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Flavonoids: A Review on Extraction, Identification, Quantification, and Antioxidant Activity

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ABSTRACT

Flavonoids are polyphenolic compounds with a 15-carbon basic scaffold (C6-C3-C6) consisting of two aromatic rings, A and B, joined by a pyran-C heterocyclic. The numerous advantages of this substance based on its antioxidant activity seem to be the reason for the high interest in flavonoid research. Since this review is a narrative review, it is written as objectively as possible, is not systematic, and does not follow any specific protocol. In this review, we first discussed flavonoid extraction techniques in the form of conventional and unconventional methods. Then, flavonoids' identification methods using chemical reactions, chromatography, and spectroscopy, followed by a review of the spectrophotometric quantification method using AlCL3 and 2,4-DNPH reagents. Finally, we discuss the methods for determining antioxidant activity that is widely applied to flavonoid compounds. This method includes both hydrogen atom transfer and electron transfer mechanisms. All the methods mentioned are accompanied by a brief procedure and the reaction mechanisms involved..

Keywords*: DPPH, AlCl3 method, Reducing Power, Hydroxyl radical*

INTRODUCTION

Flavonoids have a 15-carbon basic skeletal (C-6-C3-C6) composed of two aromatic rings (A and B) linked by a pyran heterocyclic (C) (Kumar & Pandey, 2013) (Miroslav, Bonnet, Ferreira, & Van der Westhuizen, 2010), as illustrated in figure 1. The aromatic ring's position in the function of benzopyran aids in classifying these naturally occurring compounds as 2-phenylbenzopyrans (flavonoids), 3-phenylbenzopyrans (iso-flavonoids), 4-phenylbenzopyrans (neo-flavonoids), and chalcones (Rana & Gulliya, 2019); (Ramesh, Jagadeesan, Sekaran, Dhanasekaran, & Vimalraj, 2021). Flavonoids are further classified as flavan-3-ol, flavanone, flavone, and flavanol, depending on the oxidized form of the pyran heterocyclic (Miroslav, Bonnet, Ferreira, & Van der Westhuizen, 2010) as shown in figure 2. The chemical properties of flavonoids are determined by their chemical structure, hydroxylation level, conjugation, other substitutions, polymerization (Kelly, Tagliaferro, & Bobilya, 2002), level of oxidation, glycosylation pattern, and other

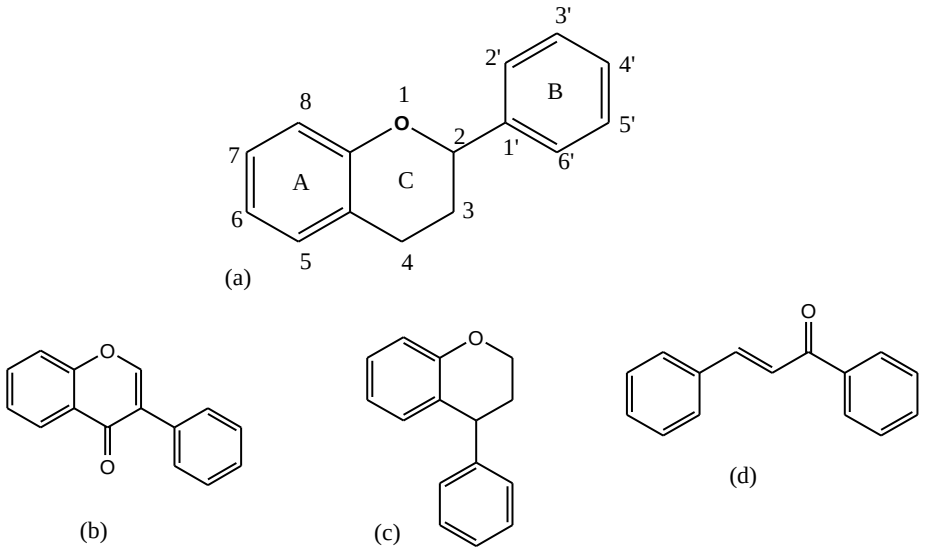

Figure 1. (a) The basic skeleton of flavonoids; (b) isoflavonoids; (c) neoflavonoids; (d) chalcones.

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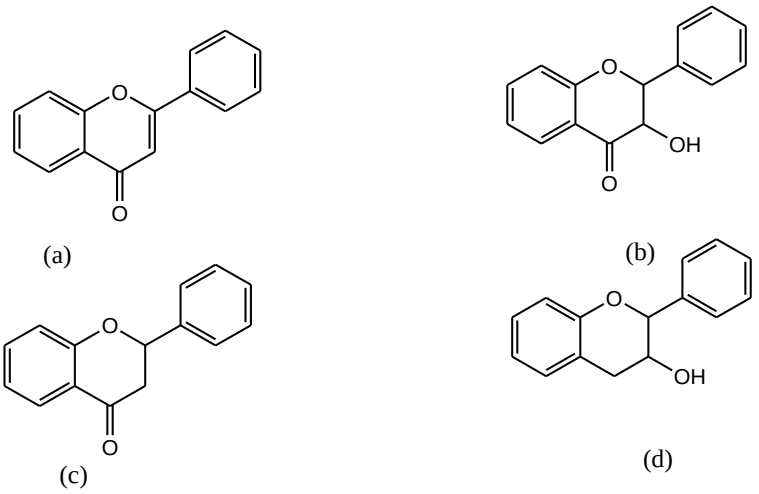

Figure 2. (a) Flavones; (b) Flavonols; (c) Flavonones; (d) Flavan-3-ol

substitutions (Santos, Maia, Ferriani, & Teixeira, 2017).

Although flavonoids have various biochemical characteristics, their capacity to function as antioxidants is one of the best known in almost every flavonoid group. The configuration of functional groups in the flavonoid core structure determines those compounds' antioxidant activity. Numerous antioxidant processes, including metal ion chelation and radical scavenging, are strongly influenced by conformation, replacement, and the overall number of OH groups (Kelly, Tagliaferro, & Bobilya, 2002) (Pandey, Mishra, & Mishra, 2012). The hydroxyl configuration of ring B is essential in regulating ROS and RNS scavenging because it transfers electrons and hydrogen to peroxy-nitrite, peroxyl, and hydroxyl radicals and generates relatively stable flavonoid radicals

(Guohua, Sofic, & Prior, 1997) (Kumar & Pandey, 2013).

