



Factors Affecting Radioprotective Efficacy of *Ocimum sanctum* (Tulsi) Extract in Mice

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PKA and BSD designed the study and wrote the protocol. Authors MT and RT carried out the animal studies while authors TP and RM did extraction and phytochemical analyses. The work is based on initial leads of authors PUD, RKS and VG took part in phytochemical analyses as well as in designing radiation protocol. Author RPT was involved in all administrative issues. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Ocimum sanctum* (Tulsi) is well reported for its medicinal values in Ayurveda. Although several chemical radioprotectors have shown excellent protection under *in vitro* conditions, lack of acceptable efficacy under *in vivo* conditions and/or undesirable toxicity has limited their applications in humans. Tulsi leave extract have shown significant normal tissue radioprotection in cell culture and animal models in preclinical studies.

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Aim: This study intends developing a standardized extraction procedure so as to minimize variations in bioefficacy of extracts.

Methods: In the present study, a number of factors related to the extract preparation were found to influence the total yield and orientin (a potential bioactive component responsible for radioprotection) level that included the season of raw material collection, geographical location of plant material, extraction method used.

Results: Although the plasma levels of orientin and radioprotective efficacy varied with the orientin levels in the extract, orientin does not appear to be the sole determinant of radioprotection afforded by the tulsi extract.

Conclusion: These results imply that careful extraction of plant material is a prerequisite for maintaining its bioefficacy and identifying at least one bioactive marker (like orientin here) may help in achieving this to a large extent.

Keywords: Herbal extract; radioprotector; tulsi; extraction method.

1. INTRODUCTION

The applications of ionizing radiation in our day-to-day life are ever increasing, starting from power generation to medical diagnostics and health care, despite its well known deleterious health effects. The most deleterious effect of radiation on the biological system includes damage to the genetic material (DNA) which can lead to strand breaks and oxidative base damages. Under the present circumstances the chances of planned or unplanned radiation exposure has increased considerably due to the increased dependency of radiation in medical practices and increased probabilities of terrorist activities. Planned exposures include predominantly medical diagnostics with external radiation source or internally administered radiotracers and tumour radiotherapy while unplanned exposures can arise from inadvertent human errors of radiation workers, accidents at nuclear power stations, terrorist event involving nuclear or radiological weapons etc. A significant amount of research efforts and resources have been utilized worldwide to protect or ameliorate the radiation effects on biological systems, especially the normal tissue injury to the human subjects [1]. However, till now only Amifostein or WR2721 has been approved by USFDA for use as a radioprotector of normal tissue under close medical supervision owing to its associated toxicity. Therefore there is a growing interest in developing radioprotector from commonly available antioxidants, herbs and other safer alternatives. Encouragingly, majority of the herbal extracts or some of their combinations have shown considerable radioprotective properties in preclinical studies involving cell culture and animal models [2-4]. However none have progressed to nonhuman primate studies or trails in human subjects. One of the main and

important reasons in the case of herbal preparations or formulations from herbal origin is degree of variations in the possible bioactive components in the extract contributed by reasons like variations from batch to batch, season to season, age of the raw plant material, geographical location (influencing soil condition) and extraction methods besides several others including lack of substantial scientific documentation and low acceptability at the global level.

Tulsi or the 'holy basil' (*Ocimum sanctum* L.) has been traditionally used against several common and life threatening ailments in India. Krishna tulsi (a variety of *O. sanctum* with darker leaves) has been extensively studied for its radioprotective efficacy by Uma Devi *et al.*, [1,5-6]. The extract of tulsi leaves has shown significant radioprotection in mice model when administered five consecutive days (once a day for five days) before irradiation. Interestingly, the protection offered by tulsi extract was selective to normal tissue and not compromising the cancer cell killing effects of radiation in tumor cells. Further studies from the same group identified two isolated compounds in the extract, namely orientin and vicenin, which could provide an equivalent degree of radioprotection in mice model [5]. Those two compounds therefore have been suggested as major bioactive principles available in the whole extract responsible for radioprotection.

The current study is a further extension of the earlier study and intends designing a better extract preparation method and administration schedule to achieve maximum radioprotection. The study used tulsi leaves collected from different geographical localities of Coimbatore, Tamil Nadu and during different

seasons. Extracts were prepared using different extraction methods and their characterization was done including the estimation of orientin content in the extract. Their efficacy was studied in mice model and was correlated with the orientin content in the extract as well as the plasma level of orientin in mice administered with the extracts.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The fresh mature leaves of Black Tulsi (*O. sanctum* L.) were collected from the different places in and around Coimbatore (Bharathiar University campus, Palakkad and Mettupalyam), Tamil Nadu, India during the three different seasons: Monsoon, Winter and Summer. The surface pollutants were removed by washing the leaves with distilled water and were shade-dried at room temperature. The dried leaves were powdered using a mixer and were used for solvent extraction.

2.2 Chemicals

All the chemicals used in this study were purchased from Sigma, Mumbai, India.

