Studies on modulation of DNA integrity in Fenton's system by phytochemicals

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ABSTRACT
Chronic exposure to oxidative stress especially to highly reactive hydroxyl radicals (HO•) could damage biomolecules, particularly DNA, that in turn would accelerate onset of degenerative diseases. In the present study a few standard phytochemicals (vitamin C, gallic acid, catechin, apigenin, naringenin and naringin) and plant extracts (Hippophae rhamnoides kernel (HRK), Syzygium cumini kernel (SCK) and Punica granatum pericarp (PGP)) were evaluated for their potential to protect/damage DNA in Fenton’s system using in vitro models. The results indicated a significant DNA protective effect for naringin and PGP whereas other phytochemicals/extracts showed DNA damaging effect similar to or more than that of control value. The phytochemicals/extracts were also evaluated for their antioxidant and iron chelation properties. In general, the phytochemicals/extracts with high antioxidant activity but without iron chelation capacity failed to protect DNA in Fenton’s system, suggesting that iron chelation was an essential requirement for the phytochemicals studied here to retard HO• generation by Fenton’s reaction. This was demonstrated by the high iron chelation capacity of naringin and PGP (83.67% and 68.67% respectively) and their DNA protective effect. Commonly consumed phytochemicals such as vitamin C and gallic acid with their high reducing power and at higher physiological concentration, could regenerate free iron for Fenton’s reaction leading to DNA damage as shown here.

1. Introduction
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously produced in all living beings, especially in higher organisms as a result of normal cellular metabolism, phagocytosis, inflammation and by other exogenous factors like ionizing radiations and xenobiotics [1]. The predominant radicals encountered in higher organisms are superoxide (O2•−), peroxyl (ROO•), nitroxy (NO•) and hydroxyl (HO•) radicals [2]. However, HO• is more reactive and is capable of causing damage to biomolecules such as lipids, proteins and DNA. It is generally recognized that in physiological system HO• is produced under aerobic condition by Fenton’s reaction [3] and its interaction with DNA causes oxidative damage. DNA damage such as single and double strand breakage, base modification, and cross-linking of DNA with other biomolecules particularly proteins are reported to be early events of diseases [4]. Chronic exposure of tissues to excess HO• therefore could predispose to cancer, cardiovascular diseases, cataract and neurological disorders [5]. Mitochondrial DNA (mtDNA) is presumed to be more susceptible to oxidation by Fenton’s reaction and thus play a key role in cellular degeneration [6,7]. Recent studies using HO• specific fluorescent probe in mammalian cell lines showed that excessive ROS caused impaired electron transport and consequent mtDNA damage [8]. The results demonstrated accumulation of the marker for oxidative DNA damage, 8-hydroxy 2-deoxy guanosine (8-oxo guanine) by several fold more in mtDNA than that in nDNA at steady state [9].
Phytochemicals are reported to have profound chemopreventive effect through modulation of molecular events that damage DNA and other biomolecules. However, studies showing the availability of phytochemicals to nDNA and mtDNA to bring about such effects are very few. Nevertheless indirect evidence for the DNA protective effects of phytochemicals based on epidemiological and in vitro studies are now available. Protective effect of epigallocatechin gallate and esculin against oxidative DNA damage caused by ultraviolet therapy [10]; inhibition of oxidant induced DNA strand breaks in cultured lung cells by green tea polyphenols [11]; protective activity of chlorophyllin against gamma-radiation induced DNA damage [12] and DNA protective activity of verbascoside under in vitro Fenton’s reaction [13] are a few examples. Studies in human volunteers and animal models also showed an association between increased plasma antioxidant level after consumption of phenolic rich food and subsequent reduced DNA damage [14–16]. Adverse effects of phytochemicals in biological systems in terms of their genotoxic potentials [17–19] and pro-oxidant activity [20,21] have also been reported. The nature of phytochemicals consumed and their serum levels thus appear to determine beneficial or adverse
effects. The studies aimed to understand the effect of phytochemicals on the integrity of DNA under oxidative stress similar to that generally observed in physiological system therefore is very important. Most of the studies on phytochemicals and plant extracts are reported in terms of their DPPH radical scavenging properties that often do not correlate with their effects in the Fenton's system.

