Chapter 4

Tumor growth inhibitory effect of juglone and its radiation sensitizing potential: *in vivo* and *in vitro* studies

Integrative Cancer Therapies (Accepted for publication)
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.

Initially acute toxicity studies were carried out to determine the LD$_{50(14)}$ value of juglone through intravenous route and was found to be around 4.18 mg/kg b. wt. Based on these studies, selection of optimum anticancer dose of juglone was performed by treating tumor bearing mice with sub-lethal doses of juglone, where a dose-dependent inhibition of tumor growth was observed (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 nucleated 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, a sensitization enhancement ratio of 1.37 was observed in the case of 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.
4.1. INTRODUCTION

The biological effects of radiation affect both neoplastic and normal tissues. The nature and extent of such effects, however, depend on select biological parameters including intrinsic resistance, oxygen supply, cell cycle etc. (Vaupel et al., 1989; Vaupel, 2004; Wilson, 2004) and can be modified by chemical agents such as radiosensitizers, radioprotectors and chemotherapeutic agents (Wilson et al., 2006). A precise control of the mode of action of the radiation is important in order to achieve the maximum effect on tumor tissue, while minimizing the effect on normal tissue. The concept of an optimal irradiation dose providing a maximally positive response of the target tissue and minimal toxicity to the perilesional area is the basis of various fractionation schedules for the radiotherapeutic treatment of tumors. In spite of vast technological advancement in this area, 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 radiotherapy with other treatment modalities may be required to attain better therapeutic outcome (Hocker et al., 2008). 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 like mitomycin C, gemcitabine, 5-fluorouracil, paclitaxel and carboplatin have been combined with radiation with varying degrees of success and many more are still under clinical evaluation (Caffo, 2001; Lawrence et al., 2003; Rich et al., 2004; Horsman et al., 2006; Wardman, 2007). Nevertheless, the side effects of such modes of treatment are severe and have resulted in the occurrence of secondary malignancies (Herskovic et al., 1992; Kirwan et al., 2003; Sijben et al., 2008; Udagawa, 2009; Busia et al., 2010) and therefore research efforts to explore compounds with better chemotherapeutic and/or radiosensitizing potential shall be continued to reduce toxic side effects of combination treatment and thereby improving the outcome of the therapy.

Herbs offer a vast source of new chemicals and some of them are structurally so complex that they cannot be synthesized even with the advanced technology at our disposal (Balunas and Kinghorn, 2005; Koehn and Carter, 2005; Gullo et al., 2006).
The herbal drugs have gained attention and popularity because of their negligible toxicity and possibly with a ray of hope that they may replace some of the available antineoplastic drugs that are highly toxic. Quinones represent one such class of pharmacologically active compounds from which several of the clinically used anticancer drugs (including mitomycin C, doxorubicin, mitoxantrone, siantopin etc) have been derived. Besides, several other plant derived quinones (including plumbagin, beta-lapachone, menadione etc) have also shown promising anticancer against various cancer models both \textit{in vitro} as well as \textit{in vivo} (Nutter \textit{et al.}, 1991; Devi \textit{et al.}, 1999; Pardee \textit{et al.}, 2002). In addition, they are also known to possess potent radiosensitizing potentials against various models systems (Taper \textit{et al.}, 1996; Devi \textit{et al.}, 1999; Nair \textit{et al.}, 2008).

Walnut (\textit{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 (Duke and Ayensu, 1985; Blumenthal, 1998; Bhatia \textit{et al.}, 2006). Herbal preparations of walnut are known to suppress the growth of spontaneous mammary adenocarcinoma in Swiss albino mice (Bhargava and Westfall, 1968). Juglone (5-hydroxy-1,4 naphthoquinone), a structural analogue of plumbagin and the main active constituent of walnut, is also reported to possess potent cytotoxic properties \textit{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) (Segura-Aguilar \textit{et al.}, 1992; Cenas \textit{et al.}, 2006; Ji \textit{et al.}, 2011). In the earlier studies (discussed in chapter 3) the effect of juglone on B16F1 melanoma cells was evaluated \textit{in vitro}, where the cytotoxic potential of juglone was found to be mediated by multifactorial mechanisms involving oxidative stress, cell membrane damage and genotoxic effect ultimately leading to cell death by both apoptosis and necrosis. In the present study, an attempt was made to evaluate the \textit{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.

