

RESEARCH COMMUNICATION

Anti-proliferative Effects of Organic Extracts from Root Bark of *Juglans Regia L.* (RBJR) on MDA-MB-231 Human Breast Cancer Cells: Role of Bcl-2/Bax, Caspases and Tp53

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Abstract

With increasing use of plant-based cancer chemotherapeutic agents, exploring the antiproliferative effects of phytochemicals has gained increasing momentum for anticancer drug design. The present study was undertaken to investigate the effect of root bark of *Juglans regia* (RBJR) organic extracts on cell proliferation, and to determine the molecular mechanism of RBJR-induced cell death by determining the expression of Bcl-2, Bax, caspases, Tp53, Mdm-2 and TNF- α in MDA-MB-231 human breast cancer cells. The results demonstrate that WNRB suppressed proliferation and induced apoptosis in a dose and time dependent manner by modulating expression of key genes. This involved characteristic changes in cytoplasmic and nuclear morphology, DNA fragmentation (TUNEL assay), levels of mRNA and expression of corresponding proteins. Real Time PCR and western blot analysis revealed that the expression of Bax, caspases, tp53, and TNF- α was markedly increased in MDA-MB-231 cells treated with the RBJR extract. In contrast Bcl2 and mdm-2 expression was down regulated after exposure. In summary, our data suggest the presence of bioactive compound(s) in WNRB capable of killing breast carcinoma cells through induction of apoptosis, and therefore a candidate source of anticancer drugs.

Keywords: *Juglans regia L.* - root bark - organic extract - breast cancer - apoptosis - DNA fragmentation - p53 - caspases

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Introduction

Juglans regia L., commonly known as walnut is an important species of deciduous trees found principally in temperate areas across the world. It is cultivated throughout eastern Asia, southern Europe, northern Africa, United States and western South America. In India it is found in Kashmir, Himachal Pradesh and Uttarakhand states. It is largely consumed as part of the diet and different parts of plant are used as local folk medicine. For example green walnuts, shells, bark, green husks (epicarps) and leaves, which have been used in the cosmetic and pharmaceutical industries (Stamper et al., 2006; Oliveira et al., 2008).

Root bark of *Juglans regia* (RBJR) is locally known as 'Dandas' in Kashmir valley and is used for cleaning and sparkling teeth. Root bark is also used by womenfolk for coloring lips (make-up) (Hamayun et al., 2006). RBJR contains naphthoquinones like juglone and bisjuglone (Pardhasaradhi and Babu, 1978). Bisjuglone had been reported for its antitumor property in mouse skin carcinogenesis (Kapadia et al., 1997).

Breast cancer incidence has been increasing among the different ethnic population all over the world, especially in areas of low incidence such as Asia. Global burden

of breast cancer has increased from 720,000 cases per year in 1985 to 1,380,000 new cases in the year 2008 (Ahmad, 2003; Ferlay et al., 2008). Breast cancer is second largest after cervix cancer among women in India, but is considered the leading cancer in certain metros such as Mumbai and Bangalore. It is estimated that approximately 80,000 cases occur annually (Harrison et al., 2010). With increase in longevity breast cancer is estimated as major cause of death among women in Gulf countries (Bin Amer et al, 2008). Even after three decades of continuous tug of war against breast cancer there is no effective cure for this disease. However, there are available reports that use of complimentary alternative medicine (CAM) had improved the conditions of breast cancer patients (Sowmyalakshmi et al., 2005).

Since, RBJR component has been reported for their antitumor effect against skin carcinogenesis, we hypothesized that anticancer phytochemicals in different organic extract may kill Breast cancer cell MDA-MB-231 in a synergistic manner. The main objective of this study to find out if different organic extract inhibits the growth of MDA-MB-231 cells through apoptosis; and to deduce the molecular mechanisms involved, focusing on related genes.

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Materials and Methods

Preparation of organic extracts with increasing polarity

Extractions were done as described earlier (Shafi et al., 2009; Ganeshan et al., 2010) with some modifications. Air dried RBJR were powdered in a milling machine. Extractions were carried out in a Soxhlet apparatus with three different solvents; methanol, chloroform and n-hexane. An extraction scheme is illustrated in Figure 1. Each organic phase was later dried under reduced pressure to obtain the residue. A rotary evaporator was used to obtain the respective lyophilized powder/paste. Extracts were weighed and stocks were prepared at a concentration of 500 µg/mL of Dimethyl Sulfoxide (DMSO).

