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Protection of hydrogen peroxide and Metal induced DNA damage by flavonoids

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Abstract: More recent research has shown that free radicals cause biomolecules to oxidatively damage. This harm has been linked to several human illnesses, diseases, and ageing. Reactive oxygen species (ROS), such as the hydroxide free radical (OH), hydrogen peroxide (H_2O_2), and superoxide ion (O^{2-}), have been proven to cause oxidative damage to DNA. Naringin, quercetin, and naringinin have been demonstrated to assist in DNA from oxidative damage. The 2,2-diphenylpicrylhydrazyl (DPPH) test was used to gauge the capacity to neutralize free radicals. The method used was founded on spectrophotometric evaluation of the modification in DPPH concentration caused by the interaction with an antioxidant. Based on the apparent contradiction in words, the study shows the impact of quercetin, naringin, and naringinin on DNA damage in the presence of cupric ions and H_2O_2 using the gel electrophoresis technique. The aforementioned flavonoids acted as a protective agent at low cupric ion concentrations. While hydroxy free radical scavenger (FRS) did not prevent DNA cleavage at greater cupric ion concentrations, flavonoids did. It follows that DNA damage brought on by flavonoids is not simply caused by oxidative stress. The current work will look at a possible mechanism for the development of Copper(II) complexes with quercetin, naringin, and naringinin that promote antioxidant activity. To properly assess the efficacy of naringenin, quercetin, and naringin when combined with chemotherapeutic drugs for the administration of human health benefits, additional in-depth investigations, including clinical trials, are required.

Introduction

By definition, a free radical is any chemical entity that has one or more mismatched electrons. In general, such free radicals are quite active. They start a series of events that can harm various elements of the living thing. For basic oxygen to become harmful, it must first be pre-activated (Richa et al., 2014). Such activation can occur in two different ways: photodynamically, primarily producing singlet oxygen; or reducing, producing radical hydroxyl and the most active chemical species in all biological systems, anion hydrogen peroxide. Transition metals like iron and copper as well as particular enzymes (monooxygenase and some oxydases) speed up the reductive process. Different cellular compartments, including the mitochondria, microsomes, peroxysomes, and cytoplasmic membrane, are where this activation occurs (Muthia, 2004). The organism fights against the possible toxicity of oxygen with a variety of anti-oxidant defence mechanisms. The first group inhibits activation processes as it works its way up the radical chain. As a result, this organisation issues warnings about the start of catastrophic reactions (Muthia, 2004). The second group breaks the chain of free radical propagation by neutralising the ones that have previously been created. This category includes detoxifying enzymes like super oxygen dismutase and catalase that work together to

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produce peroxidases, especially peroxidase glutathiones. The majority of these enzymes require minor components as cofactors. Numerous compounds that serve as an antioxidant shield or as "substrate suicide" can also be identified in this second category. Some of these compounds, including tocopherols, carotenoids, and ubiquinones, can function in the lipidic phase. Other lipophobic compounds, primarily ascorbic acid and uric acid, are functional in a moist environment. An oxidative stress condition develops when this antioxidant defence is compromised or when there is an excessive generation of radicals (Rahman et al., 2015). If left unchecked, these radicals will harm lipids, DNA, and proteins, among other biological targets. If remedial defences don't step in, disturbances in cellular metabolism will happen. An essential step is to become aware of these radical phenomena. But, free radicals have such a limited lifespan, that identification presents a significant analytical challenge. However, there are three methods that may be used to detect free radicals: directly through the use of paramagnetic in electrons resonances, or indirectly by the identification of certain more stable intermediaries. The harm to DNA leads to cancer, and lipid oxidative damage hastens the development of vascular diseases, according to the results of high-performance liquid chromatography assessment of the depicts of radical assault on molecules in the body, such as by gas-liquid chromatography or colorimetric tests, estimates of antioxidant status, for instance by colorimetric tests, resistant enzyme methods, and (Alam et al., 2014). Free radicals cause a wide range of biochemical and physiologic Lesions that lead to degenerative illnesses such as cancer, diabetes, stroke, and coronary artery disease (Cavia-Saiz et al., 2010). ROS, which is continuously created during normal physiological activities, is linked to the buildup of free radicals (Yang et al., 2014). In preventative medicine, natural antioxidants are essential (Mates et al., 2000). Our bodies have built-in antioxidant defence mechanisms to get rid of damaged particles, but these processes are ineffective (Muthia, 2004). As a result, the major endogenous sources of oxidants generated by cells in the human body—polymorphonuclear leukocytes, macrophages, and peroxisomes—were activated during aerobic respiration (Alam et al., 2014). Electrons from antioxidants produce oxidation damage to biomolecules including DNA, protein, and lipids, which is prevented and stabilised by antioxidants (Cavia-Saiz et al., 2010). ROS and reactive nitrogen species (RNS) damage is reduced by antioxidants because they scavenge free radicals. Ozone (O_3), hydroperoxyl (OOH), peroxyl (ROO), hydrogen peroxide (H_2O_2), the acid hypochlorous (HOCl), and superoxide anion (O_2) are all produced by ROS. The compounds nitric oxide (NO), peroxynitrite (ONOO), and nitrogen dioxide (NO_2) make up singlet oxygen and RNS (Rui Chen et al., 2016). Consuming fruits and vegetables with antioxidant enhancements reduces the chance of numerous ailments brought on by free radicals and safeguards cells, according to research (Ishii et al., 1996). Flavonoids, which are the compounds known as carotenoids, nutritional glutathione, vitamins (C, E), and endogenous metabolites are only a few of the many FRS compounds found in plants (Alam et al., 2014). Low molecular mass benzo-ç-pyrone derivatives known as flavonoids are a class of antioxidants that exist naturally and are found in both larger and smaller plants. The capacity of a wide variety of pharmacological and biological substances to block or regulate specific enzymes and their antioxidant effects was studied by Muthia in 2004. When it comes to organic antioxidants, phenolic compounds stand out because of their capacity to chelate metals and inhibit lipoxygenase as well as their ability to quench oxygen-derived free radicals (Ishii et al., 1996). When oxidising triglycerides, flavonoids can function as FRS agents and stop the radical chain events that develop (Ho et al., 2001).