The antioxidant activity of these polyphenolic chemicals has stimulated increased interest in this substance due to the potential health advantages. Most flavonoid research has focused on their antioxidant and anti-inflammatory properties. This literature review will discuss the methods for extracting, identifying, quantitatively assessing, and measuring the antioxidant activity of flavonoids molecules.

METHODS

This literature review is a type of narrative review, so it is not systematic and does not have a specific protocol. This article review was conducted by searching using the help of search engines, namely Google Scholar, PubMed, NCBI, and so on. The terms "flavonoids," "flavonoids extraction," "flavonoids identification," "flavonoids quantification," and "flavonoids determination" were used to search the literature. International journals provided primary data sources.

EXTRACTION

The solvents are chosen based on the polarity of the flavonoids because the solubility of flavonoids in various solvents varies. Flavonoids can be extracted using conventional and unconventional techniques. Conventional extraction techniques are maceration, percolation, reflux, and continuous reflux (Feng, Hao, & Li, Isolation and Structure Identification of Flavonoids, 2016). This standard extraction method is distinguished from other techniques by its extensive use of solvent, its low extraction yields, and lengthy extraction times. When heat is used in the extraction procedure, the flavonoids' chemical structure can degrade, leading to decreased bioactivity (Chávez-González, et al., 2020).

The most widely used unconventional extraction is ultrasound-assisted extraction (UAE) (Ali, Lim, Chong, Mah, & Chua, 2018) (Marana, S.Manikandanb, Nivethaa, & R.Dinesh, 2017), microwave-assisted extraction technology (MAE) (Alara, Abdurahman, & Olalere, 2018) (M.S.Ferreira, et al., 2019), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) (Tzanova, Atanasov, Yaneva, Ivanova, & Dinev, 2020). Many solvents are used, including ethanol, methanol, ethyl acetate, acetone, and others (Agustin-Salazar et al., 2014). High concentrations of alcohol (90-95 %) were used to extract free flavonoids, while around 60 % of alcohol was used to extract flavonoid glycosides (Feng, Hao, & Li, Isolation and Structure Identification of Flavonoids, 2016), and ethyl acetate is preferred for the extraction of highly alkylated aglycones, for instance (Dias, Pinto, & Silva, 2021).

The type of flavonoid extracted and the biological activity of the retrieved compounds are influenced by the characteristics of the extracting solvent (solvent). Because of their

greater flavonoid recovery yields, methanol and ethanol are the most frequently used agents for flavonoid extraction (Yu, Wang, Qi, Xin, & Li, 2019) (Daud, Fatanah, Abdullah, & Ahmad, 2017).

IDENTIFICATION

The identification method for flavonoid compounds can be carried out using the earliest forms, such as chemical reaction techniques, determination of specific absorbance by UV spectroscopy, or identification by thin layer chromatographic techniques with the help of $AlCl_3$ spotting reagent (Harborne, 1998).

Chemical reaction

The methods for identifying flavonoid compounds include the following:

1. A small quantity of NaOH (diluted solution) was added to a test tube containing a 1 mL stock solution, and a dark yellow hue can be seen inside the reaction tube. The presence of flavonoids is revealed when weak acids are added drop by drop to the solution, which causes it to turn colorless (Hossain, AL-Raqmi, AL-Mijizy, Weli, & Al-Riyami, 2015).
2. Robinson (1995) reported a method for identifying flavonoids in 2 mL plant extracts using concentrated HCl reagents (0.5 mL) and 0.02 mg of magnesium. A color change indicates the presence of flavonoids with concentrated Mg and HCl, producing a red, yellow, or orange color (Robinson, 1995).
3. A simple method to detect flavonoids involves adding HCl to the ethanol extract, which results in the appearance of red color (Rao, Abdurrazak, & Mohd, 2016).
4. Four milliliters of the filtrate were shaken with one milliliter of the diluted (1%) ammonia solution. The separation of the layers was permitted to occur naturally. Flavonoids are in the ammonia layer as indicated by a yellow coloration (Ukoha, Cemaluk, Nnamdi, & Madus, 2011).
5. A mild yellow coloring was observed when 1 mL of 1% aluminum chloride solution was added to 4 mL of the filter and shaken. Flavonoids are indicated by the presence of a yellow precipitate (Ukoha, Cemaluk, Nnamdi, & Madus, 2011).

Chromatographic technique

Arora and Itankar (2018) conducted a chromatographic identification procedure for flavonoids based on the method introduced by Harborne (1998) (Harborne, 1998). Briefly, the procedure can be written as follows (Aroraa & Itankar, 2018): Thin layer plates (Merck, 0.25 mm thickness) precoated with silica gel G were used. Different solvent systems were used for development, including ethyl acetate: methanol: water (100:13.5:10, v/v/v), ethyl acetate: formic acid: acetic acid: water (100:11:11:26, v/v/v), chloroform: methanol: water (70:30:4, v/v/v), toluene: ethyl acetate: diethyl amine (70:20:10, v/v/v)

and ethyl acetate: methanol: water: acetic acid (65:15:15:10, v/v/v/v). After the chromatogram was developed in the solvents, the plates were dried and sprayed with $AlCl_3$ reagents to detect flavonoids. Immediately after drying, all plates were viewed in a UV TLC viewer using UV at 254 nm and 366 nm (Aroraa & Itankar, 2018). Another solvent system is n-butanol: acetic acid: water (2:2:6) which seems to give a clear separation (Sharma & Janmeda, 2017).

If a specific flavonoid is the analysis's target, it is possible to compare the TLC profiles of the sample and the flavonoid standard. Suppose a specific flavonoid is the analysis's target. In that case, it is possible to compare the TLC profiles of the sample and the flavonoid standard, as has been reported by Calina et al. (2013). They reported the results of their work on the chromatographic analysis of the flavonoids from *Robinia pseudoacacia* species, using ruthoside as a standard compound (Călina, et al., 2013).

