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Anti-proliferative activity of *Thuja occidentalis* seed extract through regulation of BAX and BCL-X gene exprssion

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Abstract

Cancer is one of the most devastating diseases which involve uncontrolled cell growth. The conventional treatments of cancer have strong side effects which leads scientists for seeking safer treatment option from nature. Various natural plants are well documented for having anti-proliferative activity with minimal side effects. *Thuja occidentalis* is one of the common medicinal plants in our country which is successfully used as homeopathic medicine since many years to cure several diseases including wart. However, so far there is no proper scientific investigation to demonstrate these medicinal properties of this important plant. Therefore, the present investigation was designed to evaluate the anticancer activity of the crude extract of the *T. occidentalis* seed along with its antioxidant, cytotoxicity. Phytochemical examinations were also carried out to assess the types of secondary metabolites present in extract. Haemaglutination assay was performed to determine the presence of lectin protein which is considered as an effective anticancer agent. DPPH free radical scavenging assay was carried out to determine the antioxidant activity of the extract where it was compared with the ascorbic acid. EAC (Ehrlich ascites carcinoma) cells bearing mice were treated with 50mg/Kg/day of crude extract. Growth inhibition of cancer cell was calculated by hemocytometric cell counting in comparison with control after five days of treatment. Morphological changes indicative of apoptosis were evident in EAC cells which were observed using optical microscopy after DAPI staining. RNA was extracted from both control and treated cells from mice peritoneum and cDNA was prepared to observe the expression of the cancer related gene such as BAX and BCL-X by PCR amplification which specifies apoptosis process of the cancer cells. Current studies suggest that *T. occidentalis* seed extract have anti-proliferative and strong antioxidant activity which can further be studied to discover drug lead molecule for anticancer therapy.

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Introduction

Cancer is one of the leading causes of death in recent years. It is characterized by abnormal growth of cell mass which is uncoordinated and leads to continuous proliferation (Qadir *et al.* 2014). Though cancer is contemplated as a group of diseases found in developed countries but its incidence in different forms is rapidly increasing throughout the universe (Jemal *et al.* 2011).

The distinct property of cancer cells is their ability to metastasize to other organs (Minn *et al.* 2005). For example, in prostate and colon cancer, cells are metastasized or migrated to bones and liver respectively whereas in lung cancer cells are spread to adrenal glands, liver, brain, and bones, but in case of breast cancer, cells are metastasized to lungs and bones (Deep and Agarwal 2010; Ramos-Silva *et al.* 2017).

The available treatment plans of cancer are chemotherapy, radiation therapy, hormone therapy and surgery, but each of them have its own noxious side effects (Mountain 1997). Hence, the largest portion of the recent pharmacological research is dedicated to anticancer drug design customized to fit new molecular targets (Xia *et al.* 2004). Many drugs are currently in different stages of developmental pipeline while many are in clinical trials, but it is an extreme demand to develop more effective anti-cancer drug with lesser side effects. It is reported that, about 50% of all drugs in clinical use are derived from natural resources (Kim and Park 2002). Therefore, scientists are in search of new alternatives or novel compounds from plants to treat cancer (Ramos-Silva *et al.* 2017; Riaz *et al.* 2016).

Secondary metabolites of plantsare the rich sources of medicine. Many plant extracts and their bioactive compounds are well recognized for their anticancer effects (Cordell *et al.* 1991; Deep and Agarwal 2010).The major clinically useful chemotherapeutic agents are developed from natural products such as vincristine, podophyllotoxin, paclitaxel and camptothecin (Lee 1999; Newman and Cragg 2004).

T. occidentalis is an evergreen coniferous plant and widely cultivated as an ornamental plant which belongs to cupressaceae family (Caloni and Cortinovis 2015). Itis a well-known medicinal plant reported to have anti-viral and anti-wart activity and has immense pharmacological potentiality (Naser *et al.* 2005). In homoeopathy it is used to remove wart which is a form of tumor. Therefore, it is a very good candidate to have anti-proliferative activity. Current studies were aimed to evaluate the anti-proliferative activity, secondary metabolite contents, antioxidant activity, cytotoxicity and antibacterial activity of the crude seed extract of *T. occidentalis*.

Materials and methods

Collection of plant material and extract preparation

Seed of The plant material (seed) were collected from the botanical garden of the University of Rajshahi, Bangladesh during the season March-April. The experimental plant was authenticated by the experts of Botany department, University of Rajshahi, Bangladesh. Seed explants were washed with distilled water. 200g of fresh seed was suspended in 500 ml of tris-buffer, homogenized and the extract was filtered through Whatman No-1 filter paper. Subsequently, it was dried in fridge dryer and stored at 4° C until further use.

Dose Preparation

Lyophilized seed extract was dissolved in 2% DMSO (Dimethyl Sulphoxide) at the concentration of 10 mg/mL to prepare stock solution.50.0 mg/Kg/day extract was used to treat EAC cell bearing Swiss albino mice.

