALT (DEN: 141.5 ± 2.2 U/L; DEN + BC2: 124.0 ± 3.4 U/L) was observed (Figure 1.A).

3.2.2. Effect on AST

Intraperitoneal injection of DEN (3.5 µl/mg body weight once in a week for eight consecutive weeks in mice) showed tremendous increase in levels of AST (control: 87.9 ± 2.7 U/L; DEN: 474.0 ± 7.6 U/L; BC: 93.2 ± 3.5). When extract of BC was given in combination with DEN no significant increase in levels of AST was observed (DEN + BC1: 138 ± 2.7 U/L). But, intraperitoneal administration of extract of BC (150 mg/kg body weight once in a week for eight consecutive weeks) in DEN treated mice, resulted in significant decrease in level of AST (DEN: 474.0 ± 7.6 U/L; DEN + BC2: 439.3 ± 10.4 U/L) (Figure 1).

3.2.3. Effect on LDH

DEN injected intraperitoneally (3.5 µl/mg body weight once in a week for eight consecutive weeks in mice) resulted in highly significant increase in levels of LDH (control: 373.2 ± 15.0 U/L; DEN: 2165.7 ± 78.9 U/L; BC: 426.2 ± 15.8) When extract of BC was given in combination with DEN no significant increase in levels of LDH was observed (DEN + BC1: 568.9 ± 28.5 U/L). But, intraperitoneal administration of extract of BC (150 mg/kg body weight once in a week for eight consecutive weeks) in DEN treated mice, resulted in significant decrease in level of LDH (DEN: 2165.7 ± 78.9 U/L; DEN + BC2: 1835.0 ± 112.2 U/L) to significant value (Figure 1).

3.2.4. Effect on AFP

Highly significant increase in levels of tumour marker AFP was caused by DEN (3.5 µl/mg body weight in mice) once in a week for eight consecutive weeks (control: 13.5 ± 0.9 ng/ml; DEN: 63.4 ± 2.4 ng/ml; BC: 14.9 ± 0.6 ng/ml). When extract of BC was given in combination with DEN no significant increase in levels of AFP was observed (DEN + BC1: 35.4 ± 2.6 ng/ml). Significant decrease in level of AFP (DEN: 63.4 ± 2.4 ng/ml; DEN+BC2: 54.0 ± 3.0 ng/ml) was seen after intraperitoneal induction of extract of RBC (150 mg/kg body weight once in a week for eight consecutive weeks) in DEN treated mice (Figure 2).

Table 1. Effect of *Bergenia ciliata* extract on the body weight, liver and relative liver weight of different mice groups.

Group	Treatment	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Relative liver weight (Liver wt./100 g b. w.)
I	Control	156.42±1.10	244.85±0.98	7.0±0.06	2.94±0.01
II	DEN	154.71±1.56	172.57±0.68***	9.16±0.07	5.47±0.06***
III	BC	154±0.9	245.15±0.96	6.18±0.09	2.80±0.01
IV	DEN+BC1	155.28±1.5	240.57±1.25@@@	6.03±0.09	2.63±0.01@@@
V	DEN+BC2	156.28±1.18	210±1.84@@@	7.82±0.05	3.52±0.008@@@

Values are expressed as mean ± SEM of 7 mice in each group. ***p < 0.001 compared to respective control group I. @@@p < 0.001 compared with group II (DEN treated).

