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ARTICLE

Nicotine Detoxification of Rutin, Quercitrin, and Chlorogenic Acid Isolated from *Houttuynia cordata* by Reducing Reactive Oxygen Species and Inducing Conversion from Nicotine to Cotinine

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Abstract The hypothesis of the present study is that methanol extract of *Houttuynia cordata Thunb* (MEH) and its targeted bioactive components including rutin, quercitrin, and chlorogenic acid can be effective in reducing reactive oxygen species (ROS) caused by nicotine and promoting nicotine to cotinine in HepG2 cell. Oxygen radical absorbance capacity (ORAC) of bioactive components and MEH was measured to assess free radical scavenging capacity. ROS inhibition ability of bioactive components and MEH was measured by 2',7'-dichlorodihydrofluorescein diacetate assay. The conversion rate of nicotine to cotinine by bioactive components and MEH was determined by the direct barbiturate assay method. ORAC value confirmed that MEH and its bioactive components provided an antioxidant capacity ranging from 126 to 138 μMtrolox equivalents/100 g. Compared to nicotine only, pretreatment of MEH, rutin, and quercitrin was revealed to effectively inhibit ROS production in HepG2 cell by up to 9, 7.4, and 14%, respectively. Nicotine conversion to cotinine after 120 min incubation was 1.7 and 1.4 times higher in rutin and chlorogenic acid pretreatment than the control, respectively. *H. cordata* and its targeted bioactive components could be a valuable natural ingredient for inhibiting ROS formation by nicotine as well as enhancing the rate of nicotine to cotinine turnover.

Keywords cotinine · HepG2 cell · *Houttuynia cordata Thunb* · nicotine · oxygen radical absorbance capacity · reactive oxygen species

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Introduction

Nicotine, one of the main chemical components in cigarettes, has been identified to induce oxidative stress by the generation of reactive oxygen species (ROS) (Balakrishnan and Menon, 2007; Muthukumaran et al., 2008). Accumulation of excess ROS in cells by endogenous and exogenous sources are toxic to cells, which cause damage to cellular organisms, thereby resulting in accelerating diseases such as cancer and diabetes (Lee, 2003a; Lin et al., 2012). Numerous studies have provided evidence that vitamin C, vitamin E, and polyphenol could act as protectiive agents against oxidative damages and disease caused by accumulation of ROS (Lee, 2003a; Balakrishnan and Menon, 2007; Sudheer et al., 2007; Sudheer et al., 2008; Al-Malki and Moselhy, 2013). Recently, many studies have focused on the biological activities of phytochemicals derived from plants, which are potent free-radical scavengers (Lee, 2003a; Balakrishnan and Menon, 2007; Sudheer et al., 2007). For instance, polyphenols have been considered to be relatively non-toxic bioactive substances and display diverse biological effects for preventing oxidative damages and cancer (Lee, 2003a; Brunetti et al., 2013; Kang et al., 2013; Park et al., 2013). The best-described properties of all groups of polyphenols is their strong capacity to inhibit oxidation and inflammation in cells than any other clinically available compounds (Brunetti et al., 2013; Kang et al., 2013; Lee et al., 2013; Park et al., 2013).

Nicotine-derived carcinogens such as nitrosamine ketone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), carcinogen derived from nicotine, is a cause of lung cancer (Lee, 2003a). On the other hand, cotinine [(5S)-1-methyl-5-(3-pyridyl)-pyrrolidin-2-one] which is another main metabolite of nicotine does not give any negative side effects to humans (Echeverria et al., 2011; Moran, 2012). In recent studies, cotinine has been revealed to show that memory retention, attention, and the elimination of fear memory was enhanced by improving the prevention of Alzheimer's disease (Moran, 2012). According to a previous study, mint naturally

occurring in peppermint increased the nicotine to cotinine conversion ratio in urine (Ghazi et al., 2011). Besides, green tea extracts including as polyphenols enhanced conversion nicotine to cotinine (Lee, 2003a).