### 4.2. MATERIALS AND METHODS

#### 4.2.1. 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 sulphoxide (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).

#### 4.2.2. Cell lines and cultures

B16F1 melanoma cells was used throughout this study and were routinely grown in 25 cm² flasks as mentioned earlier (Chapter 3, section 3.2.2).

#### 4.2.3. 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 *in vitro* studies was chosen based on the previous studies (Chapter 3).

For *in vivo* studies, required amounts of juglone was accurately weighed and dissolved in minimum amount of alcohol and made up to required concentrations with 20% PEG in saline (vehicle). Unlike in the *in vitro* studies, drug solutions for *in vivo* studies were freshly prepared each time just prior to the drug administration.
4.2.4. 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 \(5 \times 10^5\) viable cells.

4.2.5. Irradiation

All the radiation studies were carried out using a \(^{60}\text{Co}\) gamma teletherapy facility (Theratron Atomic Energy Agency, Canada) at the Shirdi Sai Baba Cancer Hospital (Manipal, India). The radiation doses used in the present investigation were based on the initial pilot 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 juglone treatment were anaesthetized by injecting a mixture of 50 mg/kg b. wt. of Ketamine and 8 mg/kg b. wt. of Diazepam (intraperitoneally), 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 4.1).
4.2.6. Assessment of *in vivo* anticancer potential of juglone

4.2.6.1. Acute toxicity studies of juglone

The acute toxicity study was carried out to determine the LD$_{50(14)}$ dose of juglone in mice following intravenous administration and was performed as described previously (Ghosh, 1984). The fasted animals (18 h prior to juglone treatment) of either sex were divided into several groups of 10 each as follows.

Group 1: Animals in this group were injected with 100 µl of vehicle; Group 2 - 5: Animals in these groups were injected with single intravenous dose of 0.5, 1, 3, 6 and 10 mg/kg body weight (b. wt.) of juglone (prepared freshly as mentioned previously).
Immediately after the treatment, animals were provided with food and water, and the animals were monitored daily up to 14 days for mortality. These animals were also monitored for gross morphological and behavioral changes including, changes in locomotor activity, piloerection, sedation, aggressiveness as well as normal behavioral repertoire (grooming/licking/biting). LD_{50(14)} was then calculated using probit analysis as described earlier (Litchfield and Wilcoxon, 1949).

4.2.6.2. 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 (Devi et al., 2000) 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 $100 \pm 10 \text{ mm}^3$, animals were divided into following groups of 10 each:

Group 1: Tumor bearing animals in this group received 100 µl of vehicle (vehicle treated control group); Group 2 - 4: Animals in this group were injected intravenously with different doses of juglone viz 0.5, 1 and 1.5 mg/kg b. wt. 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 $100 \pm 10 \text{ mm}^3$, the tumor bearing animals were divided into following groups of 10 animals each as

Group 1 - Animals in this group received 100 µl of vehicle (vehicle treated control group); Group 2 - 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.
4.2.6.3. 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 post-treatment days. The tumor response after various treatments was then 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 (Matthews and Farewell, 1996). 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.

4.2.6.4. Alkaline comet assay

Tumors were induced in the mice by injecting $5 \times 10^5$ viable cells intradermally. Once the tumor size reached $100 \pm 10 \, \text{mm}^3$, the animals were divided into following groups of 12 each.

Group 1: Animals in this group received 100 µl of vehicle; Group 2: Animals in this group were injected intravenously with optimum anticancer dose of juglone

A subgroup of four animals from the above treatment groups were euthanized on 1, 7 and 15 post treatment days. At the indicated time intervals, the blood samples were collected in heparinized tubes by retro-orbital plexus. The tumors from these animals were also excised within a couple of minutes and immediately placed in ice-cold PBS and under reduced light to avoid any further DNA damage. Single cell suspensions were prepared by mincing the entire tumor tissue in ice-cold PBS with scissors until no pieces of ‘solid’ tissue were visible. This suspension was then filtered through a nylon mesh (30 µm) and the filtrate was washed three times with PBS (Olive et al., 2000). The resulting single cells were counted and the viability was assessed by trypan blue dye exclusion method (typically the viability was around
80%). The differential genotoxic efficacy of juglone among the nucleated blood cells and tumor samples were then studied using the alkaline version of single cell gel electrophoresis (comet) assay according to the method of Singh and co-workers (1988) as described in earlier chapter (Chapter 3, section 3.2.4.2).