High-performance liquid chromatographic (HPLC) has also been applied by several researchers to identify flavonoids (Olszewska, 2005); (Moiseev, Buzuk, & Shelyuto, 2011) and many more. In addition, new methods have been developed, such as ultra-performance liquid chromatography with photodiode array detection (UPLC-PAD) and ultra-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry method (UPLC-ESI-QTOF-MS) (Yang, et al., 2019). Kostikova et al. (2021) have also reported the results of a study on the identification of alkaloids in the leaves of Eranthis longistipitata using Liquid Chromatography with High-Resolution Mass Spectrometry (LC-HRMS) (Kostikova, et al., 2021). The application of ultra-high pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS) in plant flavonoid analyses has also been reported by (Lei, Sumner, Bhatia, Sarma, & Sumner, 2018).

Spectroscopic technique

The identification of flavonoid compounds can also be done based on the specific absorbance at the wavelength of UV light. All flavonoids have aromatic chromophores, which can be seen in their UV spectra by UV absorptions in the 250 nm range. These substances are capable of undergoing π,π^* excitation and responding from π,π^* excited states (Sisa, Bonnet, Ferreira, & Westhuizen, 2010). Since all flavonoids have the ability to absorb ultraviolet light, UV detectors can usually identify them. For flavones, flavonols, and the corresponding glycosides, it is typically detected at 254–280 nm or 340–360 nm, 520–540 nm for anthocyanidins, and 250 nm for chromones (Feng, Hao, & Li, 2017).

FLAVONOIDS QUANTIFICATION

The methods for calculating the total flavonoids in botanical materials are based on chemical extraction and combined with different analytical methods like ultraviolet spectrometry (Chen, Wang, & Wan, 2010) (Patle, et al., 2020) HPLC (Chen,

Dolnikowski, & Blumberg, 2006); (Park, Kim, Rehman, Na, & Yoo, 2016); and GC (Zhang, et al., 2009).

Spectrophotometric Quantification

a. The $AlCl_3$ Method

Woisky & Salatino were the first to present the $AlCl_3$ method (Woisky & Salatino, 1998), further modified by Chang et al. (Chang, Yang, Wen, & Chern, 2002) through the addition of potassium acetate after the addition of aluminum chloride. Chang's method obtained the maximum absorbance at a λ maximum of 415 nm against the standard compounds (quercetin). The maximum wavelength in the test can be selected based on the flavonoid standard compounds used. Denni and Mammen (Denni & Mammen, 2012) stated that flavonoid flavonols such as kaemferol, quercetin, and myricetin showed maximum absorbance at 415 nm, while other flavonoid classes showed varying maximum wavelengths.

The flavone and C-4 keto groups of flavonol and the C-3 or C-5 hydroxyl groups cause aluminum chloride to form acid-stable complexes, which is the basis for the aluminum chloride colorimetric method. Additionally, the ortho-dihydroxyl groups on flavonoids' A or B rings interact with aluminum chloride to form acid-labile complexes (Chang, Yang, Wen, & Chern, 2002) (Ahmed & Iqbal, 2018). Chang et al. (2002) developed the following procedure. The calibration curve was made using quercetin as the standard. Standard quercetin (10 mg) was dissolved in ethanol (80%) and diluted to 25, 50, and 100 g/mL concentrations. The standard aqueous solution (0.5 mL) was combined with 95% ethanol (1,5 mL), 10% aluminum chloride (0,1 mL), 1 M potassium acetate (0,1 mL), and distilled water (2,8 mL) (Chang, Yang, Wen, & Chern, 2002).

The total flavonoid concentration was calculated using a standard curve and represented as mg of quercetin or rutin equivalent per gram of weight of the sample (Baba & Malik, 2015); (Do, et al., 2014).

Several researchers developed this method by employing flavonoid standards that are specific to the type of flavonoid being analyzed, such as quercetin (Mathur & Vijayvergia, 2017); (Sembiring, Elya, & Sauriasari, 2018), and rutin (Chavan, Gaikwad, Kshirsagar, & Dixit, 2013) for the flavonol group. For the flavone class, other

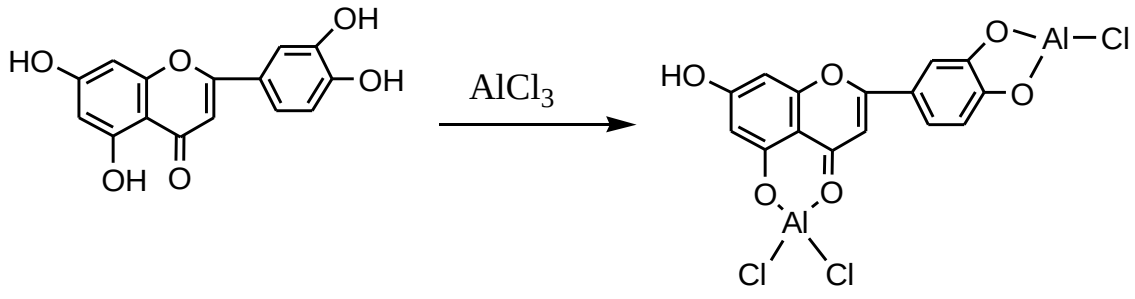

Figure 3. The Reaction of flavonoid-Aluminium Chloride

researchers used 3',4',5,7-tetrahydroxyflavone (luteolin) or 4',5,7-trihydroxyflavone (apigenin). This aluminum chloride technique did not produce any results in flavanones and isoflavones' other flavonoid groups. Similarly, the solvent used can be altered to meet the research needs. $AlCl_3$ concentrations appear to vary, ranging from 2% to 10% (Kefayati, Motamed, Shojaii, Noori, & Ghods, 2017); (Struchkov, Beloborodov, Kolkhir, Voskoboynikova, & Savvateev, 2018);

b. The 2,4-dinitrophenylhydrazine Method

The fundamental premise of this approach is that 2,4-dinitrophenylhydrazine interacts with aldehydes and ketones to generate 2,4-dinitro-phenylhydrazone. The hydrazones of all standard flavanones, namely hesperitin, naringin, and (±)-naringenin, showed maximum absorption at 495 nm, whereas flavonols, flavones, and isoflavones with C2–C3 double bonds were unable to react with 2,4-dinitrophenylhydrazine (Chang, Yang, Wen, & Chern, 2002). As an outcome, the authors can conclude that this method is only relevant to the flavanone class of flavonoids.

