Chapter 5.
Table of Contents
5.1. Introduction
5.2. Materials and Methods
5.2.1. Anthraquinone fraction (AQf) and Cyclophosphamide
5.2.2. Assays for anti-oxidant activity
5.2.3. Cells and animals
5.2.4. Induction of tumor in animals
5.2.5. Antitumor Activity of Anthraquinone fraction (AQf)
5.2.6. Anti-inflammatory Activity
5.2.7. Statistical analysis
5.3. Results
5.3.1. Action of AQf on O2\(\cdot^-\), OH\(\cdot\) and NO\(\cdot\) radicals
5.3.2. AQf prevents and regresses solid tumor and increases life span of ascites tumor bearing animals
5.3.3. Anti-inflammatory Activity of AQf
5.4. Discussion
Chapter 5.

Antitumor and Anti-inflammatory Activities of Anthraquinone fraction of *Ophiorrhiza rugosa*

5.1. Introduction

One of the major challenges in the cancer research is the identification of potent antineoplastic compounds that differentially kill tumor cells or repair or restore the altered cellular functions of a cell responsible for malignant transformation (Appel JR, et al., 1998). Nature has been serving as a wonderful source for searching such compounds. Anthraquinones are such class of compounds showing anticancer properties (Lown JW, 1993). Anthraquinones are widely distributed in many plant species of which Rubiaceae gained much attention for anticancer properties. Their anticancer activity is attributed to their very structure, position and nature of substituents to the basic anthraquinone skeleton (Sun M et al., 2000). They intercalate into DNA by virtue of their planar structure. The high redox potential of these compounds has been correlated with the production of free radicals like O$_2^\cdot$ and H$_2$O$_2$, which evoke enhanced cellular deleterious effects leading to cell death (Teich L, et al., 2004).

The extensively studied anticancer anthraquinone are emodin, aloin and aloe-emodin. The effect of emodin and aloe-emodin is through the generation of extra levels of ROS in cancer cells, which is dependent upon cell type (Jing Y, 2006). Emodin, chrysophanol and rhein, which specifically inhibit one of the carcinogenesis-related enzymes, cytochrome p450 (Pawlowska J, 2003). Kuo et al.,(2001) worked on four anthraquinones purified from *Polygonum hypoleucus* Ohwi for their activity against human mesangial cell proliferation *in vitro*. Emodin showed highest suppressing activity on cell proliferation. We studied the antioxidant, antitumor and anti-inflammatory activities of anthraquinone fraction isolated from *in vitro* cultures of *Ophiorrhiza rugosa*.

5.2. Materials and Method
5.2.1. Anthraquinone fraction (AQf) and Cyclophosphamide

Isolation of anthraquinone fraction was described in chapter 4. Cyclophosphamide (CTX) and Diclofenac were purchased from Pharmacy of Amala Cancer Hospital.

5.2.2. Assays for anti-oxidant activity

The AQf was studied for superoxide, nitric oxide and hydroxyl radical scavenging and lipid peroxidation assays. Superoxide radical scavenging activity was estimated by riboflavin photoreduction method as described by McCord and Fridovich (McCord JM and Fridovich I, 1969). Hydroxyl radical scavenging activity was carried out by the method of Ohkawa (Ohkawa H, et al., 1979). Nitric oxide radical scavenging activity was evaluated by Griess reaction (Green et al., 1982, Marcocci et al., 1994). In vitro lipid peroxidation assay was determined by thiobarbituric acid reactive substance (TBARS) (Ohkawa et al., 1979). 20-200mg/ml AQf was used for the study.

5.2.3. Cells and animals

DLA and EAC cell lines were propagated in Swiss albino mice by injecting 1 x 10^6 cells intraperitoneally. Male Swiss albino mice (20-26g) were used for the study. They were purchased from Small Animal Breeding Station, Mannuthy, Thrissur. The animals were given pelleted feed (Sai Durga Feeds and foods, Bangalore, India) and water ad libitum.

5.2.4. Induction of tumor in animals

DLA cells were aspirated from the peritoneal cavity of mice, washed with PBS and 0.1ml cell suspension containing 1 x 10^6 cells were used for injection. Solid tumor was induced on the right/left hind leg of mice by injecting 1 x 10^6 DLA cells intradermally.

Same way, injecting 1 x 10^6 EAC cells intraperitoneally induced ascites tumor in Swiss albino mice.

5.2.5. Antitumor activity of anthraquinone fraction (AQf)

Solid Tumor

The dose of anthraquinone fraction was evaluated by a toxicity study and safe doses of 100 and 200mg/kg b. wt. were determined. Animals were grouped into four with six animals in each. The first group
of this experiment was served as control, which received neither drug nor AQf. Group 2 received Cyclophosphamide (15mg/kg b.wt, i.p.) as standard drug. Group 3 and 4 received two doses of AQf, 100 and 200mg/kg b.wt., respectively. The drug treatment started simultaneous with induction of tumor. Every fourth day, the tumor volume was calculated.

