Original article

Flavonoids from *Camellia sinensis* (L.) O. Kuntze seed ameliorates TNF-α induced insulin resistance in HepG2 cells

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**Abstract**

The aim of this study is to discuss the non-catechin flavonoids (NCF) from *Camellia sinensis* (L.) O. Kuntze seed improving TNF-α impaired insulin stimulated glucose uptake and insulin signaling. Flavonoids had anti-metabolic syndrome and anti-inflammatory properties. It had widely been known for biological activity of catechins in tea, but very few research reports discussed the biological activity of non-catechin flavonoids in tea seed. We used HepG2 cell to treat with 5 μM insulin or with 5 μM insulin + 30 ng/ml TNF-α. Detecting the glucose concentration of medium, insulin decreased the glucose levels of medium meant that insulin promoted glucose uptake into cells, but TNF-α inhibited the glucose uptake effect of insulin. Furthermore, insulin increased the protein expressions of IR, IRS-1, IRS-2, PI3K-α, Akt/ PKB, GLUT-2, AMPK, GCK, pyruvate kinase, and PPAR-γ. TNF-α activated NFκB and MAPKs (p38, JNK1/2 and ERK1/2), iNOS and COX-2 which worsened the insulin signaling expressions of IR, IRS-1, IRS-2, PI3K-α, Akt/PKB, GLUT-2, AMPK, GCK, pyruvate kinase, and PPAR-γ. We added NCF (500, 1000, 2000 ppm) to cell with insulin and TNF-α. Not only glucose levels of medium were lowered, and the protein expressions of insulin signaling were increased, but p38, JNK1/2, iNOS and COX-2 were also reduced. NCF could ameliorate TNF-α induced insulin resistance through inhibiting p38, JNK1/2, iNOS and COX-2, and suggested that it might be used in the future to help control insulin resistance. This finding is the first report to present the discovery.

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**1. Introduction**

A high intake of carbohydrate or fat induced obesity and associated morbidities, such as insulin resistance, hyperglycemia and type 2 diabetes mellitus. Obesity induced chronic inflammation decreased tissues sensitive to insulin that inhibited glucose uptake and consumption (Lee et al., 2017). The inflammatory factors such as tumor necrosis factor-α (TNF-α) played key roles in the development of insulin resistance (Kang et al., 2016). The important target of the inflammatory pathways could disrupt insulin receptor substance-1 (IRS-1) and glucose transporter (GLUT) that cause insulin resistance (Shen et al., 2012). Subsequently, the risks of diabetes and cardiovascular diseases were increased by insulin resistance (Shimizu et al., 2015).

The tea plant, *Camellia sinensis* (L.) O. Kuntze, is a major crop in Taiwan, and tea is the most widely consumed beverage in the world, because of the leaves characteristic aroma, flavor and health benefits. The leaves of *Camellia sinensis* have also been used to traditional medicine to treat asthma, angina and vascular disease in ancient days (Grove and Lambert, 2010). In the present, some reports demonstrated (−)-catechins was the major component in leaves that acted as an agonist of the nuclear receptor protein peroxisome proliferator-activated receptor gamma (PPAR-γ) could be a current pharmacological target for the treatment of type 2 diabetes mellitus (T2DM) (Chen et al., 2009; Kim et al., 2008). The seeds of *Camellia sinensis* can be produced cooking oil, saponins and catechins. The rich saponins and catechins in *Camellia sinensis* seeds of *Camellia sinensis* (L.) O. Kuntze can be produced cooking oil, saponins and catechins. The rich saponins and catechins in *Camellia sinensis* seeds of *Camellia sinensis* (L.) O. Kuntze
seeds have been known intimately to lower body weight and serum lipid levels. In the present, many saponins and catechins from tea seeds findings about the anti-obesity activity, action mechanisms and potential health benefits in the treatment of obesity are reviewed (Kim et al., 2008; Song et al., 2016; Morikawa et al., 2013). However, except saponins and catechins, not much research evaluated the biological activity of the other flavonoids of the *Camellia sinensis* seeds. In this study, we excluded the saponins and catechins from *Camellia sinensis* seed by semi-preparation HPLC device, and investigate the non-catechin flavonoids (NCF) effect and mechanism on the prevention TNF-α-induced insulin resistance.

2. Materials and methods

2.1. Preparation non-catechin flavonoids (NCF) from *Camellia sinensis* (L.) O. Kuntze seed

The fresh *Camellia sinensis* (L.) O. Kuntze seed were dried separately and used for further analysis. Homogeneous *Camellia sinensis* (L.) O. Kuntze seed were extracted by a condensation reflux device with 70% (v/v) ethanol/H₂O (50 g/500 mL) for 6 h at 90 °C water bath, and the filtrates were freeze-dried. NCF were purified from above powder by a semi-preparative HPLC method. It was performed on Waters Alliance 2690 system coupled with Waters WFC III collector and the Waters 996 Photodiode Array Detector. Preparation NCF was performed on Lichrospher® 100 RP-18e column (250 mm × 10 mm i.d., 10 μm, Merck, Germany) by using a gradient mobile phase composed of Distilled water and acetonitrile, the gradient progressively started from 100% acetonitrile to 0% acetonitrile over 30 min at the flow rate 3.5 mL/min, the UV detection wavelength setup at 230 nm. The fraction of elution time at 2–6.5 min was collected and freeze-dried, the NCF powder was obtained.