Naringin

One of the primary active ingredients of Chinese herbal medicines like *Drynaria fortunei* (Kunze) J. Sm. (DF), *Citrus aurantium* L. (CA), and *Citrus medica* L. (CM), (Alrawaiq et al., 2014), is naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside, a flavanone glycoside established by the flavanone naringenin and the disaccharide neohesperidose. Naringin and a number of other glucoside flavonoids, including rhoifolin and daidzein have the capacity to induce polymorphonuclear leukocytes (PMN) activation and cause them to produce cytotoxic activity against tumour cells in vitro (Kawabata et al., 2015).

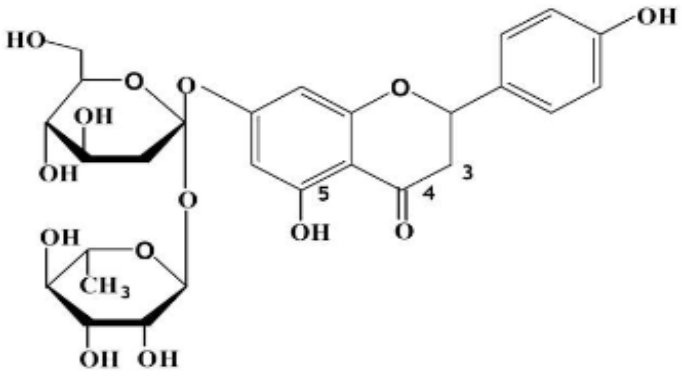

Figure 1. Chemical structure of Naringin (Rui Chen, 2016)

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Citrus fruit extracts are rich in flavonoids and have strong anti-free radical properties. Both naringin and naringenin are potent FRSs that inhibit lipid peroxidation. These flavonoids scavenge hydroxyl and superoxide radicals in vitro. In eukaryotic cells, the xanthine oxidase enzymes are physiological generators of superoxide anions. In vitro, xanthine oxidase activity was discovered to be considerably inhibited by naringin (Bernini and Velotti, 2021). The antioxidant and free radical-neutralising properties of aromatic phytonutrients are thought to contribute to their ability to improve health. By producing ROS and free radicals, which harm various cell constituents, perturbations in the standard redox state of cells can cause toxicity (Larson et al., 2012). It has been demonstrated that naringin possesses dose-dependent radical scavenging ability against tetraethyl ammonium chloride and 1,1-diphenyl-2-picryl-hydrazyl radicals. In Chinese hamster fibroblast (V79) cells, naringin demonstrated antioxidant activity and decreased the incidence of damage to DNA caused by H_2O_2 at doses of 5-2000 lM. Pretreatment with naringin (3 or 24h) restored the drop in GSH levels and increases in cytoplasmic free radicals and glucose absorption in L6 myoblast cells under oxidative stress. Chronic naringin treatment over a period of 6 weeks decreased oxidative damage in mice treated with D-galactose by lowering lipid peroxidation and nitrite concentration, restoring the lowered GSH level, and increasing SOD, catalase, and GSH-S-transferase activity (Knab et al., 2011). The similar substance kaempferol, which contains a –OH group adjacent to the keto group and is found in grapefruit, is linked to naringenin (Neamtu et al., 2022). Naringenin is 8 dimethylallyl transferase that synthesises diphosphate and 8-prenylnaringenin from dimethyl allyl diphosphate and naringenin (Pawar et al., 2022). Investigations have been done on naringenin's possible antibacterial and antifungal properties. Naringenin was said to have no antibacterial effect against *Staphylococcus epidermidis* (Yi et al., 2021). A study from 2000 demonstrated that naringenin does, in fact, have an antibacterial impact on *S. epidermidis* along with *Bacillus subtilis, S. aureus, Micrococcus luteus,* and *E. coli*, among others. However, this discovery was not confirmed in that study. Additional studies have provided evidence for antimicrobial properties against yeasts like *C. albicans* and *C. krusei*, as well as bacteria like *Lactococcus lactis, lactic acidophilus, Actinomyces, Naeslundii, Prevotella oralis, Prevotella melaninogencia,* and *Porphyromonas gingivalis* (Minocha et al., 2022). Though naringenin hasn't been found to have any influence on the microbe's urease activity, there is evidence that it has antibacterial impacts on *H. pylori*. Infectious hepatocellular (liver cells) in cell culture have also been demonstrated to produce less hepatitis C virus when naringenin is present. This appears to be a byproduct of naringenin's capacity to prevent cell release of extremely low-density lipoprotein. Naringenin's antiviral properties are now being studied in the clinic. HSV-1 and HSV-2 poliovirus antiviral effects were also reported, while virus replication has not been stopped (Haenem et al., 1997).

Quercetin

Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), a flavonoid is frequently present in fruits and vegetables. Due to its capacity to neutralise radicals that are free and bind metal ions, quercetin is a potent antioxidant. Flavonoids, which have a catechol group (B ring) and 3-OH, were discovered to be up to 10 times more powerful than ebselen, a known RNS scavenger, towards peroxynitrite (Kempuraj et al., 2005). The B-ring's dihydroxy structure, the C-ring's 3-hydroxy group, and the 2,3-double bond are responsible for quercetin's antioxidant effect. Because of this, the antioxidant activity of quercetin's glycosylated derivatives is lower than that of Aglycone (Hilliard et al., 2020). The majority of plant quercetin is found as glycosides, which are coupled to sugar molecules. After being consumed orally, the sugar moiety is first hydrolyzed. This can happen in the intestines through the activity of the lactase enzyme on the surface of the enterocytes (intestinal cells), or through the reaction of cytosolic ß-glucosidase following its transit into the enterocytes (Hilliard et al., 2020). The gut microflora and bacteria hydrolyze and further degrade glycosides that make it to the colon. The stomach, small intestine, and colon absorb all types, whether aglycone or glycoside (Leo et al., 2015). It has been discovered that Vitamin C, pectin and fat can further improve quercetin absorption (Wani et al., 2022). Regarding chemoprevention, quercetin has a variety of effects. One of the processes involves chemically altering DNA and histones to re-model chromatin. Quercetin might activate histone deacetylase in prostate cancer cells, which would reduce histone H3 acetylation and make the cells more susceptible to TRAIL-induced apoptotic (Taile et al., 2021). The capacity of quercetin to control the cell cycle via altering a number of molecular targets, including as cyclin B, p21, p27, cyclin-dependent kinase (CDKs), and a enzyme known as II, is another significant anti-cancer impact (Taile et al., 2021).

