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Comparison of Antioxidant Activities of Mono-, Di- and Tri-substituted Coumarins

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Abstract: In this study, numerous coumarin compounds were synthesized by Pechmann and Knoevenagel methods, and the substitution of the formyl group was provided by the Duff reaction. The FTIR spectra and melting points of the synthesized compounds were compared with the literature values. The structures were also confirmed by GC/MS analysis. Besides, the synthesized coumarin derivatives were compared in terms of antioxidant activity according to DPPH and CUPRAC methods. The main aim of the study is to determine the effects of substituents on antioxidant activity.

Keywords: Coumarin, Pechmann reaction, Knoevenagel reaction, Duff reaction, DDPH method, CUPRAC method.

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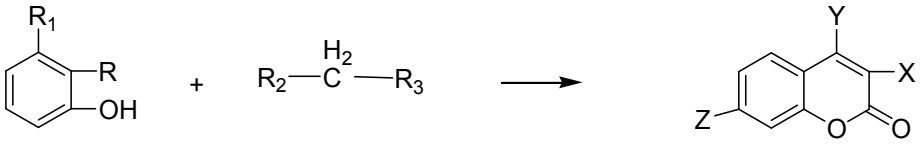
INTRODUCTION

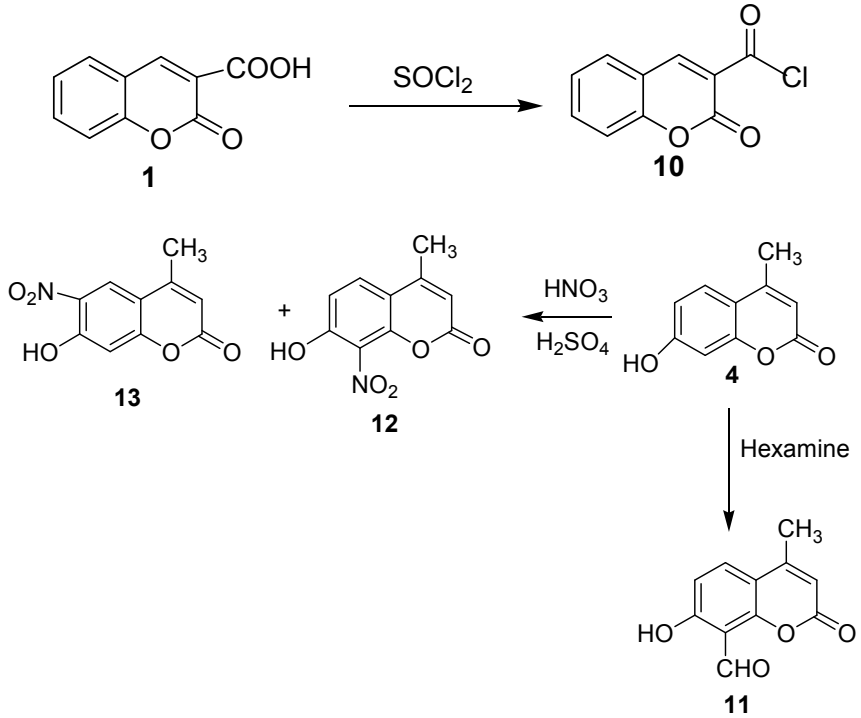
Studies on heterocyclic compounds have intensified over the past decade because they have important medicinal properties, especially small-ring heterocyclic compounds. Coumarin derivatives are also one of these and some properties such as antibacterial, antifungal, and anticoagulant agents in pharmaceutical industry were studied (1). As well as being an optical whitening reagent, it is present in the structure of fluorescent and laser dyes (2), perfumes, soaps, cleansing products and food, because of their pleasant smell and sweetening effect (3).

Coumarin is an oxygen-containing heterocyclic compound that is isolated from plants, especially from green plants. Synthesis of coumarin is usually performed with one of the reactions known as Pechmann, Perkin, Knoevenagel, Reformatsky, and Wittig. Various catalysts such as H_2SO_4, H_3PO_4, CF_3COOH, p-toluenesulfonic acid, $POCl_3$, $Bi(NO_3)_3 .5H_2O$, $AlCl_3$, $TiCl_4$, ZnI_2 and ionic liquids were used in these reactions.

Coumarin is a compound suitable for substitution at six points in the structure. The attachment of different substituents at different points adds different properties to the coumarins. Therefore, various mono-, di-, and tri-substituted coumarins have been synthesized (Scheme 1) and compared with standards for antioxidant activity according to DPPH and CUPRAC methods.



