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EVALUATION OF ANTIPROLIFERATIVE AND HEPATOPROTECTIVE EFFECTS OF WHEAT GRASS *(TRITICUM AESTIVUM)*

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This study was aimed to evaluate the pharmacological potential of various extracts (hexane, chloroform, methanol and aqueous) of dried shoots of *Triticum aestivum* (wheat grass) in terms of antiproliferative and hepatoprotective potential of *T. aestivum*. The total chlorophyll content in dried shoots of *T. aestivum* was 0.54 ± 0.016 g/L (chlorophyll-a: 0.288 ± 0.05 g/L; and chlorophyll-b; 0.305 ± 0.05 g/L), while total carotene content was 0.42 ± 0.066 g/L. In addition, the chloroform extract of dried shoots of *T. aestivum* (250 µg/mL) exhibited 87.23% inhibitory effect with potent cytotoxicity against human hepatocellular carcinoma (HepG2) cancer cell line. Moreover, chloroform and methanol extracts significantly reduced the levels of SGOT, and SGPT enzymes, as well as total bilirubin content, while raised the level of total protein in a concentration-gradient manner, confirming the potent hepatoprotective effect of *T. aestivum*. A possible mechanism of apoptosis of the chloroform extract of dried shoots of *T. aestivum* in terms of its potent antiproliferative activity against HepG2 cancer cell line can also be proposed in this study. Our findings clearly demonstrate that *T. aestivum* has a significant pharmacological potential that night be used for antiproliferative and hepatoprotective purposes.

Keywords: T. aestivum (wheat grass) – antiproliferative – hepatoprotective – apoptosis mechanism – pharmacological significance

INTRODUCTION

Triticum aestivum Linn. (wheat grass) belonging to the Gramineae family, is widely cultivated almost all over the world [18]. Wheat grass stimulates metabolism and restores alkalinity in the blood, which helps to reduce acidity in the blood and to restore healthy cells along with an ability to serve as a detoxificant agent [18]. Reactive oxygen species (ROS) are produced as by-products of various metabolic processes, mainly during respiration in living organisms. Consequently, oxidative stress is considered to be implicated in the pathophysiology of many diseases including cancer [2].

Various medicinal herbs have been used as a treatment for various ailments, including malignancies [10]. Human hepatocellular carcinoma (HCC) is one of the

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most common malignancies worldwide with an annual incidence of approximately 600,000 cases, including 55% in China [19]. Hence, patients suffering from HCC often turn to use natural treatment, largely based on the use of traditional medicinal plants. Indeed, some medicinal plant extracts have shown to display antiproliferative activities on different HCC cell lines [5].

The liver is the most important and integral part of both human and animal body system, and is highly affected primarily by toxic agents [16]. In addition, consuming large amount of drugs, intake of alcohol, and junk food is also endangering the functional ability of the liver by damaging the liver architecture. Though allopathic drugs are available, they do not represent a complete solution. However, medicinally important plants are capable of targeting these hepatic disorders because a number of plants having medicinal properties have been investigated based on the integrative approaches on drug development against liver diseases [14].

Plant extracts containing high concentration of chlorophylls and carotenoids are of significant importance because they can exhibit different protective effects by excerting different mechanisms [4]. The consumption of carotenoids and chlorophylls has been associated with protective effects against atherosclerosis, some forms of cancer, osteoporosis, cataracts, neurodegenerative diseases, mutagenesis, and oxidative stress [8]. The protective effects of carotenoids are mediated by their oxidant, antioxidant, redox sensitive cell signalling, induction of gene expression, and provitamin A properties [7]. The protective effects of chlorophylls depend on their ability to modulate the activation of the endogenous xenobiotic detoxification systems and caspases/polymerase pathway as well as by their antioxidant and mutagen trapping properties [4]. Chlorophyll and carotenoid compounds have shown enormous hepatoprotective effects via several mechanisms, which include lowering the levels of lipid peroxidation and increasing the activity of the antioxidant enzyme superoxide dismutase (SOD) [13].

Although some preliminary studies on *Triticum aestivum* (wheat grass) have been reported [18], no detailed and systematic information on antiproliferative and hepatoprotective potential of *T. aestivum* has been published so far. Hence, the present study was aimed to evaluate antiproliferative and hepatoprotective potential of various extracts derived from the dried shoots of *T. aestivum* maintained under specified growth conditions.