Houttuynia cordata Thunb. (*H. cordata*), known as 'E-Sung-Cho' in Korea, is native to Japan, Korea, southern China, and Southeast Asia (Han et al., 2009; Miyata et al., 2009; Banjerdpongchai and Kongtawelert, 2011). Representative of polyphenols in *H. cordata* are chlorogeinc acid, quercitrin, and rutin (Brunetti et al., 2013; Lee et al., 2013; Park et al., 2013). It has been used as an oriental medicine due to a wide range of biological properties including preventing mutagenesis, oxidative, allergy, virus, and bacterial infection (Shin et al., 2010; Banjerdpongchai and Kongtawelert, 2011; Jang et al., 2011; Kim et al., 2012). In particular, quercitrin showed protective effects against damages caused by hydrogen peroxide in human lymphocytes, possibly by decreasing ROS (Boligon et al., 2012). In the case of chlorogenic acid, its antioxidant activities have been associated with free radical-scavenging ability as well as inhibitory effects on lipid hyperoxidation (Ishimoto et al., 2011). Rutin presents important properties for human health due to its signicant scavenging properties on oxidizing species such as hydroxyl radical, superoxide radical, and peroxyl radical (Almeida et al., 2010).

However, very few studies have been conducted for the effects of polyphenols such as chlorogeinc acid, quercitrin, and rutin on decreasing ROS induced by nicotine and promoting the pathway from nicotine to cotinine conversion in human hepatocytes. Therefore, the purpose of the present study was to focus on detoxifying nicotine by polyphenols from *H. cordata* and its extract through scavenging ROS, promoting oxygen radical absorbance capacity, and enhancing nicotine conversion to cotinine.

Materials and Methods

Standards and chemical reagents. Standards of nicotine, cotinine, rutin, quercitrin, and chlorogenic acid were purchased from Sigma-Aldrich (USA). All chemicals were of analytical grade. Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from Gibco (Introgen Corporation, USA). Non-essential amino acids, gentamicin, and penicillin were purchased from Sigma-Aldrich.

Sample preparation. *H. cordata* (HC) was obtained from Seongju-gun, Gyeongbuk, Korea in November, 2011. They were washed with deionized water, lyophilized, and pulverized into powder. The freeze-dried HC powder (4 g) was extracted in 200 mL of 50% methanol in a sonicator for 30 min and then filtered. The residue was additionally extracted twice in 100 mL and 50 mL of 50% methanol under the same conditions. The resultant sample was concentrated using a rotary evaporator (Rotary evaporator SB-1200, EYELA, China) at 50°C with a speed of 10 rpm. Lastly, the concentrate was diluted with 10 mL of 50% methanol.

Identification and quantification of bioactive components in HC extract. Ultra-performance liquid chromatography equipped with an Accela photo diode array detector, an Accela auto sampler, an Accela 600 pump, and a LCQ fleet (Thermo Scientific, Finland) was conducted for isolation of bioactive components in 50% methanol extract of HC. A Hypersil GOLD column (50×2.1 mm, 1.9 μm, Thermo Scientific, USA) was carried out at a flow rate of 0.2 mL/min at room temperature. Mobile phases consisted of solvents A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water). The linear gradient mobile phase program was used as follows: 0−2.19 min, 100–95% B; 2.19−3.26 min, 95−90% B; 3.26−7.29 min, 90−80% B; 7.29−12 min, 80−70% B; 12−3 min, 70−100% B; and 13−5 min, 100−100%. The injection volume was 1 μL.

HepG2 cell culture. HepG2 cell lines were purchased from the Korean Cell Line Bank (KCLB; Korea). Cells were cultured by DMEM supplemented with 10% FBS, 1% non-essential amino acids, 1% penicillin/streptomycin solution, and 0.1% gentamicin. They were maintained under an atmosphere of 95% air and 5% CO_2 at 37°C. The cell culture medium was changed at 2-day intervals. Passages # 120 to 130 were used for this study.

