Radioprotective Potential of an Herbal Extract of *Tinospora cordifolia*

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**Radioprotection/Tinospora cordifolia/Cell proliferation inducer/Micronuclei/Immunostimulator/Herbal radioprotector.**

A preparation of *Tinospora cordifolia* (RTc) administered i.p. (200 mg/kg b.w.) to strain "A" male mice 1 h before whole body gamma-irradiation was evaluated for its radioprotective efficacy in terms of whole body survival, spleen colony forming units (CFU), hematological parameters, cell cycle progression, and micronuclei induction. Preirradiation treatment with RTc rendered 76.3% survival (30 days), compared to 100% mortality in irradiated control and prevented radiation induced weight loss. On 10th postirradiation day, the endogenous CFU counts in spleen were decreased with increasing radiation doses 12.0 (5 Gy), 2.16 (7.5 Gy) and 0.33 (10 Gy) but pre-irradiation administration of 200 mg/kg b.w. of RTc increased CFU counts to 31.16, 21.83 and 3.00 respectively. Pre-irradiation RTc treatment could restore total lymphocyte counts (TLC) by the 15th day to normal. It also increased the S-phase cell population that was reduced following 2 Gy irradiation in a time dependent manner. 2 Gy irradiation-induced micronuclei were also decreased by a pre-irradiation administration of RTc from 2.9 to 0.52%. Because the radioprotective manifestation of RTc observed in several systems in experimental animals can be exploited for human applications.

**INTRODUCTION**

Many synthetic or natural agents have been investigated in the recent past for their efficacy to protect against radiation damage. Among the natural radioprotective agents compounds, cystine, cysteamine, 5-hydroxytryptophan, 5-hydroxytryptamine, glutathione, and vitamins like A, C, and E⁵ have been extensively studied. Important synthetic molecules include amino-ethyl-isothiouronium bromide hydrobromide (AET), WR-2721. However, the inherent toxicity of these agents at the radioprotective concentration warranted further search of a safer and effective radioprotector. To reduce toxicity, a strategy of combining radioprotective molecules working through different modes of action has also been attempted. In fact, no radioprotective agent now available, either singularly or in combination, meets all the requisites of an ideal radioprotector.⁶ Recently several isolated plant products and crude extracts that may have a natural combination of several bio-active molecules capable of giving radioprotection through different mechanisms, have been investigated.⁵,⁶

*Tinospora cordifolia* (Family-Menispermaceae) is a glabrous, succulent, climbing shrub and is a native of India that thrives easily in plain regions. It has been widely used in the Indian system of medicine as *Rasayana* for the treatment of several ailments, such as jaundice, diabetes, rheumatoid arthritis, gout, general weakness, skin diseases, anemia, emaciation, and infections.⁹ It has also been used as a vitalizer, anti-stress and adaptogenic, anti-ulcer, and immunomodulatory agent.¹⁰ The aqueous extract of this plant possesses anti-inflammatory and anti-allergic properties. It has been proved to have hepatoprotective,¹¹ hypolipidaemic,¹² and anti-neoplastic properties.¹³ It improves the phagocytic and bactericidal capacity of polymorphs,¹⁴ protects against gastric mucosal damage,¹⁵ and scavenges free radicals.¹⁶,¹⁷,¹⁸

The whole extract of *T. cordifolia* has been reported to contain several bioactive components, such as glucoside, alkaloids, bitter principle crystalline compounds,¹²,¹⁴,¹⁵ and non-diterpene furan glucosides cordifoliside A, B, and C.²⁰ The bitter principles present in *Tinospora* have been identified as columbin, chasmanthin, and palmarin. The leaves are rich in calcium, phosphorus, protein, and alkaloids, such as protoberberine, tinosporide,²¹ tinosporic acid, and tinosporol. Phytochemical analysis indicated the presence of several diterpenes furan lactones, phenolic lignans, phenyl propane glycosides,²² and arbinogalactan.¹⁴

For radioprotection, various mechanisms such as free radical scavenging, calcium channel blocking, inhibition of lipid
peroxidation, enhancement of DNA repair, and stimulation of stem cell proliferation are considered important. Tinospora cordifolia has several of the above-mentioned properties under different experimental conditions. Therefore it became necessary to investigate its wholesome radioprotective efficacy in experimental animals in terms of whole body survival, genotoxicity, cell proliferation, and hematological parameters.