Flavonoids such quercetin, naringenin, and naringin. Chemicals like thiobarbituric acid (TBA), trichloroacetic acid (TCA), ferrous sulphate H_2O_2, ferrozine, ammonium

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molybdate, ferric chloride, butanol, and methanol Citric acid, sodium phosphate, sodium dodecyl sulphate (SDS), agarose powder, tris-EDTA (TE) buffer, tris-acetate EDTA (TAE) buffer (1X, 50X), copper sulphate, sulphuric acid, sodium chloride, ferrous salt, and magnesium chloride are all components of phosphate buffered saline, also known as PBS.

Materials and Methods

Chemicals and reagents

In vitro evaluations of phytochemicals' antioxidant activities

There are several ways to evaluate the antioxidant capacity of plant extracts or chemicals. The chemicals, substrates, experimental setup, reaction medium, and conventional analytical evaluation techniques used in these experiments vary from one another. Antioxidant tests are necessary for the evaluation of both natural and synthetic antioxidants. Due to the variation in experimental circumstances and variation in the chemical and physical properties of oxidisable substrates, precise evaluation and selection of the optimal technique are practically impossible. However, the assay may be divided into two categories: (i) aqueous antioxidant assays (DPPH, ABTS, DNA protection, etc.) and (ii) lipid-based antioxidant assays (TBARS, lipid peroxidation). There are several in vitro antioxidant techniques used, including the DPPH eliminating activity, the hydrogen peroxide scavenging (H2O2) assay, nitric oxide (NO) scavenging activity, peroxide scavenging activity, trolox comparable antioxidant activity (TEAC) method/ABTS radical cation discolouration assay, total radical-trapping antioxidants parameter (TRAP) technique, ferric reducing-antioxidant power (FRAP) the method of the capacity to absorb oxygen radicals (ORAC), the phosphomolybdenum technique, the reducing energy method (RP), technique using ferric thiocyanate (FTC), DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) technique, b-carotene linoleic acid method/conjugated diene test, TBA method, Methods include Xanthine Oxidase, Lipid Peroxidation (LPO), Cupric Ion Reducing Antioxidants Capacity (CUPRAC), and Metal Chelating Activity (Larson et al., 2012).

Activity to scavenge DPPH radicals

The molecular structure of DPPH, which has been widely used as a sensitive and quick tool for determining the FRS capacity of both hydrophilic and lipophilic antioxidants, is characterised as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole. The deep violet colour, which is characterised by a band of absorption in ethanol solution with a centre wavelength of about 517 nm, is likewise a result of electron delocalization. When a DPPH solution is combined with a substrate (RH) that has the ability to provide a hydrogen atom, the reduced form results, in losing the violet hue (Larson et al., 2012).

To determine the proportion of DPPH radical scavenging activity, the formula below was used: The % of the DPPH radical scavenging activity is equal to (A0 + A1) /A0 + 100%, where A0 is the wavelength of the control and A1 is the absorption of the extractives/standard. After that, the concentration was plotted against the % of interference, and the IC50 was calculated from the graph. The experiment was carried out three times at each concentration (Larson et al., 2012; Knab et al., 2011).

The ability to chelate metals

By building chelates with Fe^{2}+, ferrozine is capable of creating a complex with a red hue. The red colour of the ferrozine Fe^{2}+ compounds is reduced as a result of this reaction, which is limited in the existence of additional chelating agents. The binding capacity that competes with the ferrozine for the ions that are ferrous is determined by measuring the colour reduction (Bernini et al., 2015).

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Assay for total antioxidant capacity

According to Mahbubur Rahman (2015), the total capacity for antioxidants (TAC) test is based on samples reducing Mo(VI) to Mo(V) and producing a green phosphate/Mo(V) complex at an acidic pH. The following formula calculates the overall antioxidant capacity (Cavia-Saiz *et al*., 2010):

$$\% \text{ of total antioxidant capacity} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \text{ X } 100$$

Lipid peroxidation assay

Egg-yolk homogenates were utilised as a lipid-rich medium to evaluate the amount of lipid peroxide produced using a modified thiobarbituric acid-reactive species (TBARS) test. A pinkish-red chromogen with a maximum absorbance at 532 nm is produced when malondialdehyde (MDA), a byproduct of the oxidation of fatty acids that are polyunsaturated, interacts with a pair of molecules of TBA (Bernini et al., 2015).

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Preparing a sample In vitro tests for antioxidants: Each fraction was produced as a stock solution (2 mg/1 mL) in 95% methanol and diluted in accordance with various antioxidant and reducing tests. The effectiveness of standard compounds was compared to the antioxidant potency of each test. DPPH's radical-scavenging activity: (i). Using the DPPH test, the antioxidant capability of the compound and every component was evaluated. To get an absorbance at 517 nm, 2 mg of DPPH was dissolved in 100 mL of methanol. (ii) 0.075 M of $FeSO_4$ was dissolved in 20 mL of DH2O, 0.8% TBA, 1.1% SDS, 20% TCA, 50 mL of phosphate buffer saline, and 50 mL of butanol were all dissolved in 30 mL of DH2O for the lipid peroxidation assay. (iii) Metal Chelating Agent: Ferrozine (5 mM) and Fecl3 (0.2 mM) were both diluted in 20 mL of DH_2O. (iv) Total Antioxidant Assay: In 30 mL of DH_2O, 0.6 M of H_2SO_4 and 28 mM of sodium phosphate were dissolved. In the same amount of water, 4 mM of molybdate of ammonium was also dissolved. After dissolving 2 mg of ascorbic acid in 1 mL of DH_2O and 2 mg of citric acid in 1 mL of DH2O, the chemicals were combined in 50 mL of DH_2O. Experimental technique Chemical constituents' qualitative and quantitative determination: Following conventional protocols, the presence includes alkaloids, a class of compounds cardiac glycosides, coumarins, flavonoids, saponins, phlorotannins, tannins, and terpenoids that in the extract and different fractions were independently verified.

