Antioxidant capacity and radioprotective properties of the flavonoids galangin and kaempferide isolated from *Alpinia galanga L.* (Zingiberaceae) against radiation induced cellular DNA damage

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ABSTRACT

Background: *Alpinia galanga* L. belonging to the family Zingiberaceae is widely grown in the state of Kerala, India. They are effective antioxidant and free radical scavenger under both *in vitro* and *in vivo* condition. The efficacy of the isolated flavonoids in conferring protection from radiation induced damages to genomic DNA was studied. ***Materials and Methods*:** The bioflavonoids, galangin and kaempferide were isolated from the AE fractions Alpinia galanga. The isolated flavanoids - galangin and kaempferide, and the crude extracts (AE and EE) were assayed for their various free radical scavenging activities like DPPH (1,1-diphenyl-2-picryl-hydrazyl), hydroxyl and superoxide radical scavenging activities. The *in vitro* DNA damage was monitored by assessing the radiation induced relaxation of supercoiled plasmid DNA (pBR 322). Damage to cellular DNA of human peripheral blood leukocytes induced by γ-radiation (4 Gy) was monitored by alkaline single cell gel electrophoresis or comet assay. ***Results:*** The extracts and pure compounds scavenged hydroxyl radicals in a concentration dependent manner. The compounds demonstrated a concentration dependent scavenging capacity by neutralizing the superoxide radicals. A considerably lower concentration (400-1000 ppm) of the pure flavonoids showed good antioxidant capacity. The presence of 10 mM kaempferide helped in reducing the extent of DNA damage following 4 Gy gamma irradiation ($P<0.001$). Galangin (10 mM) also facilitated in reduction of comet parameters. ***Conclusion:*** The extracts of *Alpinia galanga* or the isolated bioflavonoids - galangin and kaempferide can protect DNA from radiation induced lesions resulting from radiation exposures under *in vitro* and *ex vivo* conditions.

Keywords: *Alpinia galanga, Radioprotection, DNA damage, Plasmid relaxation assay, Comet assay*

INTRODUCTION

The deleterious effects of ionizing radiation in biological systems arise directly (interaction between radiation and target macromolecules) or indirectly (mediated by the generation of free radicals) [1, 2]. The generated free radicals and related reactive oxygen species (ROS) damage vital cellular targets. The most important target

in a living cell affected by ionizing radiation is genomic DNA. The radiation induced damages to DNA may lead to cell death and increase the risk for numerous genetically determined diseases like cancer. Efforts to reduce toxicity to normal tissues and organs led to the search for radiation-protecting drugs and compounds with potential application during planned radiation exposures such as radiotherapy, diagnostic scanning, undertaking clean-up operations in nuclear accidents, space expeditions, etc and unplanned radiation exposures such as accidents in nuclear industry, nuclear terrorism and natural background radiation [3]. Many compounds with antioxidant activities are proved to be effective radio protectors [4,5] and since flavonoids and other polyphenolic compounds possess antioxidant activity the interest in these molecules as radioprotector is increased [3,5,6,7]. Flavonoids are a class of plant secondary metabolites, present ubiquitously in fruits, vegetables, and beverages. They have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens.

The present report summarizes free radical scavenging, antioxidant and radioprotective activities of two important flavonoids, viz., galangin (3,5,7-Trihydroxyflavone) and kaempferide (4'-Methylkaempferol) from *Alpinia galanga* L. rhizome.

MATERIALS AND METHODS

Chemicals

pBR 322 DNA was obtained from Bangalore Genei, India. Folin-Ciocalteu phenol reagent, Nitro blue tetrazolium (NBT), Ethylene diamine tetra acetic acid disodium salt (EDTA) were purchased from Sisco Research Lab, Mumbai, India. DPPH$^{\bullet}$(2,2-diphenyl 1- picryl hydrazyl) was purchased from Sigma Aldrich. All other chemicals were of analytical grade procured from reputed Indian manufacturers.

