Applied Chemistry Project

Project title Synthesis of hesperetin derivatives and their biological activities

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Program Bachelor of Science in Applied Chemistry

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Synthesis of hesperetin derivatives and their biological activities

by
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Saranras Thareechat

In Partial Fulfillment for the Degree of Bachelor of Science
Program in Applied Chemistry (International Program)
Department of Chemistry, Faculty of Science
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By Pakawat Sirilertpanich and Saranras Thareechat

Accepted by Department of Chemistry, Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science Program in Applied Chemistry (International Program)

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Date 30/11/2020

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Abstract

The flavonoids hesperetin and hesperidin contained in many citrus plants are claimed to provide several biological activities including anti-inflammatory, anti-hepatic fibrosis, anti-cancer and melanogenesis. They are also widely used in cosmetics industries as an active ingredient protecting skin against damages. In this research, hesperidin was isolated from dried orange peel while hesperetin was derived from hesperidin. The other six hesperetin derivatives were designed, synthesized and characterized by using NMR-spectroscopy. All compounds were evaluated for antioxidant activity using DPPH radical scavenging assay. **S11** displayed the most effective antioxidant activity which has the least IC_{50} among all synthesized compounds (IC_{50} = 0.19mM).

Acknowledgement

We would like to express appreciation to our advisor Assistant Professor Dr. Warinthorn Chavasiri for continuous support on our senior project for his patience, motivation and immense knowledge. His guidance helped us all the time of research and writing of the report.

Furthermore, we would give special thanks to all WC lab members at Chulalongkorn University for helping and supporting us in all situations, having a chance working and sharing experience with them is one of the most memorable periods.

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S01	5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2H-chromen-7-yl 6-O-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranoside
S02	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one
S03	Ethyl 2-Bromoacetate
S04	methyl2-((5-hydroxy-2-(4-methoxy-3-(2-methoxy-2-oxoethoxy)phenyl)-4-oxochroman-7-yl)oxy)acetate
S05	methyl2-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)acetate
S06	methyl(*E*)-4-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)but-2-enoate
S07	ethyl2-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)acetate
S08	ethyl 2-(5-(5,7-dihydroxy-4-oxochroman-2-yl)-2-methoxyphenoxy)acetate
S09	2-((5-hydroxy-2-(4-methoxy-3-(2-methoxy-2-oxoethyl)phenyl)-4-oxochroman-7-yl)oxy)acetic acid
S10	2-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)acetic acid
S11	(*E*)-4-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)but-2-enoic acid

Chapter 1

Introduction

1.1 Introduction to the research problem and significance

Nowadays, there are a variety of products emerging as multi-purposes skincare in the market. As the popularity of having a good skin trend is surging among Thai people, many brands are seeking advance and unique ingredients to compete in this competition. Certain skincare products contain flavonoids serving as antioxidant and anti-inflammatory agents which can be found mostly in citrus fruits like an orange which can be extracted from its peel. Now with modern technology and knowledge, many cosmetic companies have discovered the useful effect of hesperidin and became popular with many pieces of research that have focused on its performance toward its antioxidant properties in cosmetics or skincare products. Thus, certain researches widely used hesperetin derivatives for anti-inflammatory purposes. Moreover, these derivatives can act as an antioxidant and promote melanogenesis in the skin. Hesperetin and its useful derivatives can be obtained from the natural product source like orange peel. As many researches focus only on its anti-inflammatory properties. However, in this study, hesperidin, hesperetin and its derivatives will be extracted from orange peel to examine the properties of antioxidants activity by DPPH radical scavenging assay method. The results will be further analyzed to identify the relation between antioxidant activity and structure of each compound.

1.2 Research objectives

To extract hesperidin, prepare hesperetin, and design, synthesize and evaluate the most effective hesperetin derivatives based on their antioxidant activities.

1.3 Literature search

1.3.1 Flavonoids and cosmetic industries

Flavonoids or the other name "bioflavonoids" is the class name of polyphenolic secondary metabolites[1] derived from plants. Furthermore, flavonoids can be divided into subgroups according to the carbon on the C ring, the unsaturation degree and oxidation of the C ring (Figure 1.1). There are six subgroups of flavonoids including flavones, flavonols, isoflavones, flavanones and chalcones.

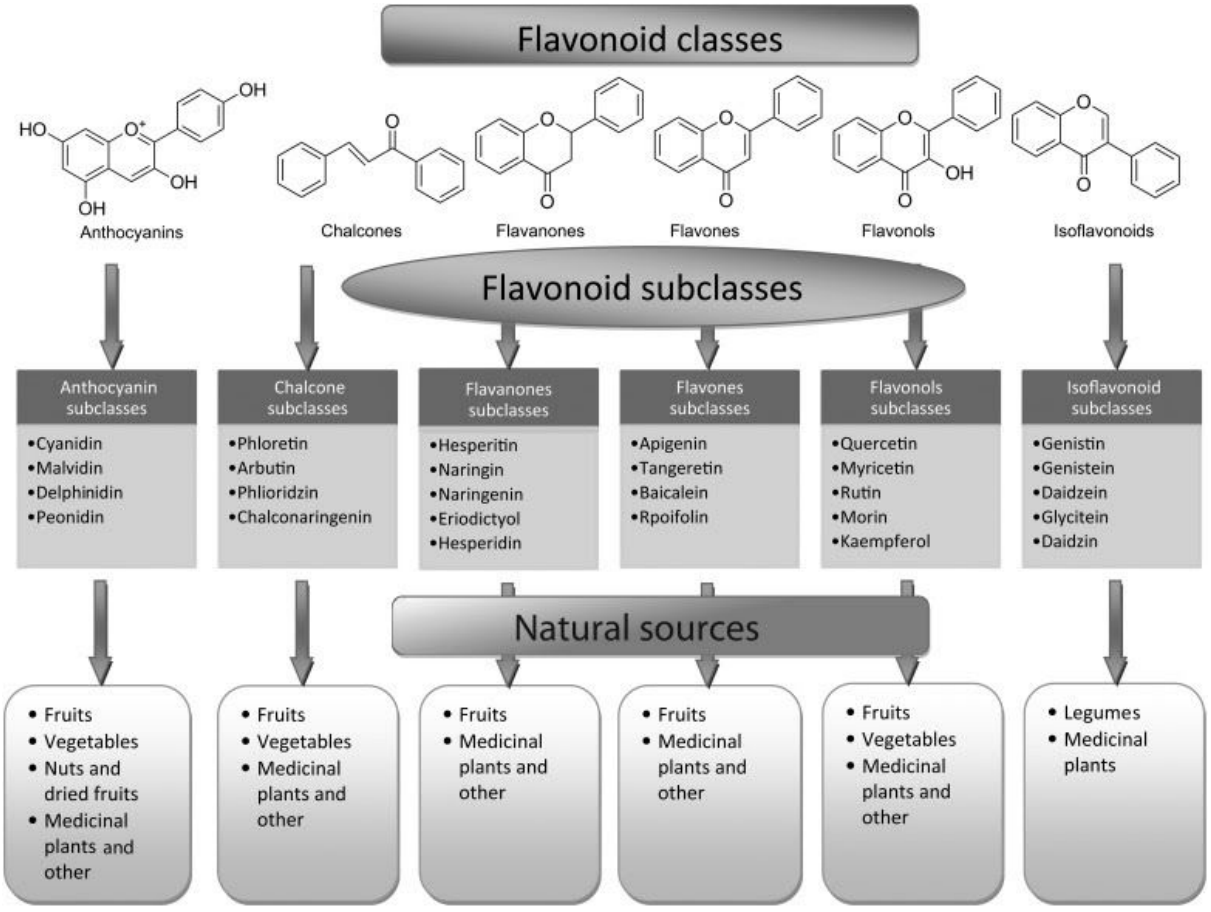
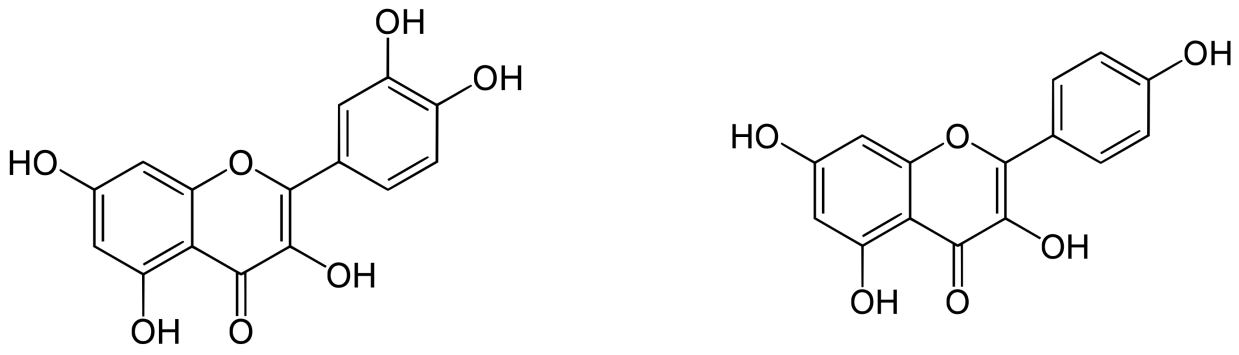


Figure 1.1 Lists of all flavonoid subclass[1]

Variety of flavonoids are formulated in present cosmeceuticals products. These flavonoids are well-known for their antioxidant and soothings action since they can strengthen the wall of blood vessels. Moreover, some flavonoids can act as antiallergic[1], anti-carcinogenic[1], anti-vira[1] and anti-inflammatory agents[1]. Many world's famous cosmetic brands like L'oreal Paris and Paula's Choice include flavonoids such as hesperidin in their products for protecting skin and maintaining the quality of the skin. Examples of the most widely used flavonoids are Quercetin[2] and Kaempferol[2] which can be provided from several fruits and vegetables (Figure 1.2).



Quercetin Kaempferol

Figure 1.2 Example of useful flavonoids

1.3.2 Hesperetin

Hesperetin is aglycone form of hesperidin (hesperetin-7-*O*-rutinoside) which is not stable, therefore it presents as a glycoside form (hesperidin) (Figure 1.3). Hesperidin is a flavonoid derived from the dried skin of citruses such as lemons, oranges, and grapefruit[13]. The human body can hydrolyze hesperidin into hesperetin in the gastrointestinal tract when ingested. Hesperetin is a lipid flavonoids molecule belonging to a group of 4'-*O*-methylated flavonoids due to the attachment between C4 atoms of flavonoid backbone and the methoxy group. Moreover, hesperetin is considered to be a trihydroxy flavanone containing three hydroxyl groups. Hesperetin

is insoluble in water but soluble in ethyl acetate and alkalis and relatively neutral. Thus, hesperetin is found in many biological fluids such as urine, blood, and feces. Hesperetin is widely used in the drugs industry as lowering cholesterol drugs due to its ability to affect lipids as it is a lipid flavonoids molecule.

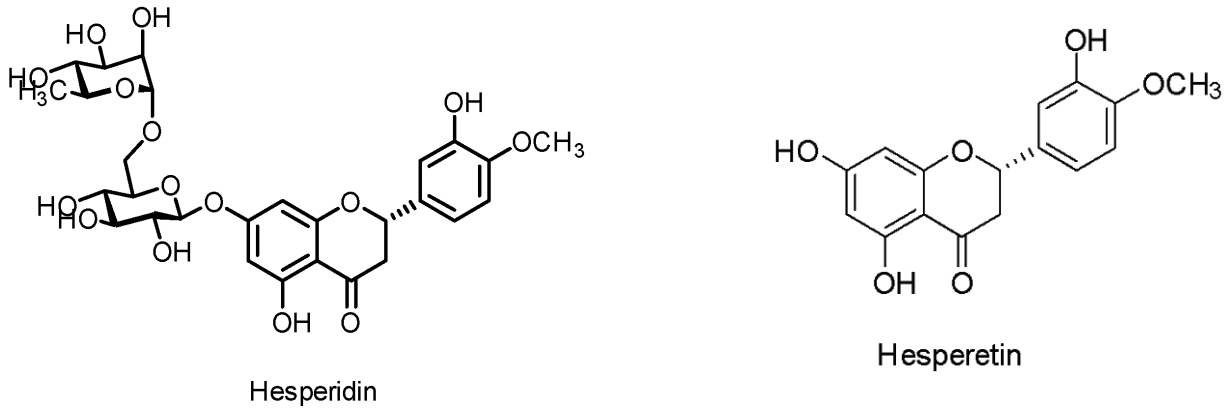


Figure 1.3 Hesperidin (left) and hesperetin (right)

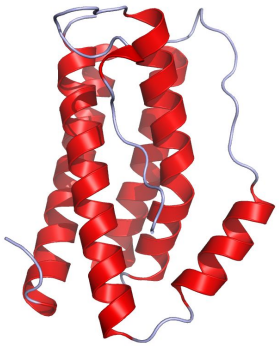
1.3.3 The biological properties of hesperidin, hesperetin and their derivatives

A. Antioxidant

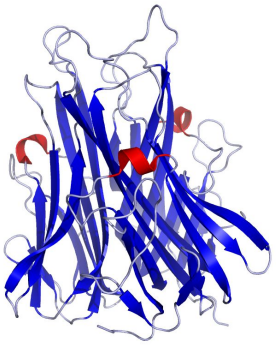
Both hesperetin and its derivatives have antioxidant properties. Hesperetin can extend its ability not only radical scavenging radical but also antioxidant against cellular damages *via* the signaling pathway called ERK/Nrf2[13]. Hesperidin was evaluated against H_2O_2 - induced membrane damage in red blood cells and the results showed that it can prevent H_2O_2 - induced oxidative against membrane damage of red blood cells. Despite this, the antioxidant ability of hesperidin was considered low when compared to activities of Ascorbic acid and trolox (vitamin E derivatives). Both hesperidin, hesperetin and their derivatives can protect proteins, DNA and tissue against internal (oncogenes) and external (inflammation and radiation) factors due to their ability to reduce tissue damages from many compounds such as nicotine, H_2O_2, CCl and various radiations.

B. Anti-inflammatory

The inflammatory process happens when the body detects injured or damaged tissue by any causes. There are mediators that release during the process including interleukin (IL-6), nitric oxide (NO) and tumor necrosis factor (TNF-α)[3] (Figure 1.4). Hesperetin derivatives have the ability to inhibit these mediators in order to stop the inflammation. Researches showed that these derivatives which were hesperetin-6-isopropylamine, hesperetin-6-tert-Butylamine and hesperetin-6-ethylpierazine can inhibit interleukin (IL-6) and tumor necrosis factor (TNF-α) in acute lung injury. The derivatives were synthesized from hesperetin through Mannich reaction. The reason why these derivatives have higher anti-inflammatory effects is due to their structures. Phenolic hydroxyl groups, the OH at position 7 of the derivatives resulted in better bioactivity which led to hypotension, vasodilatory and anti-inflammatory effect.



Interleukin (IL-6)



Tumor necrosis factor (TNF-α)

Figure 1.4 Mediators released during inflammatory process[14]

C. Anti-hepatic fibrosis

The hepatic fibrosis is a pathological feature that leads to the disease in the liver. The derivatives of hesperetin played an important role to inhibit the reaction that caused damage to the liver. These derivatives were obtained from Traditional Chinese Medicine and its monomer isolated from *Citrus aurantium L*. (Rutaceae).

Hesperetin derivatives prevented CCl_4 led to the reduction of liver injury. Furthermore, it helps in inhibiting the up-regulation of the liver fibrogenesis markers α-SMA, Col1α1, Col3α1 and TIMP-1[4] in primary hepatic stellate cells (Figure 1.5).

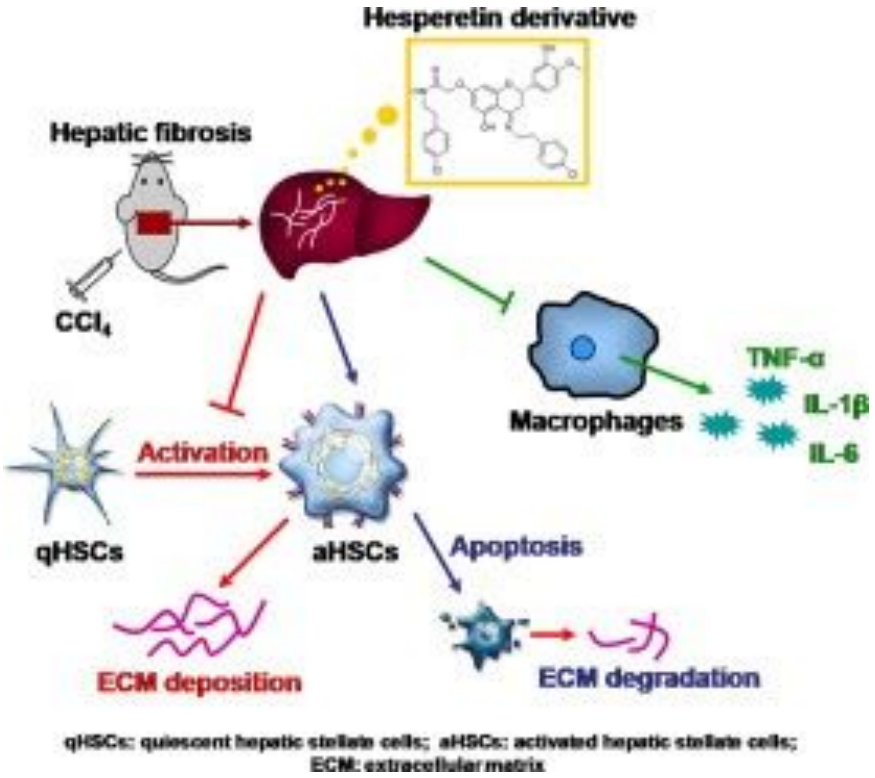


Figure 1.5 Mechanism of hesperetin derivatives as anti-hepatic fibrosis[4]

D. Anti-cancer

Cancer happens due to the malfunction of cell division, the cell starts to grow and divide uncontrollably and can be spread into other parts of the body. Mostly, cancer leads to tumor formation called malignant that can be removed. Cancer can be caused by many factors such as food and lifestyle (Figure 1.6) like smoking which contains carcinogenic agents[5]. The carcinogenic agents that enter the body, are converted by the metabolic process in the human body into reactive oxygen species (ROS)[6] and free radicals. The exuberance of these free radicals can trigger cancer and follow by many other diseases. Through the ability to inhibit glucose uptake in hesperetin, this lowers the cell viability and promotes apoptosis which exerts the anti-cancer activity. Moreover, hesperetin can promote the G1-phase cell cycle

(Figure 1.7) arrest[6] and accumulation of ROS. In lung cancer, hesperetin causes apoptosis in lung cancer cells by mitochondria-independent pathway[6]. Furthermore, in malignant tumour of liver (hepatoma HepG2 cells)[6] (Figure 1.8), hesperetin induces the gene expression of low-lipoprotein receptor (LDLr) by increasing phosphorylation of PI3K and ERK1/2[6] and results in cardioprotective effect. Hesperetin can stimulate the apoptosis of cancer cells by reducing the level of ROS.

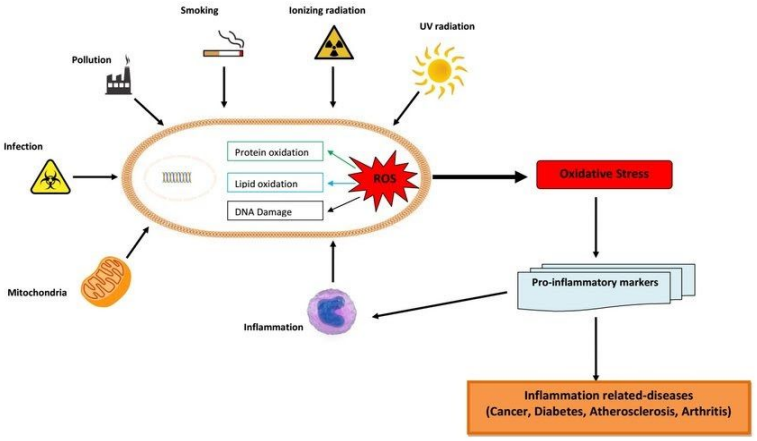


Figure 1.6 Factors causing carcinogenic agents which lead to ROS formation[6]

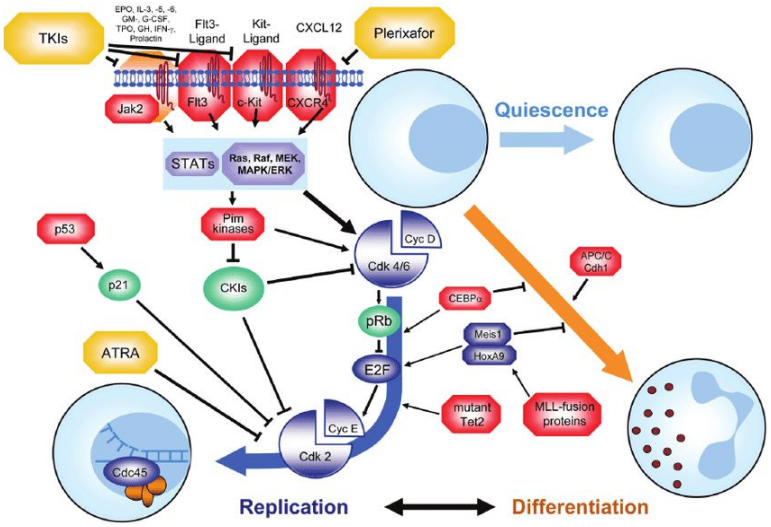


Figure 1.7 G_1 Phase Cell Cycle[13]

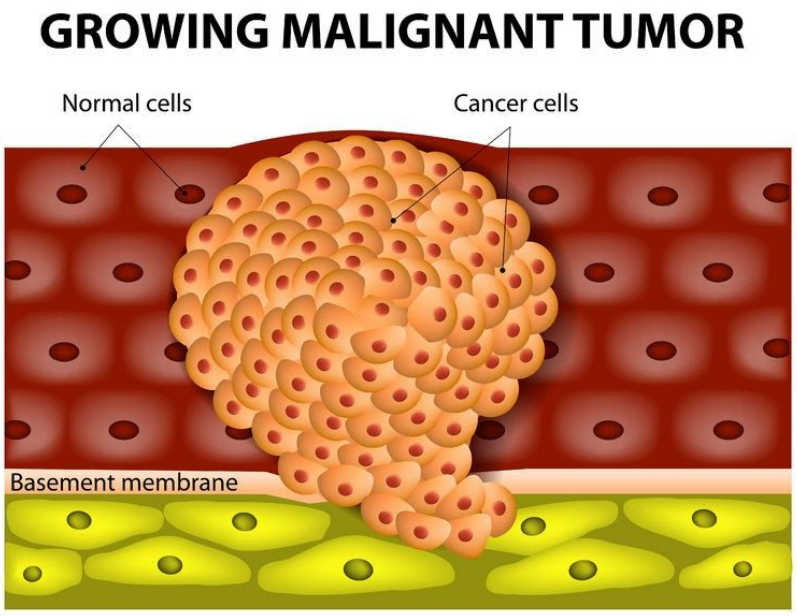


Figure 1.8 Malignant tumor[16]

E. Melanogenesis

Melanogenesis is the method of balancing skin pigmentation preventing photodamage effect by producing melanin pigments by melanocyte cells (Figure 1.9). When skin is exposed to UV light, UV induces tanning which is a defensive spontaneous process. Tanning process increases the melanin production (Figure 1.10) to protect the skin against skin UV-damaged. Researches show that hydrolyzed extracts from citrus including hesperetin have the ability to induce the protein called tyrosine and microphthalmia-associated transcription factor (MITF)[7] contributes to enhancing the synthesis activity of melanin. Furthermore, hesperetin activates phosphorylation of cAMP-responsive element binding protein (CREB), glycogen synthase kinase-3β (GSK3β) and mitogen-activated protein kinases (MAPKs) pathways[7] which helps increasing the melanin production process.