Lipid Test for Peroxidation

Egg-yolk homogenates were utilised as a lipid-rich medium to evaluate the amount of lipid peroxide produced using an altered TBA-reactive molecule (TBARS) test (Richa *et al*., 2014). A pinkish-red chromogen with a maximum absorbance at 532 nm is produced when MDA, a byproduct of the oxidisation of polyunsaturated fatty acids, interacts with a pair of molecules of TBA. In a test tube, egg homogenate (250 l, 10% in distilled water, v/v) and extract (50 l) were combined. The test tube's capacity was then increased to 500l by adding distilled water. In order to cause lipid peroxidation, 25 L of $FeSO_4$ (0.07 M) was incorporated into the aforementioned combination and kept for 30 min. After that, 25 mL of 20% TCA and 750 mL of 20% acetic acid solution (pH 3.5), 0.8% TBA (w/v), and 0.1% sodium dodecyl sulphate were added, vortexed, and cooked in a bath of boiling water for 60 minutes. 3.0 mL of one butanol was then added to each tube after chilling, and then they were subjected to centrifugation for 10 minutes at 3000 rpm. At 532 nm, the organic top layer's absorbance was evaluated against 3 mL of butanol. The extract was substituted for the blank with 50 litres of distilled water.

Assay for the chelating of ferrous ions

Different extract strengths (10–2,000 g/ml) were added to a 0.05 ml solution of 2 mM $FeCl_2$. 5 mM ferrozine (0.2 ml) was added to the mixture to start the reaction. The mixture was then violently agitated and allowed to stand at ambient temperature for 10 minutes. The solution's absorbance at 562 nm was measured spectrophotometrically after the combination had reached equilibrium. The positive control was EDTA. Triplicates of each test and analysis were performed. Inhibition (%)=[(A0-A1)/A0]100%, where A0 represented the absorption of the control substance and A1 was the absorption in the form of samples and standards, calculates the amount of inhibition of ferrozine-Fe^{2+} complex formation. The compound-forming molecules ferrozine and $FeCl_2$ were absent from the control (Knab *et al*., 2011).

Assay for scavenging DPPH radicals

This assay was used to evaluate the extracts' capacity to scavenge free radicals (Larson *et al*., 2012). The capacity of the plant extractives to donate H^+ atoms was assessed using the decolorization of a methanol solution containing DPPH. In a methanol solution, DPPH creates a violet or purple colour that, in the presence of antioxidants, fades to varying hues of yellow. 0.1 mM DPPH was dissolved in methanol to form a solution, and 2.4 mL of this solution was then added to 1.6 mL of extract in methanol at varying concentrations from 12.5 to 150 g/mL. After fully vortexing, then the reaction mixture was kept incubated at room temperature for 30 mins in the darkness. At 517 nm, the mixture's absorbance was determined spectrophotometrically. BHT was cited as a source.

Measurement of the overall antioxidant capability

This test is based on samples converting Mo(VI) to Mo(V) and producing a green phosphate/Mo(V) complex at an acidic pH. In the test tubes, 3 mL of a reaction mixture comprising 0.6 M H_2SO_4, 28 mM Na_3PO_4, and 1% ammonium molybdate was combined with 0.5 mL of samples or standards at various concentrations (12.5-150 g/mL). To finish the reaction, the experiment tubes were heated to 95°C for 10 minutes. Using a spectrophotometer and a blank solution as a reference, sample absorbance was determined at 695 nm after cooling at room temperature CA served as the benchmark.

A common blank solution contains a suitable amount of the same solvent as the samples/standard along with 3 mL of the reaction mixture. The control sample was

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incubated for 10 minutes at 95°C, and the absorbance at 695 nm was measured. The reaction mixture's enhanced absorbance is a sign of greater overall antioxidant capability. For each antioxidant experiment, we employed samples and standards at various concentrations, ranging from 12.5 to 150 g/mL. Trial and error led to the selection of concentrations that fit the range of concentrations that may accurately depict the rational change in antioxidant activity with increasing sample concentration. Additionally, we presumed that such a concentration range is helpful for a smooth determination of the IC50. Three times each at each concentration, the experiment was performed (Larson et al., 2012).

DNA splintering

By using the alkali-lysis approach, plasmid DNA (pBR322) was extracted from *E. coli*. Plasmid DNA and loading dye were added to the reaction mixture, which was loaded in a 0.8% agarose gel, and 50 mV was applied to the gel tank's 1X TAE buffer solution. These bands were obtained after the gel had run for 15 minutes (Fig. 8). DNA oxidative damage brought on by Cu^{2+}, Fe^{2+}, and H_2O_2 The reaction mixes comprise 5 l of DNA ladder (100 bp to 1 kb), 2 l of control plasmid DNA, 1 l of 25 mM H_2O_2, 2 l of 50 mM H_2O_2, 2 l of 50 mM quercetin, and 4 l of 100 mM H_2O_2. of flavonoid are added in the appropriate amounts by altering their contents in various tubes, and 10l TAE buffer is used to create the reaction mixture. After that, it is incubated at 370 degrees for 45 minutes. Before casting the gel, 1.5 l of a solution of ethidium bromide was added to create 1% agarose gel. Following incubation, each combination is given 2l of loading dye before being placed onto the gel to end the process. A gel tank containing 1X TAE buffer received a 50mV electric current. The bands were acquired after the gel had been left to flow for 15 minutes (Fig. 15 a, b, c, d, e).

Results

Activity that scavenges free radicals

Tables 4 and 5 display the DPPH-measured FRS capacity of quercetin, naringin, and naringinin. When combined, flavonoids have more antioxidant action than other substances. Ascorbic acid, which was utilised as a +ve control, had a better impact in scavenging free radicals. The antiradical efficacy was determined from the graph of the percentage of DPPH still present after the amount present achieved a constant level as a function of the mole ratio of antioxidants to DPPH (Fig. 8, 9, 10, 11), as mentioned in the methods section. Antiradical activity might be used to calculate the quantity of antioxidants required to completely reduce the original DPPH concentration. The antiradical efficiency categorization used in this work is different, and several of the chemicals examined (such as quercetin, naringin, and naringinin) rarely react with over fifty per cent of the original DPPH, even after 15 minutes and at extremely high concentrations. As a result, the anti-radical efficiency categorization was developed and is depicted in Figures 8, 9, 10, and 11. According to their concentration, all substances are arranged in Table 4,5 in an increasing sequence of ARP (Anti-Radical Power).

Figure 12, Table 6, and Table 8 display the suppression of metals chelating and overall antioxidant activities, respectively. Using a strong peroxyl radical produced by the TBARS reaction, quercetin, naringenin, and naringin inhibited lipid peroxidation in egg yolk. Table 7 and Fig 13 display the values obtained from the LPO test. Free hydroxyl radicals in C7 were discovered to be the most active substance. Additionally, flavonoids suppressed peroxyl radical activation more effectively than hydroxyl radicals.