MATERIAL AND METHODS

Reagents and chemicals

Folin-Ciocalteu's reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin, potassium ferricyanide, ferric chloride, ascorbic acid, pyrogallol, mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanine (DOPA), bovine serum albumin, P-nitrophenyl-a-D-glucopyranoside, aluminium chloride, sodium carbonate, hydrochloric acid, trypsin, ethylene diamine tetra acetic acid (EDTA), dimethyl sulphoxide

(DMSO), MTT dye, Liv-52 and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA) was obtained from Wako Pure Chemical Industries (Japan). Ferric chloride was purchased from Fluka (Steinhiem, Switzerland).

Plant material

The seeds of *T. aestivum* were collected from the Regional Agriculture Research Institute (RARI, India) in sterile polythene bags and were authenticated by a senior scientist of the institute. A voucher specimen has been deposited in the laboratory of RARI, India.

Growth conditions

The seeds of *T. aestivum* were sown during September, 2015, when the climatic conditions were suitable for the growth of wheat grass. Six plots of 1 m^2 were prepared for sowing in the Botanical garden, RARI, India. Loamy soil (70% sand + 30% clay) was prepared and conditioned with compost. The pH of the soil was optimized from 6.9–7.2, whereas germination temperature was maintained in the range of 16–21 °C, the optimum growth temperature for wheat grass. The seeds were soaked in water before sowing for 12 h at room temperature. The plants watered in alternative days and harvested on the 10^{th} day.

Preparation of organic extracts

The powdered form of dried shoots of *T. aestivum* (10 g) was successively extracted with 100 mL of each solvent, including methanol, hexane, chloroform, and distilled water, using a orbital shaker for 48 h at 37 °C. After 48 h, the supernatant was filtered and evaporated to dryness to give a viscous dark mass with a percentage yield of 1.65%, 0.88%, 9.8% and 12.41% (w/w), respectively. These dried crude extracts of *T. aestivum* were dissolved in water or the suitable solvent (1% DMSO), and used for the assessment of pharmacological activities.

Experimental animals

For our experiments adult Wistar albino rats of both sexes, weighing between 140–160 g body-weight were used. The experiments received an animal ethics approval from the institutional ethical committee via 1030/9/07/CPCSEA.

Acute toxicity assay

For acute toxicity assay, rats of both sexes were divided into different groups. After an overnight fasting, extracts of dried shoots of *T. aestivum* were administered orally in a dose range of 100–500 mg/kg body weight of each animal, following a continuous observation for the next 2 h for any toxicity or disease symptom, and for mortality up to 24 h [1].

Estimation of total chlorophyll (chlorophyll-a, chlorophyll-b) and carotene contents

Total chlorophyll (chlorophyll-a, and chlorophyll-b) and carotene contents in the extracts of dried shoots of *T. aestivum* were determined according to the method of Jayaraman [9]. Briefly, 1 g of dried shoot-leaf sample of *T. aestivum* was grinded in pestle-mortar with 5 mL of distilled water to form a paste. The contents were transferred to a centrifuge tube and the total volume was made up to 10 mL with distilled water. A 0.5 mL of supernatant from the tube was transferred to a fresh tube containing 4.5 mL of 80% acetone. The contents were centrifuged at 4,000 × *g* for 15 min. The absorbance of the supernatant was calculated to measure total contents of chlorophyll (chlorophyll-a, chlorophyll-b) and carotene (co-efficient 2500) at the wavelengths of 661.5, 645, and 450 nm, respectively, using following formulas:

$$\text{Chlorophyll-a content } (\mu g/mL) = 11.24 \times A_{661.5} = 2.04 \times A_{645.0}$$

$$\text{Chlorophyll-b content } (\mu g/mL) = 20.13 \times A_{645.0} = 4.19 \times A_{661.5}$$

$$\text{Carotenoid content } (mg/mL) = (A_{450.0} \times \text{Volume of sample taken})/2500 \text{ .}$$

Determination of antiproliferative activity against human hepatocellular carcinoma cells

