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MODULATION OF RADIATON-MEDIATED DAMAGE BY *CUSCUTA RELEXA* LINN

J. Sharma[1], R. Chawla[1], Y. Karamalakova[2], R. Kumar[1], R. K. Sharma[1], A. Zheleva[2], V. Gadjeva[2], Rajesh Arora[1, 3*]

[1]Radiation Biotechnology Group, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Marg, Delhi, India
[2]Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[3]Office of Distinguished Scientist and Chief Controller R&D (Life Sciences and International Cooperation), DRDO Headquarters, DRDO Bhawan, New Delhi, India

ABSTRACT
Ionizing radiations produces deleterious effects in the living organisms. The rapid technological advancements world over have increased the chances of human exposure to ionizing radiations enormously. Widespread use of radiation in diagnosis therapy, industry, energy sector and inadvertent exposure during air and space travel, nuclear accidents and nuclear terror attacks necessitates protection against radiation-induced alteration. This study aimed to evaluate free radical scavenging activity of an extract of *Cuscuta reflexa* Linn., an indigenous parasitic plant, on gamma radiation-induced oxidative stress leading to tissue damage. In this study, we investigated free radical scavenging (nitric oxide ion and hydroxyl ion), anti-amplification potential, anti-hemolytic and lipid peroxidation with liposome using *in vitro* assays. *Cuscuta reflexa* extract exhibited significant free radical scavenging and inhibited lipid peroxidation. The results explicitly showed that *Cuscuta* extract has high antioxidant potential and thus could be used to mitigate radiation-induced damage caused by moderate doses of radiation during unplanned exposures.

Key words: Cuscuta reflexa Linn., Ionizing radiations, Antioxidant potential

INTRODUCTION

Ionizing radiation-induced free radicals cause damaging effects on various vital cellular targets such as DNA, membrane lipids and proteins. The prophylactic administration of exogenous supplements of naturally occurring antioxidants has proven to be useful in neutralising such cascade of reactions (1). Bio-prospecting global flora has provided numerous potent leads in the past(2). Amongst synthetic drugs, Amifostine was found to be effective in modifying radiation response, however, its use has been restricted due to associated neurotoxicity (3). Consequently, there has been an upsurge of interest in harnessing the therapeutic potential of medicinal plants in reducing radiation-induced tissue injuries(4). It is essential to screen and evaluate naturally occurring bioactive components for their plausible radioprotective potential.

Cuscuta reflexa (common name) is an annual obligate stem parasite belonging to family Convolvulaceae. *Cuscuta sps.* comprises of about 100-170 species worldwide, with 12 species reported from India itself, *C. compestris* and *C. reflexa* are more common in India. This plant parasite does not have any chlorophyll and leaves, but they bear flowers. The mature vines are yellow or bright orange in colour. Various parts of *Cuscuta* have been used in Indian medicine for the treatment of diseases like fits, melancholy and insanity. A

***Correspondence to**: *Dr. Rajesh Arora, Office of Chief Controller R&D (Life Sciences and International Cooperation), DRDO Headquarters, DRDO Bhawan, Room No. 339, Rajaji Marg, New Delhi-110011, India Tel.: +91-11-23014873; Fax: +91-11-23014259, E-mail address: rajesharoradr@gmail.com*

lotion made from the *Cuscuta* stem is used in the treatment of sore heads and inflamed eyes, treatment of impotence, nocturnal emissions, vertigo, lumbago, leucorrhoea, frequent micturation, decreased eyesight, threatened abortion and chronic diarrhoea. It is also used as a purgative in the treatment of protracted fever, diaphoretic and demulcent (5). The methanolic extract is reported to show anti-steroidogenic properties (6). *Cuscuta* contains 0.2% cuscutin, 1% lactone, and large quantity of flavonoids (7). It contains high content of quinolizidine alkaloids [matrine (78.61%), sophoranol (2.98%) and methycytisine (1.32%)];kaempferol (0.1% of dried material) and a low content of kaempferol-3-o-beta-glucoside. The flavonols like quercetin and quercitin-3-glucoside have also been reported from *C. reflexa* in addition to various coumarins(8). 10% of the phenolic compounds in *C. reflexa* is caffeic acid, which includes chlorogenic acid and 3, 4-dicaffeoylquinic acid (7).