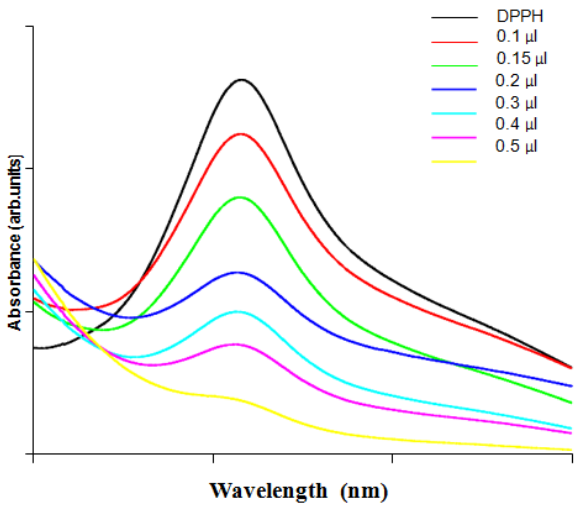

Figure 2. Optical Spectrum of DPPH with Quercetin (517nm)

Table 1. DPPH radical scavenging by quercetin

Concentration (in mM)	O.D (517 nm)
0.1218	0.1484
0.0974	0.3049
0.0731	0.3969
0.0487	0.5068
0.0243	0.8954

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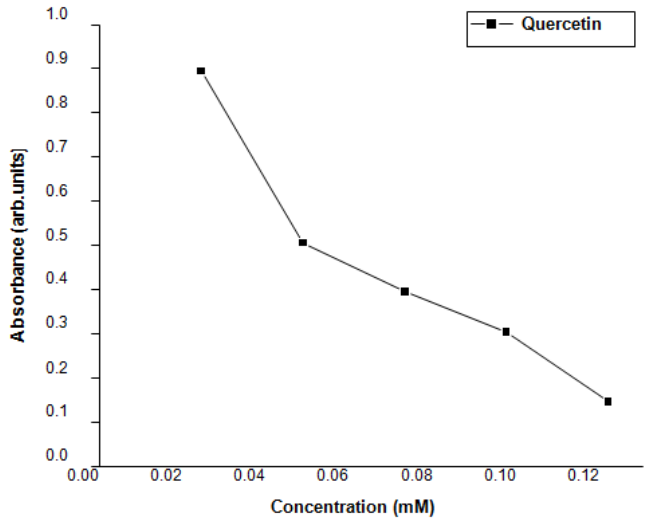

Figure 3. Graphical representation of quercetin

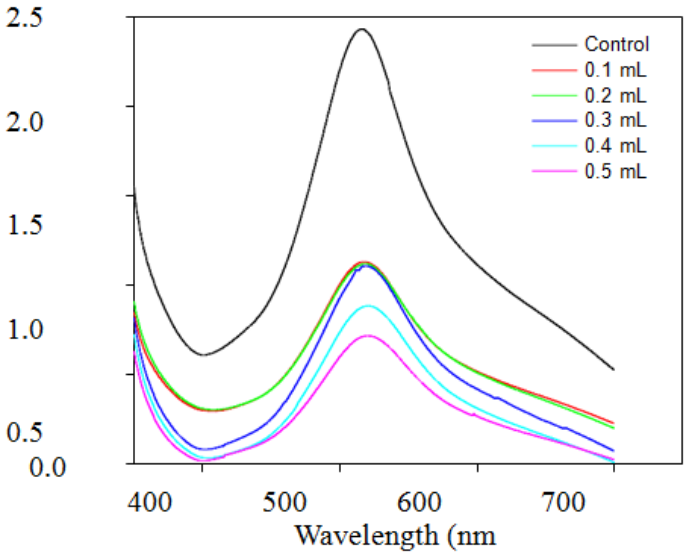

Figure 4. DPPH scavenging by naringin

Table 2. DPPH Assay with Naringin

Concentration (in mM) [NA]	O.D (517 nm)
0.1204	0.7123
0.0963	0.8774
0.0722	1.1046
0.0482	1.1187
0.0241	1.1294

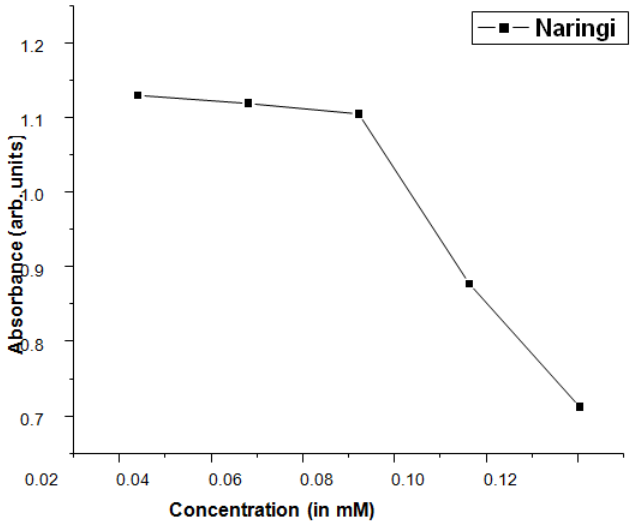

Figure 5. Graphical representation of radical scavenging by naringin

Table 3. Antioxidant activity of flavonoids (Total Antioxidant Assay) withreference to ascorbic acid

Test sample	Concentration (in mM)	O.D @ 675 nm	Total Antioxidant Capacity (%)*
Quercetin	0.441	0.202	11
Naringin	0.229	0.065	7
Naringenin	0.49	0.058	3
*Taking reference ascorbic acid as 100% capacity			

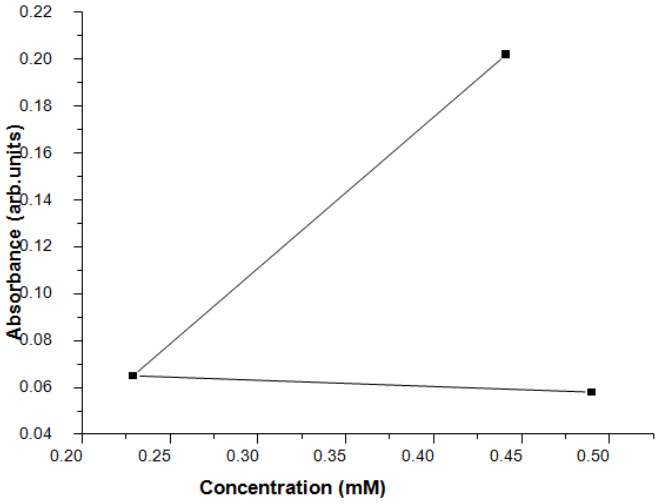

Figure 6. Graphical representation of the total antioxidant assay

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Table 4. Antioxidant activity of flavonoids (Lipid Peroxidation Assay) withreference to ascorbic acid