To evaluate the antiproliferative activity of various extracts of dried shoots of *T. aestivum*, MTT assay was used with Hep G2 (human hepatocellular carcinoma) cells, according to the modified method of Yin et al. [20]. A 90% confluent Hep G2 cells were trypsinized, counted and prepared in a suspension with 10^4 cells in 100 µL of DMEM (Dulbecco's Modified Eagle Media) and seeded in a 24 wells plate. Each extract of *T. aestivum* was dissolved in 1% DMSO and different concentrations (25, 50, 100, 150, 200 and 250 µg/mL) were prepared, and added to the wells along with the HEp-G2 cells. Treated cells were incubated at 37 °C in a humidified atmosphere for 24 h. Media was withdrawn and washed with fresh media (300 µL) and then microscopy was performed. Before and after the treatment, the cells were morphologically analyzed. Each concentration was tested in triplicates. Untreated cells were used as a negative control. After 24 h, 50 µL of MTT (2 mg/mL in PBS) was added

to each well, incubated for an additional 4 h, and the plate was centrifuged at 1,000 × *g* for 10 min. The media was removed and 100 µL of DMSO was added, dissolved and optical density (OD) was taken immediately at 570 nm by a microplate reader. The % inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \text{Control OD} - \text{Sample OD/Control OD} \times 100$$

Determination of hepatoprotective activity

Based on the effective antiproliferative results, chloroform and methanol extracts of dried shoots of *T. aestivum* were chosen for further evaluation of hepatoprotective activity. A total of 36 animals were divided into 6 groups (n = 6 in each group). The treatment period was 6 days. Group I served as a control and received normal saline (10 mL/kg p.o). Group II received CCl_4 (50 µL/kg b.w.) diluted with liquid paraffin (1:1) given orally on 3rd and 6th day, Group III received CCl_4 and standard drug Liv-52 (50 mg/kg b.w., p.o.). Similarly, Groups IV and V received CCl_4 and chloroform and methanol extracts at different concentrations (50, 100 and 200 mg/kg) once daily simultaneously for 7 days. Food was withdrawn 12 h before CCl_4 administration on the 6th day to enhance the acute liver damage in all the groups, except group I animals (control). Rats were sacrificed on 7th day, 24 h after administration of the last dose. Blood samples were collected by the abdominal aorta method in standard sampling tubes and serum was separated within 8 h at room temperature for use of assay marker enzymes and estimation of total protein.

Enzyme assays

The activities of serum hepatic marker enzymes such as serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were assayed using standard kits from Merck (Germany). The results were expressed as units/liter (U/L). The data obtained were subjected to statistical analysis.

Estimation of total serum protein and bilirubin

The level of total serum protein and bilirubin were estimated by Biuret method [15] and according to the developed protocol of Maekawa et al. [11].

Statistical data analysis

Statistical analysis was performed using an SPSS software followed by multiple Duncan's test with analysis of variance (ANOVA) to find out the significant difference at $p < 0.05$ for each parameter.

RESULTS

Total chlorophyll (chlorophyll-a, chlorophyll-b) and carotene contents

The dried leaf-shoots of *T. aestivum* were subjected to estimate total chlorophyll (chlorophyll-a and chlorophyll-b) and carotene contents. The total chlorophyll in the dried leaf-shoots of *T. aestivum* was found to be 0.54±0.016 g/L, out of which, amounts of chlorophyll-a, and chlorophyll-b were found as 0.288±0.05 and 0.305±0.05 g/L, respectively. The total carotene content was measured as 0.42± 0.066 g/L.

Antiproliferative activity against human hepatocellular carcinoma cells

The results showed that the chloroform extract of dried shoots of *T. aestivum* had a remarkable antiproliferative effect with IC_{50} value of 87.64. The absorbance values shown by the chloroform extract were 0.178±0.01, 0.156±0.02, 0.110±0.01, 0.088±0.02, 0.069±0.02 and 0.054±0.21, indicating the percentage inhibition of 16.54±1.67, 30.34±3.14, 56.18±1.98, 68.54±2.76, 79.22±4.87 and 87.64±6.07 at the concentration of 25, 50, 100, 150, 200 and 250 µg/mL, respectively (Table 1, Fig. 1A). DMEM and DMSO in a ratio of 1:1 were used as a control, and their OD value was 0.423 at 570 nm. A dose-dependent response on antiproliferative activity was observed when tested the chloroform extract of dried shoots of *T. aestivum*. Decrease in OD values of the cells represents the increase in percentage inhibition of the cancerous cells (Fig. 1B). At the highest concentration (250 µg/mL), the chloroform extract showed 87.64±6.07% inhibition of the cancerous cells.