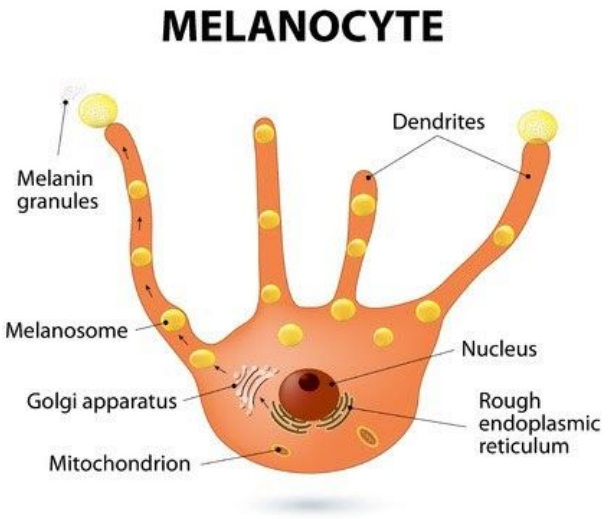


Figure 1.9 Melanocyte Cell[17]

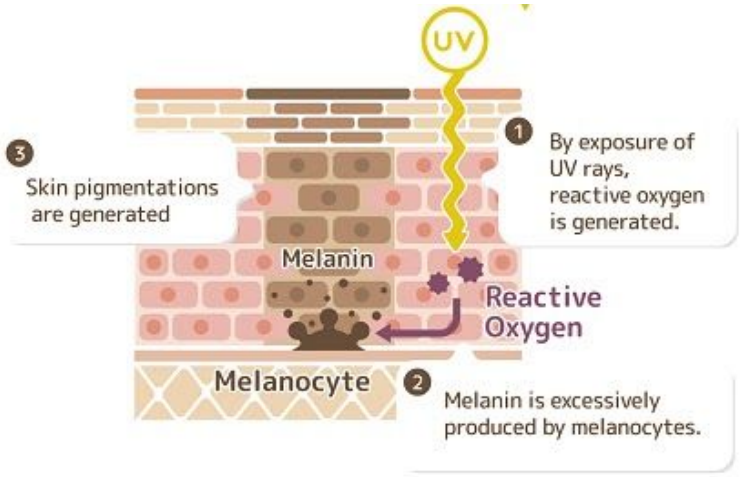


Figure 1.10 Steps of melanin production[18]

1.3.4 DPPH radical scavenging assay

This method is the most widely used for evaluation of antioxidant activity, first proposed by Blois. DPPH or 1,1'-diphenyl-2-pircrylhydrazyl[12] is one of the harmful oxidations causing damage to biological system but a stable radical due to delocalization of unwanted electrons over the molecule as a whole, therefore it is suitable to use antioxidant sensors.

When DPPH accepts an electron or hydrogen radical and becomes a stable diamagnetic molecule, the color will change from deep purple to yellow color (Figure 1.11) which can suggest that the substance has antioxidant activity.

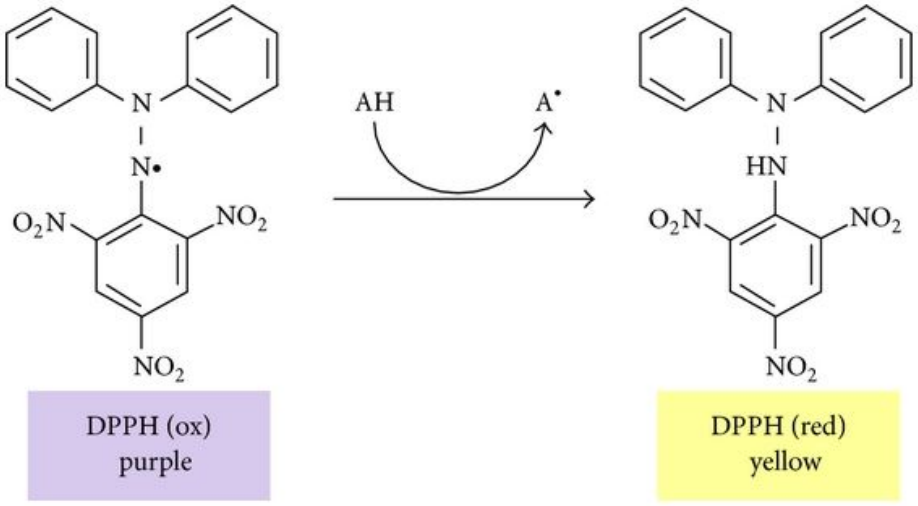


Figure 1.11 Mechanism of DPPH[15]

Chapter 2

Experimental

2.1 List of Equipment and Instrument

Rotary evaporator, round bottom flask of 50, 100, 250 and 500 mL, magnetic stirrer, beaker of 100 and 250 ml, TLC, spatula, Whatman filter paper, separatory funnel 250 and 500 mL, 2 neck round bottom flask of 50 and 100 mL, hot/stirring plate, magnetic stirrer bar, weighing scale, column for column chromatography of 250 mL, erlenmeyer flask of 50, 100, 125, 250, and 500 ml, Graduated cylinder of 10, 250, 500 ml and 1 L, glass funnel, dropper, beaker, tweezer, glass petri dish, pipette, stirring rod, clamp and stand, condenser, thermometer, oil/water/ice bath, Buchner funnel, Buchner flask, rubber sleeve, rubber tubing, vacuum filtration, 96-well plates, multichannel micropipette.

2.2 List of Chemicals and Material

Dry orange peel, methyl bromoacetate, ethyl 4-bromocrotonate, bromoacetic acid, potassium carbonate, potassium bicarbonate, sodium sulfate anhydrous, hydrochloric acid 98%, sulfuric acid 98%, sodium bicarbonate, dimethyl formamide, sodium chloride, sodium hydroxide, hexane, ethyl acetate, methanol, ethanol, dichloromethane, DI water, acetic acid, dimethyl sulfoxide, silica gel, DPPH (2,2-diphenyl-1-picrylhydrazyl), analytical grade methanol, ascorbic acid.

2.3 Extraction of Hesperidin (S01)

This is the process of obtaining hesperidin from orange peel before sugar reduction forming hesperetin. An Air-dried tangerine peel (orange peel) with an

amount of 500g was cut into small pieces, then extracted with 2 liters of water-methanol (30:70) under room temperature and maceration for 24 hours. The extract was filtered out with Whatman No.1 filter paper, then the solvent was removed by a rotary evaporator at 45°C under vacuum condition. The residue after evaporation was extracted three times with ethyl acetate (EtOAc) and water. The obtained EtOAc layer was further evaporated by a rotary evaporator at 45°C to obtain 150 mL of residue. The crude product was allowed to cool down to room temperature before checking TLC with solvent of hexane and EtOAc (2:1). The R_f value is 0.29. Hesperidin precipitated as a white brownish solid substance and filtered out by using filter paper and washed with acetone.

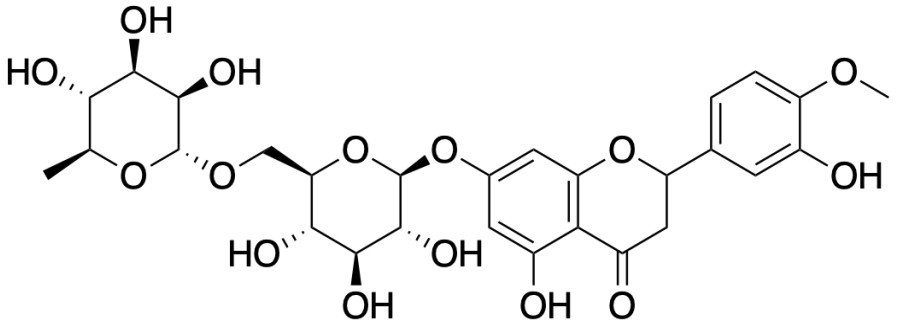


Figure 2.1 Structure of hesperidin (**S01**)

Hesperidin or 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2H-chromen-7-yl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside **^{1}H NMR** (500 MHz, DMSO-d_6): δ (ppm) 6.97 (1H, d, *J* = 2.0 Hz, H-2′), 6.88 (1H, dd, *J* = 8.0 Hz, H-5′), 6.83 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 6.14 (1H, d, *J* = 2.0 Hz, H-8), 6.13 (1H, d, *J* = 2.0 Hz, H-6), 5.50 (1H, dd, *J* = 11.0, 5.0 Hz, H-2), 4.97 (1H, d, *J* = 7.2 Hz, H-1′′), 4.54 (1H, br s, H-1), 3.78 (3H, s, 4-OCH_3),3.20–3.60 (6H, m, H-2′′ to H-6′′), 3.20–3.60 (3H, m, H-2 to H-6), 3.11 (1H, dd, *J* = 17.0, 11.0 Hz, H-3a), 2.78 (1H,dd, *J* = 17.0, 5.0 Hz, H-3b), 2.51 (1H, d, *J* = 6.0 Hz, H-5), 1.09 (3H, d, *J* = 6.0 Hz, H-6). **^{13}C NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.7, 166.3, 163.0, 162.8, 148.0,146.4, 131.9, 118.0, 114.3,

112.4, 100.6, 99.4, 96.0, 95.2, 78.4, 76.2, 75.5, 73.0, 72.1, 70.7, 69.4, 68.8, 66.7, 55.7, 42.2, 17.8.

2.4 Synthesis of hesperetin (S02)

The solution of 10 mL of 99% H_2SO_4 and 280 mL of methanol was added to 3.5 g of hesperidin, then the mixed solution was stirred at 75°C for 6 hours with reflux reaction. The mixture was allowed to cool down to room temperature before the extraction. The mixture was extracted 3 times, the first time the 100 mL of water was added together with 100 mL of EtOAc. The rest of the steps required only EtOAc for the extraction. The organic layer was obtained and evaporated by a rotary evaporator (*in vacuo*). The residue was dissolved with 70 mL of acetone and 1% of 700 mL of hot AcOH aqueous solution, the mixture stirred at 70°C for 1.5 hours. Then the product was cooled before filtration and dried. The product was tested TLC with solvent of hexane and EtOAc (2:1). The R_f value is 0.33.

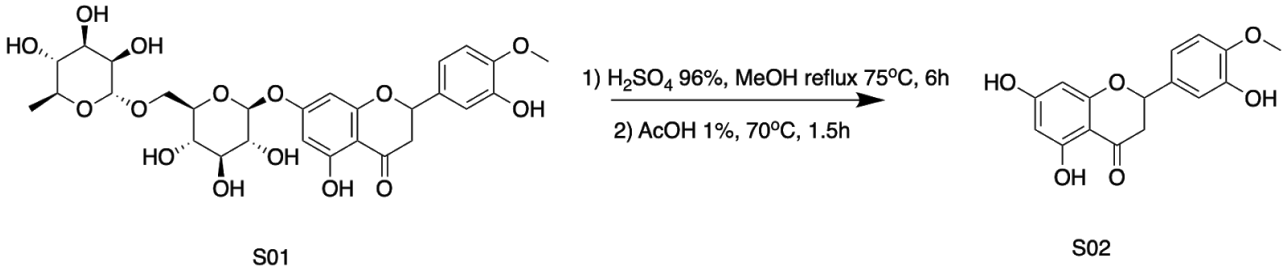


Figure 2.2 Synthesis reaction of hesperetin (**S02**)

Hesperetin or 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one (97% yield): **^{1}H-NMR** (500 MHz, DMSO-d_6): δ (ppm) 12.13 (1H, s, 5-OH), 10.82 (1H,s, 7-OH), 6.93-6.92 (2H, m, H-2', H-5), 6.86 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 5.89 (1H, d, *J* = 2.5 Hz, H-8), 5.88 (1H, d, *J* = 2.5 Hz, H-6), 5.43 (1H, dd, *J* = 12.0, 3.0 Hz, H-2), 3.77 (3H, s, 4'-OCH_3), 3.19 (1H, dd, *J* = 17.5, 12.5 Hz, H-3-trans), 2.70 (1H, dd, *J* = 17.5, 3.0 Hz, H-3-cis). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 196.3 (C4), 166.7 (C7), 163.5 (C5),

162.8 (C9), 147.9 (C4'), 146.5 (C5'), 131.2 (C1'), 117.7 (C6'), 114.1 (C2'), 112.0 (C3'), 101.8 (C10), 95.8 (C6), 95.0 (C8), 78.3 (C2), 55.7 (OCH_3), 42.1 (C3). **^{13}C NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.2, 166.7, 163.5, 162.8, 147.9, 146.5, 131.2, 117.7, 114.1, 112.0, 101.8, 95.8, 95.0, 78.2, 55.7, 39.6.

2.5 Synthesis of Ethyl Bromoacetate (S03)

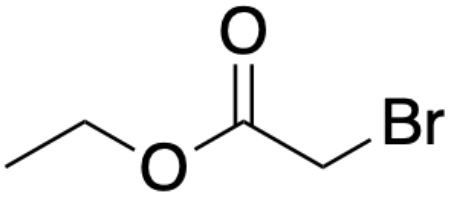


Figure 2.3 Structure of ethyl bromoacetate (**S03**)

To the round bottom flask fitted with a reflux condenser and magnetic stirrer bar 7.01 g of bromoacetic acid, 3.8 mL of 95% EtOH and 0.353 mL of 98% H_2SO_4 are added. The reaction flask was heated at 100° C and reflux was continued for 5-6 hours. At the end of the reaction the flask was allowed to cool at room temperature and then poured into a separatory funnel containing cold water. The ethyl Bromoacetate layer was separated and washed several times with water, $NaHCO_3$ until no more gas and dried with Na_2SO_4. Ethyl bromoacetate was obtained as colorless liquid.

Ethyl bromoacetate (25% yield). **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ (ppm) 4.22 (2H, q, J = 7.0 Hz, OCH_2), 3.82 (2H, s, CH_2Br), 1.29 (3H, t, J = 7.0 Hz, CH_3). **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ (ppm) 167.4 (C=O), 62.5 (OCH_2), 26.1 (CH_2Br), 14.1 (CH_3).

2.6 Synthesis of hesperetin derivatives

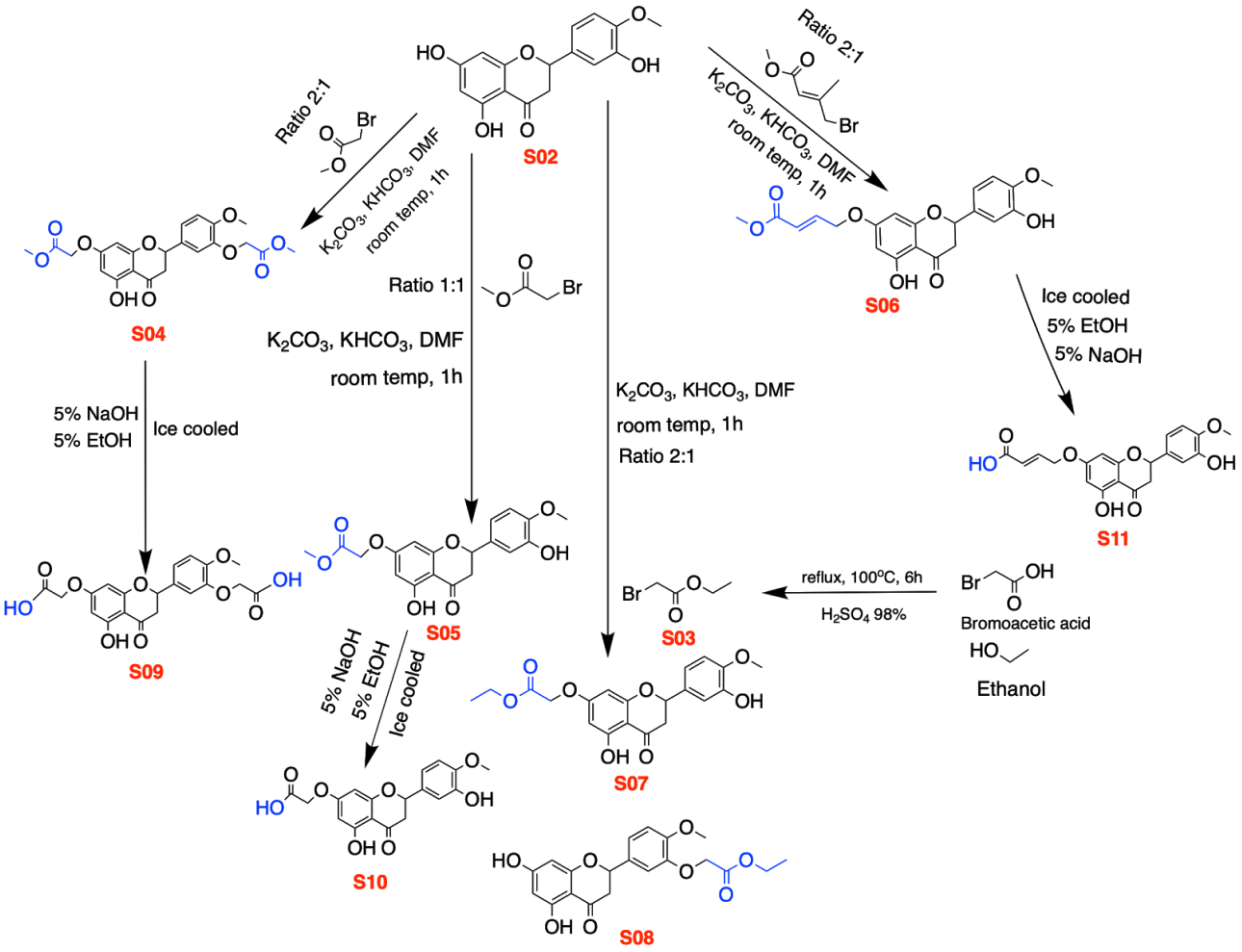


Figure 2.4 Flow Chart indicate research plan

2.6.1 The 7-O-(2-4'-methoxy-2-oxoethyl)hesperetin (S04)

The attachment of methyl bromoacetate required 1.5g pure powder of hesperetin with 1.5g of $KHCO_3$ and 1g of K_2CO_3 dissolved in 20 mL of DMF stirring at room temperature for 30 minutes. Followed by adding methyl bromoacetate (Provided by Dr. Warinthorn Chavasiri) of 1,183 microliter using a micropipette and continuing stirring for 1 hour. The reaction was compared with hesperetin and monitored by TLC with solvent hexane and EtOAc in the ratio of 3:2 respectively. The R_f value is 0.36. The reaction was acidified with dilute HCl adjusting the pH to reach around 4-5 and extracted three times with EtOAc and DI Water. The organic phase was extracted again with saturated NaCl solution and the remaining water was

removed by anhydrous Na_2SO_4, filtered and evaporated by a rotary evaporator. The residue was recrystallized by heating the product with CH_2Cl_2 and ethanol in a ratio of 1:2 respectively. The crude product of white crystalline powder was obtained.

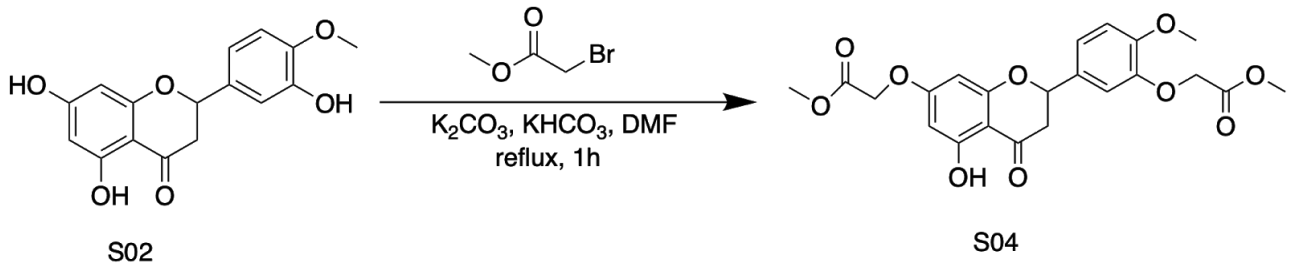


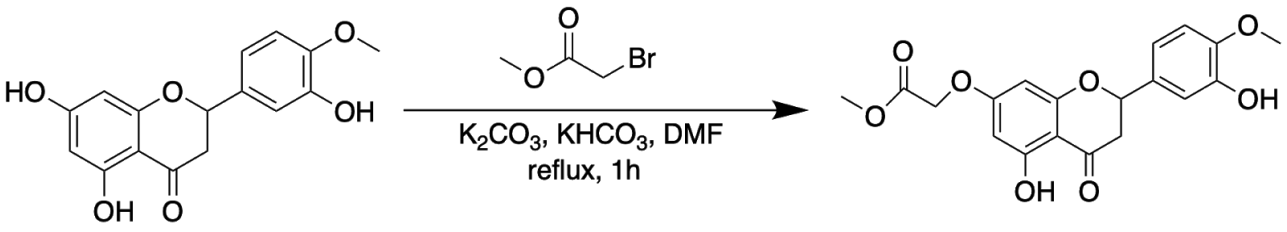
Figure 2.5 Synthesis pathway to form 7-O-(2-4'-methoxy-2-oxoethyl)hesperetin (**S04**)

Methyl 2-((5-hydroxy-2-(4-methoxy-3-(2-methoxy-2-oxoethoxy)phenyl)-4-oxo-chroman-7-yl)oxy) acetate (30% yield). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ (ppm) 11.97 (1H, s, 5-OH), 7.04 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.95 (1H, d, *J* = 2.0 Hz, H-2'), 6.93 (1H, d, *J* = 8.0 Hz, H-5'), 6.04 (1H, d, *J* = 2.0 Hz, H-6), 6.03 (1H, d, *J* = 2.0 Hz, H-8), 5.33 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 4.72 (2H, s, OCH_2), 4.63 (2H, s, OCH_2), 3.90 (3H, s, $COOCH_3$), 3.80 (3H, s, $COOCH_3$), 3.80 (3H, s, 4'-OCH_3), 3.05 (1H, dd, *J* = 17.5, 13.0 Hz, H-3), 2.78 (1H, dd, *J* = 17.5, 3.0 Hz, H-3). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.1 (C4), 169.4 (COO), 168.4 (COO), 165.9 (C7), 164.2 (C5), 162.9 (C9), 150.4 (C4'), 147.5 (C3'), 130.7 (C1'), 120.8 (C6'), 113.0 (C2'), 112.2 (C5'), 103.8 (C10), 95.8 (C6), 94.8 (C8), 79.0 (C2), 66.7 (OCH_2), 65.0 (OCH_2), 56.2 (OCH_3), 52.6 (OCH_3), 52.4 (OCH_3), 42.3 (C3).