Experimental animal

Healthy Swiss albino mice weighing 20 ± 2.0 g were obtained from the animal house of the Department of Pharmacy, Jahangirnagar University (Bangladesh. Two groups of mice each containing 6 mice (n=6) were maintained in plastic cages.They were maintained under standard conditions (25 ± 2°C) with 12 ± 1 hour dark/light cycle. The animals were fed with rat pellet having standard nutrients.

Collection of Cell line

Ehrlich ascites carcinoma (EAC) cells were collected from Protein and Enzyme Laboratory, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh. The cells were maintained *in vivo* into Swiss albino mice by intra-peritoneal transplantation. EAC cells aspirated from the peritoneal cavity of mice were washed with saline and administrated intra-peritoneally to develop more ascities cells.

Ethical clearance

This research work was authenticated by the Institutional Animal, Medical Ethics, Bio-safety and Bio-security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources, memo no: 31/320-IAMEBBC/IBSc, Institute of Biological Sciences, University of Rajshahi-6205, Bangladesh.

Chemicals and reagents

2, 2-diphenyl-1- picrylhydrazyl (DPPH), methanol, ethanol, DAPI (4, 6-diamidino-2-phenylindole) and ethidium bromide were purchased from Sigma Aldrich (California, USA). Ascorbic acid was procured fromMerck, Germany, agarose was obtained from Fluka Biochemica, Switzerland. RNAsimple Total RNA kit was purchased from Tiangen (Beijing, China). All the other reagents were of good research grade. Hemagglutination buffer (20mM Tris-Hcl buffer, 1% Nacl, 10mM $CaCl_2$, P^H 7.8), anti-coagulant (Sodium Citrate), phosphate buffer saline (PBS), TBE buffer, Trypan blue, $FeCl_3$, H_2SO_4, NaOH, Benedicts reagents, Biuret reagents, Wagner's reagents were used in different experiments.

Phytochemical screening

Medicinal plants are the important source for the development of new chemotherapeutic agents and the first step for this goal is the phytochemical screening of plant extracts (Paz *et al.* 1995).In general, phytochemicals are bioactive compounds which are present in plants and are able to decrease the disease risk through complementary mechanisms (Baskaran *et al.* 2015). The seed extracts of *T. occidentalis* was evaluated for the qualitative determination of major phyto-constituents *i.e.* alkaloids, flavonoids, tannins, saponins, glycosides etc. using standard procedure; Wagner's test for identification of alkaloids(Firdouse and Alam 2011), test for phenolic compounds (Yadav and Agarwala 2011), Salkowski's test for steroids (Yadav and Agarwala 2011), Frothing test for saponine (Oyekunle *et al.* 2006), Ferric chloride test for tannin (Parekh and Chanda 2007), test for glycosides (Trease and Evans), flavonoid (Sofowara 1993), phlobatanin (Edeoga *et al.* 2005), phytosterols (Khanna and Kannabiran 2007), anthraquinones(Akinmoladun *et al.* 2010) were carried out. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Antioxidant activity assay

Oxidation is very important to many living organisms for the production of energy through various biological processes (Brand-Williams *et al.* 1995). The DPPH scavenging activity of extracts was determined by using the method which is described by Brand-Williams *et al.*(Brand-Williams *et al.* 1995; Oktay *et al.* 2003).The experiment was carried out with slight modifications in microtiter plate. In brief, methanolic seed extract (1mg/ml stock) was taken in different test tube in different concentrations (25, 50, 100, 150 and 200 µL from stock solution). Subsequently,1.5 mL DPPH (1mg/25 mL) was taken into the test tubes and the mixture was incubated at room temperature in the dark place for 30 min. The absorbance was measured at 517 nm using spectrophotometer (GENESYS 10S UV-Vis). Ascorbic acid was used as standard to compare the activity. DPPH scavenging capacity was calculated at each concentration according to the formula below.

DPPH scavenging effect (%) = $((A_0 - A_1)/ A_0) \times 100\%$

Where, A_0 was the absorbance of the control and A_1 was the absorbance of the seed extract.

The 50% inhibition concentration of the extract, IC_{50} was determined by plotting a graph of scavenging

activity against different concentrations of the extract (Ajila *et al.* 2007).

Hemagglutination activity assay

Hemagglutination activity assay is a common method for detecting the presence of lectin protein in plant sample which has shown to haveanti cancer activity(De Mejía and Prisecaru 2005). The hemagglutination reaction was performed in 96-well microtiter U-bottomed plate which was described by Hasan et.al (2014). Fresh mice blood cells were collected and blood cells were washed for 2-3 times with PBS (Phosphate Buffer Saline). 50µl of hemmaglutination buffer was taken in each experimental well. Consequently 50µl of seed extract (from 1 mg/ml stock solution) was added into the first well and serially diluted. Finally 50µl of 2% blood cells in saline was added to each well of titer plate and the plate was vortexed by micro shaker. The plate was placed on a table at room temperature and kept for 30 minutes to complete the reaction. Hemmaglutination activity was detected by visual observation.