Maintenance of MDA-MB-231 cell line

MDA-MB-231 breast cancer cells were obtained from NCCS, Pune, India. The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured up to ~70% to 80% confluence as adherent monolayer. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ throughout the study. Cells were trypsinized to harvest after attaining confluence. Only research grade chemicals were used in this study.

Cell Viability Assay

Cell viability was assayed where and as required by trypan blue exclusion test with slight modifications (James and Warburton, 1999). Overall viability was scored more than 90%.

Cytochemical detection of apoptosis

Morphological observation and determination of apoptosis was done similarly as in earlier studies with some modification (Xiao et al., 2007; Shafi et al., 2008). MDA-MB-231 breast cancer cells were seeded at a density of 2×10^4 cells/well into 6-well plates. After 24 h adherence, the cells were treated with 50 µg/mL of methanolic, chloroform and n-hexane extracts for 72 h. Then the cells were examined with fluorescence microscope (Carl Zeiss ApoTome, Germany), phase contrast transmitted light microscope (FCTLM) (Carl Zeiss, Axiostar, Germany). The cells were stained using propidium iodide (PI) before examined under fluorescence microscope and stained by Crystal Violet before observed under FCTLM. All the observations were done at 400x and digital images were saved.

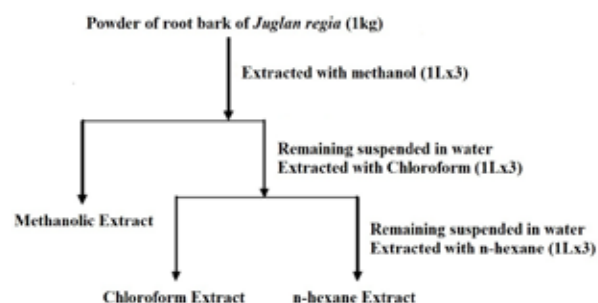


Figure 1. Extraction and Partitioning of Bioactive Compounds of Root Bark of Juglans regia

Cytochemical quantification of apoptosis by TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a beautiful blend of molecular biology and morphological observation and used largely for the quantification of apoptosis in cells (Meenakshi et al., 2003). In present study TUNEL assay was conducted to investigate the occurrence of DNA fragmentation induced by organic extracts of RBJR. The DeadEnd® TUNEL assay kit (Promega, Madison, WI) was used for studying apoptosis in a time- and dose-dependent manner. TUNEL assay was performed as described earlier (Alshatwi, 2010). Briefly, 2×10^4 cells/well were cultured in 6-well plates. Cells were treated with 50 µg/mL and 100 µg/mL of methanolic, chloroform and n-hexane extracts separately for 24, 48 and 72 hours. Culture media were aspirated after incubation period, and the cell layers were trypsinized. The trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained according to the DeadEnd fluorometric TUNEL system protocol. The stained cells were observed using a Carl-Zeiss (Axiovert) epifluorescence microscope using a triple band-pass filter. Green fluorescent (FITC stain) cells referred as TUNEL positive whereas the cells with a red nucleus (PI stained) were referred as TUNEL negative cells. To determine the cells count of TUNEL assay positive cells, 1000 cells were counted at magnification of 100x in each experiment (Shafi et al., 2009). Apoptotic cell counts in treated cells were normalized by control count. Each experiment was repeated three times.

Quantification of mRNA level of apoptosis related genes through Real-Time PCR

MDA-MB-231 cells were cultured in 6 well plates at a density of 1.5×10^5 . After 24 h of adhesion period culture media were aspirated off and cell were treated with methanolic, chloroform and n-hexane extract at a concentration of 50 µg/mL media. After 24 h of incubation media were removed and treated cells washed with ice cold PBS. Further, Fastlane® Cell cDNA kit (QIAGEN, Germany) was used to prepare cDNA directly from cultured cells, according to the manufacturer's instructions. The mRNA levels of *Caspase-3*, *Caspase-8*, and *tp53*, *bax*, *bcl2*, *mdm2*, *TNF-α* as well as the reference gene, *GAPDH*, were assayed using gene-specific SYBR Green-based QuantiTect® Primer assays (QIAGEN, Germany). Real time PCR reactions and analyses were performed on Applied Biosystems 7500 Fast (Foster City, CA).