R	R_1	R_2	R_3	X	Y	Z	Compound
CHO	H	COOH	COOH	COOH	H	H	**1**
CHO	H	$COCH_3$	$COOC_2H_5$	$COCH_3$	H	H	**2**
CHO	H	COC_6H_5	$COOC_2H_5$	COC_6H_5	H	H	**3**
H	OH	$COCH_3$	$COOC_2H_5$	H	CH_3	OH	**4**
H	NH_2	$COCH_3$	$COOC_2H_5$	H	CH_3	NH_2	**5**
H	OCH_3	$COCH_3$	$COOC_2H_5$	H	CH_3	OCH_3	**6**
H	NH_2	COC_6H_5	$COOC_2H_5$	H	C_6H_5	NH_2	**7**
H	OH	COC_3H_7	$COOC_2H_5$	H	C_3H_7	OH	**8**
H	OH	COC_6H_5	$COOC_2H_5$	H	C_6H_5	OH	**9**



Scheme 1. Synthesized compounds.

EXPERIMENTAL SECTION

Instruments

Recovery of the solvents during the recovery of the products and during the crystallization processes was carried out in a BÜCHI Rotavapor R-200 rotary evaporator. In thin layer chromatography (TLC), a Silicagel $60F_{254}$ layer (Merck) was used. The plates were illuminated with a UV lamp to make the stains on the film sheets visible. Melting points of the coumarin compounds were examined in a Buchi Melting Point B-540 melting point apparatus. Fourier Transform Infrared (FT-IR) spectra of the synthesized coumarin compounds were taken on a Mattson 1000 Series FT-IR spectrometer. Mass spectra were checked with a Shimadzu GC-MS 2010 device.

Coumarin-3-carboxylic acid synthesis (1)

The synthesis was carried out by Knoevenagel reaction. 1.05 mL (10 mmol) of salicylaldehyde and 1.56 g (15 mmol) of malonic acid were refluxed with 1 mL of KSF catalyst with 3.3 mL of H_2O at 110-120 °C for 24 hours. The reaction was checked by TLC and when a new product was formed, the reaction was terminated with TLC control about the termination of the starting material. It was cooled down to room temperature. The remaining solid was triturated with 60 mL of methanol or ethyl acetate (4).

Yield(%): 95 m.p. 192 ^{0}C. Analysis of the GC / MS revealed MS peaks at m/z 190, 173, 146,

118, 89, 77, 63. M=(190), M-OH = M^{+1}(173), M^{+1}-CO = M^{+2} (145), M^{+2}-CO = M^{+3} (117). FTIR spectrum shows a 3174 cm^{-1} (-OH) stretching, 3057 cm^{-1} (Ar, C-H) aryl stretching, 1737 cm^{-1} (C=O) lactone carbonyl stretching, 1672 cm^{-1} (C=O) acid carbonyl stretching, 1607 cm^{-1},1566 cm^{-1}(C=C) double band stretching, and 1226 cm^{-1}(C-O) stretching (4,5).

Synthesis of 3-acetylcoumarin (2)

It was synthesized by Knoevenagel reaction with triethylamine as catalyst. 2.65 mL (0.025 mol) of salicylaldehyde and 3.175 mL (0.025 mol) of ethyl acetoacetate were refluxed in 15 mL of ethanol and 5 mL of triethylamine as catalyst at 80 °C for 12 hours. The reaction was continued until TLC showed that a new product was formed. When the starting material completely finished, the reaction was terminated and cooled to room temperature, then filtered through a Büchner funnel, then the remaining solid was crystallized from ethanol (6).

Yield(%): 60 m.p. 120 °C. Analysis of the GC / MS revealed MS peaks at 188, 173, 145, 118, 101, 89, 63. M=(188), M-CH_3 = M^{+1}(173), M^{+1}-CO = M^{+2} (145), M^{+2}-CO = M^{+3} (117). FTIR spectrum shows a 3031 cm^{-1}(Ar, C-H) aryl stretching, 2983 cm^{-1} (C-H) aliphatic carbon – hydrogen stretching, 1740cm^{-1} (C=O) lactone carbonyl stretching, 1675 cm^{-1} (C=O) ketone carbonyl stretching, 1612 cm^{-1} (C=C) double band stretching, 1265 cm^{-1}(C-O) stretching (6,7).

