Study of the Antioxidant Activity of Synthetic 3-hydroxyflavone Derivatives by DPPH and Hydrogen Peroxide Methods

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Flavonoids have good efficacy as antioxidants due to their ability to scavenge free radicals within cells. In our previous study, synthetic flavonoid derivatives (A_2-A_{13}) were prepared in vitro through condensation, oxidative cyclization, alkylation and esterification reactions. The antioxidant activity of the alkyl and esterified derivatives of the flavonoid compound (A_2) 3-hydroxy-2-(4-[dimethylamino]phenyl) benzo-4-pyrone was studied by two methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition method and inhibition of hydrogen peroxide (H_2O_2) and compared to the activity of standard antioxidant compounds, ascorbic acid and quercetin. The flavonoids (prepared samples) A_2, A_5, and A_7 showed high activity approaching that of ascorbic acid and quercetin as well-known antioxidants. As for the compounds (A_3, A_6, A_8, A_9, A_{10}, A_{11}, A_{12}, A_{13}), which contain an -OH enol group, a benzyl group, and a chlorine group and the acetate group, its effectiveness has decreased significantly.

Introduction

Flavonoids are low molecular weight polyphenolic compounds [1,2] that are widely distributed in plants. They possess biological activity [3] and play an important role in photosynthetic cells [4].

3-Hydroxyflavones or flavonols form a major group of flavonoids with high biological activity as anti-inflammatory [5], anti-acetylcholinesterase [6], antioxidants [7], anti-cancer [8] and antidepressant [9], antidiabetic [10] and antimicrobial agents [11].

Free radicals are responsible for oxidative damage to biomolecules such as proteins, lipids, or nucleic acids in the structures of cell nuclei and molecular membranes. Maintaining a balance between free radicals and antioxidants is a prerequisite for maintaining health. Control of oxidative stress processes is critical in both the prevention and treatment of numerous diseases such as diabetes, atherosclerosis, coronary heart

disease, cancer, infections, liver disease and cardiovascular disease. Excess free radicals are neutralized by enzymes and non-enzymatic antioxidants, which, with a few endogenous exceptions such as glutathione, uric acid, and urisinol, must be supplied through the diet [12].

DPPH is considered to be one of the most commonly used stable free radicals, discovered by Goldsmith and Renn in 1922. DPPH is formed by changing the position of the free electron. It is a stable radical cation and does not form interference with the alcoholic solution. It forms a DPPH solution that exhibits a dark purple color with maximum absorption at 517 nm. By reacting with a chemical containing a hydrogen atom, the stable form DPPH-H is formed, and the violet color of the solution changes to yellow with a simultaneous decrease in the absorbance value. The decrease in absorbance is proportional to the amount of the oxidized form of DPPH remaining in the solution. The color change from purple to yellow can be monitored spectrophotometrically and used to evaluate the ability of antioxidants and natural products to scavenge free radicals. For the first time, a colorimetric method was described by Blois to evaluate the antioxidant properties of the amino acid cysteine, which contains the thiol group - SH as a model antioxidant. Since then, the simple and convenient colorimetric method has been widely used to evaluate the antioxidant capacity of many products of natural origin [12-18].

Hydrogen peroxide is produced by decomposition of O_2, by direct reduction of O_2, or by enzyme reactions. The presence of oxidases in mitochondria (urate oxidase, glucose oxidase, D-amino acid oxidase) can lead to the direct production of hydrogen peroxide by transferring two electrons to molecular oxygen. Hydrogen peroxide can diffuse across the cell membrane because it is lipid soluble.

These peroxides cannot easily oxidize most lipids, proteins and nucleic acids due to their low reactivity. The danger posed by H_2O_2 is that it is converted to the hydroxyl group -OH. This occurs by homologous cleavage triggered by ultraviolet light or by interaction with transition metal ions (Fenton reaction). H_2O_2 attacks the structure of Hemoglobin proteins by oxidizing iron, inactivating enzymes, oxidizing lipids, -SH groups and keto acids. The main antioxidant enzymes that can inhibit hydrogen peroxide include catalase and glutathione peroxidase [19].

Flavonoids and their derivatives have attracted much attention from organic chemists and biochemists because they are among the important groups that play a major role in nature. The aim of this research is to investigate the antioxidant activity of the prepared flavonoid derivatives and compare them with ascorbic acid and quercetin as well-known antioxidants, as well as to review the role of hydroxyl, acetate, amide, chlorine and benzyl groups in the structure of flavonoids.