Irradiation

Irradiation was carried out using a ^{60}Co-Theratron Phoenix teletherapy unit (Atomic energy Ltd., Ottawa, Canada) at a dose rate of 1.88 Gy per minute.

Plant material and extraction

Alpinia galanga L. belonging to the family Zingiberaceae is obtained from the Ayurvedic Research Institute (Poojappura, Trivandrum, Kerala, India) and was identified by their resident botanist. For the isolation of major constituents from the rhizomes of *A. galanga*, a total of 250 g of the coarsely powdered dried rhizomes were first extracted with acetone (3 × 1000 mL) at room temperature (27 °C) (we observed that crude extract obtained by extraction with acetone carried out using Soxhlet apparatus tended to polymerize during concentration procedure). The solvent was removed under reduced pressure at 40°C to get 6.8 g of the crude acetone extract. 0.5 g of this extract was kept for antioxidant assays. 6.3 g of the acetone extract was subjected to silica gel column chromatography (100-200 mesh) using increasing polarites of hexane–ethyl acetate (EtOAc-Hexane) mixture.

In order to obtain the ethanol extract, 100 g of dried and powdered rhizomes were extracted with ethanol (2000 mL, 24 h) using a Soxhlet apparatus. This extract was found to be stable and was used as such for antioxidant assays.

Phytochemical Screening

Total phenolic constituents in the ethanolic and the acetone extract of *Alpinia galanga* rhizomes was analyzed by employing the method of Slinkard and Singleton (1977) [8]. Using gallic acid as a standard, the total phenolic content of the ethanol and acetone extracts of *Alpinia galanga* rhizomes has been calculated and expressed as gallic acid equivalents (mg GAE/100 g dry rhizomes). Total flavonoid content was determined according to a colorimetric method [9] employing quercetin as the standard.

Assay for total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum

method [10] using ascorbic acid was used as the standard.

Free radical scavenging activity of ethanolic and acetone extract of Alpinia galanga

In order to determine the free radical scavenging activity of ethanolic and acetone extract of *Alpinia galanga,* the following parameters were assayed. Superoxide radical scavenging activity was done by the NBT (nitroblue tetrazolium) reduction method [11]. The deoxyribose method was used to study the hydroxyl radical scavenging property of the samples [12]. Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. DPPH radical scavenging was determined by the method of Sanchez-Moreno *et al.* (1998) [13] with minor modifications.

Isolation of compounds

The acetone extract was subjected to silica gel column chromatography and eluted with EtOAc-Hexane mixtures of increasing polarity starting from (9:1) to (1:9). The fractions were pooled according to the similarities in their thin layer chromatography plates (TLC's). Fractions 51-77 were combined and evaporated to get 1.5 g of a solid which on crystallization from chloroform–methanol yielded galangin (compound **1**) as yellow flakes (1.398 g). Fractions 78–94 were combined and evaporated to yield a solid (0.150 g) which on crystallization from chloroform–methanol yielded kaempferide (compound **2**) as pure yellow crystals (0.108 g) (figure. 1).

Effect of Galangin and Kaempferide on γ-radiation induced DNA damage

Plasmid DNA in phosphate buffer (0.1 M, pH 7.4) (100 ng) was exposed to 25 Gy γ- radiation in presence or absence of 10 mM flavanoids (*galangin or kaempferide*), on ice. After irradiation plasmid DNA was electrophoresed on 0.8% agarose in 0.08 M Tris borate/0.2 mM EDTA buffer (pH 8.3) at 55 V, 75 mA for 2 hours and the DNA damage was analyzed by Digital Gel Documentation and Analysis Software, Biotech R&D Laboratories, Yercaud, Tamil Nadu, India.