2.3 Extraction of Tulsi Leaves

The powdered Tulsi leaves were extracted using soxhlet and reflux extraction methods as described below. The extracts were further evaluated for yield, orientin content, bio-efficacy and plasma bioavailability of orientin.

2.3.1 Soxhlet extraction

The dried sample was made into a thimble and extracted with organic solvents such as petroleum ether, chloroform, acetone, methanol and double distilled water using Soxhlet apparatus.

2.3.2 Reflux extraction

2.3.2.1 Successive solvent extraction

The dried powder was extracted with organic solvents such as petroleum ether, chloroform, acetone, ethanol and double distilled water under a refluxing condition in a round bottom flask.

2.3.2.2 Direct solvent extraction

The dried powder was extracted with double distilled water under a refluxing condition.

The different extracts were concentrated by rotary vacuum evaporator (Yamato RE300, Japan) and then air dried.

2.3.2.3 Extract recovery percentage or yield

The extraction yield, a measure of the solvent efficiency to extract specific components from the original raw material, was calculated by the following formula-

$$\text{Extract Recovery (\%)} = \frac{\{\text{Extract yield of plant sample (g)}\}}{\{\text{Total amount of plant sample (g)}\}} \times 100$$

2.4 High Performance Liquid Chromatography (HPLC) Analysis of Orientin

Orientin, a known bioactive principle present in Tulsi, was diluted in a mixture of DMSO and water (1:9 v/v) for use as a standard. All the samples were filtered by 0.45 µm PVDF membrane before injection to HPLC.

The chromatographic separation was performed on a LC-6AD Shimadzu LC (UFLC) Chromatography system equipped with LC-6AD-Shimadzu LC pump system and SPD-20 A UV/VIS Detector (Spinco, USA). The HPLC analysis was performed on a Luna 5u C18 (2)-100A (250x4.60 mm) 5 µl. The mobile phases included Acetonitrile (solvent A) and 1% (v/v) acetic acid in water adjusted pH-3 with sodium hydroxide (solvent B). A gradient elution programme was used for separation as follows: 15% A (15 min), 15–40% A (10 min), 40% A (10 min), 40-0% A (10 min). The flow rate was 1 ml/min. The injection volume was 20 µl while the column temperature was maintained at 40°C. Signal was monitored at 330 nm with the UV/VIS detector [7]. The HPLC equipment was controlled by 'LC' Solution Software version 2.1.

2.5 Bio-efficacy Studies

2.5.1 Extracts and administration

Three treatment schedules were designed for the extract; single i.p dose of 50 mg/kg b.w of mice 30 min before irradiation, single i.p dose of

25 mg/kg 30 min before irradiation and 5 i.p doses of 10 mg/kg b.w in five consecutive days the last injection being at 30 min before irradiation.

2.5.2 Mice and irradiation

C 57 Bl/6 male inbred mice weighing about 20±2 g were maintained in the animal house facility of the institute under standard temperature and humidity conditions. Standard animal feed and free access to drinking tap water was available to the animal. All the experiments used 4 to 6 animals per treatment group with proper control, drug control and irradiation control groups for comparison. Use of experimental animals was strictly accordance with the Institutional Ethics Committee.

Irradiation of animals was performed using a Co60 gamma irradiator (Bhabhatron teletherapy Unit, Panacea Biotech) at a dose rate of 1 Gy/min with the SSD adjusted according to the dose rate in a field size of 35x35 mm. The doses were selected based on the end point of the experiment.

2.5.3 Survival and body weight

Animals were exposed to 10 Gy of whole body γ -radiation and observed daily for death in the next 30 days as described earlier [3]. Their body weight and visible behavioural and physiological changes were also recorded on a daily basis.

2.5.4 Spleen body weight index and endogenous spleen colony forming assay

Animals were exposed to 5 Gy whole body γ -radiation and sacrificed on 11th day for counting visible colonies on their spleen fixed with Bouin's fixative [3]. Spleen weight and body weight on 11th day were considered for calculation of spleen body weight index and was expressed as spleen weight per 100 g body weight [8].

2.6 Estimation of Plasma Orientin

Plasma was collected from blood of animals administered tulsi extracts. The extract doses and time points were based on the results of survival studies. Plasma proteins were precipitated out with methanol [9], centrifuged and the remaining solutions was 0.45 μ m filtered for HPLC analysis. Plasma orientin levels were determined using standard HPLC method on a

Waters HPLC system equipped with water 515 HPLC pump, Waters 717 plus autosampler and waters 2487 PDA detector. Separation was performed in a symmetry C18 250 mmx4.7 mm ID; 5 μ m column at a flow rate of 1 mL/min for the mobile phase 1% acetic acid: Acetonitrile 80:20 (A): methanol (B) as linear gradient run consisting of (A) 80% to 60% in 0-2 min, 60% to 50% in 3-5 min, 50% to 50% in 6-20 min. Each run was followed by a 10 min equilibrium period with 80 (A):20 (B). Absorbance was detected and recorded using a Water 2998 photodiode array from 210 to 400 nm at 1 nm intervals. Chromatograms were extracted at 348 nm (λ max for orientin) to achieve high sensitivity against endogenous compounds in the chromatograms. Respective blank and pure orientin spiked plasma samples were also run for calibration, drawing standard curve and method verification.