Experimental part

Chemicals: Dry methanol, DPPH (2,2-diphenyl-1-picrylhydrazyl from Sigma-Aldrich, Germany), ascorbic acid (produced by Spanish company Scharlau), quercetin (produced by German company Sigma-Aldrich). The tested compounds have been synthesized by us in a previous study and can be found elsewhere [20] (Figure 1).

Determination of the ability of the synthesized flavonoids to inhibit DPPH radicals

The ability of flavonoids to inhibit free radicals was determined by the DPPH assay according to the method of Blois [15]. To each test tube was added 1 mL of the solution to be tested (each flavonoid (A_2, A_3, A_5, A_6, A_7, A_8, A_9, A_{10}, A_{11}, A_{12}, A_{13}) and titer compound separately), then 5 mL of DPPH (0.2 mM/L) solution was added. The tubes were kept under stirring for 20 min in a dark place at room temperature. Absorbance was measured using UV-VIS Shimadzu at a wavelength of 516 nm (the control solution consisted of 1 mL dry methanol + 5 mL DPPH). The percentage of free radical suppression was calculated using the following arithmetic method:

$$DPPH\ \% = [(A_0 - A_1) / A_0] \times 100 \qquad (1)$$

Where A_1: the absorbance of the sample, A_0: the absorbance of the control sample. The percentage DPPH inhibition curve is plotted a percentage of concentrations, which is an even first degree equation, the IC_{50} value defined by the concentration of the solution in μg/mL is calculated to eliminate 50% of DPPH radicals [15].

The ability of flavonoids to inhibit free radicals was compared with that of standard compounds (quercetin, ascorbic acid).

Determination of the ability of flavonoids to inhibit hydrogen peroxide:

The ability of flavonoids to inhibit hydrogen peroxide was determined by the following method: To each test tube was added 1 mL of the flavonoid compound (A_2, A_3, A_5, A_6, A_7, A_8, A_9, A_{10}, A_{11}, A_{12}, A_{13}) to be tested prepared at different concentrations (250, 500, 750, 1000 μg/mL) and dissolved in methanol as solvent and 1 mL of hydrogen peroxide solution (40 mM) in a phosphate buffer (50 mM, pH=7.4). The tubes were kept in a dark place at room temperature for 10 minutes. The absorbance was measured in a UV-VIS at a wavelength of 230 nm [22]. The blank consisted of a mixture of methanol and a phosphate buffer solution without hydrogen peroxide (this is called a negative control plate (without H_2O_2)). The control solution consisted of a mixture of methanol, phosphate buffer and hydrogen peroxide (used under the same conditions: volumes and concentrations). The method described above was used to prepare the standard solutions (quercetin 250, 500, 750, 1000 μg/mL, ascorbic acid 250, 500, 750, 1000 μg/mL) [22].

The percentage of hydrogen peroxide suppression was calculated using the following arithmetic method:

$$H_2O_2\ \% = [(A_0 - A_1) / A_0] \times 100 \qquad (2)$$

Where A_1: the absorbance of the sample, A_0: the absorbance of the control sample. A curve was drawn for the percentage inhibition H_2O_2% with respect to the concentrations, which is a straight first order equation, then the value of the IC_{50} for H_2O_2 was calculated [22]. The ability of flavonoids to inhibit hydrogen peroxide was compared with that of standard compounds (quercetin, ascorbic acid).