Table 1
Antiproliferative activity of chloroform extracts *T. aestivum*

Concentration (µg/mL)	*Triticum aestivum*		
	Absorbance	% viability	% inhibition
25	0.178±0.01^{a}	83.46±1.87^{a}	16.54±1.67^{f}
50	0.156±0.02^{ab}	69.66±3.45^{b}	30.34±3.14^{e}
100	0.110±0.01^{b}	43.82±5.98^{c}	56.18±1.98^{d}
150	0.088±0.02^{c}	31.46±6.97^{d}	68.54±2.76^{c}
200	0.069±0.02^{d}	20.78±3.45^{e}	79.22±4.87^{b}
250	0.054±0.021^{e}	12.36±2.52^{f}	87.64±6.07^{a}

Superscripts in the same line not sharing a common superscript are significantly different at $p<0.05$ by Duncan's multiple range test while, Superscripts sharing a common letter in the same line are not significantly different at $p<0.05$ by Duncan's multiple range test.

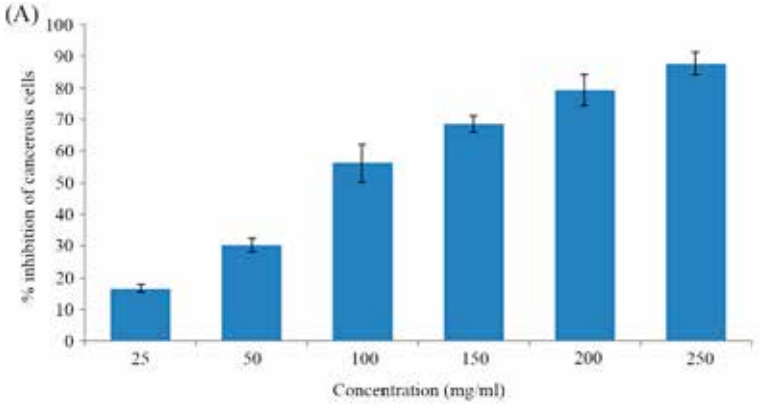

Fig. 1A. Effect of chloroform extract of dried shoots of *T. aestivum* on percent inhibition of human hepatocellular carcinoma (HepG2) cancer cell lines in MTT assay. All values are mean ± S.D., $p < 0.05$ significant

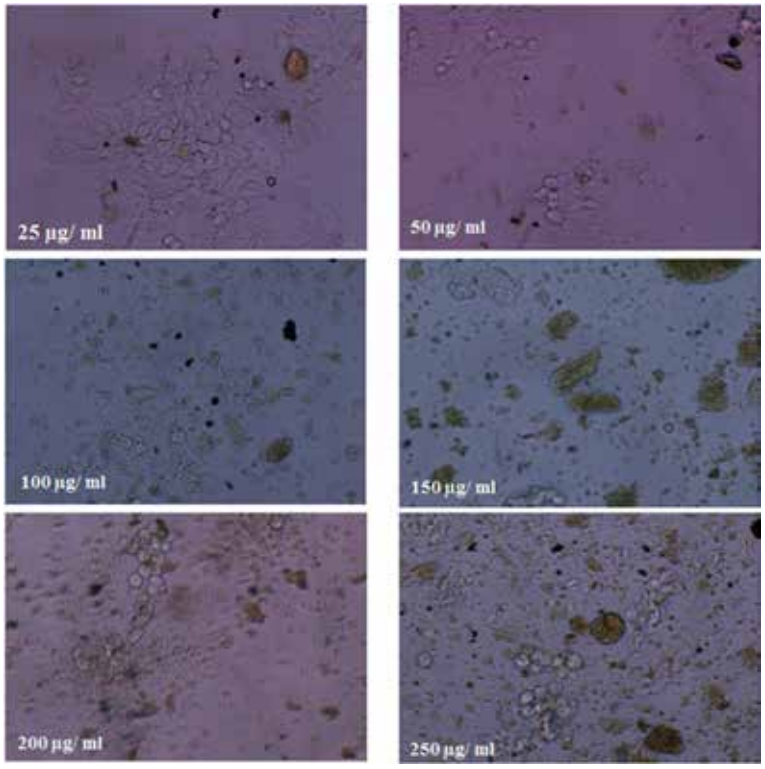

Fig. 1B. Antiproliferative potency of chloroform extract of dried shoots of *T. aestivum* at various concentrations against human hepatocellular carcinoma (HepG2) cancer cell lines