The steps involved in determining flavonoids using this approach are summarized here (Chang, Yang, Wen, & Chern, 2002): Standard (±)-naringenin (20 mg) was dissolved in methanol and diluted to 500, 1000, and 2000 µg/mL, respectively. Two milliliters of 2,4-dinitrophenylhydrazine at 1% and two milliliters of methanol were combined with one milliliter of each diluted standard solution and heated to 50 °C for 50 minutes. After being brought to room temperature, the reaction mixture was mixed with 1% potassium hydroxide (5 mL) in 70% methanol and left to sit for 2 minutes. The residue was eliminated by centrifuging 1 mL of the mixture for 10 minutes at 1,000 x g while adding 5 mL of methanol. A 25 mL dilution volume was made from the supernatant after collecting it. The absorbance of the supernatant was calculated at 495 nm.

Numerous studies have utilized this technique to compare a simplified 2, 4-dinitrophenylhydrazine test for flavonoids to a standard flavonoid assay, including Mir, Bhat, and Ahangar (2013) (Mir, Bhat, & Ahangar, 2013); (Struchkov, Beloborodov, Kolkhir, Voskoboynikova, & Savvateev, 2018). Their findings are inconsistent with Chang's report, which is thought to be due to the difference in maximum wavelengths from the reaction of different flavonoid compounds with DNP.

A different colorimetric method that uses a 2,4-dinitrophenylhydrazine reaction was found to be specific for flavanones. In contrast, the straightforward method for measuring flavonoid content that uses an aluminum chloride reaction was found to be typical only for flavones and flavonols. So that the combined results can more accurately reflect the actual content of flavonoids overall, we recommend conducting both analyses.

c. HPLC Quantification

Mass spectrometers connected to HPLC systems over the past ten years have improved the selectivity and sensitivity of flavonoid

analysis. The challenges facing all contemporary analysts include enhancing the stationary phase properties and creating more complex instruments as well as tools for quicker and more effective sample preparation. The development of more efficient and fast procedures for their identification and quantification, with HPLC remaining the most powerful technique for their separation from the complex mixtures.

The quantification of flavonoids in plant samples has been widely reported by researchers, such as the quantification of flavonoids in *Citrus reticulata* (Kumar, Ladaniya, Gurjar, Kumar, & Mendke, 2021), (Qiu & Zhang, 2020); (Chen, Zhou, Tao, Li, & Wang, 2020), etc. Kuppusami et al. (2018) performed the determination of total flavonoids with the following procedure (Kuppusamya, et al., 2018): The quantification of flavonoids by HPLC was carried out with the help of an internal flavonoid standard. Flavonoid standards like rutin hydroxide, luteolin, kaemferol, vitexin, narcissoside, and myricetin were prepared separately in four concentrations (1, 0.5, 0.25, and 0.125 mg/ml). They were then blended to produce a final concentration for each standard of 1, 0.5, 0.25, or 0.125 mg/ml. The HPLC-DAD system then carried out an analysis on 10 µl of the sample. The flavonoid concentration was calculated by preparing a calibration curve for mass concentration vs. peak area. For each standard curve, the slope of the regression line and the value of the correlation coefficient (R2) were obtained using the MS Excel program. Regression equations were calculated for both standard and pure flavonoid fractions. The pure flavonoid fraction in plant was quantified using the following equation:

$$Flavonoids\ \left(\frac{\mu g}{g}\right) = \frac{Amount_{is}\ x\ Amount_{sc}\ x\ IRF_{sc}}{Area_{is}}$$

internal response factor (IRF) is:

$$IRF = \frac{Area_{is}\ x\ Amount_{sc}}{Amount_{is}\ x\ Area_{sc}}$$

note: is = internal standard; sc = separated flavonoids

ANTIOXIDANT ASSAY

The following are the key parameters that influence flavonoids' radical-scavenging capacity (Bors & Michel, 2002); (Shahidi & Wanasundara, 1992):

1. The B ring with ortho-dihydroxy groups in the flavonoid structure is the best electron donor, thereby improving stability and electron delocalization.
2. The 4-oxo functional group in the 2-3 carbon double bond in the C ring causes electrons in the B ring to be delocalized.
3. The maximum radical scavenging potential of flavonoid compounds is determined by hydroxyl groups at positions 3 and 5 on the A ring and a 4-oxo function in the C ring.
4. Antioxidant activity depends on the 3-hydroxyl group. When compared to similar aglycones, 3-glycosylation decreases an aglycone's activity.

Antioxidants work in various ways, including scavenging free radicals and absorbing the ion of a transition metal,

breaking down hydrogen peroxide or hydroperoxide, active pro-oxidants quenching, and boosting the antioxidant defenses of endogenous substances while also repairing any cellular damage that results. As a result, testing a compound's antioxidant activity, including the flavonoid group, must consider all different antioxidant mechanisms. Based on the part antioxidant compounds play in the redox reaction mechanism, whether as HAT (hydrogen atom transfer) or ET (electron transfer), the method of testing antioxidant activity is based on these concepts (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016). DPPH, ABTS, hydrogen peroxide, hydroxyl, and superoxide radicals are methods for transferring hydrogen atoms. In the meantime, Single-electron transfer (SET) techniques consist of cupric- or ferric-reducing ability and assay for phosphomolybdenum.