Quantitative real-time PCR was performed in a reaction volume of 25 µL according to the manufacturer's instructions. Briefly, 12.5 µL of master mix, 2.5 µL of primer assay (10x) and 10 µL of template cDNA (100 µg) were added to each well. After a brief centrifugation, the PCR plate was subjected to 35 cycles of the following conditions: (i) PCR activation at 95°C for 5 minutes, (ii) denaturation at 95°C for 5 seconds and (iii) annealing/extension at 60°C for 10 seconds. All samples and controls were run in triplicates on an ABI 7500 Fast Real-time PCR system. The quantitative RT-PCR data was analyzed by a comparative threshold (Ct) method, and the fold

inductions of samples were compared with the untreated samples. GAPDH was used as an internal reference gene to normalize the expression of the apoptotic genes. The Ct cycle was used to determine the expression level in control cells and MDA-MB-231 cells treated with different extracts for 24 h. The gene expression level was then calculated as described by Yuan et al. (2006). The results were expressed as the ratio of reference gene to target gene by using the following formula: $\Delta Ct = Ct$ (apoptotic genes) - Ct (GAPDH). To determine the relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct$ (Treated) - ΔCt (Control). Thus, the expression levels were expressed as n-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic genes using the expression of $2^{-\Delta\Delta Ct}$.

Apoptosis related protein expression analysis by Immunoblotting

Immunoblotting analysis of apoptosis related proteins was performed as described by us earlier (Shafi et al., 2009) with slight modification. Briefly, 2×10^5 cells /well were cultured in 6 well plates. Cells were then treated with methanolic, chloroform and n-hexane extracts at a concentration of $50 \mu\text{g}/\text{mL}$ for 24 h. After treatment, the cells were collected and washed twice with cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 $\mu\text{g}/\text{ml}$ aprotinin, and 25 $\mu\text{g}/\text{ml}$ leupeptin) and kept on ice for 30 min. The lysates were then centrifuged at 12,000 g at 4°C for 20 min; the supernatants were stored at -70°C until use. The protein concentration was determined by the Bradford method. Aliquots of the lysates (30 μg of protein) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane using transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol [v/v]). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 h with primary antibodies, followed by 30 min with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween. Anti-human p53, caspase-3, caspase-8, Bcl-2, Bax, Mdm-2, TNF- α and GAPDH antibodies were used at a 1:1000 dilution as the primary antibodies, while horseradish peroxidase-conjugated horse anti-rabbit IgG (Sigma Chemicals, USA) was used at a 1:5000 dilution as the secondary antibody. The membrane was then exposed and protein bands were detected using Enhanced Chemiluminescence. All chemicals used during this study were of research grade.

Statistical analysis

All data were analyzed statistically through PASWA 18 software and data represented as mean \pm SEM. t-test were performed and p-value ≤ 0.05 were considered as significant.

Results

RBJR extracts induce characteristics morphological changes of apoptotic cells in MDA-MB-231

A qualitative cytochemical analysis of MDA-MB-231

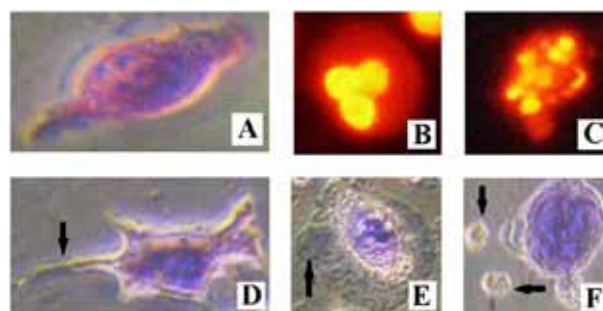


Figure 2. Microscopic Observations (400x) of Morphological Changes Associated with Apoptotic Cells. (A) Untreated cell, (B) Nuclear fragmentation- pycnosis, (C) Nuclear fragmentation- karyorrhexine, (D) Echinoid spikes (arrow), (E) surface blisters (arrow), (F) blebs and (arrow) and cellular rounding. Representative images of cells treated with $50 \mu\text{g}/\text{mL}$ of methanolic, chloroform and n-hexane extracts separately. All above were found in all treatments of extracts.

cells treated with indicated conc. of various extracts of RBJR for altered morphology related with apoptosis was carried out. During microscopic examinations echinoid spikes, surface blisters, blebs and cellular rounding were observed after crystal violet staining, whereas pycnosis and karyorrhexine were evident from propidium iodide staining. All of above mentioned morphological changes were observed in MDA-MB-231 cells treated with methanolic, chloroform and n-hexane extracts of RBJR separately. Figure 2 (B-F) summarizes all morphological events related to apoptosis, induced by three organic extracts.

