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ORIGINAL ARTICLE

Cobalt-60 lymphocytes immuno-phenotypes/myeloid-lymphoid toxicities and countermeasure effects of aqueous extracts of Parquetina nigrescens, Camellia sinensis and Telfairia occidentalis in guineapigs

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Abstract

Introduction: Radiotherapy is an outstanding and efficacious mode of cancer management. Immune dyscrasia and dyshaemopoiesis in patients being managed with radiotherapy are well documented. Currently, no ideal radio-immuno-haematologic countermeasures in clinical use especially because, of their toxicities at the optimal concentrations exists. This study assessed the countermeasure effects of *Parquetina nigrescens*, *Camellia sinensis* and *Telfairia occidentalis* on immune syndrome in irradiated guineapigs.

Methods: Thirty guineapigs were randomly assigned to nine groups: [**A1**-**A4** (Pre), **B1**-**B4** (Post) and **C** (Control)] where (n = 3)/group for countermeasure studies. Animals were exposed to 4.0 Gy whole-body Co^{60} while extracts were administered twice daily at concentrations of 400 mg/ml, 1000 mg/ml, 900 mg/ml of *C. sinensis*, *P. nigrescens* and *T. occidentalis* respectively. Peripheral whole blood was collected on days (**D**): baseline, D0 [24 hours after radiation], D3, D9 and D14. Haemogram and CD4 were analyzed.

Results: Lymphocyte immune-phenotypes (CD4, Twbc), Abs. Neutrophil and Neutrophil:Lymphocyte ratio (NLR) counts were significantly increased from day 3 to 14 except NLR that was erratic on day 14 ($p = 0.01$). Contrarily, Absolute Lymphocyte counts were significantly decreased from day 3 to 9 then increased significantly on day 14 ($p = 0.00$) with significant NLR similarly on day 14 ($p = 0.02$).

Conclusion: The results indicate a significant decrease in lymphocyte-immunophenotypes in group C as compared to groups A and B,

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suggesting that extracts showed significant ameliorating effects in groups A and B probably by minimizing the activation of ROS/NOS. The leaves' extracts of *Camellia sinensis*, *Parquetina nigrescens* and *Telfairia occidentalis* showed potent counter-effects to radiation-induced haemopoietic and immune dyscrasia syndromes in guineapigs.

Keywords: Cobalt-60, *Parquetina nigrescens, Camellia sinensis*, *Telfairia occidentalis,* Immuno-phenotypes, Myeloid-Lymphoid

Introduction

Cancer remains a leading cause of death globally, which reduces quality of life of the patients and thus represents a large socioeconomic burden[1,2]. Hematopoietic cells are highly sensitive to radiotherapy with relatively low level of exposure resulting into bone marrow failure, potential lethal hemorrhage and overwhelming infections[3]. The damaging effects of radiation on hematopoiesis have been well established[4]. Exposure to ionizing radiation also results in multi-organ dysfunction syndrome (MODS), which can lead to acute radiation syndrome (ARS) or long-term health effects like cancer or pulmonary fibrosis, depending on the radiation dose rate and total dose[5-7]. ARS includes hematopoietic and gastrointestinal (GI) sub-syndromes[8] which manifest as peripheral blood pancytopenia, depletion of bone marrow progenitor cells and impairment of intestinal crypt cell regeneration. Strategies for radiation countermeasure development are based on amelioration of peripheral blood cell depletion, restoration of bone marrow progenitor cells and regeneration of intestinal crypt cells among others[9,10].

The triad of pancytopenia (anaemia, thrombocytopenia, neutropenia) immune dyscrasia and chronic inflammatory process are indicative of hematopoietic sub-syndrome of ARS (H-ARS) that arises mainly due to the profound magnitude of radio-sensitivity of the committed progeny cells[4,7,11]. The rate of reduction in the absolute lymphocyte count following exposure to irradiation (IR) correlates well with cumulative radiation dose[12]. This decline is commonly used as a clinical surrogate marker for whole-body radiation dose[13]. Post-radiotherapy levels of lymphocytes, B lymphocytes, T lymphocytes, CD4+ and CD8+ lymphocytes remained significantly diminished[14] compared to their pre-treatment levels, while CD4+ lymphocytes

tend to recover better than CD8+ cells[15-18].