Keeping in view of such inherent potential, the present study was designed to evaluate radioprotective properties of *C. Reflexa* at *in vitro* level to provide an insight to its possible use as one of radioprotective constituents for development of standardized herbal radioprotective formulations in future.

MATERIALS AND METHODS

Irradiation

Higher radiation dose (250Gy) was delivered from a ^{60}Co gamma chamber [Gamma cell 5000, Board of Radiation and Isotope Technology (BRIT), Mumbai] at a dose rate of 1.84kGy/h. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's Chemical Dosimetry Method.

Preparation of extract

The air-dried material was ground into a coarse powder. Five hundred gramof the material was suspended in 2l of 50% ethanol, then subjected to coldmaceration for 24 h. Then, the extract was filtered through muslin cloth, and the filtrate wasevaporated under reduced pressure and vacuum-dried. The yield of the extract (designated as CSR) was 10 - 15% of the dry weight of the material. The standardised extract with 3.25% of total phenolics (estimated using Gallic Acid as standard) is referred as CSR in rest of the manuscript.

Nitric oxide ion scavenging potential

The presence of nitrite, a stable oxidized product of nitric oxide (NO) will be determined as per standard methodology of Kim et al., 2001 (9). The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent (6% sulphanilamide in 3 M HCl+ 0.3% naphthylethylenediaminedihydrochloride + 7.5% orthophosphoric acid in 1:1:1 ratio). A concentration range of CSR (0-1000µg/ml) was used for evaluation of activity. The nitric oxide scavenging activity was evaluated as decrease in %absorbance of the complex formed by diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine readable at 546nm with reference 653nm in test samples.

Anti-amplification potential

The ferrous ion chelating activity was evaluated by a standard methodology of Haro-Vincente (10)with slight modifications. The reaction was carried out in HEPES buffer (20mM pH 7.2) in which various concentrations (0-1000µg/ml) of CSR were added with12.5µm ferrous sulphate solution and the reaction was initiated by the addition of Ferrozine (75µM). The mixture was shaken vigorously and incubated for 20 min at room temperature followed by recording of absorbance at 562nm. All tests were performed three times in triplicates. EDTA was used as positive control.

Anti-hemolytic activity

Washed erythrocyte suspensions were prepared as per the procedure of Dacie and Lewis (11). Whole-blood from normal adult male volunteers was drawn using EDTA as an anticoagulant and centrifuged to remove the buffy coat layer. The cells were suspended in 0.14 M NaCl solution and then the packed cells were washed three times with the same solution.Washed erythrocyte suspensions were pre-incubated with 2mM sodium azide for 1 h at 37◦C in a shaking water-bath to inhibit any activity of the enzyme catalase. Equal volumes of erythrocyte suspension in PBS were taken and different concentrations of CSR(0-100 µg/ml) were added 30 min prior to irradiation (250 Gy), and thereafter further incubated for 1 h at 37 ◦C. Untreated control samples contained erythrocyte suspension in PBS (final volume: 1 ml) only, while in CSR control samples has different concentrations of extract. Both these control samples were not subjected to radiation, while radiation control samples

were subjected to radiation (250 Gy). Anti-hemolytic activity of CSR was evaluated in terms of inhibition of membrane degeneration or lipid peroxidation activity (12).

Hydroxyl radical scavenging

Hydroxyl radical scavenging activity was evaluated using deoxy-D-ribose degradation assay(13). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); Hydrogen peroxide (1.0 mM); ascorbic acid (100 μM) and various concentrations (0–100 μg/ml) of the test sample or reference compound. One ml each of Trichloroacetic acid (10% w/v) and thiobarbituric acid (0.5%w/v in 0.025 M NaOH) were added to each sample and re-incubated in a hot water bath (Yorco Instruments, India) at 55 °C for 15 min. The tubes were cooled to room temperature and the absorbance was recorded at 532 nm against the blank with ascorbic acid, as positive control. The percentage inhibition of degradation of deoxyribose or hydroxyl radical scavenging potential was evaluated as follows:

% Inhibition = (O.D. control - O.D. sample/ O.D. control) × 100

Membrane Protection Activity

Soya-lecithin (phospholipid) and cholesterol (1:1 molar ratio) was suspended in appropriate amount of chloroform. A thin film was developed by complete evaporation of chloroform in rotary evaporator (Buchi, USA) at an ambient temperature of 40^{o}C. The film was subjected to hydration in phosphate buffer saline (0.1M, pH = 7.4) incubating it in shaking water bath (40^{o}C) for 4hrs. The stock solution was then diluted using phosphate buffer saline (0.1M, pH = 7.4) to the final concentration of working solution as 5mg/ml (in terms of phospholipids content) (14). Four different groups with liposome only, liposome + CSR, liposome+ CSR +200Gy and liposome + 200Gy were evaluated for the levels of malondialdehyde, an end product of membrane degeneration, was measured at 535nm using standard methodology(15).

RESULTS AND DISCUSSION

Nitric oxide and superoxide anion both can cause ischemic renal injury individually, and if combined, the inflicting damage is significant. The toxicity and damage caused by NO· and O2·is multiplied as they react to produce reactive peroxynitrite (ONOO), which leads to serious toxic reactions with biomolecules including protein, lipids and nucleic acids (16). Suppression of NO· release may be partially attributed to direct NO· scavenging, as observed in case of CSR that exhibits a significant decrease in the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. As shown in **Figure 1** and **Figure 2** Nitric oxide radical scavenging activity was found to be increasing in concentration dependent manner (R^2=0.7825) with maximal activity is 35% at 500 μg/ml. These results showed that hydrophilic antioxidants are abundantly present in CSR able to provide significant free radical scavenging potential.

On the other hand, a dose-dependent site specific hydroxyl radical scavenging (R^2=0.9427) was also observed with significant 62.82% and non-site-specific hydroxyl radical scavenging (R^2=0.9445) was observed with significant 48.95%, inhibition of the formation of MDA at 1000μg/ml (**Figure 4**).

Hydroxyl radical is the most reactive among ROS and it bears the shortest half-life compared with other ROS. Hydroxyl radical scavenging of CSR exhibited significant activity. According to these results, hydrophilic phenolics are dominant in CSR and to which are attributed the radical scavenging properties (12, 17). It also reveals the direct energy impact of radiation exposure leading to generation of hydroxyl radicals from water can be directly targeted. It also indicates inherent ferrous ion chelation activity, further confirmed by CSR exhibiting the inhibition of formation of the ferrozine Fe+ complex in the presence of CSR by 48% (R^2=0.8001) at 1000μg/ml. Ferrozine can quantitatively form complexes with Fe 2% but in the presence of ion chelating agents, thecomplex formation is disrupted, resulting in a decrease in the red colour of the complex. CSR shows higher significant ability in dose dependent manner revealing its anti-amplification potential, another step to inhibit radiation induced free radical cascade.

The TBARS formation inhibitory effect of CSR is significant when compared with the MDA formed in radiation and CSR treated group of liposome (**Figure 5**)(18).

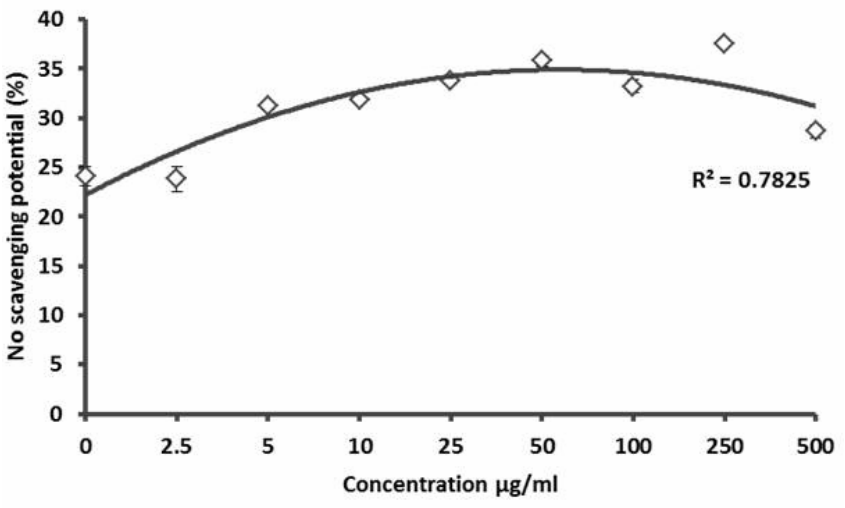

Figure 1. NO scavenging activity of CSR estimated using Griess Agent. * represents maximal activity observed at 50µg/ml. The activity difference was analysed using Student's t-test (p <005).