2.6.2 The 7-O-(2-methoxy-2-oxoethyl)hesperetin (S05)

The 1.5 g pure powder of hesperetin with 1.5g of $KHCO_3$ and 1g of K_2CO_3 dissolved in 20 mL of DMF stirring at room temperature for 30 minutes. Followed by adding 591 microliter of Methyl bromoacetate (Provided by Dr. Warinthorn Chavasiri) using a micropipette and continuing stirr for 1 hour. The reaction was compared with

starting material and monitored by TLC with solvent hexane and EtOAc in the ratio of 3:2 respectively. The R_f value is 0.36. The reaction was acidified with dilute HCl to adjust the pH around 4-5 and extracted three times with EtOAc and DI Water. The organic phase was extracted again with NaCl and removed the remaining water using anhydrous Na_2SO_4 filtered and evaporated by a rotary evaporator. The residue was recrystallized by heating the product with solvent CH_2Cl_2 and ethanol in a ratio of 1:2 respectively. The crude product of white crystalline powder was obtained.



S02 S05

Figure 2.6 Synthesis pathway to obtain The 7-O-(2-methoxy-2-oxoethyl)hesperetin (**S05**)

Methyl 2-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy) acetate(72% yield).**^{1}H-NMR** (500 MHz, DMSO-d_6): δ (ppm) 12.06 (1H, s, 5-OH), 9.12 (1H, s, 3'-OH), 7.09 (1H, d, *J* = 2.0 Hz, H-2'), 6.93 (1H, m, H-5'), 6.88 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.10 (1H, d, *J* = 2.5 Hz, H-8), 6.08 (1H, d, *J* = 2.5 Hz, H-6), 5.48 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 4.88 (2H, s, OCH_2), 3.77 (3H, s, $COOCH_3$), 3.69 (3H, s, OCH_3), 3.39 (1H, dd, *J* = 17.0, 12.5 Hz, H-3), 2.75 (1H, m, H-3). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) 197.0 (C4), 168.6 (COO), 165.6 (C7), 163.1 (C5), 162.8 (C9), 148.0 (C4'), 146.5 (C3'), 130.9 (C1'), 117.8 (C6'), 114.2 (C2'), 112.0 (C5'), 103.0 (C10), 95.2 (C6), 94.3 (C8), 78.6 (C2), 64.7 (OCH_2), 55.7 (OCH_3), 52.0 (OCH_3), 42.2 (C3).

2.6.3 The 7-O-(4-butenoate) hesperetin (S06)

S02, the attachment of ethyl 4-bromocrotonate required 1.5g of pure hesperetin with 1g of K_2CO_3 and 1.5g of $KHCO_3$ dissolve in 20 mL of DMF. The solution was stirred at room temperature for 30 minutes. Then 1467 microliter of methyl 4-bromocrotonate was added and continued stirring for 1 hour. The reaction was compared with starting material and monitored by TLC with solvent hexane and EtOAc in the ratio of 2:1 respectively. The R_f value is 0.38. The reaction was extracted three times with EtOAc and DI Water. The remaining water was removed by using anhydrous Na_2SO_4 filtered and evaporated by a rotary evaporator. The residue was further purified by silica gel column chromatography using hexane: EtOAc (5:2) to yield the desired product as white crude.

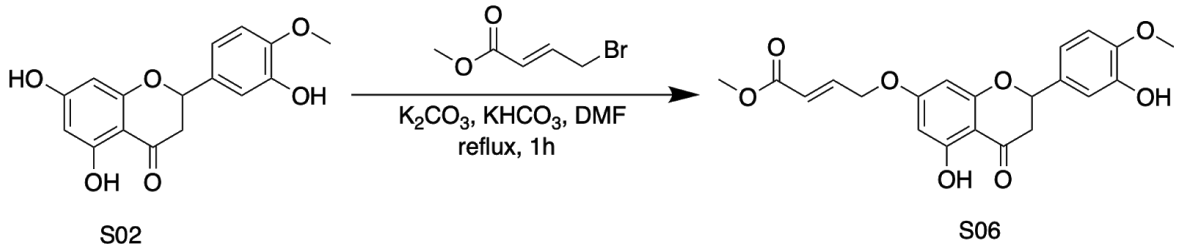


Figure 2.7 Synthesis pathway to obtain 7-O-(4-butenoate)hesperetin (**S06**)

Methyl(*E*)-4-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)but-2-enoate (60% yield). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ (ppm) 12.00 (1H, s, 5-OH), 7.04-6.99 (2H, m, H-2', C=OC=CH), 6.92 (1H, dd, *J* = 8.5, 2.5 Hz, H-6'), 6.88 (1H, d, *J* = 8.0 Hz, H-5'), 6.14 (1H, dt, *J* = 15.5, 2.0 Hz), 6.05 (1H, d, *J* = 2.0 Hz, H-6), 6.03 (1H, d, *J*= 2.5 Hz, H-8), 5.32 (1H, dd, *J* = 13.0, 3.0 Hz, H-2), 4.69 (2H, m, CH_2O), 3.91 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 3.08 (1H, dd, *J* =17.0, 13.0 Hz, H-3), 2.78 (1H, dd, *J*= 17.0, 3.0 Hz, H-3). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ (ppm) 196.2 (C4), 166.4 (COO), 166.2 (C7), 164.2 (C5), 163.1 (C9), 147.2 (C4'), 146.1 (C3'), 141.4 (CH=C-COO), 131.5 (C1'), 122.2

(C6'), 118.3 (C=CH-COO), 112.8 (C2'), 110.8 (C5'), 103.6 (C10), 95.8 (C6), 94.8 (C8), 79.1 (C2), 66.7 (OCH_2), 56.2 (OCH_3), 51.9 ($COCH_3$), 43.3 (C3).

2.6.4 The 7-O-(2-ethoxy-2-oxoethyl)hesperetin (S07)

To synthesize (7-O-(2-ethoxy-2-oxoethyl)hesperetin, 1.5g pure powder of hesperetin with 1.5g of $KHCO_3$ and 1g of K_2CO_3 dissolved in 20 mL of DMF stirring at room temperature for 30 minutes. Followed by adding lab synthesized ethyl bromoacetate of 1045 microliter using a micropipette and continue stirring for 1 hour. The reaction was compared with hesperidin and monitored by TLC with solvent hexane and EtOAc in the ratio of 3:2. The R_f values are 0.33 and 0.35 as it contains two compounds. The reaction was acidified with dilute HCl to adjust the pH around 4-5 and extracted three times with EtOAc and DI Water. The organic phase was extracted again with saturated NaCl solution and removed the remaining water using anhydrous Na_2SO_4 filtered and evaporated by a rotary evaporator. The residue was recrystallized by heating the product with solvent CH_2Cl_2 and ethanol in a ratio of 1:2 respectively and waiting until all the product dissolves in the solvent. The crude product of white crystalline powder was obtained. The product synthesized were obtained as 2 product name listed as **S07** and **S08.**

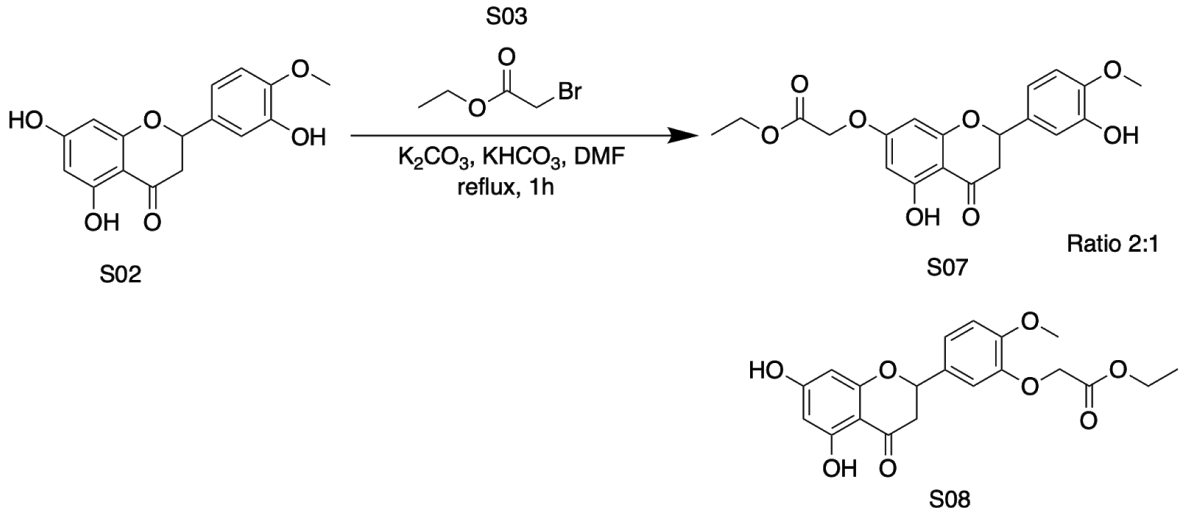


Figure 2.8 Synthesis pathway of 7-O-(2-ethoxy-2-oxoethyl)hesperetin (**S07**, **S08**)

(S07)ethyl2-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)acetate (52.3% yield). **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ (ppm) 11.98 (1H, s, 5-OH), 7.03 (1H, d, *J* = 2.0 Hz, H-2'), 6.96 - 6.92 (2H, m, H-5', H-6'), 6.03 (2H, m, H-6, H-8), 5.32 - 5.31 (1H, m, H-2), 4.61 (2H, s, OCH_2), 4.26 (2H, q, *J* = 7.0 Hz, OCH_2CH_3), 3.91 (3H, s, OCH_3), 3.10 – 3.07 (1H, m, H-3), 2.80 – 2.76 (1H, m, H-3), 1.31 – 1.29 (3H, m, CH_2CH_3). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ (ppm) 196.3 (C4), 168.0 (COO), 166.0 (C7), 164.2 (C5), 163.0 (C9), 150.4 (C4'), 146.1 (C3'), 131.6 (C1'), 118.3 (C6'), 112.8 (C2'), 110.8 (C5'), 103.8 (C10), 95.7 (C6), 94.0 (C8), 79.1 (C2), 65.1 (OCH_2), 61.8 (OCH_2CH_3), 56.2 (OCH_3), 43.3 (C3).

(S08)ethyl2-(5-(5,7-dihydroxy-4-oxochroman-2-yl)-2-methoxyphenoxy)acetate (26.7% yield). **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ (ppm) 11.96 (1H, s, 5-OH), 7.05 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 - 6.87 (2H, m, H-5', H-6'), 6.03 (2H, m, H-6, H-8), 5.34 - 5.33 (1H, m, H-2), 4.61 (2H, s, OCH_2), 4.26 (2H, q, *J* = 7.0 Hz, OCH_2CH_3), 3.90 (3H, s, OCH_3), 3.06 – 3.02 (1H, m, H-3), 2.80 – 2.76 (1H, m, H-3), 1.31 – 1.29 (3H, m, CH_2CH_3). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ (ppm) 196.1 (C4), 168.9 (COO), 166.0 (C7), 164.2 (C5), 162.9 (C9), 147.6 (C4'), 147.2 (C3'), 130.7 (C1'), 120.7 (C6'), 113.0 (C2'), 112.2 (C5'), 103.8 (C10), 95.7 (C6), 94.0 (C8), 79.0 (C2), 66.8 (OCH_2), 61.5 (OCH_2CH_3), 56.2 (OCH_3), 43.3 (C3).

2.7 Hydrolysis of hesperetin Derivatives

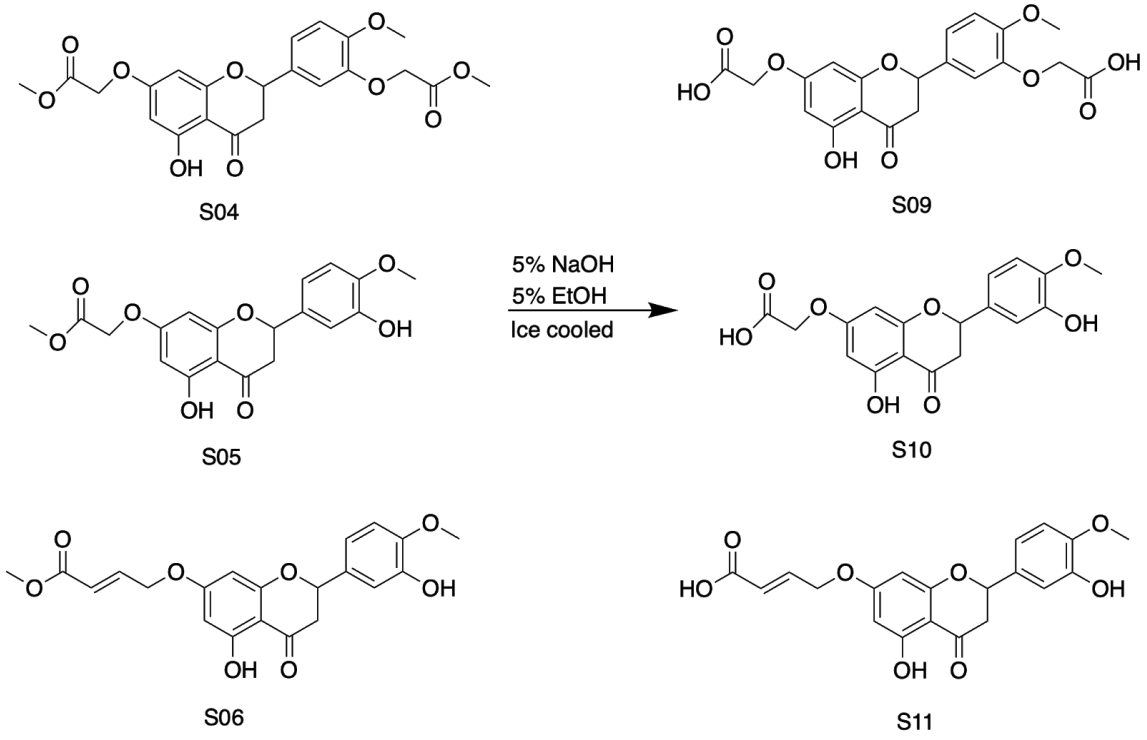


Figure 2.9 Synthesis pathway to obtain S09, S10, and S11

Each of the Hesperetin derivatives (**S04**-**S06**) was mixed with 5 mL 10% ethanol and stirred in an ice bath for 30 minutes. Followed by cooled 10% NaOH of 5 mL was dropped to the reaction and vigorously stirred for 30 minutes under cooling condition. The reaction was acidified with dilute HCl to pH 3-4 at the same temperature. The precipitate was filtered and washed with dilute HCl.

(S09)2-((2-(3-(carboxymethoxy)-4-methoxyphenyl)-5-hydroxy-4-oxochroman-7-yl) oxy)acetic acid. **^{1}H-NMR** (500 MHz, DMSO-d_6): δ (ppm) 7.08 (2H, m, H-2', H-6'), 7.04 (1H, dd, J = 8.0 Hz, H-5'), 6.08 (1H, d, J = 2.5 Hz, H-6), 6.06 (1H, d, J = 2.0 Hz, H-8), 5.52 (1H, dd, J = 13.0, 3.0 Hz, H-2), 4.75 (2H, s, OCH_2), 4.67 (2H, s, OCH_2), 3.79 (3H, s, 4'-OCH_3), 3.34 (1H, dd, J = 17.5, 13.0 Hz, H-3), 2.74 (1H, dd, J = 17.0, 3.0 Hz, H-3). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.1 (C4), 169.4 (COO), 168.4 (COO), 165.9 (C7), 164.2 (C5), 162.9 (C9), 150.4 (C4'), 147.5 (C3'), 130.7 (C1'), 120.8 (C6'), 112.2 (C2'), 112.2 (C5'), 102.9 (C10), 95.3 (C6), 94.3 (C8), 78.6 (C2), 65.1 (OCH_2), 64.8 (OCH_2), 55.7 (OCH_3), 42.2 (C3).

(S10)(*E*)-4-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)but-2-enoic acid (75% yield). **^{1}H-NMR** (500 MHz, DMSO-d_6): δ (ppm) 12.08 (1H, s, 5-OH), 6.94-6.85 (4H, m, 2′-H, 5′-H, 6′-H, C=OC=CH), 6.15 (1H, d, *J* = 2.5 Hz, H-8), 6.13 (1H, d, *J*= 2.5 Hz, H-6), 5.98 (1H, dt, *J* = 15.0, 2.0 Hz, C=OCH=C), 5.49 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 4.85 (2H, dd, *J* =4.1, 1.7 Hz, CH_2O), 3.78 (3H, s, OCH_3), 3.27 (1H, dd, *J* =17.2, 12.5 Hz, H-3), 2.75 (1H, dd, *J*= 17.1, 3.1 Hz, H-3). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.9 (C4), 166.6 (COOH), 165.8 (C7), 163.2 (C5), 162.8 (C9), 148.0 (C4'), 146.6 (C3'), 142.1 (CH=C-COOH), 130.9 (C1'), 122.4 (C6'), 117.8 (C=CH-COOH), 114.2 (C2'), 112.0 (C5'), 102.9 (C10), 95.3 (C6), 94.4 (C8), 78.5 (C2), 66.6 (OCH_2), 55.7 ($COCH_3$), 42.2 (C3).

(S11)(*E*)-4-((5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl)oxy)but-2-enoic acid (49.7 % yield). **^{1}H-NMR** (500 MHz, DMSO-d_6): δ (ppm) 12.08 (1H, s, 5-OH), 6.94-6.85 (4H, m, 2′-H, 5′-H, 6′-H, C=OC=CH), 6.15 (1H, d, *J* = 2.5 Hz, H-8), 6.13 (1H, d, *J*= 2.5 Hz, H-6), 5.98 (1H, dt, *J* = 15.0, 2.0 Hz, C=OCH=C), 5.49 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 4.85 (2H, dd, *J* =4.1, 1.7 Hz, CH_2O), 3.78 (3H, s, OCH_3), 3.27 (1H, dd, *J* =17.2, 12.5 Hz, H-3), 2.75 (1H, dd, *J*= 17.1, 3.1 Hz, H-3). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ (ppm) 196.9 (C4), 166.6 (COOH), 165.8 (C7), 163.2 (C5), 162.8 (C9), 148.0 (C4'), 146.6 (C3'), 142.1 (CH=C-COOH), 130.9 (C1'), 122.4 (C6'), 117.8 (C=CH-COOH), 114.2 (C2'), 112.0 (C5'), 102.9 (C10), 95.3 (C6), 94.4 (C8), 78.5 (C2), 66.6 (OCH_2), 55.7 ($COCH_3$), 42.2 (C3).

2.8 Antioxidant activity evaluation

Sample stocks of **S01**, **S02**, **S04**-**S06**, **S09**-**S11** are prepared each 3 mM and in 50 microliter analytical grade MeOH and 50 microliter DMSO. Stock of DPPH was prepared by weighing 2 mg DPPH dissolve in 40 mL analytical grade MeOH. Followed by adding all blank, negative control, blank samples as instructed in **Table 2.1.**

Table 2.1 Direction guideline for testing antioxidant in 96-well plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	BK
B	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	BK
C	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	BK
D	BS	BS	BS	BS	BS	BS	BS	BS	BS	BS	BS	BK
E	BS	BS	BS	BS	BS	BS	BS	BS	BS	BS	BS	NC
F	SD	SD	SD	SD	SD	SD	PS	PS	PS	PS	PS	NC
G	SD	SD	SD	SD	SD	SD	PS	PS	PS	PS	PS	NC
H	SD	SD	SD	SD	SD	SD	PS	PS	PS	PS	PS	NC

BK: Blank (150 microliter MeOH)

NC : Negative control (100 microliter of 0.05mg / ml DPPH + 50 microliter MeOH)

BS : Blank Sample (50 microliter of sample + 100 microliter MeOH)

SD : Sample DPPH (50 microliter of sample + 100 microliter of 0.05mg / ml DPPH)

PS : Positive control (50 microliter of ascorbic acid+100 microliter of 0.05mg / ml DPPH)

For sample DPPH (SD) insert 100 microliter of 3 mM sample into column of well No.1 and No.7 followed by insert 50 microliter of MeOH into column of well No.2 to 6 and No.7 to 11. The variation of concentration 1, 0.5, 0.25, 0.125. 0.0625 from each row were done by dilution using multichannel micropipette. From this 50 microliter from column of well No.1 and No.7 were transferred into columns of well No.2 and 8. Mixed the solution by using multichannel micropipette and continued the

process to column of well No.6 and 11 respectively. For PS the Ascorbic acid of 12 millimolar was prepared in 1mL MeOH and dilute to 300 micromolar in 1mL MeOH and the same procedure has been done with the test sample. The free radical scavenging activity of each sample was measured from the reduction of DPPH radical using different concentrations of samples in methanol. The plate was incubated at room temperature for 30 minutes in the dark and then the absorbance was measured at 520 nm using a microplate reader. Ascorbic acid was used as a standard reference. Percentage of DPPH inhibition can be calculated from the following formula and half-maximal inhibitory concentration (IC_{50}) at 50% was determined for each sample.

$$\mathrm{SA}_{\mathrm{DPPH}}.\left(\%\right)=\frac{A_{\mathrm{control}}-A_{\mathrm{sample}}}{A_{\mathrm{control}}}\times 100\%$$

Figure 2.10 Formula for calculating scavenger effect

SA_{DPPH} = Scavenging effect of DPPH

$A_{control}$ = Absorbance of control

A_{sample} = Absorbance of sample

Chapter 3

Results and discussion

3.1 Extraction of hesperidin

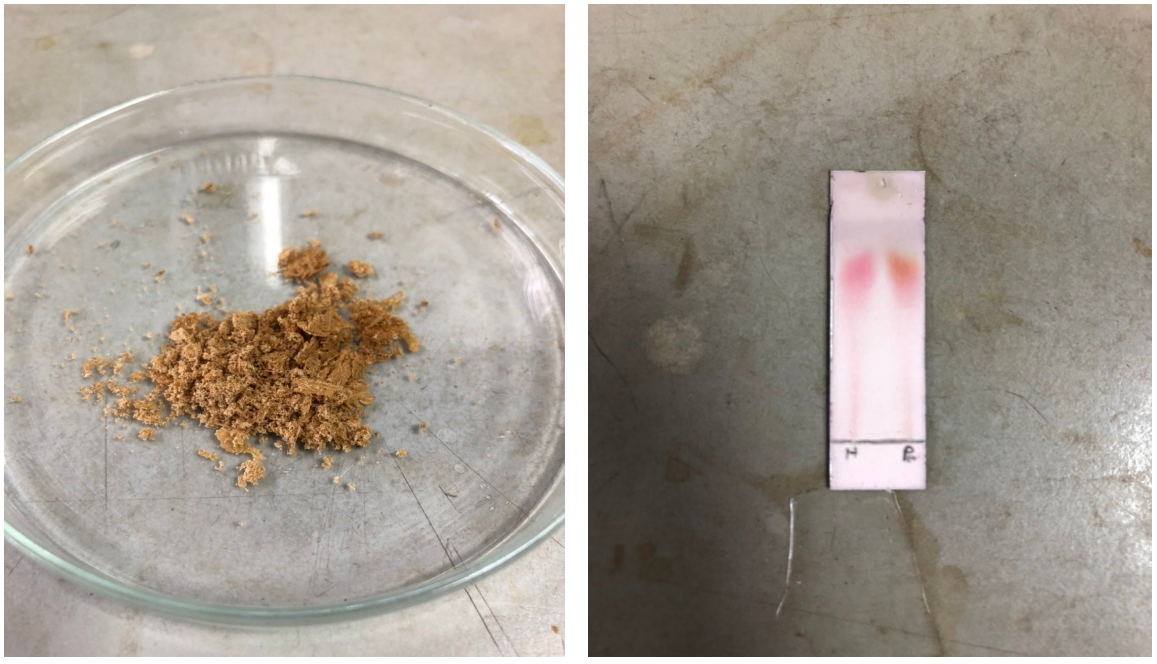


Figure 3.1 (a) Hesperidin obtained from dried orange peel and (b) TLC of synthesized hesperidin compared with commercial hesperidin

Hesperidin was derived from 500 g of dried orange peel and approximately 3.8g of hesperidin was obtained. As written in the research paper, only 1 L of water-methanol (30:70) and 8 hours of maceration was required. However, the mentioned ratio could not be used. The working ratio was 2 L of water-methanol (30:70) and 24 hours of maceration instead. Moreover, 500 mL of petroleum ether was not used for extraction as mentioned in the research paper, only water and EtOAc were used. The crude product of hesperidin was solid yellow-brown color. The TLC result showed that the obtained hesperidin was pure when compared to commercial hesperidin as shown in Figure 3.1.