Human blood samples were collected from 3 healthy non-smoking volunteers. The blood samples were exposed to 4 Gy gamma-radiation at ambient temperature in presence or absence of 10 mM of galangin or kaempferide. The blood samples were subjected to alkaline single cell gel electrophoresis. The DNA strand breaks in human peripheral blood leucocytes were measured using alkaline single cell gel electrophoresis performed using method given by Chandrasekharan *et al.* (2009) [1]. The comets were visualized after silver staining [14]

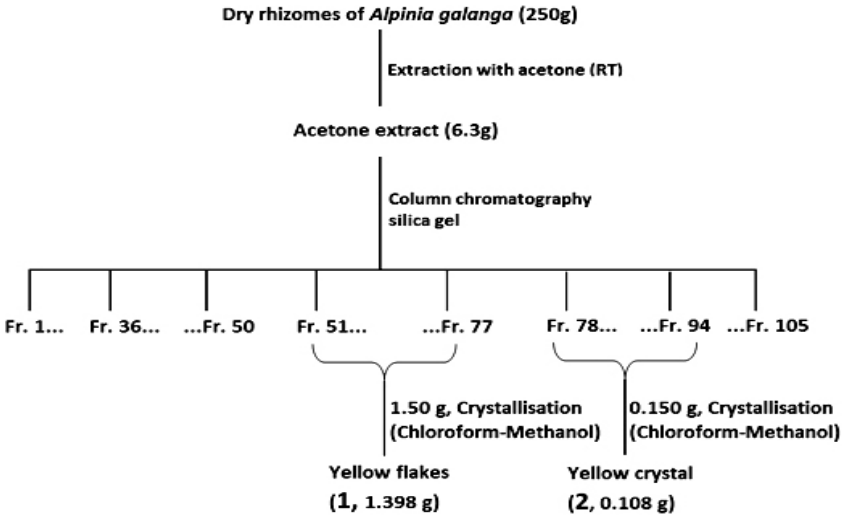

Figure 1. Pictorial representation of isolation of Compound 1 (Galangin) and Compound 2 (Kaempferide).

and the images captured were analyzed using the software 'CASP' which gives % DNA in tail, tail length, tail moment and olive tail moment directly. The parameter tail moment (TM) is the product of tail length and % DNA in tail and olive tail moment (OTM) is the product of the distance between the centre of the head and the centre of the tail and % DNA in tail [15]. The results are presented as mean ± SD of the studied groups. Statistical analyses were performed using ANOVA with Tukey – Kramer multiple comparisons test.

RESULTS

Phytochemical Screening

The total amount of phenolics in *A. galanga* showed 1.93 ± 0.7 g GAE /100 g of dry rhizomes in the ethanol extract and 2.17 ± 0.3 g GAE /100 g of dry rhizomes in the acetone extract. The total flavonoids content expressed in grams as the number of equivalents of quercetin for the ethanolic extract of the rhizomes of the *A. galanga* was 3.3 ± 0.9 and the acetone extract contained 0.01 ± 0.004.

Total antioxidant capacity

The ethanol extract of *Alpinia galanga* rhizomes showed antioxidant capacity of 9.6 ± 1.6 g ascorbic acid equivalents/100 g dry weight of the rhizomes whereas the acetone extract showed a higher antioxidant capacity of 11.1 ± 4.8 g ascorbic acid equivalents/100g dry weight of the rhizomes.

Antioxidant activity of A. galanga ethanol extract (EE), acetones extract (AE), galangin and kaempferide

The antioxidant capacity of the extracts and pure compounds against three reactive species, *viz*., the superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$) and DPPH free radicals (DPPH$^{\bullet}$) were studied by comparing with the standard antioxidants.

The compounds demonstrated a concentration dependent scavenging capacity by neutralizing the superoxide radicals. A considerably lower concentration (400-1000 ppm) of the pure flavonoids showed good antioxidant capacity and their activity was compared with the standard flavonoid, quercetin. Kaempferide showed the highest superoxide scavenging property with an EC_{50} value of 868 ppm compared to galangin (EC_{50} value, 903 ppm) and Quercetin (EC_{50} value, 1560 ppm). At this concentration, the extracts did not show any superoxide radical scavenging capacity. Therefore the activity of AE and EE were evaluated at higher concentrations (2000-8000 ppm) along with Tocopherol as the standard. Of these, tocopherol showed highest superoxide scavenging capacity with an EC_{50} value of 3663 ppm whereas EE and AE showed EC_{50} value of 7033 ppm and 10509 ppm respectively (table 1).