Brine shrimp lethality test

Brine shrimp (*Artemias alina*) lethality assay is a common biological method to evaluate the cytotoxic effect of bioactive compounds of plants. This method is a primary toxicity screening system of plant extracts (Oberlies *et al.* 1998; Sarah *et al.* 2017). It represents a simple, rapid and inexpensive bioassay for checking plant extracts bioactivity which correlates with cytotoxic and anti-tumor properties (Krishnaraju *et al.* 2005; McLaughlin *et al.* 1993).The brine shrimp lethality bioassay was carried out to evaluate the cytotoxicity of plant extracts by using brine shrimps (*Artemiasalina*) that were hatched in a beaker (1L) filled with NaCl solution (38 gm/L). After hatching, 10 nauplii were taken by a glass capillary and kept in each of the 10 test tube which contained 10 ml of brine solution. Seed extract was added to these 10 test tubes at 10 different concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 µg/mL) and maintained at room temperature for 24 hours with proper aeration system. After 24 hours, the live and dead shrimps were counted and LC_{50} value was calculated using regression line.

Cell growth inhibition

Determination of *in vivo* cancer cell growth inhibition was performed using the method described by Sur *et al.* (Sur and Ganguly 1994). To determine the cell growth inhibition, two groups of Swiss albino mice (n=6) were used. For therapeutic evaluation 1.72×10^6 EAC cells were inoculated into each mice on day "0". Treatments were started after 24 hours of cell inoculation and continued for five days. Mice in each group were sacrificed on day six and total intra-peritoneal EAC cells were aspirated and diluted in normal saline (0.98% NaCl). Viable cells were first identified by using trypan blue and then counted by using haemocytometer through the following formula:

$$\text{Cells/ml} = \frac{\text{the average count per square} \times \text{dilution factor}}{\text{depths of fluid under cover slip} \times \text{area counted}}$$

Then the total number of viable cells in the treated groups were compared with those of control (EAC cell only) group and the cell growth inhibition was calculated using the following formula (Al-Mamun *et al.* 2016).

% Cell growth inhibition = (1-Tw/Cw) × 100 %

Where, Tw =Mean of number of EAC cells of the treated group of mice,

Cw = Mean of number of tumor cells of the control group of mice.

Observation of cellular morphology

Morphological observation of cells in the presence and absence of seed extract of *T. occidentalis* was studied using an optical microscope (Olympus iX71, Korea). After 5 days of treatment EAC cells with treated and control (EAC cell only) were collected from mice peritoneum and were washed twice with phosphate buffer saline (PBS). The cells were then stained with DAPI (4',6-diamidino-2-phenylindole). Successively, the cells were washed with phosphate buffer saline (PBS) and re-suspended in PBS for observation of morphological changes under fluorescent microscope.

Gene expression analysis

In gene expression analysis, total RNA from both control and treated EAC cells were isolated by using RNA simple Total RNA kit (Tiangen, Beijing, China). cDNA was created through reverse transcription PCR method using Tianscript MMLV reverse transcriptase (Tiangen, Beijing, China). The gene expression analysis was carried out by PCR amplification using two cancer related gene primers BAX and BCL-X where GAPDH gene primer was used as internal control.

PCR was carried out in 20 µl reaction containing, 10X polymerase buffer2.00 µl, dNTPs 0.40 µl (10mM), forward primer 0.40 µl (10mM), reverse primer 0.40 µl (10mM), DNA polymerase 0.20 µl, template 0.50 µl (25 ng), distilled water 16.10 µl.

Table 1 shows the sequences of primers used for PCR amplification and annealing temperature used during PCR. Cycling condition was set as initial activation of 5 min at 94°C, followed by 35 cycles of 94°C/30 sec., annealing at (50-55)/30 sec.,72°C/1min.A final extension of 72°C for 10min was also given. Amplified PCR products were run into 1% agarose gel where Tiangen 1KB plus DNA ladder (Beijing, China) was used as marker. After 50-60 minutes of running at 80V the gel was visualized under UV illuminator using gel documentation system (ProteinSimple, Alphaimager mini, USA).

Statistical analysis

All the experiments were performed in triplicates. Data are expressed as mean ± SD. The significance tests were carried SPSS-16 using one way ANOVA followed by a Dunnett Post hoc test compare with control. The significant test were set up at 5% level, 1% level and 0.1% level where P*<0.05, P**<0.01 and P***<0.001respectively. The Microsoft Excel 2007 was used for the statistical and graphical presentation of data.

Results

Phytochemical screening

Phytochemical screening of the plant extracts were performed using standard qualitative methods described earlier(Ahmad and Beg 2001; Fadeyi *et al.* 1989; Odebiyi and Sofowora 1977; Rizk and Bashir 1980; Vogel *et al.* 1989). The seed extract of *T. occidentalis* was used to check the presence of biologically active compounds like alkaloids, phenolic compounds, steroids, saponins, glycosides, flavonoids, tannins, phlabotannins, phytosterols etc. The qualitative results of phytochemical screening of the seed extract are shown in Table 2. Experimental results suggest that the seed extract of *T. occidentalis* contains higher amount of glycosides, flavonoids andphytosterols, whereas alkaloids, steroids, saponine and phenolic compounds were found in moderate level. Level of tannins was low. Interestingly, phlobatannins and anthraquinones were not detected in the seed extract of *T. occidentalis*.