Oxygen radical absorbance capacity (ORAC) assay. The ORAC assay was implemented according to Huang et al. (2002). Briefly, a fluorescein stock solution (0.004 mM) was made in 10 mM phosphate buffer (pH 7.4) prepared immediately before use. The working solution was diluted with 10 mM phosphate buffer (pH 7.4) for final concentration of 10 nM. The diluted sodium fluorescein solution was made fresh daily. A 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was dissolved with 10 mM of phosphate buffer (pH 7.4) to adjust to a final concentration of 240 mM. A 150 μL of sodium fluorescein solution was added in each well. For calibration of standard curve, 25 μL of stock solution of trolox standard solutions (12.5 to 200 μM) were used. A 25-μL of samples (rutin, quercitrin, chlorogenic acid, and methanol extract of HC (MEH)) were filled in each well. In order to react, the plate was incubated for 30 min at 37°C. Reactions were then started by the addition of 25 μL of AAPH solution for a final volume of 200 μL. The fluorescence was measured by using a microplate reader (Varioskan Flash) at 485 nm for excitation and at 520 nm for emission. The fluorescence was read 10 times for 100 min. ORAC values were calculated using following equation.

$$AUC_{net} = AUC_{sample/standard} - AUC_{blank}$$

(AUC = the area under the curve)

$$\text{Trolox standard curve} = 15629x + 11125,\ R^2 = 0.904$$

Measurement of intracellular ROS. According to the method performed by Gong et al. (2010), 2',7'-dichlorodihydrofluorescein diacetate (2'7'-DCFH-DA) was implemented to measure the capacity of nicotine to generate ROS in a HepG2 cell. In order to confirm a non-cytotoxic concentration range of treatments, IC_{50}

was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay, measuring cell cytotoxicity derived from mitochondrial dysfunction in HepG2 cells. In brief, about 5×10^4 cells were seeded into a black 96-well plate. When the cells were confluent, cell media were removed and washed with PBS. For the treatment, rutin (100, 250, and 500 μM), quercitrin (100, 250, and 500 μM), chlorogenic acid (50, 100, and 250 μM), and MEH (100 mg/mL) were treated into cells for 2 h in non-cytotoxic concentration ranges. Subsequently, the cell media containing IC_{50} value of nicotine were suspended into the cells and sustained for 24 h in an incubator. After treatment, the cell media were sucked, and cells were detached by trypsin EDTA, followed by addition of 2'7'-DCFH-DA. After 30 min of reaction, the cell fluorescence was measured by using a micro plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland) at 488 nm for excitation and at 525 nm for emission.

Measurement of nicotine degradation ability. The quantity of cotinine converted from nicotine was measured by the direct barbiturate assay according to the manufacturer's instructions (Lee, 2003a). When the HepG2 cells approached 100% confluency, cell media were removed, and media containing nicotine was added into cells. For the treatments, rutin (100, 250, and 500 μM), quercitrin (100, 250, and 500 μM), chlorogenic acid (50, 100, and 250 μM), and MEH (100 mg/mL) were treated into the cells under the non-cytotoxic concentration ranges. They were incubated for 2 h, which is the half-life of nicotine. At various time points (10, 60, and 120 min), cell media were removed and washed three to four times with PBS. The cells were separated by treating trypsin EDTA and then centrifuged for 5 min at 1100 rpm. The supernatant was removed, and the cells were washed with PBS. The cells were sonicated for 30 s in 100 μL PBS and then added with 100 μL of distilled water. To measure the quantity of cotinine, 100 μL of 4 M sodium acetate buffer (pH 4.7), 40 μL of 1.5 M potassium cyanide, 40 μL of 0.4 M chloramin T, a mixture of 100 μL of 78 mM barbituric acid and acetone (50:50) were added into the cells and mixed well for 10 s. It was added into 40 μL of 1 M sodium metabolism before incubated for 15 min at room temperature. Absorbance at 490 nm was measured by a micro plate reader. This experiment was repeated three times, and the amount of cotinine was calculated by a standard curve.

$$\text{Cotinine standard curve} = 0.0626x - 0.0259,\ R^2 = 0.9503$$

Statistical analysis. The results were presented as representative data from triplicate sets of experiments. Data were expressed as the mean ± standard error of mean. Statistical analysis for comparison among groups was performed by using analysis of variance followed by Turkey's post-hoc test using Graphpad Prism 3.0 software (Graphpad, USA). Difference among means was considered statistically significant at $p<0.05$.