4.2.7. Assessment of radiosensitizing potential of juglone

4.2.7.1. In vivo studies

Tumors were induced in the mice by injecting $5 \times 10^5$ viable cells intradermally. Once the tumor size reached $100 \pm 10 \text{ mm}^3$, the animals were divided into following groups of 10 each

Group 1: Animals in this group received 100 µl of vehicle; Group 2-3: Animals in this group received local tumor irradiation with different doses of $\gamma$-radiation (10 Gy and 30 Gy); Group 4-5: Animals in this group received optimum dose of juglone followed by local tumor irradiation with 10 Gy and 30 Gy dose of $\gamma$-radiation respectively.

The tumor growth parameters after various treatments were then studied as previously described (section 4.2.6.3).

4.2.7.2. In vitro studies

4.2.7.2.1. Clonogenic assay

Cell survival following gamma radiation with or without juglone treatment was evaluated using clonogenic assay (Puck and Marcus, 1955). Exponentially growing B16F1 melanoma cells with or without juglone treatment (5 µM for 1 h) were exposed to different doses of $\gamma$-radiation (1, 3, 6 and 8 Gy). Cells were then trypsinized and appropriate numbers of viable cells were plated in 60 mm$^2$ 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 (Chapter 3, section 3.2.3.3). 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 (Chou and Talalay, 1984) as described by elsewhere (Pauwels et al., 2003) using the following formula:

\[
\text{Combination index (CI)} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}
\]

where \((Dx)_1\) and \((Dx)_2\) 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 inhibits x% (iso-effect). CI < 1, CI = 1 and CI > 1 indicates synergistic, additive and antagonistic effect respectively.

4.2.7.2. 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, 6 and 24 h post-treatment repair incubations. At the end of incubation times, DNA strand breaks were quantified using the alkaline comet assay (Singh et al., 1988) as described earlier (Chapter 3, section 3.2.4.2).

4.2.7.2.3. 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 in a CO₂ incubator (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.

4.2.8. Statistical analysis

The survival studies were performed by Kaplan–Meier survival analysis using GraphPad Prism® version 3.00 (California, USA) and the median survival time was reported. In order to compare the significance between various treatments, student t-test (to compare two groups) as well as One way ANOVA followed by Bonferroni’s post-hoc test (where more than two groups were to be compared) was used to.

4.3. RESULTS

4.3.1. In vivo anticancer potential of juglone

4.3.1.1. Acute toxicity studies in mice

Acute toxicity studies were carried out in C57BL/6J mice by injecting different doses of juglone intravenously (Figure 4.2).

![Figure 4.2. Acute toxicity studies for intravenously administered juglone using Probit method of analysis](image)
Treatment with juglone resulted in 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, 20 % mortality was observed accompanied by 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 LD$_{50(14)}$ value for juglone was calculated using the probit method and was found to be 4.18 mg/kg b. wt. when administered intravenously.

4.3.1.2. 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 4.3A and table 4.1.

**Figure 4.3.** A) In vivo optimum dose selection studies for juglone administered as single dose via intravenous route of administration; B) Comparison between single dose (1 mg/kg b. wt.) and repeated dose juglone (1 mg/kg b. wt. on 1, 3 and 5 days) treatment administered intravenously.
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 4.3A, table 4.1). 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. These results were further substantiated from the dose dependent increase in the VDT, 5X as well as GD values (Table 4.1)