The action of antioxidant flavonoids has been assessed against several reactive oxygen and nitrogen species, including 1,1-diphenyl-2-picrylhydrazyl radical DPPH• (Nanjo, et al., 1996) (Hidalgoa, Sánchez-Morenob, & Pascual-Teresaa, 2010), hydroxyl (HO•), superoxide ($O_2^{\bullet -}$), 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) cation, otherwise known as ABTS (Saeed, Khan, & Shabbir, 2012), peroxyl (ROO•) (Alton J. Dugas Jr., Bonin, Price, Fischer, & Winston, 2000) and hypochlorite (Firuzi, Mladênka, Petrucci, Marrosu, & Saso, 2004). Furthermore, it has been observed that antioxidant capacity testing using the reaction reduction by flavonoids based on the transformation of Fe^{3+} to Fe^{2+} (Saeed, Khan, & Shabbir, 2012); (Dibacto, et al., 2021) an assay of antioxidant capacity equivalent to Trolox (TEAC), an assay of oxygen radical absorption capacity (ORAC) (Apak, et al., 2007), and total antioxidant capacity utilizing the phosfomolybdate method (Abdel-Gawad, Abdel-Aziz, El-Sayed, El-Wakil, & Abdel-Lateef, 2014) based on the technique popularized by Prieto at al (Prieto, Pineda, & Anguilar, 1999).

a. 1,1-Diphenyl-2-picrylhydrazyl free radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable radical species having an unpaired electron on its nitrogen atom (Eklund, et al., 2005); (Sharma & Bhat, 2009). The odd electron of the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants and converting it to the equivalent hydrazine (Kedare & Singh, 2011). This method is the most popular for determining the antioxidant activity of a sample. For the DPPH assay of the antioxidant activity of extract or compound with different polarities, methanol or buffered methanol was a suitable solvent. Ascorbic acid, BHT, and propyl gallate, three common antioxidants used as comparison standards for evaluating antioxidant potential, were also measured for their ability to scavenge DPPH radicals (Ricci et al., 2005; Kano, Takayanagi, Harada, Makino, & Ishikawa, 2005). With these three standard

antioxidant compounds. It was discovered that the time required for the reaction of scavenging free radicals was different for each compound. Based on the rate of the reaction, it can be said that ascorbic acid reacts with DPPH in a fast time, followed by gallic acid. BHT reacts more slowly as time passes and starts to decline after 90 minutes of observation (Sharma & Bhat, 2009).

Using the equation, the level of DPPH color change as a free radical scavenger was calculated (Delgado, et al., 2014):

$$\% \, Scavengging \, effect = [\frac{A_{DPPH} - A_S}{A_{DPPH}}] \, x \, 100$$

Where As is the sample solution's absorbance and A_{DPPH} is DPPH solution absorbance (blank).

According to (Sharma & Bhat, 2009), who compared the reaction products using methanol and buffered methanol (40 ml of buffered methanol was mixed to create buffered methanol), methanol or buffered methanol is the most appropriate solvent for DPPH, based on how easily the compound under investigation dissolves. The buffered methanol solution was prepared by mixing 0.1 M acetate buffer solution pH 5.5 with methanol (40:60) (Sharma & Bhat, 2009). The spectrophotometric absorbance accuracy range is 0.2-0.8, corresponding to a transmittance range of 20-80 percent; thus, the concentration of DPPH is sufficient to suit that range.

The standard antioxidant compounds that are often used are ascorbic acid (Esmaeili, Taha, Mohajer, & Banisalam, 2015), BHT (Kiani, Arzani, & Maibody, 2021) (Alma, Mavi, Yildirim, Digrak, & Hirata, 2003), propyl gallate, rutin (Esmaeili, Taha, Mohajer, & Banisalam, 2015), Trolox (Zhang, Luo, Wang, He, & Li, 2014) and quercetin (Siatka & Kašparová, 2010). The DPPH solution had a concentration of 50–100 μM and 0.5 to 3 mL in volume.

Briefly, different concentrations of sample solutions were combined with DPPH solutions. The mixture of sample solution and DPPH was allowed to settle for 30 minutes at room temperature and in complete darkness. After 30 minutes, the absorbance value at 517 nm was measured and converted to the percentage of radical scavenging.

An antioxidant compound must donate electrons in the DPPH free radical neutralization reaction. The reaction at 517 nm is accompanied by a change in the color of DPPH, which is a sign of antioxidant activity, as shown in Figure 4. This test makes it easier to understand various chemical phenomena through its use. It has many benefits, including low cost, simplicity in experimentation, reproducibility, use at room temperature, and the potential for automation. (Munteanu & Apetrei, 2021).

The reaction in the picture above shows a positive result as an antioxidant which is indicated by the formation of a pale yellow color on a purple background. The formation of yellow spots after treatment is caused by the presence of compounds that can donate

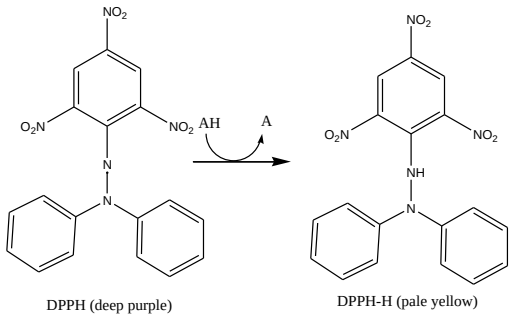

Figure 4 The Reaction of flavonoid-DPPH radical.

hydrogen atoms in the extract, so that it can result in reduced DPPH molecules followed by a purple color change from the DPPH solution to clear yellow

b. ABTS Radical Scavenging Assay

Radical cation 2,2'-azinobis(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS•+) is a chromophore with maximum absorption at 734 nm and a blue-green color. The color intensity of the chromophore will decrease if it reacts with appropriate antioxidant compounds. As previously mentioned, the 734 nm maximum wavelength of the blue-green ABTS chromophore is the product of the reaction between the ABTS radical cation and potassium persulfate (Miller & Rice-Evans, 1996); (Re, et al., 1999). Potassium persulfate causes ABTS2 to oxidize, and antioxidants convert the anion ABTS• radical into the colorless ABTS2 product ((Huang, Ou, & Prior, 2005); (Shahidi & Zhong, 2015), as depicted in the following reaction:

The duration of the reaction, sample concentration, and intrinsic antioxidant activity all affect the degree of blue-green discoloration, which is indicated by a significant decrease in absorbance to 734 nm (Munteanu & Apetrei, 2021). Because it is soluble in water and organic solvents, the ABTS radical can be used to assess the antioxidant activity of both lipophilic and hydrophilic substances. Lipophilic substances like carotenoids, tocopherols, etc., the presence and effectiveness of lipid-soluble antioxidants on lipids were assessed using homogeneous solutions. Furthermore, because the ABTS radical scavenging assay can be examined throughout a wide pH range, it is helpful to investigate how pH influences antioxidant processes for food-related components.