Table 1. The sequences of primers used for PCR amplification.

Sl. No.	Gene Name	Primer Sequence	Annealing temp.
01	GAPDH	Forward: 5´- GTGGAAGGACTCATGACCACAG - 3'	
		Reverse: 5´-CTGGTGCTCAGTGTAGCCCAG-3´	52°C
02	BAX	Forward: 5'- CGCCCACCAGCTCTGAGCAGA - 3'	
		Reverse: 5'- GCCACGTGGGCGTCCCAAAGT - 3'	50° C
03	BCL-X	Forward: 5' - TTGGACAATGGACTGGTTGA - 3'	
		Reverse: 5'- GTAGAGTGGATGGTCAGTG - 3'	55° C

The qualitative results are tabulated in table 2 and is expressed as (+ + +) for the presence at high level, (+ +) for moderate level, (+) for low level and (-) for the absence of phytochemicals.

Antioxidant test

Antioxidant activity of *T. occidentalis* seed extract was evaluated using DPPH free radical scavenging assay. To find a new natural source of antioxidant. DPPH radical is a commonly used substrate for fast

evaluation of antioxidant activity because of its stability in the radical form and simplicity of the assay (Bozin *et al.* 2008; Jothy *et al.* 2011). The principle of this assay is the color change of DPPH solution from purple to yellow (Parthasarathy *et al.* 2009).

Table 2. Phytochemical screening results of seed extract of *T. occidentalis*.

Sl. No.	Name of Compound	Result
01	Alkaloids	+ +
02	Phenolic compounds	+ +
03	Steroids	+ +
04	Saponine	+ +
05	Glycosides	+ + +
06	Flavonoids	+ + +
07	Tannins	+
08	Phlobatannins	-
09	Phytosterols	+ + +
10	Anthraquinones	-

The changes of color were measured by taking absorbance in a spectrophotometer (GENESYS 10S UV-Vis) at 517 nm. Significant antioxidant activity was found in seed extract and the IC_{50} value was 24.54 ± 0.12µg/ml where the IC_{50} value of ascorbic acid (standard) was 16.24 ± 0.42 µg/ml. The DPPH free radical activities of seed extract of *T. occidentalis* and ascorbic acid were shown in Figure 1.

Hemagglutination assay

Plant derived lectin protein is well known for anticancer activity (Fu *et al.* 2011). Hemagglutination activity assay is a common and simple method to check the presence of lectin protein in plant samples (Kabir *et al.* 2013). The Hemagglutination activity assay of seed extract of *T. occidentalis* was shown in figure 2. The seed extract showed the significant agglutination activity which was able to agglutinate the red blood cell at the concentration up to 3.12µg/ml (figure 2) as shown in by black circle.

This result indicates that the seed extract of *T. occidentalis* has high level of agglutination activity which ensures the presence of high or highly active level lectin or lectin like proteins.

Brine shrimp lethality test

The brine shrimp lethality assay is widely used technique to assess the bioactivity of the plant extract (Sam 1993). This assay proved that it is a convenient system for evaluating the biological activities of plants (Krishnaraju *et al.* 2005).In our present study, cytotoxic activity of the seed extracts of *T. occidentalis* was determined using the method described by Meyer *et al.* (Meyer *et al.* 1982) with slight modification.

The LC_{50}values of the shrimp was obtained by linear regression analysis. The LC_{50} value of seed extract was 175.92 ± 2.99µg/ml which is shown in figure 3.

Cell growth inhibition

The simplest most convenient and cheapest way of determining the numbers of cells in a sample is to use a haemocytometer under a microscope (Lund *et al.* 1958; Strober 2001). In the present studies, the percentage of EAC cell growth inhibition was calculated after five days of treatment with seed extract of *T. occidentalis*. The number of viable EAC cells counted by hemocytometer using trypan blue demonstrated decreased number of cells in treated mice in comparison with control which is shown in figure 4 (A) and (B). The percentage of cell growth inhibition by seed extract of *T. occidentalis* was calculated as 55.24 ± 3.02 % where the percentage of cell growth inhibition in treated mice by standard anticancer drug Bleomycin was 78.92 ± 2.43 %. So, a significant cell growth inhibition was found in the peritoneum of treated albino mice by *T. occidentalis* seed extract which is shown in figure 4 (C).

Table 3. Antibacterial activity of seed extracts of *T. occidentalis* against different bacteria.