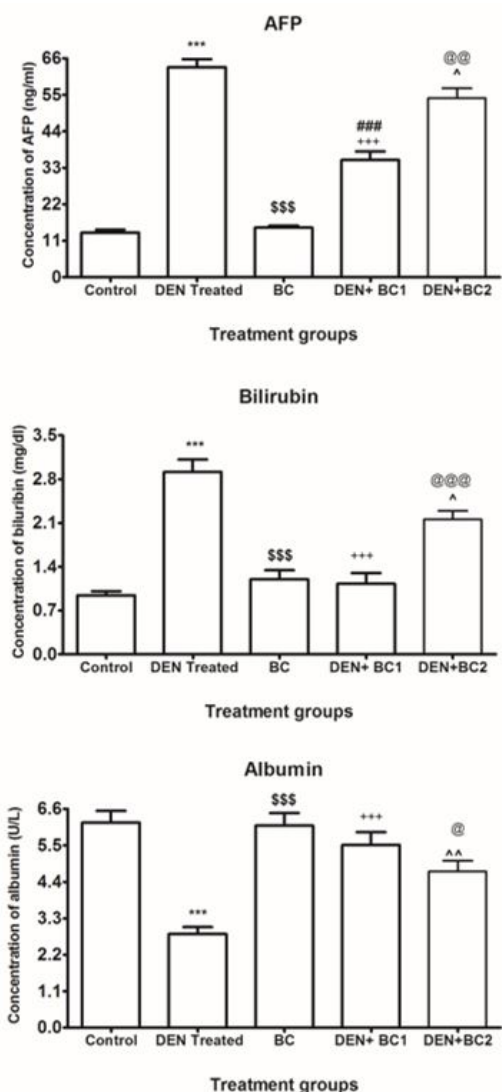
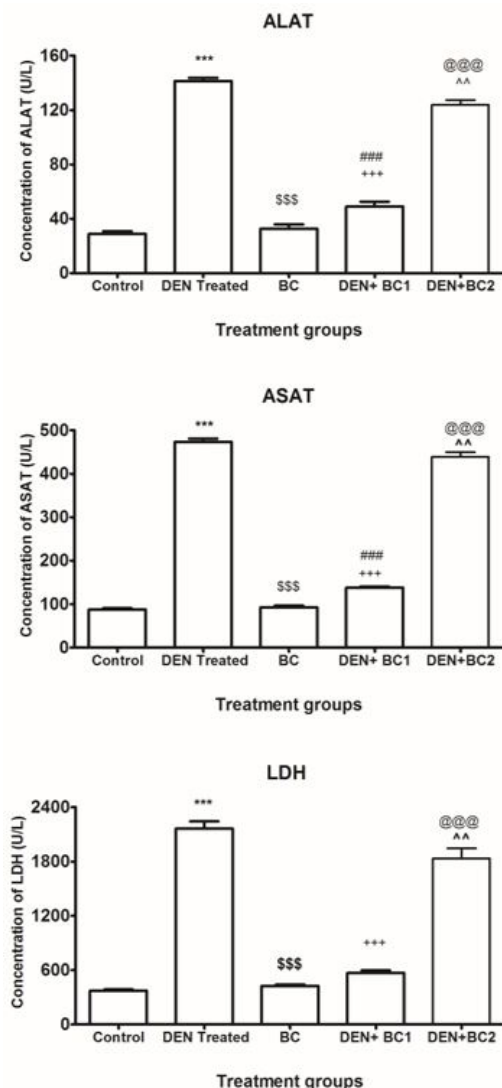


Figure 1. Analysis of Alanine Transaminase (ALAT), Aspartate Transaminase (ASAT) and Lactate Dehydrogenase (LDH). Keys: DEN, Diethylnitrosoamine; BC, extract of *Bergenia ciliata*; DEN+BC1, DEN and BC given at a time; DEN+BC2, treatment of DEN injected mice after eight weeks using BC *depicts differences between control and DEN treatment groups. #depicts differences between control and BC1 extract treatment groups. @depicts differences between control and BC2 extract treatment groups. \$depicts differences between DEN and BC extract treatment groups. +depicts differences between DEN and BC1 extract treatment groups. ^depicts differences between DEN and BC2 extract treatment groups. Each bar represents the mean value of seven replicates and SEM. Statistical icon ^ = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, @ = p ≤ 0.05, @@ = p ≤ 0.01, @@@ = p ≤ 0.001, \$\$\$ = p ≤ 0.001.

