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Radioprotective Properties of Polyphenols from *Phyllanthus amarus* Linn

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Radioprotection/Antioxidants/*Phyllanthus amarus*/Ellagitannins/Flavonoids.

Radioprotective activity of pure compounds isolated from the plant *Phyllanthus amarus* was studied using rat liver mitochondria and pBR322 plasmid DNA as an *in vitro* model system. These compounds were ellagitannins namely amariin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl (DHHDP)-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, and flavonoids namely rutin, and quercetin 3-O-glucoside. The activity was then correlated with their hydroxyl and superoxide radical scavenging activity. Both ellagitannins and flavonoids effectively prevented lipid peroxidation and protein oxidation in mitochondria. The compounds also prevented radiation induced single strand breaks in pBR322 plasmid DNA. The radioprotective activity of ellagitannins and flavonoids could be due to their ability to scavenge different radicals more or less efficiently, relieving the oxidative stress. Protection conferred by flavonoids, rutin and quercetin 3-O-glucoside to rat liver mitochondria and plasmid pBR322 DNA from radiation induced damage was due to their strong hydroxyl radical scavenging activity. The inhibitory effect of ellagitannins on lipid peroxidation in liver mitochondria was due to their efficient superoxide radical scavenging ability. This is the first report about the radioprotective activity of pure ellagitannins from *Phyllanthus amarus*.

INTRODUCTION

Radiation induced damage to living cells are mediated by the generation of free radicals and related reactive oxygen species (ROS) that damage vital cellular targets such as DNA, membrane lipids and proteins. Naturally occurring antioxidants are also effective radioprotectors due to their ability to scavenge free radicals or neutralize their reactions.[1,2] These natural radioprotective compounds are of great interest to health management due to their potential applications during radiotherapy in cancer treatment, diagnostic scanning and cleaning operations in nuclear accidents. *Phyllanthus amarus* is a tropical medicinal plant, widely distributed, with many reported beneficial effects. These include antiviral, anti-inflammatory,[3,4] hypoglycemic, hypocholesteremic,[5] antibacterial,[6] antifungal[7] and radioprotective[8] activities. Extract of the plant is known to inhibit gastric carcinogenesis[9] and HIV replication *in vitro* and *ex vivo*.[10] The antioxidant activities of the methanolic extracts of *Phyllanthus* were recently demonstrated.[11]

The major chemical constituents of *Phyllanthus amarus* are highly heterogeneous and complex comprising of lignans like phyllanthin and hypophyllanthin,[12,13] alkaloids, flavonoids and hydrolysable tannins.[14–16] Among these compounds only a few such as gallic acid, ellagic acid[17] rutin and quercetin[18–21] have been studied extensively for their biological activities. The hydrolysable tannins have been shown to inhibit protein kinases[22] and their activities are compiled on the website[23] and reviewed by Okuda.[24] The pharmacological actions of polyphenolic compounds may stem mainly from their free radical scavenging and metal chelating properties as well as their effect on cell signaling pathways and gene expression.[25] In the present study radioprotective effect of ellagitannins namely amariin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl (DHHDP)-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, and flavonoids namely rutin, and quercetin 3-O-glucoside isolated from this plant (Fig. 1) against ionizing radiation induced damage was examined. The study focuses on the effect of these compounds on radiation induced DNA damage, and on rat liver mitochondria by examining oxidation in membrane