2.2. Identification of NCF by TLC, HPLC, LC- Q- TOF-MS

TLC (Thin layer chromatography) experiment was carried out in the rectangular developing chamber (catalog no. Z126195, Sigma-Aldrich, USA), the development solvent were consisted of chloroform: methanol: Distilled water (65:35:10, v/v). NCF powder and saponins standard (from Labtools International Trading Limited, CAT No: LTR01508) were dissolved in methanol and directly applied on silica gel (SiO₂)-coated TLC plates (catalog no. 105626, Merck Millipore, Germany) with an Eppendorf micropipette. After developing on the TLC system, the spraying reagents as 10% ethanolic sulphuric acid, was used to identify the respective TLC results of NCF (a), tea saponins standard (b).

![TLC analysis with NCF (a) and standard saponins (b).](image)

The HPLC results of catechins (a), NCF (b)

![HPLC analysis with standard catechins (a) and NCF (b).](image)
compounds. The color of the spots was noted and Rf values were calculated.

HPLC (High Performance Liquid Chromatography) analysis was performed as follow: NCF powder and Catechin derivatives (Gallocatechin, Epigallocatechin, Catechin, Epicatechin, Epigallocatechin gallate, Gallocatechin gallate, Epicatechingallate, Catechin gallate) were dissolved in 10 mL 0.1% phosphoric acid and passed through 0.45 μm filter for HPLC analysis. HPLC conditions as follow: the separation was finished on the Lichrospher® 100 RP-18e column (250 mm × 4.6 mm i.d., 5 μm, Merck, Germany) and the UV detection wavelength setup at 280 nm, the mobile phase composed of 0.1% formic acid/99.9% Distilled water (A) and 0.1% formic acid/99.9% acetonitrile (B), the gradient progressively started from 100% A to 0% A over 30 min at flow rate 1.0 mL/min.

Flavonoids of NCF was identified by Acquity UPLC system (Waters Co., Massachusetts, USA) coupled with Quadrupole and Time of flight mass spectrometry (Q-TOF-MS), UPLC was used as a separation means equipped with a binary solvent delivery system, an auto-sampler, and an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.7 μm, Waters, USA). A gradient elution was achieved using two mobile phases: 5 mM ammonium formate

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**Table 1**

The flavonoid contents in extract of *Camellia sinensis* (L.) O. Kuntze seed determined by aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods.

| Flavonoid content (mg/g) | AlCl₃ | 2,4-D | Total |
|--------------------------|-------|-------|-------|
| NCF<sup>d</sup>          | 9.6 ± 1.2 | 31.3 ± 1.2 | 40.9 ± 2.4 |

<sup>a</sup> All results were presented as mean ± SD (n = 3). Flavonoid content (%) of solid samples = flavonoid (μg/mL) × total volume of ethanol extract (mL) × sample weight (g) × dilution factor × 10<sup>-6</sup> (g/μg) × 100. Flavonoid contents (%) of tinctures = flavonoid (μg/mL) × dilution factor × 10<sup>-6</sup> (g/μg) × 100.

<sup>b</sup> Levels calculated as quercetin equivalents.

<sup>c</sup> Levels calculated as naringenin equivalents.

<sup>d</sup> non-catechin flavonoids from *Camellia sinensis* (L.) O. Kuntze seed.
(A) and acetonitrile (B) at a flow rate of 0.3 mL/min with the following gradient conditions: 0 min, 95% A; 5 min, 40% A; 8.0 min, 20% A; 18.0 min, 2% A. The sample injection volume was 5 μL and the column temperature was maintained at 30 °C. Mass spectrometric analyses were performed on Waters VION LC Q-TOF equipped with an electrospray ionization (ESI) source in positive ion mode. The optimized parameters for the mass spectrometric analysis were as follows: capillary voltage, 1.5 KV; cone voltage, 20 V; desolvation gas flow rate, 1000 l/h; desolvation gas temp, 500 °C; collision energies, 6 eV. All mass data acquisition was performed on MassLynx software (Version 4.1, Waters, Cicero, USA).

2.3. Estimation of flavonoids by colorimetric method

It was very difficult to quantitate all flavonoids in NCF by HPLC and LC-Q-TOF-MS. Colorimetric methods were often used to estimate the flavonoids. Four major groups of flavonoids were defined by Mabry et al (1970), flavones and flavonols were found to complex stably with aluminum chloride, while flavanones and flavanonols reacted better with 2,4-dinitrophenylhydrazine (Nagy and Grancai, 1996; Chang et al., 2002; Woisky and Salatino, 1998). To estimate total flavonoids of NCF, the aluminum chloride and 2,4-Dinitrophenylhydrazine colorimetric method were used and modified from the procedure reported by Chang et al (2002). In this study, we found NCF can be fully dissolved in the 80% ethanol, so the extraction procedure refer as Chang et al (2002) was simplified to one step.

