Volume-04
Issue-06
June-2019

ISSN: 2455-3085 (Online)
RESEARCH REVIEW International Journal of Multidisciplinary
www.rrjournals.com[UGC Listed Journal]

Structure activity Relationship Study of Simple Hydroxyl Coumarins as Antioxidant to Scavenge Hydrogen peroxide

[*1]Nagaretnam Prahadeesh and [2]Kiruthika Arulmoly

[1,2]*Assistant Lecturer, Department of Chemistry, Eastern University Sri Lanka, Chenkalady, Sri Lanka*

ARTICLE DETAILS

Article History
Published Online: 12June 2019

Keywords
Coumarin, Hydroxyl coumarin, Antioxidant, Hydrogen peroxide assay.

[*]Corresponding Author
Email:shapirah[at]live.com

ABSTRACT

Coumarins are heterocyclic compounds; structurally constructed by α-pyrone ring, fused with benzene ring. Coumarins are very interested because of their abundance in nature and their tremendous pharmacological properties such as anticoagulant, anticancer, antibacterial, anti-inflammatory, anti-tuberculosis, antioxidant, etc.The need for the antioxidant is very important because of the role reactive oxygen species and its contribution to many deleterious effects in human body. Hydrogen peroxide is such a reactive oxygen species. This study focused on synthesizing the parent compound,Coumarin and its hydroxyl derivatives (7-hydroxy coumarin and 4-hydroxy coumarin) and studying the positional effect hydroxyl group at 7th and 4^{th}positions to scavenge the Hydrogen peroxide. The different coumarins were synthesized by using standard methods and werecharacterized by using UV-visible, IR, ^1H and ^{13}C NMR spectra. The antioxidant activity of the synthesized coumarins was studied by using standard Hydrogen peroxide scavenging assay. Hydroxyl coumarins showed higher activity than parent coumarin. The 7th positioning hydroxyl group was more effective than 4th positioning hydroxyl group in coumarin to scavenge the Hydrogen peroxide. But all the studied coumarins showed poor hydrogen peroxide scavenging activity when compared to standard ascorbic acid.

1. Introduction

Coumarins are very important class of heterocyclic comounds. They are α -benzopyrone, where a benzene ring is fused with an alpha pyronering.The IUPAC nomenclature of the parent compound, Coumarin is 2*H*-1-benzopyran-2-one (Figure 1.1).Coumarins are being an attractive highlight among the researches for many years because of their diversity in nature and their wide range of pharmacological properties such as antibacterial, antifungal, anticoagulant, anti-inflammatory, antitumor, antioxidant, etc. Coumarins are also widely used as additives in cosmetics and food products. Coumarin owed its name from “coumarou” which is the vernacular name of the Tonka bean (*Dipteryxodorata*Wild). It was first isolated by Vogel of Munich in 1820 (Bruneton.1999) and was first synthesized by William Henry Perkin in 1868via his classical reaction (Perkin1868).

Coumarins are highly distributed among the plant kingdom such plant families areRutaceae, Umbelliferae, Guttiferae, Clusiaceae, Oleaceae, Apiacea, and Nyctaginaceae.Scopoletin, Umbelliferone and Esculetinare the most widely distributedcoumarins in nature (Ribeiro*et al.* 2002). Some coumarins are also found in microorganisms such as Novobiocin, Coumermycin and Aflatoxins (Cooke 1999). Coumarins are famous for their wide range of pharmacological properties such as vasorelaxant,antimicrobial, antidepressant, anticoagulant, anticonvulsant, antihistaminic, anti-inflammatory, anti-tumor,anticancer, antiasthmatic, antituberculosis, etc. (Venugopala*et al.* 2013).

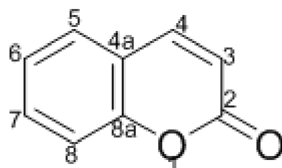


Figure 1.1: Structure and IUPAC numeration of Coumarin.

In recent years,oxidative stress have become a major health issueand being the main subject for plenty of researches. Oxidative stress,which arises as a result of an imbalance between reactive oxygen species (ROS) production and antioxidant defenses in our body, is commonly associated with unfavourable damage to a wide range of molecular species including lipids, carbohydrates, proteins, and nucleic acids. Higher oxidative stress can lead to the deleterious effects to the human body such as cancer, atherosclerosis, inflammatory diseases (eg, arthritis, vasculitis, glomerulonephritis, lupus erythematous),ischemic diseases, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), etc. (Harman 1992).The major cause for the higher oxidative stress is ROS. Reactive oxygen species consist of free radicals and non-radical oxygen species which are highly reactive and generally formed by the partial reduction of oxygen.The most harmful oxygen-containing free radicals are hydroxyl radical ($OH^•$), alkoxy radical ($OR^•$), superoxide anion radical ($O_2^{-•}$) and peroxy radicals ($OOH^•$, $OOR^•$). Hydrogen peroxide (H_2O_2)

RRIJM 2015, All Rights Reserved

and singlet oxygen are the examples for non-free radical ROS (Hayyan*et al.* 2016). But hydrogen peroxide also has the tendency to produce highly reactive hydroxyl radical, when it is partially reduced (Figure 1.2)(Turrens 2003). H_2O_2 does not cause damage directly to the DNA but produces hydroxyl radical that attacks sugars, phosphate backbone and the bases of the DNA helix (Friedberg *et al.* 1995).The hydroxyl radical is very reactive and pulls out the electron from any molecules on its path, converts that molecule into free radical. Thus propagating a free radical mediated chain reaction.