Synthesis of 3-benzoylcumarin (3)

It was synthesized by Knoevenagel reaction with pyridine as catalyst. 1.05 mL (0.01 mol) of salicylaldehyde and 3.47 mL (0.020 mol) of ethylbenzoylacetate were refluxed with 10 mL of ethanol with 0.08 mL of pyridine as catalyst at 80 °C for 12 hours. The reaction was checked by TLC and when a new product was formed and the starting material completely finished, the reaction was terminated. It was cooled to room temperature and filtered through a Büchner funnel. The remaining solid was crystallized from ethanol (8).

Yield(%): 60 m.p. 138 ^{0}C. Analysis of the GC / MS revealed MS peaks at 250, 222, 194, 173, 105, 77, 51. M=(250), M-CO = M^{+1}(222), M^{+1}-CO = M^{+2} (194). IR spectrum shows a 3046 cm^{-1}(Ar, C-H) aryl stretching, 1716 cm^{-1} (C=O) lactone carbonyl stretching, 1658 cm^{-1} (C=O) ketone carbonyl stretching, 1608 cm^{-1} (C=C) double band stretching, 1264 cm^{-1}(C-O) stretching (8).

Synthesis of 4-methyl-7-hydroxycoumarin (4)

It was synthesized by Pechmann reaction in the presence of a catalytic amount of oxalic acid. 1.1 g (10 mmol) of resorcinol (1,3-dihydroxybenzene) and 2.54 mL (20 mmol) ethylacetoacetate were reacted at 0.09 g (1 mmol) of oxalic acid catalyst and at 80 °C for 12 hours with some molecular sieve. The reaction was checked by TLC and when a new product was formed and the starting materials completely finished, the reaction was stopped and allowed to cool. As the reaction cooled, ice was added and then filtered on a Büchner funnel. The remaining solid was crystallized from ethanol (9).

Yield(%): 60. m.p.118 °C. Analysis of the GC / MS revealed MS peaks at 176, 148, 133, 116, 105, 91, 74, 65. M=(176), M-CO = M^{+1}(148), M^{+1}-CH_3 = M^{+2} (133), M^{+2}- OH= M^{+3} (116). FTIR spectrum shows a 3225 cm^{-1}(-OH) stretching, 3077 cm^{-1} (Ar, C-H) aryl stretching, 1673 cm^{-1} (C=O) lactone carbonyl stretching, 1586 cm^{-1} (C=C) double band stretching, 1272 cm^{-1}(C-O) stretching (9,10).

Synthesis of 4-methyl-7-amino-, 4-methyl-7-methoxy-, 4-phenyl-7-aminocoumarin

They were synthesized by Pechmann reaction with oxalic acid as catalyst. (10 mmol) of 3-substituted phenol and (12 mmol) of ethyl acetoacetate or ethylbenzoyl acetate were reacted at 0.09 g (1 mmol) of oxalic acid catalyst and a quantity of molecular sieve at 80 °C for 12 hours. The reaction was checked by TLC and when a new product was formed and the starting materials completely finished, the reaction was turned off and allowed to cool. As the reaction cooled, ice was added and then filtered through a spinner funnel. The remaining solid was crystallized from a mixture of ethanol-water (9:1) (11-13).

4-Methyl-7-aminocoumarin (5): Yield(%): 80 m.p. 223,8 °C. Analysis of the GC/MS revealed MS peaks at 175, 147, 132, 116, 91, 73, 65, 44. M=(175), M-CO = M^{+1}(147), M^{+1}-CH_3 = M^{+2} (132), M^{+2}- NH_2= M^{+3} (116). FTIR spectrum shows a 3249 cm^{-1} (-NH_2) stretching, 1661 cm^{-1} (C=O) lactone carbonyl stretching, 1603 cm^{-1} (C=C) double band stretching, and 1294 cm^{-1}(C-O) stretching (9,14).

4-Methyl-7-methoxycoumarin (6): Yield(%): 80 m.p.159,2 °C. Analysis of the GC / MS revealed MS peaks at 190, 162, 147, 116, 91, 77, 65. M=(190), M-CO = M^{+1}(162), M^{+1}-CH_3 = M^{+2} (147), M^{+2}-OCH_3 = M^{+3} (116). FTIR spectrum shows a 3028 cm^{-1}(Ar, C-H) aryl stretching, 1711 cm^{-1} (C=O) lactone carbonyl stretching, 1605 cm^{-1} (C=C) double band stretching, 1264 cm^{-1} (C-O) stretching (12,15).