Test sample	Concentration (in mM)	O.D @ 532 nm	% inhibition of lipid peroxidation
Quercetin	0.220	1.716	24
Naringin	0.114	2.390	5.1
Naringenin	0.245	2.237	1.4

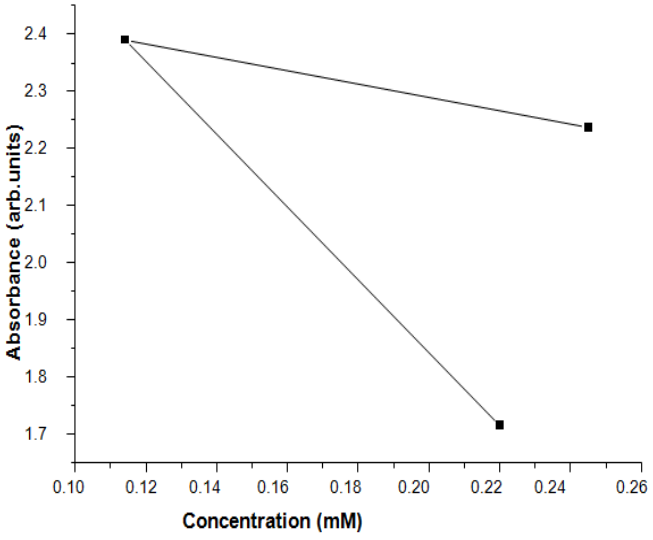

Figure 7. Graphical representation of lipid peroxidation assay

Table 5. Antioxidant activity of flavonoids (Metal Chelating Assay) withreference to ascorbic acid

Quercetin Concentration (in mM)	O.D @ 562 nm	% inhibition
0.0017	0.250	67.5
0.0008	1.000	29.8
0.0004	1.120	31.3

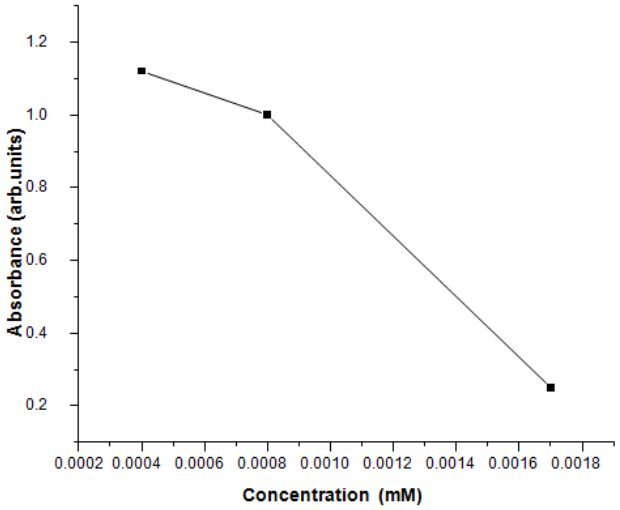

Figure 8. Graphical representation of metal chelating assay

DNA fragmentation studies :

Analysis of Plasmid DNA

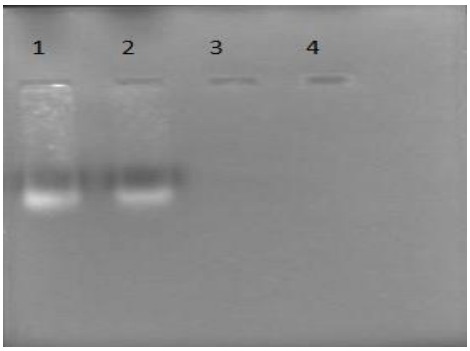

Figure 9. Lane 1 and lane 2 contain plasmid (1: Analysis of plasmid DNA)

Figure 15 shows that the undamaged double-stranded plasmid pBR322 exists in a compact supercoiled formation

Copper (Cu^{2}+) and H2O2 induced DNA damage

Figure 15 (a)indicated that in the formation of strand breaks, the supercoiled form of DNA was disrupted into a cupric ion plus H_2O_2 caused the cleavage of SC DNA into CU DNA and L DNA. In the presence of H_2O_2 DNA cleavage induced by cupric ion, as seen from the formation of linear DNA. L2, L4 and L6 were completely damaged by the Cu^{2}+, H_2O_2 and the flavonoids are not protected and induce DNA damage.

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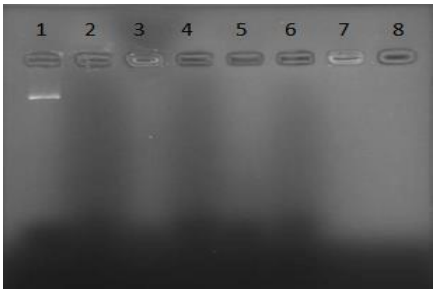


Figure 9(a). L1 contains plasmid DNA; L2 contains DNA+Cu^{2+}; L3 contains DNA+ H_2O_2 , L4 contains DNA+ Cu^{2+}+ H_2O_2 , L5 contains DNA+Cu^{2+}+ H_2O_2 , L6 contains DNA+ Cu^{2+}+ H_2O_2 + 25µM quercetin , L7 containsDNA+ Cu^{2+}+ H_2O_2 + 50µM quercetin

Dose dependent protection of DNA fragmentation by quercetin

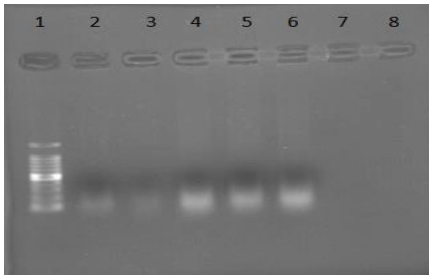


Figure 9 (b). L1 contains DNA ladder (100 bp – 1Kb) , L2 contains DNA control , L3 contains DNA + 25 µM H_2O_2 + 100 µM quercetin , L4 contains DNA + 25 µM H_2O_2 + 50 µM quercetin, L5 contains DNA +50µM H_2O_2 +50 µM quercetin , L6 contains DNA + 50µM H_2O_2 + 100 µM quercetin

Figure 9 (b) demonstrates the DNA fragmentation induced by H_2O_2 with varying concentrations of quercetin. L4, L5 and L6 contain varying concentrations of H_2O_2 and quercetin. L4 and L5 were protected to a lesser extent when compared to lane 6. The intensity of the band is more when compared to all the lanes in the gel. Thus, increasing concentration of quercetin was found to protect by hydrogen peroxide and it was seen in the supercoiled form.