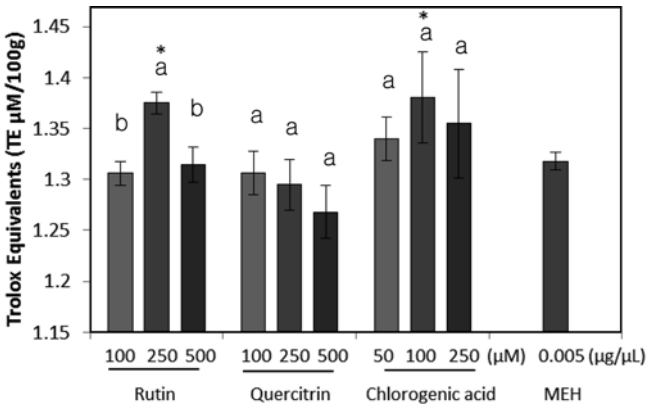

Fig. 1 Peroxy radical scavenging capacities of rutin, quercitrin, chlorogenic acid, and MEH, expressed as micoromole trolox equivalents. Different letters indicate a significant difference among treatments at $p<0.05$, *$p<0.05$, compared with MEH.

Results and Discussion

Oxygen radical absorbance capacity of rutin, chlorogenic acid, quercitrin, and MEH. Oxygen radical absorbance capacity (ORAC) of rutin, quercitrin, chlorogenic acid, and MEH were measured and then expressed as μM trolox equivalents (TE)/100 g (Fig. 1), ranging from 126 to 138 μM TE/100 g for various concentrations of rutin, quercitrin, chlorogenic acid, and MEH treatment. Chlorogenic acid (100 μM) showed the highest ORAC value (138 μM TE/100g), followed by 250 μM of rutin (137 μM TE/100 g), 250 μM of chlorogenic acid (135 μM TE/100 g), 50 μM of chlorogenic acid (134 μM TE/100 g), MEH (132 μM TE/100 g), 500 μM of rutin (131 μM TE/100 g), 100 μM of rutin (130 μM TE/100 g), 100 μM of quercitrin (130 μM TE/100 g), 250 μM of quercitrin (129 μM TE/100 g), and 500 μM of quercitrin (127 μM TE/100 g). ORAC value from quercitrin decreased with increasing quercitrin concentration. ORAC value of MEH was significantly higher than 500 μM of quercitrin while it was significantly lower than the others ($p<0.05$).

When polyphenols having the same structural backbone of C6-C3-C6, antioxidant activity was determined by the number of hydroxyl groups on their structure (Jo et al., 2009; Kang et al., 2010). The more the hydroxyl groups in the backbone structure, the stronger scavenging activity against peroxyl radicals (Jo et al., 2009; Kang et al., 2010; Sueishi et al., 2012; Zhang et al., 2013). For instance, myricetin and kaempferol have the same structure but ORAC value of myricetin was higher than that of kaempferol, because hydroxyl groups of myricetin have much more than kaempferol (Sueishi et al., 2012). In a similar pattern, rutin and quercitrin have the same backbone structure while the number of hydroxyl groups is different. Results from a current study revealed