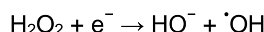


Figure 1.2:Partial reduction of Hydrogen peroxide produces hydroxyl radical (•OH)

H_2O_2 can be produced intracellularly.Cellular respiration is such a major source for the H_2O_2 production in body (Han *et al.* 2001). Oxidative phosphorylation is the key step in the production of ATP of cellular respiration, occurs in the mitochondrial cristae which comprises the electron transport chain. The electron transport chain is a series of protein complexes that transfer electrons from electron donors to electron acceptors via redox reactions. The last acceptor for the electron along this chain is molecular oxygen. Normally this molecular oxygen is reduced into water but there are significant chances for the partial reduction of the molecular oxygen. This produces superoxide (Figure1.3) (Li *et al.* 2013).

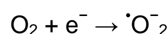


Figure 1.3: Partial reduction of molecular oxygen (O_2)

Dismutation of superoxide produces H_2O_2 which is catalyzed by enzyme, superoxide dismutase (Figure 1.4). It involves the disproportionation of super oxide (Borgstahl*et al.* 1992).

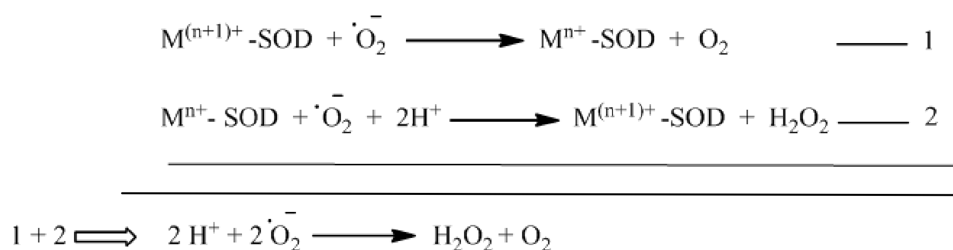


Figure 1.4: Dismutation of superoxide; whereM- SOD: Metal- Superoxide dismutase complex

The produced H_2O_2 can be converted into water and molecular oxygen by catalase enzyme. Glutathione peroxidase is another enzyme present in our body which catalyzes the reduction of hydrogen peroxide into water (Figure 1.5) (Bhabak *et al.* 2010). However, when the production of H_2O_2 becomes higher and the self-defense of body become weak then there will be a need for the exogenous source for the defense - the antioxidants.

$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$(Catalase)
$2GSH + H_2O_2 \rightarrow GS\text{–}SG + 2H_2O$ (Glutathione peroxidase)

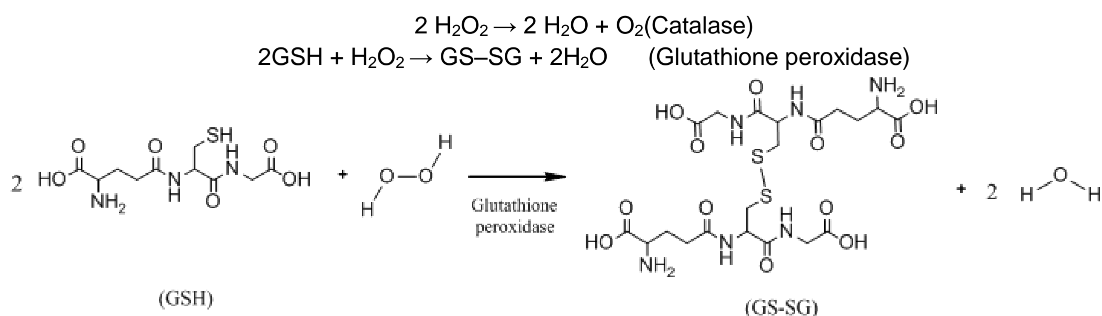


Figure 1.5: Enzymatic action on Hydrogen peroxide; where, GS-SG - Glutathione disulfide; GSH -Glutathione