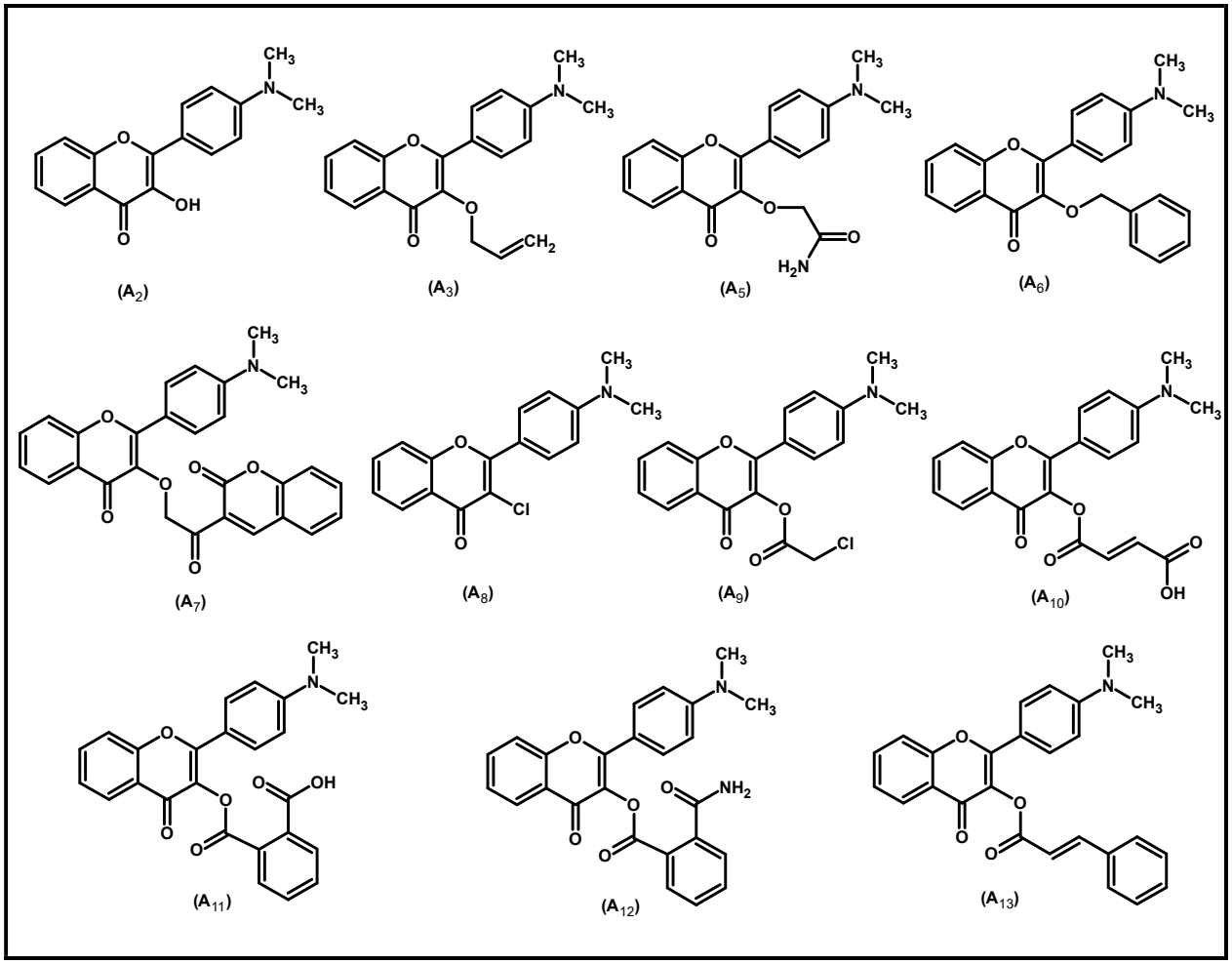

Figure 1. Chemical formulas for the alkyl and esterified derivatives of synthetic flavonoids (A_2-A_{13}) [20]

Results and Discussion:

Results

The inhibition ability of flavonoids was measured by the DPPH and hydrogen peroxide methods in four different concentrations, that is, at (250, 500, 750 and 1000 µg/mL) at a wavelength of 516 nm by the DPPH method and at a wavelength of 230 nm by the hydrogen peroxide method. The percentages of the ability of flavonoids (A_2-A_{13}) to inhibit DPPH and H_2O_2

were calculated by the relationships (1) or (2) as shown in **Table 2**.

The value of IC_{50} for each compound was calculated using the equation of the inhibition ratio graph as a function of concentration and summarised in **Table 3**.