MATERIALS AND METHODS

Preparation of plant extract
The fresh stems of Tinospora cordifolia were collected from Maranda Distt, Kangra, Himachal Pradesh, India, at an altitude of 3,000–3,200 ft. An ethnobotanist identified the samples; the stems were thoroughly washed with water and shade dried. A known quantity of the dried material was extracted with absolute alcohol and triple-distilled water (50:50, v/v; three changes), filtered, lyophilized and stored at 4°C. The extract was tested for endotoxins, using Lal assay kit as described by the manufacturer, and was found to be free from endotoxins. It was assigned a code name RTc. For standardization purposes, the HPLC spectra of RTc extracted from three lots of Tinospora cordifolia obtained during different periods were compared (Fig. 1). An HPLC profile of RTc was obtained by using C-18 column (4.6 × 250 mm; Water’s HPLC system), using a gradient mobile phase of 2% water in acetonitrile to 85% water in acetonitrile. The flow rate of mobile phase was kept between 0.3 to 1.5 ml/min.

Animals
Swiss albino strain ‘A’ male mice (10–12 weeks) weighing about 25 ± 2 g were maintained under controlled laboratory conditions (25 ± 2°C; RH 60 ± 5%; 12 h photo-period), fed standard animal food pellets (Amrut Laboratory Animal Feed, India), and tap water ad libitum. Animal experiments were conducted according to the guideline of the institutional ethical committee.

Administration of plant extract
The lyophilized preparation was dissolved in triple-distilled water for an administration of desired concentration, and the doses were expressed in mg/kg b. w. referring to the weight of lyophilized extract (RTc). Different doses of RTc were administered to mice through intraperitoneal (i.p.) route in a maximum volume of 0.2 ml. Control animals received 0.2 ml of normal saline.

Maximum tolerated dose (MTD)
An acute toxicity of RTc was studied in terms of percent survival, changes in behavior, alterations in neuromuscular coordination, and respiratory disorder for two days after the administration of singular doses of different concentrations of RTc. The maximum concentration of RTc, which yielded no toxic manifestation, was considered as MTD.

Irradiation of animals
Desired doses of gamma-radiation were delivered to mice by 60Co gamma chamber (Model-220, Atomic Energy of Canada Ltd.) at a dose rate ranging from 0.64 to 0.59 Gy/min during the experimental period. Dosimetry was carried with Baldwin Farmer’s secondary dosimeter and Fricke’s chemical dosimetry. Each mouse was kept in a perforated plastic container and irradiated individually with a continuous supply of fresh air through a rubber pipe to avoid hypoxia.

Animal survival and body weight
The effects of time interval between administration of different concentrations of RTc and irradiation on survival and body weight of mouse were investigated. Survival was observed daily up to 30th post-irradiation day, and data was expressed as % survival. Mice used for this study were distributed into different treatment groups, including normal control, irradiated control, drug control, and drug plus radiation group. The body weights of the animals were recorded every alternate day. The change in the average weights of the animals at different time intervals due to various treatments was calculated in consideration of the initial body weight of the animal (zero day of experiment) as 100%.

Endogenous spleen colony forming unit (CFU) Assay
The mice were sacrificed by cervical dislocation on the 10th post-irradiation day in all groups. For endogenous CFU assay spleens were dissected out and fixed in Bouin’s solution for 24 h. Macroscopic colonies (CFU) visible to naked eyes were scored from each spleen.

Haematological studies
The mice were distributed into 4 groups:
(i) The control animals were treated with 0.2 ml normal
saline
(ii) The irradiation group animals were exposed to 10 Gy
(iii) The drug-alone group animals were administered 200
mg/kg RTc, and
(iv) The drug + irradiation group animals were irradiated
(10 Gy), 1 h after an administration of 200 mg/kg RTc.
Haemoglobin and total lymphocyte counts (TLC) were
studied in peripheral blood drawn from the heart of mouse on
the 7th, 10th, or 15th post-irradiation day.

Micronucleus assay
The effect of 3 doses of RTc (150, 200, and 250 mg/kg
b.w.) on 2 Gy induced MN formation in mice bone marrow
cells was studied at 24 and 48 h time intervals as described
earlier. The epiphyses of femur bones were cut, and bone
marrow cells were flushed out with 0.5 ml PBS into 15 ml
centrifuge tubes. The cells were centrifuged once at 1,000
rpm and resuspended in a few drops of fetal calf serum.
Smears of the cells were drawn on clean glass slides, fixed
with methanol for 30 min, and stained with Giemsa stain. At
least 2,000 cells were scored from each animal to determine
the ratio of polychromatic and normochromatic erythrocytes
(PCE and NCE). The number of micronucleated PCEs was
expressed in a percentage value to depict the MN frequency.