Bacteria	Diameter of zone of inhibition (mm) at different concentration (µl/disc)			Antibiotic (Gentamycin)	Results
	50	100	200		
E. coli	8.2 ± 0.3	13.3 ± 0.2	16.3 ± 0.3	19.6 ± 0.4	Susceptible
Acetobacter sp.	-	9.4 ± .03	7.4 ± 0.3	16.5 ± 0.3	Resistant
Pseudomonas sp.	-	8.5 ± 0.4	7.6 ± 0.2	17.6 ± 0.4	Resistant
RVM	-	10.6 ± 0.2	11.3 ± 0.4	17.1 ± 0.2	Intermediate
RCA	-	6.7 ± 0.3	7.3 ± 0.2	18.5 ± 0.3	Resistant

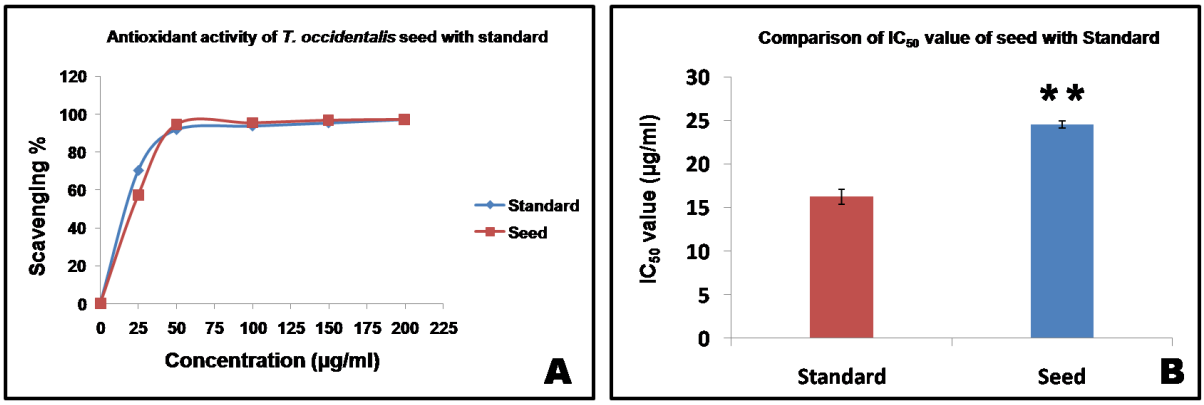

Fig. 1.DPPH radical scavenging activity of seed extract of *T. occidentalis* and ascorbic acid. (A) Scavenging % of seed extract and ascorbic acid. The 50% inhibition concentration, IC_{50} value of seed extract is 24.54 ± 0.12 µg/ml, where the IC_{50} value of ascorbic acid (standard) is 16.24 ± 0.42 µg/ml (B) Comparison of IC_{50} value between seed sample and standard. All data expressed as mean ± SD (n = 3) for all tested dosages. Significant differences of values are compared to values of standard and sample which marked as *p<0.05, **p<0.01, and***p<0.001.

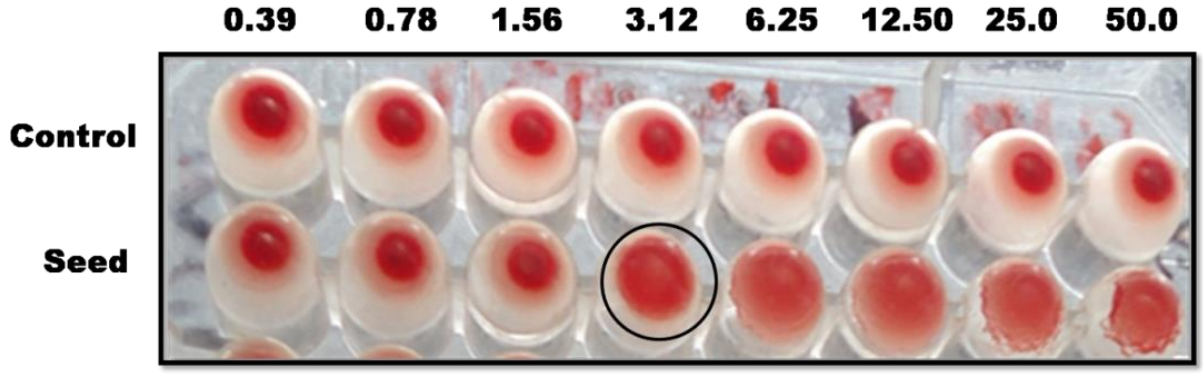

Fig. 2. Hemagglutination activity assay of seed extract of *T. occidentalis*. The seed extract was able to agglutinate the red blood cell at the concentration up to 3.12 µg/ml. But in control wells, no agglutination activity was observed.

Observation of cellular morphology

The morphological changes of the cells were observed after five days of treatment with seed extract under optical microscope by using DAPI dye staining system. Appearance of EAC cells from control mice were found to be round in shape and with normal

physical appearance were found in control mice (figure 5A). On the contrary, the EAC cells from treated mice showed significance morphological changes such as cell membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and aggregation of apoptotic bodies etc as shown by arrows in figure 5 (B). So the current studies implies that experimental plant extract has the ability to change the morphological structure of cells as similar as apoptotic features.