2.7 Statistics

Kaplan-Meier survival assay was performed using SPSS software. All other data were analysed using student's *t* test and *P*<0.05 was considered significant.

3. RESULTS

3.1 Yield of Whole Tulsi Extracts

Hot solvent extraction methods (Soxhlet extraction and reflux method) showed higher recovery of the total leaf extract and better extraction of the polyphenolic compounds (Fig. 1). Among the different solvents used for extraction, methanol, ethanol and water showed higher degrees of extraction (Figs. 1a and 1b, Table 1), with the maximum yield (27.56%) in the water extract of leaves from winter followed by summer (13.73%). Since the nature of soil and nutrition are important determinants of plant metabolites, we examined the yield from leaves collected in 11 different regions in and around Coimbatore. The total yield from water extracts of leaves collected in the summer season from four different regions showed a moderate variation with 12% in the leaves from Somayampalyam and 17.5% from Bharathiar as well as Karamadai. The yield was generally lower in the monsoon season with a maximum value of 11.5% and highest in the winter where up to 31% was observed. These results strongly suggest that the yield of total extracts vary significantly with the leaves collected in different seasons, while it varies moderately in leaves from different regions.

3.2 Orientin Content of Tulsi Extracts HPLC Analysis

Since orientin has been suggested as a potential active component responsible for radioprotection from the Tulsi extracts [5,6] we also examined the extracts of leaves from different seasons and regions with respect to their orientin content. Fig. 2 shows typical HPLC finger prints of Tulsi extracts with identified peak of orientin from ethanol extract of leaves collected in winter season. Orientin content in the extracts were estimated from the integrated area under the orientin peak in different extracts and the standard curve generated with the standard orientin in the concentration range of

100-400 µg/ml. Different extraction methods showed significant variations in their orientin content as shown in Table 1. Among the different solvent extractions, the water extract (TWM) showed the highest amount of orientin content (9.91 mg/g). With the reflux method of extraction, successive ethanol extraction (TEW) gave a higher orientin content (7.30 mg/g) than the successive water extract (TWW, 3.30 mg/g) and direct water extract (TWS, 4.15 mg/g).

Based on their orientin content, three water extracts of leaves from three different seasons viz. monsoon (TWM), winter (TWW) and summer (TWS) as well as ethanol extract from winter (TEW) were selected for bio efficacy studies.

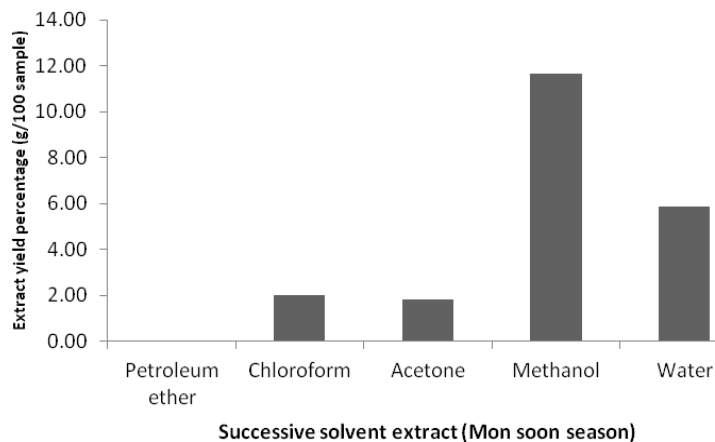


Fig. 1(a)

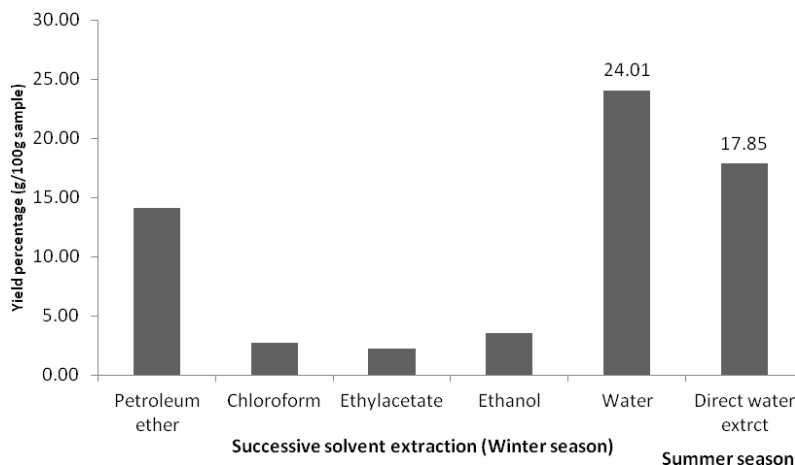


Fig. 1(b)