TUNEL assay favors highest apoptosis inducing potency of chloroform fraction of RBJR

A cytochemical quantification of apoptosis and microscopic scoring of apoptotic MDA-MB-231 cells after treatment with indicated conc. of methanolic, chloroform and n-hexane extracts of RBJR separately was carried out. After 24 h of exposure, out of 1000 cell counts, chloroform extracts treatment showed 332 ± 39 and 384 ± 41 TUNEL positive cells for $50 \mu\text{g}/\text{mL}$ and $100 \mu\text{g}/\text{mL}$ concentration respectively. No significant differences were observed between the TUNEL positive cell counts for $50 \mu\text{g}/\text{mL}$ (48 h) and $50 \mu\text{g}/\text{mL}$ (72 h) treatments. Similarly difference between $100 \mu\text{g}/\text{mL}$ (48 h) and $100 \mu\text{g}/\text{mL}$ (72 h) of cell counts were non-significant for chloroform extract treatments. However after 72 h of exposure TUNEL positive cell counts were 735 ± 80 and 803 ± 98 for $50 \mu\text{g}/\text{mL}$ and $100 \mu\text{g}/\text{mL}$ concentration respectively. TUNEL positive cell counts for chloroform extract were followed by methanolic extract and then n-hexane extract. After 72 h of treatment, methanolic extract accounted for 316 ± 22 and 573 ± 60 for $50 \mu\text{g}/\text{mL}$ and $100 \mu\text{g}/\text{mL}$ concentration respectively, whereas for the same two concentrations n-hexane extract accounted for 285 ± 32 and 297 ± 31 TUNEL positive cells. Figure 3 represents each treatment and their microscopic examination at 100x. Figure 4 summarize the results of TUNEL assay.