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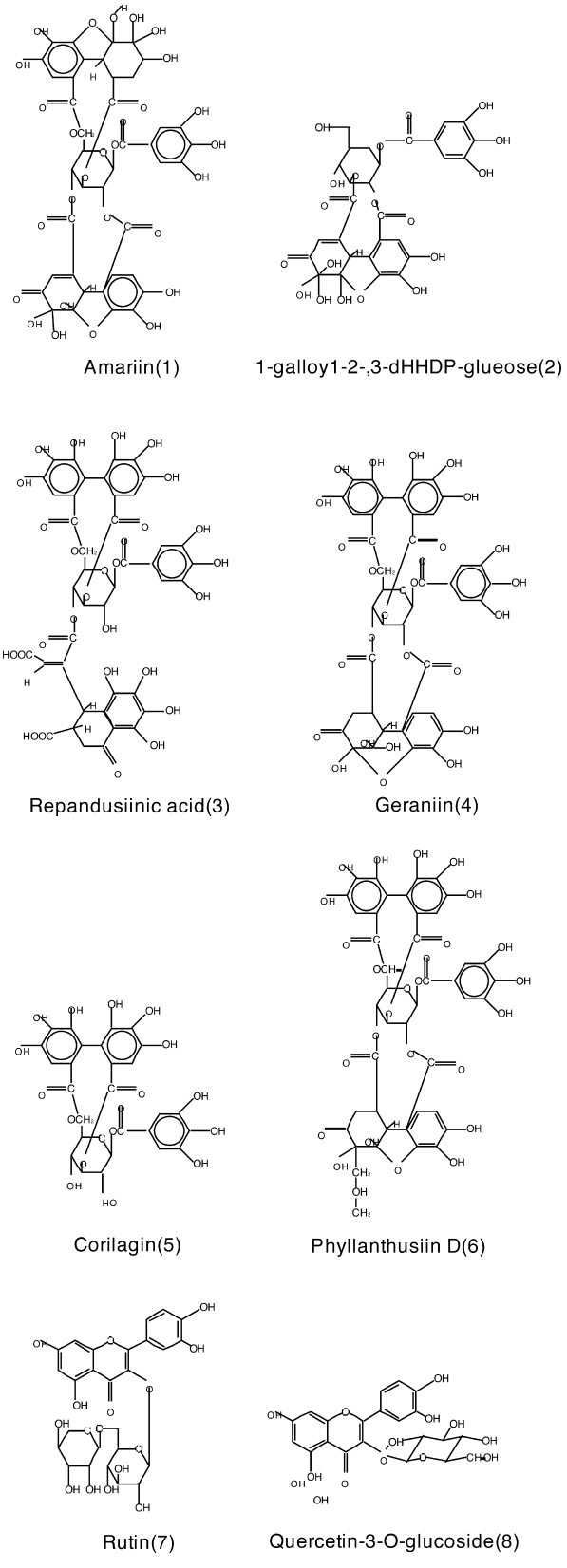

Fig. 1. Structures of the compounds purified from *Phyllanthus amarus* and used in this study.

lipid and protein. The possible mechanisms of radioprotective activity of compounds in terms of their radical scavenging activity were also studied.

MATERIALS AND METHODS

Thiobarbituric acid (TBA), and 2,4-dinitrophenylhydrazine (DNPH), xanthine, xathine oxidase were purchased from Sigma Chemicals, USA. Plasmid isolation midi kit was purchased from Qiagen, Germany. Guanidine hydrochloride, trichloroacetic acid (TCA), ethylene diaminotetraacetic acid (EDTA), Nitrobluetetrazolium (NBT), solvents were purchased from Sisco research laboratories, India or Merck, Germany. All the compounds from *Phyllanthus amarus* used, were purified by Dr Foo,[14–16] in his laboratory at the New Zealand Institute for Industrial Research and Development, Industrial Research Ltd, Gracefield Research Centre, New Zealand.

Pulse radiolysis:

In this assay hydroxyl radicals ($^{\bullet}OH$) are formed by the radiolysis of water. The linear accelerator (LINAC) electron pulse radiolysis system at the 'National Center for Free Radical Research, University of Pune, Pune, was used in this study. Irradiation of water with 7 MeV electron pulse (50 ns pulse width) and dose rate 17 Gy/pulse generates hydroxyl radicals, hydrated electrons, and hydrogen atoms. To measure only the reactions of the $^{\bullet}OH$, all solutions were presaturated with nitrous oxide (N_2O) to remove dissolved oxygen gas and to quantitatively convert the hydrated electrons and hydrogen atoms to $^{\bullet}OH$. Generated hydroxyl radicals were made to react with different concentrations (0.1, 0.2, 0.3, 0.4 mM) of ellagitannins. The first order rate constants for formation of ellagitannins radicals were measured and found to vary linearly with the concentration. The slope of linear plots for rate constants vs. concentration of compounds gave the second order rate constant. However, for flavonoids, rutin and quercetin 3-O-glucoside absorption of radical formed being very low, it was difficult to measure the rate constants. Therefore, their ability to scavenge hydroxyl radicals was measured by comparing it with a standard such as potassium thiocynate (KSCN) using competition kinetics.[26] In this method, $^{\bullet}OH$ is made to react with 1mM KSCN in the absence and in the presence of different concentrations of rutin and quercetin 3-O-glucoside. $^{\bullet}OH$ reacts completely with SCN^- to produce $(SCN)_2^{\bullet-}$ which absorbs at 480 nm. In the presence of flavonoid, decrease in the absorbance was measured. The difference between rate constant of $(SCN)_2^{\bullet-}$ in presence and absence of flavonoids was calculated. The rate of hydroxyl radicals scavenging was the slope of linear plot of this difference vs. concentration of flavonoids.