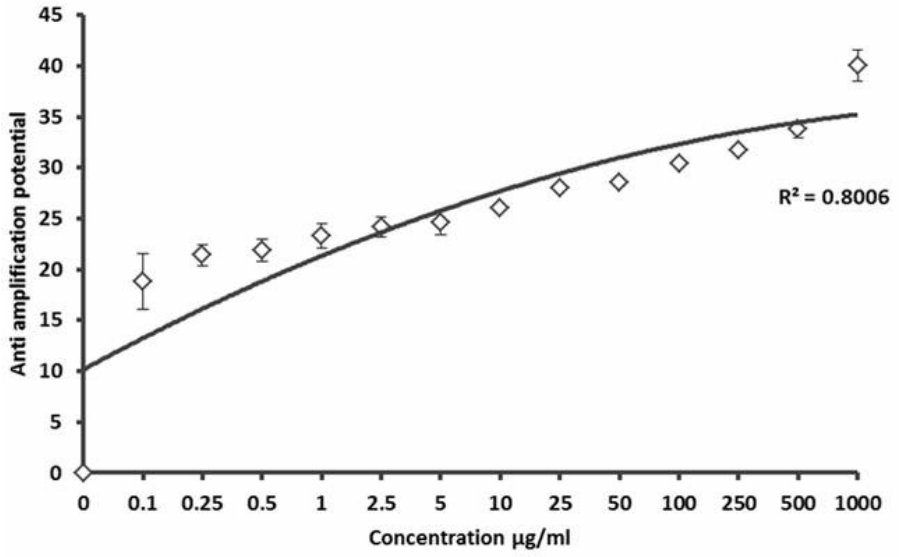

Figure 2. Dose dependent effect of anti-amplification potential of CSR evaluated using Ferrozine. * represents maximal activity observed at highest tested dose. The dose dependent correlation value was found to be R^2 = 0.8006.

The membrane protection of CSR was evaluated using liposomal system at a radiation dose of 200 Gy (dose rate =1.24KGy/hr). It exhibited significantly ($P<0.05$) higher membrane protection potential at all the concentrations (10-250µg/ml) with maximal activity of 40% at 250mg/ml. The role of aryl-tetralinlignans in providing radiation protection by virtue of its membrane protection ability has been reported (19)in coherence with outcome of present study. It indicates inherent protective potential of CSR in cellular milieu. This has been further confirmed by its ability to provide protection erythrocyte against radiation -induced lysis of erythrocytes was monitored and efficacy was tested in the concentration range of 10 – 100 µg/ml. CSR exihibited significant protection against radiation-induced hemolysis (R2=0.9799) 55% at 100µg/ml (**Figure 3**).Management of radiation induced hematopoetic syndrome(3) requires such potential agents that can be used to mitigate radiation impact.

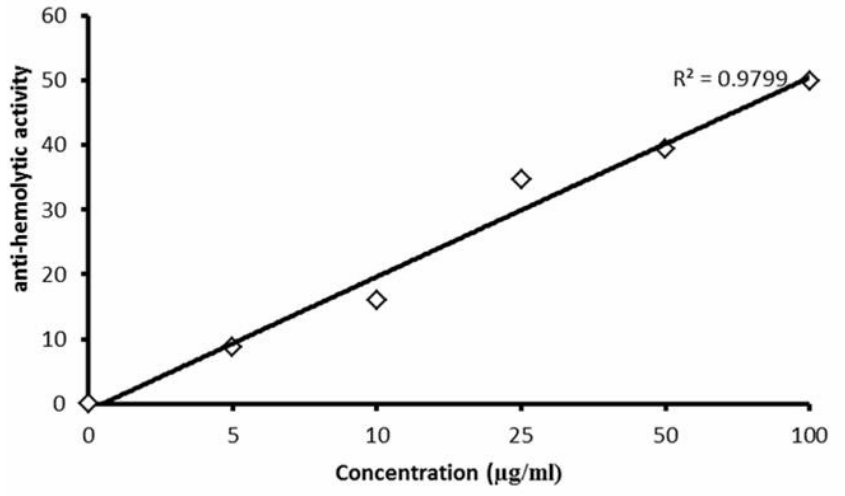

Figure 3. Hydroxyl radical scavenging (site / non-site specific using deoxy D-ribose degradation) of CSR with >90% correlation, significant at p <0.05. * indicates significant difference in site-specific activity as compared to non-site specific activity at p<0.05, evaluated using One-Way ANOVA followed by post hoc test.