The following can be used to carry out the testing procedure: A stock solution of the ABTS radical cation with a dark color was created by treating the 7 mM ABTS solution with 2,45 mM potassium persulfate solution and keeping it for 12–16 hours without being exposed to light. An initial absorbance of roughly 0.70 (0.02) at 745 nm at a temperature of 30 °C was achieved by adding the ABTS stock solution with 50% methanol (Esmaeili,

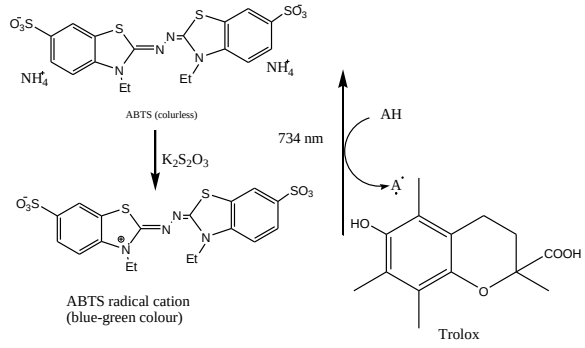

Figure 5. The ABTS color change reaction mechanism

Taha, Mohajer, & Banisalam, 2015). Measurements of absorbance were taken 6 to 30 minutes after the sample solutions of different concentrations (0.05 to 10 mg/ml) had been mixed. Trolox, BHT, and ascorbic acid are typical antioxidants frequently used in ABTS (Zheleva-Dimitrova, Nedialkov, & Kitanov, 2010).

The following equation calculates the antioxidants' radical-scavenging percentage:

$$\%\ Scavengging\ effect = \left[\frac{A_{ABTS} - A_S}{A_{ABTS}}\right] x\ 100$$

An ABTS represents the control ABTS's absorbance and AS represents the absorbance of the sample.

c. Hydrogen Peroxide Scavenging Activity

Few enzymes are directly inactive by weak oxidizing agents such as hydrogen peroxide, which typically works by oxidizing thiol (-SH) groups. H_2O_2 readily penetrates cell membranes and, once inside, is likely to interact with Fe^{2+} or Cu^{2+} ions to create hydroxyl radicals that have various harmful consequences. Therefore, controlling the amount of H_2O_2 cells allowed to build up is advantageous from a biological perspective.

According to Adjimani and Asare (2015), hydrogen peroxide can be reduced to water (H_2O) by accepting protons (H^+) or electrons (Adjimani & Asare, 2015). Flavonoid or polyphenolic compounds are effective electron and proton donors, enabling them to convert hydrogen peroxide radicals into water molecules. Figure 6 depicts a hypothetical reaction exemplified by the compound quercetin. A 50 mM phosphate buffer solution with a pH of 7.4 comprised a 2 mM hydrogen peroxide solution. A total of 0.4 mL of 50 mM phosphate buffer (pH 7.4) was added to the test tubes after 0.1 mL aliquots of the extracted sample at various 50–300 g/mL concentrations were transferred. After adding 0.6 mL of H_2O_2 solution and vortexing for 10 minutes, a blank was used as a reference point to compare the solution's absorbance at 230 nm (Esmaeili, Taha, Mohajer, & Banisalam, 2015). Hydrogen peroxide's reducing ability is calculated using the following equation (Bhatti, Ali, Ahmad, Saeed, & Malik, 2015):

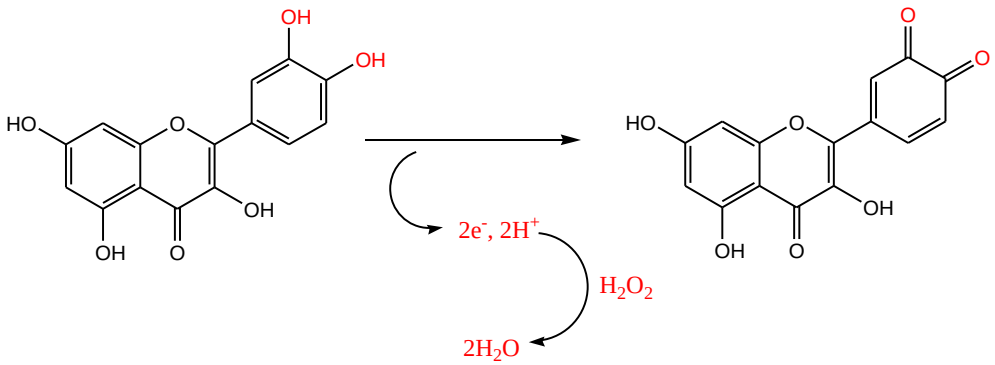

Figure 6. Reaction mechanism of hydroperoxide radical scavenging by quercetin

$$\%\ H2O2\ scavengging\ = \frac{A_0 - A_1}{A_0}\ x\ 100$$

Where A_0 is the control absorbance and A_1 is the sample absorbance.

d. Reducing Power Assay

The potential of flavonoid compounds to behave as reducing agents can be used to evaluate their antioxidant qualities. In this case, using reductants in a redox-related colorimetric technique of easily reduced oxidants can provide a quick way to assess this ability. Benzie and Strain (Benzie & Strain, 1996) created the ferric reducing antioxidant power (FRAP) test as an easy and accurate colorimetric method for calculating total antioxidant capacity based on this reducing ability. This procedure depends on the power of antioxidants to convert Fe^{3+} to Fe^{2+}.