Figure 2. Analysis of Alpha Fetoprotein (AFP), Bilirubin and Albumin. Keys: DEN, Diethylnitrosoamine; BC, extract of *Bergenia ciliata*; DEN+BC1, DEN and BC given at a time; DEN+BC2, treatment of DEN injected mice after eight weeks using BC *depicts differences between control and DEN treatment groups. #depicts differences between control and BC1 extract treatment groups. @depicts differences between control and BC2 extract treatment groups. \$depicts differences between DEN and BC extract treatment groups. +depicts differences between DEN and BC1 extract treatment groups. ^depicts differences between DEN and BC2 extract treatment groups. Each bar represents the mean value of seven replicates and SEM. Statistical icon: @, ^ = p ≤ 0.05, @@, ^^ = p ≤ 0.01, ***, ###, @@@, \$\$\$ = p ≤ 0.001.

3.2.5. Effect on Albumin

Albumin level was significantly decreased (control: 6.2 ± 0.4 U/L; DEN: 2.8 ± 0.2 U/L; BC: 6.1 ± 0.4 U/L) by intraperitoneal administration of DEN (3.5 μl/mg body weight once in a week for eight consecutive weeks

in mice). When extract of BC was given in combination with DEN no significant decrease in levels of albumin was observed (DEN + BC1: 5.5 ± 0.4 U/L). But intraperitoneal induction of extract of BC (150 mg/kg body weight once in a week for eight consecutive weeks) in DEN treated

with DEN induction demonstrated a significant increment in serum transaminases ALT, AST and LDH activities. Over generation of these proteins in tumor cells, is due to alterations caused by DEN in the permeability of cell membrane thus, bringing about leakage of these proteins into serum (Ramakrishnan et al., 2007). The increment of these enzymatic activities was characteristic of the toxic effects of DEN on the liver tissue. It is referred to that N-nitroso compounds have been found as cancer causing agents in different warm blooded animals including primates (Swenberg et al., 1991). DEN has been appeared to be processed by cytochrome P-450 (CYP 2E1) to its dynamic ethyl radical metabolite, which interacts with DNA causing change and carcinogenesis (Anis et al., 2001). Discoveries of present research were consistent with the findings of (Ramakrishnan et al., 2007; Swenberg et al., 1991; Mittal et al., 2006; Vozarova et al., 2002). Then again, extract of *B. ciliata* rhizome significantly reduced the elevated activities of these proteins which may be because of the capacity of constituents in *B. ciliata* to maintain parenchymal cell recovery in liver, in this way ensuring membrane integrity, along with diminishing enzymatic leakage (Jadon et al., 2007).

A significant increment in the level of serum bilirubin was seen in DEN administered mice, which might be due to mass hindrance of the conjugation response and release of unconjugated bilirubin from damaged hepatocytes (Raj Kapoor et al., 2006). Decline in serum bilirubin after treatment with *B. ciliata* demonstrated the viability of the plant in keeping up the healthy status of the liver.

Five nucleotidase is a fundamental protein in the extracellular pathway, since it produces adenosine from AMP and is communicated in a wide range of tissues (Zimmermann, 1992). Studies confirm that elevation of 5-nucleotidase is related with an exceedingly obtrusive phenotype (Sadej et al., 2006), tranquilize resistance and tumor-advancing capacities (Ujházy et al., 1996). Besides producing adenosine, 5-nucleotidase may take part in bonding and interacting with extracellular matrix proteins. Adenosine is accounted for to be available at high levels in strong tumors, with high concentration in extracellular tumor microenvironment and is accounted for tumor development and angiogenesis (Spychala, 2000). Extracts of *B. ciliata* treatment significantly diminished the levels of 5-nucleotidase in serum, which may be because of the capacity of the molecules in *B. ciliata* to successfully restrain expansion.