**Table 4.1.** Tumor growth kinetics and survival studies for optimum dose selection studies 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 ± SD (days)</th>
<th>5 X ± SD (days)</th>
<th>GD ± SD (days)</th>
<th>Median survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>1.57 ± 0.53</td>
<td>3.55 ± 0.76</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Juglone 0.5 mg/kg b. wt. (single dose on day 1)</td>
<td>1.67 ± 0.19</td>
<td>4.14 ± 0.41</td>
<td>0.59 ± 0.35</td>
<td>22</td>
</tr>
<tr>
<td>Juglone 1 mg/kg b. wt. (single dose on day 1)</td>
<td>1.83 ± 0.25</td>
<td>5.8 ± 0.85*</td>
<td>2.29 ± 0.63</td>
<td>26</td>
</tr>
<tr>
<td>Juglone 1.5 mg/kg b. wt. (single dose on day 1)</td>
<td>1.96 ± 0.57</td>
<td>6.22 ± 0.98*</td>
<td>2.67 ± 0.92</td>
<td>27</td>
</tr>
<tr>
<td>Juglone (1 mg/kg b. wt.) (three doses on 1, 3 and 5 days)</td>
<td>2.86 ± 0.69*</td>
<td>5.84 ± 0.63*</td>
<td>2.55 ± 0.82</td>
<td>28</td>
</tr>
</tbody>
</table>

Significance levels – * = $P<0.001$, compared to vehicle treated controls.
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 (figure 4.4A). 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 4.3B. Treatment of mice with repeated doses of 1 mg/kg b. wt. juglone on day 1, 3 and 5 resulted in an improved antitumor activity compared to single dose administration with consequent improvement in animal survival evidenced by the Kaplan Meier analysis (Figure 4.4B) of survival where repeated dose treatment resulted in further increase in survival from 26 days (single dose) to 28 days (repeated dose administration).

Figure 4.4. Effect of juglone treatment on the survival pattern of C57BL/6J mice bearing B16F1 tumor model A) single dose treatment; B) multiple dose treatment of 1 mg/kg b. wt., on 1, 3 and 5 days

4.3.1.3. Assessment of DNA damage in vivo

The effect of juglone treatment on the DNA damage levels in the tumors were studied (Figure 4.5A). At different intervals of time after treatment, the tumors were
excised and processed immediately for comet assay. A significant elevation in the OTM levels ($P<0.01$) in the tumor tissue was observed 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 4.5B. On day 1 post-treatment, there was 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.

![Figure 4.5](image)

**Figure 4.5.** 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 nucleated 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.
4.3.2. 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 4.6. and table 4.2. A significant increase in the volume doubling time as well as tumor growth delay was observed 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.

**Figure 4.6.** Radiosensitizing potential of juglone *in vivo* against B16F1 melanoma cells A) Radiation dose of 10 Gy B) radiation dose of 30Gy

Analysis of the survival patterns (Figure 4.7 and table 4.2) 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.
Table 4.2: Tumor growth kinetics and survival studies for radiosensitizing potential 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>1.43 ± 0.47</td>
<td>3.64 ± 0.50</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Juglone (1 mg/kg b. wt.)</td>
<td>1.83 ± 0.25</td>
<td>5.8 ± 0.85</td>
<td>2.55 ± 0.82</td>
<td>26</td>
</tr>
<tr>
<td>Radiation alone (10 Gy)</td>
<td>2.34 ± 0.54</td>
<td>6.58 ± 1.13</td>
<td>2.93 ± 1.13</td>
<td>31</td>
</tr>
<tr>
<td>Radiation alone (30 Gy)</td>
<td>3.82 ± 1.13</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 ± 0.77*</td>
<td>8.9 ± 1.01*</td>
<td>5.26 ± 1.01*</td>
<td>34</td>
</tr>
<tr>
<td>Radiation (30 Gy) + juglone</td>
<td>9.50 ± 1.96**</td>
<td>17.03 ± 1.74**</td>
<td>13.38 ± 1.74**</td>
<td>46</td>
</tr>
</tbody>
</table>

Significance * = \( P < 0.05 \); ** = \( P < 0.001 \), compared to respective radiation alone group

Figure 4.7. Radiosensitization potential of juglone treatment – Kaplan-Meier analysis of survival in C57BL/6J mice bearing B16F1 tumor model A) 10 Gy radiosensitization; B) 30 Gy radiosensitization.
4.3.3. Radiosensitizing potential of juglone *in vitro*

4.3.3.1. Juglone increases radiation sensitivity of melanoma cells

The effect of juglone on the radiation survival curves was studied using colony-forming assay (Figure 4.8). Cells were pre-treated with 5 µM juglone (selected based on the studies described in chapter 3) for 1 h before treating with different doses of gamma radiation (1 - 8 Gy). As shown in Figure 4.8, 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 ($SF_2 = 0.303$, $D_0$ value = 1.16 Gy) compared to radiation alone group ($SF_2 = 0.545$; $D_0$ value = 1.59 Gy) suggesting the potential of juglone to increase the radiosensitivity of melanoma cells. The SER calculated based on the $D_0$ value was found to be 1.37.