Extracts of RBJR alter the mRNA level of apoptosis related genes

To investigate the molecular mechanism of apoptosis

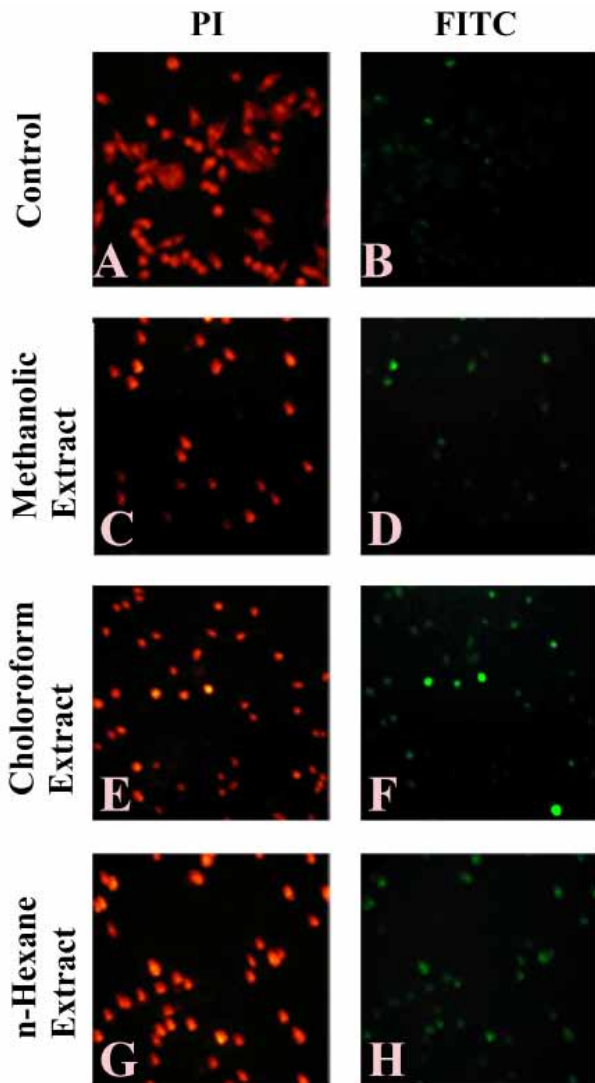


Figure 3. Morphological Observation with Propidium iodide/FITC Double Staining by Fluorescence Microscope (100x). Cells were prepared in 6 well plates. Controls cells were untreated (A, B). Test cells were subjected to treat with 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ of methanolic (C,D), chloroform (E,F) and n-hexane extracts(G,H) separately for three different incubation periods (24 h, 48 h, 72 h). TUNEL assay was performed and TUNEL positive cells (green fluorescent) were scored out of 100 cell counts. Representative images from three independent experiments for 72 h incubation periods were shown.

induced by RBJR extracts in MDA-MB-231 cells, the mRNA expression levels of some apoptosis related genes were analysed. The relative quantification of *tp53*, *Caspase-3*, *-8*, *bax*, *bcl-2*, *TNF- α* , *mdm-2* and a housekeeping gene (*GAPDH* as reference) mRNA expression levels was performed by SYBR Green-based quantitative real-time PCR (RT-PCR) using a 7500 Fast Real Time System (Applied Biosystems). Figure-5 summarize the gene expression changes of *tp53*, *Caspase-3*, *-8*, *bax*, *bcl-2*, *TNF- α* , *mdm-2*. RBJR extracts increased the transcripts of *tp53*, *Caspase-3*, *-8*, *bax* and *TNF- α* by several fold. Highest level of mRNA expression of pro-apoptotic genes were found in cells treated with chloroform extract, followed by methanolic extract and n-hexane extract. The expression levels of *tp53*, *Caspase-3*, *-8*, *bax*, *TNF- α* , in MDA-MB-231 cells treated with 50 $\mu\text{g}/$

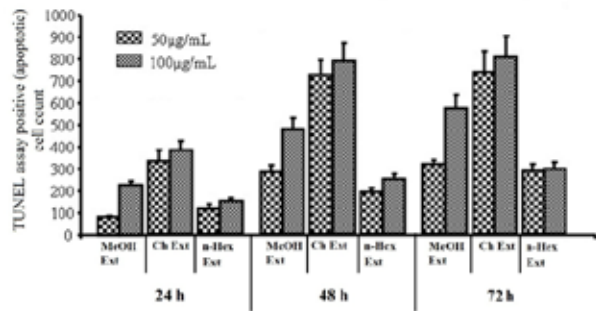


Figure 4. Graphical Representation of Score of TUNEL Positive Cells. 1000 cells were counted after each incubation period after staining with propidium iodide and FITC and TUNEL positive (green fluorescent) cells were scored. Data shown are Mean \pm SEM for three independent experiments. [In illustration MeOH Ext=Methanolic Extract, Ch Ext=Chloroform Extract and n-Hex Ext= n-Hexane Extract]

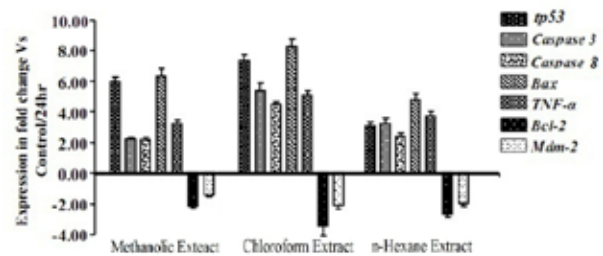


Figure 5. Graphical Representation of Fold Change in Expression of mRNA of Apoptosis Related Genes Against Control for 24 h.

Real-time polymerase chain reaction analysis of apoptosis related genes showed *tp53*, *caspase-3*, *-8*, *bax* and *TNF- α* were up regulated in all three extracts treatment to cells but *bcl-2* and *mdm-2* were down regulated. Data represented Mean \pm SEM of three independent experiments.