For the present study, Fenton's reaction was selected because the hydrogen peroxide as its reactant is generated by the dismutation of superoxides, a range of oxidase enzymes and beta oxidation of fatty acids continuously. The hydrogen peroxide thus available is decomposed to HO\(^\bullet\) by Fe\(^{2+}\) which is known as Fenton’s reaction. The Fe\(^{2+}\) for the Fenton’s reaction could normally be available from iron binding proteins under acute oxidative stress [22–25]. The hydroxyl radicals produced through the Fenton’s reaction is most reactive free radical that can damage DNA in its proximity. Some of the dietary phytochemicals are capable of either sequestering Fe\(^{2+}\) and/or scavenging HO\(^\bullet\) radicals thus preventing damage to DNA. However, predictability of such phytochemicals based on their structure-function is poor. Studies to establish an association between iron sequestering and free radical scavenging properties of phytochemicals with modulation of DNA damage are fragmented. The objective of this study was to establish such an association, if any. The phytochemicals selected for the study therefore were based on their commonality in the diet, their antioxidant properties and biological activities. The six standard phytochemicals (Fig. 1) selected for the study represent different classes, namely, gallic acid (phenolic acid), catechin (flavan-3-ol), apigenin (flavone), naringenin (flavonone), and naringin (flavonone glycoside). Vitamin C was included as the most common antioxidant and essential nutrient for human body. The plant extracts used in this study were with high antioxidant activity as observed in preliminary studies conducted in our laboratory.

2. Materials and methods

2.1. Chemicals

Chemical abstract number (CAS no.) of each item is given in parenthesis. Lambda DNA (91080-14-7), tris buffer, tris acetate EDTA (TAE) buffer, ethidium bromide (1239-45-8) and agarose (9012-16-6) were purchased from Bangalore-Geneti (Bangalore, India). Vitamin C (50-81-7), catechin (18829-70-4), naringin (90236-47-2), naringenin (67604-48-2), gallic acid (149-91-7), apigenin (520-36-5) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (1898-66-4) were procured from Sigma–Aldrich (Bangalore, India). Hydrogen peroxide (7722-84-1), ferrous chloride (7758-94-3), ferrozine (69898-45-9) and potassium ferri cyanide (13746-66-2) were from Sisco Research Laboratories (Mumbai, India). All other chemicals and solvents were of analytical grade.

2.2. Sample preparation

The standards (catechin, apigenin, naringin, naringenin, vitamin C, and gallic acid) were prepared in tris buffered (50 mM, pH 7.0) 0.01% dimethyl formamide (TBDMF) as stock solution of 5.0 mM concentration. The plant materials used here were H. rhamnoides kernel (HRK), S. cumini kernel (SCK), and P. granatum pericarp (PGP). The plant materials were selected based on their high antioxidant activity obtained from the studies conducted in our laboratory. The methanol extracts were prepared from powdered raw materials. The extraction was performed with material to solvent ratio of 1:5 (w/v) for five times. The extracts thus obtained from each material were pooled and concentrated to dryness using vacuum rotary evaporator at reduced temperature and pressure (50 °C, −700 mm Hg). The alcohol free extracts were then dissolved in TBDMF, filtered, and adjusted to 5.0 mM phenolic content. Phenolic content of extracts was estimated using Folin’s reagent [26].

2.3. Induction of oxidative DNA damage and evaluation of samples

Oxidative DNA damage was induced by adopting two methods reported previously with slight modification [27,28]. A dose–response pattern of oxidative DNA damage was standardized by treating lambda DNA with Fenton’s reactants to establish the reproducibility of oxidation and quantification of DNA damage. The method in brief was as follows: the reaction system contained lambda DNA (2.5 μg), ferrous chloride (0.25 mM) and various concentrations of hydrogen peroxide (0.05–0.35 mM; step value, 0.05). Fenton’s reaction was initiated by addition of hydrogen peroxide. Final reaction volume of the assay system was maintained at 20 μL. The tubes were sealed and incubated at 37 °C for 2.0 h in a constant temperature water bath. After incubation, the reaction was stopped by addition of 10 μL of 4× gel loading buffer.