4-Phenyl-7-aminocoumarin (7): Yield(%): 75 m.p.222,6 °C. Analysis of the GC / MS revealed MS peaks at 237, 209, 193, 116. M=(237), M-CO = M^{+1}(209), M^{+1}–NH_2 = M^{+2} (193), M^{+2}–C_6H_5=

M^{+3} (116). FTIR spectrum shows a 3245 cm^{-1}(N–H) stretching, 1661 cm^{-1} (C=O) lactone carbonyl stretching, 1598 cm^{-1} (C=C) double band stretching, 1293 cm^{-1}(C-O) stretching (13).

Synthesis of 4-propyl-7-hydroxycoumarin (8)

It was synthesized by Pechmann reaction in the presence of oxalic acid as catalyst. 1.1 g (10 mmol) of resorcinol (1,3-dihydroxybenzene) and 1.6 mL (10 mmol) of ethylbutyryl acetoacetate were added to the reaction mixture at 0.08 g (1 mmol) of oxalic acid and a quantity of molecular sieve were introduced. The reaction was checked by TLC and when a new product was formed and the starting materials completely finished, the reaction was turned off and allowed to cool. As the reaction cooled, ice was added and then filtered on a Büchner funnel. The remaining solid was crystallized in ethanol. Column chromatography was performed for purifying using chloroform: methanol (5: 1) as the eluent phase (16).

Yield(%): 65 m.p.133 °C. Analysis of the GC/MS revealed MS peaks at 204, 189, 176, 161, 148, 133, 105, 91, 77, 44. M=(204), M-CH_3 = M^{+1} (189) M-CO = M^{+1}(176), M^{+1}- C_3H_7 = M^{+2} (133). FTIR spectrum shows a 3253 cm^{-1} (O-H) stretching, 1691 cm^{-1} (C=O) lactone carbonyl stretching, 1614 cm^{-1} (C=C) double band stretching, and 1310 cm^{-1}(C-O) stretching (16,17).

Synthesis of 4-phenyl-7-hydroxycoumarin (9)

It was synthesized by Pechmann reaction with oxalic acid as catalyst. 2.0 g (12 mmol) of ethylbenzoyl acetate were reacted with 1.1 g (10 mmol) of resorcinol (1,3-dihydroxybenzene) and 0.314 g (1 mmol) of amberlyte 15 catalyst and a quantity of molecular sieve at 80 °C for 12 hours. The reaction was checked by TLC and in case a new product was formed, the starting materials were waited to finish completely, the reaction was turned off and allowed to cool. As the reaction cooled, ice was added and then filtered on a Büchner funnel. The remaining solid was crystallized from ethanol (18).

Yield(%): 65 m.p. 249 °C. Analysis of the GC / MS revealed MS peaks at 238, 210, 181, 152, 139, 127, 76, 63, 51. M=(238), M-CO = M^{+1}(210), $C_3H_7^+$ = (76). FTIR spectrum shows a 3115 cm^{-1}(O–H) stretching, 1684 cm^{-1} (C=O) lactone carbonyl stretching, 1592 cm^{-1} (C=C) double band stretching, 1268 cm^{-1}(C-O) stretching (18).

Synthesis of coumarin-3-carboxylic acid chloride (10)

To 1.9 g (0.1 mol) of coumarin-3-carboxylic acid, 10 mL of thionyl chloride ($SOCl_2$) was added to a round bottom flask, a few drops of pyridine was introduced, and the reboiler was fitted with a 5% NaOH solution in a hose over the cooler. It was refluxed at 80°C for three hours and cooled to room temperature. The white precipitate was washed several times with dry n-hexane (19).

Yield(%): 95 m.p. 145 °C. Analysis of the GC / MS revealed MS peaks at 208, 173, 145, 101, 89, 63. M=(208), M–Cl = M^{+1}(173), M^{+1}-CO = M^{+2} (145). FTIR spectrum shows a 3038 cm^{-1}(Ar, C–H) aryl stretching, 1747cm^{-1} (C=O) lactone carbonyl stretching, 1693 cm^{-1} (C=O) acyl carbonyl stretching, 1605 cm^{-1} (C=C) double band stretching, 1179 cm^{-1}(C–O) stretching and 759 cm^{-1} (C–Cl) stretching (19,20).