Cell cycle analysis
The mice were sacrificed at 8, 24, 48, or 72 h time intervals
after various treatments, and bone marrow cells were col-
lected as described earlier. Treating the cells with RBC
lysis buffer containing ammonium chloride for 2–3 min
removed the RBCs, and the remaining nucleated cells were
fixed with 70% chilled alcohol. Approximately 1 million cells
from each animal were suspended in 1 ml PBS, treated with
200 mg/ml RNase A for 30 min at 37°C, and stained with pro-
pidium iodide (50 µg/ml) for another 30 min at 37°C under
dark conditions. A minimum of 10,000 cells from each sam-
ple was acquired on a FACS Calibure (Beckton, Dikinson)
flow cytometer equipped with suitable optics, and the data
were analyzed by using Cell-Quest software.

Statistical analysis
The data were subjected to statistical analysis, and signifi-
cance was determined by use of a Student’s t-test. The MN
frequency was expressed as percentage value. The level of
significance was determined by an ANOVA test; p < 0.05
was considered significant.

RESULTS

Maximum tolerated dose (MTD)
The single doses of RTc up to 400 mg/kg b.w. were toler-
ated well by mice with no adverse manifestations, except for
the mice being slightly drowsy for 3 to 5 min. However, RTc
dose of 500 mg/kg b.w. or above manifested a lack of activity
and mortality within 2–3 post-treatment days in a dose-depen-
dent manner. The death rate of mice treated with 500, 550, or
600 mg/kg b.w. RTc was 40, 60, and 100%, respectively. The
dose of 400 mg/kg b.w. therefore was considered as MTD.

Survival studies and body weight
All irradiated animals without RTc treatment died by the
12th post-irradiation day (Fig. 2). The administration of RTc
(200 mg/kg b.w.) 1 h before 10 Gy whole body gamma-irra-
diation rendered 76.3 ± 3.71% (30 days) survival. Doses of
RTc less than or more than 200 mg/kg b.w. were less effec-
tive. The effect of time intervals between the administration
of RTc and irradiation was also studied in terms of 30 days
postirradiation survival. For the dose of 200 mg/kg b.w of
RTc, an interval of 1 h between the administration of RTc and
Irradiation was found to be optimal (Fig. 3). The relative change in the average weight of animals depicted in Fig. 4 demonstrated that RTc administration before irradiation allowed a recovery of the loss in body weight faster than the untreated irradiated mice.

Colony forming unit (CFU) assay

The effect of various doses of radiation on endogenous CFU and its modulation by a pre-irradiation administration of RTc (200 mg/kg b.w., –1 h) are depicted in Fig. 5. CFU counts in spleen decreased with increasing radiation doses (5, 7.5, and 10 Gy). Animals given RTc with no other treatment rendered no significant change in CFU counts. Pre-irradiation treatment with RTc rendered significantly higher \( (p < 0.05) \) CFU counts in comparison to the corresponding irradiated groups. An estimation of DRF (dose reduction factor) on the basis of CFU counts gave an approximate value of 1.7 (the effect produced by 5 Gy without RTc treatment could be expected with ~8.5 Gy dose in the case of pre-irradiation administration of RTc). However, a more detailed study is required to make an accurate estimation of the DRF.

Hemoglobin (Hb)

Changes in the amount of Hb in different treatment groups have been shown at different post-irradiation intervals (Fig. 6a). RTc alone depressed (statistically non-significant) the Hb level during the first week of treatment, but later it became normal. In the irradiated group Hb, decreased continuously up to the 10th post-irradiation day, and the data for later periods could not be collected because all the irradiated animals had died by that time. In RTc + 10 Gy group, the Hb level decreased initially up to the 10th post-treatment day, but recovered steeply thereafter.

Total leukocyte counts (TLC)

The administrations of only a single dose of RTc (200 mg/kg b.w.) increased TLC \( (5.9 \times 10^3 \pm 173.74) \) up to the 7th post-treatment day, but attained control value gradually by the 10th day (Fig. 6b). Irradiation decreased TLC sharply up to the 10th day, and data for the 15th day could not be obtained because of the death of all animals by that time. Pre-irradiation treatment with RTc did not exhibit change in TLC in comparison to the irradiated group up to the 10th day. However, the recovery became evident thereafter, and the TLC values overshot the control value by the 15th day. The changes in differential leukocyte count (DLC) (lymphocyte, polymorph, and monocyte), in animals of all four groups, were similar to the changes in TLC.