Also, it is increasingly apparent that neutrophils and other myeloid cell populations play an important role in cancer progression and treatment response. Impairment of adaptive immune responses during chronic inflammation has been postulated to favour promotion of tumour growth, angiogenesis and cancer cell survival[19,20]. Neutrophil-Lymphocyte ratio (NLR) is regarded as a marker of the body's immune response to offending agents. It is also regarded as a rapid and simple parameter indicative of systemic inflammation and stress[21]. High NLR points to a predominance of inflammatory factors in the aetio-pathogenesis of different conditions and predicted poorer overall survival in gastric cancer patients, in certain gynaecological, sensorineural hearing loss, gastrointestinal cancer and cardiovascular diseases[22].

The main specific therapeutic principles and experimental investigations of radiation haemopoiesis deficiencies sequellae have focused on replacement with blood products, the administration of cytokines [G-CSF and GM-CSF], stem cell transplants and bone marrow transplantation[23]. However, such treatments are costly, and are not without certain risks[10]. With the recognition that normal tissue protection during radiotherapy is as important as the destruction of cancer cells, the focus of protection research therefore became more therapy oriented. Despite improvements in supportive care, together with treatment using cytokines such as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), mortality from radiation toxicities remain high[9]. Meanwhile, multiple animal models of the hematopoietic syndrome have been developed to study radiation countermeasures that could enhance survival after total body irradiation (TBI)[6]. Several chemical compounds like 5-androstenediol (5-AED)[12] and angiotensin peptides[13] and botanicals[14] have been screened for their radioprotective and therapeutic potentials. However, high toxicity at optimum protective doses and inability to provide post-irradiation protection precluded their clinical use[24,25].

Recently, researchers have reported that dietary supplementation with a mixture of antioxidants comprised of l-selenomethionine (SeM), vitamin C, vitamin E succinate, α-lipoic acid and *N*-acetyl cysteine (NAC) was effective as a preventative measure prior to total-body X-irradiation or as a treatment after TBI[26]. However, in spite of several studies on radiation countermeasures, FDA has not approved any as safe, cost effective and readily available[26,27]. Numerous studies have demonstrated that extracts of *Camellia sinensis, Telfairia occidentalis* and *Parquetina nigrescens* possess erythropoietic[14,28-30]; anti-inflammatory[31]; antidiabetic[32-34]; hypolipidemic[35]; effective chemo-preventive properties against toxic chemicals and carcinogens[13,36]; anti-sickling[37,38]; nutritive[39] and anti-oxidant potentials[40]; and are non-toxic to haematological and biochemical tissues[41,42]. Our previous studies on the leave extracts of *Camellia sinensis, Telfairia occidentalis* and *Parquetina nigrescens* have established their potential effects in promoting and increasing the proliferation and differentiation of bone marrow haematopoietic stem cells in a dose-dependent pattern[43-47], hence this study.

Materials and methods

Ethical considerations: The study proposal was reviewed and approved by the Ethics and Scientific Committees of our Institute during the sitting of the Faculty Board Meeting on 31st of March 2015 and as recorded in the Minute 1 dated 10/04/2015 and with reference number 4/2015. Also, ethical clearance and approval for the use of guineapigs in this study was given by the University Ethical Review Committee (UERC), University of Ilorin, Ilorin with

reference number UERC/ASN/2018/1109.

Study design: The study is an experimental, interventional and prospective one that was designed to compare benefits of an intervention with no treatment in radiation-induced dyshaemopoietic and immune dyscrasia in an animal model.

Collection, identification, and authentication of plants' materials: Fresh leaves of *Parquetina nigrescens* and *Telfairia occidentalis* were collected from our University Plant Garden while a refined product of *Camellia sinensis* (purity and authentication certified by the regulatory body in Nigeria- National Agency for Food and Drug Administration and Control {NAFDAC}) was procured from an accredited pharmaceutical premises in Ilorin. The plants were identified, authenticated, and respectively assigned voucher numbers by Mr. Bolu-Ajayi, the Curator in the herbarium of the Department of Plant Biology, University of Ilorin, Ilorin. *Parquetina nigrescens* was given Serial Number 876 and Ledger Number 67 while *Telfairia occidentalis* was given Serial Number 959 and Ledger Number 150. Thereafter, collected samples were deposited in the herbarium for future references.