2. Materials and methodology

2.1. Chemicals and Instruments:

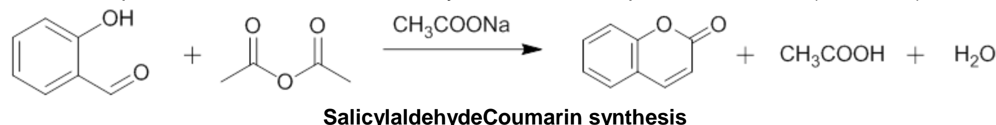
Standard chemicals (Sigma-Aldrich) were obtained from Chemical laboratory, Department of Chemistry, EUSL. The chemicals were purified prior to its use. (Malic acid was purified by recrystallization from acetone and then ethyl acetate by adding petroleum ether (60-70 ^{O}C); resorcinol was purified by recrystallization from Toluene). Precoated TLC plates were used to monitor the reaction. Melting point apparatus (Gallenhamp) was used to find the melting points of the crystals. Infrared spectra were recorded by using ATR– Thermo scientific Nicolet IS10 spectrometer. The ^{1}H spectra and ^{13}C spectra were recorded by usingBruker NMR spectrometer (400 MHz) at room temperature (solvent- methanol-d_4). UV-Visible absorption spectra were recorded (as absorption versus wavelength) by using Double beam scanning UV-Vis Spectrophotometer; BK-D580, by using 10mm Quarts cuvette in the range of 190-700 nm.

2.2. Methodology:

RRIJM 2015, All Rights Reserved

2.2.1. Synthesis of Coumarin

Fused sodium acetate (5 g), salicylaldehyde (4.0 g) and acetic anhydride (10.0 ml (0.104 mol)) were placed in a three neck flask. The mixture was refluxed at 180 ^{O}C for 6 hours and was TLC monitored. At the top of the condenser, Calcium chloride drying tube was used. Then the mixture was steam distilled. The obtained residue was rendered to basic with solid $NaHCO_3$. Then it was cooled, filtered and washed with cold water. The crude coumarin was boiled in water with activated charcoal. Then it was filtered and concentrated. Then cooled product was collected and recrystallized from aqueous methanol (Kar 2004).



SalicylaldehydeCoumarin synthesis

Figure 2.1: Reaction scheme of Coumarin synthesis

2.2.2.Synthesis of 4-hydroxy coumarin

Part 1

Synthesis of acetyl methyl salicylate:

Concentrated H_2SO_4 (0.3 ml) was added to a mixture of methyl salicylate (40 g) and acetic anhydride (40 g). Then it was mixed thoroughly and was allowed to stand at room temperature for 40 minutes. The mixture was added to cold water (3000 ml) and well stirred for30 minutes. The resulting mixture was allowed to stand for 6 hours. The obtained product was filtered and washed with water. Then it was dried in desiccator.

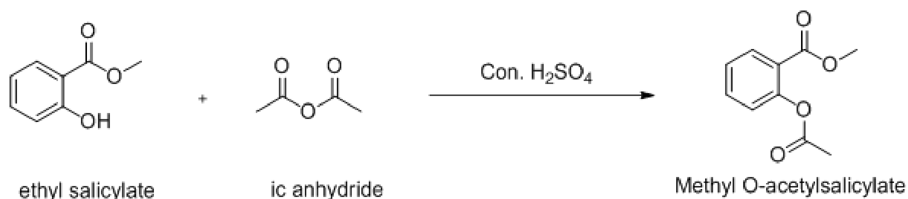


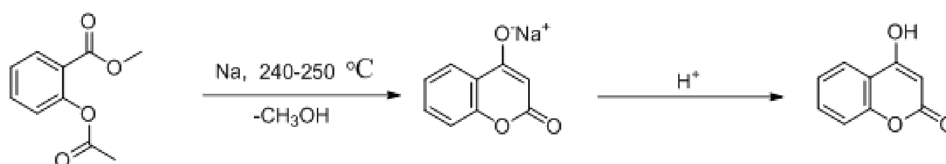
Figure 2.2: Reaction scheme of Acetyl methyl salicylatesynthesis

Part 2

Synthesis of 4-hydroxy coumarin:

Metal sodium (4.8 g) and paraffin (60 ml) were added into a three neck flask, equipped with a short fractionation column with condenser. It was heated to 250 °C. Then dried acetyl methyl salicylate (40 g) was added portion wisely for 40 minutes. The reaction mixture was maintained between 240-250 °C for 2 hours. Then the hot reaction mixture was filtered under suction. The obtained crude product was cooled and washed with low boiling petroleum fraction. The dried product was added slowly into the distilled water (200 ml) with vigorous stirring and maintained at about 60-70 °C. Then the pH of the solution was reduced to 5.5 by adding diluted HCl. Flocculants were removed by skimming and discarded.

The resulting mixture was shaken well with equal volume of ethyl ether. Then the aqueous phase was separated by using separating funnel. The aqueous solution was acidified with concentricHCl to pH 1.5. The obtained crude was filtered and recrystallized from water twice (Mark*et al.* 1949).



2.2.3.Synthesis of 7-hydroxy coumarin

Malic acid (5.948 g) and resorcinol (4.4 g) were added into concentrated H_2SO_4 (10.8 ml) with stirring. It was maintained at 120 °C till the effervescence ceased. Then the hot solution was poured into crushed ice with stirring. The mixture was allowed to stand for 24 hours and filtered under suction. The obtained crude product was recrystallized from aqueous ethanol twice. Then it was again recrystallized from aqueous acetic acid (Pechmann*et al.* 1884).