Hepatoprotective activity

Based on effectiveness of chloroform and methanol extracts of dried shoots of *T. aestivum* observed in various *in vitro* antioxidant assays (data not shown), *in vivo* hepatoprotective activity of both the extracts was investigated using three different

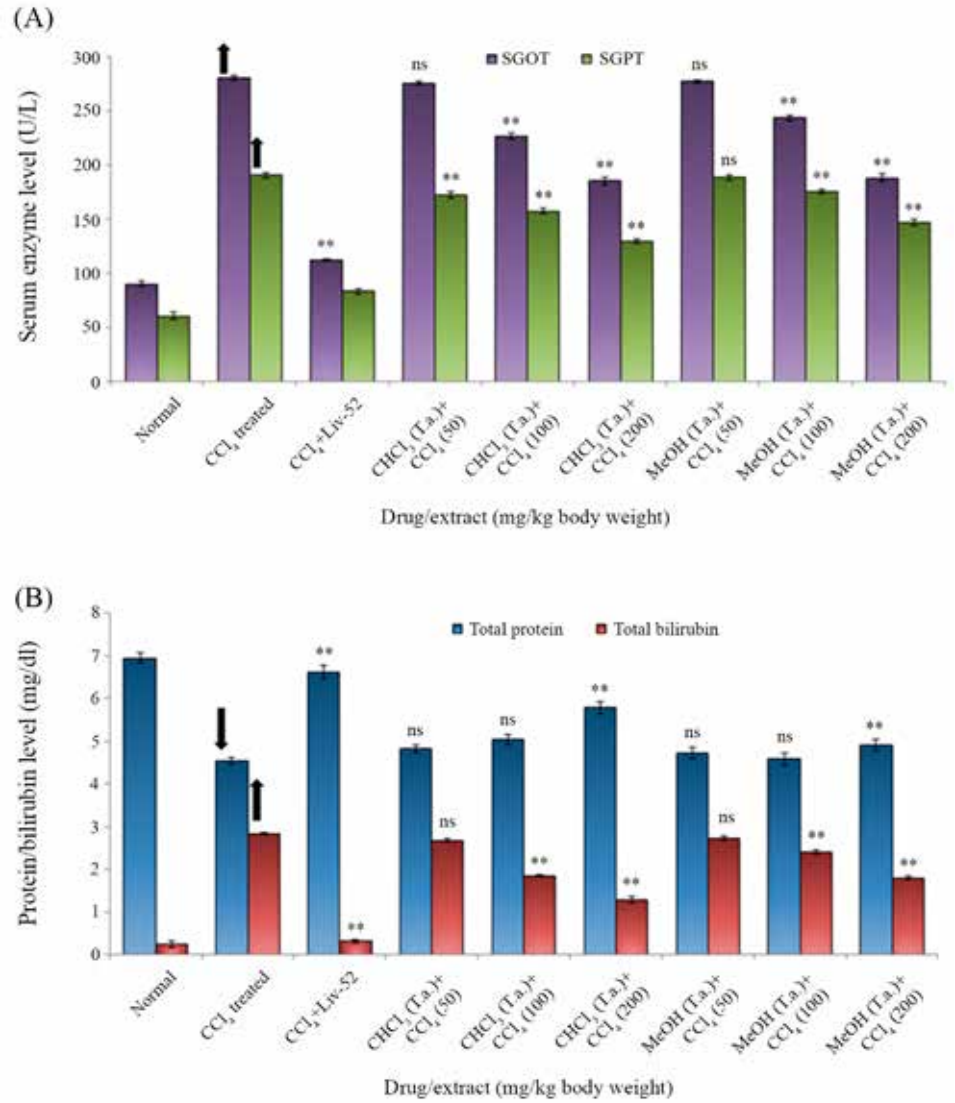

Fig. 2. Hepatoprotective activity of chloroform and methanolic extracts of dried shoots of *T. aestivum* in terms of evaluation of levels of serum enzymes (A) and protein/bilirubin (B). SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; $CHCl_3$: Chloroform extract; MeOH: Methanol extract; *T.a.* = *Triticum aestivum*. Values are mean ± S.D. p value; **p < 0.01; ns = non-significant; ▲= Increase; ▼= Decrease