The extracts and pure compounds scavenged hydroxyl radicals in a concentration dependent manner. The activity was compared with that of the standards, butylated hydroxy anisole (BHA) and quercetin. The high hydroxyl radical scavenging activity of the flavonoids and the extracts could be attributed to the

Table 1. The antioxidant capacity expressed as EC_{50} values of the AE, EE, galangin, Kaempferide and the standards.

Sample	Radical scavenging capacity, EC_{50} (ppm)		
	Superoxide	Hydroxyl	DPPH$^{\bullet}$
Alpinia galanga ethanol extract (EE)	7033	1.51	895
Alpinia galanga acetone extract (AE)	10509	1.70	851
Galangin	903	1.40	442
Kaempferide	868	1.41	541
BHA	-	1.39	139
Tocopherol	3663	-	-
Quercetin	1560	2.00	1.67

active hydrogen donating ability. All the tested samples showed more or less the same EC_{50} values ranging from 1.39-1.51 ppm (table 1).

The stable free radical DPPH with characteristic absorption at 515 nm was reduced by the flavonoids, galangin and kaempferide resulting in decrease in the absorption, which is directly related to the electron scavenging capacity of the flavonoids (table 1).

Isolation of galangin (1) and kaempferide (2)

Based upon the observation that the acetone extract of *A. galanga* showed higher phenolic content, higher flavonoid content as well as high total antioxidant capacity, the flavonoids galangin (**1**, 1.398 g) and kaempferide (**2**, 0.108 g) were isolated and characterized. The compounds isolated corresponded to different spectral values with that reported in the literature [16] and found to possess the same melting points as galangin (214-215 °C) and kaempferide (198-199°C) [17]. The concentration of galangin and kaempferide in the rhizomes of *A. galanga* were 5.592 g/kg and 0.432 g/kg of the plant material respectively. The structures of the isolated compounds are given in figure 2.

Effect of Galangin and Kaempferide on γ-radiation induced DNA damage

The agarose gel electrophoresis pattern of pBR 322 DNA exposed to 25 Gy γ-radiation in the presence or absence of 10 mM galangin, and kaempferide is given in figure 3(a). Exposure of plasmid pBR 322 DNA to gamma radiation resulted in strand breaks by which the super coiled/ covalently closed circular form (ccc) of plasmid DNA was converted to open circular (oc) form or linear form. The disappearance of ccc form of plasmid DNA following exposure to gamma radiation can be taken as an index of DNA damage induced by radiation. Both the flavonoids offered protection to the plasmid DNA against radiation induced strand breaks as can be seen in figure 3(b).

The results of comet assay performed on human blood leukocytes irradiated *ex vivo* in the presence or absence of 10 mM kaempferide or galangin is shown in figure 4. Exposure of human peripheral blood leucocytes to 4 Gy gamma radiation caused damage to the cellular DNA as evident from the increase in the comet parameters. Percentage DNA in tail, tail length, tail moment and Olivetail moment were increased from 3.56±0.90, 3.15±0.53, 0.12±0.04 and 0.51±0.24 to 11.10±2.77, 9.31±0.73, 4.26±0.40, 2.65±0.80 in the control irradiated group. The presence of 10 mM kaempferide helped in reducing the extent of DNA damage as can be seen from the comet parameters which were brought down to 4.87 ± 0.58, 4.41±1.65, 0.45±0.16, and 0.47 ±0.04 respectively ($P < 0.001$). Galangin (10 mM) also facilitated in reduction of comet parameters to 6.07 ±0.52, 7.28 ±1.55, 0.55 ±0.14 and 0.53 ±0.17 respectively.