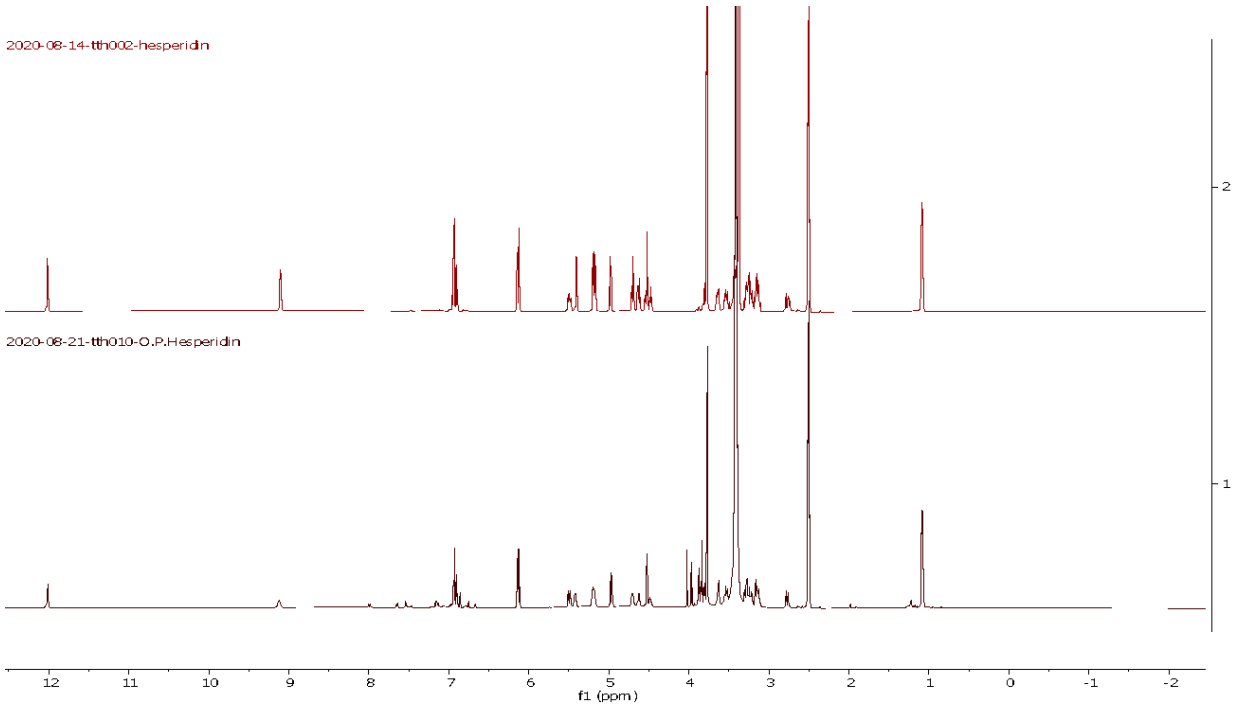


Figure 3.2 The ^{1}H NMR data (DMSO-d_6, 500 MHz) comparison of commercial hesperidin (upper) and synthesized **S01** (lower)

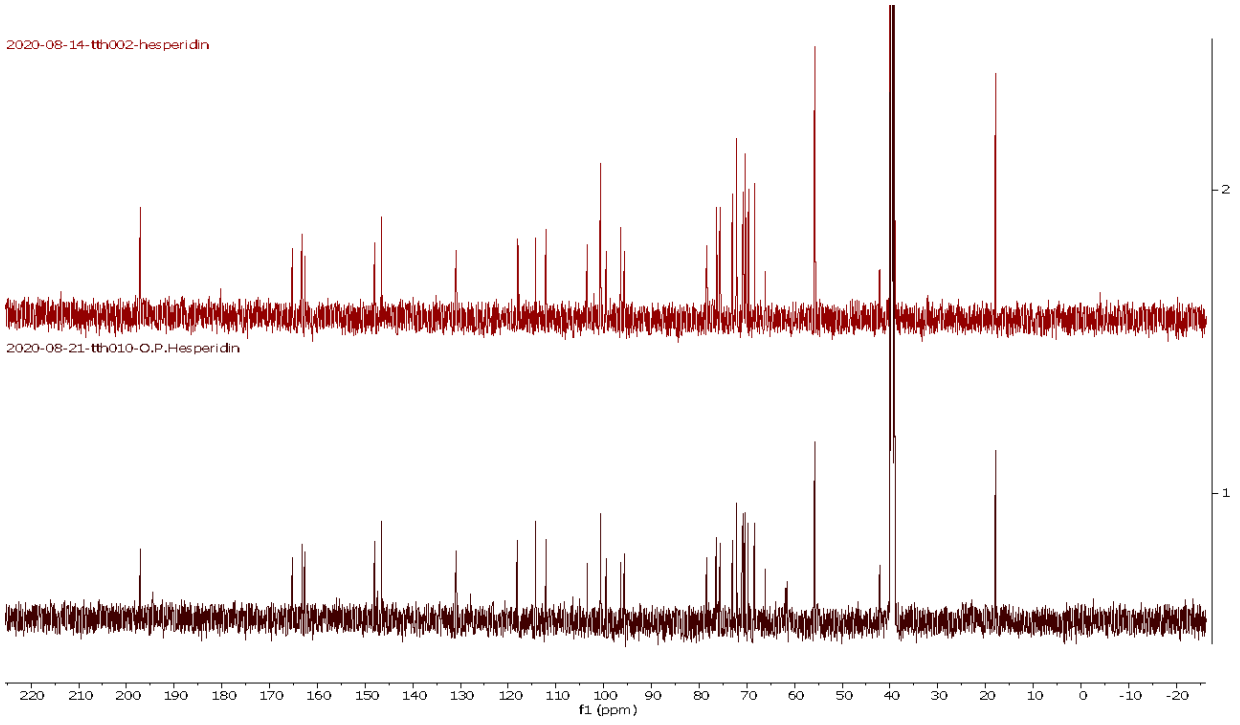


Figure 3.3 The ^{13}C NMR data (DMSO-d_6, 100 MHz) comparison of commercial hesperidin (upper) and synthesized **S01** (lower)

3.2 Synthesis of hesperetin

Hesperetin was synthesized by hydrolysis of hesperidin. The hydrolyzed form of hesperidin that results in the reduced sugar form by using H_2SO_4, MeOH, and AcOH. The yield of hesperetin was 97% or around 3.4 g can be obtained. During the extraction method, only 400 mL of water was required for four times. However, only water could not separate the compound, thus the method was changed to using 100 mL of water and 100 mL of EtOAc for the first time and the rest steps only added EtOAc. Then the organic layer could be completely separated. Hesperetin was white-yellow color solid (Figure 3.4). The mixture of the solution was pure as shown in ^{1}H NMR figure 3.5.

Figure 3.4 (a) Bottle of synthesized hesperetin and (b) product view of dry yellow solid hesperetin

The TLC showed that the synthesized hesperetin was pure compared to commercial hesperetin. Moreover, it was clearly seen that the physical color of obtained hesperetin shown on TLC was different from hesperidin color after reducing the sugar group.

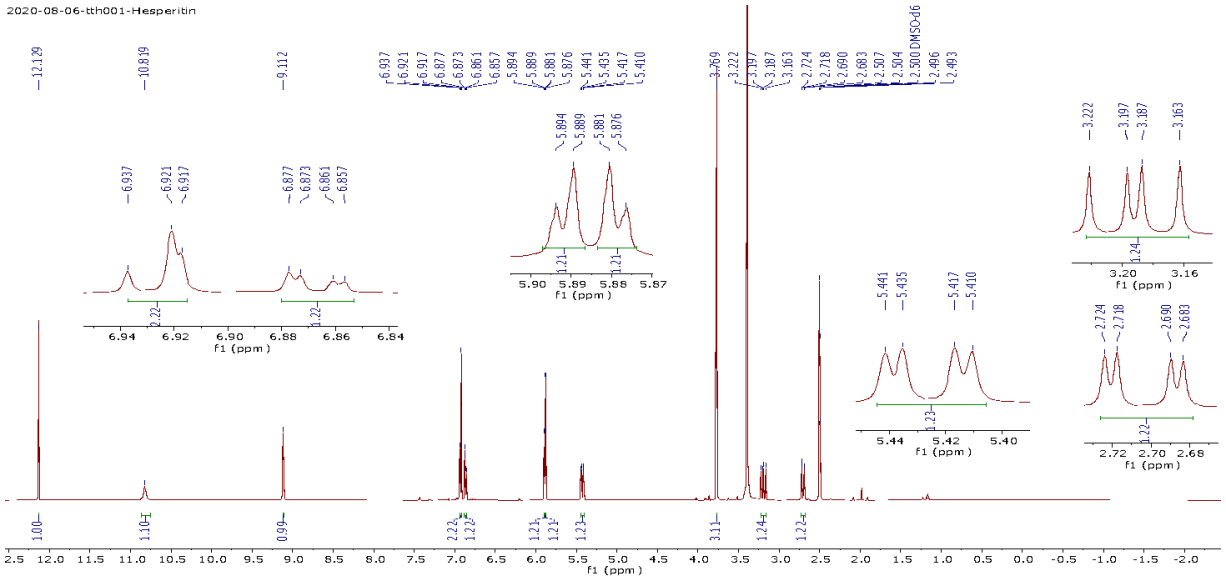


Figure 3.5 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S02**

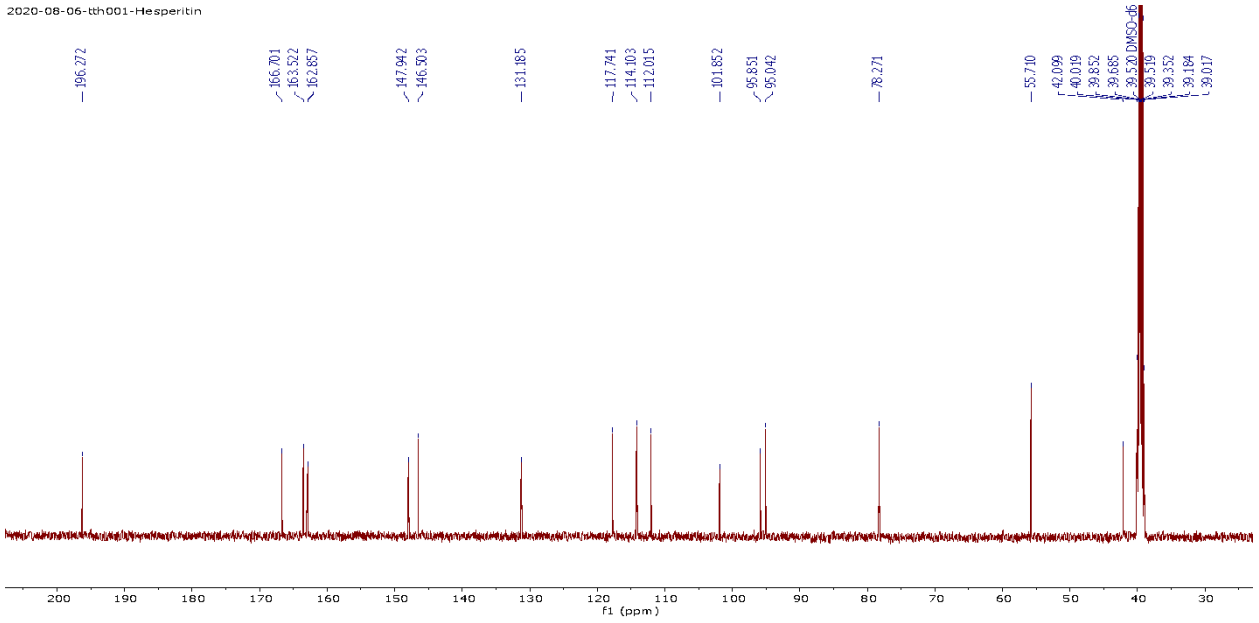


Figure 3.6 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S02**

3.3 Synthesis of Ethyl bromoacetate

Since ethyl bromoacetate is forbidden in Thailand, therefore it is needed to be synthesized to ensure that, with the same ratio, methyl bromoacetate and ethyl bromoacetate can give the same product either di or mono substitution. With starting material of bromoacetic acid and EtOH under acidic condition of H_2SO_4 were heated as shown in Figure 3.7.

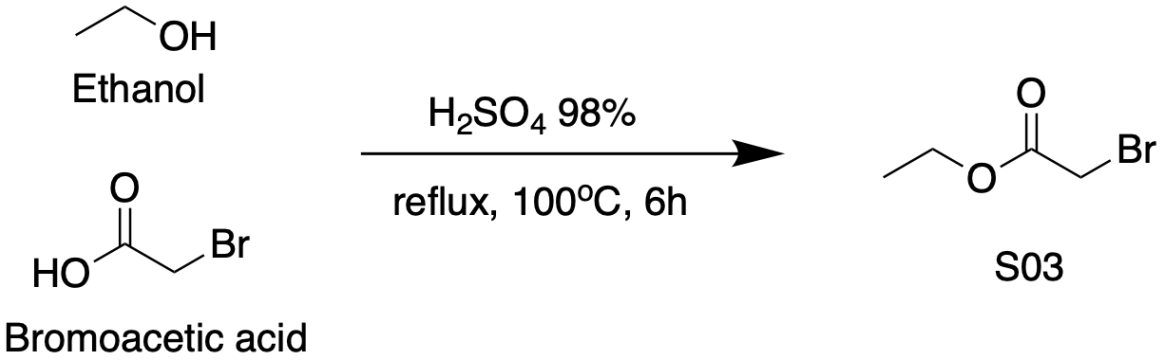


Figure 3.7 Synthesis pathway of ethyl bromoacetate (**S03**)

Total of 1045 microliter of Ethyl bromoacetate was obtained as colorless liquid (Figure 3.8) which can cause severe eye irritation. Thus, the reaction should be done inside the fume hood.

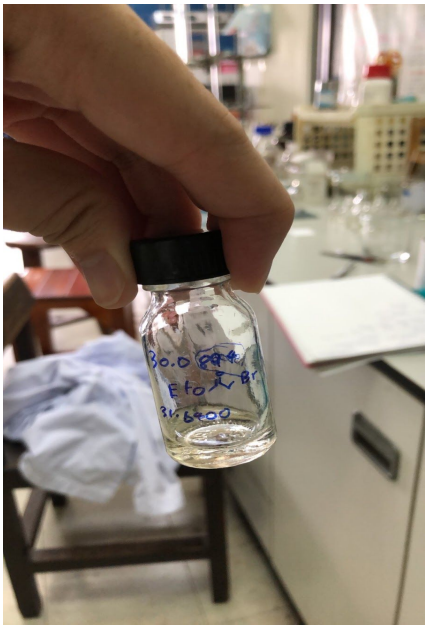


Figure 3.8 Colorless liquid of ethyl bromoacetate

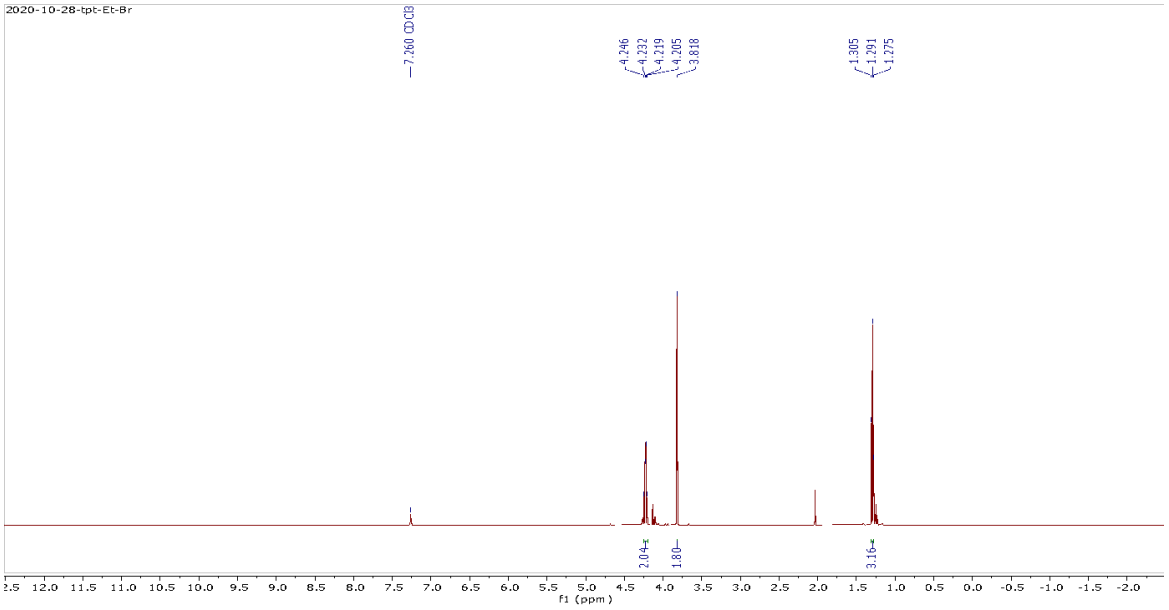


Figure 3.9 The ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of **S03**

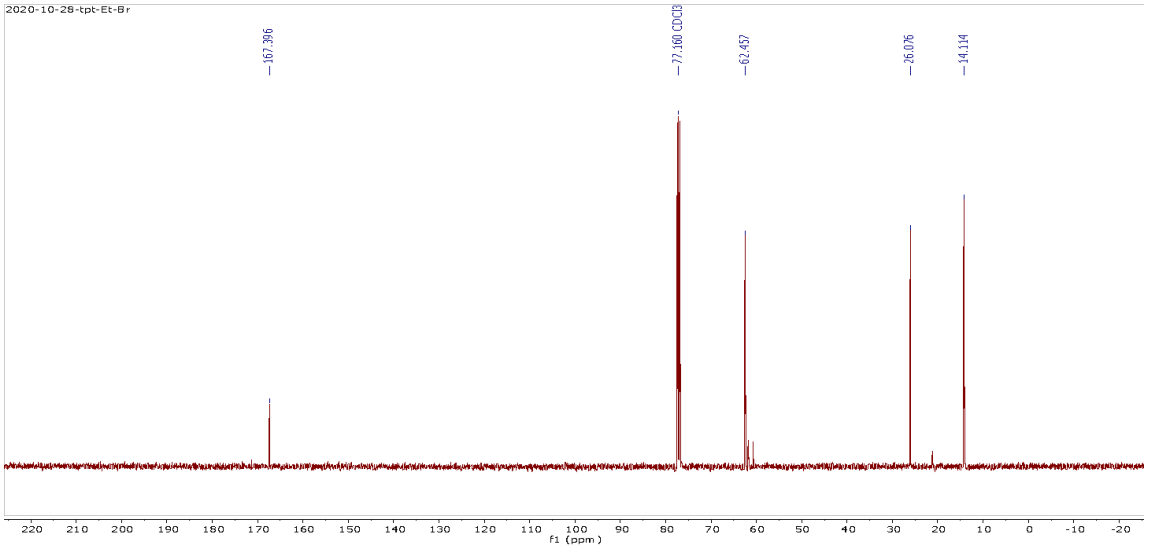


Figure 3.10 The ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **S03**

3.4 Synthesis of S04 and S09

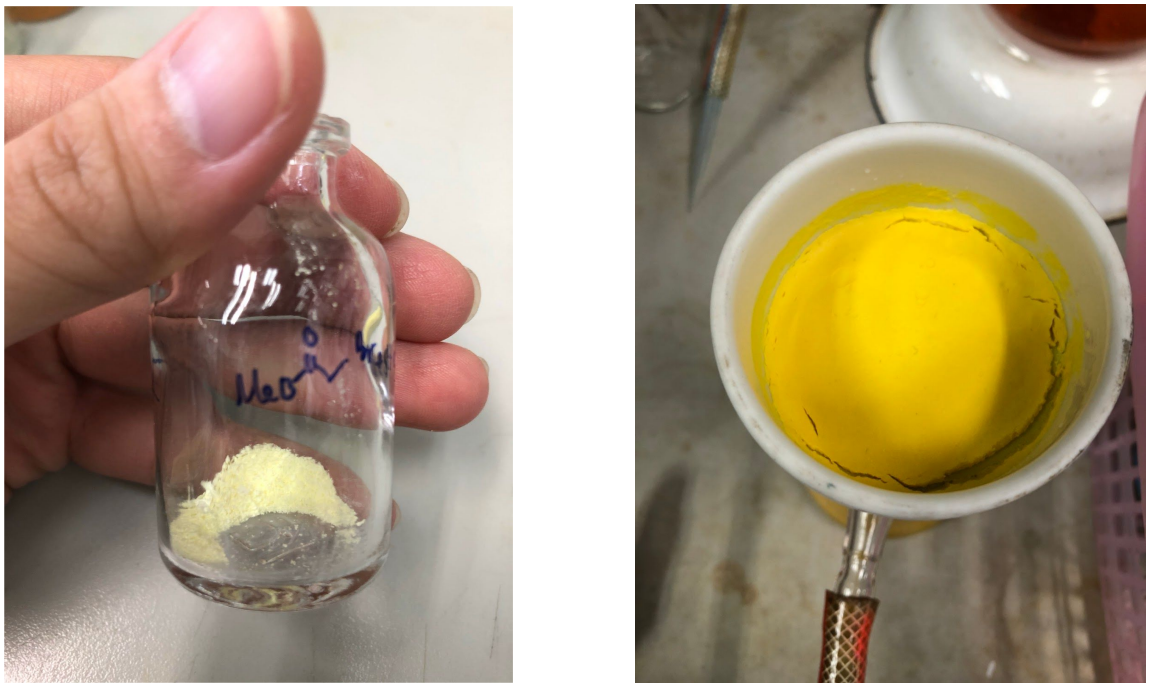


Figure 3.11 S04 before hydrolyzed (left) and hydrolyzed form of **S09** (right)

S04 synthesized from hesperetin and methyl bromoacetate was obtained as a deep yellow solid with 30% yield (Figure 3.11). However, the ratio of methyl bromoacetate and hesperetin was 2:1 , the product occurred in di substitution as shown in Figure 3.12.

