List of Publications

Thesis related


Other publications


Conference Proceedings

1. Bagged the Young Scientist foreign travel grant from Department of Science and Technology (Govt. of India) to attend and present a paper entitled “In vitro cytotoxic and genotoxic potential of juglone against melanoma cells proceeds via oxidative stress mediated mechanisms” at the 2009 AAPS Annual Meeting and Exposition held at Los Angeles, California during November 2009.

2. Presented a poster entitled “Cytotoxic and clastogenic effect of juglone against melanoma cells – An in vitro study” at the International Conference on Radiation Biology & Translational Research in Radiation Oncology held at Jaipur in November 2008 and won the Best Poster Award for the same.
Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells

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Abstract

This study demonstrates cytotoxic and genotoxic potential of juglone, a chief constituent of walnut, and its underlying mechanisms against melanoma cells. MTT assay and clonogenic assay were used to study cytotoxicity, micronucleus assay to assess genotoxicity, glutathione (GSH) assay and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay to evaluate the oxidative stress induction. Apoptosis/necrosis induction was analysed by flow cytometry. We observed a concentration-dependent decrease in cell survival with a corresponding increase in the lactate dehydrogenase levels. A dose-dependent increase in the frequency of micronucleated binucleate cells indicated the potential of juglone to induce cytogenetic damage in melanoma tumor cells. Moreover, results of the micronuclei study indicated division delay in the proliferating cell population by showing decrease in the cytokinesis blocked proliferation index. Further, juglone-induced apoptosis and necrosis could be demonstrated by oligonucleosomal ladder formation, microscopic analysis, increase in the hypodiploid fraction (sub Go peak in DNA histogram), as well as an increased percentage of Annexin V(+) /PI(+) cells detected by flow cytometry. A significant concentration-dependent decrease in the glutathione levels and increase in dichlorofluorescein (DCF) fluorescence after juglone treatment confirmed the ability of juglone to generate intracellular reactive oxygen species. The cytotoxic effect of juglone can be attributed to mechanisms including the induction of oxidative stress, cell membrane damage, and a clastogenic action leading to cell death by both apoptosis and necrosis.

Keywords: Juglone; Melanoma; Genotoxicity; Reactive oxygen species; Apoptosis; Necrosis

1. Introduction

Quinones represent a broad category of widely distributed quinoid compounds in nature. Many quinones have been associated with a range of biological activities, including antitumor activity (Babula et al., 2007). Although, there are many clinically important agents containing a quinone nucleus with excellent antitumor activity (e.g. anthraquinone, mitoxantrone, and saitopin), many other quinones require testing for their antitumor activity (Kim et al., 2006). Although, DNA

represents the main target of quinoids, the exact contribution of the quinone moiety to the cytotoxic effect is not clear. However, most of them belong to the groups of DNA intercalating and/or alkylating agents, and/or topoisomerase inhibitors (Rowley and Halliwell, 1983; Yamashita et al., 1991). In general, quinone toxicity has been attributed to the ability to undergo reversible oxidation-reduction reactions, as well as to its electrophilic nature leading to the formation of free radicals (Bachur et al., 1979; Giulivi and Cadenas, 1994).

Plants possess diverse principles, which are of immense nutritional and medicinal value. Plant-derived quinones are among the compounds that are extensively studied for their potential as cytotoxic/anticancer agents. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a compound derived from the...
roots of *Plumbago zeylanica,* is among the most extensively studied quinones. Our previous studies gave some insight into the anti-tumor and radiosensitizing properties of plumbagin (Nareesh et al., 1996; Prasad et al., 1996; Devi et al., 1998; Tiwari et al., 2002). Other natural naphthoquinones, such as lapachone and shikonin, also possess promising cytotoxic/anticancer properties (Wu et al., 2004; Reincke et al., 2005).

Juglone (5-hydroxy-1, 4-naphthoquinone), a structural analogue of plumbagin is a pigment that occurs as a natural product in the roots, leaves, nut-hulls, bark and wood of black walnut (*Juglans nigra* L.), European walnut (*Juglans regia* L.) and butternut (*Juglans cinerea* L.) of the Family Juglandaceae (Botanical Dermatology Database, 1999). Herbal preparations of walnut have been extensively used in folk medicine for the treatment of acne, inflammatory diseases, ringworm, bacterial, viral, fungal infections as well as cancer (Duke and Ayensu, 1985; Blumenthal, 1998). Walnut contains juglone as the principal component, with other constituents such as alpha-hydrojuglone (1,4,5-trihydroxynaphthalene) and its glycoside beta-hydrojuglone, along with caffeic acid, ellagic acid, hyperin, and kaempferol. Herbal preparations of walnut are reported to suppress the growth of spontaneous mammary adenocarcinoma in Swiss albino mice (Bhargava and Westfall, 1968). Sugie and co-suppress the growth of spontaneous mammary adenocarcinoma with other constituents such as alpha-hydrojuglone (1,4,5-trihydroxynaphthalene) and its glycoside beta-hydrojuglone, along with caffeic acid, ellagic acid, hyperin, and kaempferol. Herbal preparations of walnut are reported to suppress the growth of spontaneous mammary adenocarcinoma in Swiss albino mice (Bhargava and Westfall, 1968). Sugie and co-workers (1998) found that juglone reduced the formation of ascorvymethane induced intestinal tumors in F344 rats and concluded that juglone could be a promising chemopreventive agent. Although few groups have studied the in vitro cytotoxic activity of juglone against cancer cell lines (Segura-Aguilar et al., 1992; Cenas et al., 2006), the exact mechanism remains doubtful. Therefore, we investigated the cytotoxic potential of juglone and its underlying mechanisms using a relatively chemo-resistant cell line (B16F1 melanoma) growing in vitro.

2. Materials and methods

2.1. Chemicals and reagents

Juglone purchased from Sigma (St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) diluted with Eagle's minimum essential medium (MEM) to the required concentration (the final concentration of DMSO was <0.02%). All drug solutions were prepared freshly before use due to the instability of juglone in medium. 3-(4,5-dimethyl tetrazolium-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2',7'-dichlorofluorescein diacetate (DCFH-DA), annexin-V-FITC apoptosis detection kit, RNase A, Proteasease-K, Nonidet P40, sodium dodecyl sulphate (SDS), Cytoclasin-B, glutathione, MEM, L-glutamine, gentamycin sulfate, fetal calf serum and DMSO were obtained from Sigma (St. Louis, MO, USA). Cytoclasin-B was dissolved in DMSO at 1 mg/ml, stored at −80°C and diluted with PBS immediately before use.

2.2. Cell line and culture

B16F1 melanoma cell line obtained from the National Centre for Cell Science (Pune, India) was grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% l-glutamine and 80 μg/ml gentamycin. Cells were cultured in 75 cm² flasks with loosened caps and incubated in 5% CO₂ in humidified air at 37°C (NuAire, Plymouth, MN, USA).

2.3. MTT assay for assessment of cytotoxicity

The cytotoxic potential of juglone was quantified using MTT assay. Briefly, 5 × 10⁴ cells per well were seeded in 96-well plates and incubated. Twenty-four hours later, cells were treated with juglone within a range of 0–20 μM for 1, 24 and 48 h. After treatment, cells were washed with PBS and incubated a further 4 h with 100 μl (1 mg/ml) of MTT. The formazan crystals formed were dissolved in DMSO (100 μl) and absorbance measured at 540 nm using a microplate spectrophotometer system (Biotek ELx 800, USA). Each treatment was completed in quadruplicate (with each experiment being repeated at least twice) and IC⁵₀ (concentration of drug that inhibits cell growth by 50%) values were determined from the concentration versus percent viability curve.

2.4. Lactate dehydrogenase (LDH) leakage assay

LDH released in the medium was measured according to the procedure of Wroblewski and Ladue (1955). Briefly, cells were treated with juglone (0–20 μM) for 1 h and the medium from cell culture flasks of control and treated groups was collected. The media were centrifuged and 50 μl of the supernatant transferred to individual tubes containing Tris-EDTA-NADH buffer, followed by 10 min incubation at 37°C and the addition of trypan blue solution. Absorbance was read at 539 nm using a UV–Vis spectrophotometer (UV-260, Shimadzu Corp., Tokyo, Japan) and the data were expressed as units/liter (U/l).

2.5. Clonogenic assay

Clonogenic cell death was measured using colony-forming assay according to the method of Pack and Marcus (1955). Briefly, exponentially growing cells were treated with juglone (0–20 μM) for 1 h. After treatment, appropriate number of cells was seeded into culture petri dishes in triplicate and left undisturbed for 10–12 days for colony formation. After fixation, the colonies were stained with crystal violet and the viable colonies containing 50+ cells were counted, from which the fraction surviving was calculated (Satish Rao et al., 2009).

