DPPH Free Radical Scavenging Activity of Cinnamic Acid Derivatives

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Abstract

In this research, ester derivatives of cinnamic acid and benzenediol were synthesized, purified and then elucidated by FT-IR and NMR spectroscopy. Three compounds were synthesized examined for their antioxidant activity as free radical scavengers by DPPH assay. Among these compounds, compound **5c** showed the best antioxidant activity against DPPH with IC_{50} value of 56.35 µg/ml, followed by **5a** (26.10 mg/ml) and **5b** (47.90 mg/ml).

Keywords: cinnamic acid derivatives / antioxidant / DPPH scavenging activity

บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์เพื่อสังเคราะห์อนุพันธ์กลุ่มเอสเทอร์ของกรดทรานส์ซินนามิกโดยนำมาทำปฏิกิริยากับสารกลุ่มเบนซีนไดออล และนำมาศึกษาฤทธิ์การต้านอนุมูลอิสระ โดยทำการทดสอบฤทธิ์การต้านอนุมูลอิสระด้วยวิธี DPPH และวัดผลด้วยค่า IC_{50} จากผลการศึกษาพบว่าสารที่สังเคราะห์ได้ทั้งสามตัว มีฤทธิ์ต้านอนุมูลอิสระโดยที่อนุพันธ์ **5c** มีฤทธิ์การต้านอนุมูลอิสระดีที่สุดด้วยค่า IC_{50} 56.35 ไมโครกรัมต่อมิลลิลิตร ตามด้วยอนุพันธ์ **5a** (26.10 มิลลิกรัมต่อมิลลิลิตร) และ **5b** (47.90 มิลลิกรัมต่อมิลลิลิตร) ตามลำดับ

คำสำคัญ: อนุพันธ์กรดทรานส์ซินนามิก / สารสังเคราะห์ / ฤทธิ์การต้านอนุมูลอิสระ

Introduction

Cinnamic acid and its derivatives are known ingredients commonly used in cosmetics as fragrance ingredients and sunscreen agents. It is mainly found in Cinnamomum cassia BLUME and Panax ginseng. Cinnamic acid derivatives possess a wide range of biological activities (Gruenwald, 2010; Budavari, 1996). The antioxidant activities of the cinnamic acid derivatives are also studied. The antioxidant capacity relative to trolox represented that the cinnamic acid derivatives have more antioxidant ability compare with benzoic acid in the same substituted functional group (Natella, 1999).

Benzenediols, also known as dihydroxybenzenes are phenolic compounds having the antioxidant activity. Hydroquinone shows the highest redox potential of three benzenediols and possesses stronger inhibitory activity of melanin synthesis in melanocytes than resorcinol

and pyrocathechol. Hydroquinone is frequently used as a topical ingredient in skin whitening product to whiten the skin color. However, this use is banned in many countries because there are some carcinogenic effect evidences in animal test (Enguita & Leitao, 2013; Gillbro & Olsson, 2011; Matsumoto, 2016; Shibata et al., 1991). Some of lower cytotoxicity hydroquinone derivatives are developed and widely used in several whitening cosmetic products. In this study we had chosen to synthesize a series between cinnamic acid and benzenediols via acylation reaction. Oxalyl chloride had been used as an efficient and cheap acid activator reacted with cinnamic acids to produce an acyl chloride. Then the acyl halide was used acylating agents to acylate alcohols to form ester products and had been evaluated for their antioxidant activities.

Objectives

The objective of this research focuses on synthesizing a series of cinnamic acid ester derivatives bearing benzenediol moiety as potential skin whitening and antioxidant activities.

Scope of the study

Synthesize the cinnamic acid ester derivatives bearing benzenediol moiety, and investigate antioxidant activities as free radical scavengers by DPPH assay.

Materials and methods

Materials

Hydroquinone, resorcinol and pyrocatechol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cinnamic acid was obtained from Thermo Fisher Scientific Inc., (Waltham, MA). All other reagents were analytical grade.