Synthesis of 4-methyl-7-hydroxy-8-formylcoumarin (11)

This compound was synthesized by Duff reaction. 6.5 g of 4-methyl-7-hydroxycoumarin, 12 g of hexamethylene tetramine (hexamine), 61.5 mL of glacial acetic acid were added and the mixture was refluxed at 85-90 °C for 12 hours. 92.18 mL of 20% HCl solution (49.83 mL of HCl, 42.33 mL of distilled water) was added and refluxed for a further 4 hours. The reaction was checked by TLC and when a new product was formed, it was cooled to room temperature and extracted with diethyl ether. The resulting extract was dried in sodium sulfate (Na_2SO_4) for 15 min. The solvent was removed on the evaporator to give a pale yellow solid. The residue was purified by crystallization from a solvent mixture of ethanol-1,4-dioxane (9:1) (21).

Yield(%): 65 m.p. 176 °C. Analysis of the GC/MS revealed MS peaks at 204, 176, 148, 119, 91, 77, 65, 44 M=(204), M–CO = M^{+1}(176), M^{+1} –CO = M^{+2}(148). FTIR spectrum shows a 3125 cm^{-1}(O-H) stretching, 1742 cm^{-1} (C=O) lactone carbonyl stretching, 1644 cm^{-1} (C=O) aldehyde carbonyl stretching, 1518 cm^{-1} (C=C) double band stretching, 1268 cm^{-1}(C-O) stretching (21-23).

Syntheses of 4-methyl-7-hydroxy-8-nitrocoumarin and 4-methyl-7-hydroxy-6-nitrocoumarin

1.2 g of 4-methyl-7-hydroxycoumarin was dissolved in 10 mL of H_2SO_4 at 0-5 °C. 1.5 mL of H_2SO_4 and 0.5 mL of HNO_3 were added dropwise to the reaction flask. It was stirred at 0-5 °C for four hours and then filtered on a Büchner funnel. When crystallized from ethanol, the solid residue on the filter paper was separated as 4-methyl-7-hydroxy-6-nitrocoumarin. The 4-methyl-7-hydroxy-8-nitrocoumarin which remains in the filtrate is cooled in the refrigerator and crystallized, then filtered through Büchner funnel (24).

4-Methyl-7-hydroxy-8-nitrocoumarin (12): Yield(%): 40 m.p. 254,6 °C. Analysis of the GC/MS revealed MS peaks at 221, 193, 176, 147, 135, 119, 91, 77, 65, 51, 44. M=(221), M–CO = M^{+1}(193), M^{+1}– OH = M^{+2}(176). FTIR spectrum shows a 3265 cm^{-1}(O-H) stretching, 1703 cm^{-1} (C=O) lactone carbonyl stretching, 1569 cm^{-1} (C=C) double band stretching, and 1314 cm^{-1}(C-O) stretching (24,25).

4-Methyl-7-hydroxy-6-nitrocoumarin (13): Yield(%): 40 m.p. 261 °C. Analysis of the GC/MS revealed MS peaks at 221, 193, 147, 135, 119, 91, 77, 65, 51, 44. M=(221), M-CO = M^{+1}(193), M^{+1} – NO_2 = M^{+2}(147). FTIR spectrum shows a 3244 cm^{-1}(O–H) stretching, 1727 cm^{-1} (C=O) lactone carbonyl stretching, 1615 cm^{-1} (C=C) double band stretching, 1290 cm^{-1}(C-O) stretching (24,25).

DPPH assay

Concentrations of standards and coumarin compounds were studied at 250, 500, and 1000 µg/mL. In the experiment, 1 mL of sample solution was mixed with 1 mL of DPPH solution in 0.002% concentration prepared in methanol. After standing for 30 minutes in the dark, absorbance was measured at 517 nm. The spectrophotometer was made zero with methanolic solution. The mixture prepared from 1 mL of methanol with 1 mL of DPPH solution was measured as control sample (26).

The DPPH solution was prepared daily, fresh and kept in the dark and at 4 °C, and covered with aluminum foil around the flask during use. DPPH inhibition is calculated by the following formula:

$$IC(\%) = \left[\frac{(A_0 - A_t)}{A_0}\right] \times 100$$

A_0: Absorbance of the control sample
A_t: The absorbance value of the sample being tested

CUPRAC assay

1 mL of a solution of $0.01.10^{-2}$ M copper(II) chloride, 1 mL of a solution of $7.5.10^{-3}$ M neocuproine, 1 mL of a 0.1 M ammonium acetate buffer (pH= 7.0) and 1 mL of a sample solution (250, 500, 1000 µg/mL) were placed in the tube and mixed. The mixture was allowed to stand at room temperature for 30 minutes, in the dark and absorbance was read at 450 nm. The reading was performed by zeroing against the blank, which was prepared by placing 1 mL of methanol instead of 1 mL of sample (27).