Table 1. Absorption values for synthesised flavonoids (A_2 - A_{13}):

Control abs		**1.897 (DPPH·)**				**1.388 (H_2O_2)**			
Concentration (µg/mL)	**0**	**1000**		**750**		**500**		**250**	
Comp		**DPPH·**	**H_2O_2**	**DPPH·**	**H_2O_2**	**DPPH·**	**H_2O_2**	**DPPH·**	**H_2O_2**
A_2	0	0.561	0.365	0,601	0.387	0.642	0.401	0.661	0.421
A_3	0	0.987	0.756	1.057	0.812	1.123	0.877	1.195	0.923
A_5	0	0.816	0.567	0.820	0.588	0.846	0.601	0.890	0.670
A_6	0	0.945	0.755	1.009	0.813	1.055	0.897	1.124	0.945
A_7	0	0.757	0.678	0.812	0.724	0.896	0.796	0.957	0.813
A_8	0	0.641	0.802	0.685	0.836	0.880	0.911	1.214	0.968
A_9	0	1.140	0.789	1.151	0.858	1.155	0.923	1.167	0.986
A_{10}	0	1.156	0.712	1.213	0.745	1.242	0.803	1.310	0.856
A_{11}	0	1.047	0.724	1.087	0.768	1.134	0.799	1.199	0.845
A_{12}	0	1.116	0.853	1.170	0.896	1.205	0.969	1.240	1.005
A_{13}	0	1.256	0.969	1.387	1.012	1.443	1.056	1.480	1.125
Quercetin	0	0.115	0.103	0.116	0.112	0.118	0.114	0.129	0.118
Ascorbic acid	0	0.552	0.298	0.559	0.308	0.559	0.322	0.561	0.335

Table 2. Results of percentage inhibition of DPPH and H_2O_2 for synthetic flavonoids (A_2-A_{13}):

Concentration µg/mL	**1000**		**750**		**500**		**250**	
Compound	**DPPH· %**	**H_2O_2 %**	**DPPH· %**	**H_2O_2 %**	**DPPH· %**	**H_2O_2 %**	**DPPH· %**	**H_2O_2 %**
A_2	70.42	73.70	68.31	72.11	66.15	71.10	65.15	69.66
A_3	47.97	45.53	44.28	41.49	40.80	36.81	37.00	33.50
A_5	56.98	59.14	56.77	57.63	55.40	56.70	53.08	51.72
A_6	50.18	45.60	46.88	41.42	44.38	35.37	40.74	31.91

A_7	60.09	51.15	57.19	47.83	52.76	42.65	49.55	41.42
A_8	66.20	42.21	63.89	39.76	53.61	34.36	36.00	30.25
A_9	39.90	43.15	39.32	38.18	39.11	33.50	38.48	28.96
A_{10}	39.06	48.70	36.05	46.32	34.52	42.14	30.94	38.32
A_{11}	44.80	47.83	42.69	44.66	40.22	42.43	36.79	39.12
A_{12}	41.17	38.54	38.32	35.44	36.47	30.18	34.63	27.59
A_{13}	33.79	30.18	26.88	27.08	23.93	23.91	21.98	18.94
Quercetin	93.93	92.57	93.88	91.93	93.77	91.78	93.19	91.49
Ascorbic acid	70.90	78.53	70.53	77.80	70.53	76.80	70.42	75.86

Table 3. The values of the IC_{50} for synthetic flavonoids (A_2 - A_{13}):

Compound	**IC_{50} (μg/mL)**	
	DPPH·	**H_2O_2**
A_2	430.451	378.230
A_3	887.021	968.282
A_5	617.494	599.597
A_6	818.403	974.739
A_7	619.295	807.632
A_8	594.664	1049.734
A_9	1076.346	1056.178
A_{10}	1157.117	852.701
A_{11}	947.643	874.864
A_{12}	1078.139	1195.176
A_{13}	1488.965	1594.087
Quercetin	169.177	182.776
Ascorbic acid	385.669	314.496