2.6. Micronucleus assay for assessment of genotoxicity

Micronucleus assay was performed as by Fenech and Morley (1985), with minor modifications (Rao et al., 2006). Briefly, exponentially growing cells were treated with juglone for 1 h, after which the medium was replaced with fresh medium containing cytoclasin-B (final concentration 3 μg/ml). After incubation for 36 h, the cells were harvested with mild trypsin treatment (0.1%) and centrifuged (1000 rpm for 5 min). The supernatant was discarded and the resuspended...
pellet was given mild hypotonic treatment (0.75% ammonium oxalate) for 2–4 min, followed by fixation with Carnoy’s fixative (3:1 methanol:acetic acid). The fixed cells were gently dropped on to pre-cleaned glass slides, air-dried, stained with acridine orange (0.002% w/v dissolved in Sorenson’s buffer, pH 6.8) and observed under a fluorescence microscope (Photomicroscope III, Carl Zeiss, Germany) using 40X neofluor objective. Slides were coded to avoid observer bias and 1000 binucleate cells with well-preserved cytoplasm were scored for the presence of micronuclei from each group according to the established criteria of Fenech et al. (2003). Data on cell proliferation was also obtained by counting the frequencies of mono-, bi-, tri- and tetraneucleate cells, and the cytokinesis blocked proliferation index (CBPI) was calculated as described earlier (Kumar et al., 2009).

2.7. Glutathione (GSH) assay

GSH levels with or without juglone treatment were monitored, which served as an index of oxidative stress. Exponentially growing cells were treated with juglone in the range of 0–20 μM for 1 h. The cells were washed with PBS and lysed at 4 °C for 2 h using chilled 5% w/v metaphosphoric acid to extract the cellular GSH. The suspension was centrifuged at 13,000 rpm for 5 min, and GSH levels were determined using the standard method of Moron et al. (1979). Briefly, the supernatant was mixed with 0.2 M sodium phosphate buffer (pH 8.0) and 0.04 mM DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) and kept at room temperature for 10 min. Absorbance of the samples was recorded against a reagent blank at 412 nm in the standard method of Moron et al. (1979). Briefly, the cell lysate was treated with sodium dodecyl sulfate (1%) and RNase A (100 μg/ml) overnight at 37 °C, followed by proteinase K (50 μg/ml) treatment at 56 °C for 8 h. At the end of extraction, DNA was precipitated overnight at −20 °C by adding a half volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol. The DNA thus obtained was resolved on a 1.5% agarose gel, visualized and photographed under UV illumination after staining with ethidium bromide. Untreated cells processed in the same way served as control.

2.8. Measurement of intracellular ROS

The effect of juglone on the intracellular ROS levels was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay of Bai and Cederbaum (2003). In brief, exponentially growing cells were treated with different concentrations of juglone for 1 h followed by incubation with 5 μM DCFH-DA in MEM for 30 min in the dark. The cells were washed, resuspended in PBS, and the fluorescence intensity was measured using a microplate reader (InfiniteM200, TECAN) at excitation and emission wavelengths of 488 nm and 525 nm, respectively. Results were statistically measured using an annexinV-FITC apoptosis detection kit (Sigma–Aldrich, St. Louis, USA) by the manufacturers’ recommended protocol. Cells (1 × 10⁶) were treated with 5 and 10 μM juglone for 12 and 24 h. At the end of treatment, both floating as well as attached cells were collected, washed with PBS twice, and resuspended in 1× binding buffer (part of the kit) at a cell density of 1 × 10⁶ cells/ml. The cells were double stained in the dark for 10 min with the fluorescein isothiocyanate (FITC)-labeled annexin V (5 μl) and PI (10 μl) before being analyzed flow cytometrically using FACSCalibur™ flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA, USA). At least 10,000 events were acquired, cell debris was excluded by gating and the percentage of sub-G0 cell population (hypodiploid fraction) was determined using WinMDI version 2.9 software.

2.9.2. Assessment of DNA fragmentation by agarose gel electrophoresis

Exponentially growing cells (2 × 10⁶) were treated with indicated concentrations of juglone and DNA fragmentation assay was performed according to Kumar et al. (2009). Briefly, the cell lysate was treated with sodium dodecyl sulfate (1%) and RNase A (100 μg/ml) overnight at 37 °C, followed by proteinase K (50 μg/ml) treatment at 56 °C for 8 h. At the end of extraction, DNA was precipitated overnight at −20 °C by adding a half volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol. The DNA thus obtained was resolved on a 1.5% agarose gel, visualized and photographed under UV illumination after staining with ethidium bromide. Untreated cells processed in the same way served as control.

2.9.3. Analysis of DNA content by flow cytometry

Juglone-induced apoptosis was assessed flow cytometrically using propidium iodide staining (Chang et al., 1999). In brief, melanoma cells (5 × 10⁶) after treatment with juglone for 12 and 24 h were harvested and fixed using 70% ice-cold ethanol overnight at 4 °C. The cells were washed twice with cold phosphate-buffered saline (PBS), subjected to RNase treatment (100 μg/ml) at 37 °C for 1 h. The cellular DNA was stained with PI (50 μg/ml) in PBS for 15 min at 4 °C in the dark and analysed by FACS Calibur™ flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA, USA). At least 10,000 events were acquired, cell debris was excluded by gating and the percentage of sub-G0 cell population (hypodiploid fraction) was determined using WinMDI version 2.9 software.

2.9.4. Flow cytometric measurement of apoptosis/necrosis using annexin V-FITC

The extent of apoptosis and/or necrosis was flow cytometrically measured using an annexin V-FITC apoptosis detection kit (Sigma–Aldrich, St. Louis, USA) by the manufacturers’ recommended protocol. Cells (1 × 10⁶) were treated with 5 and 10 μM juglone for 12 and 24 h. At the end of treatment, both floating as well as attached cells were collected, washed with PBS twice, and resuspended in 1× binding buffer (part of the kit) at a cell density of 1 × 10⁶ cells/ml. The cells were double stained in the dark for 10 min with the fluorescein isothiocyanate (FITC)-labeled annexin V (5 μl) and PI (10 μl) before being analyzed flow cytometrically using FACSCalibur™. At least 10,000 events were acquired and the percentage of cells in different quadrants was analyzed using quadrant statistics (WinMDI Version 2.9).
2.10. Statistical analysis

The experimental data were expressed as mean ± SEM. The plating efficiency of cells was determined and the data points of surviving fraction was fitted on to a linear quadratic model, SF (surviving fraction) = \( \exp(-\alpha D + \beta D^2) \). The percentage of micronucleated binucleate cells (MNBC) in different treatment groups was compared with the control by One-way ANOVA using GraphPad InStat, Software, USA. The dose response curve for micronuclei was fitted on a linear model (Y = \( \alpha + \beta X \)).

3. Results

3.1. Cytotoxic assays

3.1.1. Cytotoxic effects of juglone

The cell killing effect of juglone on murine B16F1 melanoma cells after 1, 24 and 48 h treatment were studied and results expressed as IC50 (Fig. 1). A concentration-dependent reduction in the viability of melanoma cells at all treatment regimens was observed. The IC50 values for 1 h exposure, 1 h exposure followed by 24 h incubation, 24 h and 48 h exposure were 9.9 \( \mu M \), 7.74 \( \mu M \), 7.46 \( \mu M \) and 6.92 \( \mu M \), respectively.

3.1.2. LDH levels

To study the cell membrane damaging potential of juglone against B16F1 melanoma cells, LDH leakage into the supernatant of culture following 1 h treatment was measured (Fig. 2). Treatment with juglone produced a significant increase in LDH levels at 5, 10, 15 and 20 \( \mu M \) in comparison to vehicle-treated control. The increase in the LDH levels was concentration-dependent up to a dose of 20 \( \mu M \) and fitted well into linear model with an R-value of 0.9922.

3.1.3. Effect on clonogenic survival

Fig. 3 summarizes the effect of juglone on the clonogenic survival of melanoma cells. The cell survival curve was fitted using a linear quadratic equation. The dose-response curve for juglone had a definite shoulder region at lower doses (5 and 10 \( \mu M \)). Treatment of melanoma cells with juglone caused a concentration-dependent reduction in their colony forming ability, which followed a polynomial regression pattern. In comparison to vehicle-treated controls, the lowest concentration of juglone (5 \( \mu M \)) reduced the mean surviving fraction to 0.62 ± 0.10 (a 37% reduction) and the highest concentration (20 \( \mu M \)) caused a drop in the mean surviving fraction to 0.0032 ± 0.0006 (a 99% drop).

3.2. Juglone-induced micronuclei formation

This experiment was carried out using lower concentrations of juglone in the range of 0–10 \( \mu M \), because the agent severely reduced the percentage of binucleate cells beyond 10 \( \mu M \) concentration. Treatment of melanoma cells resulted in a concentration-dependent increase in the frequency of MNBC (Fig. 4). The increase in the frequency of MNBC was statistically significant in comparison to vehicle-treated controls at all treatment doses. At 0.5, 1, 2.5, 5 and 10 \( \mu M \) juglone, we observed a 1.25, 1.57, 1.84, 3.16 and 4.61-fold increase in the mean frequency of MNBC, respectively. The increase in the frequency of MNBC with one MN, two MN, multiple MN and total MN fitted well to linear model with R-values of 0.9960, 0.9626, 0.9806 and 0.9935, respectively.
3.3. Cytokinesis blocked proliferation index (CBPI)

Treatment of melanoma cells with juglone resulted in a significant concentration-dependent increase in the frequency of mononucleate cells (Table 1). We observed a 9-fold and 29-fold increase in the number of mononucleate cells (in comparison to vehicle-treated control) at 5 and 10 µM concentrations of juglone, respectively. Conversely, the frequency of binucleate cells as well as polynucleate cells decreased in a concentration dependent manner in comparison to vehicle treated controls. The CBPI, which is considered an index of cell proliferation, showed a significant decrease in a concentration-dependent manner.