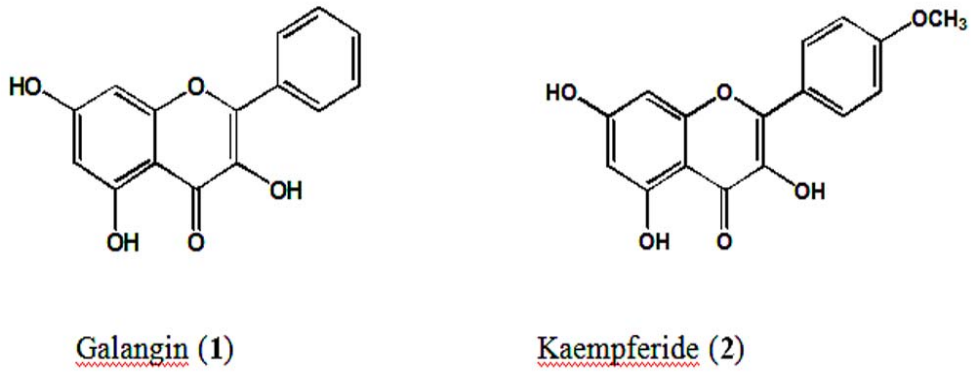

Figure 2. Structure of galangin (**1**) and kaempferide (**2**).

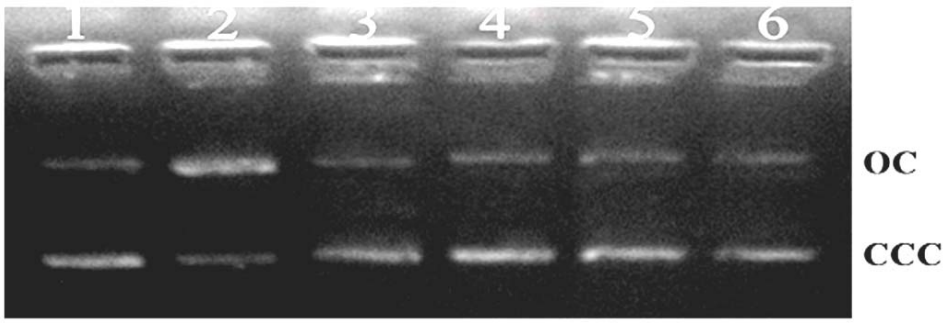

Figure 3 (a). Effect of Galangin and Kaempferide (10 mM) on Plasmid DNA (pBR 322) damage induced by Gamma irradiation. Lane 1: 0 Gy, Lane 2: 25 Gy, Lane 3: Galangin +0 Gy, Lane 4: Kaempferide +0 Gy, Lane 5: Galangin +25 Gy, Lane 6: Kaempferide +25 Gy. oc represent open circular form of pBR, ccc represent covalently closed circular form of pBR.

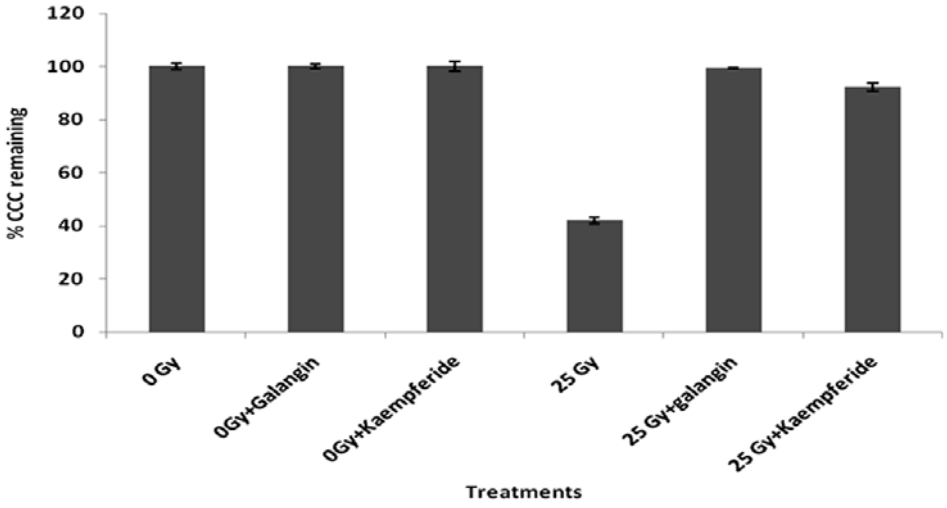

Figure 3(b). Protection of plasmid pBR 322 DNA by Galangin and kaempferide (10 mM) against different doses of gamma radiation (25 Gy).