The hydrolysis of hesperetin was conducted as follows. First, the solution was not dissolved in 10% EtOH, but by adding 10% NaOH which it started to dissolve slowly. Then, the pH was adjusted to around 3-4, which helped promote the precipitate before filtration. The product was obtained as orange solid with 79% yield.

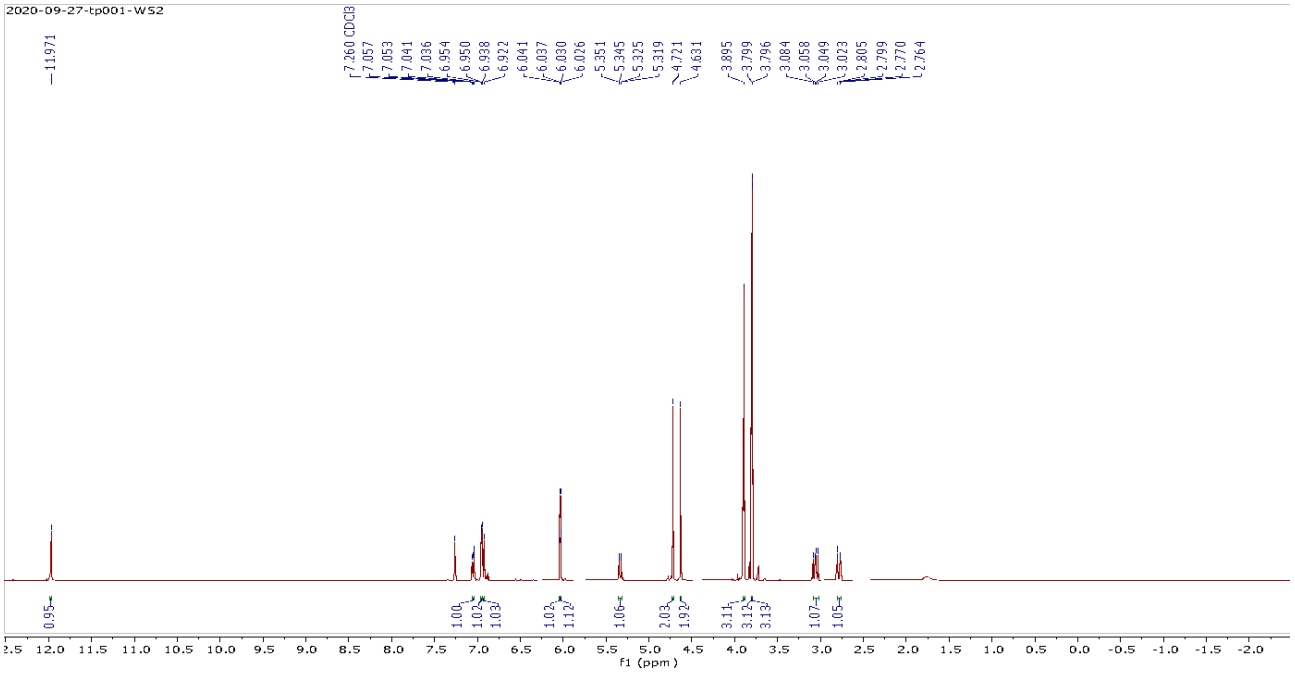


Figure 3.12 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S04**

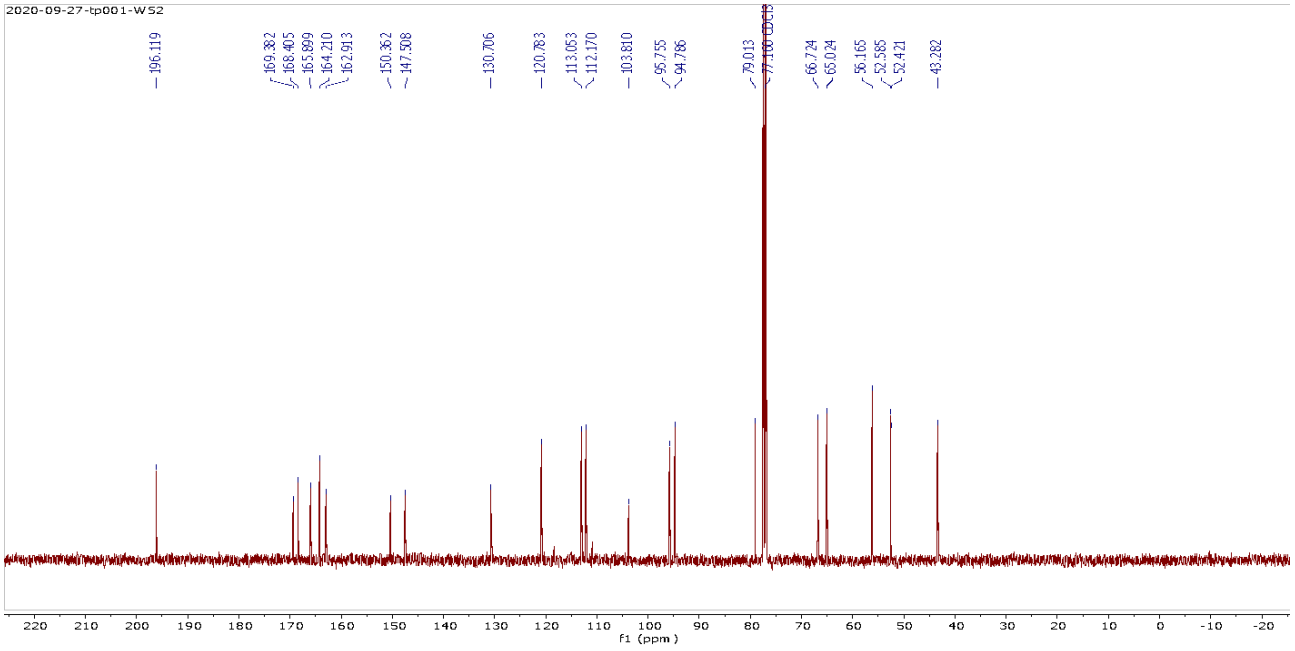


Figure 3.13 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S04**

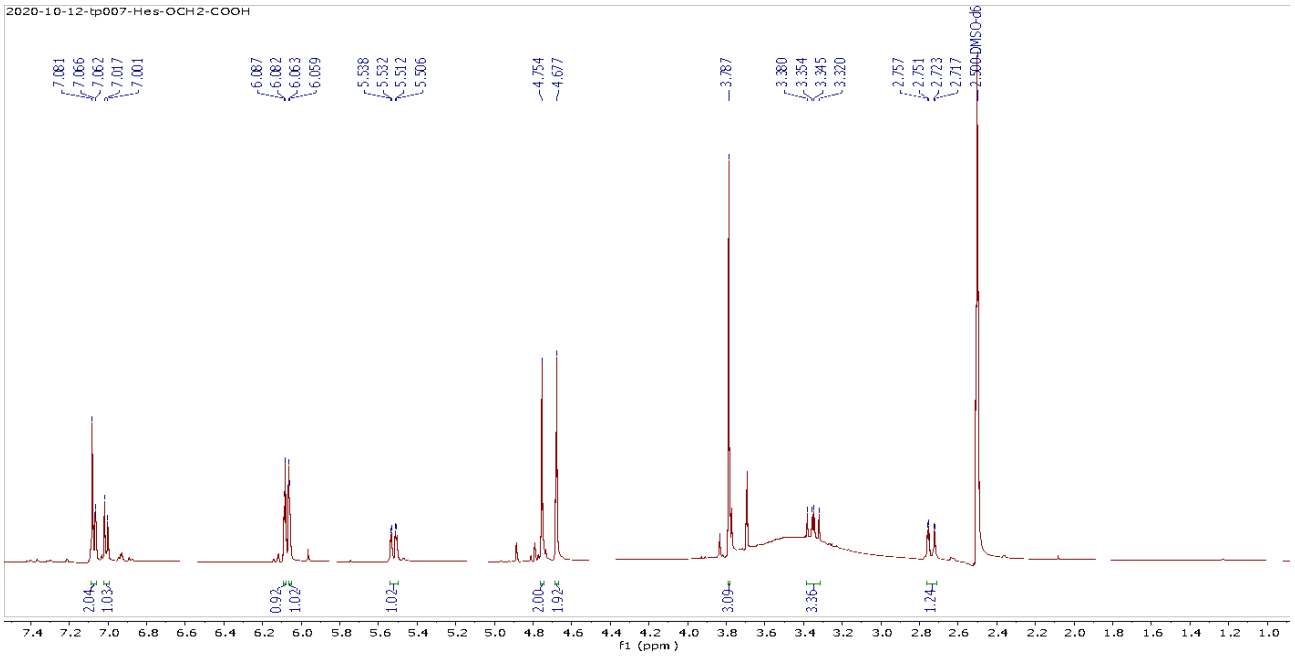


Figure 3.14 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S09**

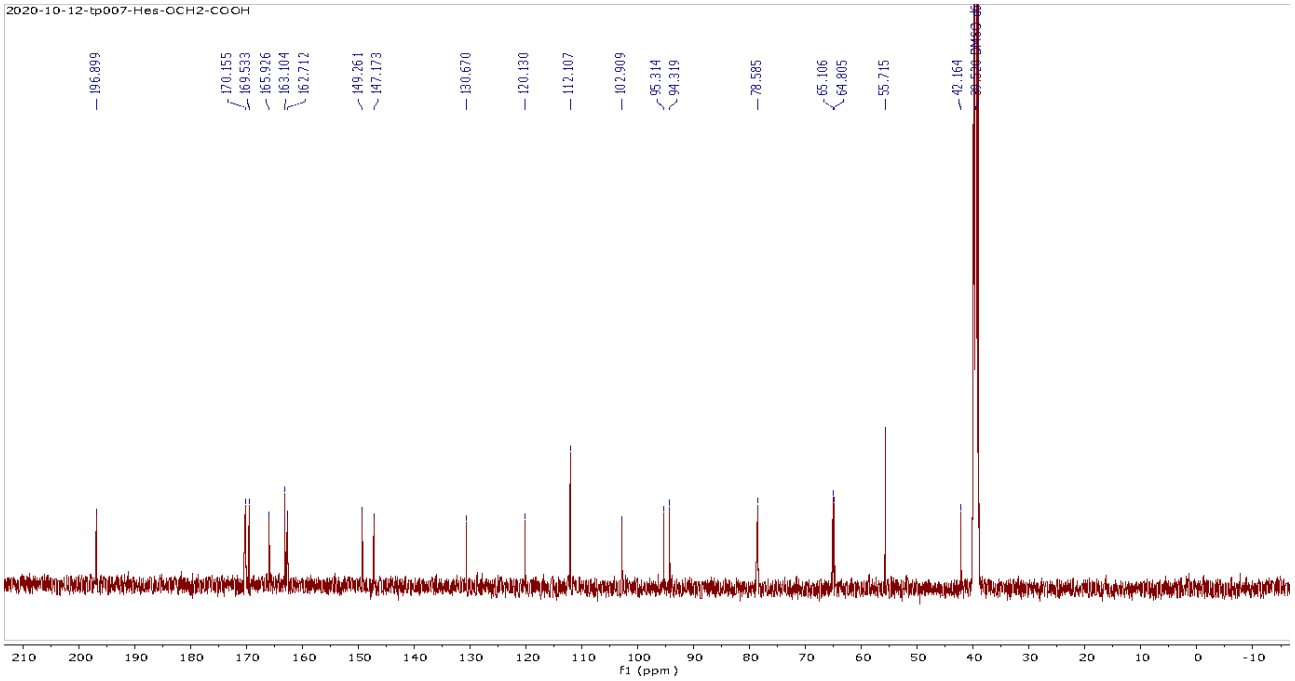


Figure 3.15 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S09**

3.5 Synthesis of S05 and S10

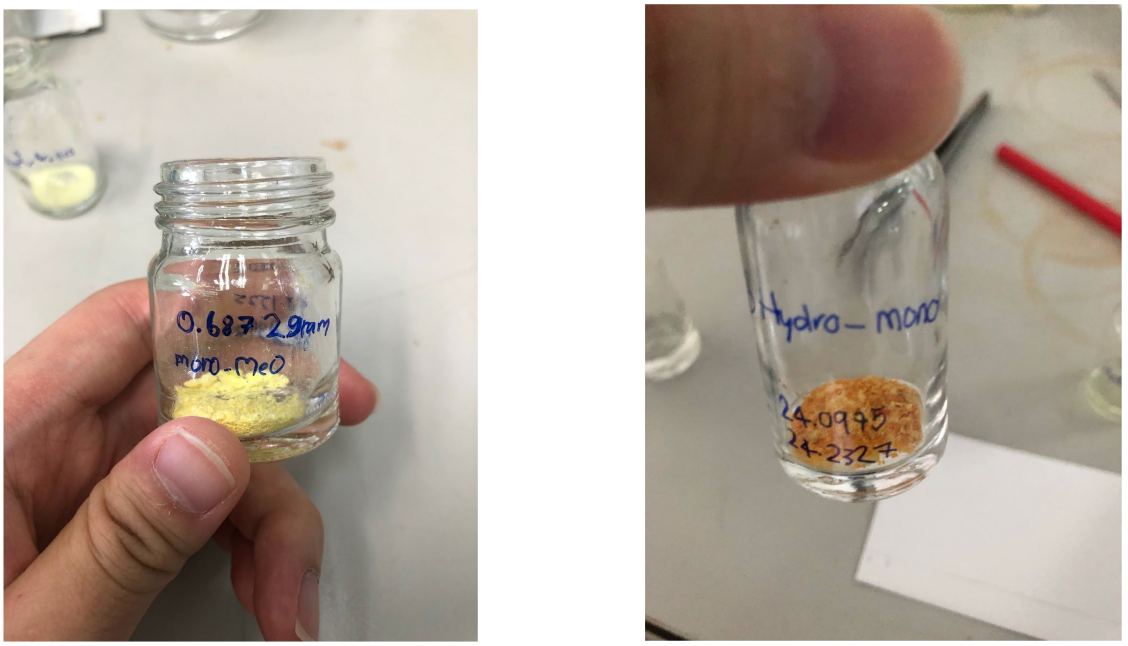


Figure 3.16 Product of **S05** before hydrolysis (Left) and hydrolyzed form **S10** (Right)

The ratio of methyl bromoacetate and hesperetin was reduced to 1:1 to ensure that the ratio has a significant effect on the product formation. The result obtained from TLC with the solvent of hexane: EtOAc (3:2) indicated that the product was pure. Followed by ^{1}H and ^{13}C NMR showed that the obtained product was mono ester (Figure 2.5) with the 56% yield. The product has a physical appearance as crude yellow solid (Figure 3.16).

After the hydrolysis of **S05**, TLC could not be done due to its high polarity. Therefore, the product was sent for ^{1}H and ^{13}C NMR and the result showed some impurities in the final product. The product was obtained as yellow solid with 75% yield.

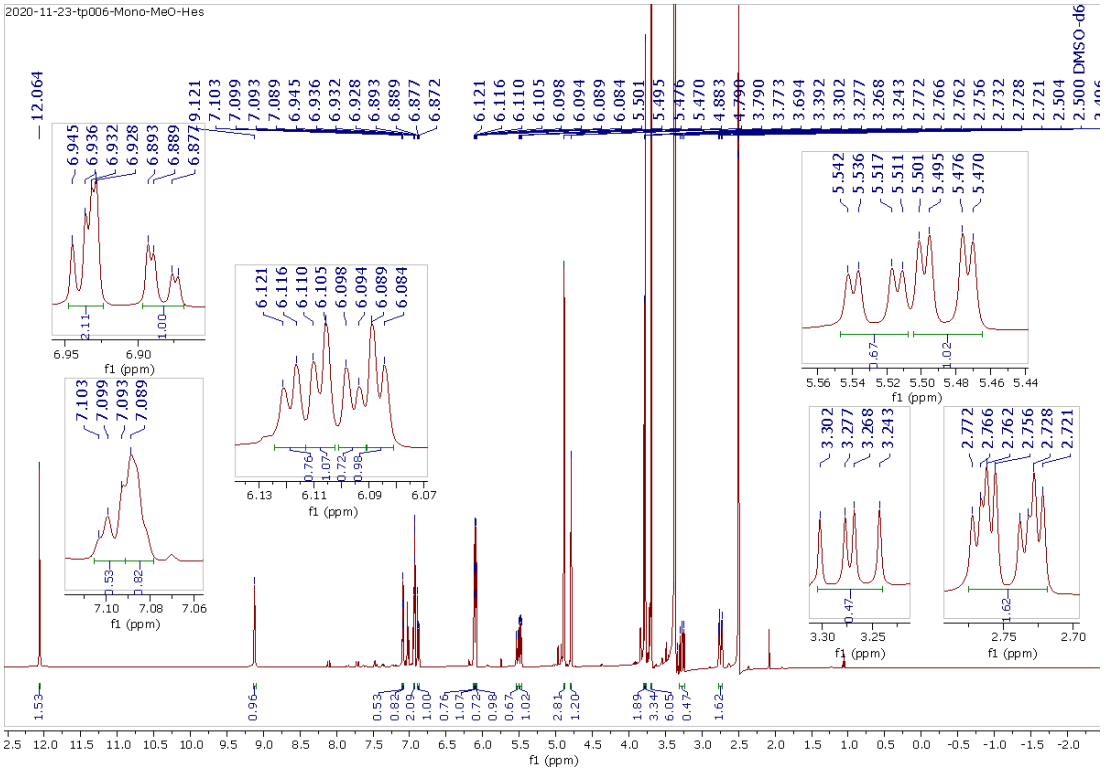


Figure 3.17 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S05**

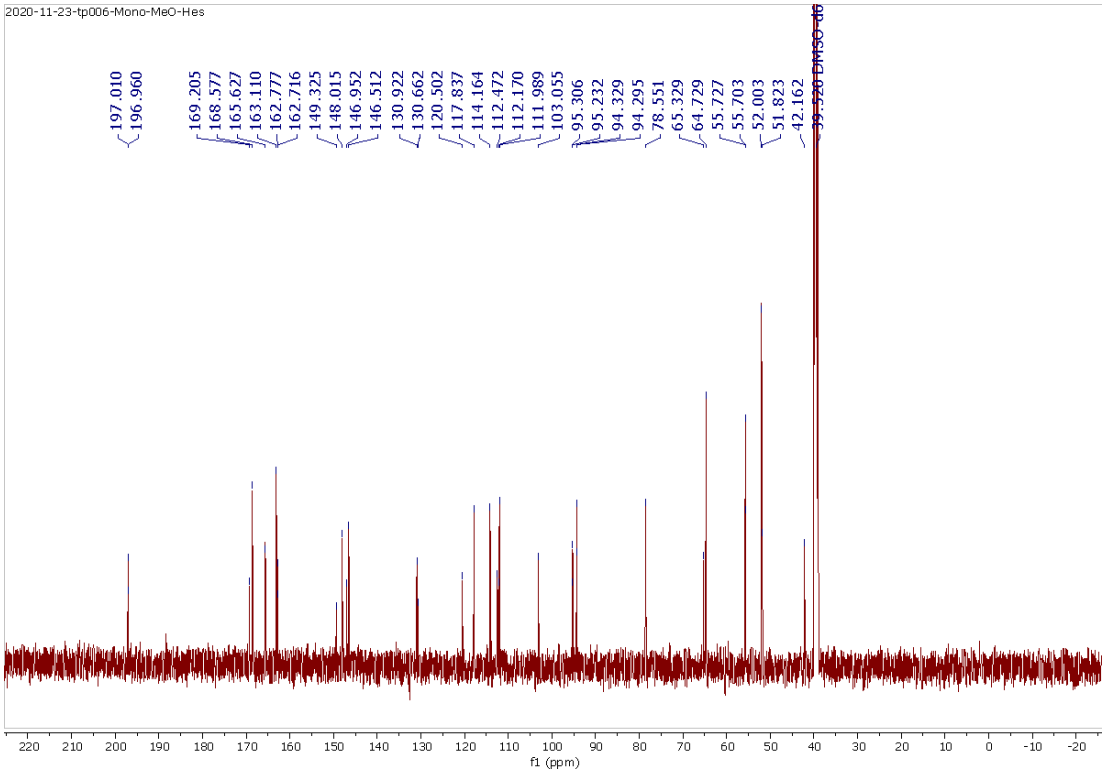


Figure 3.18 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S05**

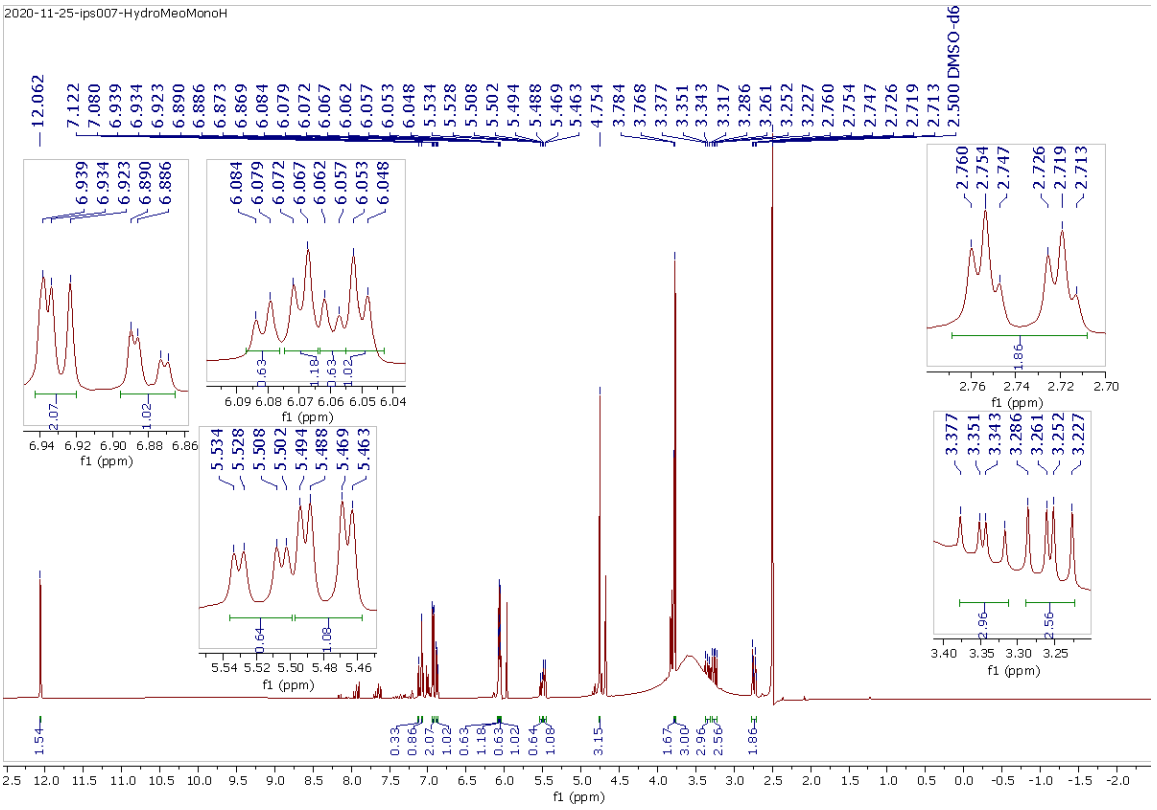


Figure 3.19 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S10**

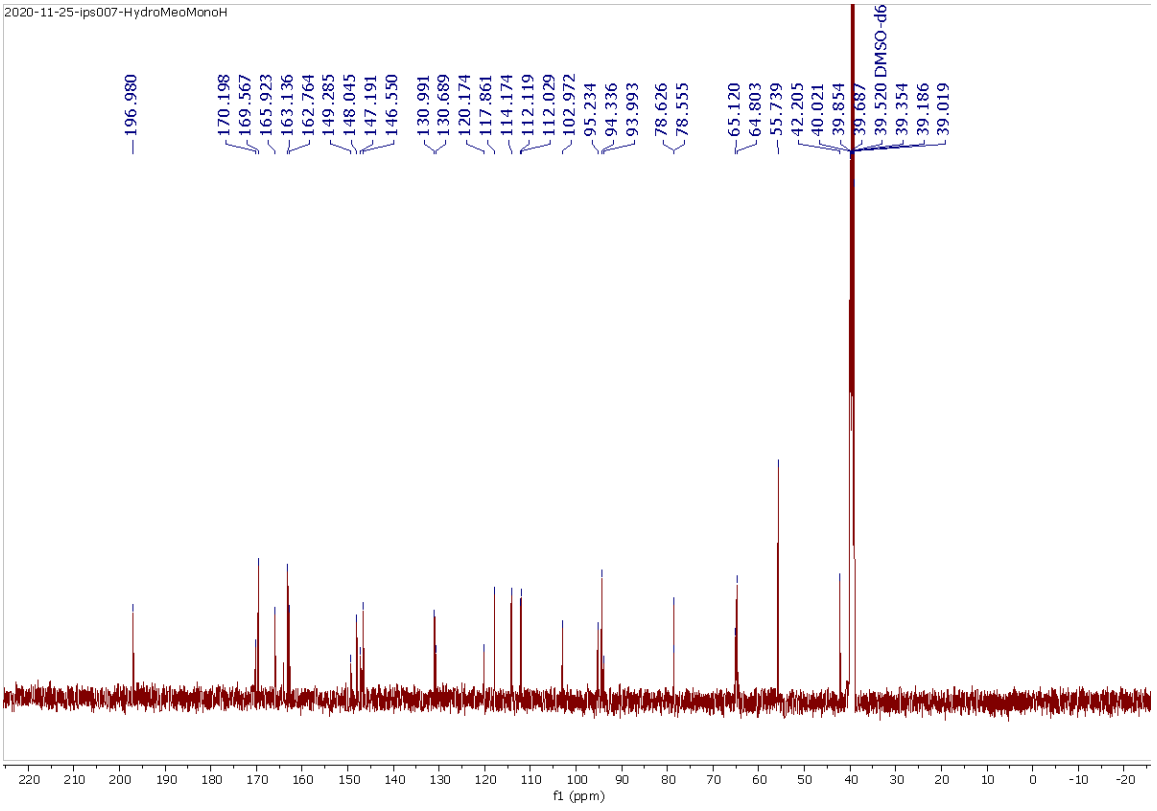


Figure 3.20 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S10**

3.6 Synthesis of S06 and S11

Figure 3.21 Product of **S06** before hydrolysis (Left) and hydrolyzed form **S11** (Right)

The ratio used in this reaction was 2:1 of hesperetin and methyl 4-bromocrotonate. The product was obtained as white solid crude with 49.7% yield. The TLC result showed that this compound was impure and needed to be further purified by the column chromatography.