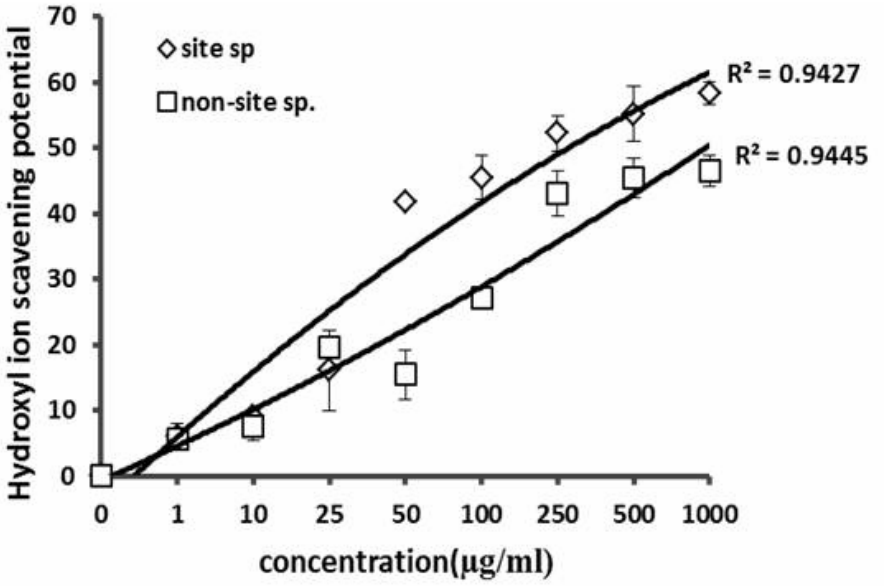

Figure 4. Concentration dependent membrane protection estimated by evaluation of % inhibition of lipid peroxidation. * indicated maximal protection observed at 250µg/ml.

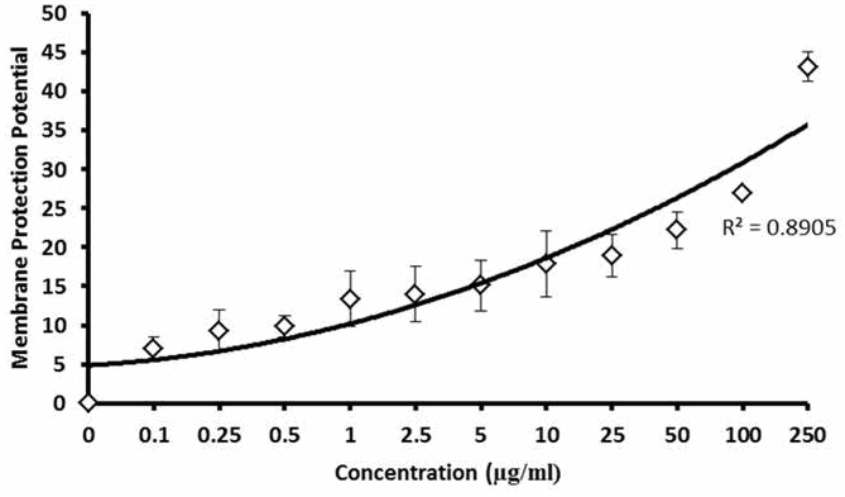

Figure 5. Anti-hemolytic potential of CSR (3.25% polyphenolics) between 0-100µg/ml with maximal protection at highest test dose (100µg/ml).

CONCLUSION

The present study provides an *in vitro* radiation protection potential of CSR and could be utilised to develop lead drug candidate for radiation mitigation research. Another dimension that can be explored in providing augmented care to radiotherapy patients to reduce deleterious side effects of defined radiation exposure. Further studies are warranted at *in vivo* level to ensure replication of such activity potential in radiation mitigation both in pre- and post-disaster scenario.

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