Extraction processes of the plants' materials: Four hundred grams and 350 g of the powdered leaves of *Telfairia occidentalis* and *Parquetina nigrescens* respectively were each soaked in distilled water in closable containers. The finished product (fine granules) of *Camellia sinensis* was also weighed and soaked in distilled water. These were shaken for about 5 minutes and left to extract by means of maceration (shaking the mixture intermittently) at 28 °C for 72 hours. The mixtures were filtered into a porcelain crucible using a fine mesh. The sub-natant was concentrated below 40°C using rotary evaporator and then freeze-dried (Mitsubishi GOT 1000®). The extracts were stored separately at 4°C in freeze-dried form in the refrigerator for subsequent use in the experiment. A stock concentration of 400 mg/ml, 1000 mg/ml, 900 mg/ml of *Camellia sinensis*, *Parquetina nigrescens* and *Telfairia occidentalis* were prepared respectively for subsequent oral administrations into the experimental animals.

Animals procure, handling and care: Healthy male guineapigs of weight range 350 – 400 g were selected for radiation toxicity and radiation counter-measure studies. All Animals were kept in the animal holding of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria. The animals were kept in plastic cages (34 × 47 × 18 cm^3) at animal house of the Department, in an air-conditioned environment with three mice in each cage and one guineapig in each cage. Animals were maintained at room temperature of (25 ± 2) °C with relative humidity (60 % ± 10 %) under REW12 hour night and day-light cycle. They had free access to standard pellets as basal diet and water *ad libitum*. Animals were habituated to laboratory conditions for two weeks prior to experimental protocol to minimize any non-specific stress. All experimental protocols followed the guidelines of our University Ethical Review Committee (UERC), the Guideline for Care and Use of Laboratory Animals, National Institute of Health, Department of Health Services Publication, USA, (NIH, 1985) and European Union Directive 2010/63/EU Guidelines for handling animals used for scientific purposes.

Plants' extracts administration regimen: Animals were broadly categorized into two groups as it relates to treatment pattern, viz; Pre-irradiation groups and Post-irradiation

groups. Pre-irradiation means extracts were administered twice a day, 72 hours before exposure to radiation and continue twice daily after irradiation. Post-Irradiation means extracts were administered twice daily 24 hours after exposure to radiation and continue throughout the period of treatment (14 days).

Animals grouping for irradiation study: Thirty-five male guineapigs of approximately 450 g were obtained from the animal house of University of Ilorin, Ilorin. Thirty were randomly assigned to nine groups (**1-9**) with 3 animals (n=3) per group for countermeasure study. **Group A1:** Irradiated, 1400 mg/kg dose treated with *C. sinensis*; Pre-irradiation, **Group A2**: Irradiated, 4000 mg/kg treated with *P. nigrescens*; Pre-irradiation, **Group A3:** Irradiated, 3500 mg/kg treated with *T. occidentalis*; Pre-irradiation, **Group B1:** Irradiated, 1400 mg/kg dose treated with *C. sinensis*; Post-irradiation, **Group B2:** Irradiated, 4000 mg/kg treated with P. *nigrescens*; Post-irradiation, **Group B3:** Irradiated, 3500 mg/kg treated with *T. occidentalis*; Post-irradiation, **Group A4**: Irradiated, Combine doses of the extracts treated ; Pre-Irradiation, **Group B4:** Irradiated, Combine doses of the extracts treated; Post-Irradiation. **Group C: (Control)**: Irradiated, non-extracts treated **(exposed)**.

Method of irradiation of the guineapigs*:* Irradiation was done at the Department of Radiology, University of Ibadan, Ibadan, Nigeria. The method described by Shittu *et al*., 2019[48] was adopted. Following induction of general anesthesia with intra-muscular ketamine at 5 mg/kg (body weight) and 1 mg Atropine, each guineapig was placed in a cotton-gauze bag and in a position lying on its side. Each animal was given 400r (4.0Gy) whole-body gamma-irradiation under general anaesthesia, using a ^{60}Co therapy unit as a source. The radiation technique is Source Skin Distance (SSD) at the depth of 4 cm and dose rate of 3Gy/1.53 minute.