Micronucleus (MN) assay

The effect of irradiation on micronucleus (MN) induction and its modification by treatment with different doses of RTc has been depicted in Table 1. MN frequency in the control group (Group-I) was 0.33 and 0.35% at 24 and 48 h respectively. Exposure to 2 Gy gamma-irradiation (Group-II) resulted in a significant \( (p < 0.01) \) increase in the MN frequency (2.75 and 2.9% at 24 and 48 h, respectively) in comparison to the control value. Administration of different doses of RTc alone was found to render dose dependent increase in micronuclei frequency (groups 3–6), as compared to untreated controls at all time intervals. Doses of RTc (150, 200, and 250 mg/kg b.w.) rendered 0.4, 0.45, and 0.49% MN at 24 h, respectively. At 48 h the corresponding MN frequencies were 0.43, 0.47, and 0.51%, respectively. Treatment with 150, 200, or 250 mg/kg b.w. of RTc 1 h before irradiation (groups 7–10), however significantly \( (p < 0.05) \), reduced the frequency of radiation-induced MN, in comparison to the radiation alone group. A pre-irradiation administration of 200 mg/kg b.w. of RTc was most effective, and it reduced the radiation-
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Induced MN frequency up to 0.52 and 0.54% at 24 and 48 h time intervals, respectively.

In the control group, the PCE/NCE ratio (Table 1) was 0.98 at both time intervals. Different doses of RTc alone reduced the PCE/NCE ratio in a dose-dependent manner. At 24 h post-treatment time, the values were 0.94, 0.92, and 0.89 for 150, 200 and 250 mg/kg b.w. of RTc, respectively. The corresponding values at 48 h were 0.92, 0.91 and 0.87 respectively.

Two Gy irradiation rendered significant ($p < 0.05$) bone marrow depression, and the PCE/NCE values were 0.65 and 0.59 at 24 and 48 h, respectively. A pre-irradiation administration of different doses rendered a dose-dependent increase in PCE/NCE values at all time intervals in comparison with the irradiated group. The values were 0.76, 0.85, and 0.79 at 24 h and 0.77, 0.89 and 0.80 at 48 h for 150, 200 and 250 mg/kg b.w. of RTc respectively.

**Table 1.** The effect of different doses of RTc on 2 Gy-induced MN frequency and bone marrow suppression in mice. Smears of bone marrow cells were fixed with methanol and stained with Giemsa stain. At least 2,000 cells were scored from each animal. The values are mean ± SD of three independent experiments performed with three animals in each group; $p < 0.05$ was considered significant.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Time interval (h)</th>
<th>PCE/NCE ± SD</th>
<th>%MN frequency ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>0.98 ± 0.1</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.98 ± 0.09</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>2 Gy</td>
<td>24</td>
<td>0.65 ± 0.07</td>
<td>2.75 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.59 ± 0.05</td>
<td>2.91 ± 0.07</td>
</tr>
<tr>
<td>RTc alone</td>
<td>150 mg/kg</td>
<td>0.94 ± 0.05</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.92 ± 0.07</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.92 ± 0.08</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>150 mg/kg</td>
<td>0.91 ± 0.05</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.89 ± 0.06</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.87 ± 0.09</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>RTc + 2 Gy</td>
<td>150 mg/kg</td>
<td>0.76 ± 0.07</td>
<td>1.1 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.77 ± 0.06</td>
<td>1.4 ± 0.06</td>
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<tr>
<td></td>
<td>48</td>
<td>0.85 ± 0.08</td>
<td>0.52 ± 0.02</td>
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<td>0.54 ± 0.02</td>
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<tr>
<td></td>
<td>24</td>
<td>0.79 ± 0.04</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.8 ± 0.06</td>
<td>0.98 ± 0.04</td>
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</table>

**DISCUSSION**

The present study revealed that a pre-irradiation administration of a single dose of RTc (200 mg/kg b.w., −1 h) rendered 76.3% survival in mice exposed to (10 Gy) whole body lethal gamma-irradiation, but irradiated mice without RTc treatment suffered from 100% mortality within 10–15 days. It also protected significantly against radiation-induced loss in body
weight, with a dose of 200 mg/kg b.w. being most effective. Most of the synthetic and herbal radioprotective agents often render maximum radioprotective effect at doses approaching MTD. However, RTc rendered significant radioprotection at 50% concentration of its MTD. Therefore it might render potential benefits in clinical applications. Most radiation damages arise from and interaction of the radiation-induced free radicals with the biomolecules. Molecules with the ability to scavenge free radicals, therefore, can prevent radiation damage. Since the free radicals are short-lived, it is necessary for such radioprotective molecules to be present in the cellular milieu in sufficient concentrations at the time of irradiation. RTc has already been shown to scavenge free radicals. In this study, a time interval of 1 h between RTc treatment and irradiation was found to be maximally effective. Possibly the major mechanism of the action of RTc in rendering radioprotection is the scavenging of free radicals generated by irradiation, and to achieve the optimal cellular concentration of the free radical scavenging constituents of RTc it takes about 1 h.