The hydrolyzed product of **S06** was obtained as brown orange color solid with 36% yield. However, it could not be further purified or performed TLC due to its high acidity and polarity. Therefore, the product was sent for ^{1}H and ^{13}C NMR to characterize the compound with predicted structure, but the result showed some impurities.

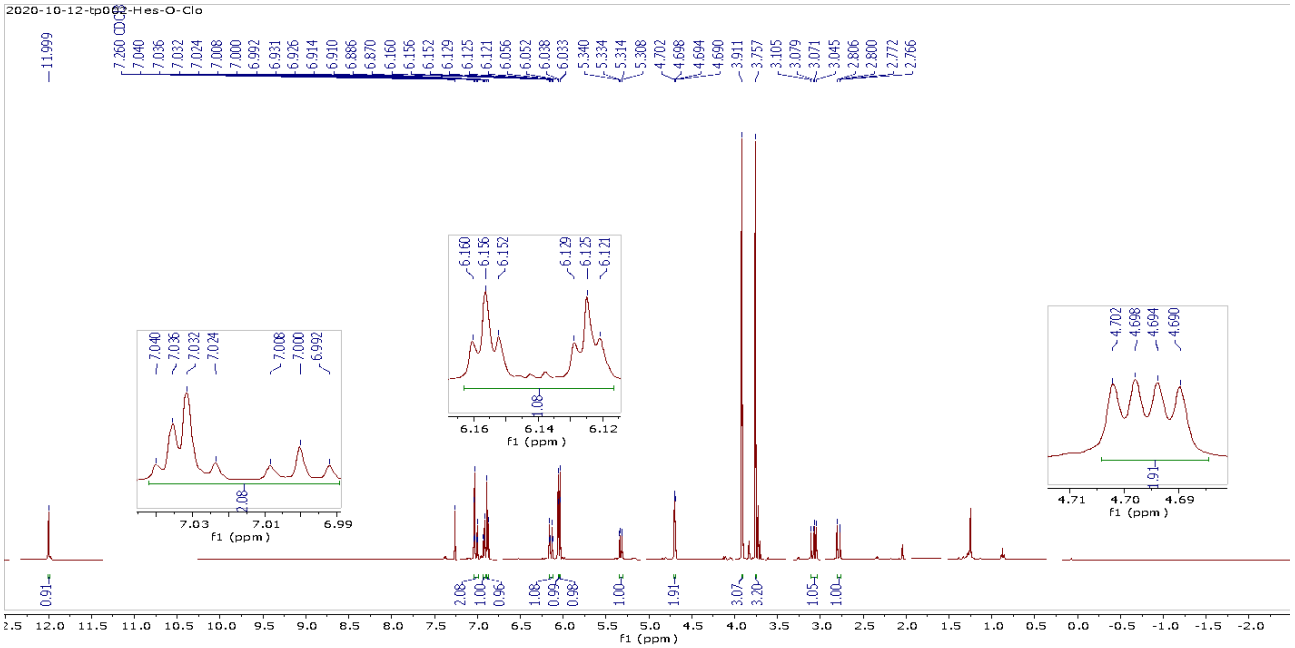


Figure 3.22 The ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of **S06**

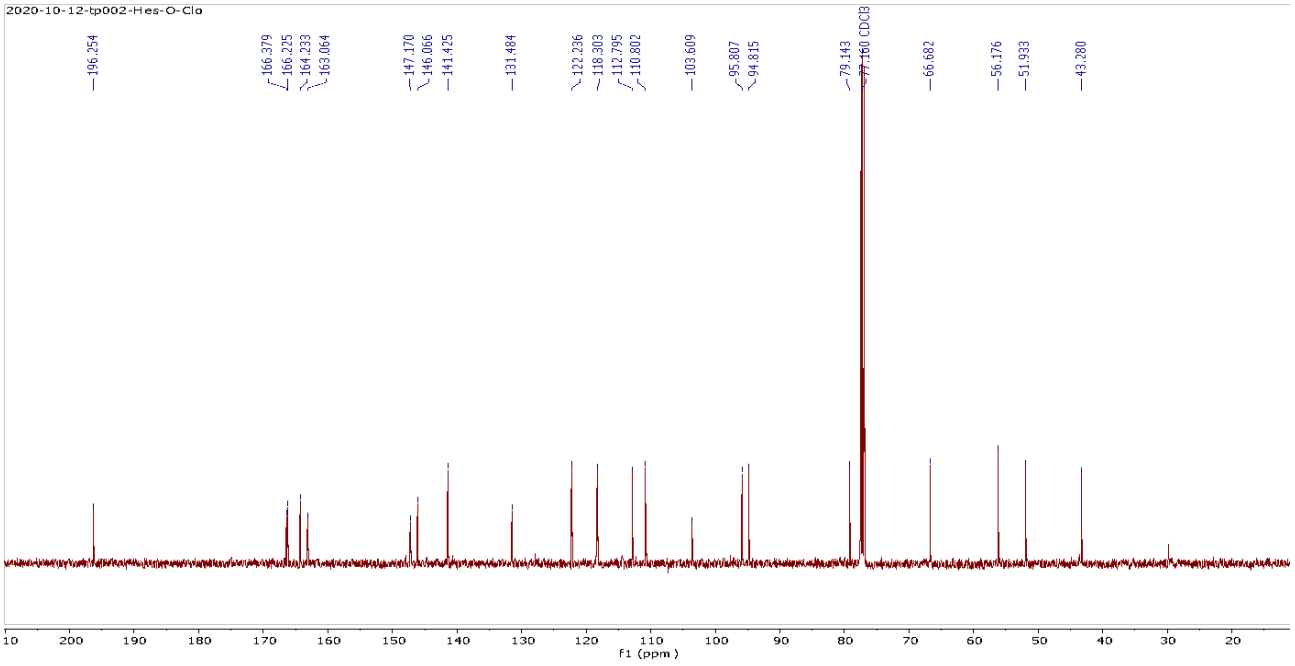


Figure 3.23 The ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of **S06**

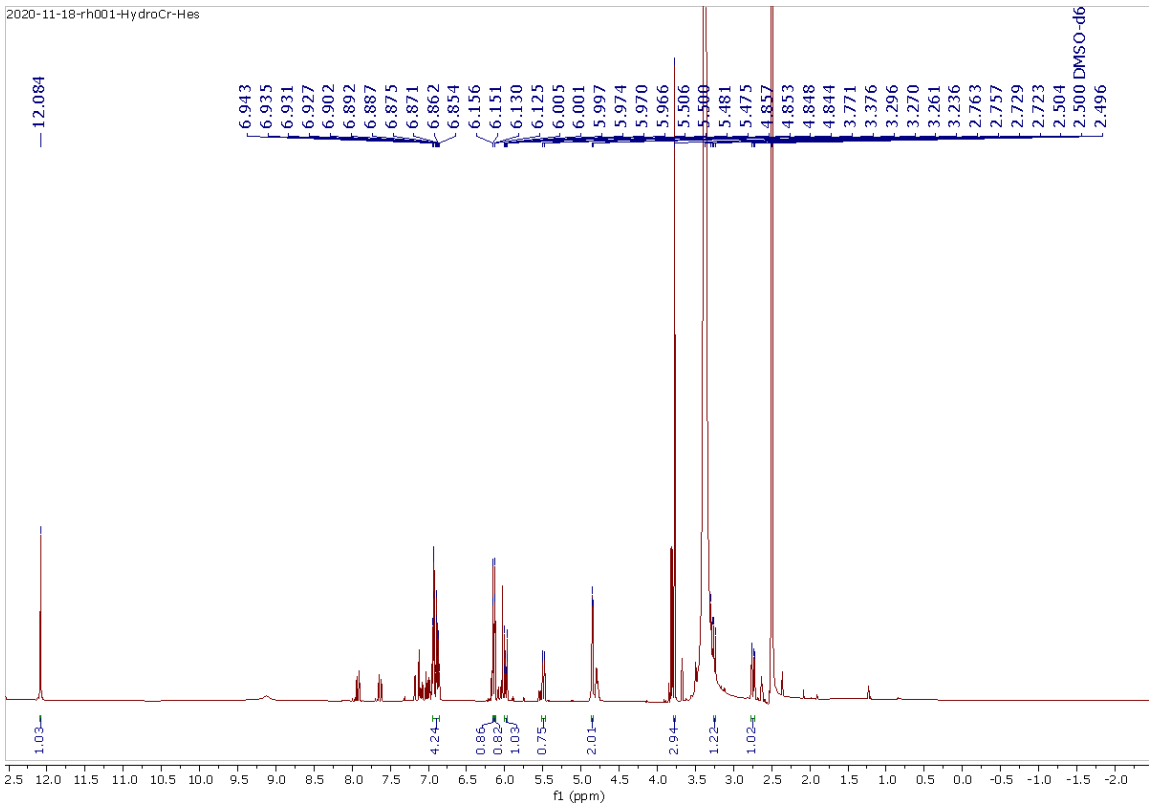


Figure 3.24 The ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of **S11**

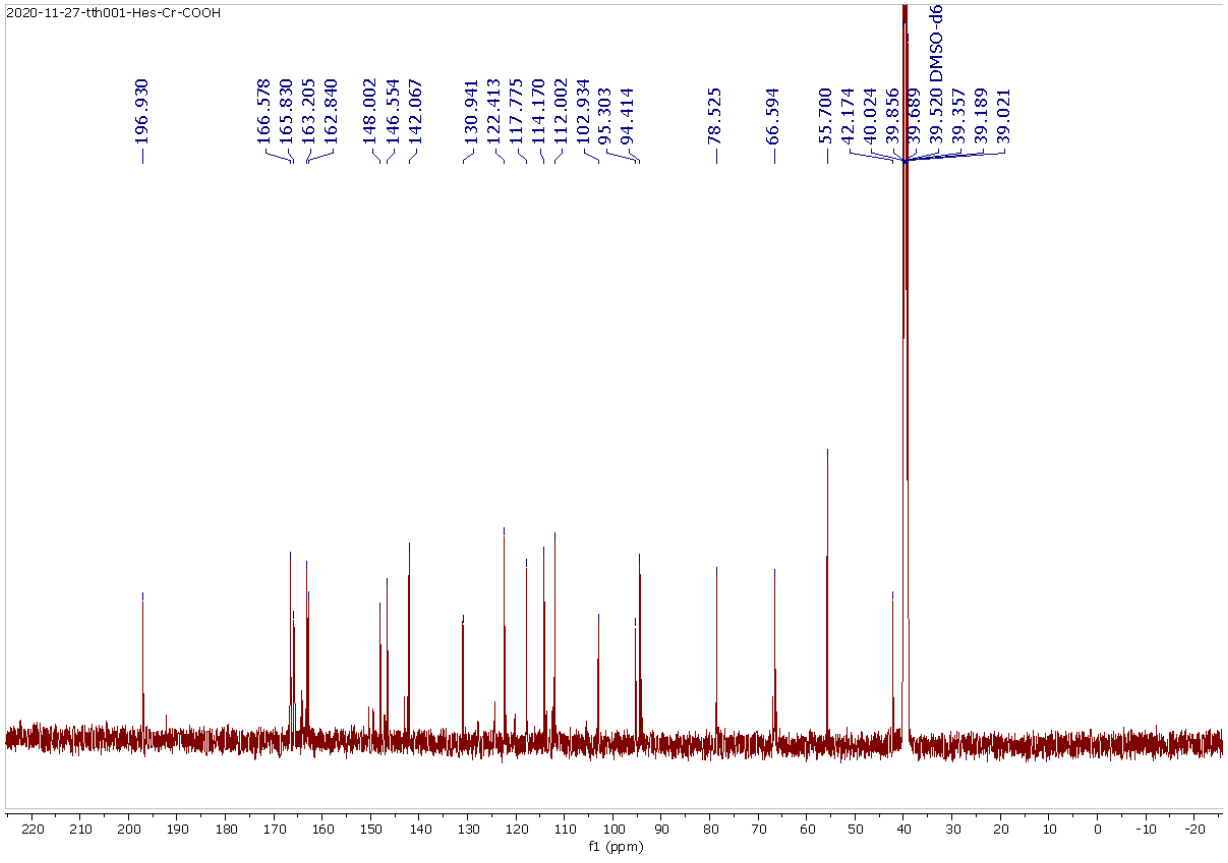


Figure 3.25 The ^{13}C NMR (DMSO-d_6, 100 MHz) spectrum of **S11**

3.7 Synthesis of S07 and S08

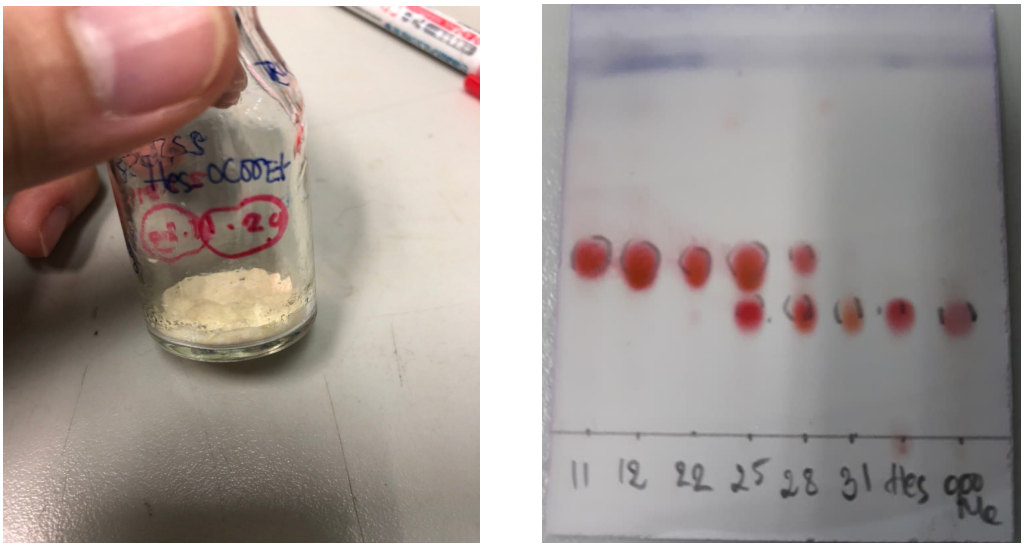


Figure 3.26 Product obtained from **S07** and **S08**

This reaction was done only to ensure that with the same ratio of methyl bromoacetate, the product will form as mono or di substitution. The result obtained as two different compounds with 80% yield of **S07** and **S08** as shown in Figure 3.8.

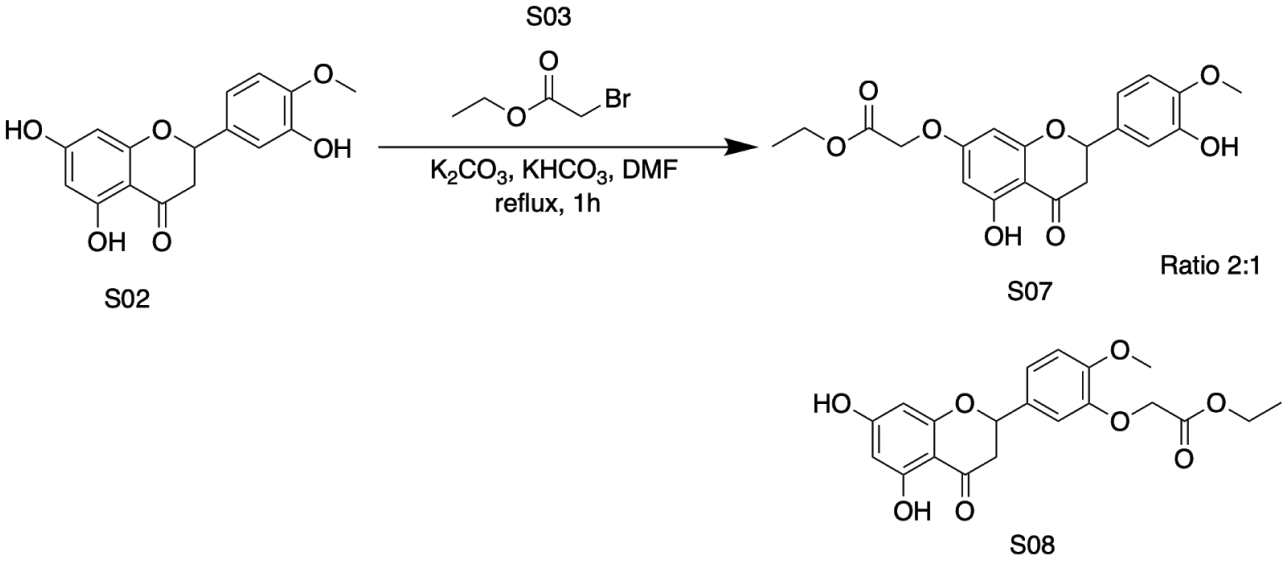


Figure 3.27 The reaction of S.2 with methyl bromoacetate

Then TLC was performed with solvent hexane: EtOAc (3:2), notice that there were two spots of different intensity under UV light. The column chromatography was performed using hexane: EtOAc (2:1) to separate two compounds and TLC was performed again under UV light and stained with vanillin reagent, it showed different spots but with the same color. The NMR result showed that there were two different compounds with a ratio of 2:1.