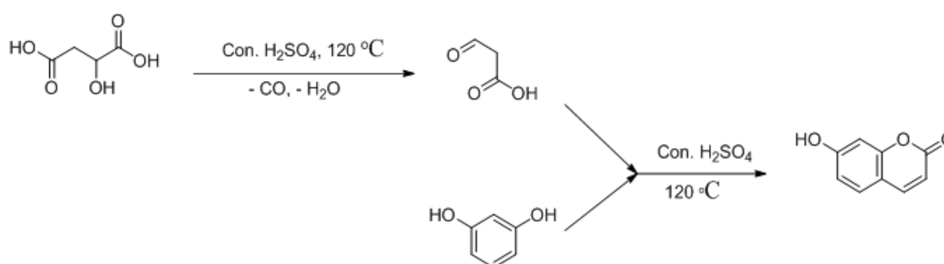


Figure 2.4: Reaction scheme of 7-hydroxy coumarin synthesis

2.2.5. Measuring antioxidant activity (AOA)

Hydrogen peroxide (H_2O_2) scavenging activity:

The hydrogen peroxide scavenging activity of the synthesized coumarins was measured by using standard H_2O_2 scavenging assay method (Ruch*et al.* 1989). H_2O_2 solution (40mM) was prepared in phosphate buffer saline (10x, pH 7.4). Different concentrations (20 ppm, 200 ppm, 400 ppm, 600 ppm, 800 ppm and 1000 ppm) of the synthesized compounds were prepared in ethanol (95%). Test coumarin solution (1 ml) was added to hydrogen peroxide solution in phosphate buffer (40 mM) and the absorbance at 230 nm was determined after 10 minutes of addition against blank solution containing the phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard reference. All the experiments were repeated three times in dark condition. Hydrogen peroxide scavenging ability was calculated as follows:

$$H_2O_2 \text{ Scavenging ability } \% = [(A_i - A_t) / A_i] \times 100$$

Where A_i is the absorbance of control and A_t is the absorbance of test.

IC_{50} value for H_2O_2 Scavenging ability was calculated by plotting graph Concentration of test versus absorbance.

3. Results and Discussion

Characterization of the products

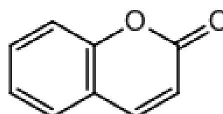
Coumarin (P_1)**:**

IR (cm^{-1}): 1704 (C=O), 3057(C-H aromatic), 1277 and 1259 (C-O lactone's ester), 1618 (C=C), 1601and 1562 (C=C aromatic).

UV-Visible (nm): 311 and 272 (n→π * of carbonyl chromophoreand π→π* aromatic compound).

Melting point: 69 ^{0}C, Yield : 40%

Structure:



4-hydroxy coumarin (P_2)**:**

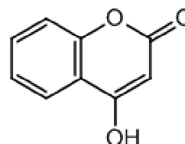
IR (v, cm-1): 3354 (O-H), 1660 (conjugated C=O), 1294 and 1273 (C-O lactone's ester), 1597 (C=C), 1553 (C=C aromatic).

^{1}H-NMR (CD_3OD, 400MHz) (δ, ppm): δ 5.65 (s, 1H, -C=C-H of lactone (3-H)), δ7.92 (dd, 1H, ^1j=7.7 Hz, ^2j=1.5 Hz, 5-H), δ 7.64 (triplet of doublets, 1H, ^1j=7.7 Hz, ^2j=1.7 Hz,6-H), δ 7.3638 (m, 2H, 7-H and 8-H).

^{13}C NMR (CD_3OD, 400MHz) (δ, ppm): δ 166.94 (4-C), 164.63(2-C), 153.82(8a-C), 132.54(7-C), 123.88(5-C), 123.19(6-C), 2 signals at 116.16 (4a-C and 8-C), 90.30(3-C).

Melting point: 214 ^{0}C, Yield: 21 %

Structure:



7-hydroxy coumarin (P_3)**:**

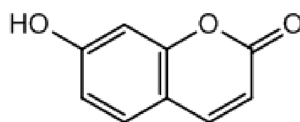
IR (v, cm^{-1}): 3199 (O-H), 1697(C=O), 1228 and 1257(C-O lactone's ester), 1602(C=C), 1562 and 1516 (C=C aromatic).

^{1}H-NMR (CD_3OD, 400MHz) (δ, ppm):6.19 (d, 1H, ^1j=9.3 Hz, 3-H), 6.72(d, 1H, ^2j= 1.5 Hz, 8-H), 6.81(dd, 1H, ^1j= 8.4 Hz, ^2j= 1.5Hz, 6-H), 7.46 (d, 1H, ^1j= 8.4 Hz, 5-H), 7.86 (d, 1H, ^1j= 9.3 Hz, 4-H).