### Table 1

<table>
<thead>
<tr>
<th>Juglone (µM)</th>
<th>Frequency/1000 Binucleates</th>
<th>CBPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mononucleates</td>
<td>Polynucleates</td>
</tr>
<tr>
<td>0.5</td>
<td>277.30 ± 2.20</td>
<td>2.02</td>
</tr>
<tr>
<td>0.5</td>
<td>113.30 ± 2.88</td>
<td>0.02</td>
</tr>
<tr>
<td>1</td>
<td>410.30 ± 2.10</td>
<td>0.02</td>
</tr>
<tr>
<td>2.5</td>
<td>498.30 ± 2.30</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>2513.30 ± 15.60</td>
<td>0.02</td>
</tr>
<tr>
<td>10</td>
<td>8121.30 ± 25.80</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* P < 0.05.
* * P < 0.01.
* * * P < 0.001.
* ** P < 0.0001 in comparison to vehicle-treated control. No symbol = non significant.

3.4. Juglone induced changes in GSH levels

Experiments were designed to determine the role of juglone in oxidative stress induction by measuring GSH levels, a major intracellular antioxidant enzyme. Fig. 5A shows the changes in GSH levels upon treatment of melanoma cells with different doses of juglone. As the concentration of juglone was increased, there was a corresponding drop in GSH levels, which was statistically significant (P < 0.01 to P < 0.001) in comparison to vehicle-treated controls. Treatment of melanoma cells with 5, 10, 15 and 20 µM juglone resulted in a 37, 58, 71 and 83% drop in mean intracellular GSH levels, respectively. The concentration-dependent drop in GSH levels was linear with an R-value of 0.97.

3.5. Juglone induced changes in intracellular ROS levels

From the results of the DCFH-DA assay (Fig. 5B) a significant dose-dependent elevation in DCF fluorescence (indicative of intracellular ROS levels) up to 10 µM doses of juglone was detected. Exposure of melanoma cells to 2.5, 5, 7.5 and 10 µM doses of juglone caused a 2.54, 4.71, 8.32 and 10.12 fold increase in the mean DCF fluorescence, respectively.

3.6. Apoptosis/necrosis assays

3.6.1. Altered nuclear morphology induced by juglone

The nuclei of untreated control cells were found to be intact, round in shape and were stained green. In contrast, cells treated with 5 µM juglone (for 12 h) had some cells stained green and some red. Membrane blebbing, chromatin condensation as well as nuclear fragmentation was evident in juglone treated cells, indicating apoptotic mode of cell death. At a higher dose of
juglone (10 μM), an increase in the number cells with red colored nuclei (necrotic/late apoptotic) was observed (Fig. 6).

### 3.6.2. DNA fragmentation induced by juglone

To ascertain whether apoptosis could be the mechanism by which juglone induces cytotoxic effect, melanoma cells were treated with juglone for various time periods and the extracted DNA was resolved on agarose gel. The results of the DNA fragmentation/ladder assay (Fig. 7) show a typical oligonucleosomal ladder pattern. DNA laddering was seen as early as 12 h after treatment of melanoma cells with juglone, and the intensity of these ladders increased in a time-dependent manner (data not shown).

### 3.6.3. Increase in sub-G0 fraction induced by juglone

We observed a dose- and time-dependent increase in the sub-G0 (hypodiploid) fraction (Fig. 8). After 12 h exposure to 5 and 10 μM juglone, the percentage of cells having DNA content <2 n were 3.77% and 50.75%, respectively. The corresponding values for 24 h exposure were 26.27% and 74.08%.

### 3.6.4. Juglone induced late apoptosis and necrosis

Phosphotidyl serine translocation from inner part of plasma membrane to outer part is believed to be an early event in apoptosis. Binding of Annexin V to phosphotidyl serine in presence of calcium ions results in green fluorescence. During late apoptosis or necrosis, owing to increased membrane permeability, PI also enters the cell and binds to cellular DNA, staining the nucleus red. Results from the present study (Fig. 9) show that juglone treatment resulted in significant elevation in the percentage of Annexin V-FITC (+)/PI (+) cells (upper right quadrant), both in a time- and concentration-dependent manner, indicating late apoptotic/secondary necrotic death. We observed 30.6% cells in upper right quadrant.
quadrant after treatment with 5 mM juglone for 12 h. This figure drastically increased to 67% after 24 h incubation with juglone. However, at a higher dose (10 mM) of juglone, no significant time-dependent increase was observed in the percentage of Annexin V-FITC(+)/PI(+) cells.

4. Discussion

Walnut has been used in traditional medicines for various ailments and some extracts of walnut are also reported to possess anticancer properties (Duke and Ayensu, 1985; Blumenthal, 1998). Juglone is reported to be the main active ingredient in walnut (Duke and Ayensu, 1985). However, not much has been reported about the mechanisms underlying the cytotoxic potential of juglone. In the present study, treatment of melanoma cells with juglone resulted in immediate loss of viability in a concentration-dependent manner, which was statistically significant in comparison to the vehicle treated controls. Earlier, Segura-Aguilar and co-workers (1992) compared the effect of juglone and other quinones on human leukemic (HL-60) cells and doxorubicin-resistant human leukemic (HL-60R) cells and concluded that multidrug resistance that develops in the doxorubicin-resistant HL-60R cells had no effect on the cytotoxicity of juglone indicating its therapeutic possibilities. Although juglone treatment resulted in a time-dependent reduction in cell viability as observed in our study, the IC50 value for different treatment regimens did not change significantly. Earlier, juglone produced a concentration-dependent but not time-dependent cytotoxicity against HaCaT keratinocytes (Inbaraj and Chignell, 2004), which is similar to what we have observed with malignant melanoma cells. Recently, plumbagin a structurally related naphthoquinone was shown to cause a dose- as well as time-dependent cytotoxicity against cervical cancer cells (Srinivas et al., 2004).

The LDH assay used in the present investigation is based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in cells. LDH assays have been used to quantify cell-mediated cytotoxicity as well as to identify mediators that induce cytolysis (Korzeniewski and Callewaert, 1983). The results of MTT assay gave us an indication about the immediate cytotoxic effects of juglone against B16F1 melanoma cells. To study whether cell membrane damage could be the reason behind the immediate cytotoxicity of juglone, we used the LDH leakage assay. Treatment of melanoma cells with juglone resulted in a significant concentration-dependent increase in the LDH levels which further support the findings of MTT assay. This indicates that the cytotoxicity of juglone against melanoma cells could be attributed, at least in part, to the cell membrane damaging effects of juglone.

Any compound that can cause damage to the clonogenic potential of the tumor cell could be an anticancer agent. Various drugs of plant origin, such as vincristine and taxol, produced a dose-dependent reduction in tumor cell survival (Mujagic et al., 1983; Helson et al., 1993). However, there have been no previous reports on the effect of juglone on the clonogenic survival in melanoma cells. Treatment of melanoma cells with juglone caused a dose-dependent reduction in the cell survival, which was statistically significant in comparison to vehicle-treated controls. The survival curve for juglone against melanoma cells had a linear coefficient (α) value of 0.00753 ± 0.0154 and with a ”p value” of 0.00012. These results indicate that the juglone severely affects the clonogenic potential of melanoma cells.

The micronucleus assay is one of the most widely used methods to study the genotoxic potential of a test compound and several previous studies have shown that clonogenic survival and micronuclei induction are closely related (Geard and Chen, 1990; Mariya et al., 1997). Micronuclei are formed...
from whole or fragmented chromosomes that lag behind during the cell division (Fenech, 2007). To see whether the observed reduction in the clonogenic survival of melanoma cells after treatment with juglone is related to its genotoxic potential, we used the cytokinesis blocked micronucleus assay.

Exposure of melanoma cells to juglone caused a concentration-dependent increase of MN up to 10 μM. The increased micronuclei frequency indicates that a substantial part of the genome is lost which may have a major impact on the cell division as well as cell death. Another important feature of this

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**Fig. 8.** Flow cytometric analysis of DNA content as an index of juglone-induced apoptosis. Panel A: percentage of apoptotic cells (sub-G0 hypodiploid fraction) after 12 h incubation with juglone; Panel B: percentage of apoptotic cells (sub-G0-hypodiploid fraction) after 24 h incubation with juglone.

**Fig. 9.** Flow cytometric analyses of apoptosis and necrosis using Annexin V-FITC/PI dual staining. Cells in lower right quadrant (Annexin V-FITC (+)/PI (+)) represent apoptotic population and cells in upper right quadrant (Annexin V-FITC (+)/PI (+)) represent late apoptotic/necrotic population. Panel A: cells treated with indicated concentration of juglone for 12 h, Panel B: cells treated with indicated concentration of juglone for 24 h.
assay is that it provides data on the proliferative activity of a cell population. In the present study, treatment of melanoma cells with juglone caused a significant delay in cellular proliferation, as evidenced by a dose-dependent reduction in the Clonogenic assay (Table 1). Taken together, the genotoxic potential of juglone may have contributed to the observed dose dependent reduction in clonogenic survival of melanoma cells.