The survival against irradiation, in fact, is a result of several factors, such as the prevention of damage through inhibition of free radical generation or efficient scavenging of free radicals, repair of DNA, membrane and other damaged target molecules, and the replenishment of severely damaged or dead cells. The recruitment of cells to substitute the apoptotic and necrotic cells could add to survival. This process significantly contributes toward the recovery of several target systems, such as bone marrow, gastrointestinal tract and skin. Chemicals or other agents, which enhance stem cell proliferation, also can therefore yield an appreciable recovery of the damaged tissue following radiation exposure and thereby contribute to the survival. The enhancement of CFU counts in the spleen of RTc treated irradiated mice in comparison to irradiated control (Fig. 5) indicates the role of RTc in protecting the stem cells and/or stimulating the proliferation of the surviving cells. The ability of RTc to enhance cell proliferation is further supported by the cell cycle studies. RTc alone or its preirradiation administration rendered increased cell population in the S-phase (Fig. 7), indicating increased DNA synthesis. However, the cell proliferation enhancing ability of

Fig. 7. The effect of RTc on 2 Gy-induced cell cycle perturbations was studied in mouse bone marrow cell flow cytometrically. Alcohol-fixed cells were treated with RNase and stained with propidium iodide to quantify the DNA content. Three animals were taken in each group, and the experiments were repeated thrice. The flowcytograms are representative of all animals in each group.
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RTc needs further confirmation. The hemoglobin content was observed to decrease in RTc alone, as well as in RTc treated irradiated animals initially. Irradiation might have caused damage to the RBCs; liver and spleen might have sequestered the defective RBCs resulting in the initial decrease of Hb. The initial reduction in the Hb content in spite of the preirradiation treatment with RTc may also be explained on this basis. In Ayurvedic literature, Tinospora has been reported to be a blood purifier that possibly acts by stimulating liver and spleen, which remove defective and damaged RBCs from peripheral blood circulation. The feedback mechanism, however, stimulated haemopoiesis in the bone marrow, and therefore higher Hb levels were observed on the 10th and 15th post-treatment days (Fig. 6a). RTc pre-irradiation administration rendered a significant increase in TLC that was reduced because of irradiation. The increase in CFU counts in spleen associated with the increase in TLC in RTc treated irradiated animals (Fig. 6b) in comparison to irradiated control animals indicated the immunostimulatory role of RTc and could therefore be attributed to the already known immunomodulatory constituents present in T. cordifolia. Since immunosuppression following radiation exposure and subsequent opportunistic infections are the major drawbacks of radiation therapy, the use of RTc in radiotherapeutic applications can also be exploited. T. cordifolia has already been reported to contain a large number of bio-active molecules, which elicit protection against several stress and pathological conditions by acting through different mechanisms, such as antioxidant, stimulation of cell proliferation, immunomodulation and anti-inflammatory activity. RTc has been demonstrated to manifest antioxidant properties both in vitro and in vivo. The present study rendering increased CFU counts, TLC, and survival could also be the result of the above said properties of T. cordifolia.

The present study dealing with different doses of RTc (Table 1) in protecting against radiation-induced genotoxicity as observed by MN frequency depicts that 200 mg/kg b.w. doses were maximally effective. This corroborates the findings of whole body survival experiments. However, RTc alone also induced MN in bone marrow cells in a dose-dependent manner. Therefore RTc possibly renders some toxicity when present with no other stress, but renders protection against subsequent exposure to various stress conditions, a mechanism by which most flavonoids act. It is also possible that RTc stimulates the cellular repair machinery and thereby reduces the MN frequency induced by radiation. No direct evidence in support of DNA repair enhancing efficacy by RTc is, however, presently available. Although, radiation-induced bone marrow suppression was protected by RTc, different doses of RTc alone were found to induce bone marrow suppression, indicating that the doses were toxic to the haemopoietic system. These data are corroborated by the Hb content analysis. However, its pre-irradiation administration could protect against radiation-induced bone marrow suppression that could significantly contribute to radioprotection.

The enhancement of cell proliferation, immunomodulation, stimulation of haematopoiesis, and protection against radiation-induced genotoxicity together could contribute to the radioprotective efficacy of RTc. The radioprotective manifestations need to be further investigated in other model systems to assess its potential utility for human applications. The identification and characterization of individual constituents of RTc would be also a necessary step in this direction.

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