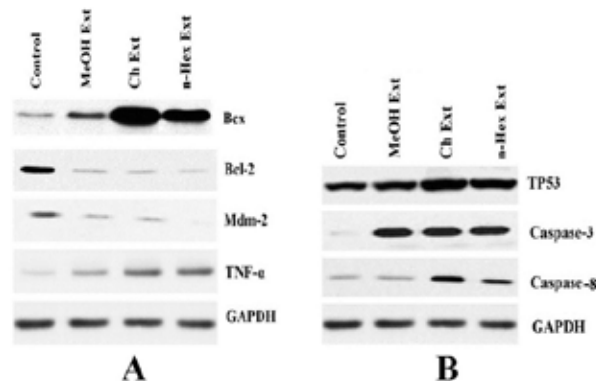


Figure 6. Protein Immunoblotting/Western Blotting of Apoptosis Related Proteins.

Protein expression study showed the up regulation of *TP53*, *Caspase-3*, *-8*, *Bax* and *TNF- α* whereas down regulation of *Bcl-2* and *Mdm-2*. [In illustration MeOH Ext=Methanolic Extract, Ch Ext=Chloroform Extract and n-Hex Ext= n-Hexane Extract]

mL of chloroform extracts of RBJR for 24 h increased by 7.35 \pm 0.36, 5.41 \pm 0.48, 4.48 \pm 0.22, 8.24 \pm 0.49, 5.11 \pm 0.27 fold, respectively, as compared to the levels in untreated control cells. Similarly, the increase in the mRNA level in the cells treated with 50 $\mu\text{g}/\text{mL}$ methanolic and n-hexane extracts were 5.93 \pm 0.35, 2.24 \pm 0.11, 2.14 \pm 0.19, 6.36 \pm 0.49, 3.22 \pm 0.27 and 3.05 \pm 0.32, 3.21 \pm 0.07, 2.42 \pm 0.19, 4.8 \pm 0.38 and 3.7 \pm 0.31 folds for *tp53*, *Caspase-3*, *-8*, *bax* and *TNF- α* respectively, as compared to the levels in untreated control

cells. A decrease in *bcl-2* and *mdm-2* mRNA expression was observed in all three extracts treatments. The lowest value of *bcl-2* and *mdm-2* mRNA expression was found in chloroform extract treated MDA-MB-231 cells (-3.44 ± 0.61 and -2.09 ± 0.24 , respectively). Interestingly *tp53* and *bax* expression significantly increased, whereas *mdm-2* and *bcl-2* in all three RBJR extracts treated cells, indicating that the RBJR extracts treatment induced apoptosis by shifting the *tp53:mdm-2 bax:bcl-2* ratio in favor of apoptosis.

RBJR extracts modulates apoptosis related protein expression

Although mRNA expression analysis data indicated that cell death has taken place through apoptosis. However, for further confirmation for translation of apoptosis related gene's mRNAs in to apoptosis related proteins, immunoblottings was performed. Figure 6 (A and B) summarize the results of protein immunoblottings. It was clear from blot results that the expression of *tp53*, *Caspase-3*, *-8*, *Bax* and *TNF- α* was higher in chloroform extract ($50 \mu\text{g/mL}$) treated cells, followed by methanolic extract and then n-hexane extracts treated cells compared to respective controls. Although, *Bcl-2* and *Mdm-2* were expression was down regulated in cells treated with all the extracts (Figure 6A and B). However, highest down regulation of these proteins were found in cells treated with n-Hexane extract followed by chloroform and then methanolic extracts. (Figure 6 B). This suggested that chloroform, methanolic and n-hexane extracts of RBJR contains some bioactive compounds which can down regulate the expression of *Bcl-2* and *Mdm-2* as well as up regulate the expression of *tp53*, *Caspase-3*, *-8*, *Bax* and *TNF- α* .

Discussion

An anticancer agent that suppresses the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. While many anticancer agents have been developed, unfavorable side effects and resistance are serious problems (Khan and Mlungwana, 1999). Since the most common tumors in adults are resistant to almost all presently available anticancer drugs the most of the available drugs have limited anti-solid tumor activity (Parasnis, 2004). Hence there is a need for more effective anticancer agents. Over past few decades, screening of medicinal plants and/or their parts for their anticancer potencies is a major field of interest for scientists working in field of medicine and chemistry. Mostly such medicinal plants are selected on the basis of ethnopharmacological, chemosystemic and ecological information. In present study cancer of our interest was breast cancer and root bark of Walnut (*Juglans regia L.*) (RBJR) was the plant material for evaluation of their anticancer property against MDA-MB-231 cells. For this study RBJR was subjected to extraction in different organic solvents (methanol, chloroform and n-hexane).

