CHAPTER IX

EFFECT OF WITHANIA SOMNIFERA ALONG
WITH THERMO, CHEMO AND RADIOTHERAPY
ON SOLID TUMOUR DEVELOPMENT
I. Introduction

Cancer can be described as the growth of cells in a disorganised fashion and it is the tendency of tumour cells to invade and to discriminate. Chemotherapy, radiotherapy and surgery are the three important treatment modalities of cancer. Both chemo and radiotherapies cause toxic symptoms such as nausea, vomiting, diarrhoea, mucosal ulceration etc. Besides these, immunosuppression is the major drawback in cancer therapy (1).

It has been reported that hyperthermia was found to potentiate the immune system (76). Use of hyperthermia alone or in combination with radiation, chemotherapeutic agents or other treatment modalities continues to be an area of great interest for improving cancer therapy. Heat shock prior, during or immediately after radiation synergistically increases cell killing, a phenomenon termed hyperthermic radiosensitization is due to heat induced DNA damage (77).

In the present chapter we have made a detailed study on the effect of Withania somnifera on the tumour development along with other treatment modalities such as cyclophosphamide, radiation and hyperthermia.

II. Materials and Methods

II.1 Animals

Swiss albino mice (6-8 weeks old; 20gm-25gm) were used.
II.2 Cell lines

Dalton’s lymphoma ascites cells (DLA) and Ehrlich ascites tumour cells (EAC) were used.

II.3 Chemicals

Cyclophosphamide (CTX), Hexadecyl trimethyl ammonium bromide and O-Dianisidine hydrochloride were used. All other chemicals used were of analytical reagent grade (Chapter II).

Radiation

Radiation was given to animals using a (CO$_6$) gamma source teraton 780 Telecobalt unit, Atomic Energy Canada Ltd) at Amala Cancer Hospital.

II.4 Treatments

1. Solid tumour induction

Daltons lymphoma ascites cells (DLA) (1x10$^6$) were injected subcutaneously on the hind limb of mice to develop solid tumour. The diameter of the tumour was measured on every third day for 1 month and the tumour volume was calculated using the formula \( V=\frac{4}{3} \pi r_1^2 r_2^2 \) where \( r_1 \) and \( r_2 \) are the radii of tumour in two directions (Chapter II).

2. Radiation treatment

Animals were exposed to a single dose of whole body radiation (600 rads/animal), prior to drug administration.
3. **Chemotherapy**

Cyclophosphamide (CTX) (25mg/kg b wt; i.p) was given to animals prior to the administration of Withania extract or after radiation exposure.

4. **Hyperthermia Treatment**

Whole body hyperthermia was given to animals by keeping at 43°C for 40 minutes.

II.5 **Determination of the effect of *Withania somnifera* on solid tumour development.**

Four groups (6nos/grp) of animals were used in this study. All animals were treated with DLA cells (1x10^6). Group I animals received tumour cells alone. Group II animals were treated with 5 doses of Withania extract (20mg/dose/animal, i.p). Group III animals were treated with 5 doses of Withaferin A (500µg/dose/animal, i.p.) and Group IV animals were treated with 5 doses of Withanolide D (500µg/dose/animal, i.p). The tumour volume was calculated on 7th day after the tumour induction and on every 3rd day thereafter for 1 month.

II.6 **Determination of the effect of *Withania somnifera* on ascites tumour reduction.**

Groups of six Swiss albino mice (20-25gm) (6nos/grp) were used for animals experiments. Ehrlich ascites tumour cells (1x10^6 cells) were given intraperitoneally to develop ascites tumour, after 24h; 5 doses of Withania extract (20mg/dose/animal, i.p.), Withaferin A (500µg/dose/animal, i.p) and Withanolide D (500µg/dose animal, i.p.) was given to the animals.
on continuous days. The mortality of animals dying of tumour were noted and the percentage increase in life span was calculated from the formula

\[ \% \text{ILS} = \frac{T-C}{C} \times 100 \]

Where T is the average number of days of the treated animals survived and C is the average number of days of the control animals survived.

### 11.7 Determination of the effect of *Withania somnifera* on tumour development along with Radiohyperthermia treatment.

Six groups (6nos/group) of mice were used in this study and all the animals were induced solid tumours with DLA cells as described above. After 7 days of tumour induction, the different groups of animals were treated as follows. Group 1 was kept as untreated control. Group 2 animals received hyperthermia treatment. Group 3 animals were treated with radiation; Group 4 animals were subjected to hyperthermia 20-40 minutes later followed by radiation; Group 5 animals were treated with hyperthermia; Group 6 animals were simultaneously treated with hyperthermia and radiation; Group 5 and 6 animals were continued with *Withania* administration half an hour later after hyperthermia treatment for 10 consecutive days.