Infrared spectral measurements were recorded on an ATR-FTIR spectrum 100 (PerkinElmer, USA). NMR spectra were acquired on an Ultrashield 400 MHz nuclear magnetic resonance spectrometer (BRUKER, USA). Absorbance spectra were obtained from 96-well Thermo Scientific™ Sterilin™ Clear Microtiter™ Plates with EZ Read 2000 microplate reader (Biochrom, England)

Experimental

Synthesis

Fifty millimole of Cinnamic acid was dissolved in 10 mL of CH_2Cl_2 in round-bottomed flask, and stirred gently at room temperature. Thirty drops of Oxalyl chloride were slowly added and a drop of DMF was then added. The reaction flask was stoppered with a glass stopper to prevent the solvent from evaporating and then stirred in room temperature for an hour. A piece of blue litmus paper was held over the vapor in the flask to check if the HCl gas was presented in the reaction. Small amount of $NaHCO_3$ was added until a blue litmus paper did not change to red anymore. Then 50 mmol benzenediol in 5 mL of THF and 15 mL of CH_2Cl_2 were added. The reaction mixture was stirred at room temperature for 2 hours. The reaction was checked by TLC technique using 30% ethyl acetate: hexane as the mobile phase system. The reaction mixture was poured to a separatory funnel and 50 mL of 10% $NaHCO_3$ solution was added. The separatory funnel was stoppered, shook gently and then vented to release the pressure in the funnel until no more gas release. The organic layer was collected and the solvent was removed by the rotary evaporator to give the pale yellow crude product which was purified by column chromatography using silica gel 60 (0.040 – 0.063 mm) as the

stationary phase and using a gradient elution of ethyl acetate: hexane (starting with 100% hexane) as the mobile phase. The products obtained were identified by IR and NMR spectroscopy

2-hydroxyphenyl cinnamate (**5a**)

IR (ν, cm^{-1}): 3381 (OH), 1702 ($C=O_{ester}$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, *J* = 16), 6.9 (1H, d), 7.0 (1H, dd), 7.1 (2H, m), 7.4 (3H, t), 7.6 (1H), 7.8 (1H, d, *J* = 16)

3- hydroxyphenyl cinnamate (**5b**)

IR (ν, cm^{-1}): 3337 (OH), 1702 ($C=O_{ester}$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, *J* = 16), 6.7 (3H, m), 7.2 (1H, t), 7.4 (3H, t), 7.6 (2H), 7.8 (1H, d, *J* = 16)

4- hydroxyphenyl cinnamate (**5c**)

IR (ν, cm^{-1}): 3391 (OH), 1699 ($C=O_{ester}$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, *J* = 16), 6.8 (2H, d, *J* = 7), 7.0 (2H, d, *J* = 7), 7.4 (3H, t), 7.6 (2H), 7.8 (1H, d, *J* = 16)

Antioxidant capacity assay

The antioxidant activity of cinnamic acid derivatives were measured on the basis of the radical-scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by DPPH assay, as previously reported (Alam, 2013) with minor modifications. The solutions of cinnamic acid derivatives (**5a**-**5c**) at various concentrations in DMSO were prepared. The test solution consisted of adding 50 µl of 1mM DPPH in methanol, a series of 10-120 µl of samples and total volume of solution were adjusted to 300 µl with DMSO in the 96-well microplate (Thermo Fisher Scientific). Then test solution was kept in the dark for 30 minutes and the absorbance was measured at 517 nm against a blank. The percentage of the DPPH radical scavenging was calculated using the equation as given below:

$$\%Inhibition = \frac{(Abs._{Control} - Abs._{Sample})}{Abs._{Control}} \times 100$$

Cytotoxicity Test

This assay was performed in 96-well plate in quadruplicate. First, plates were seeded with 200 µl of cell suspension or blank medium into well, and incubated at 37ºC humidified incubator with 5% CO_2 for 48 hours. Subsequently, culture medium was replaced with 200 µl of fresh medium containing test-compounds or 1%DMSO, and plates were further incubated for 24 hours. After incubation period, the plates were added with 50 µl of 125 µg/ml resazurin solution and incubated at 37 ºC humidified incubator with 5% CO_2 for 4 hours. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths by using the bottom-reading mode of fluorometer. The signal of test wells was subtracted with that of blank wells before calculations. Percent of cytotoxicity was calculated by the following equation:

$$\%Cytoxicity = [\,1 - (FU_T / FU_C)\,] \times 100$$

Whereas FU_T and FU_C were the mean fluorescent unit from cells treated with test compound and that treated with 1% DMSO, respectively.

The CC_{50} value was derived from dose-response-curve that was plotted between % cytotoxicity versus the sample concentrations by using SOFTMax Pro software (Molecular Devices, USA).

Results and discussion

The Synthesis of Cinnamic Derivatives

The cinnamic acid derivatives were synthesized according to Figure 1. Cinnamic acid was reacted with excess oxalyl chloride to give cinnamoyl chloride which was subsequently reacted with benzenediol without isolation. The obtained ester products were purified by column chromatography and characterized by IR and NMR spectroscopic technique.