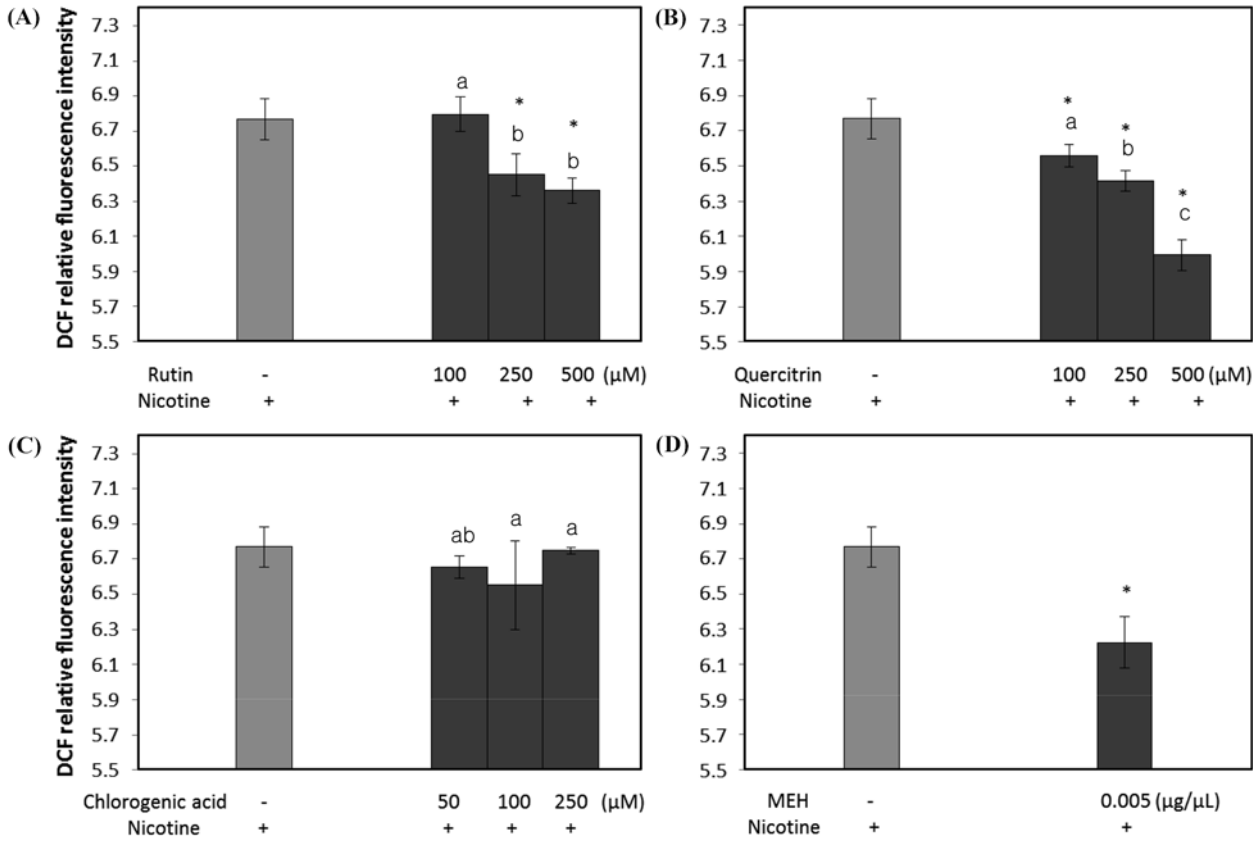

Fig. 2 Inhibitory effects of rutin, chlorogenic acid, quercitrin, and MEH on oxidative stress induced by nicotine in HepG2 cell. Data are presented as means ± SD. Different letters indicate a significant difference among treatments at $p < 0.05$, * as well as significant difference between negative control and treatments.

that the ORAC value of rutin was greater than that of quercitrin due to having more hydroxyl groups. Chlorogenic acid consisting of the ester between caffeic acid and (−)-quinic acid was revealed to provide a remarkable radical scavenging capacity greater than those of rutin and quercitrin (Fig. 1). MEH consists of 1% of rutin, 4% of quercitrin, and 0.8% of chlorogenic acid, implying that MEH appeared to provide less antioxidant capacity compared to rutin and chlorogenic acid alone (Fig. 1).