Fig. 1. Depicts the yield percentages based on extraction method used on Tulsi leaves collected in various seasons. A) Soxhlet method of extraction with successive solvent on leaves collected in monsoon season, B) Reflux method of extraction both successive solvent and direct solvent methods on leaves collected in winter and summer seasons

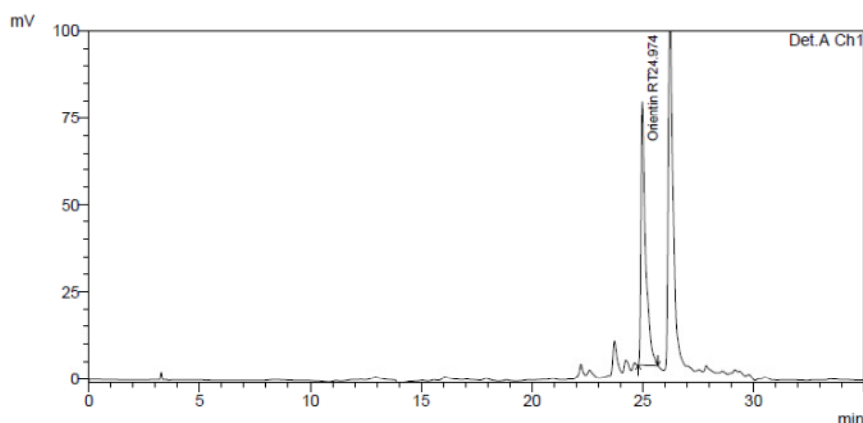


Fig. 2. Depicts representative HPLC chromatograms of ethanolic extract of tulsi leaves collected in winter season (TEW)

Table 1. Yield and orientin content of Tulsi extracts

Samples	Solvent	Method of extraction	% Yield (g)	Orientin (mg/g extract)
TWM ¹	Water	Soxhlet extraction	5.87	9.91
TEW ²	Ethanol	Reflux extraction	3.5	7.30
TWW ³	Water	Reflux extraction	27.56	3.30
TWS ⁴	Water	Reflux extraction	13.73	4.15

Extract yield per 100 g of powdered sample and the orientin content in the extract measured by HPLC method are depicted for the extracts selected for further bioefficacy studies. ¹TWM: Tulsi water extract- Monsoon season, ²TEW: Tulsi ethanol extract- Winter season, ³TWW: Tulsi water extract-Winter season, ⁴TWS: Tulsi water extract- Summer season. It may be noted that soxhlet extraction method with water as solvent resulted in highest orientin content with moderate yield while reflux extraction with ethanol also resulted in high orientin content but the overall yield was less

3.3 Bio Efficacy Studies

Our earlier studies have shown significant protection (70% survival) against lethal radiation dose of whole body irradiation with aqueous extracts of Tulsi leaves (10 mg/kg b.w.) from Southern India when administered for 5 days before irradiation [6]. More recently we observed that a single administration of the extract from Tulsi leaves of plant growing in Mana Hills (in the Himalaya region of India) at a dose of 50 mg/kg offered 75% (Data not shown). Therefore, we evaluated the efficacy of three dosage regimen viz. 25 and 50 mg/kg b.w. 30 min before irradiation, and 10 mg/kg b.w. for 5 days before irradiation. Table 2 summarises the results of bio efficacy in terms of animal survival at lethal radiation dose (10 Gy; 0% survival), CFUs, body weight and spleen body weight index. The water extract from monsoon season (TWM) at 50 mg/kg b.w administered 30 min before irradiation was most effective with 57% survival (Table 2, Fig. 3a) with an overall increase of nearly 8% in animal body weight (Table 2, Fig. 4a). The dosage of 25 mg/kg b.w. was effective only for TWM while in all other extracts animals survived

for 17 to 20 days (Fig. 3a). The CFUs number remained high (37.33) as well as the spleen body weight index, which was 479.89 (Table 2). Although the extract from winter leaves (TWS) gave 30 days survival (50%) comparable to monsoon (TWM) (Fig. 3b, Table 2), it was associated with a nearly 17% body weight loss (Table 2), low spleen body weight index of 312.5 (Table 2) and low CFUs number of 11.66 (Table 2).

3.4 Estimation of Plasma Orientin

In order to examine if differences in the bio-efficacy (radioprotection) was related to the variations in the levels of the proposed bioactive component (orientin), we estimated the plasma orientin levels following the treatments (except for 25 mg/kg b.w that did not afford significant protection compared to other dosage schedules, Table 2). The plasma level of orientin in the mice blood 30 min after the last administration (i.e. the time of exposure to radiation) of the extract was studied by HPLC method in un-irradiated mice. The results are summarised in Table 3 and representative HPLC chromatogram shown in

Fig. 5. The TWM extract resulted in a plasma orientin level of $8.95 \pm 0.79 \mu\text{g/ml}$ 30 min after administration compared to $4.60 \pm 0.36 \mu\text{g/ml}$ for extract of TWS at the same time and dose. The plasma orientin levels corroborated well with the animal survival and also the orientin levels in the extract (9.91 mg/g for TWM) and (4.15 mg/g for TWS; Table 1).