For evaluation, concentrations of oxidants and standard compounds/extracts were set at equimolar level. The standard antioxidant compounds and the samples were added to the Fenton’s system so as to maintain a final concentration of 0.25 mM. Molarity of the extracts were calculated based on the assumption of average molecular weight of plant phenolic compounds as 350. The concentrations of ferrous chloride and hydrogen peroxide were kept at 0.25 mM in total volume of 20 μL. Working solutions of hydrogen peroxide and ferrous chloride were prepared freshly before each batch of experiments.

2.4. Electrophoresis

The contents of the reaction tubes were mixed well with gel loading buffer and loaded on to 0.7% agarose gel equilibrated with TBE buffer in a horizontal electrophoretic system (Genei, Bangalore, India). Immediately after sample loading, electrophoresis was carried out for the first 15 min at 20 mA and continued for the next 30 min at 40 mA. The gel was then removed and stained in 0.2 μg/mL ethidium bromide solution for 30 min. The staining conditions were kept identical for all subsequent experiments.

Fig. 1. Phytochemicals evaluated for their protective/damaging effect on double stranded DNA in Fenton’s system.

Catechin

Vit.C

Gallic acid

Naringenin

Naringin

Apigenin

[Diagram of phytochemical structures]
2.5. Imaging and data analysis

Uniformly stained gels were placed on a UV trans-illuminator at 254 nm and photographed to tagged image file format (TIFF) with a 4 mega-pixel digital camera (Canon power shot, Japan). The image captured in RGB format was imported into image processing software. The image was then transformed to 8-bit grayscale format. The grayscale image was then normalized based on the blank lane by adjusting the brightness and contrast tool. The fluorescent region of each lane was then selected using free-hand selection tool and measured the average intensity of all pixels in the selected region. The numeric values of white and black pixels are 255 and 0 respectively. The value of a pixel lies between 0 and 255 based on their signal strength. The values of all pixels in the selected area were computed and the average value per pixel was arrived at for each lane. Average fluorescence in the blank lane was considered 100% (0% DNA damage) and the difference between the blank and treated DNA was taken as the extent of damage in percentage.

2.6. Iron chelating effect

Iron chelating properties of standard compounds and plant extracts were evaluated using the method reported by Pascual et al. [29] with slight modifications as follows: Standard compounds and plant extracts (2.0 mg) dissolved in 0.5 mL methanol were treated with 0.25 mL ferrous chloride (1.0 mM) and 1 mL ferrozine (5 mM). Volume of the assay was then made up to 2.0 mL with methanol. Final concentration of the standards and extracts in the assay was 1.0 mg/mL. The reaction mixture was shaken well and incubated for 10 min at room temperature (25–26 °C) for ferrous–ferrozine complex formation. Absorbance of the color developed due to the ferrous ferrozine complex was measured using spectrophotometer (Shimadzu UV-2450, Shimadzu Corporation, Kyoto, Japan) at 560 nm against reagent blank. Percentage iron chelation was calculated with respect to blank, where ferrous ferrozine complex formed was taken as 100%. The extend of reduction in color was taken as iron chelating activity of the standard compounds/extracts used here under the assay conditions.

2.7. Total reducing power

Reducing power of the standard compounds and extracts was estimated following the method reported previously [30]. The method in short was: 125 μg of standard compounds and samples dissolved in minimum volume of methanol (50 μL) was made up to 2.5 mL with sodium phosphate buffer (0.2 M, pH 6.6) followed by addition of 2.5 mL potassium ferriyanide (1.0%). The reaction mixture was incubated at 50 °C for 30 min. The reaction was terminated by addition of 2.5 mL of 10% trichloroacetic acid and centrifuged at 3000 × g for 20 min. The supernatant (2.5 mL) was collected and mixed with equal amount of water and 0.5 mL of 0.1% FeCl₃ solution. Absorbance of the color developed was measured using spectrophotometer (Shimadzu UV-2450) at 700 nm against reagent blank.