ROS scavenging activity by rutin, chlorogenic acid, quercitrin, and MEH in HepG2 cell. The scavenging effect of rutin, chlorogenic acid, quercitrin, and MEH pretreatment on ROS production induced by nicotine are shown in Fig. 2. ROS production was reduced up to 7.4% by rutin pretreatment, showing a dose independent manner. There was a significant difference in ROS scavenging ability between rutin (250 and 500 μM) and the control (Fig. 2A). A significant inhibition in ROS production ranging from 4.5 to 14% was observed with increasing concentration of quercitrin (Fig. 2B). In the case of pretreatment of chlorogenic acid, ROS scavenging capacity was effective up to 100 μM. At 250 μM of chlorogenic acid, the data shows that there was no inhibitory effect towards ROS (Fig. 2C). ROS scavenging capacity in pretreatment of quercitrin was stronger than rutin and chlorogenic acid. MEH containing 1% of rutin, 4% of quercitrin, and 0.8% of chlorogenic acid decreased ROS production induced by nicotine, indicating it was 9% lower than that of the control (Fig. 2D). The protective effect of MEH on ROS caused by nicotine was higher than those of pretreated rutin and chlorogenic acid. These results suggest that rutin, chlorogenic acid, quercitrin, and MEH enhanced capacity on the decrease of ROS generation caused by nicotine in HepG2 cells.

ROS, potent intracellular oxidants have been proposed as critical regulators of apoptosis (Gong et al., 2010). Results from the oxidative stress produce many unfavorable responses to the endothelial cells and thus is regarded as a critical pathogenic factor in the development of cancer, cardiovascular diseases, and brain dysfunction (Muthukumaran et al., 2008; Gong et al., 2010). Nicotine has been suggested as an inducer of apoptosis in several types of cells and also has been identified as a major factor to induce ROS production (Lee, 2003a; Erat et al., 2007; Tu et al., 2011). Several studies indicated that polyphenols could protect cells from toxic materials (Chen et al., 2000; Alía et al., 2006; Kim and Jang, 2009; Gong et al., 2010; Hoelzl et al., 2010; Jung et al., 2010; Yang et al., 2013). For instance, a previous study indicated that rutin reduces the cytotoxic effect induced by H_2O_2