4. DISCUSSION

The objective of extracting phenolic compounds from their plant sources is to release these compounds from the vacuolar structures where they are found, either by rupturing plant tissue or by a diffusion process [10]. Normally, a high extraction yield is required for an efficient process, although it will not necessarily ensure a high concentration of bioactive components.

Since, some bioactive components are very sensitive to oxygen and heat [11], more care should be taken to prevent their oxidation and thermal degradation. Therefore, the extraction yield and the bioactive components characteristics should also be considered when an extraction method is selected. Classical techniques for the solvent extraction of nutraceuticals from plant matrices are based on the choice of solvent coupled with the use of agitation and/or heat. Many factors such as solvent characteristics, ratio of solvent and sample, extraction time and temperature, should be taken into account when the extraction method is chosen [12]. Selection of a suitable solvent is the most important step in optimizing the recovery of desire components from a complex matrix.

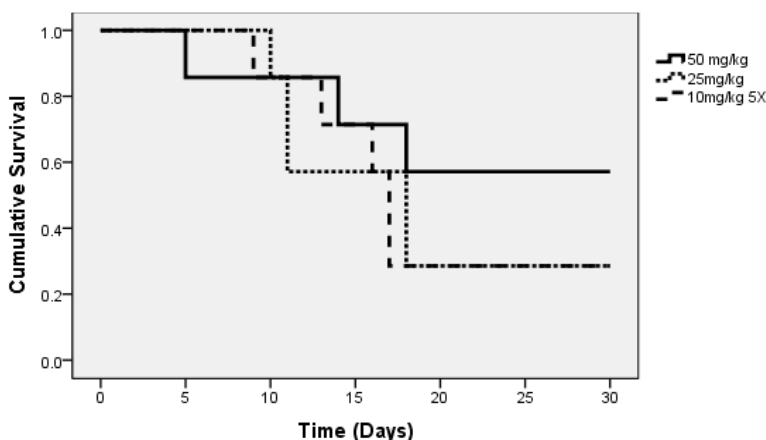


Fig. 3(a)

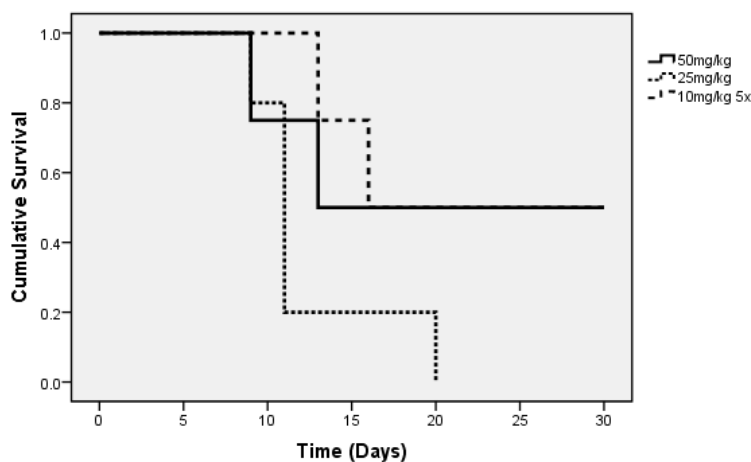


Fig. 3(b)

Fig. 3. Depicts the Kaplan Myer survival plot of mice after 10 Gy whole body gamma irradiation: A) TWM and B) TEW

Table 2. Bioefficacy evaluation of various Tulsi extracts

Samples	Dose (mg/kg)	Survival (%)	Body weight change (%)	Spleen body weight index (mg/100 g)	CFU-s count
TWM ¹	25	25	-4.64	421.38±28.12	7.66±2.34
	50	50	8.03	479.89±32.11	37.33±7.17
	5x10	25	7.9	444.26±30.24	24.33±7.67
TEW ²	25	0	na	370.75±25.25	6.33±1.17
	50	50	8.18	471.10±35.15	12.66±2.33
	5x10	50	6.26	465.63±33.17	10.66±2.84
TWW ³	25	0	na	446.47±24.03	16.66±3.14
	50	25	10.11	224.61±10.09	19.33±4.77
	5x10	50	0.44	333.17±32.33	14.00±3.50
TWS ⁴	25	0	na	391.10±20.41	5.50±1.50
	50	50	-17.64	312.50±13.50	11.66±2.33
	5x10	25	-11.53	331.15±11.07	9.66±2.33

Bioefficacy in terms of 30 day survival, change in body weight over 30 days against lethal whole body 10 Gy irradiation, spleen body weight index and endogenous CFU-s in 5 Gy whole body exposed mice were studied as described in material and methods section. A negative sign indicated reduction in body weight and NA indicates no animals surviving for estimating body weight change. ¹TWM: Tulsi water extract-Monsoon season, ²TEW: Tulsi ethanol extract- Winter season, ³TWW: Tulsi water extract- Winter season, ⁴TWS: Tulsi water extract-Summer season