Superoxide radical scavenging activity

Superoxide radical scavenging activity was assayed spectrophtometrically by xanthine/ xanthine oxidase according to Ukeda[27] with slight modification. The enzymes xanthine oxidase catalyzes the oxidation of xanthine to uric acid. During this reaction, molecular oxygen acts as an electron acceptor, producing superoxide radicals. The extent to which the compounds inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radical was monitored at 560 nm. The 1ml of reaction mixture contains 50 mM potassium phosphate buffer (pH 8), 0.1 mM xanthine, 1mM EDTA, 75 µM NBT, 0.1 mM sample. The reaction was started by adding xanthine oxidase. The absorbance was read at 560 nm after 30 minutes of incubation in dark at room temperature.

Isolation of rat liver mitochondria and exposure to ionizing radiation

Three months old female wistar rats were used for the preparation of mitochondria as described by Katyare and Rajan.[28] In brief, rat liver was excised, homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3000 × g for 10 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at 10,000 × g for 10 min to sediment mitochondria. This pellet was washed with 5 mM potassium phosphate buffer, pH 7.4, to remove sucrose. Protein was estimated by the method of Lowry *et al.*[29] and pellet was suspended in the above buffer at the concentration of 10 mg protein/ml.

Ionizing radiation (^{60}Cobalt - gamma source)

Oxidative damage was induced in mitochondria by exposure to γ-rays from a ^{60}Co source. Mitochondria from rat liver treated with or without test compounds *in vitro* were exposed to 450 Gy gamma radiation at a dose rate of 7 Gy/min whereas plasmid pBR322 DNA was exposed to 12 Gy gamma radiation at a dose rate of 4.6 Gy/min.

Lipid peroxidation

Lipid peroxidation was measured in terms of nmoles of malondialdehyde equivalents formed.[30] Briefly, samples were boiled with TBA reagent (20% TCA, 0.5% TBA, 2.5 N HCl and 6 mM EDTA) for 20 min in a boiling water bath. After cooling, the pink colour representative of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm and was expressed as nmoles of TBARS formed per mg protein.

Estimation of protein carbonyls

This method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenylhydrazine, which can be measured at 366 nm. Amount of carbonyls formed were expressed as nmoles of protein carbonyls formed per mg protein.[31]

Protection to plasmid pBR322 DNA against radiation

Plasmid pBR322 DNA was isolated from *E coli* using, plasmid midi kit (Qiagen, U.S.A.). Radiation mediated DNA strand breaks were measured by the conversion of supercoiled double strand DNA to open circular form, according to the procedure described by Zhao *et al.*[32] Briefly 0.5 µg DNA was exposed to 12 Gy gamma radiation with or without test compounds. Following radiation exposure, the samples were immediately loaded onto 1% agarose gel and electrophoresed. The gel was stained with ethidium bromide and the fluorescence was observed under UV and the intensity of the bands was measured using Gel documentation system (Alpha Innotech Corporation, USA).

Statistical analysis

All experiments were repeated at least three times and data presented is average of these replicates. The one-way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance of the differences among experimental groups. Dissimilar alphabets a, b, c and d in superscript in the Fig. 2, 3, 4, 5 and 7 indicate statistically significant difference at 0.05 level. All the statistical analysis was done using SPSS 10.0 software.

RESULTS

Pulse radiolysis

Hydroxyl radical scavenging activity of the compounds was checked by pulse radiolysis. Rate constant for the reaction of the any antioxidant with free radicals indicates its reactivity towards the free radical. In general flavonoids showed higher hydroxyl radical scavenging activity ($p < 0.05$) than ellagitannins. Flavonoid, quercetin 3-O-glucoside exhibited highest radical scavenging activity, while among ellagitannins, repandusinic acid and geraniin exhibited higher ($p < 0.05$) radical scavenging activity (Fig. 2).