Iron (Fe^{2}+) and H_2O_2 induced DNA damage and its protection by flavonoids

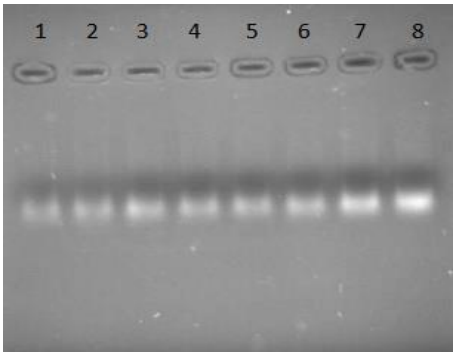


Figure 9(c). L1 contains DNA control , L2 contains DNA + 50µM H_2O_2 + 50µM Fe^{2+} , L3 contains DNA + 25 µM H_2O_2 +50µM Fe^{2+}, L4 contains DNA + 100 µM H_2O_2+ 50 µM Fe^{2+}, L5 contains DNA +100µM H_2O_2 + 50 µM Fe^{2+} + 50 µM quercetin, L6 contains DNA + 100µM H_2O_2+ 50 µ M Fe^{2+} + 50 µM ascorbic acid, L7 contains DNA + 100µM H2O2 + 50 µM Fe^{2+} + 50µM naringenin, lane 8 contains DNA + 100µM H_2O_2 + 50 µM Fe^{2+} + 50 µM naringin

Figure 9(c) shows the effects of flavonoids by various concentrations of induced H_2O_2 in DNA damage. Lane 5, and lane 6 contain quercetin and ascorbic acid which are found to be less protected whereas lane 7 and lane 8 contain naringenin and naringin and the intensity of their bands is more. Therefore, lane 8 containing naringin the high-intensity band was supercoiled.

Combinatorial defence against DNA fragmentation

Double-strand breaks are significantly more likely to develop when quercetin and naringin are present in increasing amounts [Figure 15(d)]. When compared to lane 8, which includes both naringin and quercetin, lanes 5 and 6 have different amounts of quercetin and exhibit a minor supercoiled band. As a result, it was discovered that it harms DNA less than the other lanes. A study of DNA fragmentation used flavonoids' synergistic effects. Double-strand breaks were more likely to arise when quercitin and narigin were combined [Figure 15(e)]. Flavonoids work together to provide the effects seen in lanes 7 and 8.

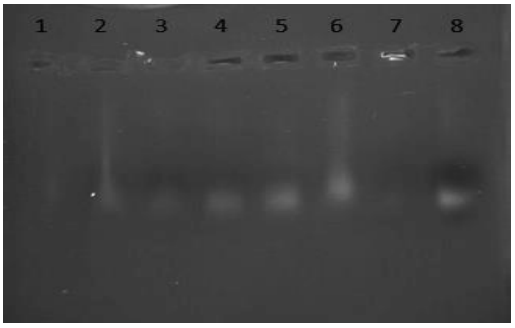
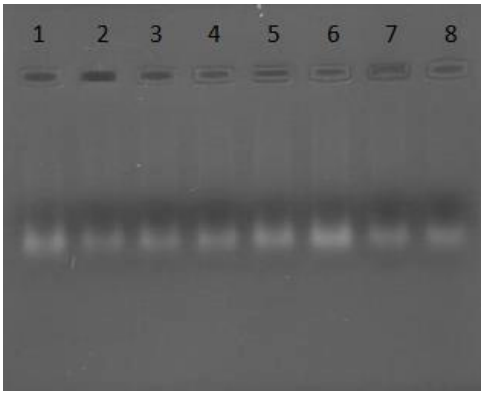


Figure 10 (d). L2 – DNA Control, L3 – DNA + 25 µM H_2O_2 +100 µM quercetin, L4 – DNA + 25 µM H_2O_2 + 50 µM quercetin, L5 – DNA +50µM H_2O_2 + 50 µM quercetin, L6 – DNA + 50µM H_2O_2 + 100 µM quercetin, L7– DNA + 50µM H_2O_2 + 50 µM naringin, L8 - DNA + 50µM H_2O_2+ 50 µM (naringin and quercetin).



(5B) Synergistic protection of DNA fragmentation

Figure 10 (e). L1 contains DNA control, L2 contains DNA + 500µM H_2O_2 +100µM Fe^{2+}, L3 contains DNA + 500 µM H_2O_2 +100µM Fe^{2+}, L4 contains DNA + 500 µM H_2O_2 +100 µM Fe^{2+} + 250µM naringenin, L5

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contains DNA + 500µM H_2O_2 + 100 µM Fe^{2+} + (250 µM) naringin, L6 containsDNA + 500µM H_2O_2 + 100 µ M Fe^{2+} + 250 µM quercetin, L7 contains DNA + 500µM H_2O_2 + 100 µM Fe^{2+} + 250 µM (quercetin and naringenin), L8 contains DNA + 500µM H_2O_2 +100 µM Fe^{2+} + 250 µM (Quercetin and Naringin)

Discussion

When DPPH receives an electron or hydrogen radical, it transforms into an eternal diamagnetic molecule (Larson et al., 2012). DPPH solution produced a rich purple colour with a maximum absorption at 517 nm, however, it usually wears off in the presence of antioxidants. The next study confirms that antioxidant activity has a direct correlation with the overall phenol or anthocyanin amount in various plants (Larson et al., 2012). Compared to quercetin, naringin and naringinin exhibited a greater ability to reduce the DPPH radical (Table 5). Total phenolic content and the ability of DPPH to neutralise free radicals have been shown to be highly correlated (Masood, et al., 2020). Some flavonoid-type compounds, one of the most varied and prevalent classes of natural phenolics, were the source of antioxidant activity (Masood et al., 2020). Because MeOH has a larger phenolic content (quercetin, naringin, and naringenin), it may be able to reduce DPPH and hydroxyl radicals more effectively and have a greater scavenging impact on these radicals.