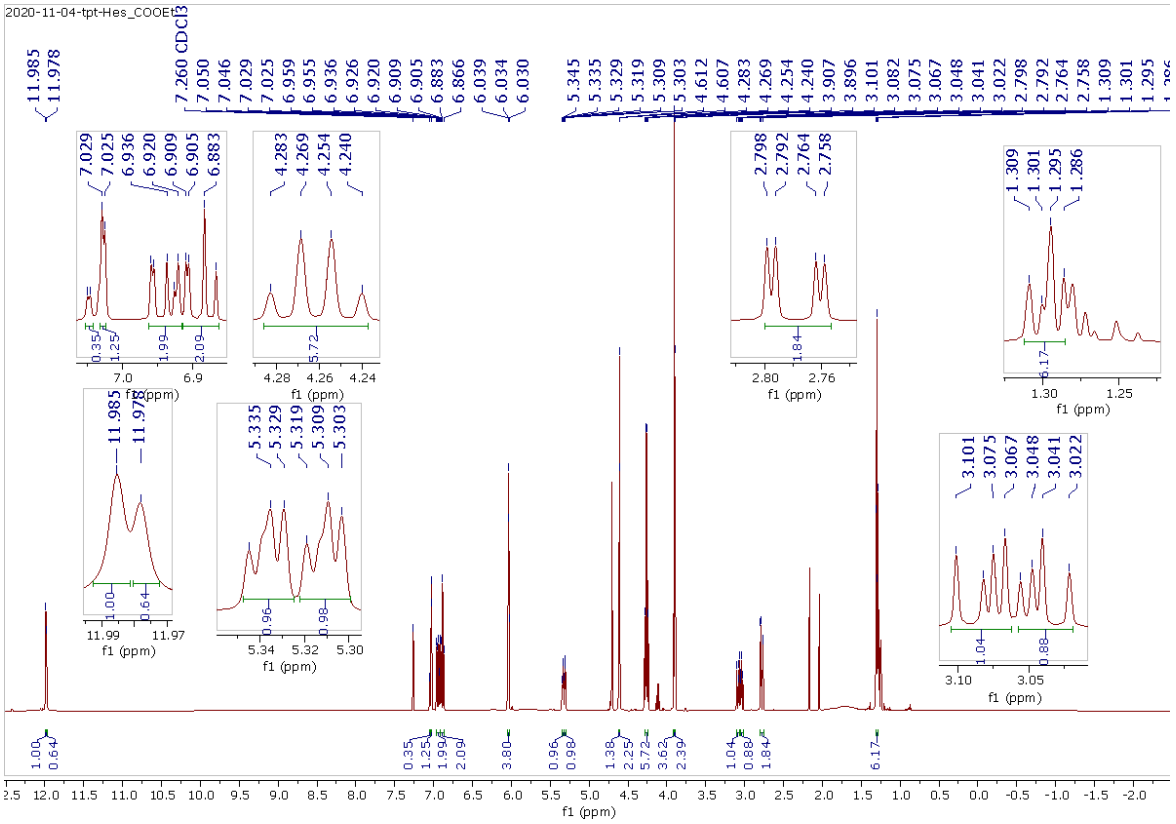


Figure 3.28 The ^{1}H NMR ($CDCl_3$, 500 MHz) spectrum of the mixture of **S07** and **S08**

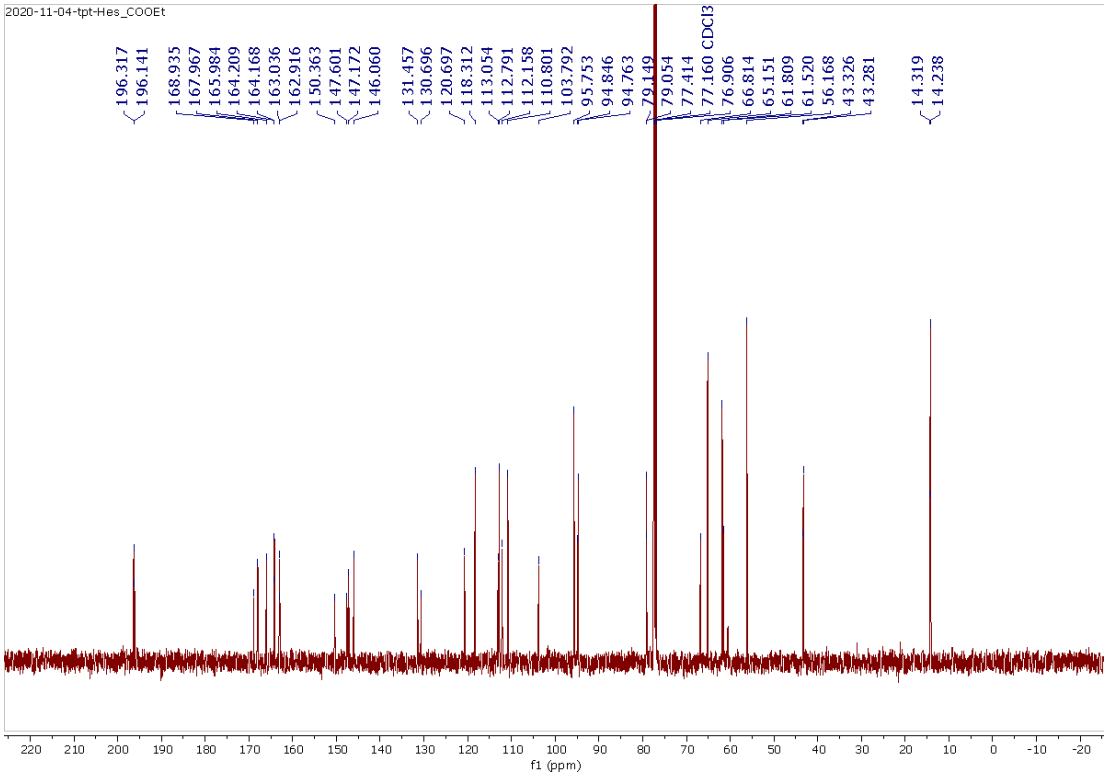


Figure 3.29 The ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum of the mixture of **S07** and **S08**

3.8 Antioxidant Activity of Synthesized Compounds

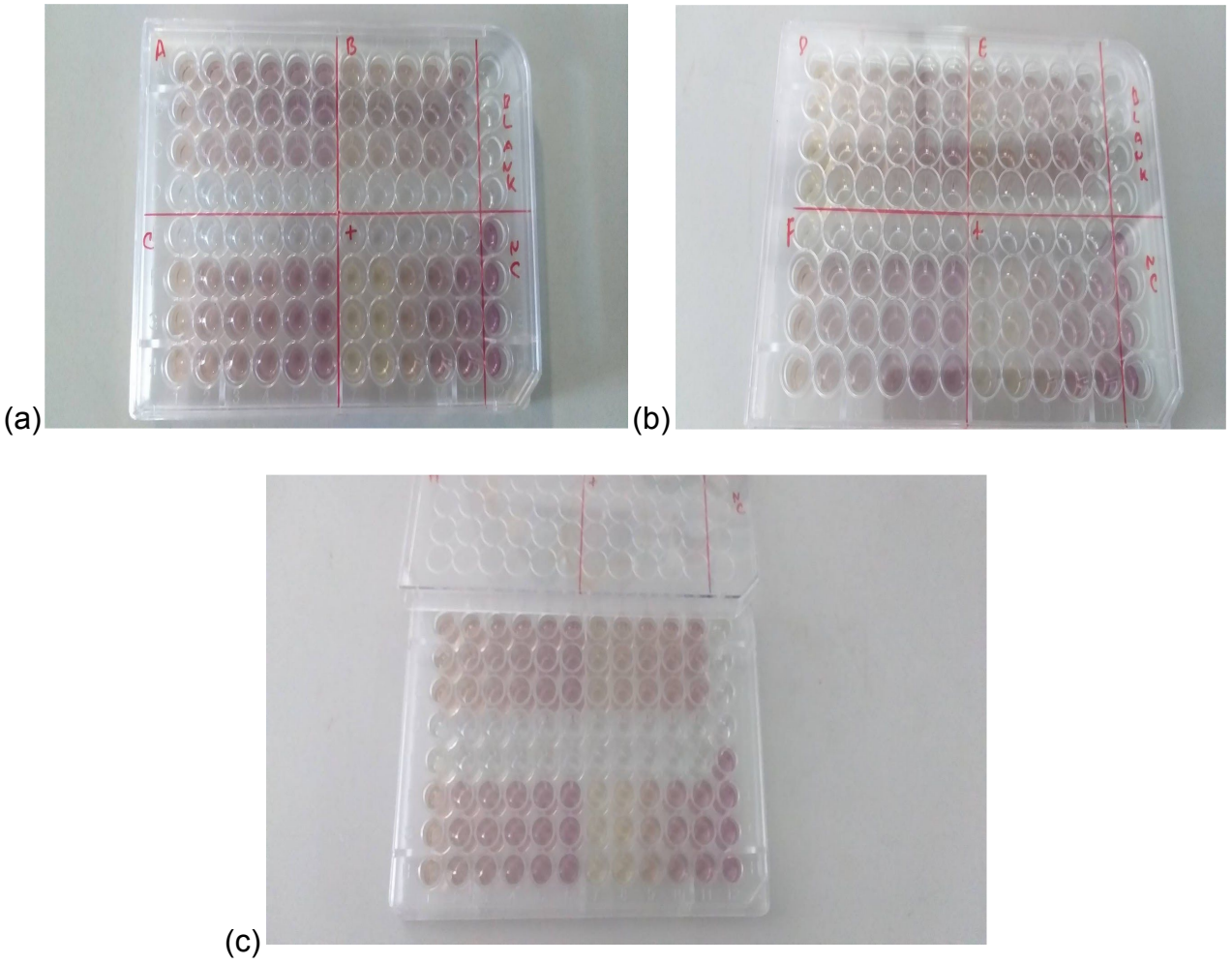


Figure 3.30 Antioxidant activity of synthesized compounds with DPPH A(01), B(02), C(06), D(011), E(05), F(S10), G(S04), H(S09)

The synthesized compounds of **S01**, **S02**, **S04**-**S06** and **S09**-**S11** were tested for antioxidant activity using DPPH radical scavenging assay and the results showed that all compounds have antioxidant activities. According to the results, the most effective compound is **S11** with the lowest IC_{50} (0.19mM) and **S01** is the least effective with the highest IC_{50} (0.78mM). **S11** showed the best Inhibition percentage due to the presence of hydroxyl groups. As figure 3.25 illustrates the effect of structure to explain the inhibition effect. **S11** shows the best result due to its natural number of hydroxyl groups that do not contain hydrogen bonds with other neighbor atoms and the double bond that may have an inductive effect to make the proton radical to form easier which then reacts with DPPH radical. In comparison, the

structure of **S09** and **S10** contained hydrogen bonding that could affect the inhibition concentration. The structure of **S06** shows the second best yield, this can be applied for the same reason with **S11** as it contains double bond. When compared to the starting compounds, **S01** and **S02**. **S01** shows the least antioxidant activities since it contains disaccharide with phenolic ring which demote electron-donating ability which results in less antioxidant activity. While **S02** does contain phenolic rings but its antioxidant effect is slightly less effective than **S11** due to absence of double bond.

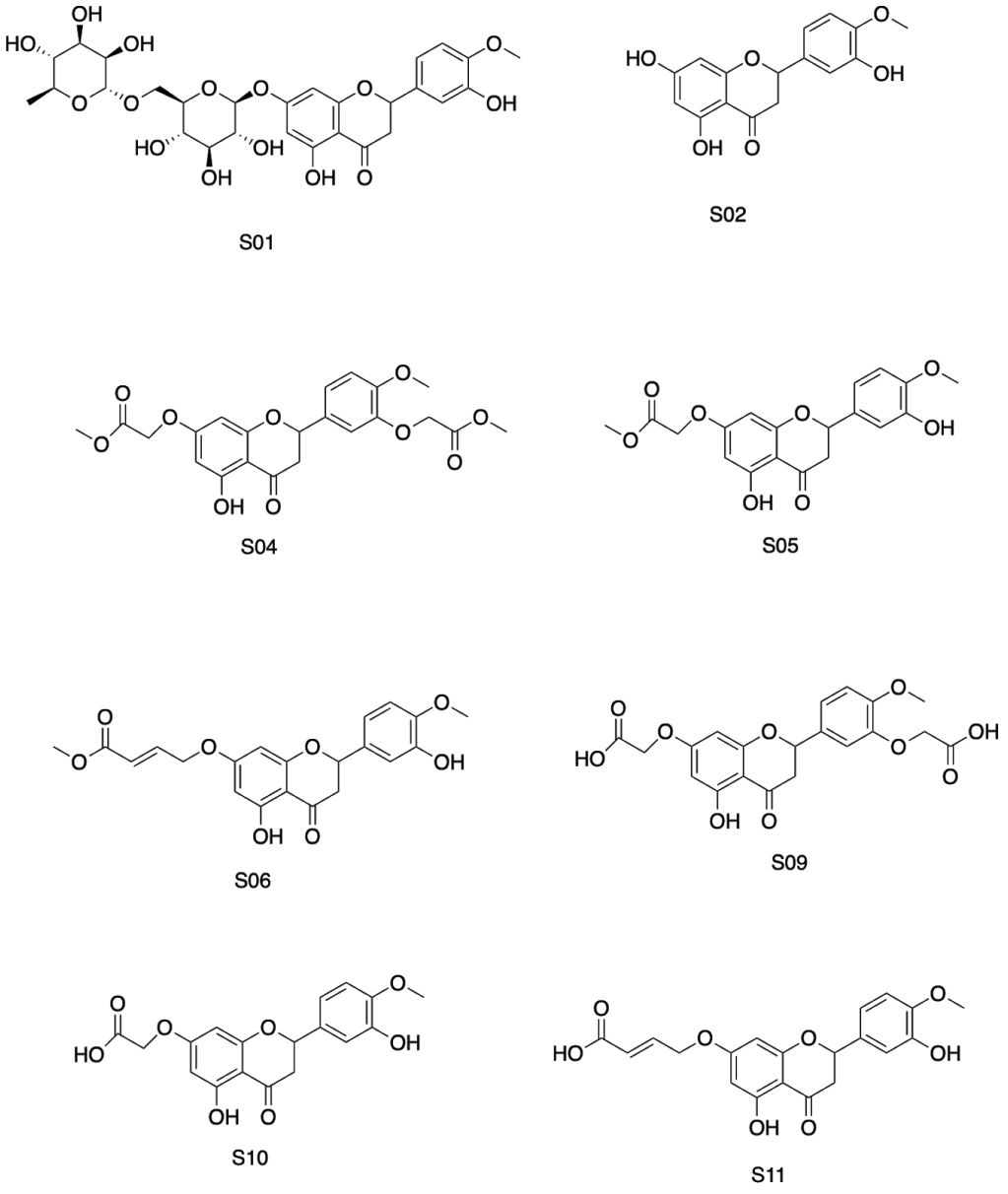


Figure 3.31 List of compounds tested for antioxidant activity

Table 3.1 Inhibition percentage of each hesperetin derivatives

	Concentration (millimolar)						
	1	0.50	0.25	0.125	0.0625	0.03125	
Compound	Inhibition Percentage (%)						IC_{50} (mM)
S01	53.09	47.41	39.14	29.01	19.63	11.11	0.78
S02	63.46	56.30	48.52	38.15	27.28	-	0.26
S04	59.06	51.24	43.55	30.65	22.70	11.17	0.52
S05	58.46	47.79	40.56	31.25	20.96	-	0.63
S06	64.44	57.78	50.74	40.56	32.04	19.63	0.23
S09	57.38	46.03	43.80	34.68	24.26	14.95	0.63
S10	55.88	48.90	42.65	32.90	21.14	15.44	0.63
S11	71.81	62.50	55.64	45.10	32.48	24.51	0.19

Table 3.2 Inhibition percentage of Ascorbic acid as positive control

	Concentration (micromolar)						
	100	50	25	12.5	6.25	-	
Compound	Inhibition Percentage (%)						IC_{50} (mM)
Ascorbic Acid	96.48	90.93	68.15	33.70	16.85	-	18.39

According to the %inhibition vs. concentration graph of each compound, the IC_{50} can calculated by the equation the linear equation solving for x

$$y = mx + c$$

$$x = \frac{y-c}{M}$$

While y is always equal to 50, m is the slope and c is the y-intercept. In order to plot %inhibition vs. concentration graph, only 3 out of 6 consecutive datas of each compound was chosen to ensure that R^2 value is around 0.90-1 make the calculated IC_{50} to be in the range of concentration of 3 chosen datas.