After-care of the irradiated animals: To minimize the two major post-irradiation complications (i.e., the danger of internal haemorrhage from minor trauma and the risk of infection), resulting from the effects of irradiation on haemopoietic tissues, each irradiated animal were kept in a separate cage and excessive handling avoided until it was due for sacrifice. Each animal was adequately fed and had free access to water supply.

Peripheral blood collection: Two milliliters of venous blood were collected aseptically from the lateral saphenous vein from each animal using the method described by Malene *et al*., 2004 [49]. The samples were dispensed into bottles containing EDTA and analyzed immediately.

Protocol: Laboratory Evaluation: CBC & CD4 Counts*:*

Complete Blood Counts (CBC): The collected blood was well mixed using an automatic spiral mixer that gently rotates sample in the bottle and inserted into Sysmex 2000i machine with 200µL of blood aspirated using the open tube method. The specimen was analyzed, and the counts were displayed on the screen. Total White Blood Cell Counts (Twbcs), absolute neutrophils, Absolute lymphocyte counts, neutrophil/lymphocyte ratio were recorded Pre- and Post-irradiations.

CD4 Count: Analysis was determined by Flowcytometric method using PartecCyflow Counter.

Assay procedure:

1. 20µL of EDTA whole blood delivered to sample tube
2. 20µLof CD4 mAb PE added into the sample and mixed gently
3. Mixture incubated for 15minutes at room

temperature in the dark
4. 80µLof no lyse buffer added and swirl gently
5. CD4 measurement script loaded, and the sample aspirated
6. Start menu was pressed and CD4 result gated.

Data collection, handling and analysis: All relevant data were recorded immediately in a hard copy form, screened and transcribed into an electronic version. Data analysis was performed using Statistical Package for the Social Sciences, version 20. The results were expressed as mean ± standard error of mean (Mean ± SEM), Mean ± standard deviation (Mean ± SD), mean difference and percentage difference. Statistical differences between tests and controls and between pre- and post-irradiation values were calculated using student paired sample t-test and ANOVA. A p value of < 0.05 was considered significant.

Results

Mean difference of Immune Response Parameters between the extracts treated and control groups Pre-Irradiation

Increased mean differences that were statistically significant were observed for CD4 counts, Total White Blood Cells and Absolute Lymphocyte counts in all the treated groups pre-irradiation with an individual extracts and also display of significant positive synergistic effects when combined. Values were expressed as Mean Difference between the test and control (n=5). $P < 0.05$ indicates significant difference (Table 1).

Mean difference of Immune Response Parameters between the extracts treated and control groups Post-Irradiation

Increased mean differences that were statistically significant were observed for CD4 counts, Total White Blood Cells and Absolute Lymphocyte counts in all the treated groups post-irradiation with an individual extracts, so also significant positive synergistic effects when combined. Values were expressed as mean difference between the test and control (n=5). P < 0.05 indicates significant difference (Table 2).

Table 1: Mean difference of immune response parameters between the extracts treated and control groups pre-irradiation