![Graph showing radiosensitizing potential of juglone](image)

**Figure 4.8.** Radiosensitizing potential of juglone *in vitro* against B16F1 melanoma cells assessed using clonogenic assay.
4.3.3.2. 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 (Figure 4.9). 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) levels respectively in comparison to untreated. Further, treatment of cells with juglone before irradiation showed significantly higher OTM levels (7.89-fold) compared to independent treatments ($P<0.05$ versus juglone alone; $P<0.01$ versus radiation alone) as well as the control ($P<0.001$). This increased OTM levels in the cells treated with the combination treatment was greater than the additive effect of individual treatment.

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 and repair, the alkaline comet assay was performed after incubating the cells with fresh medium for 0, 3, 6 and 24 h after various treatments (Figure 4.9).

![Figure 4.9](image)

Figure 4.9. 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
From these results, it can be seen that the cells treated with radiation alone repaired almost completely within 3 h of incubations. However, although the repair proficiency of the cells treated with juglone alone was slightly reduced as evidenced by the incomplete repair of DNA damage at 3 h incubation, at 6 h incubation period, the DNA damage levels reached the baseline value. 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 around 21.5 (4-fold higher than control values) even at 6 h incubation time. However, at 24 h incubation time point, the OTM values reached to baseline levels in all the treatment groups.

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

In order to investigate the relationship between ROS generation and the observed radiosensitizing effect of juglone, ROS-sensitive dye DCFH-DA was employed to detect ROS production fluorimetrically (Figure 4.10). From the results of this study, 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.

![Graph showing ROS levels](image)

**Figure 4.10.** Effect of juglone, radiation and combination treatment on the intracellular ROS levels in melanoma cells assessed using DCFH-DA assay. Significant levels *-P<0.001 compared to controls and the independent treatments.
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.

4.4. DISCUSSION

A long-standing paradigm in radiation biology has been that many effects induced by ionizing radiation, including the ability to kill cancer cells as well as its carcinogenic effects, are the result of DNA damage arising from the actions of radiation in cell nuclei, especially interactions of ionizing radiation and its products with nuclear DNA (Iliakis, 1991; Goodhead, 1994). Consistent with this view, ionizing radiation is known to damage DNA by directly ionizing DNA itself (as in the case of high LET radiations) and by indirect processes (low LET radiations) in which DNA reacts with numerous radiolytic reactive products, e.g., $^\cdot$OH, $^\cdot$H, $^\cdot$O$_2$¯, and $^\cdot$H$_2$O$_2$, that are generated in aqueous fluid surrounding DNA. In the past, many attempts have been made to increase the effect of ionizing radiation on the tumor cells by combining hypoxic cell sensitizers with radiation. Although often effective in experimental models, the results obtained when these combinations are applied in a clinical setting have generally not been encouraging. Since these sensitizers did not meet expectations, another approach has been to combine radiotherapy with chemotherapeutic drugs routinely used in clinics.

To that effect, several chemotherapeutic agents (including cisplatin, carboplatin, gemcitabine, paclitaxel, docetaxel, topotecan etc.) have been combined with radiation and have been proved beneficial in solid neoplastic disorders in randomized clinical trials (Schaake-Koning et al., 1994; Blackstock et al., 1999; Sharma and Wilson, 1999; Dunton et al., 2002; Chen et al., 2003; Groen et al., 2004; Perrotti et al., 2008). However, chemoradiotherapy using conventional chemotherapeutic agents is often associated with significant toxicity (Bischoff et al., 2009) and therefore necessitates the search for novel anticancer compounds with radiosensitizing properties.
Plants, by virtue of their wide usage in the traditional medicine and less toxic implications have been drawing the attention of researchers around the world in the recent past (Brower, 2008). Walnut has been used in traditional folk medicines for the treatment of cancer. In the present study, an attempt was made to investigate the \textit{in vivo} anticancer and radiosensitizing potential of the main active constituent of walnut, juglone, against B16F1 melanoma cells.