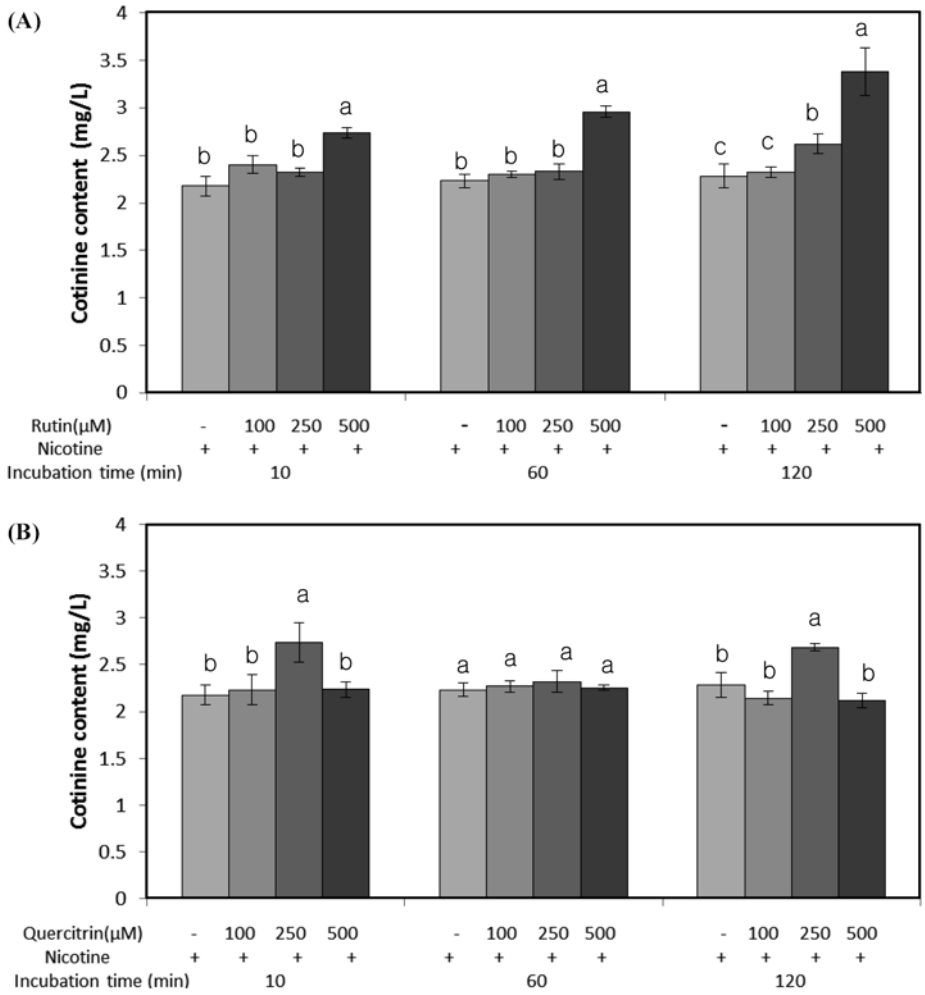

Fig. 3 Measurement of nicotine degradation ability (NDA) from nicotine to cotinine by rutin, chlorogenic acid, quercitrin, and methanol extract of *H.cordata* (MEH). Different letters indicate a significant difference among treatments at $p < 0.05$.

in human umbilical vein endothelial cells (HUVECs) against injury induced by H_2O_2 (Gong et al., 2010). A previous study suggested that quercitrin caused a significant protective effect against MeHg-induced ROS generation (Wagner et al., 2010). Chlorogenic acid also could protect HUVECs from H_2O_2-induced damage throughout its antioxidant properties (Wu et al., 2012). Likewise, results from our study revealed that pretreatment of rutin and quercitrin remove ROS generated in the presence of nicotine.

Measurement of cotinine formation converted from nicotine by rutin, chlorogenic acid, quercitrin, and MEH in HepG2 cell. Cotinine is converted from nicotine in the human liver by cytochrome P450, particularly CYP2A6. In order to examine the effect of rutin, chlorogenic acid, quercitrin, and MEH on the conversion of nicotine to cotinine, cotinine content (mg/L) was measured according to incubation time (10, 60, and 120 min) in HepG2 cells (Fig. 3). Overall, rutin treatment tended to promote nicotine to cotinine according to incubation time (Fig. 3A). Cotinine content of 500 μM rutin at 10 (2.7 mg/L) and 60 (3.0 mg/L) min were significantly higher than the control at each incubation time (Fig. 3A) ($p < 0.05$). At 120 min of incubation, 250 μM rutin (2.6 mg/L) and 500 μM rutin (3.4 mg/L) were significantly higher than the control (Fig. 3A) ($p < 0.05$). In the case of quercitrin, it did not show an effect on conversion from nicotine to cotinine between treatment and control (Fig. 3B). Fig. 3C shows the effect of chlorogenic acid on the conversion rate of nicotine to cotinine. The conversion effects of nicotine to cotinine were enhanced as the concentration increased at 120 min of incubation; 50, 100, and 250 μM of cotinine contents were 10, 12, and 14% higher than the control, respectively (Fig. 3C). MEH treatment promotes cotinine content during incubation (Fig. 3D). Cotinine content in the treatment of MEH were 1.1, 1.2, and 1.5 times greater than control at each incubation time (Fig. 3D). After 120 min incubation, 500 μM of rutin and 500 μM of chlorogenic acid were 1.7 and 1.4 times higher than control, respectively (Fig. 3A and 3C).