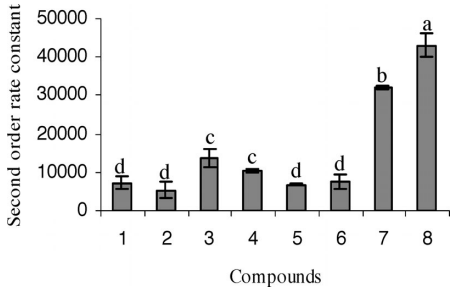

Fig. 2. Scavenging of hydroxyl radicals in terms of second order rate constant of different compounds by pulse radiolysis. 1-8: amariin, 1-galloyl-2,3-DHHDP-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin 3-O-glucoside respectively at 0.1 mM concentration Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

Superoxide radical scavenging activity

Superoxide radical scavenging activity of these compounds was estimated by nitroblue tetrazolium reduction by xanthine-xanthine oxidase method. The ellagitannins compared with flavonoids rutin and quercetin 3-O-glucoside exhibited higher activity ($p < 0.05$). Among the ellagitannins repandusinic acid, geraniin and phyllanthusiin D showed higher ($p < 0.05$) superoxide radical scavenging activity than amariin, corilagin and 1-galloyl-2,3-(DHHDP)-glucose (Fig. 3).

Analysis of mitochondrial membrane damage

Lipid peroxidation is the most important consequence after radiation induced damage to cell or mitochondria. Malondialdehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid (TBA) to give a pink coloured species that absorbs at 532 nm. Figure 4 shows the inhibition of formation of TBARS by ellagitannins and flavonoids isolated from *Phyllanthus amarus* at 0.01 mM concentration. In control, unirradiated mitochondria 1.65 ± 0.4 nmoles of TBARS/mg protein were formed which increased to 5.38 ± 0.09 nmoles of TBARS/mg protein on exposure to radiation. All the compounds showed protection against radiation induced damage to mitochondria. The ellagitannins, amariin and repandusinic acid and flavonoid, rutin showed higher inhibition of TBARS formation compared to ($p < 0.05$) corilagin, 1-galloyl-2,3-(DHHDP)-glucose, geraniin, and quercetin 3-O-glucoside while phyllanthusiin D showed very less activity.

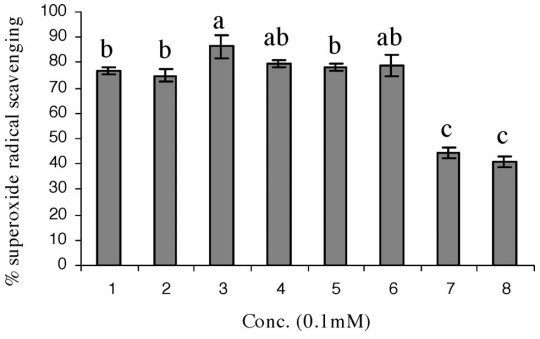

Fig. 3. Superoxide radical scavenging activity of different compounds at 0.1 mM concentration generated by xathine-xanthine oxidase method. 1-8 are compounds amariin, 1-galloyl-2,3-DHHDP-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin 3-O-glucoside respectively. Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

Inhibition of protein carbonyl formation in rat liver mitochondria

Protein carbonyl is one of the products of oxidatively damaged protein. Figure 5 shows the formation of protein carbonyls in rat liver mitochondria in presence or absence of these compounds following exposure to radiation. Protein carbonyl formation increased to 14.18 ± 0.7 nmoles/mg protein after radiation compared to 3.24 ± 0.7 nmoles/mg protein in un-irradiated-mitochondria. Phyllanthusiin D, geraniin, 1-galloyl-2,3-DHHDP-glucose, rutin and quercetin 3-O-glucoside showed higher protection ($p < 0.05$) compared to repandusinic acid, corilagin and amariin.