^{13}C NMR (CD_3OD, 400MHz) (δ, ppm): 162.27 (7-C), 161.73 (2-C), 155.84 (8a-C), 144.63 (4-C), 129.25 (5-C), 113.09 (6-C), 102.00 (8-C), 2 signals at 110.96 (4a-C and 3-C).

Melting point: 229 ^{0}C, Yield : 42 %

Structure:



H_2O_2 scavenging activity:

P_1 is the parent compound coumarin which has no substitution on it. P_2 and P_3 are hydroxyl coumarins they have hydroxyl group (OH) substitution at 4th and 7th position of the parent coumarin skeletal respectively. Coumarins are lactones constructed by a benzene ring fused with α-pyrone ring, and possess electron conjugated skeletal system with rich electron and good charge transfer properties. Conjugated skeletal system allows this compound to delocalize the electron and giving additional stability for it. The hydroxyl functional group has potential to be an active scavenger for ROS. Its mode of action is by giving its hydrogen from the hydroxyl functional group (OH) to ROS. The donated hydrogen ("H") neutralizes the ROS and reduces the harmful oxidative breakages. Thus it also terminates the free radical mediated chain reaction which has the tendency to cause higher oxidative stress. When a hydroxyl group contained compound donates its hydrogen eventually that generates the compound into an unstable free radical containing compound. This formed free radical should be stabilized itself. This could also be possible when the compound has the ability to delocalize the free radical and making the free radical containing intermediate into stable form. Since the coumarins have electron delocalizing skeletal system it acts as a good antioxidant.

H_2O_2 scavenging activity of the tested simple Coumarins, P_1, P_2 and P_3 is shown in Figure 3.1, 3.2 and 3.3 respectively. All the tested coumarins showed hydrogen peroxide scavenging activity. IC_{50} value of the tested Coumarins is shown in Table 3.1. IC_{50} value is the concentration of the test sample (antioxidant) required to scavenge 50% of H_2O_2. An antioxidant with lower IC_{50} value indicates a good potent antioxidant.

H_2O_2 scavenging antioxidant activity of the three synthesized simple coumarins in decreasing order: P_3> P_2> P_1. The IC_{50} value of P_3, P_2 and P_1 was 7029 ppm, 9150 ppm and 24902 ppm respectively(Table 3.1).The hydroxyl coumarins (P_3 and P_2) showed higher activity than the parent compound P_1.