To study the ability of juglone to induce oxidative stress in melanoma cells, we carried out the GSH and DCFH-DA assays. GSH is an important intracellular reducing agent, which is responsible for the maintenance of antioxidant molecules and the thiol groups on intracellular proteins which is responsible for the maintenance of antioxidant activity, by participating in the elimination of reactive interme-
diates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching (Jewell et al., 1986; Liu et al., 2002). Therefore analysis of changes in intracellular GSH status produced by toxic compounds may provide important information about the mechanism of toxicity of the test compound. A significant concentration-dependent drop in GSH levels was seen after treatment with juglone, indicating the induction of oxidative stress. These results are consistent with a recently reported study where juglone and plumagin were shown to cause oxidative stress in keratinocytes (Ishibaraj and Chignell, 2004). Further, menadione, another plant-
derived quinone with anticancer property, exerts its activity via depletion of GSH (Rahula et al., 2007).

Juglone-induced oxidative stress was further confirmed by measuring the intracellular levels of ROS using DCFH-DA assay with the result of DCFH-DA assay gives an indication of the ability of juglone to induce formation of intracellular ROS, which substantiated our findings in the GSH studies. A concentration-dependent depletion of GSH levels with corresponding elevation in intracellular ROS levels after treatment with juglone indicates that oxidative stress could be an important mechanism by which juglone exerts its toxic activity against melanoma cells as well.

Apoptosis and/or necrosis are among the key mechanisms by which most compounds exert their cytotoxic effects, especially anticancer agents (Romney et al., 1998). Overload of intracellular ROS induces apoptosis or necrosis or a combina-
tion of both (Higuchi, 2003; Pelciano et al., 2004). Many of the other well-known cytotoxic/anticancer agents belonging to anthracyclins, alkylating agents, epipodophyllotoxins and camptothecins induce apoptosis through oxidative stress-mediated mechanisms (Muller et al., 1997; Conklin, 2004). Using different assays, an attempt was made to ascertain whether the cell death induced by juglone in melanoma cells could be due to apoptosis and/or necrosis. Microscopic staining revealed membrane blebbing, chromatin condensation and/or fragmentation, which are considered to be hallmarks of apoptosis. The increase in the number of cells stained red with or without fragmented DNA at higher doses of juglone suggests the possibility of late apoptosis as well as necrotic cell death. Also, gel electrophoresis of extracted DNA revealed characteristic oligonucleosomal ladder, which further confirms the mechanism of apoptosis. The DNA content analysis using flow cytometry also revealed prominent (dose- and time-
dependent) sub-Go-hypo-diploid fraction (<2 n DNA content), which is again associated with apoptosis (Renouze et al., 1998). Flow cytometric analysis using Annexin V-FITC/PI dual staining showed the ability of juglone to induce time as well as concentration-dependent increase in the percentage of late apoptotic/necrotic cells. Recently, Cenas et al. (2006) studied the effect of several chemicals including juglone on a couple of cancer cell lines in vitro where juglone was potent inducer of apoptosis. However, results from the present study demonstrate that cell death caused by juglone is due to both apoptosis as well as necrosis.

 Traditionally, necrotic cell death is recognized as an unregulated form of cell death. However, few of the recent studies have shown that necrosis could be a self-determined fate of cell, induced via several mechanisms such as stress stimuli, ion channel activation or DNA damage. Moreover, in cases where apoptotic pathways are deficient or absent (as seen in cancer), necrosis is known to occur as an alternate form of cell death (Sun et al., 2006). Recent literature illus-
trates that the use of compounds which specifically induce necrosis in tumor cells could also be a promising therapeutic strategy to counter the defective apoptotic pathways typically found in cancerous tissue. Other structurally related naph-
thaquinones with established anticancer activity such as beta-lapachone, shikonin, etc. also exert their cytotoxic activity via necrosis as their predominant mechanism (Sun et al., 2006; Han et al., 2007). In view of this, juglone induced apoptotic and necrotic cell death observed in chemoresistant melanoma cells may have some clinical relevance.

In general, the toxicity of quinones is known to depend mainly on two mechanisms, redox cycling resulting in produc-
tion of semiquinone radicals, and reactive oxygen species leading to depletion of glutathione in the cells (Ishibaraj and Chignell, 2004), with the former leading to the latter. Further, the structure-activity relationship studies on the toxicity of naphthaquinones have revealed that 1,4-naphthaquinones with a hydroxyl group at position 5 (as seen in plumagin and juglone) or at position 5- and 8- (as in naphthazarin) in the aromatic ring significantly increase toxicity against rat hepatopo-
cytes (Ollinger and Brunmark, 1991) compared to 1,4-naph-
thoquinone itself, due to increased efficiency of redox cycling. On the other hand, methyl substitution of 1,4-naphthaquinone at position 2 (as in plumagin and menadione) reduces its cyto-
toxicity owing to lower rate of redox cycling or lower rate of electrophic addition (Ross et al., 1986).

In conclusion, this study for the first time has shown the potential of juglone to reduce the clonogenic survival as well as the ability to induce micrometastases in melanoma cells grown in vitro. In addition it demonstrates that juglone-induced cell death proceeds via multifactorial mechanisms such as induc-
tion of oxidative stress, cell membrane damage and clasto-
genic effect, ultimately resulting in cell death both by apoptosis and necrosis. Juglone might be categorized as a potential anticancer compound based on the criteria estab-
lished by the National Cancer Institute (Pisha et al., 1995) that
any compound with IC50 value of <4 µmol/l has a potential to be an anticancer compound. In view of these present findings, and the existing reports of its use in traditional folk medicine as an anticancer agent, juglone deserves further studies to justify its potential as an anticancer agent, using a spectrum of preclinical models.

Acknowledgements

The authors are thankful to Dr Satyamoorthy K, The Director, Manipal Life Sciences Centre, Manipal University, Manipal for providing the facilities and encouragement during this study. The financial support from Indian Council of Medical Research (ICMR), New Delhi, India (IRIS No: 2005-00150), to carry out this study is gratefully acknowledged.

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Yamashita Y, Kawada S, Fujii N, Nakano H. Induction of mammalian DNA topoisomerase I and II mediated DNA cleavage by anticancer, a new anti-
Tumor growth inhibitory effect of juglone and its radiation sensitizing potential: in vivo and in vitro studies

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Abstract
The present study was aimed to evaluate the anticancer and radiosensitizing potential of juglone against a chemo- and radio-resistant tumor (B16F1 melanoma) growing on C57BL/6J mice. Volume doubling time, growth delay and median survival were used to assess in vivo anticancer and radiosensitizing potential of juglone. In vitro radiosensitizing potential of juglone was studied using clonogenic, comet and ROS induction assays. Treatment of tumor bearing mice with sub-lethal doses of juglone caused a dose-dependent inhibition of tumor growth as evident from the growth delay and median survival values. Comet assay using tumor tissue and blood showed differential toxicity of juglone where higher levels of DNA damage was seen in tumor tissue compared to blood cells. Pre-treatment of tumor bearing mice with optimum dose of juglone before radiation resulted in significant tumor growth inhibition compared to radiation alone. From the clonogenic assay, we observed a sensitization enhancement ratio of 1.37 for the combination treatment compared to radiation alone group. Further, comet assay studies revealed the potential of juglone to enhance the radiation induced DNA damage and cause a delay in its repair. Juglone pre-treatment before radiation also resulted in a significant elevation in the intracellular ROS levels compared to radiation alone. In conclusion, this study has shown the potential of juglone to inhibit the growth of melanoma in vivo. The study also revealed the potential of juglone to augment the radiation induced cell death of melanoma cells which may be attributed to oxidative stress mediated DNA damage and its delayed repair.

Key words
juglone, melanoma, anticancer, radiosensitization, DNA damage, reactive oxygen species

Introduction
As per the recent World Health Organization (WHO) report, cancer remains a major global health threat with an estimated worldwide death of 12 million by the year 2020. The effectiveness of radiation in combating cancer was established over 100 years ago and it still continues to be one of the mainstays of cancer therapy with approximately half of all cancer patients receiving radiotherapy in some form during the course of their illness. Chemotherapy on the other hand has developed more recently and since then extensive research efforts in the field of drug discovery and development has yielded a respectable armamentarium of potent anticancer agents. Nevertheless, the treatment of most human solid tumors remains largely palliative and thus the need for more effective antineoplastic agents still remains. Intrinsic tumor resistance and normal tissue toxicity appear to be two major biological determinants in the failure of chemo- as well as radiotherapy. It is now generally believed that the effective treatment of most solid tumors is less likely to be achieved with one “magic bullet” and that a combination approach involving second generation chemotherapeutics along with other treatment modalities may be required to attain better therapeutic outcome. One of the strategies that have been proposed to improve the therapeutic outcome in cancer is to manipulate radiation-induced cell kill by combining a radiosensitizer or a chemotherapeutic agent along with radiotherapy. Many chemotherapeutic drugs have been combined with radiation with varying degrees of success and many more are still under clinical evaluation. Nevertheless there is still a need to look for novel chemotherapeutic agents that could effectively be combined with radiation to improve the cancer therapeutic outcome.