Lipid peroxidation, a series of events known as reactive oxygen species (ROS) causes harm to membranes by peroxiding lipid molecules, notably polyunsaturated fatty acids (Masood et al., 2020). A second radical is produced by the original reaction, and this radical can then interact with another macromolecule to start a chain reaction that disrupts cellular function. Through the production of OH radicals in the Fenton reaction, ferric ion and potassium chloride are caused by in vitro lipid peroxidation in egg yolk. The most significant indicator of antioxidant activity was thought to be the suppression of lipid peroxidation. TPL's ability to reduce lipid peroxidation here was greater than that of other extracts (Masood et al., 2020) (Fig. 13). These findings demonstrated that quercetin, naringin, and naringenin can inhibit the lipid peroxidation chain events that lead to cellular abnormalities brought on by free radicals (Kempuraj et al., 2005; Maniko et al., 2009). Because they are rich sources of natural antioxidants, quercetin, naringin, and naringenin can be utilised to treat a variety of ailments brought on by free radicals. Lipid peroxidation has the potential to significantly alter biological membranes' characteristics, causing major cell damage and being a key factor in the aetiology of disease (Yi et al., 2021). The genetically augmented amounts of proteins like cytokines have also been linked to certain lipid peroxidation products. Numerous investigations have demonstrated that naringenin and naringin in this situation lowered the quantity of lipid peroxidation (Fig. 7, 18). However, a comparison of the two flavonoids was not offered in these interactions since flavanones were used in various systems and situations. On the other hand, it is well known that a substance's ability to suppress lipid peroxidation depends on the type of oxidant used. The findings demonstrated that -OH in C-7 improved the effectiveness of lipid peroxidation inhibition and that the concentration of naringenin required to achieve 50% inhibition when utilising peroxyl or hydroxyl radical as the inducing system was lower than that of the glycoside (Xu et al., 2021). It was shown that naringin and naringenin are powerful defences against lipid oxidative damage as a result.

In this work, the Copper(II) complex of naringin, naringinin, and quercetin was examined for its effects on oxidant harm to DNA, DNA binding, and anticancer activity. Previous findings show that flavonoids in the copper complex may efficiently encourage the breakdown of plasmid DNA via an oxidative pathway at normal pH and temperature. Regarding the additive effects of naringin and naringinin complexes with metals, the speed continuous of the oxidised DNA cleavage discovered in this work is significantly larger than the rate constant of the hydrolytic DNA cleavage discovered in our earlier investigations (Shafabakhsh and Asemi, 2019). Studies conducted in vitro demonstrated that the production of ROS in a mixture of copper (II) and H_2O_2 caused DNA damage at the thymine and guanine bases (Shafabakhsh and Asemi, 2019; Yabalak et al., 2023). A reactive copper (II)-hydroperoxo complex is created when copper activates H_2O_2 to cause oxidative DNA damage.

It was also proposed that the combination can intercalate into DNA due to the ligands' favourable planarity, quercetin, naringin, and naringinin and that the copper cation could work together with the negatively charged oxygen atom in DNA's phosphodiester backbone, displace a water molecule, and thus increase the affinity for binding between the complex and DNA. The effects of a number of other agents on the cleavage of DNA were studied in order to understand the mechanism by which the $Cu(Que)_2(H_2O)_2$ complex induces the cleavage of pBR322 DNA.

(Murakami et al. 2006), although bathocuproine disulfonic acid, DMSO, and glycerol had less of an inhibitory impact on DNA breakage, it was not implied

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that the system was devoid of hydroxyl radicals and Copper(I). It was possible that the ligand quercetin would sterically block the chelation of bathocuproine sulfonic acid and Copper(I). However, H_2O_2 and catalase considerably accelerated and prevented, respectively, the strand breaking of plasmid pBR322 DNA caused by the complex (Ho et al., 2001). It is claimed that both ROS, particularly H_2O_2, participated in DNA breakage and that this DNA cleavage was significant. There is evidence to suggest that the chemopreventive benefits of polyphenols like quercetin may not fully be explained by their antioxidant properties (Shi and Williamson, 2015). It was proposed that these polyphenols' antioxidant properties may be necessary but insufficient for their efficacy against tumour promotion. The most recent research also showed that quercetin, a tyrosine kinase inhibitor, was ineffective as a cancer treatment (Yanez et al., 2007). However, the $Cu(Que)_2(H_2O)_2$ complex was more hazardous to cancer cells than quercetin by itself, which may be explained by the fact that the complex intercalated DNA and effectively caused oxidative DNA damage. Additionally, the neighbouring deoxyribose ring in the DNA backbone is attacked by the hydroxyl radical generated by the peroxide complex, which exhibits modest specificity in oxidative DNA damage. In other words, the $Cu(Que)_2(H_2O)_2$ complex's affinity for intercalating into DNA determines the type of selective oxidative harm to DNA it causes (Yanez et al., 2007). However, more thorough research is required to confirm the actual apoptosis process. Free radicals cause oxidative DNA damage, altering the biomolecule's structural or functional properties. DNA lesions caused by oxygen radicals that are free like hydroxyl radicals include oxidised bases, basic sites, and breaks in DNA strands (Choudhury et al., 1999). When DNA integrity was assessed using agarose its separation, naringenin and naringin therapy had a concentration-dependent protective effect on the DNA. At concentrations greater than 1 mol L-1, both flavanones inhibited oxidant-induced DNA strand breaking. In mouse bone marrow, naringin has been shown to decrease radiation-induced DNA damage, ferric iron DNA oxidation, and DNA damage (Erlund et al., 2001). In contrast, Sahu showed that naringenin (Uzel et al., 2005) caused DNA strand breakage and concentration-dependent oxidation of nuclear lipids in the membrane in an experimental system composed of separated rat liver nuclei. However, Lyu et al., 2005 found that the citrus flavonoid naringenin accelerated the repair of DNA in prostate cancer cells.

Conclusion

The current findings unmistakably shown that antioxidant activity in the free radical removal and DNA damage assays were improved by quercetin, naringenin, and naringin. In particular, tumours with inherent drug resistance that is mediated by MRPs may benefit from the combined use of quercetin, naringin, and naringenin with chemotherapeutic drugs. To properly assess the usefulness of naringin and naringenin in conjunction with chemotherapeutic drugs for the management of various human illnesses, however, more in-depth investigations, including animal studies, are required.

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Conflict of interests

The author declare no conflict of interests.

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