In another set of experiment, after 10 days of tumor induction, the treatment has started for the next ten days. Grouping was same as in first experiment. The tumor volume was calculated using the formula \( V = \frac{4}{3}\pi r_1^2 r_2 \) on every fifth day of experiment.

**Ascites Tumor**

Injecting EAC cells into the intraperitoneal cavity of mice produced ascites tumor. The animal grouping and AQf treatment followed as in solid tumor model.

The death pattern of the animals due to tumor burden was noted and percentage increase in life span (ILS) was calculated (Mazumdar UK, *et al.*, 1997).

\[
\%\text{Increase in life span (ILS)} = \frac{\text{Mean survival time of treated group} - \text{Control Group}}{\text{Mean survival time of control group}} \times 100
\]

**5.2.6. Anti-inflammatory activity**

Male Swiss albino mice (20-26g) bred in our institute were used for the study.

*Carrageenan induced acute inflammation*

The animals were grouped into 4 groups with six animals in each. In animals of each group acute inflammation was induced by subdermal injection of 20μl of freshly prepared 1% suspension of carrageenan in normal saline in the right hind paw of mice (Winter CA, *et al.*, 1962) and paw thickness was measured using a vernier calipers before and intervals of 1hr for four times after the challenge with carragenan. Animals were given two doses of AQf (100 and 200mg/kg b.wt) half an hour before the carragenan insult. Diclofenac is administered as standard drug.
5.2.7. Statistical analysis

Values are recorded as mean ± SD. The data were analyzed by one-way ANOVA followed by Dunnet comparison Test using InStat statistical software package.

5.3. Results

5.3.1. Action of AQf on O2·−, OH· and NO· radicals

AQf does not found to have any scavenging activity over O2·− and NO· radicals. But it dose dependently scavenged the OH· radicals. But in contrast, O2·− radicals are generated by the AQf, it could be clear from the oxidation of NBT molecule. The NBT upon oxidation with O2·− radicals were converted to blue colored diformazan. The intensity of color is proportional to the concentration of O2·− generated (figure 5.1.a). Here we found that AQf treated groups contain more oxidized NBT than control groups. It suggested the pro-oxidant activity of AQf. AQf showed OH· radical scavenging activity. It dose-dependently scavenged OH· radicals. The IC50 for OH· radical scavenging was 190μg/ml (Figure.5.1.b). No considerable NO· radical scavenging activity was found for AQf (Figure.5.1.c). AQf also inhibited the in vitro lipid peroxidation (Figure.5.1.d).

5.3.2. AQf prevents and regresses solid tumor and increases life span of ascites tumor bearing animals

Two regimens of AQf studied for their antitumor activity. In the simultaneous treatment group, there had an increase of tumor volume until 17th day post tumor inoculation. After that a regression in the tumor volume was found for both concentrations of AQf. The tumor decreased and became almost normalized on 27th day. Compared to control and CTX treated groups, the tumor volume were lesser for AQf treated groups on 7th day itself. It points that AQf treatment blocks the formation of tumor mass rather more effectively than CTX(Figure 5.2.a).

AQf decreased the tumor volume when it is administered after the development of solid tumor. Not a complete remission was noticed for all the two concentration of AQf(Figure 5.2.b).
The effect of AQf to increase the life span of ascites bearing animals was studied. The simultaneous treatment of AQf (200mg/kg b. wt) increased the life span up to 80.65% compared to control group. The standard drug CTX increased the life up to 90.32%. This was comparable with the percentage increase in life span of 200mg/kg b. wt treated group.

The administration of AQf after the development of tumor increased the life span only up to 54.4%, while the increase in the life span by CTX treated group was 72.7%. AQf at 100mg/kg b. wt didn’t increase the life span of animals significantly compared to control (Table. 5.1).

5.3.3. Anti-inflammatory activity of AQf

AQf treatment, half an hour prior to carrageenan insult reduced the edema formation in the paw. At the first hour, diclofenac and two concentrations of AQf(100 & 200mg/kg.b.wt) didn’t show any significant reduction of paw edema. In the diclofenac treated group, at second hour significant reduction of edema was noticed, while there was no such reduction by AQf groups. At third hour and fourth hour significant reduction of edema was noticed for AQf treated groups. No significant change was noticed depending upon the concentration. Both the concentrations did the same level effect on paw edema(Figure 5.3).

5.4. Discussion

Anthraquinones are phenolic compounds showing wide range of pharmacological properties, which are the basis for different applications in the broad area of pharmacy and medicine(Igarashi Y, et al., 2007; Savarino L, et al., 2007; Taccoen A, 1993). Here we tested the antioxidant, antitumor and anti-inflammatory activities of crude anthraquinone (AQf) from in vitro cultures of O. rugosa.