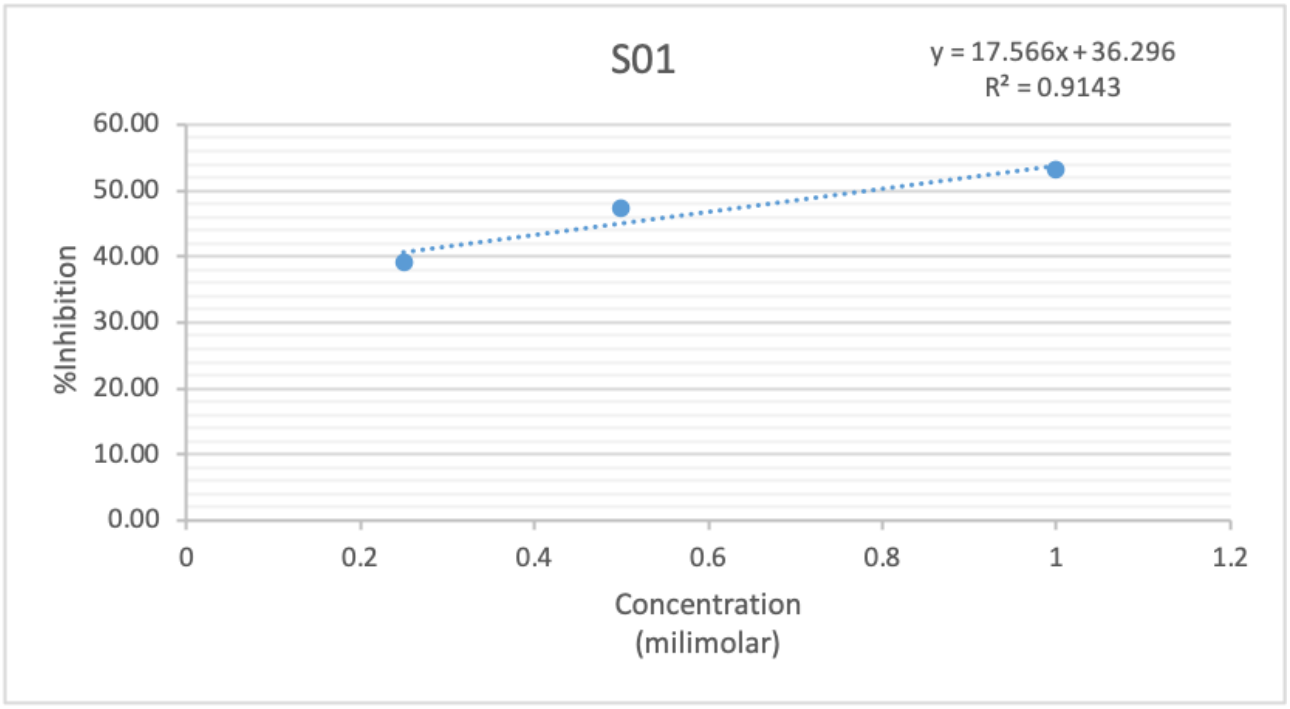


Figure 3.32 Inhibition concentration graph of **S01**

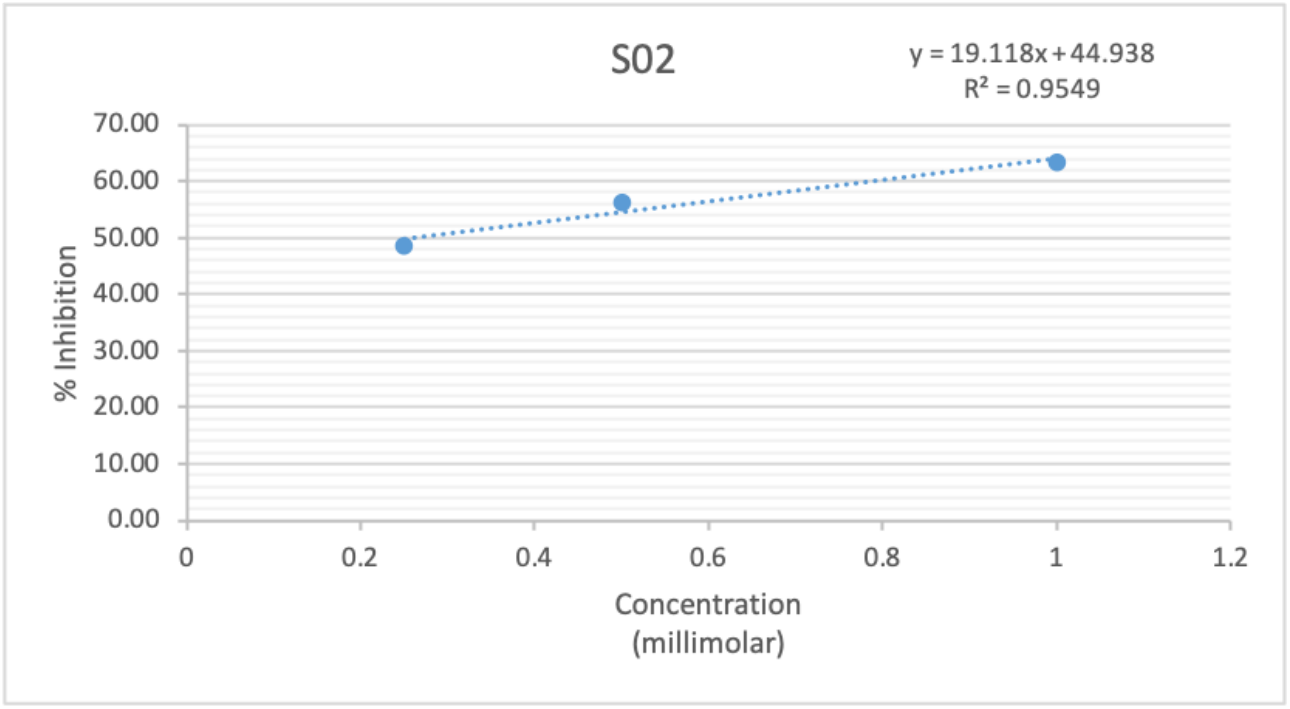


Figure 3.33 Inhibition concentration graph of **S02**

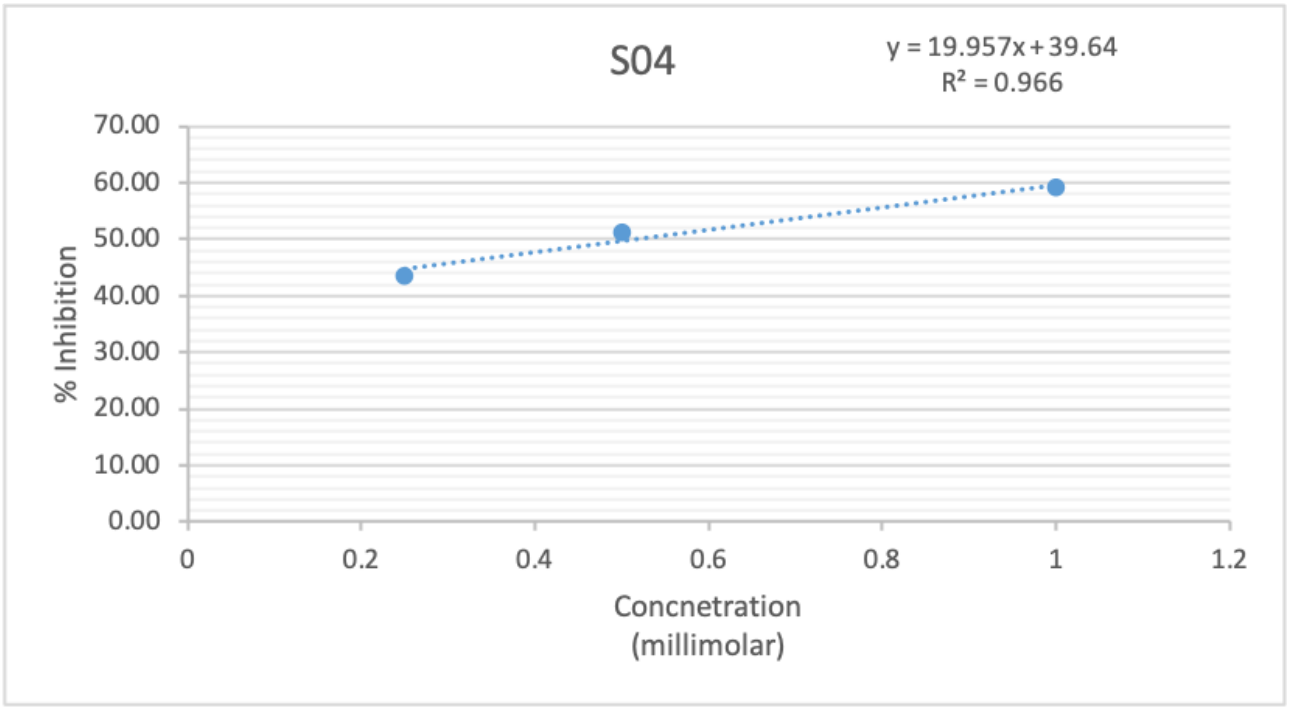


Figure 3.34 Inhibition concentration graph of **S04**

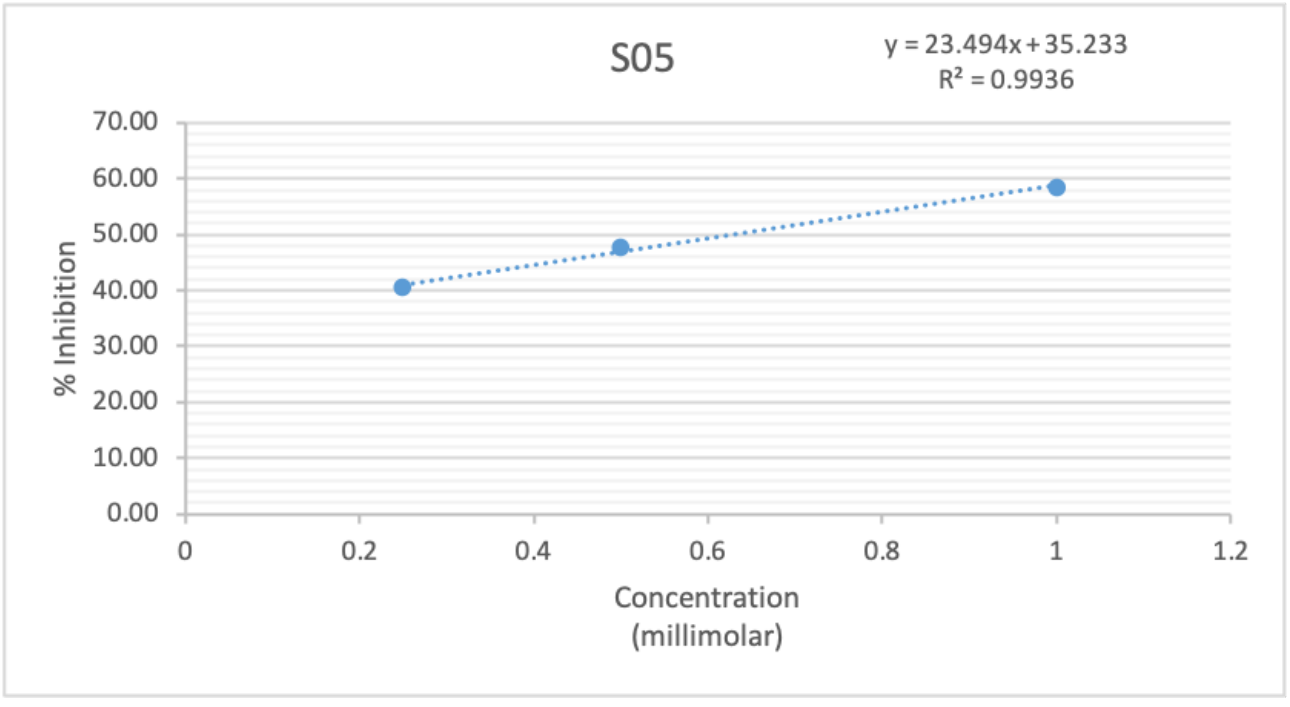


Figure 3.35 Inhibition concentration graph of **S05**

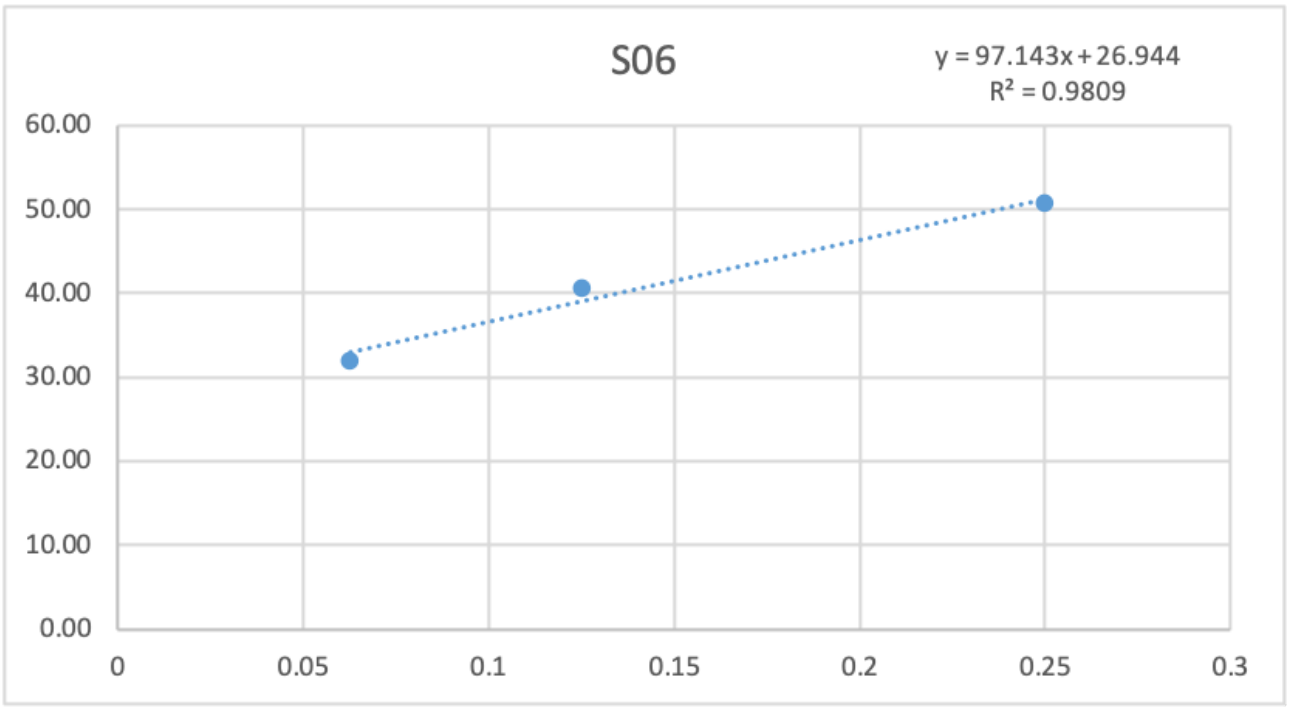


Figure 3.36 Inhibition concentration graph of **S06**

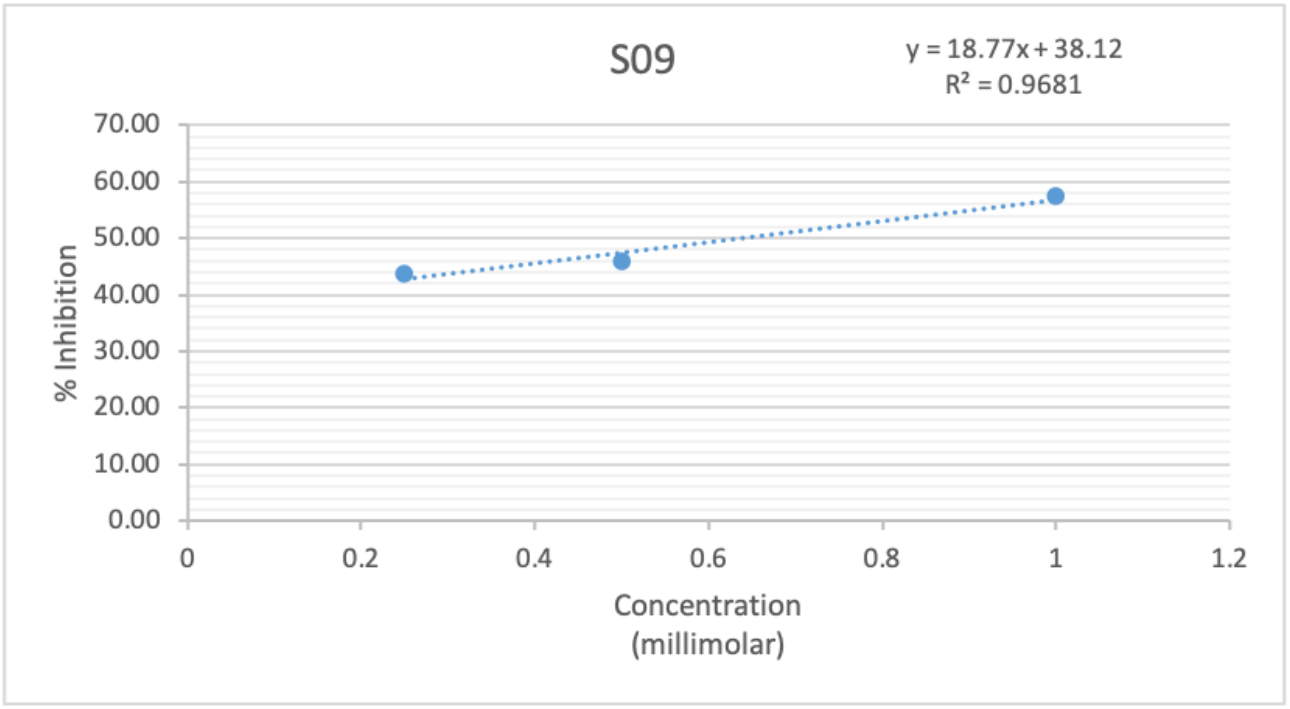


Figure 3.37 Inhibition concentration graph of **S09**

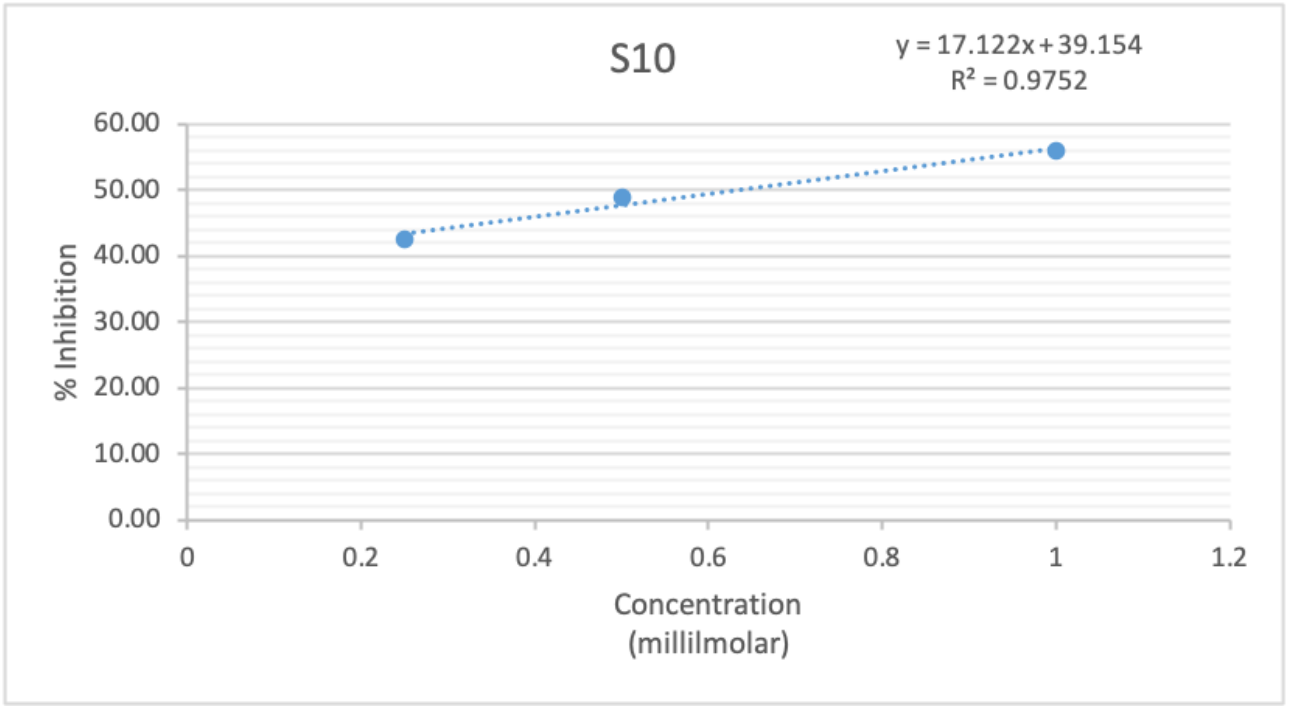


Figure 3.38 Inhibition concentration graph of **S10**

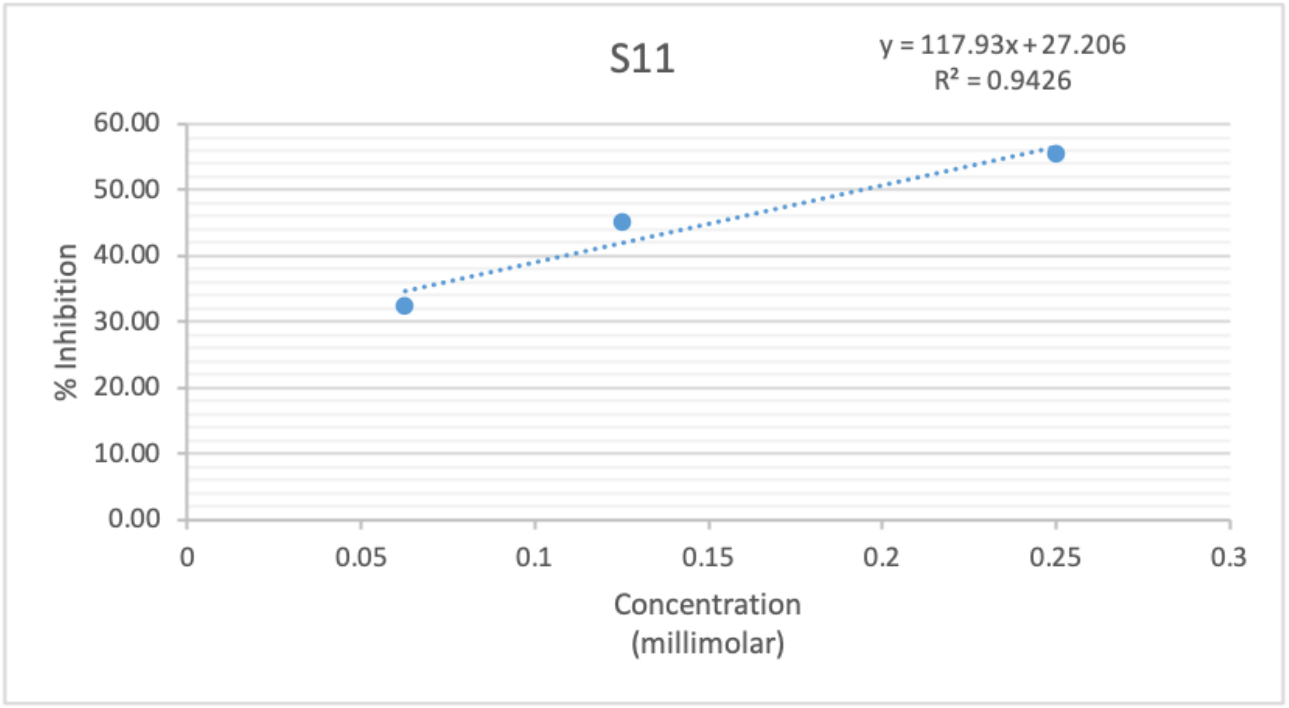


Figure 3.39 Inhibition concentration graph of **S11**

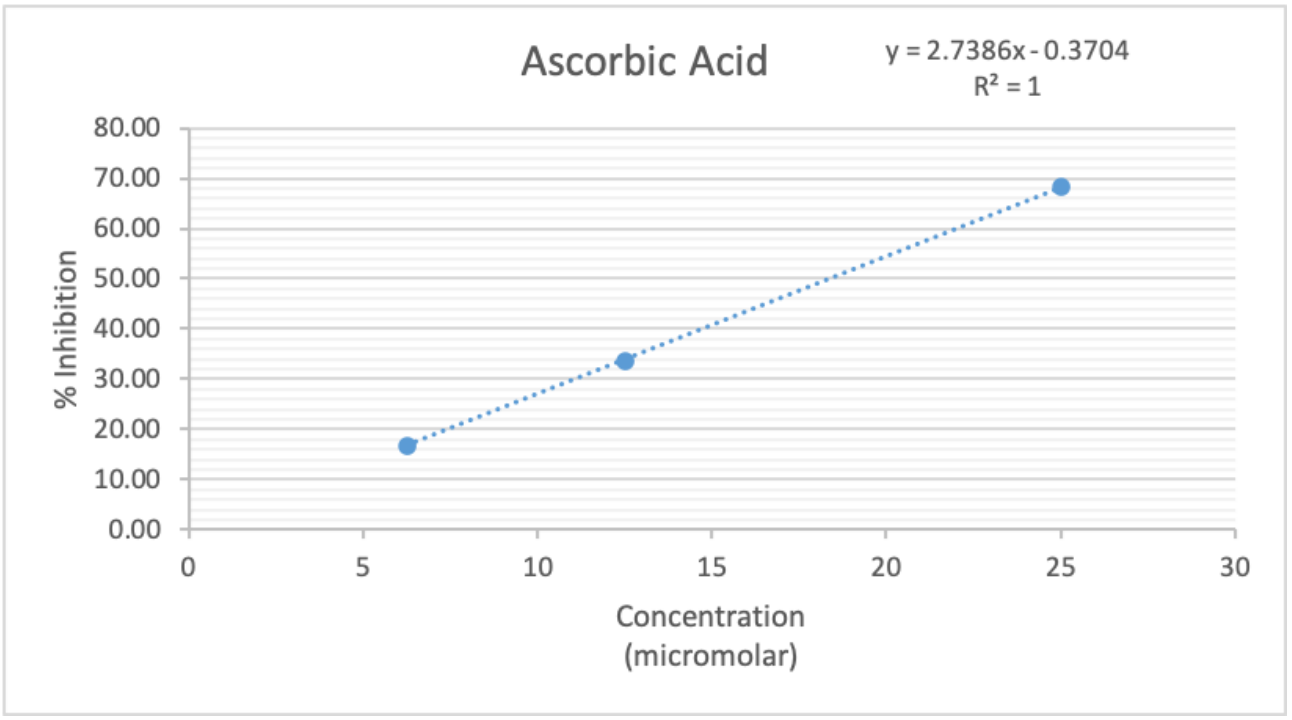


Figure 3.40 Inhibition concentration graph of Ascorbic Acid

Table3.3 Rank of compounds based on antioxidant activities

Rank	Compound
1	S11
2	S06
3	S02
4	S04
5	S10
6	S09
7	S05
8	S01

Chapter 4

Conclusion

Hesperetin and its derivatives are widely formulated in the cosmetic industry due to their well known activities of antioxidant and anti-inflammatory protecting the skin from both external and internal damages. In this research, ten hesperetin derivatives were successfully synthesized and characterized by NMR spectroscopy. In addition, eight derivatives were further evaluated for their antioxidant activities. According to DPPH radical scavenging assay, all compounds displayed favorable antioxidant activities. **S11** and **S06** were the most effective compounds to inhibit oxidation against free radicals of DPPH as their structure contains hydroxyl groups with no hydrogen bonding with other neighbour atoms and also the double bond presented in the structure may have inductive effect to form the proton radical easier which can react with DPPH radical. The IC_{50} results of **S11** and **S06** are 0.19 mM and 0.23 mM, respectively.

References

1. Panche, A.N.; Diwan, A.D.; Chandra, S.R. Flavonoids: an overview. *JOURNAL OF NUTRITIONAL SCIENCE*. **2016**, *5*, 1-15.
2. E.Brand-Garnys, E.; Denzer, H.; Meijer, H.; M.Brand, H. FLAVONOIDS: A REVIEW FOR COSMETIC APPLICATION. *J. Appl. Cosmetol*. **2007**, *25*, 145-159.
3. Ding, H.W.; Huang, A.L.; Zhang, Y.L.; Li, B.; Huang, C.; Ma, T.T.; Meng, X.M.; Li, J. Design, synthesis and biological evaluation of hesperetin derivatives as potent anti-inflammatory agent. *Fitoterapia.* **2017**, *121*, 212-222
4. Chen, X.; Li, X.F.; Chen, Y.; Zhu, S.; Li, H.D.; Chen, S.Y.; Wang, J.N.; Pan, X.Y.; Bu, F.T.; Huang, C. Hesperetin derivative attenuates CCl4-induced hepatic fibrosis and inflammation by Gli-1-dependent mechanisms. *International Immunopharmacology*. **2019**, *76*
5. Prakash, S.; Elavarasan, N.; Subashini, K.; Kanaga, S.; Dhandapan, R.; Sivanandam, M.; Kumaradhas, P.; Thirunavukkarasu, C.; Sujatha, V. Isolation of Hesperetin - A flavonoid from *Cordia sebestena* flower extract through antioxidant assay guided method and its antibacterial, anticancer effect on cervical cancer via *in vitro* and *in silico* molecular docking studies. *Journal of Molecular Structure.* **2020**, *1207*
6. Wang, Y.; Liu, S.; Dong, W.; Qu, X.; Huang, C.; Yan, T.; Du, J. Combination of Hesperetin and platinum enhances anticancer effect on lung adenocarcinoma. *Biomedicine & Pharmacotherapy*. **2019**, *113*
7. Huang, Y.C.; Liu, K.C.; Chiou, Y.L. Melanogenesis of murine melanoma cells induced by Hesperetin, a Citrus hydrolysate-derived flavonoid. *Food and Chemical Toxicology.* **2020**, *50*, 653-659
8. Zhang, Y.; Zheng, Y.; Shi, W.; Guo, Y.; Xu. T.; Li, Z.; Huang, C.; Li, J. Design, Synthesis and Investigation of the Potential Anti-Inflammatory Activity of 7-O-Amide Hesperetin Derivatives. *Molecules*. **2019**, *24*, 3663

9. Hidayat, M.A.; Fitri, A.; Kuswandi, B. Scanometry as microplate reader for high throughput method based on DPPH dry reagent for antioxidant assay. *Acta Pharmaceutica Sinica B.* **2017**, *3*, 395-400
10. Parhiz, H.; Roohbakhsh, A.; Soltani, F.; Razaee, R.; Iranshah, M. Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and Hesperetin: an updated review of their molecular mechanisms and experimental models. *Phytother Res*. **2015,** *29*, 323-331.
11. Jung, K.Y.; Park, J.; Han, Y.S.; Lee, Y.H.; Shin, S.Y.; Lim, Y. Synthesis and biological evaluation of Hesperetin derivatives as agents inducing apoptosis. *Bioorganic & Medicinal Chemistry*. **2017**, *25*, 397-407.
12. Usach, I.; Talens-Visconti, R.; Magraner-Pardo,L.; Jose-Esteban, P. Hesperetin Induce Melanin production in adult human epidermal melanocytes. *Food and Chemical Toxicology*. **2015**, *80*, 80-84.
13. Schnerch, D.; Yalcintepe, J.; Schmidts, A.; Becker, H.; Follo, M.; Engelhardt, M.; Wasch, R. Cell cycle control in acute myeloid leukemia. *American journal of cancer research.* **2012**, *2,* 508-528
14. Wikipedia - Interleukin 6 [online] https://en.wikipedia.org/wiki/Interleukin_6 (Accessed Aug 15, 2020)
15. Teixeira, J.; Gaspar, A.; Garrido, EM.; Garrido, J.; Borges F. Hydroxycinnamic acid antioxidants: an electrochemical overview. *Biomed Research International.* **2013**, *2013,* 1-11
16. Bassem, A.G.; Begnin or malignant? What is the difference? [online] https://www.spineuniverse.com/resource-center/spinal-tumors/benign-or-malignant-what-difference (Accessed Nov 12, 2020)

17. Gertrude, E.C.; Gopinathan, K.M. Know your skin cells III. The melanocytes [online] http://www.thecosmeticchemist.com/education/skin_science/know_your_skin_cells_iii_the_melanocytes.html (Accessed Oct 24, 2020)
18. Advaitaa, R.; What is Hyperpigmentation - causes, treatment, prevention tips [online] https://skinkraft.com/blogs/articles/hyperpigmentation (Accessed on Oct 29, 2020)

Biography

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