The FRAP method directly measures a substance's reducing power, which is crucial in determining whether a compound is a good antioxidant, in contrast to other methods that assess antioxidant capacity. According to the reaction mechanism, the FRAP assay relies on the antioxidants in the sample quickly reducing ferric-tripyridyltriazine (Fe (III)-TPTZ) to ferrous-tripyridyltriazine (Fe (II)-TPTZ), a blue product at acidic pH conditions (Santos & Silva, 2020):

To make FRAP reagent, combine 0.3 M acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM 2,4,6-tris(2,4-pyridyl)-S-triazine in 40 mM hydrochloric acid (10:1:1) in (v/v/v). A 2 mL premixed amount of FRAP reagent is combined with 0.1 mL of the sample solution, and the mixture is then allowed to sit at room temperature for 30 min. The sample absorbance is then measured at 593 nm. The equivalents of Trolox, gallic acid, ascorbic acid, quercetin, or -tocopherol can be used to measure FRAP activity (Sadeer, Montesano, Albrizio, Zengin, & Mahomoodally, 2020). The $[Fe(III)(TPTZ)_2]^{3+}$ complex will be reduced as well if there are species in the sample solution with a lower redox potential than Fe(III) (0.70 V), which leads to inaccurate measurement results (Benzie & Strain, 1996). To solve this issue, care must be taken to add the reagents in the correct order when producing FRAP reagents. As an illustration,

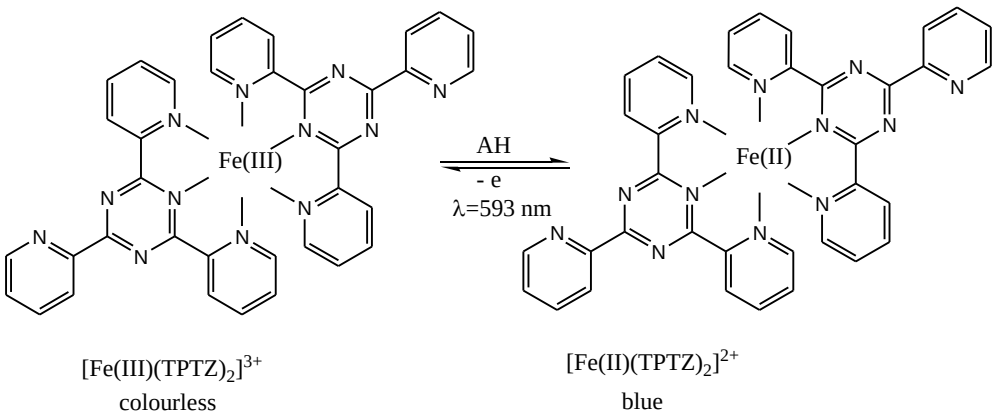

Figure 7. The reaction scheme involved in FRAP assay.

acetate buffer is added. TPTZ is added after adding $FeCl_3$ (Sadeer, Montesano, Albrizio, Zengin, & Mahomoodally, 2020).

e. Cupric Reducing Antioxidant Capacity (CUPRAC)

Antioxidants (like β-carotene and α-tocopherol) that are hydrophilic and lipophilic can be measured using this technique. The hydrophilic and lipophilic antioxidants in the serum can be measured independently of one another by extracting the serum in hexane, followed by discoloration in dichloromethane (Munteanu & Apetrei, 2021). To analyze the reactivity of antioxidants in tissues, Bean et al. (2009) discovered that the CUPRAC test was significantly more helpful because it was sensitive to lipophilic, hydrophilic, and thiol (SH) antioxidants at physiological pH (Bean, Florian Radu, Schuler, Leggett, & Levin, 2009).

Apak et al. (2004) have created a technique for measuring antioxidant capacity based on reducing Cu^{2+} to Cu^{1+} (APAK, GUCLU, OZYUREK, & KARADEMIR, 2004). The oxidizing reagent in the CUPRAC assay, cation (Cu(II)-Nc), bis(neocuproine) copper (II), functions as an electron donor. The reduction of oxidizing agents by antioxidant compounds yields the CUPRAC chromophore cation (Cu(I)-Nc), bis(neocuproine) copper(I). The reaction is helpful at neutral pH in ammonium acetate medium with an incubation period of 30 minutes. A redox reaction involving the antioxidant polyphenols produced Cu(I)-chelate, which had its absorbance at 450 nm measured (Mustafa Özyürek, et al., 2011).

The reactive Ar-OH groups of polyphenol antioxidants are effectively converted to quinones in this reaction by reducing Cu(II)-Nc to the orange-yellow colored $Cu(Nc)^{2+}$ chelate. The CUPRAC antioxidant capacity of various polyphenols and flavonoids was assessed using Trolox as a standard antioxidant (Mustafa Özyürek, et al., 2011).

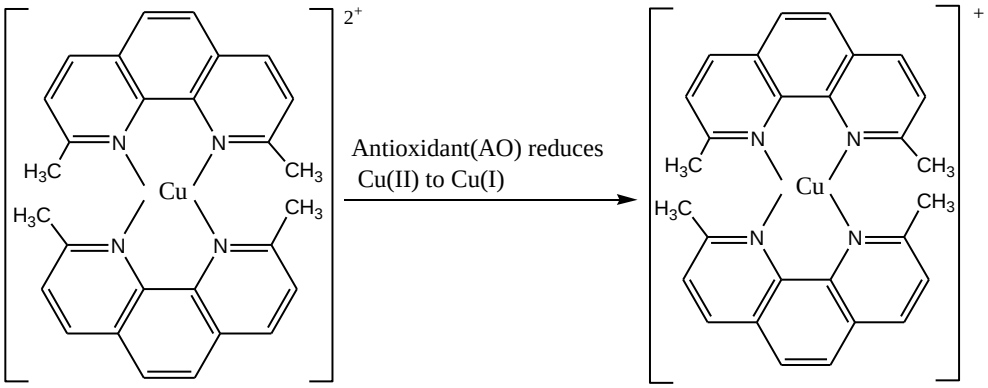

Figure 8. The reaction involved in CUPRAC Assay

f. Superoxide Anion Radical Scavenging Assay

Superoxide (O2•-), one of the ROS, can harm cells and DNA, resulting in many inflammatory and degenerative diseases (Fazilatun, Nornisah, & Zhari, 2005). Robak and Gryglewski (1988), in their study of seven flavonoid compounds (Myricetin, Quercetin, Quercitrin, troxerutin,Rutin, Meciadonol, Cyanidol) and three non-flavonoid antioxidants, developed this assay method. In the xanthine-xanthine oxidase system and the phenazine methosulphate-NADH system, the superoxide anion was produced both enzymatically and non-enzymatically. The reduction of nitro blue tetrazolium measured its concentration (Robak & J.Gryglewski, 1988).