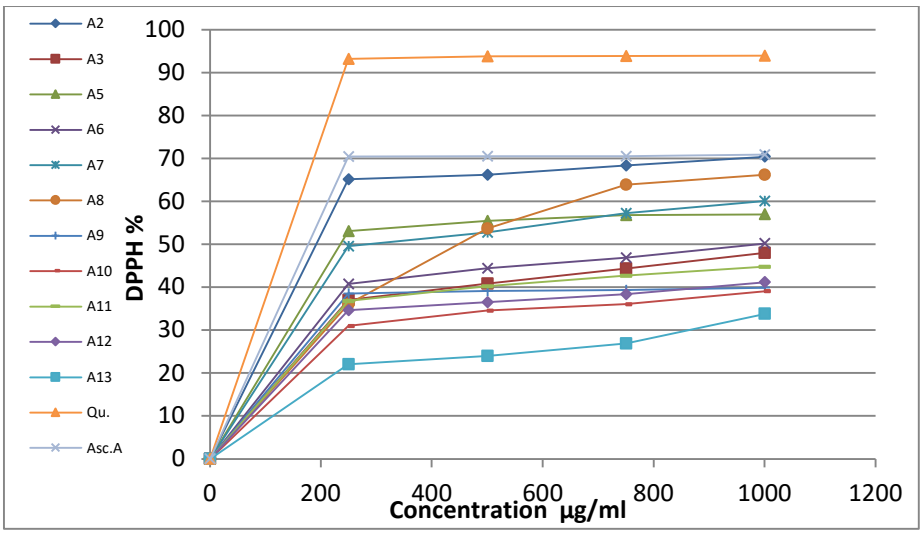

Figure 2. Results of the DPPH % test for flavonoids (A_2 – A_{13})

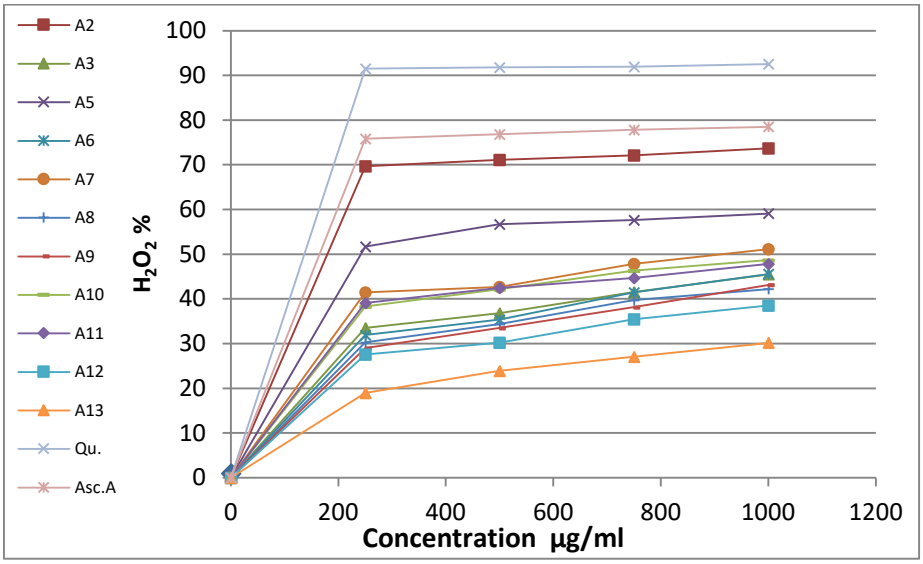

Figure 3. Results of the H_2O_2% test for flavonoids (A_2 – A_{13})

The results showed the inhibitory effect of synthesized flavonoids (A_2 - A_{13}) on DPPH and hydrogen peroxide. The percentage of free radical inhibition was variable, some of them being high and others being medium compared to the two standard compounds, ascorbic acid and quercetin. A decrease in the percentage of free radical inhibition was observed in compound A_2, which has the - OH group at the C_3 site as it reached (70.42, 73.70%). The observation of IC50 values of 430.451 µg/mL and 378.230 µg/mL, respectively, using two radical inhibition methods (DPPH and H2O2), was compared to the standard compounds. In **Table 2**, it can be seen that the percentage values of radical suppression decreased when the allyl, amide and coumarin groups were introduced into the compounds (A_3, A_5, A_7) compared to flavonoid compound A_2, and the IC_{50} values were higher than the IC_{50} value of flavonoid compound A_2,

which means that they are less effective compounds as antioxidants.

A slight decrease in the percentage of free radical suppression by the DPPH method was observed. When the group -OH in compound A_8 was replaced by a chlorine group -Cl, the percentage reached 66.20% and the value of IC_{50} = 594.664 µg/mL. A significant decrease in the percentage of free radical inhibition by the H_2O_2 method was also observed for the above compound, as it reached 42.21% and the value of IC_{50}= 1049.734 µg/mL. The percentages of free radical inhibition were medium when acetyl, maleic, phthalic and phthalimide groups were added to compounds A_9, A_{10}, A_{11} and A_{12} by the above two methods. While the percentage of free radical inhibition by the two methods (DPPH, H_2O_2) decreased when the cinnamic group was added to compound A_{13}, where the values of IC_{50}= 1488.965 and 1594.087 µg/mL show that A_{13} is a less effective as antioxidant. The smaller the IC_{50} value, the greater the effectiveness of the compound as an antioxidant.