2.8. DPPH radical scavenging activity

DPPH radical scavenging activity was estimated by the method reported by Brand-Williams et al. [31]. The assay contained 2.9 mL of 0.1 mM DPPH in methanol and various concentrations of standard compounds and plant extracts with total volume of 3 mL. The contents were mixed well immediately and the degree of reduction of absorbance was recorded for 30 min using spectrophotometer (Shimadzu UV-2450) at 520 nm on a time course mode. The difference in absorbance at time zero and at 30th minute was used for calculating percentage radical scavenging activity (RSA). A dose–response curve was plotted with percentage RSA against sample concentration and IC₅₀ value (amount of substance required to scavenge 50% DPPH radicals in the system) was obtained by regression analysis.

2.9. Statistical analysis

All experiments were repeated at least three times and results are expressed as mean ± S.D. or at 95% confidence level. Mean, standard deviation and confidence levels were calculated using Microsoft excel. Correlation study was performed to understand the relation between given oxidative treatment versus DNA damage. Antioxidant activity of standard phytochemicals/plant extracts versus their effect on DNA damage in Fenton's system was also correlated. Correlation coefficient (p value) less than 0.05 was considered statistically significant. Correlation analyses were performed using the statistical software Microcal Origin.

3. Results

3.1. DNA damage studies

Fenton's reaction refers to iron-catalyzed decomposition of hydrogen peroxide and generation of highly reactive hydroxyl radicals. DNA damage caused in response to various levels of oxidative stress by Fenton’s reaction is shown in the electrophoretic pattern (Fig. 2A and B). Ethidium bromide was the fluorescent probe used in this study to quantify the amount of detectable DNA in agarose gel with respect to blank. Ethidium bromide intercalates with double stranded DNA and produces bright fluorescence on irradiation at 254 nm. The fluorescence intensity is proportional to the amount of double stranded DNA which intercalates the dye. The high resolution images of the ethidium bromide stained gel illuminated at 254 nm was captured using a digital camera and quantified the fluorescence intensity of each lane by an image processing software. The fluorescent region was selected and quantified as average intensity of pixels. Since the image was 8-bit grayscale format, the intensity of each pixel, which ranges from black to white, was in the scale of 0–255 respectively. The average signal strength, as integers, obtained was proportional to the amount of double stranded DNA. Lane 1 is the blank in which the DNA was treated with ferrous chloride (0.25 mM) alone and therefore no oxidative damage was expected. No difference in total fluorescence was observed between DNA alone with buffer and DNA with ferrous chloride (data not shown). Therefore, the total fluorescence in lane 1 was considered as nil damage and used as blank for calculating relative DNA damage in presence of both ferrous chloride and hydrogen peroxide. Lanes 2–8 are the electrophoretic pattern of DNA treated with 0.05–0.35 mM (step: 0.05) H₂O₂ and 0.25 mM ferrous chloride. A linear relation was observed between DNA damage and concentration of hydrogen peroxide used here (r = −0.93, n = 12, p < 0.01) (Fig. 2B). The assay conditions of lane 6 (0.25 mM FeCl₃ and 0.25 mM H₂O₂) was considered as optimum and used as control for the subsequent evaluation of standard compounds and samples. This intermediate oxidative state of DNA was selected so that antioxidant or pro-oxidant action of test samples could be measured quantitatively in terms of reduced or increased DNA damage.