Treatments	Parameters	DAY 0 Mean Diff.	DAY 0 t-test	DAY 3 Mean Diff.	DAY 3 t-test	DAY 9 Mean Diff.	DAY 9 t-test	DAY 14 Mean Diff.	DAY 14 t-test	*p*-value
	CD4	4.97	2.56	11.00	6.10	12.70	8.87	12.73	4.47	0.01
Camellia	TWBC	-0.13	-0.21	0.97	5.21	1.40	2.84	3.70	5.81	0.00
Sinensis	Abs.LYM	-0.01	-0.92	-1.38	-4.84	0.71	1.66	4.49	8.08	0.00
	Abs.NEU	-0.08	-0.28	1.13	4.76	-1.70	-2.47	-1.17	-3.13	0.04
	NLR	0.10	0.94	1.43	13.59	3.36	15.02	0.63	9.92	0.00
	CD4	6.90	3.75	16.53	5.43	14.50	6.92	28.20	21.18	0.00
	TWBC	0.40	0.65	3.33	7.91	1.60	1.83	4.90	3.03	0.04
Parquetina	Abs.LYM	0.00	0.03	-1.67	-6.02	0.17	0.14	0.30	0.46	0.67
Nigrescens	Abs.NEU	0.39	0.74	1.61	3.64	-0.26	-0.83	4.18	3.84	0.02
	NLR	0.29	0.51	2.80	9.32	1.32	6.99	-0.06	-0.87	0.44
	CD4	9.67	3.49	19.20	9.52	23.00	9.60	29.20	23.64	0.00
	TWBC	0.53	0.72	3.17	3.31	5.93	3.26	3.07	5.56	0.01
Telfairia	Abs.LYM	0.03	0.27	-1.26	-3.84	1.65	2.47	5.97	4.00	0.02
Occidentalis	Abs.NEU	0.47	1.15	1.23	8.09	-0.49	-1.03	-0.52	-0.53	0.62
	NLR	0.22	0.61	1.63	4.29	2.75	9.92	0.49	5.62	0.00
	CD4	9.07	5.54	15.90	8.55	20.10	8.36	29.27	24.80	0.00
COMBINED	TWBC	0.40	0.57	1.03	3.30	1.77	1.94	2.67	4.06	0.02
EXTRACTS	Abs.LYM	0.06	1.57	-0.44	-1.09	-0.14	-0.47	1.77	2.88	0.05
	Abs.NEU	-0.07	-0.29	1.25	5.15	1.67	1.86	-0.76	-1.24	0.28
	NLR	-0.08	-0.71	0.77	2.65	1.26	5.00	-0.13	-1.94	0.12

Table showing increased mean differences that were statistically significant were observed for CD4 counts, Total White Blood Cells and Absolute Lymphocyte counts in all the treated groups pre-irradiation with an individual extracts so also display of significant positive synergistic effects when combined. Values were expressed as Mean Difference between the test and control (n=5). $P < 0.05$ indicates significant difference.

Table 2: Mean difference of immune response parameters between the extracts treated and control groups post-irradiation.

Treatments	Parameters	DAY 0 Mean Diff.	t-test	DAY 3 Mean Diff.	t-test	DAY 9 Mean Diff.	t-test	DAY 14 Mean Diff.	t-test	*p*-value
	CD4	0.00	0.00	11.80	6.34	15.50	9.42	20.31	10.33	0.00
	TWBC	0.03	0.12	-0.03	-0.12	1.80	3.43	2.50	5.08	0.01
Camellia	Abs.LYM	0.04	0.91	0.03	0.10	1.03	2.02	2.18	5.27	0.01
Sinensis	Abs.NEU	0.14	0.62	0.24	2.13	-0.86	-2.26	-0.33	-0.41	0.70
	NLR	-0.01	-0.09	0.06	0.93	1.48	10.13	0.28	3.87	0.02
	CD4	0.00	0.00	16.33	5.26	27.97	11.63	33.87	56.53	0.00
	TWBC	0.10	0.34	0.73	2.05	1.67	2.34	3.53	14.16	0.00
Parquetina	Abs.LYM	0.04	0.69	-0.38	-1.15	-0.10	-0.36	2.55	5.03	0.01
nigrescens	Abs.NEU	0.11	0.50	0.46	3.43	-1.25	-2.74	2.41	3.44	0.03
	NLR	0.15	0.76	0.34	2.28	2.43	5.65	-0.15	-2.31	0.08
	CD4	0.00	0.00	17.17	8.60	21.57	6.04	31.67	22.90	0.00
	TWBC	0.03	0.27	0.53	1.49	1.57	3.30	4.33	6.49	0.00
Telfairia	Abs.LYM	0.02	1.01	-0.47	-1.67	1.51	3.81	3.63	6.00	0.00
occidentalis	Abs.NEU	-0.04	-0.23	0.32	2.85	-0.86	-1.57	-0.48	-0.69	0.53
	NLR	0.14	0.69	0.31	3.53	1.02	7.64	0.83	3.19	0.03
	CD4	0.00	0.00	12.50	5.87	19.47	6.61	33.07	33.77	0.00
COMBINED	TWBC	0.03	0.13	0.70	2.94	1.20	2.71	2.93	10.92	0.00
EXTRACTS	Abs.LYM	0.01	0.52	-0.46	-1.25	0.34	0.87	2.22	6.59	0.00
	Abs.NEU	0.02	0.09	0.60	1.86	0.01	0.03	-0.39	-0.55	0.61
	NLR	-0.07	-0.67	0.46	2.49	0.62	6.22	0.12	0.68	0.53

Table showing increased mean differences that were statistically significant were observed for CD4 counts, Total White Blood Cells and Absolute Lymphocyte counts in all the treated groups post-irradiation with an individual extracts and display of significant positive synergistic effects when combined. Values were expressed as Mean Difference between the test and control (n=5). $p < 0.05$ indicates significant difference.