To measure the absorbance of the reaction mixture, which included the sample solution, phosphate buffer pH 4.7 (1 mL + 2 mL), 20 μM PMS (1 mL), 156 μM NADH, and 25 μM NBT, also in a phosphate buffer solution, absorbance measurements were taken at 560 nm against control after two minutes of incubation at 25°C (Fazilatun, Nornisah, & Zhari, 2005). The concentration of the sample solution is 12% v/v in DMSO (Hatano, et al., 1999). The formula used to calculate the % scavenging activity is as follows:

$$Scavengging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sampel}{Absorbance\ of\ control}\right) x\ 100$$

g. Hydroxyl radical scavenging assay

Hydroxyl radicals are chain reaction products of stress conditions caused by excess superoxide anion radicals, which release Fe^{2+} ions from intracellular proteins such as ferritin and ceruloplasmin, which react with H_2O_2 according to the Fenton and Haber-Wiess reactions (Haber & Weiss, 1934); (Fenton, 1894):

$H_2O_2 + Fe^{2+} \rightarrow OH\bullet + OH^- + Fe^{3+}$ (Fenton Reaction)

$O_2\bullet^- + H_2O_2 \rightarrow OH\bullet + OH^- + O_2$ (Haber-Wiess reaction)

The most reactive and harmful radicals in biological systems are the hydroxyl radicals (OH•). These free radicals have a powerful ability to interact with biomolecules like DNA, proteins, lipids, and carbohydrates and can harm cells more severely than other ROS. (Halliwell, 1987).

In a study on *Spondias pinata's* antioxidant and anti-free radicals abilities, the hydroxyl radical scavenging capacity was examined using the breakdown product of 2-deoxyribose produced by condensation with TBA (Hazra, Biswas, & Mandal, 2008). The system composed of Fe^{3+}, ascorbate, EDTA, and H_2O_2 (the Fenton reaction) produced hydroxyl radical. The first step in this procedure is the formation of a hydroxyl radical through the Fenton reaction, composed of Fe3+, ascorbate, EDTA, and H2O2. Furthermore, the reaction mixture contained various concentrations of the test sample or reference substance (0–200 μg/ml) in addition to 2-deoxy-2-ribose (2.8

mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 μM), EDTA (100 μM), H2O2 (1.0 mM), and ascorbic acid (100 mM) in a final volume of 1 ml. One milliliter of the solution was added to one milliliter of 2.8 percent TCA, followed by one milliliter of 1 percent aqueous TBA, and then incubated at 37°C for 1 hour before being heated to 90°C for 15 minutes to produce the color. After cooling, the absorbance was measured at 532 using mannitol as the reference OH scavenger against an adequate blank solution.

$$\% \, Scavengging \, activity = \left(1 - \frac{absorbance \, of \, sample}{absorbance \, of \, control}\right) x \, 100$$

h. Phosphomolybdate Assay

The phosphomolybdenum test's fundamental premise is that antioxidant-rich plant extracts can reduce Mo (VI) to Mo (V), which is how antioxidant capacity is measured. Prieto et al. (1999) developed this method, which is based on the analyte sample's reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate/Mo (V) complex at an acidic pH (Prieto, Pineda, & Aguilar, 1999).

The following steps were taken by Khan et al. (2012) and have been adopted by numerous researchers: The reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and four mM ammonium molybdate) was combined with 0.1 ml of the test solution and 1 ml of it in a vial for the experiment. After being sealed, the vial spent 90 minutes resting in a water bath that had been heated to 95 °C. Samples were incubated and cooled to room temperature, and their absorbance at 765 nm was compared to a control (Khan, Khan, Sahreen, & Ahmed, 2012).

CONCLUSION

The fifteen-carbon base framework (C6C3C6) of the polyphenolic compounds known as flavonoids is composed of two benzene rings (A and B) linked by a heterocyclic pyran ring (C). Flavonoids can be extracted by conventional and modern methods, with the most common solvents being ethanol, methanol, an ethanol-water mixture, and a methanol-water combination. Identification of flavonoids can be done in several ways, such as through chemical reactions, chromatographic techniques, and ultraviolet spectroscopy techniques. Several reagents for identifying flavonoid compounds in the extract are dilute NaOH, concentrated HCl + magnesium, dilute ammonia, and aluminum chloride.

Quantitative analysis methods of flavonoid compounds have been reported using spectrophotometric (The total flavonoid levels can be determined using $AlCl_3$ and 2-4 dinitrophenylhidazine methods) and chromatographic techniques.The technique of determining antioxidant activity is based on the role of antioxidant compounds in the reaction mechanism, either as hydrogen atom transfer (HAT) or electron transfer (ET). The HAT mechanism includes DPPH, ABTS, hydrogen peroxide, hydroxyl, and superoxide

radicals, while electron transfer (ET), including cupric or ferric reduction ability, and test for phosphomolybdenum. In addition, other modes of action of antioxidants, include radical scavenging, transition metal ion capture, hydrogen peroxide or hydroperoxide breakdown, and active extinction of pro-oxidants.

The working pH is one of the critical factors to consider when choosing an antioxidant test. The tests are run in neutral (CUPRAC) and acidic (FRAP) environments. The use of antioxidant assays for both hydrophilic and lipophilic antioxidants is also a crucial factor. Hydrophilic and lipophilic antioxidants can be measured using the ABTS and CUPRAC methods, whereas the FRAP method only measures hydrophilic antioxidants, and DDPH only applies to the hydrophobic system. Therefore, combining more than one method in testing the antioxidant activity of flavonoid compounds is necessary to obtain complete information.

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