Exploring the natural resources for the development of new anticancer agents has proven to be extremely beneficial. It is estimated that about 67% of pharmaceuticals approved for cancer therapy during the period between 1974 and 1994, were derived from natural sources. Quinones comprise a broad class of phenolic compounds that exhibit an array of pharmacological properties and are widely distributed in nature. Plants that
are rich in quinones have been extensively used in traditional folk medicine for the treatment of various ailments including cancer. These compounds are of great interest to researchers, because of the fact that several important anticancer drugs like doxorubicin, daunorubicin, mitomycin C etc. contain the quinone nucleus. Many other natural compounds with quinone nucleus such as β-lapachone, plumbagin, etc have also shown promising anticancer activity both in vitro and in vivo. Besides, several groups including ours have shown plumbagin to possess radiosensitizing potential as well.

Walnut (Juglans regia L) belonging to the family Juglandaceae has a long history of use in traditional folk medicine for the treatment of various ailments. Different parts of the tree are reported to possess antimicrobial, anti-inflammatory, immuno-modulatory, diuretic, laxative as well as anticancer properties. The aqueous extracts of walnut are also reported to possess strong antioxidant properties. Herbal preparations of walnut are known to suppress the growth of spontaneous mammary adenocarcinoma in Swiss albino mice. The major active ingredients of walnut include naphthoquinones such as juglone, α- and β-hydrojuglone alongside others such as vitamin C, caffeic acid, ellagic acid, hyperin, and kaempferol. Juglone (5-hydroxy-1,4 naphthoquinone), a structural analogue of plumbagin is reported to possess potent cytotoxic properties in vitro against human cancer cell lines including, human leukemia (HL-60) cells, doxorubicin resistant human leukemia (HL-60R) cells, human lung carcinoma (A549), human gastric cells (SGC-7901). We earlier reported the effect of juglone on B16F1 melanoma cells in vitro and concluded that the cytotoxic potential of juglone was mediated by multifactorial mechanisms involving oxidative stress, cell membrane damage and clastogenic effect ultimately leading to cell death by both apoptosis and necrosis. In the present study, we evaluated the in vivo anticancer potential of juglone against a chemo and radioresistant B16F1 melanoma solid tumor model. To our knowledge, there are no previous reports about the radiosensitizing potential of juglone. Therefore, the present research work was also aimed to evaluate the in vitro and in vivo radiosensitizing property of juglone against B16F1 melanoma cells.

Materials and Methods

Chemicals and reagents

Juglone, ethidium bromide, agarose (normal melting as well as low melting), ethylenediamine tetra acetic acid (EDTA), Eagle’s minimum essential medium (MEM), l-glutamine, gentamycin sulfate, fetal calf serum, polyethylene glycol (PEG), Triton-X, Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals such as sodium bicarbonate, sodium chloride, potassium di-hydrogen phosphate, di-sodium hydrogen phosphate, sodium hydroxide, potassium chloride and hydrochloric acid were purchased from Qualigens Fine Chemicals (Mumbai, India).

Cell lines and cultures

B16F1 melanoma cells procured from National Centre for Cell Sciences (Pune, India) were routinely grown in 25 cm² flasks containing MEM supplemented with 10% fetal bovine serum, 1% l-glutamine and 80 µg/ml gentamycin sulfate at 5% CO₂ in humidified air at 37 °C (NuAire, Plymouth, MN, USA).

Preparation of juglone solutions

For in vitro studies, juglone was dissolved in absolute alcohol at a stock concentration of 10 mM and stored at -80 °C. Working concentrations were prepared from the stock aliquots by serially diluting with MEM to the required concentrations. The concentration of juglone for the in vitro studies was chosen based on our earlier studies. For in vivo studies, juglone was dissolved in minimum amount of alcohol and made up to the required concentrations with 20% PEG in saline (vehicle).

Animals and tumor model

C57BL/6J mice, procured from National Institute of Nutrition (Hyderabad, India) were maintained under controlled conditions of temperature (23 ± 2 °C), humidity (50 ± 5 %), light (14 h and 10 h light and dark cycles respectively) and were allowed access to sterile food and water ad libitum. The animal care and handling were according to the guidelines of World Health Organization (Geneva, Switzerland) and the Indian National Science Academy (INSA) (New Delhi, India). For experimental purposes, eight to twelve week old mice from either sex, weighing 20 - 25 g from an inbred colony were selected. All the animal experiments were carried out with the prior approval from the institutional animal ethics committee (Kasturba Medical College, Manipal, India). Solid tumors were grown on the dorsal side of the mice by an intradermal injection of 5 × 10⁷ viable cells.

Irradiation

All radiation studies were carried out using a Co gamma teletherapy facility (Theratron Atomic Energy Agency, Canada) at the Shirdi Sai Baba Cancer Hospital.
The radiation doses used in the present investigation were based on our earlier studies (unpublished observation).

For in vitro experiments, exponentially growing cells with or without juglone treatment were exposed to gamma radiation at a dose rate of 1 Gy/min and source to surface distance (SSD) of 73 cm. For in vivo experiments, local tumor irradiation was carried out at a dose rate of 1.33 Gy/min in a field size of 4 x 4 inch (10.1 x 10.1 cm).

Tumor bearing animals with or without drug treatment were anaesthetized (50 mg/kg b. wt. of Ketamine and 8 mg/kg b. wt. of Diazepam), immobilized in a specially designed well-ventilated perspex box in such a way that only tumors protrude out into the radiation field. All the other parts of the body were shielded with 4 half value layer (HVL) of lead (5 cm thick), kept in a shielding tray fitted to the collimator (Figure 1).

**Assessment of in vivo anticancer potential of juglone**

**Acute toxicity studies of juglone**

The acute toxicity study of juglone was performed as described previously. Briefly, animals (fasted for 18 h prior to juglone treatment) were divided into several groups of 10 each. Each group of animals was injected intravenously with different doses of juglone (prepared freshly as mentioned previously) viz., 0.5, 1, 3, 6 and 10 mg/kg body weight (b. wt.). Immediately after the treatment, animals were provided with food and water, and the animals were monitored daily up to 14 days for mortality. LD\(_{50}\) was then calculated using probit analysis as described by Litchfield and Wilcoxon.

**Selection of optimum dose for antitumor activity**

Tumors were induced in the mice by injecting \(5 \times 10^5\) viable cells intradermally on the dorsal side. When the tumors became palpable, the tumor diameter in three perpendicular directions was measured using a vernier caliper and the tumor volume was calculated as previously described using the following formula

\[
V = \frac{\pi}{6} (D_1 \times D_2 \times D_3)
\]

where \(D_1, D_2\) and \(D_3\) are tumor diameters in three perpendicular planes. Once the tumor size reached 100 ± 10 mm\(^3\), animals were divided into following groups of 10 each:

- **Group I:** Tumor bearing animals in this group received 100 µl of vehicle (vehicle treated control group).
- **Group II - IV:** Animals in this group were injected intravenously with different doses of juglone viz 0.5, 1 and 1.5 mg/kg b. wt. (prepared freshly under sterile conditions) as single administration.

The best optimum dose from this study was used to evaluate the anticancer activity of juglone administered as multiple doses. Once the tumor size reached 100 ± 10
mm³, the tumor bearing animals were divided into following groups of 10 animals each as:

Group I: animals in this group received 100 µl of vehicle (vehicle treated control group);

Group II: animals in this group received repeated injections of the optimum dose of juglone dissolved in 100 µl of vehicle on day 1, 3 and 5 consecutively.

Assessment of tumor response

The tumor diameters were measured every alternate day using a vernier caliper and the tumor volume was calculated and plotted against days post-treatment. The tumor response after various treatments was assessed using a) Volume doubling time (VDT) – time required for the tumors to reach double the initial volume and b) Growth delay (GD) – the difference in time between the treated and untreated tumors to reach 5 times the initial volume. The animals were further monitored for survival and the median survival times were determined from the Kaplan Meier analysis of survival. During the course of experimentation, animals that were under stress due to excess tumor burden were terminated from the study (due to humane considerations) and were considered as censored in the survival analysis.

Alkaline comet assay

Tumors were induced in the mice by injecting 5 × 10³ viable cells intradermally. Once the tumor size reached 100 ± 10 mm³, the animals were divided into following groups of 12 each.

Group I: Animals in this group received 100 µl of vehicle

Group II: Animals in this group were injected intravenously with optimum anticancer dose of juglone (1 mg/kg b. wt.)