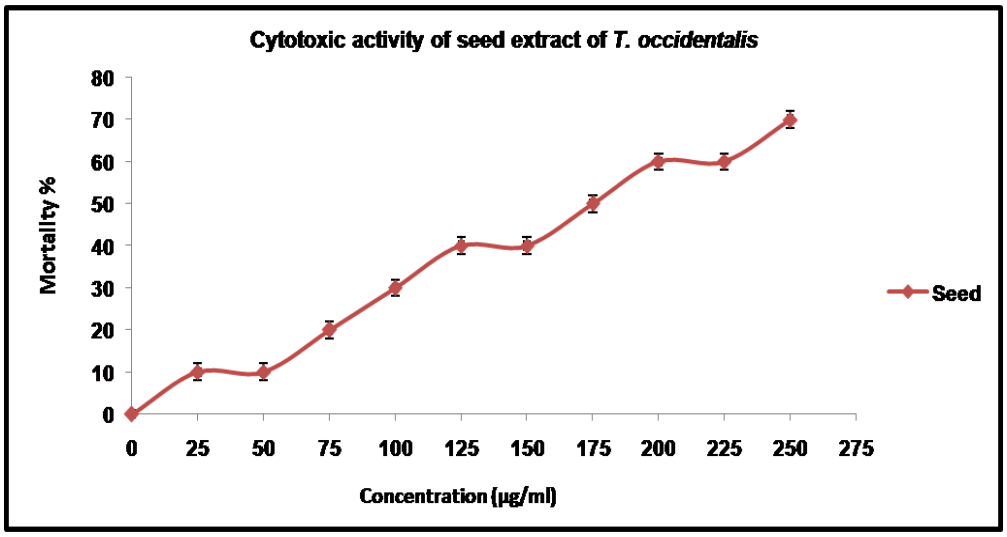

Fig. 3. Cytotoxic activity of seed extract of *T. occidentalis*. Lethality percentage was increasing with high concentration. Each value is expressed as mean ± SD (n=3) and significance was set at P<0.05 (*), P <0.01 (**) and P <0.001 (***).

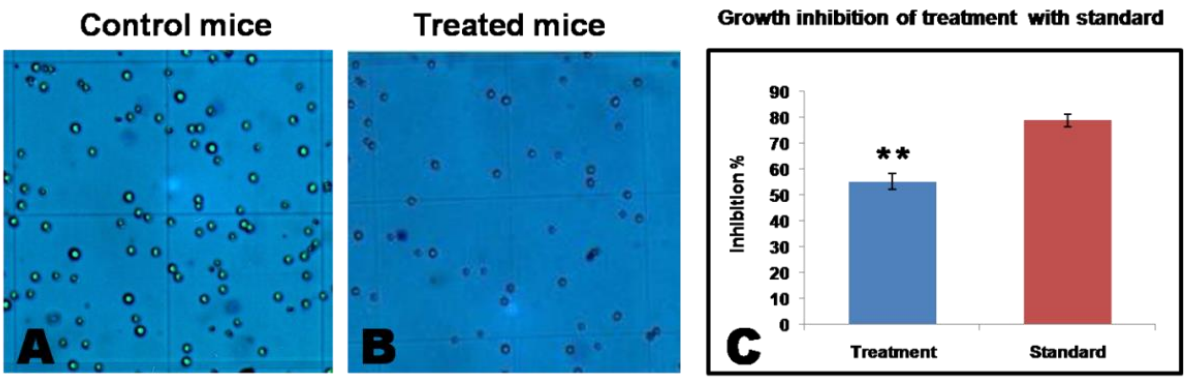

Fig. 4. The cell growth inhibition of seed extract of *T. occidentalis*.(A) The haemocytometric observation of the EAC cells control mice and (B) EAC cellsfrom *T. occidentalis* seed extract treated mice under optical microscope. (C) Percentage (%) of cell growth inhibition by the seed extract of *T. occidentalis* in comparison with standard anticancer drug (Bleomycin). All Data are expressed as mean ± SD (n = 5) and significant differences of values are compared to values of standard and sample and marked as (*p<0.05, **p<0.01, and***p<0.001).

Gene expression analysis

RNA extraction

EAC cell total RNA was extracted from both control and treated mice. Both the extracted RNA yielded two distinct bands of 28s and 18s in 1% agarose gel (Figure 6A). These two bands were appeared at the same level with minimal smearing in the gel which indicates that the RNA of both control and treated EAC cells were good. Quantity of RNA was checked using a Nanodrop and 260/280 ratio of the RNA was found within the range of 1.7 to 1.9.

PCR amplification of GAPDH, BAX and BCL -X gene

GAPDH is a housekeeping gene which was amplified to ensure the cDNA quality from RNA. In our experiment, the bands from both control and treated samples were appeared at the same level with nearly identical intensity on the gel at the position near about 400 bp which is shown in figure 6B.