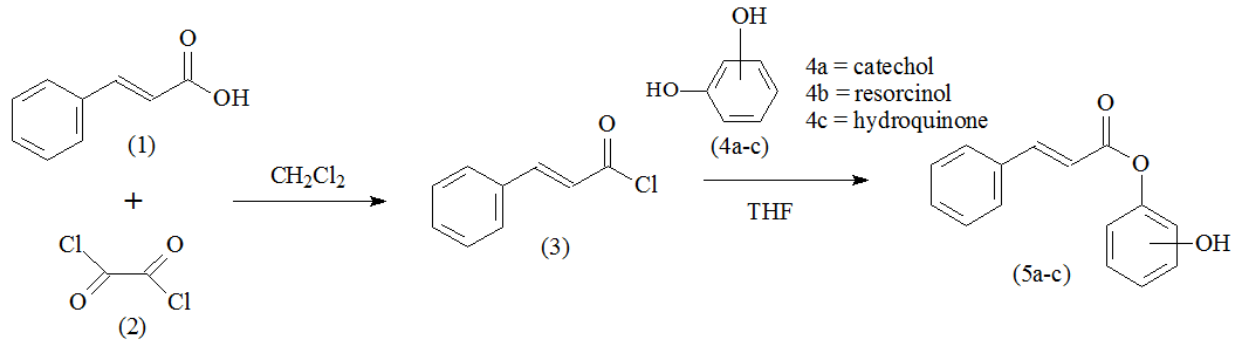

Figure 1: The synthetic routes to the cinnamic acid derivatives bearing phenolic moieties

The IR spectrum of each compound showed strong absorption peak at 1702, 1702 and 1699 cm^{-1} represented ester carbonyl functionality and also showed hydroxyl absorption peaks at 3381, 3337 and 3391 cm^{-1} as shown in Figure 2. The chemical structures of compound 5a-5c were confirmed by 1H-NMR as indicated previously. All three 1H-NMR spectra showed characteristic trans vinylic protons at δ 6.6 and 7.8 ppm with coupling constant of 16 Hz (J=16) represented alkene moiety of cinnamic acid molecule. Compound 5a-5c showed different peaks around δ 6.6-7.2 ppm. In 5a spectrum showed δ 6.9 (1H), 7.0 (1H), 7.1 (2H) represented chemical shift of protons of catechol moiety. In 5b spectrum showed δ 6.7 (3H) and 7.2 (1H) represented protons of resorcinol moiety and in 5c spectrum showed δ 6.8 (2H), 7.0 (2H) represented of hydroquinone moiety.

Antioxidant Studies: DPPH Assay

The antioxidant activities by DPPH assay of **1**, **4a**, **4b**, **4c**, **5a**, **5b** and **5c** were determined. The graphs of % inhibition against the concentration (g/ml) were plotted and the free radical scavenging activity of each compound was evaluated as the IC_{50} value, as shown in Figure 3. The IC_{50} of test compounds were summarized in Table 1.

Table 1: IC_{50} and CC_{50} value of selected compounds

compound	IC_{50} (µg/ml)	CC_{50} (µg/ml)
1	-	-
4a	45.90	-
4b	28.60	-
4c	1.81	20.23
5a	26100.00	-
5b	47900.00	-
5c	56.35	>100.00