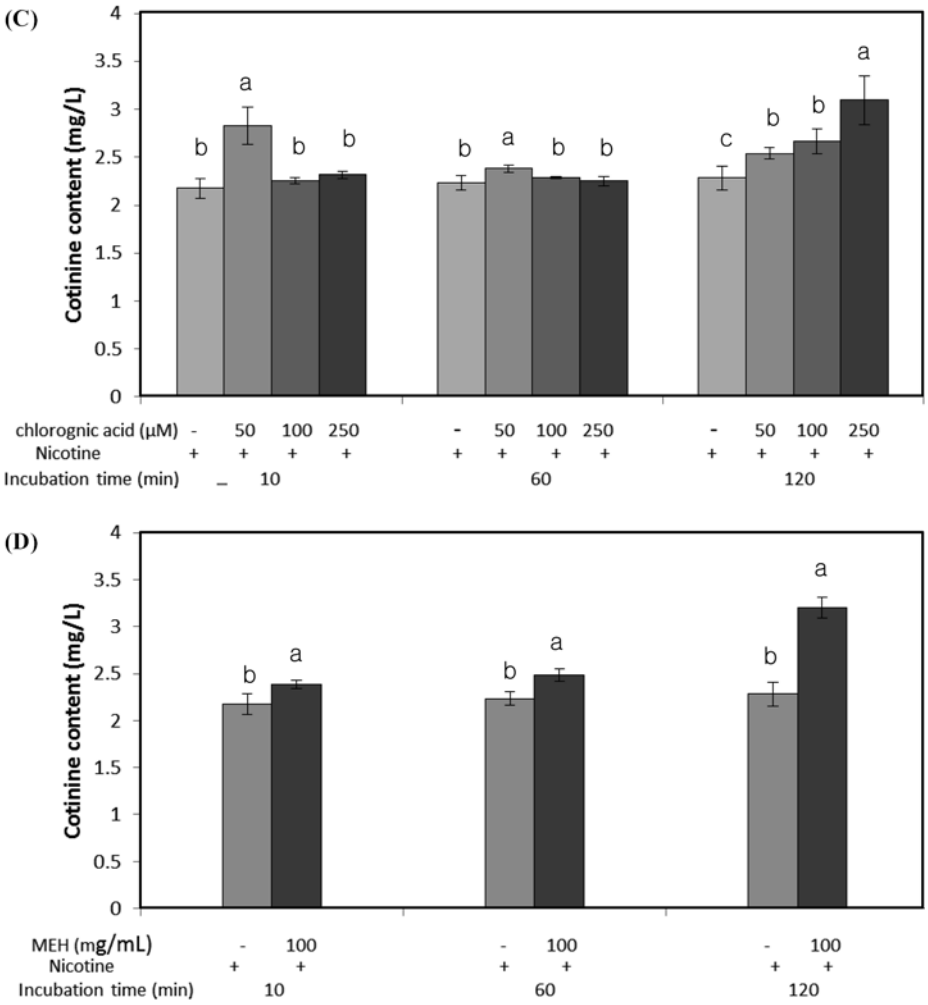

Fig. 3 Continued.

Previous studies have reported that polyphenol and herbs enhance the conversion of nicotine to cotinine (Lee, 2003a; Lee, 2003b). However, there are a limited number of studies showing that polyphenols have an effect on the turnover of nicotine to cotinine. The tea polyphenols were shown to positively affect conversion of nicotine to cotinine, indicating that its metabolites account for 50–60% of nicotine metabolism in humans (Lee, 2003a). Thus, it was suggested that polyphenols could inhibit the nicotine conversion into NNK causing lung cancer (Lee, 2003a). The present study also revealed that rutin, quercitrin, chlorogenic acid, and MEH have an effect on the turnover of nicotine to cotinine. Rutin has one more hydroxyl group than quercetrin. A previous study reported that the hydroxyl group could donate hydrogen atoms to the active radical and inhibit continuous radical reaction (Hong et al., 2003); thereby making the polyphenols easier to combine with nicotine (Hong et al., 2003). For these reasons, the cotinine content of rutin was shown to be higher than that of quercitrin. Kim et al. (2002) reported that phyto-extract mixture enriching phenolic compound have a positive effect on conversion of nicotine to cotinine. A similar pattern was found in the present study.

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