concentrations (50, 100 and 200 mg/kg b.w.) against the liver toxicity induced by CCl_4 in albino Wistar rats. The group treated with CCl_4 only, was more vulnerable to oxidative injury and thereby produced high liver marker enzymes in blood, whereas the group receiving the co-administration of extracts exhibited significant protection. As a result, the chloroform extract of dried shoots of *T. aestivum* significantly decreased the level of serum SGOT (275.87±1.43, 226.56±2.57, 185.37±3.45 U/L) and SGPT (172.55±2.88, 157.66±2.76, 129.56±2.34 U/L) at the concentration of 50, 100, and 200 mg/kg b.w., respectively. Also, the chloroform extract (50, 100, and 200 mg/kg b.w.) significantly reduced the level of total bilirubin (2.68±0.04, 1.85±0.03, 1.28±0.08 mg/dL), while the level of total protein was elevated (4.82±0.09, 5.04±0.12, 5.78±0.15 mg/dL) (Fig. 2A, B). The results were compared with the standard (Liv-52) treated group along with CCl_4 group, which showed a significant reduction in the elevated enzyme levels (112.56±1.22 U/L, 83.56±2.45 U/L), total bilirubin (0.32±0.04 mg/dL) and elevation in a level (6.62±0.16 mg/dL) of protein (Fig. 2A, B).

On the other hand, the methanolic extract of dried shoots of *T. aestivum* also reduced the level of SGOT (277.12±1.23, 243.76±2.65, 188.34±3.12 U/L) and SGPT (188.72±2.54, 175.55±1.76, 147.22±3.38 U/L) at the concentrations of 50, 100 and 200 mg/kg b.w., respectively (Fig. 2A). Decreased level of total bilirubin was also evoked by the methanolic extract (2.72±0.05, 2.41±0.05, 1.79±0.04 mg/dL), while the increased level of total protein in the methanolic extract treated group was found to be 4.72±0.14, 4.98±0.15, 5.52±0.13 mg/dL at the extract concentrations of 50, 100 and 200 mg/kg b.w., respectively (Fig. 2B). Interestingly, both the chloroform and methanolic extracts of dried shoots of *T. aestivum* displayed dose-dependent hepatoprotective activity with reduced SGOT and SGPT enzyme, as well as total bilirubin levels. The total protein level was enhanced in a concentration-gradient manner. At the tested concentrations of 100 and 200 mg/mL, the chloroform and methanolic extracts of dried shoots of *T. aestivum* showed statistically significant differences ($p < 0.05$) when compared with control groups (Fig. 2A, B).

DISCUSSION

Nowadays, there is a huge increase in using phenolic compounds in food and pharma industries, due to their potent ability to retard oxidative degradation of lipid, thereby improving the nutritional quality of a variety of foods as well as they show the multitude of pharmacological properties [3]. The mechanism of the cytotoxicity might be based on some reasons other than oxidative stress. Hence, various prospective epidemiological studies on individual and in the natural complex form of phytochemicals should be performed to investigate the effects on chronic diseases including cancer and cardiovascular diseases.

Previously, the chemoprotective ability of chlorophyll and its derivatives obtained from *T. aestivum* (wheat grass), has been determined using cell cultures and animal experimentation [15]. Chlorophyll is reported to show the antioxidant, anti-mutagen-