Table 3. Plasma content of orientin in mice treated with Tulsi extracts

Samples	Dose (mg/kg b.w)	Mean±SE (µg/ml)
TWM ¹	5x10 mg	1.62±0.14
	50 mg	8.95±0.79
TWS ²	5x10 mg	1.15±0.097
	50 mg	4.60±0.36

Mice were administered different doses of Tulsi extracts and plasma orientin level at 30 min post-treatment was evaluated through HPLC method

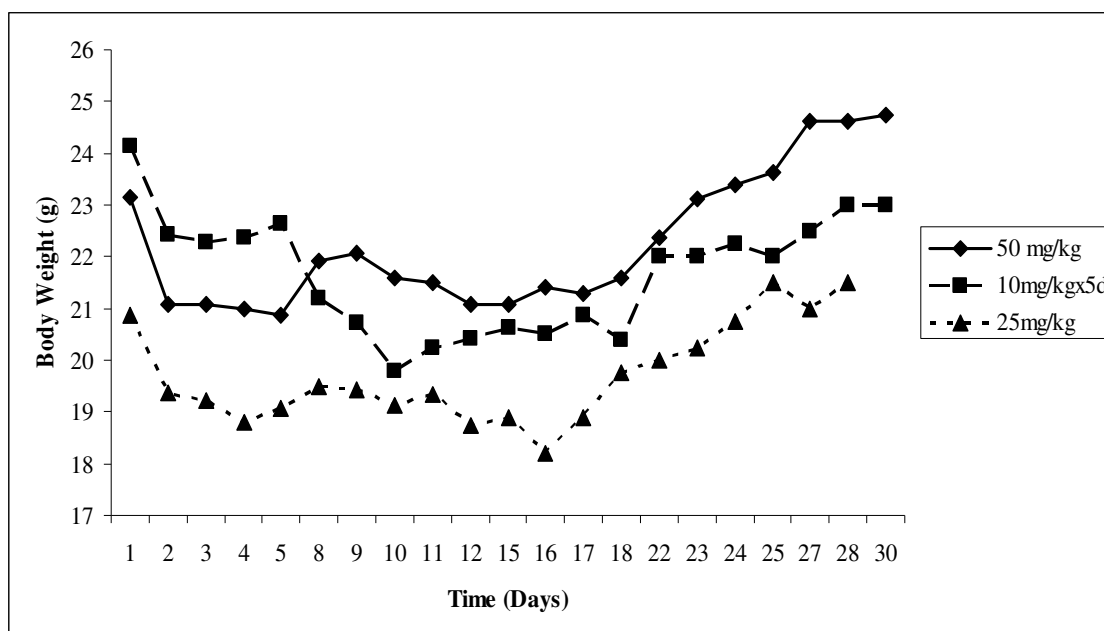


Fig. 4(a)

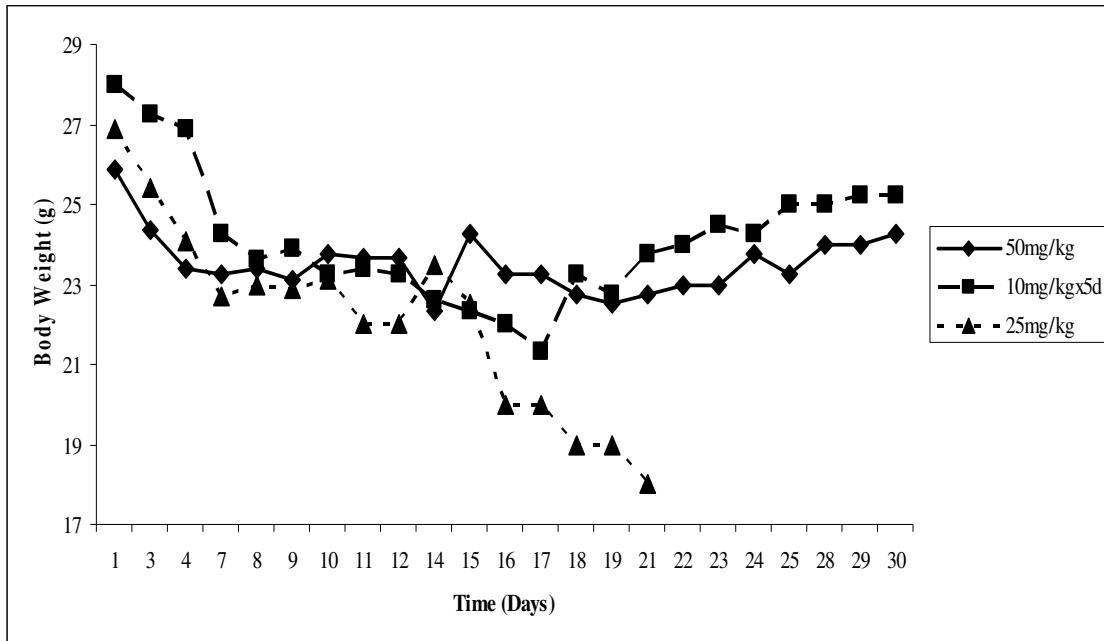


Fig. 4(b)