Figures 4, 5, 6, 7 and 8 show the proposed mechanisms of compounds (A_2, A_3, A_5, A_6) as antioxidants.

Figure 9 shows the IC_{50} values for flavonoids (A_2-A_{13}) according to the two DPPH methods. and H_2O_2.

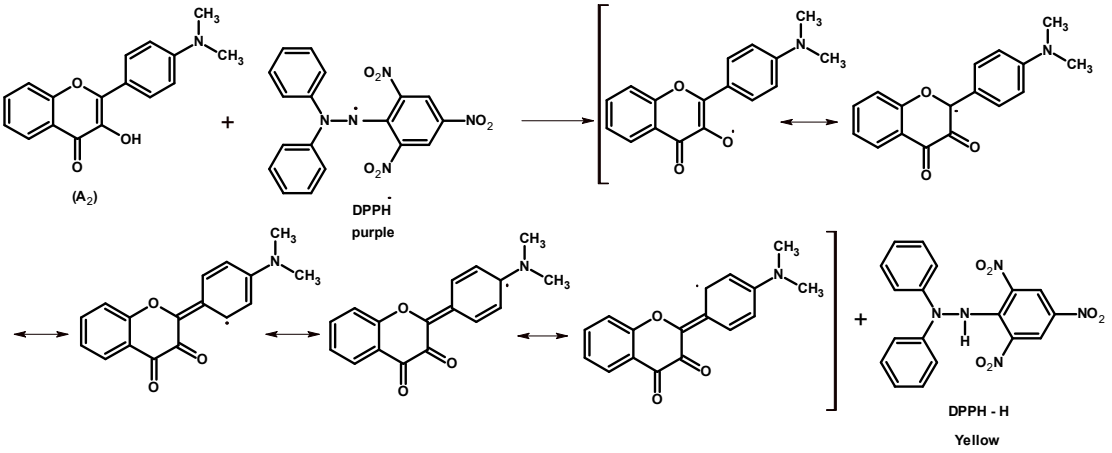

Figure 4. Proposed mechanism of compound A_2 as an antioxidant

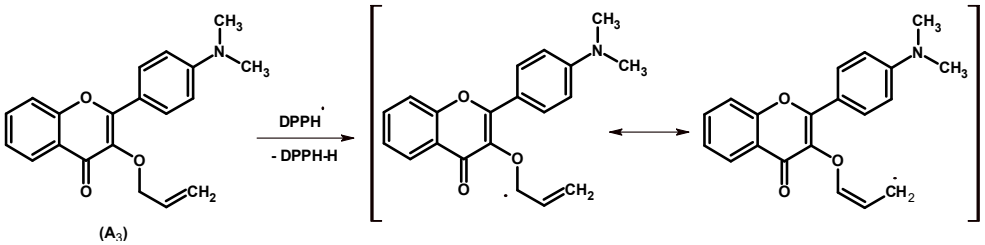

Figure 5. Proposed mechanism of compound A_3 as an antioxidant