### 11.8 Determination of the effect of *Withania somnifera* on solid tumour development along with Radiochemotherapy.

Six groups (6nos/group) of animals were induced solid tumour and after 7 days the different groups of animals were treated as follows. Group 1 animals were treated with DLA cells alone; Group 2 animals were subjected to radiation; Group 3 animals were treated with CTX (25 mg/kg b.wt i.p) for 10 consecutive days; Group 4 animals were treated with CTX,
Group 5 animals were subjected to radiation; Group 6 animals were treated with radiation; followed by CTX and group 4, 5, & 6 animals were continued with Withania (20mg/dose/animal, i.p) for 10 continuous days.

II.9 Determination of the effect of Withania somnifera along with Radiohyperthermia Chemotherapy.

Six groups (6 nos/group) of animals were induced solid tumour and after 7 days of different groups of animals were treated as follows - Group 1 served as untreated control; Group 2 animals were treated with Withania (20mg/dose/animal, i.p) on 10 consecutive days, Group 3 animals were treated with radiation followed by 10 doses of CTX (25mg/kg b.wt) for 10 consecutive days. Group 4 animals were simultaneously treated with hyperthermia followed by CTX, Group 5 animals were received hyperthermia then 20-40 minutes later radiation followed by CTX; Group 6 animals were similar to that of group 5 but continued with Withania administration as that of group 2.

II.10 Determination of effect of Withania somnifera on Myeloperoxidase activity.

Three groups of Swiss albino mice (6nos/ group) were injected with DLA cells (1x10^6) alone. Group 1 animals were treated with DLA cells alone. Group 2 animals were treated with whole body hyperthermia (43° c for 40 minutes); Group 3 animals were treated same as that of group 2 but continued with Withania administration (20mg/dose/animal i.p) for 10 consecutive days. The animals were sacrificed on 10th day and the tumour tissue (200-400mg) was homogenised in cold Hexadecyl trimethyl ammonium bromide (0.5% in 50mM phosphate buffer). The
cell suspensions were sonicated on ice for 10s and centrifuged at 40000 rpm for 15 minutes. The supernatant was assayed for Myeloperoxidase (MYP) activity spectrophotometrically (79). Supernatant (0.1 ml) was mixed with 2.9 ml of 50 mM phosphate buffer (pH, 6.0) containing 0.167 mg/ml O-Dianisidine hydrochloride and 0.0005% H₂O₂. The change in absorbance was measured at 460 nm. One unit of MyP activity is defined as degrading 1 μmol of peroxide/minute at 25°C.

III  Results

III.1.  Effect of Withania somnifera on solid tumour development.

The effect of Withania somnifera on solid tumour reduction is given in Figure IX.1. The tumour volume of untreated control animals was found to be 4.03 cm³ on 30th day. Withania somnifera administration showed significant reduction in the tumour volume 1.51 cm³ on the same day. Withaferin A treated group showed tumour reduction up to 0.609 cm³ on the same day. Administration of Withanolide D also showed a significant inhibition in the tumour development (1.8 cm³).

III.2.  Effect of Withania somnifera along with Radiohyperthermia on solid tumour Development.

Effect of Withania somnifera along with radiohyperthermia on solid tumour reduction is given in Fig IX.2. In the case of animals treated with tumour alone, the tumour volume was found to be 5.1 cc. which was reduced to 3.1 cc by hyperthermia treatment. In radiation treated
group the tumour volume was found to be 1.89cc. Tumour volume was significantly reduced to 0.73cc by simultaneous administration of radiation and hyperthermia tumour volume in the hyperthermia and Withania treated group was found to be 1.3cc. When Withania was continued along with hyperthermia and radiation tumour volume was found to be significantly reduced to 0.59 cc.

III.3. Effect of Withania somnifera along with Radiochemotherapy on solid tumour development.

Effect of Withania somnifera along with radiochemotherapy on solid tumour development given in Figure 1X.3. Tumour volume of the untreated control was found to be 5.1cc. The tumour volume in animals treated with CTX was 1.72cc. and that of mice treated with radiation was 2.49cc. Administration of Withania along with CTX or radiation significantly reduced tumour volume to 0.90cc. and 1.23cc respectively. Administration of Withania could significantly reduce the tumour volume when CTX and radiation was given synergistically. (0.60cc).

III.4. Effect of Withania somnifera along with Radiohyperthermia Chemotherapy on solid Tumour Reduction.

Effect of Withania somnifera along with radiohyperthermia chemotherapy is given in Figure 1X 4. The tumour volume of the untreated control group was found to be 5cc. on 30th day which was reduced to 1.5cc when Withania was administered for 10 consecutive days. The tumour volume was found to be significantly inhibited upto 1.18cc. when CTX and radiation was given synergistically. The simultaneous treatment of hyperthermia and CTX was found to be significantly reduced the tumour volume upto 1.09cc. There was a significant inhibition in
the tumour volume when radiation, CTX and hyperthermia was given simultaneously (0.51 cc) and when Withania was given along with all the three treatments tumour volume was reduced maximally to 0.35 cc.