To understand the effect of the selected standard phytochemicals, Fenton’s system containing DNA and phytochemicals was...
Fig. 3. (A) Electrophoresis pattern of DNA in Fenton’s system treated with six standard phytochemicals and (B) bar chart showing percentage DNA damage/protection. Final concentration of all phytochemicals was 2.5 mM; lane 1, blank (lambda DNA alone); lane 2, control (DNA + Fenton’s reactants); lane 3, DNA + Fenton’s reactants + naringenin; lane 4, DNA + Fenton’s reactants + naringin; lane 5, DNA + Fenton’s reactants + apigenin; lane 6, DNA + Fenton’s reactants + catechin; lane 7, DNA + Fenton’s reactants + vitamin C; and lane 8, DNA + Fenton’s reactants + gallic acid.

evaluated under equimolar conditions. Fig. 3 is the results of λ DNA treated with naringenin, naringin, apigenin, catechin, vitamin C, and gallic acid at 0.25 mM concentration in optimized Fenton’s system. The blank (lane 1, λ DNA alone in buffer) was assumed as 0.0% damage. Lane 2 shows the DNA damage in control (λ DNA and Fenton’s reagent). With respect to blank, the mean DNA damage in control was 51.0 ± 6.0%. Lane 3 displays the pattern of DNA treated with Fenton’s reagent and naringenin and percentage of DNA damage observed was 52.0 ± 7.0, which was similar to that of control. The mean DNA damage observed for naringin (lane 4) was 19.0 ± 5.0 suggesting that naringin afforded 32% protection to DNA as compared to that of control. The mean DNA damage observed for apigenin (lane 5) was 52.0 ± 7.0% and 54.0 ± 8% respectively indicating that they had no protective effect against the oxidation of DNA. Interestingly, vitamin C and gallic acid, the two well-known antioxidants caused mean DNA damage of 68.0 ± 4.0% and 72.0 ± 9.0% respectively, the values being significantly greater than that of control.

Among the plant extracts tested (Fig. 4A, lanes 4 and 5), both SCK and HRK were found to damage DNA (SCK, 79.0 ± 3.0%; HRK, 68.0 ± 5.0%). The electrophoresis pattern and corresponding histogram (Fig. 4B) showed that the total DNA damage observed for SCK and HRK were greater than that of control by 25.0% and 14.0% respectively. Among the plant extracts tested, only PGP showed protective effect with 18.0 ± 3.0% DNA damage compared to the control value of 51.0 ± 6.0%.

3.2. Iron chelation

Bivalent transition metal ions play a very important role in the process of Fenton’s reaction that leads to generation of highly reactive hydroxyl radicals [32]. The agents that can attenuate the action of these bivalent metal ions have been classified as secondary antioxidants which retard the rate of radical initiation reaction by means of eliminating initiators [5]. Iron chelation properties of the standard molecules and plant extracts were evaluated and the results are presented at 10.0 mg/mL concentration (Table 1). Among the six standard compounds evaluated here, naringin showed more than 80% iron chelation. Activities of naringenin, apigenin and catechin were 20.83 ± 1.39%, 13.47 ± 1.36% and 11.17 ± 1.6% respectively. No measurable iron chelation was observed for vitamin C and gallic acid. Among the plant extracts, PGP was found to be more active in chelating iron (68.67 ± 2.27%) followed by SCK (73.0 ± 1.27%) and HRK (60.3 ± 1.87%).