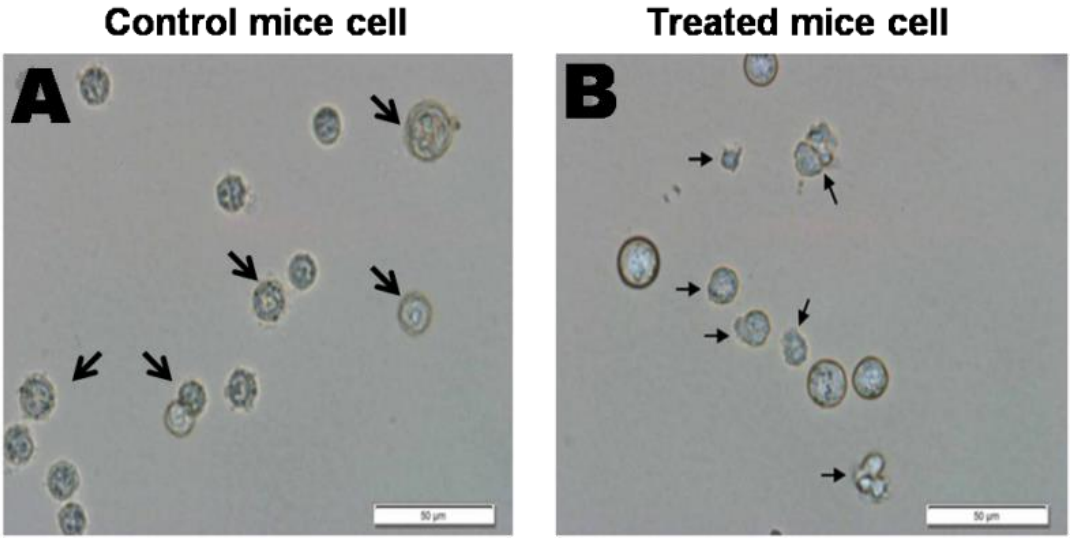

Fig. 5. Optical microscopic observation of EAC cells for control mice and treated mice. (A) In control mice normal and round shaped cells were appeared. (B) In treated mice, the arrows indicate the cells having apoptotic features

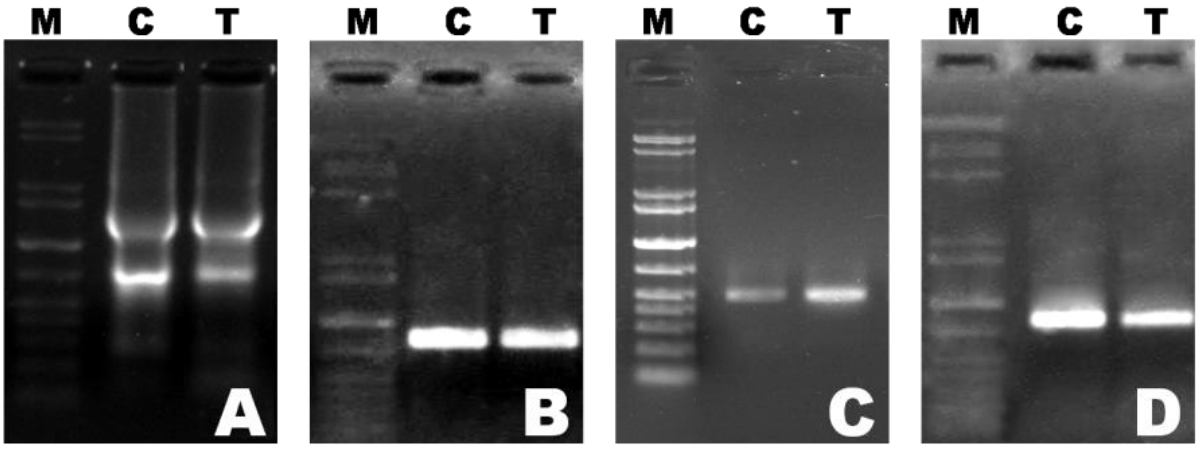

Fig. 6. Gene expression analysis. M, C and T represent molecular marker, control and treatment respectively. (A) RNA isolated from both control and treated EAC cells. (B) Agarose gel electrophoresis of GAPDH gene which shows a similar expression pattern in both control and treated EAC cells (C) Expression pattern of BAX gene shows higher expression in treatment than control (D) Gel electrophoresis of BCL-X gene shows the down regulation in treated EAC cells in compared to control.

BAX and BCL-X are well studied apoptosis regulatory genes. Current experiment clearly shows the up-regulation of BAX gene and the down regulation of BCL-X gene in comparison to control which indicate that the experimental sample is able to induce apoptosis to inhibit the cells growth which was shown in figure 6 (C) and figure 6 (D).

Expression level of amplified genes

The free software Gel Analyzer was used to observe

the expression level of apoptotic genes such as BAX, BCL-X and housekeeping gene GAPDH based on the band intensity. These results demonstrated that the EAC cells treated with the seed extract show up-regulation of BAX mRNA and down regulation of BCL-X mRNA when compared with their respective controls. (Figure 7) which indicates the mitochondria mediated apoptosis of EAC cells was taken place. The plants which show more antioxidant activity indicate that these plants are more biologically active. In this experiment, the seed extract of *T. occidentalis* showed excellent antioxidant activity with IC_{50} value of 24.54 ± 0.12µg/ml which is near to the value of standard (Figure 1).

Discussion

Cancer is a death causing and one of the most devastating diseases all over the world. Due to its unfavorable treatment system, scientists are trying to develop a novel drug to treat cancer from natural resources. *T. occidentalis* is an ornamental plant which has multiple medicinal value.