III.5. Effect of *Withania somnifera* on the tumour reduction of Ehrlich ascites tumour bearing animals.

The effect of *Withania somnifera* on the reduction of Ehrlich ascites tumour is given in Table IX 2. Administration of *Withania somnifera* significantly inhibited the tumour development. For Withania treated group, the average number of days of survival was found to be 25 and increase in life span was found to be 34%. For Withaferin A treated group, average number of day of survival was found to be 23 and increase in life span was found to be 29%. Treatment with Withanolide D showed an average number of survival 21 and increase in life span was found to be 18%.

III.6 Effect of *Withania somnifera* on Myeloperoxidase (MYP) activity in tumour bearing mice.

The MYP level in the tumour tissue of animals was significantly enhanced by the administration of *Withania somnifera* (Table IX 1). DLA alone treated group showed a MYP level of $7 \pm 1.35$ U/mg protein whereas tumour and hyperthermia treated group showed a significant enhancement in MYP activity of $(28.3 \pm 2.21$ U/mg protein). When Withania was administrated to the hyperthermia treated group, there was a significant enhancement in MYP level upto $32.3 \pm 2.23$ U/mg protein.
IV. Discussion

Withania could reduce the solid tumour development induced by DLA cells as well as ascites tumour induced by Ehrlisch Ascites cells. Both Withaferin A and Withanolide D could also reduce the solid tumour development induced by DLA cells and ascites tumour development induced by EAC cells.

Present study clearly indicates that Withania could act as an adjuvant during chemotherapy, radiotherapy and thermotherapy. Most interestingly it was found that Withania could synergistically reduce the solid tumour volume in the presence of radiation, cyclophosphamide and hyperthermia and the combined action of these modalities produced significant reduction in tumour volume. Even mild heat treatment at 43°C for 40 minutes could reduce the solid tumour development very effectively without any stromal injury. Whole body hyperthermia can protect mice from lethal dose of gamma radiation and extent of protection depending on the time at which mice were exposed to radiation. It was speculated that hyperthermia resulted in further accumulation of neutrophils and active oxygen species (79).

Myeloperoxidase is an enzyme found in neutrophils and in much smaller quantities in monocytes and macrophages (80). MYP activity is a product of neutrophils and is proportional to the number of neutrophils present. Present study also shows that hyperthermia along with Withania could stimulate the immune system and enhances MYP level in DLA induced mice.
During inflammation, neutrophils produce super oxide and other highly reactive oxygen products capable of inducing cellular injury. Stimulation of neutrophils also induces release of granules containing lysosomal enzymes which result in cell injury.

Present study clearly indicates that Withania could act as an adjuvant during chemotherapy radiotherapy and thermotherapy. Hence Withania could be used in the treatment of cancer as response modifier of other treatment modalities.
Figure IX.1 Effect of *Withania somnifera* on solid tumour reduction induced by DLA cells
Figure IX.2 Effect of *Withania somnifera* along with Radiohyperthermia on solid tumour development
Figure IX.3  Effect of *Withania somnifera* along with Radiochemotherapy on solid tumour development

![Graph showing the effect of different treatments on tumour volume over days](image-url)
Figure IX.4  Effect of *Withania somnifera* along with Radiohyperthermia Chemotherapy on solid tumour development
Table IX - I

Effect of *Withania somnifera* on Myeloperoxidase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MYP (Unit/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Tumour alone</td>
<td>7 ± 1.33</td>
</tr>
<tr>
<td>Tumour + Hyperthermia</td>
<td>28.3 ± 2.21*</td>
</tr>
<tr>
<td>Tumour + Hyperthermia + Withania</td>
<td>32.3 ± 2.22*</td>
</tr>
</tbody>
</table>

*P<0.001

All the animals were treated with DLA cells(1x10^6). Whole body hyperthermia was given to group 2 & 3 (43°C for 40’) before Withania administration. Withania treated group received 10 doses of the drug (20mg/dose/animal,i.p.).
Table IX - 2

Effect of *Withania somnifera* on the tumour reduction of Ehrlich Ascites tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average number of days survived</th>
<th>% increase in life span ($\frac{T - C}{C} \times 100$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Tumour + Withania</td>
<td>25 ± 2.67</td>
<td>34</td>
</tr>
<tr>
<td>Tumour + Withaferin A</td>
<td>23 ± 2.97</td>
<td>29</td>
</tr>
<tr>
<td>Tumour + Withanolide D</td>
<td>21 ± 1.33</td>
<td>18</td>
</tr>
</tbody>
</table>

All the animals were treated with tumour cells. (Ehrlich ascites tumour cells (1x10⁶).

Treated animals received 5 doses of the drug.

*Withania* (20mg/dose/animal, i.p)

*Withaferin A* (500μg/animal, i.p)

*Withanolide D* (500μg/animal, i.p)