A subgroup of four animals from the above treatment groups were euthanized on 1, 7 and 15 post treatment days. At the indicated time periods, the blood samples were collected in heparinized tubes by retro-orbital plexus. The tumors from these animals were also excised immediately and single cell suspension was prepared in ice-cold PBS as described elsewhere. The differential genotoxic efficacy of juglone among the blood and tumor samples were then studied using the alkaline version of single cell gel electrophoresis (comet) assay as previously described. Briefly, microscopic slides were coated with 1.5 % normal melting agarose, and left overnight to dry. Cells suspensions (tumor as well as blood) were mixed with 190 µL of 0.75 % low-melting-point agarose (LMPA) and were distributed on the coated slide. The slides were left to gel for 10 min at 4 °C, before a third layer of 200 µL 0.75 % LMPA was added to the slide and left for 10 min at 4°C. The slides were then dipped in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base and 0.2 M NaOH, 1% Triton X, 10 % DMSO; pH 10) for at least 2 h at 4°C. The slides were then transferred to an electrophoresis unit containing electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH ≥ 12.3), and were left immersed in the solution for 20 min to allow DNA unwinding before being subjected to electrophoresis. Electrophoresis was carried out for 20 min at a voltage of 0.7 V/cm and a current of 300 mA. Next, the slides were rinsed with neutralization buffer (0.4 M Tris; pH 7.4) and stained with ethidium bromide. The stained cells were then observed and photographed at 20x magnification using a fluorescence microscope (Olympus BX51, Olympus Microscopes, Tokyo, Japan) equipped with a 515–535 nm excitation filter, a 590 nm barrier filter and a CCD camera (Cool SNAP-Procf Digital Color Camera Kit Version 4.1, Media Cybernetics, Silver Spring, Maryland, USA). Images of a minimum of 100 cells per treatment were analyzed using the Komet software (Version 5.5, Kinetic Imaging Ltd., Bromborough, UK). The mean olive tail moment (OTM) was used as a parameter to assess the level of DNA damage.

Assessment of radiosensitizing potential of juglone

In vivo studies

Tumors were induced in the mice by injecting 5 × 10⁵ viable cells intradermally. Once the tumor size reached 100 ± 10 mm³, the animals were divided into following groups of 10 each:

Group I: Animals in this group received 100 µl of vehicle

Group II-III: Animals in this group received local tumor irradiation with different doses of γ-radiation (10 Gy and 30 Gy),

Group IV-V: Animals in this group received optimum dose of juglone followed by local tumor irradiation with 10 Gy and 30 Gy dose of γ-radiation respectively.

The tumor growth parameters after various treatments was then studied as previously described.

In vitro studies

Clonogenic assay

Cell survival following gamma radiation with or without juglone treatment was evaluated using clonogenic assay. Exponentially growing B16 melanoma cells were treated with 5 µM juglone for 1 h followed by different doses of γ-radiation (1, 3, 6 and 8 Gy). Cells were then trypsinized and appropriate numbers of viable cells were plated in 60 mm² petri-dishes (in triplicate) and were left undisturbed for 10 – 12 days. Thereafter, colonies
containing at least 50 cells were counted as clonogenic survivors. All colony counts were corrected for plating efficiency (PE) to yield survival values of 100% for untreated controls and the surviving fractions were calculated and plotted using linear quadratic model as mentioned previously. Sensitizing efficiency of juglone was then expressed in terms of SF2 value (defined as the survival fraction of exponentially growing cells at 2 Gy radiation dose) and the sensitizer enhancement ratio (SER) (defined as the ratio of D0 untreated cells/D0 treated cells, where D0 value represents the radiation dose that leads to 37% survival). In order to evaluate the nature of the interaction between juglone and gamma irradiation, the combination index (CI) was calculated according to the method of Chou and Talalay as described by elsewhere using the following formula

\[
\text{Combination index (CI)} = \frac{(D_1)}{(D_{x1})} \times \frac{(D_2)}{(D_{x2})}
\]

where \((D_{x1})\) and \((D_{x2})\) in the denominators are the doses (or concentrations) of juglone and radiation alone that give x% inhibition, whereas \((D_1)\) and \((D_2)\) in the nominators are the doses of juglone and radiation in combination that also inhibit x% (iso-effect). CI <1, CI = 1 and CI >1 indicates synergistic, additive and antagonistic effect respectively.

**DNA damage and repair studies using comet assay**

B16F1 melanoma cells were incubated with or without juglone (5 µM) for 1 h before irradiation (6 Gy) and during 3 and 6 hour post-treatment repair incubations. At the end of incubation times, DNA strand breaks were quantified using the alkaline comet assay as described earlier.

**Analysis of the intracellular Reactive oxygen species (ROS) levels**

Effect of various treatments on the intracellular ROS levels was studied using a fluorescent probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA). Briefly, cells were seeded in 6-well plate at a density of 2 x 10⁵/well. Twenty four hours later, cells were incubated with 10 µM concentrations of DCFH-DA for 30 min at room temperature in the dark. The unincorporated fluorescence probe was removed from the cells by two washes with PBS. The DCFH-DA loaded cells were then treated with juglone alone (5 µM) or radiation alone (6 Gy) or in combination. At the end of various treatments, the cells were trypsinized, washed, re-suspended in appropriate volume of PBS and transferred to 96-well black plates. The fluorescence intensity of the cells was then measured using a microplate fluorescence reader (Infinite® M200, TECAN) at excitation and emission wavelengths of 488 nm and 525 nm, respectively.

**Statistical analysis**

The experimental data were expressed as Mean ± SEM (standard error of the mean). The plating efficiency of cells was determined and the data points of surviving fraction was fitted on to a linear quadratic model, SF (surviving fraction) = exp[-(αD + βD²)]. Survival studies were performed by Kaplan–Meier survival analysis using GraphPad Prism version 3.00 (California, USA) and the median survival time was reported. For all other studies, One way ANOVA followed by Bonferroni’s post-hoc test was used to compare the significance between various treatments.

**Results**

**In vivo anticancer potential of juglone**

**Acute toxicity studies in mice**

Acute toxicity studies were carried out in C57 mice by injecting different doses of juglone intravenously (Figure 2).

**Figure 2: Acute toxicity studies for intravenously administered juglone using Probit method of analysis**

We observed a dose dependent increase in the animal mortality with animal death occurring within 48 h of drug administration. Animals injected with 0.5 and 1 mg/kg b. wt. juglone did not show any signs of toxicity and no mortalities were seen during the 14 days of observation period. However, in animals injected with 3 mg/kg b. wt. juglone, we observed 20% mortality along with toxic symptoms like reduced activity, anorexia etc. Administration of 6 and 10 mg/kg b. wt. juglone resulted in 60 and 100% mortality respectively. The LD₅₀ value
for juglone was calculated using the probit method and found to be 4.2 mg/kg b. wt. when administered intravenously.

**Optimum dose selection for anticancer activity**

Tumor bearing animals were injected with single sub-lethal doses of juglone viz. 0.5, 1 and 1.5 mg/kg b. wt. and the tumor growth kinetics were monitored and recorded as shown in Figure 3 and Table 1. The tumor size in the vehicle treated mice increased steeply (approximately 66-fold) and reached an average size of 6600 mm$^3$ within 14 days. On the other hand, juglone treatment resulted in inhibition of tumor growth in a dose dependent fashion. While the tumor size in mice treated with 0.5 mg/kg b. wt. juglone reduced only marginally, the tumor size in mice treated with 1 and 1.5 mg/kg b. wt. reduced significantly ($p<0.001$) on day 14 compared to vehicle treated controls (Figure 3, Table 1). However, there was no significant reduction in the tumor volume when the dose of juglone was increased from 1 to 1.5 mg/kg b. wt. Animal survival analysis revealed that the treatment of tumor bearing mice with 0.5, 1 and 1.5 mg/kg b. wt. juglone resulted in a median survival of 22, 26 and 27 days respectively compared to 19 days in vehicle treated group.

Based on these studies, 1 mg/kg b. wt. juglone was considered optimum dose for further studies. Studies were also carried out to determine the effect of repeated dose juglone treatment on the tumor growth kinetics and the results are shown in Figure 3 (inset). Treatment of mice with repeated doses of juglone on day 1, 3 and 5 resulted in a significant ($p<0.01$) improvement in antitumor activity compared to single dose administration.

**Table 1: Tumor growth kinetics and survival studies for optimum dose selection studies of juglone in vivo.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VDT ± SE (days)</th>
<th>5 X ± SE (days)</th>
<th>GD ± SE (days)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>1.57 ± 0.17</td>
<td>3.55 ± 0.24</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Juglone 0.5 mg/kg b. wt. (single dose)</td>
<td>1.67 ± 0.06</td>
<td>4.14 ± 0.13</td>
<td>0.59 ± 0.11</td>
<td>22</td>
</tr>
<tr>
<td>Juglone 1 mg/kg b. wt. (single dose)</td>
<td>1.83 ± 0.08</td>
<td>5.8 ± 0.27</td>
<td>2.29 ± 0.20</td>
<td>26</td>
</tr>
<tr>
<td>Juglone 1.5 mg/kg b. wt. (single dose)</td>
<td>1.96 ± 0.18</td>
<td>6.22 ± 0.31*</td>
<td>2.67 ± 0.29*</td>
<td>27</td>
</tr>
<tr>
<td>Juglone (1 mg/kg b. wt.) (three doses)</td>
<td>2.86 ± 0.22</td>
<td>5.84 ± 0.20*</td>
<td>2.55 ± 0.26</td>
<td>28</td>
</tr>
</tbody>
</table>

Significance levels: *$p<0.001$, compared to vehicle treated controls.