3.3. Total reducing power

Total reducing potential (TRP) of the standard compounds and samples were evaluated at 25 μg/mL (Table 1). The reducing potential was proportional to color developed and expressed in absorbance unit. The iron (III) reduction method employed here is often used to indicate electron transfer potential of
<table>
<thead>
<tr>
<th>Samples/standards</th>
<th>Antioxidant activity</th>
<th>Iron chelation (%)</th>
<th>DNA damage</th>
</tr>
</thead>
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<td></td>
<td>DPPH (IC50) a</td>
<td>TRP (OD) b</td>
<td></td>
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<tr>
<td>Control</td>
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<td>NA</td>
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<tr>
<td>Naringenin</td>
<td>25.0 ± 4.0</td>
<td>0.10 ± 0.01</td>
<td>20.83 ± 1.39</td>
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<td>Naringin</td>
<td>327.0 ± 13.0</td>
<td>0.01 ± 0.00</td>
<td>83.67 ± 3.51</td>
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<tr>
<td>Apigenin</td>
<td>267.0 ± 7.7</td>
<td>0.05 ± 0.01</td>
<td>13.47 ± 1.36</td>
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<tr>
<td>Catechin</td>
<td>6.1 ± 1.3</td>
<td>0.74 ± 0.04</td>
<td>11.17 ± 1.65</td>
</tr>
<tr>
<td>Vitamin.C</td>
<td>7.0 ± 0.7</td>
<td>0.97 ± 0.04</td>
<td>nd</td>
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<tr>
<td>Gallic acid</td>
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<tr>
<td>SCK</td>
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<td>HRK</td>
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<tr>
<td>PGP</td>
<td>5.0 ± 0.8</td>
<td>0.81 ± 0.07</td>
<td>68.67 ± 2.27</td>
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a DPPH radical scavenging activity.
b Total reducing power (optical density) at 25 μg/mL.
c Percentage iron chelation at 1.0 mg/mL concentration.
d Confidence interval at 95% level; nd, not detected; NA: not applicable; SCK: S. cumini Kernel; HRK: H. rhamnoides kernel; PGP: P. granatum pericarp.

Antioxidant activity of the standard compounds and extracts were studied using the stable free radical DPPH, which is purple in color (λmax, 520 nm) and its conversion to non-radical form by hydrogen atom abstraction from the added test compound(s) was quantified photometrically [35]. Conversion of DPPH into non-radical form is characterized by decrease in absorption at 520 nm. Hydrogen atom donation capacity, the characteristic feature of primary antioxidants [36–38], of the standard compounds and plant extractants was found to be the most potent (1.32 ± 0.1) followed by vitamin C (0.97 ± 0.04) and catechin (0.74 ± 0.04). Reducing capacity of naringin, naringenin and apigenin were relatively very less. In the case of plant extracts, the results were comparable with those of reference standards. Extracts such as SCK and PGP showed high reducing activity 0.89 ± 0.07 and 0.81 ± 0.07 respectively followed by HRK (0.71 ± 0.04).

3.4. Antioxidant activity

Antioxidant activity of the standard compounds and extracts were studied using the stable free radical DPPH, which is purple in color (λmax, 520 nm) and its conversion to non-radical form by hydrogen atom abstraction from the added test compound(s) was quantified photometrically [35]. Conversion of DPPH into non-radical form is characterized by decrease in absorption at 520 nm. Hydrogen atom donation capacity, the characteristic feature of primary antioxidants [36–38], of the standard compounds and plant extractants was found to be the most potent (1.32 ± 0.1) followed by vitamin C (0.97 ± 0.04) and catechin (0.74 ± 0.04). Reducing capacity of naringin, naringenin and apigenin were relatively very less. In the case of plant extracts, the results were comparable with those of reference standards. Extracts such as SCK and PGP showed high reducing activity 0.89 ± 0.07 and 0.81 ± 0.07 respectively followed by HRK (0.71 ± 0.04).

**Fig. 5.** Dose dependent DPPH radical scavenging activity of standard phytochemicals. (A) naringenin; (B) naringin; (C) apigenin; (D) catechin; (E) vitamin C; and (F) gallic acid.
extracts were evaluated here. Fig. 5 is the dose dependent radical scavenging activity (RSA) of the above-mentioned standard compounds. Among the six standard compounds studied here, gallic acid was found to be most active with IC\textsubscript{50} value of 1.4 ± 0.3 μg/mL. Catechin, vitamin C and naringenin showed the next highest activity with IC\textsubscript{50} values of 6.1 ± 1.3 μg/mL, 7.0 ± 0.7 μg/mL and 25.0 ± 4.0 μg/mL respectively (Table 1). Apigenin was relatively less active in DPPH\textsuperscript{•} scavenging (267.0 ± 7.7 μg/mL) and the lowest activity was observed for naringin (327.0 ± 3.0 μg/mL).