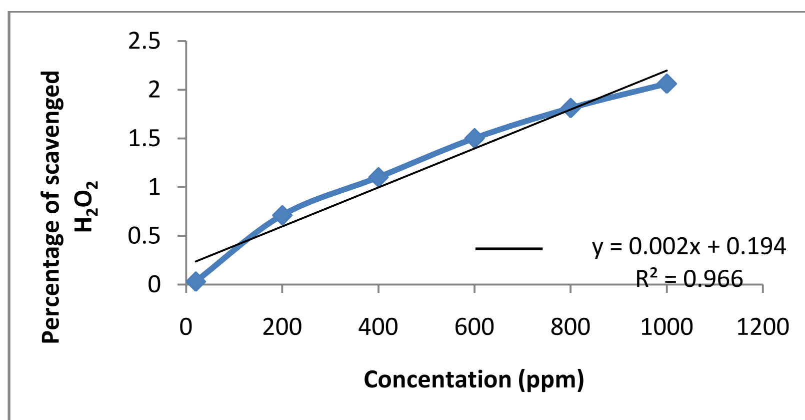


Figure 3.1:H_2O_2 Scavenging activity of P_1

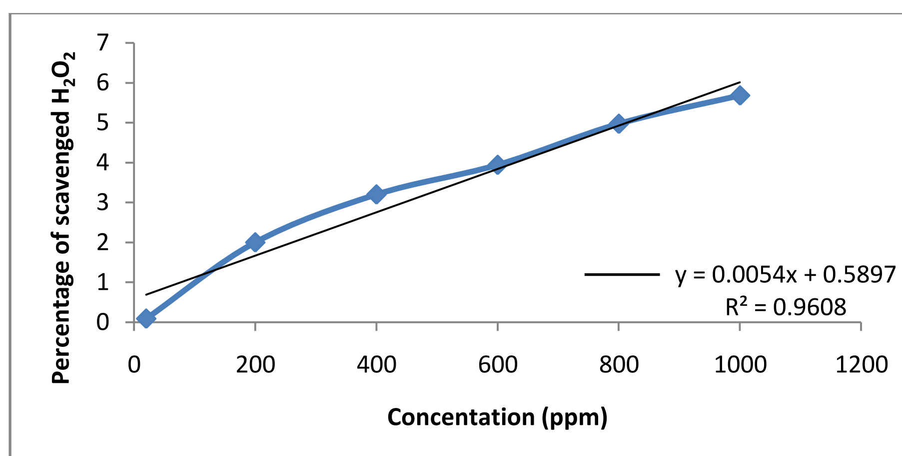


Figure 3.2: H_2O_2 Scavenging activity of P_2

RRIJM 2015, All Rights Reserved

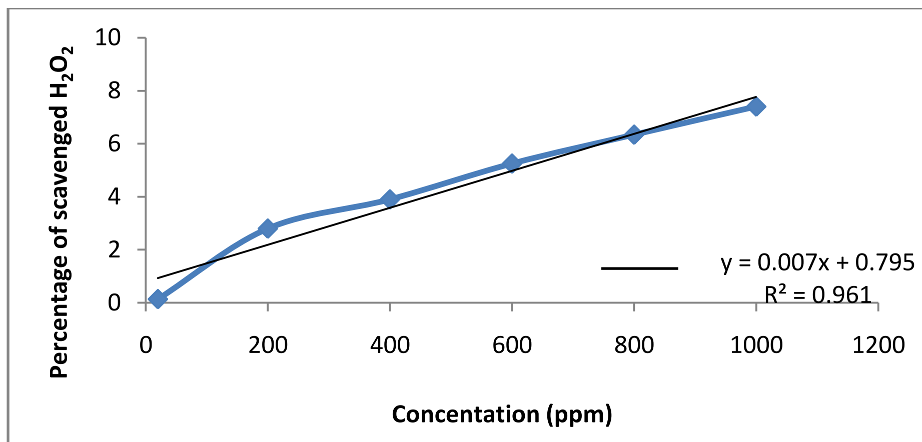


Figure 3.3: H_2O_2 Scavenging activity of P_3

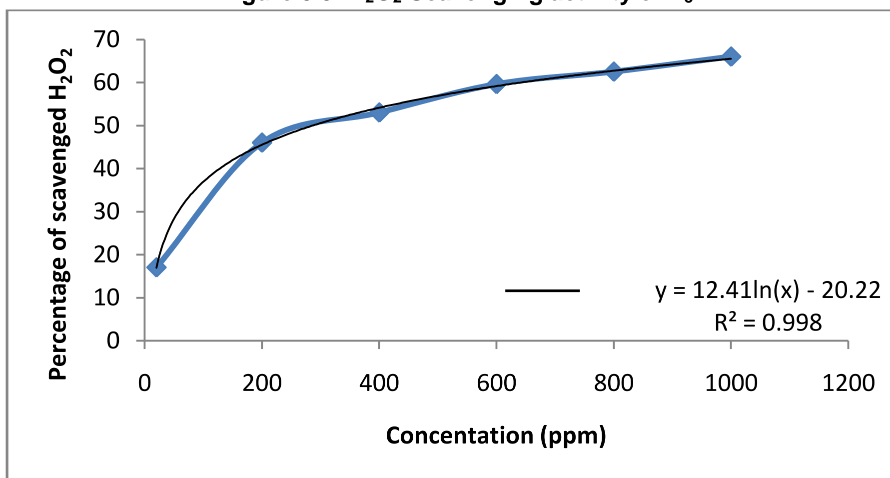


Figure 3.4: H_2O_2 Scavenging activity of Ascorbic acid

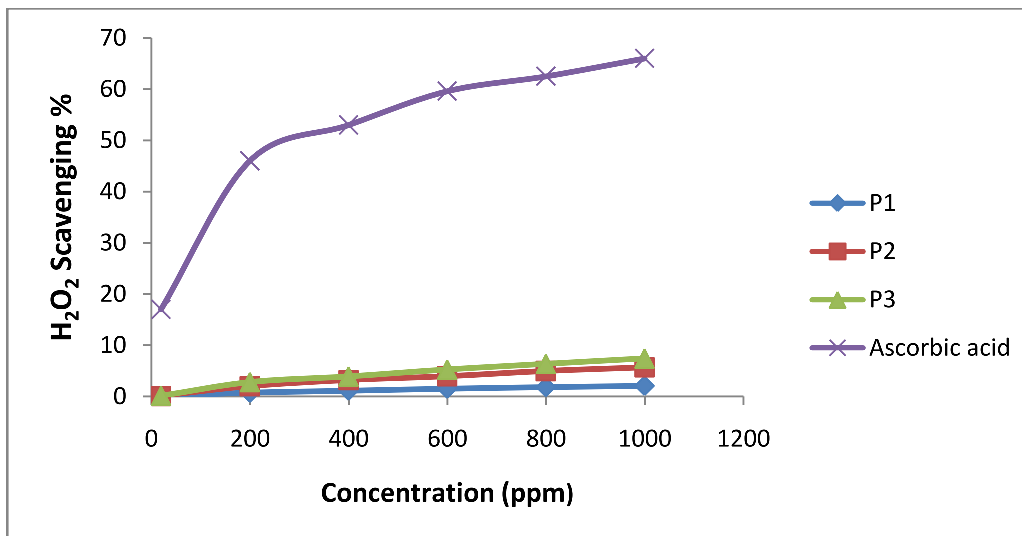


Figure 3.5: Comparison of H_2O_2 Scavenging activity of P_1, P_2, P_3 and Ascorbic acid