DISCUSSION

Flavonoid's potential health benefits are due to their antioxidant effects which can be attributed to the phenolic hydroxyl groups attached to the flavonoid structure [18]. Flavonoids due to their free radical scavenging properties has been suggested to have radio-protective activities [19,20]. The present study focuses on free radical scavenging and radioprotecting properties of *Alpinia galanga* and the two bio-flavonoids isolated *viz*. galangin, and kaempferide.

A. galanga extract has been reported to possess several pharmacological activities. Previous studies have shown that it exhibits antioxidant properties [21]. Moreover *A. Galanga* also found to have anti inflammatory [22], immune-stimulating [23], and anticancer [24] activities.

The extract of *Alpinia galanga* rhizome prepared in acetone had higher content of phenolics, flavonoids and total antioxidant capacity than the extract prepared in ethanol. The current study indicates that rhizome extracts and the isolated flavonoids effectively scavenged superoxide and hydroxyl radicals in a dose dependent manner. These radicals are generated inside the body during the normal metabolism or in presence of xenobiotics. The stable free radical DPPH was also scavenged by the extracts and flavanoids.

The search for non-toxic, selective and effective cytoprotective compounds that preferentially protect normal tissues, without protecting malignant tissue, is of prime concern in radiation biology. Such compounds could protect against the genetic damage, mutation, immune system alterations, as well as the teratogenic effects of radiation. Several phytochemicals have been shown to be effective radioprotectors [4,5].

In living systems DNA constitutes the primary vital target for cellular inactivation by ionizing radiation. The damages to cellular DNA produced by exposure to ionizing radiation are mainly lesions in DNA like double and single – strand breaks, base damages, elimination of bases, sugar damage, DNA- DNA and DNA-protein cross-links. The evaluation of these lesions is an essential step in the examination of the sequence of events leading to mutagenic, carcinogenic and other lethal effects of radiation. In the present study it is clear that the presence of the galangin or kaempferide protected plasmid DNA from the radiation induced damages. Thus the flavonoids effectively protect DNA against ionizing radiation in a system devoid of repair and replication machinery.

Alkaline comet assay is a sensitive technique to monitor strand breaks and alkali labile DNA lesions and is widely used to study genotoxicity, cellular DNA lesions such as single strand breaks or double strand breaks, apoptosis and DNA repair [1,25,26]. When the human peripheral blood leukocytes were exposed to γ-radiation *ex vivo*, the cellular DNA undergoes damage, as reflected in the increase in comet parameters (tail length, % DNA in tail, tail moment and olive tail moment). Presence of galangin or