The plant extracts evaluated here showed that HRK and PGP scavenged more than 75.0% radicals at 10.0 μg/mL concentration. SCK showed slightly less activity as compared to those of other extracts (Fig. 6). IC\textsubscript{50} values (μg/mL) for DPPH\textsuperscript{•} scavenging activity of HRK, PGP and SCK were 9.0 ± 1.0, 5.5 ± 0.3 and 5.0 ± 0.8 respectively (Table 1).

3.5. Correlation analyses

The effect of phytochemicals and plant extracts in Fenton-DNA system was correlated with their corresponding DPPH\textsuperscript{•} scavenging activity (IC\textsubscript{50} values). A weak inverse correlation was observed between the IC\textsubscript{50} values and percentage DNA damage as shown in Fig. 7 (\(r = -0.806; n = 8; p < 0.01\)). PGP (grated in the figure) was excluded from the correlation analysis, as it exhibited high antioxidant as well as high iron chelation property. The iron chelation property of PGP was dominant over its reducing potential and hence showed less DNA damage, by retarding Fenton’s reaction. With inclusion of PGP, the correlation between reducing power of phytochemicals/extracts and DNA damage was further weakened (\(r = -0.5; n = 8; p = 0.12\)) suggesting that reducing power of PGP did not have direct influence on DNA damage in Fenton’s system.

4. Discussion

In physiological system, redox regulation is a vital process for homeostasis and presently studies are focused to understand its detailed mechanism. Normally a balance in the level of ROS/RNS by endogenous and exogenous antioxidant defense system is maintained [39]. The optimum levels of these ROS/RNS are known to facilitate several signal transduction and inter cellular communications [40]. However, excessive production of ROS/RNS is deleterious to tissues. Among the various ROS in physiological system, HO\textsuperscript{•} is the most reactive and it can inflict damage to biomolecules in its vicinity. Hydroxyl radicals are known to be produced by Fenton’s reaction and combinations of several factors are warranted for the Fenton’s reaction to take place. Availability of H\textsubscript{2}O\textsubscript{2} and free iron (Fe\textsuperscript{2+}) is the most critical elements for initiation of Fenton’s reaction. Hydrogen peroxide is generated \textit{in vivo} by dismutation of superoxide radicals by a range of oxidase enzymes including glycollate and monoamine oxidases as well as the beta-oxidation of fatty acids. The free iron required for the Fenton’s reaction could be generated by radiation, xenobiotics or excessive oxidative stress [41–43]. The free iron thus available under such conditions promotes Fenton’s reaction by liberating hydroxyl radicals spontaneously from H\textsubscript{2}O\textsubscript{2}. Hydroxyl radicals produced in close proximity to DNA cause strand break, base modification and DNA protein cross-linking [44]. Under oxidative stress, this mechanism may be dominating and the protective mechanism could be by scavenging the HO\textsuperscript{•} at very faster rate either by antioxidants or by chelation of the free iron.