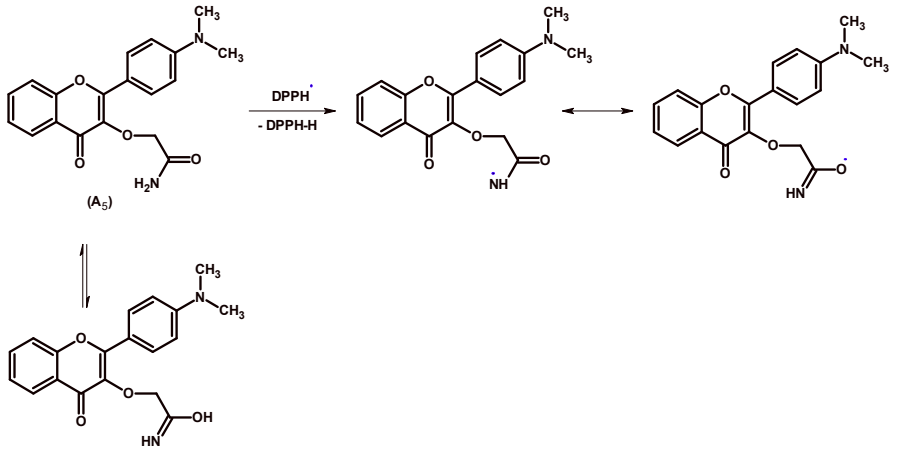

Figure 6. Proposed mechanism of compound A_5 as an antioxidant

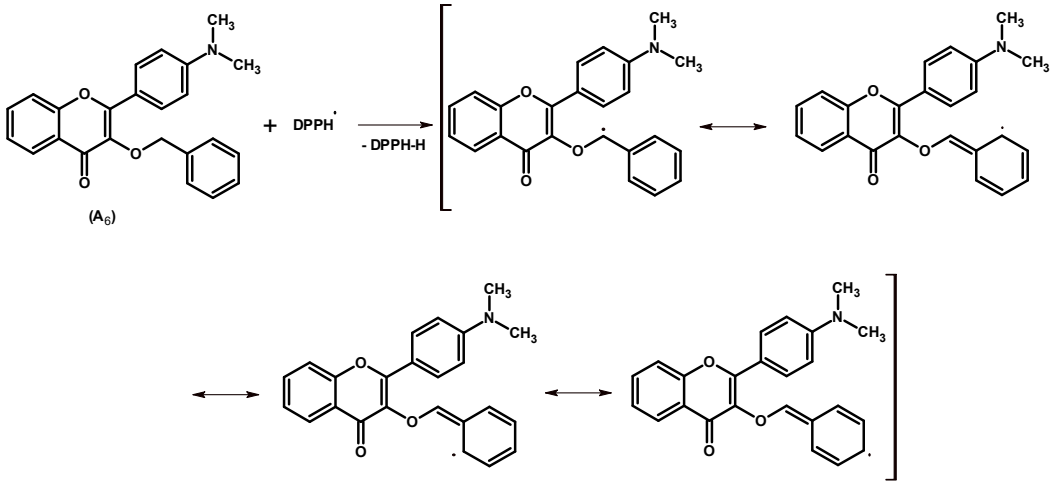

Figure 7. Proposed mechanism of compound A_6 as an antioxidant

The following figure shows the proposed mechanism for the ability of flavonoids to inhibit hydrogen peroxide:

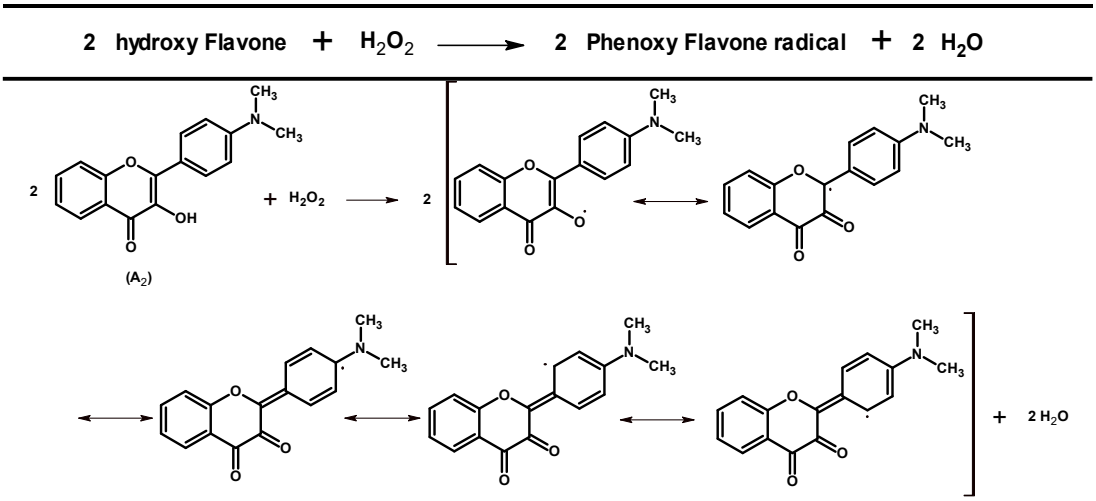

Figure 8. Proposed mechanism for the ability of flavonoids to inhibit hydrogen peroxide