Estimation of DNA damage

Plasmid DNA was exposed to ionizing radiation in the presence or absence of these compounds. In Fig. 6 lane 1 is untreated pBR322 DNA, where majority of the DNA (81%, Fig. 5,) is in the supercoiled form. Substantial increase in the nicked circular form (66.7%, Fig. 5) and decrease in the supercoiled form (33.3%, Fig. 5) due to damage by ionizing radiation is seen in lane 2. Lane 3 to 10, shows DNA damage induced in presence of 0.1 mM compounds namely amariin, 1-galloyl-2, 3-DHHDP-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin 3-O-

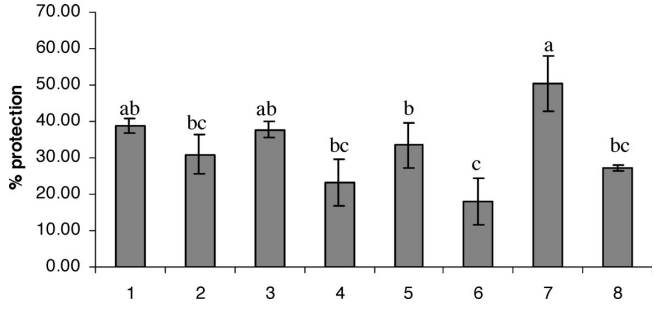

Fig. 4. Percent protection to rat liver mitochondria by different compounds against radiation induced damaged (450 Gy) in term of inhibition of lipid peroxidation. The concentration used was 0.01 mM for all compounds. Compound 1to 8: amariin, 1-galloyl-2,3-DHHDP-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin–3-O-glucoside respectively. Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

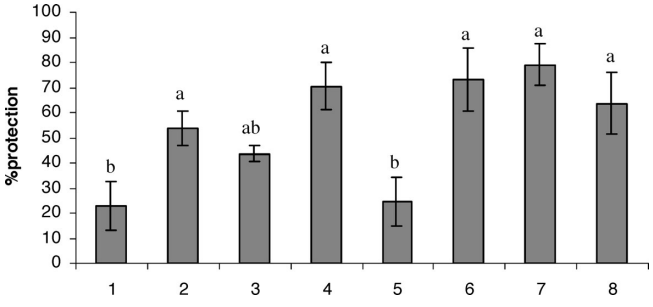

Fig. 5. Inhibition of protein oxidation measured in rat liver mitochondria by different compounds of *Phyllanthus amarus*. Compounds 1 to 8 amariin, 1-galloyl-2,3-DHHDP-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin 3-O-glucoside respectively at 0.1 mM concentration against radiation induced damaged (450 Gy). Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

glucoside respectively. In the presence of each of these compounds alone substantial amount of DNA remained in supercoiled form (data not shown). Highest protection to the DNA against radiation induced damage was offered by Quercetin 3-O-glucoside followed by rutin. Among ellagitannins corilagin, geraniin, 1-galloyl-2, 3-DHHDP-glucose exhibited higher activity ($p < 0.05$), followed by repandusinic acid, phyllanthusiin D and amariin (Fig. 7).

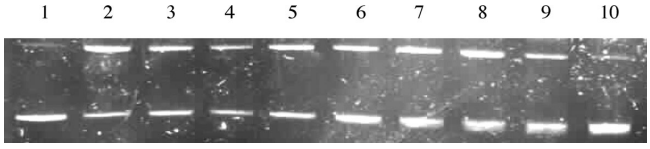

Fig. 6. Agarose gel electrophoretic pattern showing protection to pBR322 against radiation induced damage by compounds. Lane 1: Control pBR322 DNA; Lane 2: Radiation damaged DNA; Lane 3 to 10: Radiation damaged DNA in presence of amariin, 1-galloyl-2,3-DHHDP- glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D, rutin, and quercetin 3-O-glucoside respectively at 0.1 mM concentration.

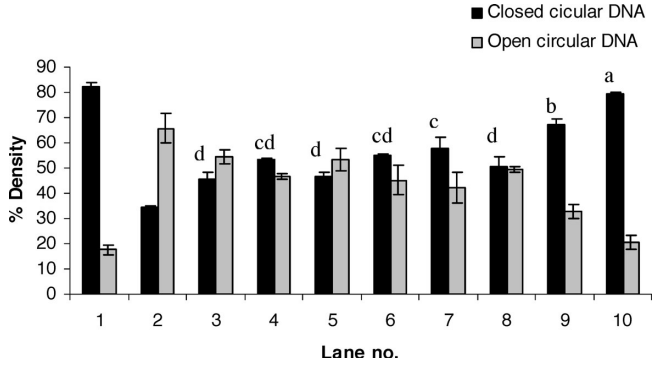

Fig. 7. Amount (%) of supercoiled and nicked circular DNA after exposure by ionizing radiation in presence and absence of compounds estimated from densitometric measurements of electrophoreogram (Fig. 5). Dissimilar alphabets in superscript indicate significant difference at 0.05 level.