Table 3.1: IC_{50} value for H_2O_2 scavenging activity of tested coumarins and Ascorbic acid

Compound	H_2O_2 scavenging activity IC_{50} value (ppm)
Coumarin (P_1)	24902
4-hydroxy Coumarin (P_2)	9150
7-hydroxy Coumarin (P_3)	7029
Ascorbic acid	286

RRIJM 2015, All Rights Reserved

The mode action of the hydroxyl coumarin could be similar to Glutathione. Glutathione is a endogenous antioxidant found in our body that reduces H_2O_2 into H_2O by using its thiol(-SH) group. It donates hydrogen ($H^{\cdot}$) to reduce the H_2O_2. But insteadof thiol group, hydroxyl coumains(P_2 and P_3) have hydroxyl group (-OH) with similar function from which the hydrogen is donated for the reduction of H_2O_2. Two molecules of hydroxyl coumarin (P_2orP_3) reacts with one mole of H_2O_2 and producing 2 moles of respective phenoxy radical and 2 moles of water. The mechanism for this reduction of H_2O_2 is shown in Figure 3.6

2 Hydroxyl coumarin + H_2O_2 → 2 Coumarinicphenoxy radical + 2 H_2O

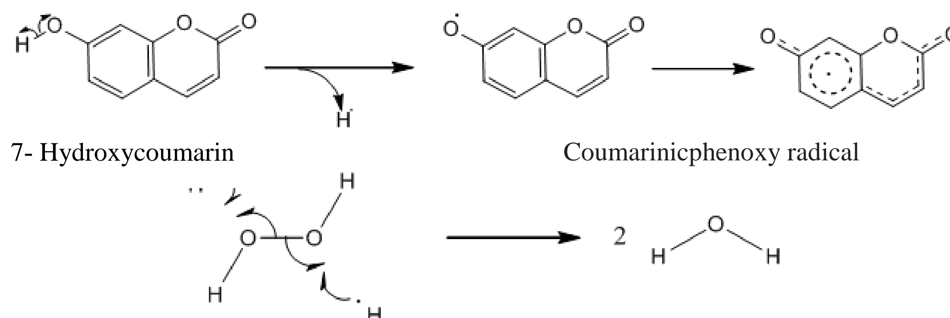


Figure 3.6:Reaction mechanism for the reduction of Hydrogen peroxide.

Among the hydroxyl coumarins, 7-hydroxy coumarin (P_3) showed higher activity than 4-hydroxy coumarin (P_2). It reveals that the compound having hydroxyl group at 7^{th}positionis more effective than the compound having hydroxyl group at the 4th position. This could be due to the extended resonance stability of the formed phenoxy radical of the P_3 than P_2. Formed phenoxy radical of P_3 due to the donation of "H" undergoes delocalization in the benzene moiety and also it is extended to the alpha pyrone ring (Figure 3.7).However, in the case of P_2 electron delocalizes within the alpha pyrone ring only (Figure3.8). The extended resonance of P_3phenoxy radical comprises more numbers of resonance structures. When the number of resonance structure of the phenoxy radical increases, the stability also increases. Thus formed phenoxy radical of P_3 is stabilized much than that of P_2.Therefore, the hydroxyl group at 7th position is effective than that of 4th position.

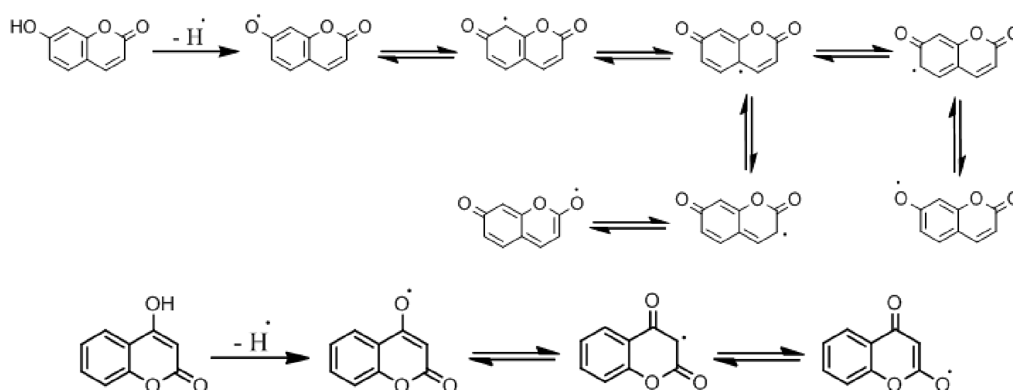
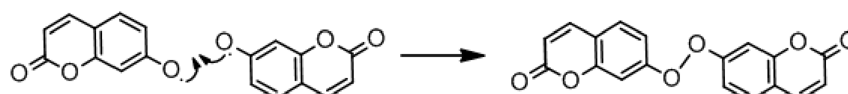