AQf was isolated from in vitro cultures of O. rugosa. This crude fraction of AQs was tested for antioxidant and cytotoxicity potential. The antioxidant assays showed interesting results that the generation of superoxide radical but AQf scavenged hydroxyl radicals. We didn’t find any significant scavenging activity for nitric oxide radical. The AQf fraction effectively inhibited in vitro lipid peroxidation. Anthraquinones are high redox compounds, which can generate superoxide radicals by
electron transfer to acceptors. Physico-chemical studies employing electron paramagnetic resonance and electrochemistry of emodin showed that this compound could be easily reduced chemically, photochemically or enzymatically to its corresponding semiquinone. In the presence of oxygen the semiquinones generated reactive oxygen species (ROS), mainly superoxide (Rahimipour S, et al., 2001). Intracellular increase in ROS often leads to apoptosis.

The cytotoxic activity in DLA and EAC cells prompted us to study the antitumor activity of AQf in vivo. The antitumor activity was evaluated in two model systems; solid tumor and ascites tumor. AQf treatment reduced the solid tumor burden very effectively. In the simultaneous treated experiment, a good response was observed as reduction in the tumor volume. In another experiment where AQf treatment was started after 10 days post tumor induction, only 200mg/kg.b.wt. AQf resulted in marked decrease in tumor volume. Many previous reports are regarding the antitumor activities of anthraquinones isolated from different plant species (Zagotto G, et al., 2000; Morreal CE, et al., 1999).

Treatment with AQf (200mg/kg.b.wt.) increased the life span of ascites tumor bearing animals. But simultaneous treatment, 80.65% increase in the life span was observed. The treatment after development of tumor resulted in 54.4% increase in life span. No significant increase of life span was noticed for 100mg/kg.b.wt in those groups where treatment was started after 10 days post tumor induction.

Anti-inflammatory activity was observed in AQf treated groups. Inflammation is a complex, difficult to control, self-perpetuating process that is responsible for development of diseases like atherosclerosis and cancer (Ferencik M, et al., 2007). The recent withdrawal of popular anti-inflammatory drugs like Vioxx and Celebrex from market because of safety concerns underscores the need for finding more suitable replacements, especially in terms of health related hazards (Kerr DJ, et al., 2007; van Adelsberg J, et al., 2007; Salzberg DJ & Weir MR., 2007).

There is only few works have been done on the anti-inflammatory activities of anthraquinones. Our study shows that AQf has scavenging
effect only on OH’ Radical and not significant level of scavenging activity against NO’ was observed. But it generated O$_2$’ radicals. The inhibition of lipid peroxidation and OH’ radical scavenging may be correlated the anti-inflammatory action of AQf. Further studies are required to confirm this postulation.
**Table 5.1.a. Effect AQf on Life Span of Ascites Tumor Bearing Animals**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg.b.wt</th>
<th>No. of animals with tumor</th>
<th>No.of Days Survived</th>
<th>%ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Simultaneous</td>
<td>Developed</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>6/6</td>
<td>15.5±1.7</td>
<td>16.5±1.6</td>
</tr>
<tr>
<td>CTX</td>
<td>15</td>
<td>6/6</td>
<td>29.5±1.2**</td>
<td>28.5±1.5*</td>
</tr>
<tr>
<td>AQf</td>
<td>100</td>
<td>6/6</td>
<td>20±2.6**</td>
<td>17.5±5.7 ns</td>
</tr>
<tr>
<td>AQf</td>
<td>200</td>
<td>6/6</td>
<td>28±2.3**</td>
<td>25.5±1.5 ns</td>
</tr>
</tbody>
</table>

Values are Median±SD. **p<0.01, *p<0.05; ns p>0.05
Fig. 5.1.a. Represents the superoxide generation by AQf. Significant increase in superoxide was noticed. Different alphabet shows statistical significance. p<0.01

Fig. 5.1.b. Represents the hydroxyl radical scavenging effect of AQf. Significant level of scavenging was noticed. Different alphabet shows statistical significance. p<0.01
Fig. 5.1.c. Effect of AQf on NO. Radical scavenging

![Chart showing effect of AQf on NO. Radical scavenging](image)

Fig. 5.1.c. Represents nitric oxide scavenging by AQf. ns, p>0.05; *p<0.05  **p<0.01

Fig. 5.1.d. Inhibition of Lipid Peroxidation by AQf

![Chart showing inhibition of lipid peroxidation by AQf](image)

Fig. 5.1.d. Represents the inhibition of Lipid peroxidation (LP) by AQf. AQf significantly reduced LP. **p<0.01
**Fig. 5.2.a.** Solid tumor reducing activity of AQf - Simultaneous Treatment

**Fig. 5.2.b.** Effect of AQf on Developed Tumor
Fig. 5.3: Antiinflammatory Activity of AQf
Plate 5.1. Antitumor Activity of AQf

Solid Tumor

Ascites Tumor