Discussion

Increased mean differences that were statistically significant were observed for CD_4 counts, total white blood cells and absolute lymphocyte counts in all the treated groups pre- and post-irradiation with each extract when administered individually. Also, significant positive synergistic effects were displayed when the extracts were administered into the animals as a polyherbal mixture. More so, decreased mean differences were observed to be statistically significant for absolute neutrophil count and neutrophil/lymphocyte ratio in all the treated groups pre- and post-irradiation. Interestingly, consistent significant mean differences were noticed when *Parquetina nigrescens* and *Telfairia occidentalis* were used as single treatments and even when the three extracts were combined with highest enhancing potentials than when *camellia* only was administered (Tables 1 and 2).

These findings reflect the potent innate immune modulatory effects of the plants in generating and possibility of maintaining anti-tumour immune responses through their interactions with cytotoxic T lymphocytes, B lymphocytes, macrophages and NK cells[50-52]. The effects of the plants on the adaptive immune responses is reflected in the increased number of the absolute lymphocyte count with low absolute neutrophil count and low NLR because elevated NLR could predict poorer survival in cancer patients on radiotherapy[53,54]. In a study conducted by Tadej *et al.*, 2018, the levels of lymphocytes, B lymphocytes, T lymphocytes, CD4+ and CD8+ lymphocytes post-irradiation exposure remained significantly diminished compared to their pre-treatment levels[18,54-56].

This current study however showed that the plants leaves extracts corrected the induced post-radiotherapy lymphocytes diminished syndrome and further lend credence to the local use of these plants for hematologic-immune stimulants[14,43-47,57]. Classically, these results generally were thought to be the *sine qua non* of hematologic-immune responses of these plants with an insight to the possible mechanisms of actions[58], thus, further strengthened our previous findings on the haemopoietic multipotent stem cell proliferation and differentiation[46]. Furthermore, comparative analysis of the outcome between pre- and post-irradiation clusters revealed that administration of the extracts-initiated pre-irradiation had significant advantage over the post-irradiation treatment intervention as like some previous reports[12,59,60].

Conclusion

Our study highlighted that the leaves' extracts of *Parquetina nigrescens, Camellia sinensis* and *Telfairia occidentalis*, showed significant, dose-dependent protection and recovery from radiation-induced haemopoietic and immunosuppression syndromes sequalae radiotherapy, when administered daily pre- and post-irradiation. The extracts also displayed demonstrable synergistic activity when administered pre-irradiation, rather than post-exposure to Cobalt-60 radiation. These findings are novel as no study previously explored the counter-measure effects of these plants either as individuals or in a combined form to Cobalt-60 induced irradiation. It is recommended therefore to explore the extracts' counter-measuring potentials in non-human primates (NHPs) model. This may provide the research community a good understanding of their mechanism(s) of actions and pharmacokinetic profiles before human clinical trials are conducted.

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animals to Cobalt-60 radiation source.

Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors contributions

L.O.O. conceived the original idea, designed the experiments, performed irradiation of the animals, verified the analytical methods, analyzed and interpreted the data, drafted the manuscript and oversaw the entire study. E.G.A. encouraged L.O.O. and supervised the project. A.A.A., F.D.O., S.A.L., S.A.B., K.T.O., M.A.O. and L.I.K. contributed to the design and implementation of the research. S.A.A. assisted in the design and planning of the project, carried out plants collection, processing and preparation of extracts, dosing of the animals with extracts, involved in the irradiation of the animals, and critical review of the original manuscript. All authors provided critical feedback, discussed the results and contributed to the final manuscript.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Disclaimer

A statement that the views expressed in the submitted article are his or her own and not an official position of the institution or funder.

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