Figure 3.7: Resonance stability of phenoxy radical of 7- hydroxycoumarin (P_3)

The formed phenoxy radical (Figure 3.9 and 3.10) also could be stabilized by forming the dimer of the respective compound in both cases(P_2 and P_3) as like the cellular antioxidant-Glutathione stabilizes its formed radical (Figure 1.5). However, the possibility for the formation of dimer compound could be low, since the coumarins skeletal having the electron conjugating system and the formed phenoxy radical readily undergoes delocalization and gets stabilized. However, The H_2O_2 scavenging activity of the synthesized hydroxyl coumarins was poor when compared to that of standard ascorbic acid (Figure 3.5).



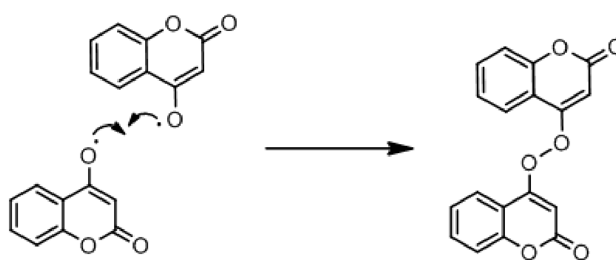


Figure 3.8: Resonance stability of phenoxy radical of 4-hydroxy coumarin (P_2)

4. Conclusion

Coumarins have H_2O_2 scavenging activity. Hydroxyl substitution on coumarin increases its H_2O_2scavenging activity. The compound having hydroxyl group at 7th position skeletal is more effective to scavenge the H_2O_2 than that of the 4th position. However, the H_2O_2 scavenging activity of 7-hydroxy coumarin and 4- hydroxyl coumarin is poor with compared to ascorbic acid.

References

1. Bhabak KP, Mugesh G. 2010. Functional mimics of glutathione peroxidase: bioinspired synthetic antioxidants. *Accounts of Chemical Research*.43(11): 1408–19. doi:10.1021/ar100059g
2. Borgstahl GE, Parge HE, Hickey MJ, Beyer WF, Hallewell RA, Tainer JA. 1992. The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles.*Pubmed*.71(1): 107–18. doi:10.1016/0092-8674(92)90270-M
3. Bruneton J. 1999. Pharmacognosy, Phytochemistry, Medicinal Plants.2nd Ed., Hampshire UK, Intercept Ltd, 263-277pp.
4. Cooke D. 1999.Studies on the Mode of Action of Coumarins (Coumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and Esculetin) at a Cellular Level.PhD Thesis, Dublin City University, Dublin, Ireland. DCU online research access service, http://doras.dcu.ie/18438/ (Accessed 2017.08.17)
5. Friedberg EC, Walker GC, Siede W. 1995.DNA Repair and Mutagenesis ASM Press, Washington, DC.
6. Han D, Williams E, Cadenas E. 2001. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembranespace.*The Biochemical Journal*.353 (2): 411–6. doi:10.1042/0264-6021:3530411
7. Harman D. 1992.Role of free radicals in aging and disease, Annals of the New York Academy of Sciences.673: 126–141.
8. Hayyan M, Hashim MA, AlNashef IM. 2016. Superoxide Ion: Generation and Chemical Implications. *Chemical Reviews*. 116 (5): 3029–85. doi:10.1021/acs.chemrev.5b00407.
9. Kar A. 2004. Advanced Practical Medicinal Chemistry, 1st Ed., New Age International Publishers, 184-205pp.
10. Li X, Fang P, Mai J, Choi ET, Wang H, Yang XF. 2013.Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. *Journal of Hematology & Oncology*.6 (19): 19. doi:10.1186/1756-8722-6-19.
11. Mark AS, Karl. 1949. United states patent office.Synthesis of 4-hydroxy coumarin serial no.456, 710.
12. Pechmann HV, Duisberg C. 1884. Synthesis of coumarins by condensation of phenols with beta keto ester in the presence of Lewis acid catalyst.*Chem. Ber*.17: 929.
13. Perkin WH. 1868. On the artificial production of coumarin and formation of its homologues, *Journal of Chemical society*.21: 53–63. doi:10.1039/js8682100053.
14. Ribeiro CV. Kaplan MA. 2002. Evolutionary tendency of coumarin-bearing families in angiospermae, *QuímicaNova*.25(4): 533-538.
15. Ruch RJ, Cheng SJ, Klaunig JE. 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea, *Carcinogeesis*.10: 1003-1008.
16. Turrens JF. 2003. Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*.552: 335–44. doi:10.1113/jphysiol.2003.049478.
17. Venugopala KN, Rashmi V. Odhav B.2013.Review on Natural Coumarin Lead Compounds for their Pharmacological Activity. *BioMed Research International*.1-14. DOI:10.1155/2013/963248.