**Assessment of DNA damage in vivo**

The effect of juglone treatment on the DNA damage levels in the tumors of mice was studied (Figure 4A). At different intervals of time after treatment, the tumors were excised and processed immediately for comet assay. We observed a significant elevation in the OTM levels ($p<0.01$) in the tumor tissue one day after juglone treatment compared to corresponding vehicle treated controls. On day 7 post treatment, the damage largely got repaired with OTM values remaining only slightly higher than its vehicle treated counterpart. However, on day 15 following juglone treatment, the OTM levels reached the baseline levels.

The same sets of animals were used to study the differential genotoxic effect by measuring the DNA damage levels (OTM) in the peripheral blood (as an index of DNA damage in lymphocytes). The results of this study are depicted in Figure 4B. On day 1 post-treatment, we observed a significant increase ($p<0.05$) in the OTM levels of animals treated with juglone compared to the respective controls. However, on day 7 and thereafter, these levels got back to normal levels seen in the control animals.
Radiosensitizing potential of juglone in vivo

The optimum dose of juglone (1 mg/kg b. wt.) from the previous study was then combined with radiation (10 and 30 Gy) to evaluate its radiosensitizing potential in vivo. Tumor response was measured in terms of volume doubling time and growth delay as mentioned in the materials and methods section. The results of this study are depicted in Figure 5 and table 2. We observed a significant increase in the volume doubling time as well as tumor growth delay with combination therapy (juglone plus radiation) in comparison to radiation alone group at both 10 Gy (p<0.05) and 30 Gy (p<0.001) radiation doses. Analysis of the survival patterns (table 2) revealed that the treatment of tumor bearing mice with 10 and 30 Gy radiation resulted in median survival values of 31 and 40 days respectively. In addition, pretreatment of tumor bearing mice with juglone before local tumor irradiation with 10 and 30 Gy caused further increase in the median survival to 34 and 46 days respectively.

Figure 4: Effect of juglone (1 mg/kg b. wt.) treatment on DNA damage levels A) in tumor cells of mice treated with optimum dose of juglone B) in blood cells of mice treated with optimum dose of juglone. Significant levels - * - p<0.05; ** - p<0.01 in comparison to respective vehicle treated control cells.

Figure 5: Radiosensitizing potential of juglone in vivo against B16F1 melanoma A) Radiation dose of 10 Gy B) radiation dose of 30 Gy
Table 2: Tumor growth kinetics and survival studies for radiosensitizing potential of juglone in vivo. VDT – Volume doubling time (Time required for the tumors to reach double the initial volume); 5X – Time required for the tumors to reach 5 times the initial volume; GD – Growth delay (The difference in time between the treated and untreated tumors to reach 5 times the initial volume)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VDT ± SE (days)</th>
<th>5 X ± SE (days)</th>
<th>GD ± SE (days)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>1.43 ± 0.15</td>
<td>3.64 ± 0.16</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Juglone (1 mg/kg b. wt.)</td>
<td>1.83 ± 0.08</td>
<td>5.8 ± 0.27</td>
<td>2.55 ± 0.26</td>
<td>26</td>
</tr>
<tr>
<td>Radiation alone (10 Gy)</td>
<td>2.34 ± 0.17</td>
<td>6.58 ± 0.36</td>
<td>2.93 ± 0.26</td>
<td>31</td>
</tr>
<tr>
<td>Radiation alone (30 Gy)</td>
<td>3.82 ± 0.64</td>
<td>12.31 ± 1.02</td>
<td>8.66 ± 1.02</td>
<td>40</td>
</tr>
<tr>
<td>Radiation (10 Gy) + juglone</td>
<td>4.11 ± 0.15*</td>
<td>8.9 ± 0.32*</td>
<td>5.26 ± 0.32*</td>
<td>34</td>
</tr>
<tr>
<td>Radiation (30 Gy) + juglone</td>
<td>9.50 ± 0.66**</td>
<td>17.03 ± 0.55**</td>
<td>13.38 ± 0.55**</td>
<td>46</td>
</tr>
</tbody>
</table>

Significance levels – * = p<0.05; ** = p<0.001, compared to respective radiation alone groups

Radiosensitizing potential of juglone in vitro

Juglone increases radiation sensitivity of melanoma cells

The effect of juglone on the radiation survival curves was studied using colony-forming assay. Cells were pre-treated with 5 µM juglone (selected based on our previous studies) for 1 h before treating with different doses of gamma radiation (1 - 8 Gy).

![Radiosensitizing potential of juglone in vitro against B16F1 melanoma cells assessed using clonogenic assay.](image)

As shown in Figure 6, treatment of melanoma cells with juglone prior to exposure to different doses of radiation resulted in a clear dose dependent decrease in radiation induced cell survival (SF₂ = 0.303; D₀ value = 1.16 Gy) compared to radiation alone group (SF₂ = 0.545; D₀ value = 1.59 Gy) suggesting the potential of juglone to increase the radiosensitivity of melanoma cells. The SER calculated based on the D₀ value was found to be 1.37.

Juglone enhances radiation induced DNA damage

The effect of juglone on the radiation induced DNA damage in melanoma cells was determined using alkaline comet assay. Melanoma cells treated with 5 µM juglone alone or radiation (6 Gy) alone showed a 5.14 - and 2.19 fold increase in the OTM (indicative of DNA damage) respectively in comparison to untreated (Figure 7). Further, treatment of cells with juglone before irradiation showed significantly higher OTM levels compared to independent treatments (p<0.05 versus juglone alone; p<0.01 versus radiation alone) as well as the control (p<0.01).

Juglone slows down the repair of radiation induced DNA damage

In order to study if juglone has any effect on the radiation induced DNA damage repair, we performed the alkaline comet assay after incubating the cells with fresh medium for 0, 3 and 6 h after various treatments. The results of this study are depicted in Figure 7. From these results, it can be seen that the cells treated with radiation alone repaired almost completely within 3 h of incubation. Further, cells treated with juglone alone showed significant reduction in OTM levels (indicative of DNA repair) at 3 h compared to the respective 0 h interval. However, near-complete repair was only seen at 6 h incubation time. On the other hand, cells treated with a combination of juglone followed by radiation showed a remarkable slow down in the DNA damage repair kinetics with mean OTM values hovering around 21.5 % (4-fold higher than control values) even at 6 h incubation time.
Radiosensitization potential of juglone against melanoma cells in vitro assessed using alkaline comet assay. Significance levels: * - p<0.01 in comparison to respective radiation alone groups

Elevated levels of intracellular ROS by a combination of juglone and radiation

Recently we demonstrated the potential of juglone to induce ROS in melanoma cells. In order to investigate the relationship between ROS generation and the observed radiosensitizing effect of juglone, we employed a ROS-sensitive dye DCFH-DA to detect ROS production fluorimetrically.

From the results of this study (Figure 8), treatment of melanoma cells with either juglone (5 µM) or radiation (6 Gy) resulted in 3.2- and 2.9-fold increase in the levels of intracellular ROS respectively compared to untreated control. Further, a combination treatment of juglone followed by radiation resulted in 5.26-folds increase in ROS levels compared to untreated controls. The increase in the levels of intracellular ROS upon combination treatment was statistically significant (p<0.001) in comparison to individual treatments as well as untreated controls.

Discussion

Intrinsic chemo/radio-resistance as well as the presence of hypoxic cells makes effective treatment of most solid tumors a daunting task. In order to improve the therapeutic outcome in cancer, several attempts have been made to combine chemical agent/radiosensitizer with radiotherapy. However, clinical usefulness of most sensitizers remains ineffective and controversial. As a result, efforts are now being directed at combining the chemotherapeutic agents with radiation to improve the therapeutic outcome. In this regard, various chemotherapeutic agents such as cisplatin, gemcitabine, paclitaxel, docetaxel, irinotecan etc. have been combined with radiation with varying degrees of success. However, chemoradiotherapy with conventional chemotherapeutic agents is associated with toxicity which necessitates the search for novel anticancer compounds with radiosensitizing properties.