The phytochemical screening test is one of the most well known process which is used to identify the medicinal and physiological activity of plant's extracts (Sofowara 1993). In the current study, the phytochemical test reported that, the experimental plant extract is rich in glycosides, flavonoids and phytosterol but the phlobatannins and anthraquinone were absent (Table 2).

Antioxidant activity assay is the most widely used to determine the bioactivity of plant materials and their phytochemical constituents (Wolfe and Liu 2008).

The result of hemagglutination test showed that, the seed extract of *T. occidentalis* contain lectin or lectin like protein which has the ability to destroy cancer cells (Beuth *et al.* 1992). The experimental extract can agglutinate the red blood cell (RBC) up to 3.12 µg/ml concentration (Figure 2).

The brine shrimp lethality bioassay is an well used technique to check the of cytotoxicity for screening pharmacological activities in plant extracts (Carballo *et al.* 2002). This bioassay reported that, the experimental plant was rich in bioactive compound. Figure 3 showed the IC_{50} value of this plant extract as 175.92 ± 2.99µg/ml.

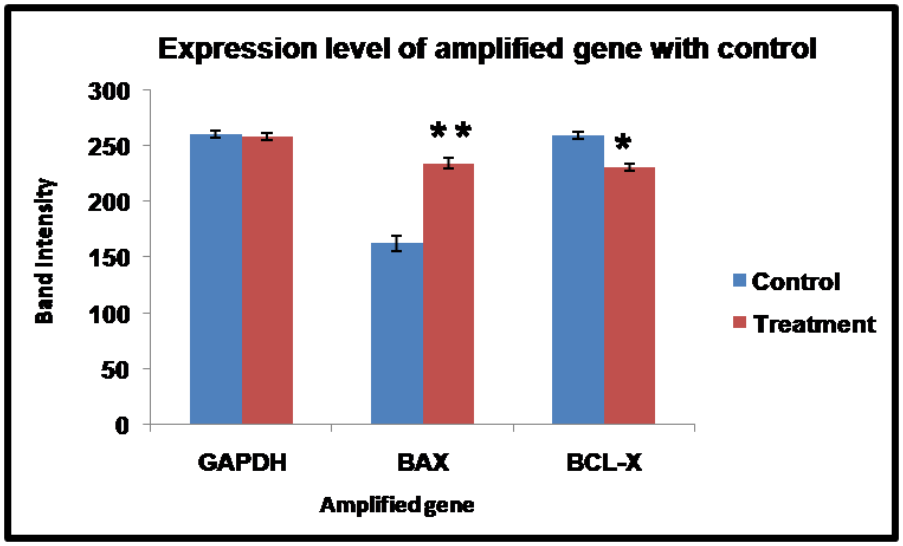

Fig. 7. Expression level of amplified genes based on their band intensity using the software GelAnalyzer. The band intensity of control and treated mice for GAPDH gene were 260.33 ± 3.06 and 258.33 ± 2.52 respectively. In case of BAX gene bands intensity were162.67 ± 6.81 and 234.33 ± 4.93whereas BCL-X bands intensity were 259.33 ± 2.09 and 230.67 ± 3.51respectively. Data are expressed as mean ± SD (n = 3) for all tested dosages. Significant differences of values are compared to values of control and treated samples and marked as (*p<0.05, **p<0.01, and***p<0.001).

The most interesting finding of the current studies is that the seed extract of *T. occidentalis* inhibited the growth of cancer cells significantly and cell growth inhibition by the seed extract was 55.24 ± 3.02 % where standard (Bleomycin treated mice) was 78.92 ± 2.43 % which showed in figure 4. This result indicates that the seed extract can significantly reduce the number cell which can be used to treat cancer.

The morphological experiment showed that seed extract of *T. occidentalis* treated cells lost their original rounded shape and turned into shrinked shape which is a hallmark of apoptosis, whereas the cells from control mice retained their original rounded shape (Figure 5). So it is suggested that the seed extract possibly arrested the cell growth due to having apoptosis inducing compounds(Fathy *et al.* 2013; Guo *et al.* 2009).

The BAX and BCL-X genes are regular member of the Bcl-2 gene family and it is believed that these gene play an important role in regulating apoptosis associate with Bcl-2 gene (Apte *et al.* 1995).

The present study revealed that seed extract of *T. occidentalis* up-regulates BAX gene and down regulates BCL-X gene which confirms that the cells are undergoing apoptosis (Figure 6 and figure 7).

Conclusion

Now-a-days researchers prefer natural medicinal source to treat cancer due to having adverse side effects of the conventional methods. *T. occidentalis* is one of the good natural sources of high antioxidant which can be utilized to discover drug leads to treat cancer because it containslectin or lectin like protein which has the ability of terminatingcancer cells. The seed extract of *T. occidentalis* successfullyand significantly inhibit the growth ofEAC cells through apoptosis which was confirmed by the fluorescence microscopy and expression pattern of some cancer related genes. In conclusion the seed extract of *T. occidentalis* is can be a potential natural source of anticancer drug lead compound.

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