Gamma-glutamyl transferase (YGT) is a membrane bound enzyme, which shows a tissue specific expression and is modified under different physiologic and pathologic conditions, for example, development and carcinogenesis (Yao et al., 2004). It is most elevated in developing fetus livers and declines quickly to the least levels after birth, and is an enzyme that has been widely examined in connection to hepatocarcinogenesis. A few reviews have shown that YGT level was elevated during development of HCC, which may demonstrate the fundamental tumor load (Tang et al., 1999). Though, *B. ciliata* treated mice

demonstrated diminished level of YGT in contrasted to tumor-bearing mice, which shows diminished tumor load in *B. ciliata* treated mice.

AFP is a well-known representative tumor marker of HCC. High levels of AFP are believed to be strongly suggestive of HCC (Endo et al., 1975) because greater than seventy percent of HCC patients have high serum concentration of AFP because of the tumor secretion. AFP is also most extensively used in the diagnosis of HCC (Ramakrishnan et al., 2007). The increased level of AFP observed in DEN-induced animals is an indicative of HCC. Extract of *B. Ciliata* treatment significantly reduced the levels of AFP, which revealed the anti-tumor effect of the *B. ciliata* against HCC.

Information exhibited in the present research shows that DEN altogether diminished serum albumin. Vandenberghe (1996) revealed that, hypoalbuminemia may be result of liver damage, which is associated with decrease in albumin synthesis. Cross et al. (1987) ascribed the hypoalbuminemia state to the increased rate of catabolism as compared to disability of synthesis. Due to the toxic effect of cancer causing agents; there is an increased production of ROS. These free radicals are equipped for harming biomolecules, for example, proteins that affect cell activities, membrane capacities and structure. Treatment of *B. ciliata* returned the albumin level to ordinary, which mirrors the well working of hepatocytes protein.

Glucose-6-phosphate dehydrogenase (G6PDH) is the key enzyme that catalyses the first step of the pentose phosphate pathway to produce NADPH and ribose-5-phosphate (Ulusu and Tandogan, 2006). This regulatory house-keeping enzyme is localized to the cytosol and mitochondria and is an antioxidant that preserves the cytosolic redox status by producing the cellular reductant, NADPH (Jain et al., 2003; Frederiks et al., 2003; Stanton et al., 1991). The literature suggests that under proliferative conditions, G6PD gene expression increases both in foetal hepatocytes (Molero et al., 1994) and in cultured mature liver cells (Stanton, 2012). Our results based on the biochemical parameters are consistent with previous reports in which DEN shows a significant increase in LFT proteins due to hepatic intoxication (Mukherjee and Ahmad, 2015; Latha and Latha, 2014). *B. ciliata* treatment returned the levels of G6PDH to normal levels in DEN treated mice. Therefore, *B. ciliata* administration seems to be a highly promising agent for protecting hepatic tissue against oxidative damage and in preventing hepatic injury and dysfunction.

5. Conclusion

Hepatocellular carcinoma is highly aggressive cancer, characterized by rapid growth and early vascular invasion. Present study proposed that besides various chemotherapeutic drugs are being used for the treatment of HCC, the vital role was played by *B. ciliata* in prevention and treatment of hepatocellular carcinoma. Methanolic extracts of *B. ciliata* contains cytotoxic effects and have apoptotic properties against hepatocellular carcinoma

in vivo. *B. ciliata* decreased the levels of tumor markers: aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alpha-fetoprotein, gamma glutamyl transferase, 5' nucleotidase and glucose-6-phosphate dehydrogenase. It was concluded that bioactive compounds *B. ciliata* is a source of anticancer drug.

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Authors contributions

Kamran Khurshid Dar and Shaukat Ali proposed the thought and framework of this paper and wrote this paper. Shukat Ali implemented the thought with software, analyzed the data. Mubashair Ejaz, Sundas Nasreen, Fiza Gillani, Sobia Safer and Naheeda Shafi maintained animals in Lab, Muhammad Adeeb Khan and Tafail Akbar Mughal provided medical guidance of this paper, Saiqa Andleeb provided guidance in preparation of extracts. All authors read and approved the final manuscript.