Acute toxicity studies are preliminary drug development studies that provide basic safety information about an investigational chemical product early in the development process. The data generated are generally used to determine the maximal tolerated dose and appropriate doses for future non-clinical and clinical studies. The small number of animals used reduces the number of animals subjected to potential overt toxicities while still obtaining the necessary information. From the present study, 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 C57/BL6J mouse. However, Westfall and co-workers (1961) 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 (Boelkins \textit{et al.}, 1968). From the present study, the animals injected intravenously with 3 mg/kg b. wt. juglone exhibited toxic symptoms including weight loss, reduced activity etc. However no toxic effects were seen in animals treated with lower doses of juglone (upto 1.5 mg/kg b. wt.).

Okada and co-workers (1967) 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 and co workers (Sugie \textit{et al.}, 1998), wherein male F344 rats
administered with juglone had lower incidence and multiplicity of azoxymethane induced intestinal tumors. On the contrary, another study (Monks et al., 1990) 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 (Van Duuren et al., 1978), 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 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 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 the earlier findings (Okada et al., 1967; Sugie et al., 1998).

Reactive oxygen species (ROS) are generally very small, highly reactive molecules that can be generated as by-products of cellular metabolism or exogenous insult. Although, moderate levels of ROS are essential for signaling of many cellular pathways, 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 (Fruehauf and Meyskens, 2007). 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 (Trachootham et al., 2009). 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 (Hsu et al., 2006), redox cyclers (Wondrak, 2007), organic endoperoxides (Efferth, 2005). 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 (Ji et al., 2011). Based on the existing literature as well as from earlier studies (chapter 3), the observed anticancer potential of juglone 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. (Valko et al., 2004). 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 (Fairbairn et al., 1995). In order to study if the observed tumor growth inhibition could be a result of DNA damage induced by juglone, an attempt was made to 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 in vivo and this DNA damage in the tumor tissue was reported to be closely associated with the tumor cell kill (Dorie et al., 1999). 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 (Kopjar et al., 2002). Therefore, to evaluate the effect of juglone on the DNA damage levels in the nucleated 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 genotoxicity which however
may be mitigated by formulating juglone using various drug targeting strategies as was demonstrated for plumbagin in an earlier report (Mandala Rayabandla et al., 2010).

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 in vitro (Devi et al., 1996; Prasad et al., 1996). Further, earlier studies have also demonstrated the radiosensitizing potential of these plant based compounds against various cancer models in vivo (Devi et al., 1999; Devi et al., 2000). 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. A sensitizer enhancement ratio of 1.37 was observed which may be considered as a significant level of sensitization based on the earlier reports where SER values of > 1.1 were considered significant (Chung et al., 2009). 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 (Seiwert et al., 2007). These findings further corroborate the results of the 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. 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 (Boothman et al., 1989). In the present investigation, the effect of juglone on the radiation induced DNA damage and repair was studied. At zero time (immediately after various treatments), the OTM levels in the combination treatment group was significantly higher in comparison to the individual treatments. Results from the repair studies shows 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. 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. From the previous studies (Chapter 3), juglone treatment was found to cause depletion of the intracellular GSH levels and thereby 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 (Ollinger and Brunmark, 1991; Murakami et al., 2010). 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 \textit{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 \textit{in vitro} and \textit{in vivo}. However, considering the fact that juglone induced some toxicity to the nucleated blood cells \textit{in vivo}, it may be necessary to formulate juglone using targeted delivery approaches aimed at improving the anticancer activity and thereby reducing the associated toxicity.
4.5. REFERENCES


Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53


Ollinger K, Brunmark A. Effect of hydroxy substituent position on 1,4-naphthoquinone toxicity to rat hepatocytes. Journal of Biological Chemistry. 1991;266:21496-503.


Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research. 1988;175:184-91.