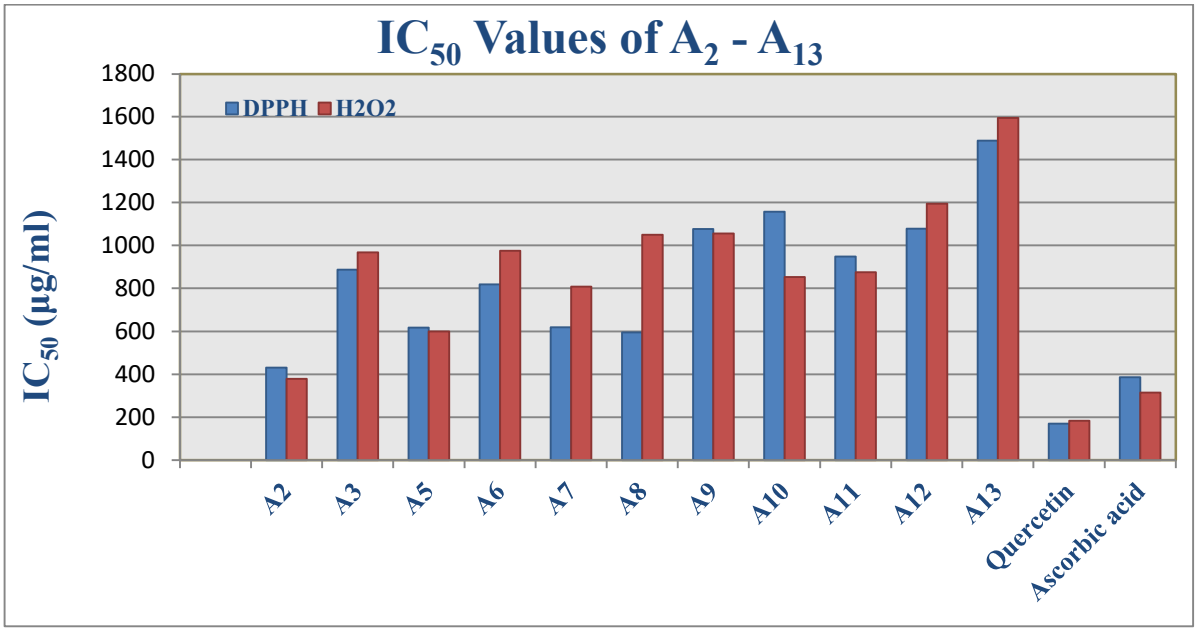

Figure 9. IC_{50} values for flavonoids (A_2-A_{13}) by DPPH and H_2O_2 methods

Discussion

The compound is considered an antioxidant when the phenoxy radicals it produces are stabilized. If it fails to stabilise unstable radicals, it sets off a free radical-mediated chain reaction that can lead to harmful effects in the human body. However, the stabilization of unstable free radicals can be easily achieved in phenolic compounds (especially flavonoids) by delocalising the electron, since they contain a conjugated structural system either in the aromatic ring or in the pyran ring (as in the mechanism proposed in **Figure 4**). Thus, the formed phenoxy radicals undergo electron delocalization at the flavonoid structure and transform the unstable radicals into a stable form.

Some reference studies suggest that flavonoids containing an unsaturated bond at the C_2 and C_3 sites in conjugation with the 4-oxo group are more potent antioxidants than flavonoids lacking the double bond. The conjugation between the A and B rings allows a resonance effect for the aromatic nucleus, which gives stability to the flavonoid radical. The antioxidant activity of flavonoids in scavenging free radicals is enhanced by the presence of each of the above elements [23].

The heterocycle of flavonoids contributes to antioxidant activity through the coupling between the two aromatic rings A and B and the presence of the hydroxyl group -OH at the C_3 site. Removal of the -OH group from the C_3 site leads to a decrease in the antioxidant activity of flavonoids [24], which is consistent with our current study.

The results presented in **Tables 2 and 3** show that the compounds (A_2, A_5, A_6) are

effective as antioxidants because they contain hydroxyl, amides and benzyl groups that donate hydrogen to the free radical DPPH (inactivating it into the stable form diphenylpicrylhydrazine DPPH-H) or to hydrogen peroxide (turning it into water molecules). The results show that the inhibitory effect of flavonoid compounds on the DPPH free radical is higher than on hydrogen peroxide. The reason for this could be that the DPPH free radical is an intermediate (unstable) compound compared to hydrogen peroxide (stable compound) and DPPH is therefore characterised by high activity as it reacts faster with flavonoids than with hydrogen peroxide.

Conclusions

The results show the presence of antioxidant activity of some flavonoid compounds. Compounds containing enolic hydroxyl group, amide group and benzyl group in their structure have an increased antioxidant activity. Flavonoids that contain an acetate group in their structure are significantly less efficient antioxidants.

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