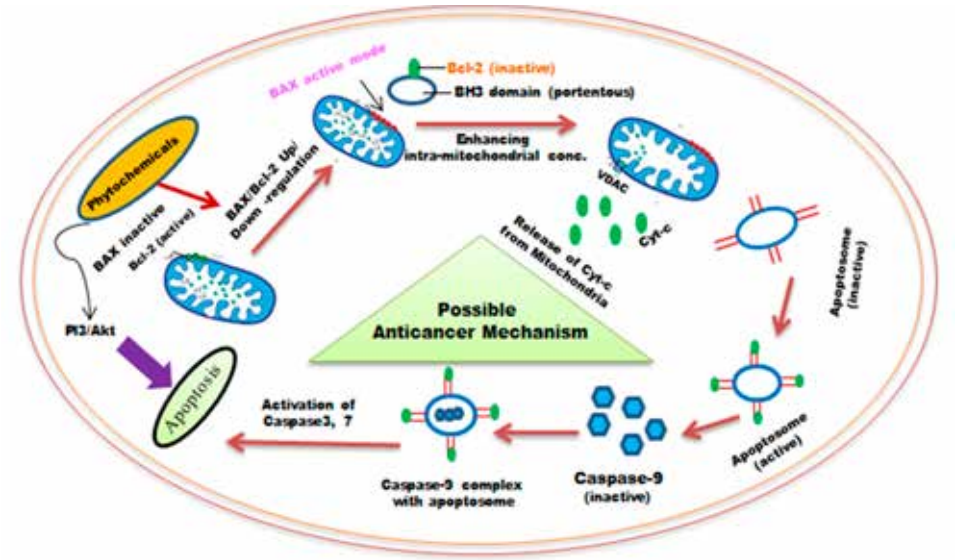

Fig. 3. Proposed apoptosis mode of action of bioactive phytochemicals of plant extracts. Bcl-2: B-cell lymphoma 2 protein, BAX: Bcl-2 associated X proteins, Cyt-c: Cytochrome C, VDAC: Voltage dependent anion channels, PI3/Akt: Phosphoinositide 3/protein kinase B pathway. Bioactive phytochemical chloroform extract of *T. aestivum* might up-regulate BAX protein in a dose-dependent manner while down-regulate the expression of Bcl-2 mRNA, which might be responsible causes and sustained elevation of calcium levels in mitochondria followed by cell apoptosis

ic and chemopreventive properties [3, 6, 15], which may be possible candidates of the anticancer activity of the chloroform extract of dried shoots of *T. aestivum,* as observed in this study.

Based on the present results, it can be stated that the chloroform extract of dried shoots of *T. aestivum* may act as an inducer of apoptosis and can exhibit anticancer activity via similar pathway. In anticancer pathway, Bcl-2 genes encode the Bcl-2 anti-apoptotic proteins acting as a checkpoint in the regulation of apoptosis. In addition, phytochemicals present in the chloroform extract of dried shoots of *T. aestivum* may lead to up-regulation and down-regulation of BAX and Bcl-2 proteins, which may substantially elevate the calcium level in the mitochondria (Fig. 3), suggesting the possibilities of the anticancer effect of *T. aestivum* derived phytochemicals. Although natural products have been reported to exhibit anticancer or anti-proliferative action very frequently, their various modes of actions have not been defined well to certain extended levels. However, based on the available research findings, we hypothesize and outline a possible molecular mechanism by which plant extracts or their active phytochemicals may exhibit anticancer effects (Fig. 3).

In this study, a significant increase of liver enzymes level in the plasma was observed after CCl_4 administration, which was significantly lowered by the treatment with the chloroform and methanolic extracts of dried shoots of *T. aestivum.* The level

of total protein was increased in the extracts of dried shoots of *T. aestivum*-treated group when compared to CCl_4-treated group. Since the extracts of dried shoots of *T. aestivum* possessed rich amounts of bioactive substances including chlorophyll and carotene contents, the protective effects of chloroform and methanolic extracts of *T. aestivum* were observed against hepatotoxicity induced by CCl_4. In addition, the hepatoprotective efficacy of chloroform and methanolic extracts of dried shoots of *T. aestivum* may be attributed to free radical scavenging activity of chlorophyll and carotene contents, as it was also confirmed previously [17]. The results obtained in the present study show that the chloroform and methanolic extracts of dried shoots of *T. aestivum* are promising sources of antiproliferative and hepatoprotective agents, and are rich in carotene and chlorophyll, which can act either individually or synergistically with a multitude of therapeutic potential. Further research strategies on isolation and identification of individual bioactive compounds from wheat grass (*T. aestivum*) and their efficacy on cellular and molecular levels need to be elaborated, in order to identify their exact action of mechanism.

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