DISCUSSION

Most of the cellular alterations induced by ionizing radiation are mainly due to the generation of free radicals, derived from oxygen. Free radical scavengers therefore, have a key role in radioprotection. Previous report on radioprotective effect of *Phyllanthus amarus* suggested that *P. amarus* extracts could increase the antioxidant status in mice and thereby protect the animals from radiation-induced cellular damage.[8] In this study ellagitannins and flavonoids isolated from *Phyllanthus amarus* have been shown to scavenge hydroxyl and superoxide radicals and thereby protect rat liver mitochondrial lipids, proteins from radiation induced damage. pBR322 DNA was also protected from radiation induced damage by these purified compounds.

Water, the most abundant intracellular material decomposes, after exposure to ionizing radiation and generates primary $^{\bullet}OH$ radicals and secondary superoxide radicals. Radioprotective substances scavenge any or all radicals and minimize the damaging threat. In this study flavonoids rutin and quercetin showed good hydroxyl radical scavenging activity generated by radiolysis of water in pulse radiolysis study as compared to ellagitannins (Fig. 2), which exhibited better superoxide radical scavenging activity (Fig. 3).

Both Flavonoids and ellagitannins protected lipids from oxidative damage. Maximum protection was offered by flavonoids rutin and ellagitannin repandusinic acid (Fig. 4). The action of ROS on proteins has been shown to increase the formation of carbonyl groups[33,34] and the level of carbonyl groups in circulating proteins is considered as a useful marker of oxidative stress. These oxidation products are formed relatively early and are more stable.[35] The compounds corilagin, phyllanthusiin D, rutin and quercetin 3-O-glucoside exhibited higher activities in protecting proteins against oxidative damage followed by 1-galloyl-2,3-DHHDP-glucose, repandusinic acid, amariin and corilagin (Fig. 5).

Ionizing radiations induce damage to cellular DNA, which is of prime biological significance. The types of damage include strand breaks, base damage, elimination of bases and sugar damage.[36] Oxidative damage to plasmid DNA caused by free radicals generated from radiation was measured in terms of conversion of supercoiled to nicked circular form. Protection offered by the compounds was calculated by quantifying the amount of DNA in both nicked circular and supercoiled form in presence and absence of these compounds. The present study reveled that DNA is protected from deleterious effects of ionizing radiation by all the compounds used. Among these compounds rutin followed by quercetin 3-O-glucoside offered maximum protection.

In conclusion, all the eight compounds, ellagitannins and flavonoids isolated from *Phyllanthus amarus* showed radioprotective activities due to their ability to scavenge different radicals more or less efficiently generated after radiation exposure. Protection conferred by rutin and quercetin 3-O-glucoside to rat liver mitochondria and plasmid pBR322 DNA from radiation induced damage could be due to their strong hydroxyl radical scavenging activity. Whereas the inhibitory effect of ellagitannins on lipid peroxidation in liver mitochondria is due to their efficient superoxide radical scavenging. Tannins have been reported to inhibit lipid peroxidation in mouse ocular lens induced by xanthine-xanthine oxidase which generates superoxide radicals.[37] Additionally tannin fraction from barks of *Pinus caribaea* was shown to be antigenotoxic against gamma-rays when the *Escherichia coli* cells were pretreated or simultaneously

treated with this extracts, but not during post-irradiation treatments, suggesting that the antigenotoxic action is through free radical scavenging mechanisms.[38)]

Earlier studies of radioprotective effect of an extracts of *Phyllanthus amarus* were done using adult BALB/c mice. In this, treatment with extracts increased the activity of antioxidant enzymes in blood and tissues thereby protecting the animals from radiation induced cellular damage.[8)] Our results showed that ellagitannins and flavonoids isolated from *Phyllanthus amarus* protected rat liver mitochondria and pBR322 DNA from oxidative damage induced by radiation. Ellagitannins scavenged superoxide radicals more efficiently than the flavonoids while the latter compounds were more efficient in scavenging the hydroxyl radicals thereby relieving the oxidative stress as a result of radiation. This is the first report about the radioprotective activity of pure ellagitannins from *Phyllanthus amarus*.

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