Fig. 4. Depicts the change in body weight for 10 Gy whole body irradiated mice during 30 days of observation treated with different doses of tulsi extract: A) TWM and B) TEW

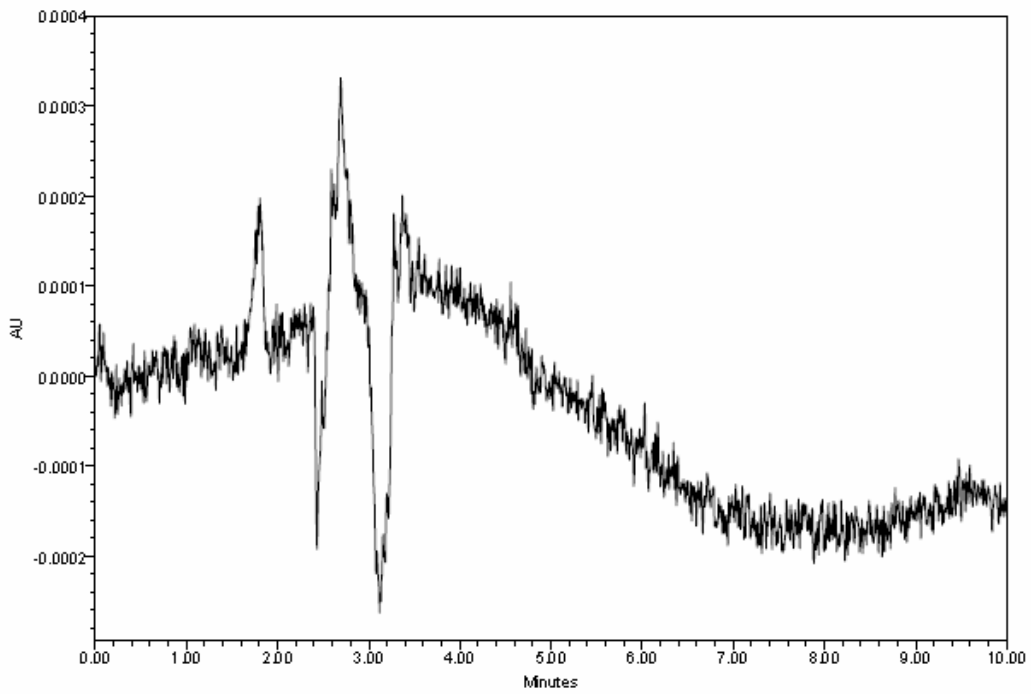


Fig. 5(a)

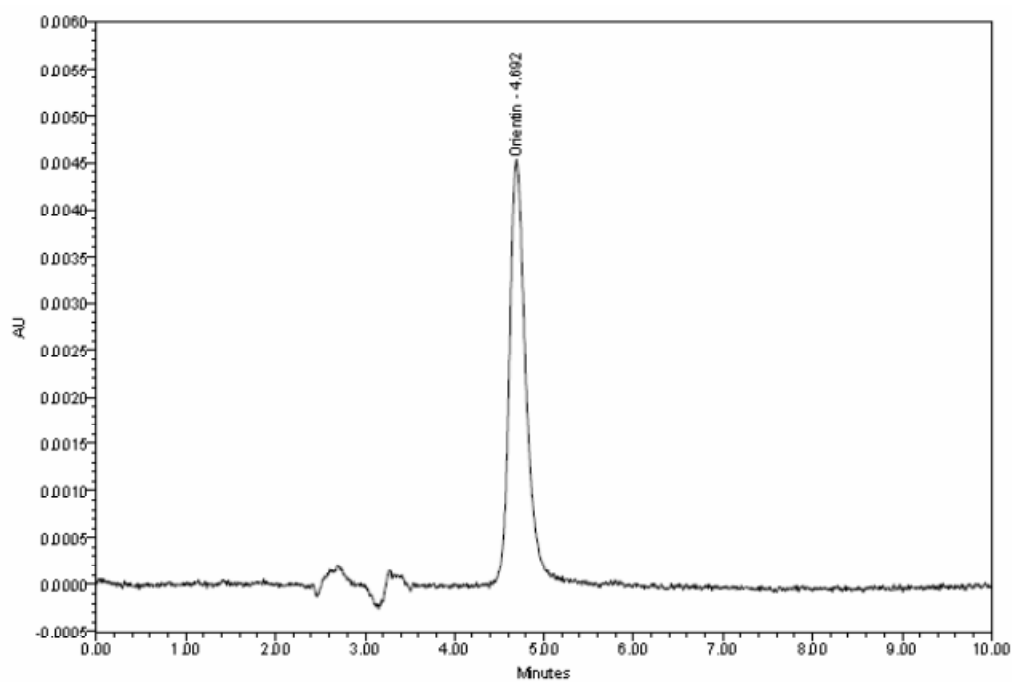


Fig. 5(b)