In the present study, six standard compounds and three plant extracts were evaluated for their effect on the integrity of double stranded DNA in Fenton’s system. The results obtained were then correlated with their iron chelation property, total reducing power and DPPH radical scavenging activity to understand the mode of action (primary or secondary) as antioxidants and their possible role in the DNA-Fenton’s system. Among the six phytochemicals and three plant extracts studied, two were protecting DNA (naringin and PGP), four were promoting DNA damage (gallic...
acid vitamin C, HRK and SCK) and three of them were neutral (catechin, apigenin and naringenin). Naringin and PGP exhibited high iron chelation activity while the other test materials showed poor iron chelation under the assay conditions used here. The iron chelation property of the test materials could also be attributed to their secondary antioxidant activity which is their ability to retard or block upstream event of electron transfer from Fe (II) to hydrogen peroxide that triggers hydroxyl radical generation. To be an efficient inhibitor of Fenton’s reaction, the compound in the assay system should either chelate iron and prevent the electron transfer or should accept the electron from Fe (II). It is obvious from the results of this study that in the control assay system (Fenton’s reactants and DNA alone) the DNA damage was caused due to electron transfer from Fe (II) to hydrogen peroxide as it did not contain any chemical agent to chelate iron. However, in the presence of naringin and PGP, the percentage DNA damage was significantly less than that of control suggesting that the Fenton’s reaction was retarded by naringin and PGP with their higher efficiency to chelate iron at 83.67 ± 35 and 68.69 ± 2.27% respectively (Table 1). Naringin is a major flavonoid glycoside of many citrus fruits reported to have high iron chelation capacity [45]. DNA protective activity of naringin in cell lines are also reported due to its ability to chelate iron [46]. The protective activity exhibited here by PGP could be attributed to its high tannins [47,48] that are known to chelate iron thereby limiting the availability of free iron for Fenton’s reaction. Though naringin and PGP had high iron chelation capacity they behaved differently for their TRP and DPPH* scavenging activity. While PGP showed powerful antioxidant and iron chelation property, naringin exhibited poor activity for TRP and DPPH* and strong iron chelation suggesting that iron chelation property should be dominant to retard Fenton’s reaction. Vitamin C, gallic acid, SCK and HRK were excellent antioxidants but they were found to promote DNA damage probably by regenerating Fe^{2+} from the product of Fenton’s reaction (Fe^{3+}) [49–52]. This may not happen under normal physiological condition except in the event of much higher concentration of iron with other factors being favorable for Fenton’s reaction. Vitamin C, gallic acid, SCK and HRK were very poor iron chelators but all of them had high reducing potential. The DNA damaging effect observed in the presence of gallic acid, vitamin C, SCK and HRK therefore were due to the lack of their iron chelation ability.

Naringenin, apigenin and catechin showed neutral effect in Fenton-DNA system that they neither promoted nor protected DNA damage though they had poor iron chelation properties. In the case of catechin, its high reducing potential comparable with that of vitamin C did not promote DNA damage like vitamin C, gallic acid, HRK and SCK. The mechanism for promotion of DNA damage by phytochemicals with high reducing power therefore is unclear and studies related to their kinetics may throw some light on this. Neutral or DNA damaging effect of phytochemicals did not have correlation with their chemical structure or antioxidant potential. To confirm this, the results were further subjected to statistical analysis. A weak negative correlation (r = −0.83, n = 8, p = 0.01) between antioxidant activity (IC50) and percentage DNA damage (Fig. 7) was observed when PGP were excluded. With inclusion of PGP, the correlation further weakened (r = −0.5, n = 9, p = 0.12) indicating that phytochemicals with very high reducing power without iron chelation ability may damage DNA in Fenton’s system.

Thus, chronic exposure to oxidative stress, particularly HO* may lead to cellular and tissue degeneration, in the context of factors favorable for Fenton’s reaction. The HO* produced by Fenton’s reaction under the conditions described elsewhere cannot only damage DNA, but it can also react with proteins, lipids and carbohydrates. Prolonged exposure to such oxidation is presumed to be early events of cancer, CVD, diabetes, cataract, and neurologi-cal disorders. Phytochemicals of dietary and non-dietary origin can intercept at various levels and retard the progress of tissue degeneration. However, factors such as bioavailability, stability, access to the site of oxidation, blood–brain barrier, and effective concentration, may limit efficacy of phytochemicals.

The method employed here for measuring DNA damage may have deficiencies like low sensitivity and accuracy. However, the results are convincing due to the large difference between the protective and damaging effects of the phytochemicals and extracts used. The results were also analyzed using appropriate statistical tool to further establish the confidence level at 95% and above. The fact that we selected only six phytochemicals is also another limitation of the study as numerous other phytochemicals of dietary and non-dietary origins that we consume may exert synergistic and antagonistic effects as far as DNA damage is concerned. It may be worthwhile therefore to conduct studies using more number of phytochemicals that we consume and their interactions to further establish the findings reported here.

**Conflict of interest**

None.

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