RESULTS AND DISCUSSION

The physical and spectroscopic values of the synthesized coumarin derivatives are in accordance with the literature values. The DPPH activity results of the coumarin derivatives are shown in Table 1, and the CUPRAC activity results are shown in Table 2.

In DPPH antioxidant capacity determination, as shown in Table 1, DPPH activity was observed to increase more as the concentration of only coumarin-3-carboxylic acid was decreased. DPPH activities decreased as concentration decreased in other substances. Especially some of the coumarin compounds which are coumarin-3-carboxylic acid chloride, 4-methyl-7-aminocoumarin and 4-phenyl-7-aminocoumarin, showed values close to DPPH activity at concentrations of 1000 µg /mL of α- tocopherol, NDGA, BHT, BHA and ascorbic acid as standard antioxidants.

In the determination of CUPRAC antioxidant capacity, as shown in Table 2, as the concentration decreased, CUPRAC reduction activities decreased as well. The compounds 4-Methyl-7-hydroxy-8-formylcoumarin and 4-methyl-7-hydroxy-8-nitrocoumarin showed values close to the CUPRAC reduction activity exhibited by BHA and ascorbic acid. CUPRAC reduction activity of only four of the synthesized compounds was observed. In other compounds, values are too small to be considered, so they are not added to the table.

In particular, 3-carboxylic acid chloride, 4-methyl-7-amino coumarin, 4-phenyl-7-aminocoumarin, 4-methyl-7-hydroxy-8-formyl, 4-methyl-7-hydroxy-8-nitro coumarin compounds have been found to be more effective in terms of antioxidant activity. These coumarin compounds can be evaluated with antioxidant activity properties.

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Table 1. The DPPH activity results of the coumarin derivatives

Entry	Sample	%DPPH (1000 µg/mL)	%DPPH (500 µg/mL)	%DPPH (250 µg/mL)
1	3-Benzoylcoumarin	5.6	0.4	0
2	Coumarin-3-carboxylic acid	3.2	4.4	5.2
3	3-Acetylcoumarin	6	2.8	0
4	Coumarin-3-carboxylic acid chloride	**84**	64.4	42.8
5	4-Methyl-7-hydroxycoumarin	18.4	11.2	10.4
6	4-Propyl-7-hydroxycoumarin	13.6	6.4	5.2
7	4-Phenyl-7-hydroxycoumarin	22	11.6	6.4
8	4-Methyl-7-methoxycoumarin	4.4	2	1.6
9	4-Methyl-7-hydroxy-8-formylcoumarin	14.4	11.6	7.6
10	4-Methyl-7-hydroxy-8-nitrocoumarin	28	13.6	3.6
11	4-Methyl-7-aminocoumarin	**82**	78	70.8
12	4-Phenyl-7-aminocoumarin	**82.8**	75.2	69.6
13	α-Tocopherol	96.4	93.6	93.2
14	NDGA*	95.6	93.2	92
15	BHT*	95.6	95.2	94.8
16	BHA*	96.8	94	93.6
17	Ascorbic acid	97.2	97.2	96.8

*BHA (Butylated Hydroxy Anisole) BHT (Butylated Hydroxy Toluene) NDGA (Nordihydroguaiaretic Acid)

Table 2. The CUPRAC activity results of the coumarin derivatives

Entry	Sample	%Absorbance (1000 µg/mL)	%Absorbance (500 µg/mL)	%Absorbance (250 µg/mL)
9	4-Methyl-7-hydroxy-8-formylcoumarin	**0.664 ± 0.0202**	0.199 ± 0.0263	0.022 ± 0.0021
10	4-Methyl-7-hydroxy-8-nitrocoumarin	**0.457 ± 0.0025**	0.226 ± 0.0036	0.116 ± 0.001
11	4-Methyl-7-aminocoumarin	0.209 ± 0.0035	0.136 ± 0.0062	0.095 ± 0.0043
12	4-Phenyl-7-aminocoumarin	0.196 ± 0.0020	0.142 ± 0.0037	0.098 ± 0.0049
16	BHA*	0.734 ± 0.0117	0.489 ± 0.001	0.456 ± 0.0035
17	Ascorbic acid	0.476 ± 0.0041	0.443 ± 0.001	0.432 ± 0.0007

*BHA (Butylated Hydroxy Anisole)Values are means ± SD.