Owing to their ease of availability, cost effective nature and safer toxicity profiles, screening of plant and other natural products has gained considerable interest in modern drug discovery programs. Walnut has been used in traditional folk medicines for the treatment of cancer. In the present study, we investigated the in vivo anticancer and radiosensitizing potential of the main active constituent of walnut, juglone, against B16F1 melanoma cells. The acute toxicity studies carried out using the probit analysis revealed that juglone administered by intravenous route, had a LD$_{50}$ value of about 4.2 mg/kg b. wt. To our knowledge, there are no previous reports of any toxicity studies of juglone administered via intravenous route in mouse. However, Westfall et al., reported a LD$_{50}$ value of 2.5 mg/kg b. wt in mice when administered through the oral route. Registry of Toxic Effects of Chemical Substances (RTECS, National Library of Medicine) reported a LD$_{50}$ value of 25 mg/kg b. wt for juglone in mice administered via intraperitoneal route. In another report, administration of juglone in dogs at a dose of 5 mg/kg b. wt. intravenously was found to cause slight hemorrhages in the lungs with no direct effect on cardiovascular system.
Okada and co-workers\textsuperscript{36} studied the effect of juglone \textit{in vivo} against Ehrlich ascites tumor cells transplanted in Swiss mice and reported that juglone induced mitotic abnormalities like decreased mitotic figures with concomitant metaphase arrest, indicating its therapeutic potential. Similar observation was also made by Sugie et al\textsuperscript{37} wherein male F344 rats administered with juglone had lower incidence and multiplicity of azoxymethane induced intestinal tumors. On the contrary, Monks et al.\textsuperscript{38} reported that juglone promoted the 7, 12-dimethylbenz[a]anthracene (DMBA) initiated skin carcinomas in SENCAR mice when applied topically for 40 weeks. In another study\textsuperscript{39}, juglone treatment promoted the skin tumors in female ICR/Ha Swiss mice pretreated with DMBA. However, treatment of these mice with juglone alone (without DMBA pretreatment) did not induce any tumors in these mice. This shows that there is still quite a lot of ambiguity regarding the \textit{in vivo} anticancer potential of juglone, which formed the basis of the present investigation. In the present study, based on the acute toxicity data, sub-lethal doses of juglone were chosen for evaluation of its \textit{in vivo} anticancer activity. Treatment of tumor bearing mice with juglone (1 mg/kg b. wt.) resulted in significant inhibition (p < 0.001) in tumor growth parameters as evidenced by the 5X, growth delay as well as median survival values. Results from the present investigation indicate the potential of juglone as an anticancer agent, in agreement with earlier findings\textsuperscript{36-37}.

Reactive oxygen species (ROS) are generally very small, highly reactive molecules that can be generated as by-products of cellular metabolism. The overproduction of ROS can cause damage to various biomolecules, including lipids, DNA, proteins, ultimately leading to cell death. It is well documented that increased generation of reactive oxygen species (ROS) leading to an altered redox homeostasis is a common feature in most cancer forms.\textsuperscript{40} Taking that into consideration, it has been hypothesized that pro-oxidant compounds that induce ROS in the cells result in the dysregulation of already altered ROS levels in cancer cells leading to their loss of viability. In contrast, untransformed normal cells having a normal redox status are believed to be more resistant to the ROS insults. This diversity in the biochemical property of cancer and normal cells are now being exploited for their possible beneficial role in cancer therapy.\textsuperscript{41} To that effect, numerous studies have been carried out to evaluate the preferential sensitivity of cancer cells to small molecule pro-oxidants like quinone based electrophiles\textsuperscript{4}, redox cyclers\textsuperscript{42}, organic endoperoxides\textsuperscript{43}. Juglone, a small molecule pro-oxidant compound belonging to the quinone class, induces apoptotic and necrotic cell death in cancer cells essentially by oxidative stress mediated mechanisms\textsuperscript{19,21}. Based on the existing literature as well as from our previous studies, the observed anticancer potential of juglone \textit{in vivo} may be attributed at least in part to its ROS generating ability.

Reactive oxygen species are known to induce DNA damage by causing single strand breaks, double strand breaks etc. The alkaline version of the single cell gel electrophoresis (comet) assay is a versatile method to detect DNA single-strand breaks, double-strand breaks, or cross-links in virtually any cell type\textsuperscript{44}. In order to study if the observed tumor growth inhibition could be a result of DNA damage induced by juglone, we measured the DNA damage levels in the tumor tissue. Juglone treatment caused a significant (p < 0.01) elevation in the DNA damage levels of tumor tissue on day 1 post treatment. On day 7 post treatment, the OTM levels were only slightly higher than the control levels indicating repair of the damaged DNA. Thereafter, the OTM levels reached normal values. Tirapazamine, a well known chemotherapeutic agent was also shown to induce DNA damage in the tumor cells \textit{in vivo} and this DNA damage in the tumor tissue was reported to be closely associated with the tumor cell kill\textsuperscript{45}. From the present study, the DNA damage induced by juglone in the tumor tissue could have resulted in the tumor cell kill ultimately resulting in the observed delay in tumor growth parameters.

Conversely, many chemotherapeutic agents are also reported to induce DNA damage in blood lymphocytes at their therapeutic doses\textsuperscript{46}. Therefore, to evaluate the effect of juglone on the DNA damage levels in the blood cells, alkaline comet assay was performed using the blood cells of the animals treated with optimum dose of juglone. Results indicate that on day 1 post treatment, juglone induced significant (p < 0.05) increase in the OTM levels which however reversed back to normal levels on day 7 and thereon. The present results suggest that juglone also causes moderate levels of initial normal tissue toxicity which however may be mitigated by formulating juglone using various drug targeting strategies as was demonstrated for plumbagin in our earlier report\textsuperscript{47}.

As discussed earlier, bimodality approach involving a combination of chemotherapeutic agent along with radiation may help in improving the therapeutic outcome in cancer. Many of the potent phytochemicals like plumbagin, withaferin A etc have been shown to augment the radiation induced cell killing against various cell lines \textit{in vitro}\textsuperscript{12,48}. Further, our earlier studies have also demonstrated the radiosensitizing potential of these plant
based compounds against various cancer models in vivo. To our knowledge, there are no earlier reports about the potential of juglone to modify the radiation induced cell killing. In the present study, exposure of melanoma cells with juglone prior to treatment with different doses of γ-radiation resulted in a significant reduction in the surviving fraction of melanoma cells in comparison to radiation alone group (Figure 6). A sensitizer enhancement ratio of 1.37 was observed which may be considered significant levels of sensitization based on the earlier reports where SER value of > 1.1 were considered significant. Also, the analysis of the clonogenic assay data using the method of Chou and co-workers gave a mean combination index (CI) value of 0.99 suggesting that the interaction between juglone with gamma radiation was more of an additive nature and not synergistic. Several other clinically approved anticancer agents are also known to elicit an additive response in combination with radiation. These findings further corroborate the results of our in vivo studies where pretreatment of mice with juglone before local tumor irradiation, resulted in significant delay in the tumor growth parameters as compared to independent treatments (Table 2 and Figure 5). These results clearly indicate the ability of juglone to enhance the radiosensitivity of melanoma cells both in vitro as well as in vivo.

Effective repair of the damaged DNA is a critical event that determines the radiosensitivity of cancer cells and inhibition of DNA damage repair is proposed to be one of the key mechanisms in radiosensitization. Beta-lapachone, a plant based quinone with established anticancer properties, was shown to enhance the radiosensitivity of cancer cells by inhibition of DNA repair mechanisms. In the present investigation, we studied the effect of juglone on the radiation induced DNA damage and repair. At zero time (immediately after various treatments), we observed significantly elevated OTM levels in the combination treatment compared to the individual treatments. Results from the repair studies show significantly higher levels of OTM in combination treatment compared to the independent treatments even at 6 h incubation. However, at the 24 h incubation time point, the DNA damage levels in all the treatment groups reached baseline levels (data not shown). These results clearly indicate the potential of juglone to slow down the repair of radiation induced DNA damage in melanoma cells which may have resulted in the observed radiosensitizing effect of juglone.

It is a known fact that under aerobic conditions, radiation causes generation and accumulation of ROS such as superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radical and peroxyl radical which could damage DNA as well as hinder its repair process. The intracellular antioxidant defense mechanisms normally help to restore the elevated oxidative status in the cells. However, the presence of an agent that could deplete the cellular antioxidant defenses before subjecting to radiation could enhance the cell kill in cancer. We earlier reported the potential of juglone to deplete the intracellular GSH and induce oxidative stress in melanoma cells. From the present study, combined treatment of melanoma cells with juglone followed by radiation resulted in significant increase in the intracellular ROS levels compared to the control as well as individual treatments which may have resulted in the observed radiosensitization effect by enhancing DNA damage and slowing down its repair.

The toxicity of quinones is generally attributed to their ability to undergo redox cycling with consequent production of semiquinone radicals and ROS leading to oxidative stress mediated cell death. Although the exact mechanism of radiosensitization potential of juglone is not clearly understood, the observed additive nature of its radiosensitizing potential against melanoma cells may be attributed to the ability of juglone to induce reactive oxygen species mediated DNA damage (comet assay) leading to cell death (as evidenced by clonogenic assay). Also, the presence of hydroxyl group on the position 5 of the naphthoquinone ring may have contributed to the observed ROS levels which resulted in the radiosensitizing effect of juglone. Similar findings were also reported in earlier studies where juglone with hydroxyl group in 5th position was reported to considerably increase the toxicity against rat hepatocytes. However, the involvement of other mechanistic pathways in the radiosensitizing potential of juglone needs further investigation.

In conclusion, the present study has shown the potential of juglone to inhibit the growth of melanoma cells in vivo. Also this study has for the first time shown the potential of juglone to enhance the radiation induced cell kill in melanoma cells both in vitro and in vivo. In view of these findings, and the existing earlier reports of its use in traditional folk medicine, juglone deserves further studies on its toxicity aspects using a spectrum of preclinical models.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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