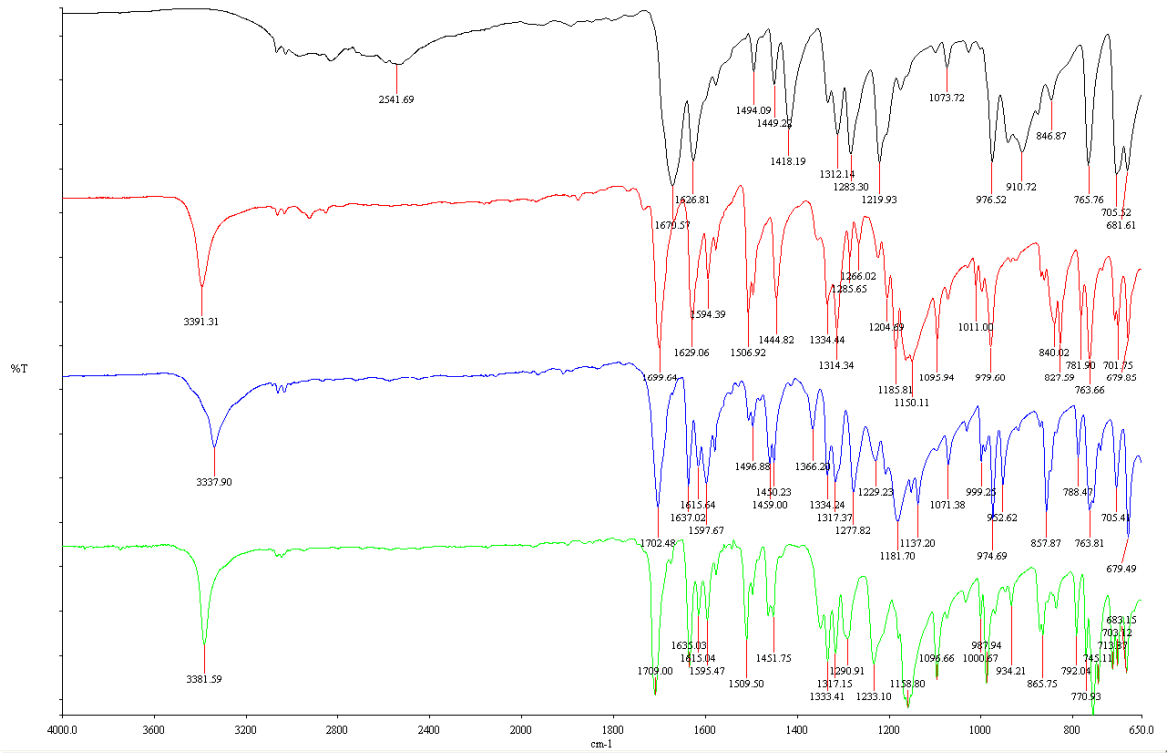

Figure 2: ATR-FTIR spectrum of compound 1, 5a-5c.

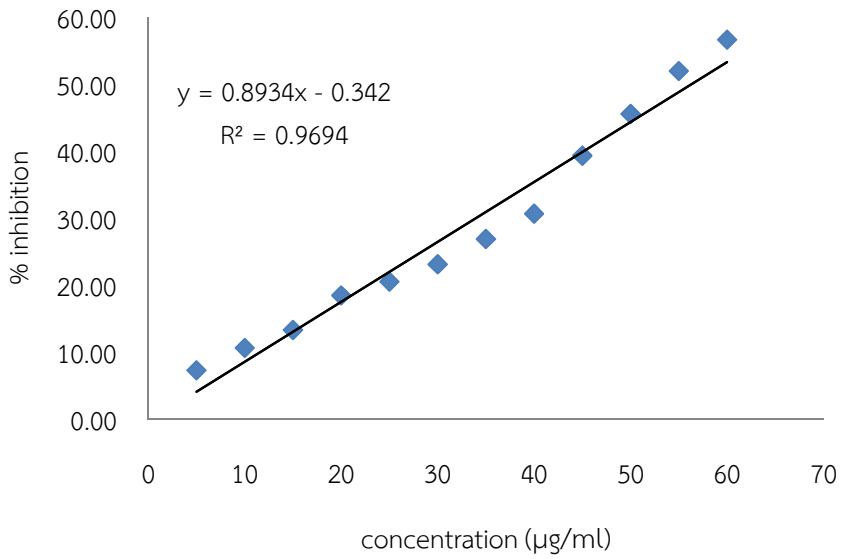

Figure 3: Correlation between antioxidant activity and concentration of compound 5c

All of the test compounds except compound **1** exhibited inhibitory activity. Compound **4a** (hydroquinone) showed the highest DPPH scavenging activity followed by **4b** (resorcinol), **4a** (pyrocatechol), **5c**, **5a** and **5b**, respectively. Among the 3 synthesized isomeric compound **5c** showed the highest antioxidant activity indicating that phenolic –OH at para position was the most active isomer.

Cytotoxicity

Compound **5c** and **4c** were selected for the cytotoxic test. The CC_{50} value of **5c** was higher than 100 µg/ml and 4c was 20.23 µg/ml, respectively.

Conclusion

The results of the present study showed that all the synthesis compounds between cinnamic acid and benzenediols exhibited antioxidant activity. Among the three compounds, compound **5c** had the highest potency of DPPH free radical scavenging activity, with the IC_{50} value of 56.35 µg/ml, followed by compound **5a** (26.10 mg/ml) and compound **5b** (47.90 mg/ml). Even the compound **5c** displayed lower potency of DPPH free radical scavenging activity than that of hydroquinone; it posed lower cytotoxicity than hydroquinone and higher chemical stability. Thus, ongoing studies are being conducted to test other properties and modified some functional group to increase some activities as a whitening agent in cosmetic product.

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