There are many methods to test apoptosis. Frequently used methods for test of apoptosis are morphological

study, biochemical assay, Flow cytometric analysis and so on. However, morphological study is the most reliable one. Catchpoole and Stewart (1995) reported that the appearance of DNA ladder is detected after the appearance of characteristic morphological changes of apoptosis in cells by morphological observation. Hence, the morphological microscopic study, especially in the early stage of apoptosis, is very important for apoptosis test (Xiao et al., 2007). In our study we found many characteristic morphological changes of apoptosis. For example, spikes, surface blisters, blebs and cellular rounding and nuclear disintegration (pycnosis and karyorrhexine) were observed frequently in all three organic extracts treated cells (Barrett et al., 2001) (Figure 2).

TUNEL assay is an efficient combination of molecular biology and morphological observation and used largely for the quantification of apoptosis in cells (Meenakshi et al., 2003). Therefore, we further confirmed our results by TUNEL assay. Once the morphological microscopic observation confirmed that extracts have ability to induce apoptosis, we switch on our study for TUNEL assay for quantification of apoptosis inducing ability of extracts (Xiao et al., 2007). A dose and time dependent treatment was given to MDA-MB-231 cells. Chloroform extracts shown highest TUNEL positive cell counts in all dose and time dependent treatments (735 ± 80 and 803 ± 98 counts for $50 \mu\text{g/mL}$ and $100 \mu\text{g/mL}$ after 72 h incubation), followed by methanolic and n-hexane extracts (Figure 3 and 4).

Real-time polymerase chain reaction (RT-PCR) is another way to analyze the alteration of apoptosis related gene mRNAs for confirmation of apoptosis. Hence, in our study we subjected the cells for treatment of extracts and gone for RT-PCR analysis of level of mRNA expressed. Chloroform extract showed highest level of expression of mRNA for *tp53*, *caspase-3*, *-8*, *bax*, *TNF- α* and lowest level of expression of mRNA *bcl-2* and *mdm-2*, followed by methanolic and n-hexane extracts (Figure 5).

A complex interplay between regulatory proteins from the *Bcl-2* superfamily of proteins is involved in apoptosis (Ramos, 2007). These pro- and anti-apoptotic proteins are principal regulators of the intrinsic pathway of apoptosis (Williams and Smith, 1993). Previous reports have shown that the ratio of *Bax* to *Bcl-2* determines, in part, the susceptibility of cells to death signals (Marzo and Naval, 2008). Changes in the *Bcl-2/Bax* ratio have been reported to be caused by downregulation of *Bcl-2* and slight downregulation of *Bax* (Chang et al, 2005), downregulation of *Bcl-2* and upregulation of *Bax* (Cha et al., 2004; Paris et al., 2007), and downregulation of *Bcl-2* with no change in the level of *Bax* (Han et al., 2008). Over expression of *Mdm-2* inhibit upstream apoptosis pathways. Hence under expression of *Mdm-2* may be a support for acceleration of process of apoptosis (Arden et al., 2007). *TNF- α* is an activator of extrinsic pathway of apoptosis (Wang et al., 2009). Whereas presence of *Caspase-3*, *-8* etc. indicate that mitochondrial release of cytochrome-c has took place.

In this study, we demonstrated that *Bcl-2* and *Mdm-2* expression was significantly inhibited while *Bax*, *tp53*, *Caspase -3*, *-8* and *TNF- α* expression was markedly

increased in all extract treatments. Interestingly, expression of Bax, *Tp53*, *Caspase -3,-8* and *TNF- α* were highest in cells treated with chloroform extract, followed by methanolic and n-hexane extract, whereas *Bcl-2* and *Mdm-2* expression found down regulated maximum in n-hexane extract, followed by chloroform and methanolic extracts. This findings suggests that there are bioactive compounds in n-hexane which down regulates the expression of *Bcl-2* and *Mdm-2* proteins whereas chloroform, methanolic and n-hexane extracts have bioactive compounds which up regulate expression of Bax, *Tp53*, *Caspase -3,-8* and *TNF- α* . Although this study is a preliminary study, this report may help to investigate some bioactive compounds (particularly from chloroform extract of RBJR) for their anti cancer effects.

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