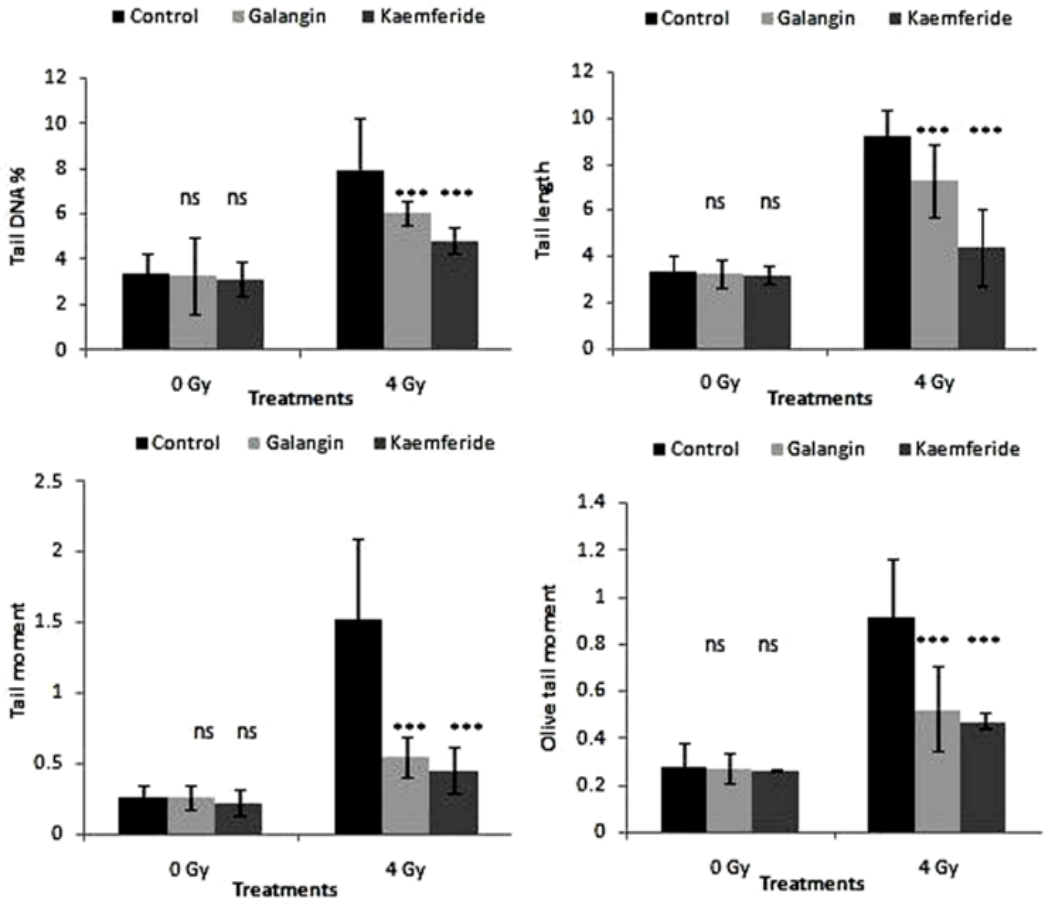

Figure 4. Effect of Galangin and Kaempferide (10 mM) on DNA damage in human peripheral blood leukocytes induced by exposure (*ex vivo*) to gamma radiation (4 Gy) assessed by comet assay. Percentage DNA in tail, tail length, tail moment and olive tail moment are presented as mean ± sd. 'ns' indicate not significant and *** indicate $p < 0.001$ when compared with respective control.

kaempferide during irradiation of the cells decreased the comet parameters indicative of its radioprotecting ability.

The protection of DNA by the flavonoids is possibly due to the scavenging of radiation-derived primary as well as secondary reactive oxygen species. More over the study also revealed that these flavonoids do not induce any DNA damage by itself. Earlier it was reported that *in vitro* or *in vivo* treatment of lymphocytes with galangin suppressed the induction of chromosome aberrations induced by bleomycin in a dose-dependent manner [27]. Murray *et al.* (2006)[28] have reported that galangin inhibited transition of cells from the G0/G1 to the S phases of cell growth, through total elimination of cyclins D3,A and E and also inhibits Hs578T cell proliferation and the activity of the AhR, (a transcription factor implicated in the initiation and growth of mammary tumors).

Thus the present work showed the ability of isolated bioflavonoids, galangin and kaempferide in protecting DNA against ionizing radiation induced damages under *in vitro* and *ex vivo* conditions. The mechanism of radioprotection by these compounds could be ascribed to its antioxidant and free radical scavenging activities. The present study suggests the possibility of using the extract of *Alpinia galanga* or the isolated bioflavonoids for the prevention of deleterious effects of ionizing radiation in situations of radiation exposure.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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