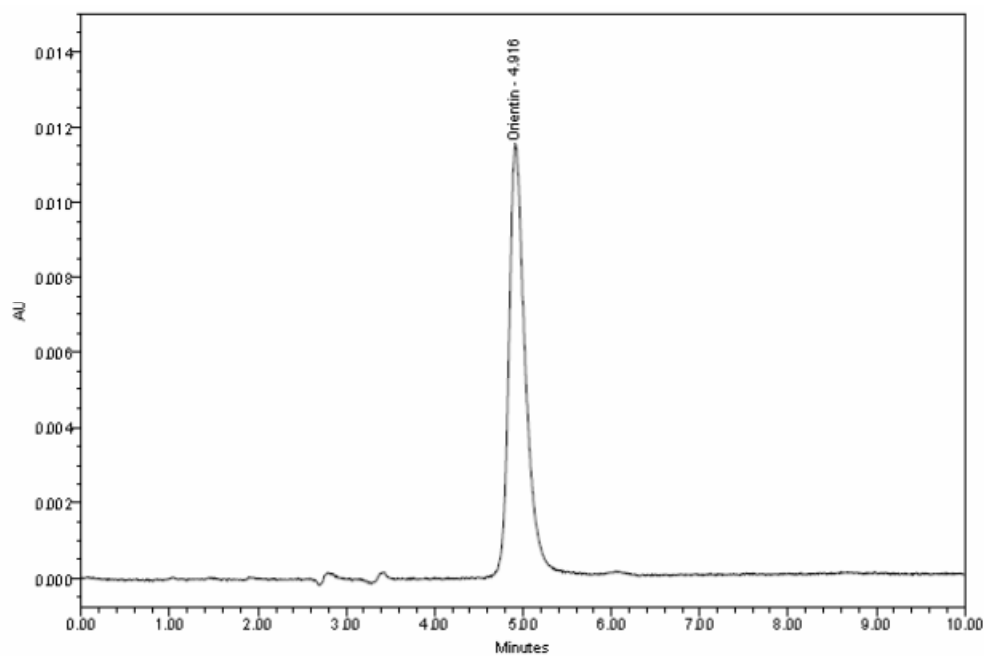


Fig. 5(c)

Fig. 5. Depicts the HPLC chromatograms of mice plasma with different treatments: A) plasma of control mice with no orientin, B) mice plasma spiked with standard orientin and C) mice plasma from animal treated with tulsi extract (TWM)

In the present study, water and alcoholic extracts showed higher yield of the total extract, which are also well known for their effectiveness in extracting polyphenolic compounds from plant extracts [13]. Amongst the polyphenols, Orientin is one of the major flavonoids found in alcohol and water extracts of Tulsi leaves suggested to be largely responsible for radioprotection [6]. It appears that different extraction methods lead to significant differences in the levels of polyphenolic compounds like orientin that could be recovered from the tulsi extracts (Fig. 1) and therefore, the choice of extraction method(s) should be based on the source of the material and components of interest for a particular bio-effect. Radioprotection, the focus of bio-efficacy in the present studies showed higher survival rate against radiation induced mortality with extracts having higher orientin content. The alcoholic extract having a higher orientin level, though showed a considerably higher survival rate equivalent to its aqueous counterpart, was not able to produce a higher CFUs and also showed a significant weight loss of about 17% body weight over 30 days of observation period. Furthermore, aqueous extract has an additional advantage from the therapeutic view point as it is widely accepted as a drug unlike the alcohol preparation that has associated toxicity and other application issues. Therefore, the aqueous extract of tulsi appears to be a better candidate as a radio-protective agent than the alcoholic extract despite having equivalent or higher orientin content, as reported earlier [6]. The extent of weight loss and CFUs induction may also be dependent on other bioactive components present at a sufficient level in the aqueous extract compared to the alcoholic extract, which needs further investigation. The plasma level of orientin appears to be independent of the solvent used for extraction, as the plasma levels observed were nearly proportionate to the levels in the crude extract. Interestingly, a dose of 50 mg/kg body weight of the extract could build up a higher plasma orientin level (Table 3), which was nearly 5 times of 10 mg/kg extract given over 5 days, justifying the single injection protocol.

5. CONCLUSION

Taken together, results of the present studies indicate that the method of extraction, the choice of solvent, the geographical location of the raw plant material (that determines the nature of the soil and nutrition, besides the associated microbes) and the season of collection of Tulsi

leaves may have significant influence on the bio efficacy (radioprotection) of the preparation. However, appropriate bioactive principles can be used as internal standards for optimizing the overall preparation for intended bio efficacy, like for example the orientin level in the present studies with protection against radiation-induced lethality as an end point. Since the aqueous extract resulted in a better recovery of the animal weight in addition to 30 day survival similar to alcoholic extract, a reliable signature of aqueous extraction coupled with orientin level can together serve as markers for ensuring reproductive bioefficacy. A more detailed study on the minimum content of the bioactive principle(s) in the extract itself and the plasma level of the bio-effective component correlating the extract bioefficacy from lot to lot can be useful in optimizing the preparation.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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