2.4. Cell culture

The methods were referenced our previous study (Chen et al., 2018). The HepG2 cell line was purchased from the National Development Center of Biotechnology (Taipei, Taiwan). The cells were cultured in 6-well plates at a density of 1 × 10⁴ cells/well and grown for 48 h to 80–100% confluence. The cells were then incubated for 2, 4 or 6 h in fresh medium containing 5 μM insulin only, 5 μM insulin and 30 ng/ml TNF-α, or 5 μM insulin and 30 ng/ml TNF-α into cells added with NCF (500, 1000, 2000 ppm).

2.5. MTT assay

The NCF (0–2000 ppm) was added in HepG2 cell (5 × 10⁴ per well) for 3 days, respectively. After washing, cells were incubated for 2 h with 0.5 mg/ml of MTT and added DMSO (100 μl), gentle shaking for 10 min. The solutions were transferred in 96-well plates and absorbance was recorded at 595 nm.

Table 2

| Glucose levels (nmole) in media | 2 h        | 4 h        | 6 h        |
|--------------------------------|-----------|-----------|-----------|
| Control                        | 48.53 ± 0.76 | 38.37 ± 1.19 | 37.57 ± 1.99 |
| Insulin (5 μM)                 | 44.69 ± 0.79* | 33.83 ± 1.32* | 32.80 ± 0.10* |
| Insulin + TNF-α (30 ng/ml)     | 47.52 ± 1.53* | 38.42 ± 0.72* | 37.25 ± 0.70* |
| Insulin + TNF-α + NCF (2000 ppm)| 41.28 ± 2.08 | 31.31 ± 2.16 | 29.76 ± 2.15* |
| Insulin + TNF-α + NCF (1000 ppm)| 40.72 ± 0.66* | 32.41 ± 0.46* | 31.87 ± 0.26* |
| Insulin + TNF-α + NCF (500 ppm) | 41.60 ± 1.36* | 36.36 ± 1.32 | 35.50 ± 0.43 |

* P < 0.05 vs control group.
# P < 0.05 vs insulin group.
* P < 0.05 vs insulin + TNF-α group.
2.6. Glucose uptake assay

At 2, 4, and 6 h, 30 μl of the medium was withdrawn and centrifuged at 500g for 5 min. 5 μl supernatant was mixed with 250 μl of glucose kit (BioVision, Milpitas, California, USA) at 37°C for 10 min. Absorbance at 500 nm was measured (Molecular Devices, Sunnyvale, California, USA).

2.7. Western blot analysis

The proteins expressions were identified by IR, IRS-1, IRS-2, PI3K-α, Akt/PKB, GLUT-2, AMPK, GCK, pyruvate kinase, PPAR-γ, p65, JNK1/2, p38, ERK1/2, iNOS and COX-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The analysis of relative expression of those proteins was followed as previously described (Chen et al., 2018).

2.8. Statistical evaluation

All statistical operations and figures scheming were performed by SigmaStat: Version 2.03 and SigmaPlot: Version 8.0 (Systat Software, Point Richmond, CA, USA). Data were indicated as mean ± SE. Statistical differences were determined by independent and paired Student’s t-test in unpaired and paired samples, respectively, and then used Dunnett’s or Student-Newman-Keuls test for further analysis if a significant difference was found. A p value < 0.05 was considered significant.

3. Results

3.1. Identification of NCF

Besides seed oil, many researches point out that the tea seed has much saponins and polyphones such as the Gallocatechin, Epigallocatechin, Catechin, Epicatechin, Epigallocatechin gallate, Gallocatechin gallate, Epicatechingallate, Catechin gallate. In this study, TLC, HPLC and UPLC-Q-TOF-MS were used to identify NCF, the results of TLC and HPLC were showed as in Figs. 1 and 2. NCF was neither saponins nor catechins, and the result with the high resolution UPLC-Q-TOF-MS system, 8 major flavonoids were identified, which were showed as in Fig. 3. These flavonoids have the similar structure as naringenin, including naringin, eriodictyol, luteoforol, hesperitin, naringenin-6-C-glucoside, proanthocyanidine, eriodictyol.

3.2. The flavonoids contents of NCF

Estimate the total flavonoids in NCF by the aluminum chloride and 2,4-Dinitrophenylhydrazine colorimetric method, the results were showed as in Fig. 5.
revealed that the flavonoids contents of NCF were 40.09 ± 1.04 mg/g in NCF (Table 1).

3.3. The cytotoxicity effect of NCF

After incubating 2–6 h, or 1–3 day, the Hep G2 cell was intact following treat NCF (500, 1000, 2000 ppm), respectively (Fig. 4).

3.4. The glucose uptake effect of NCF

The concentration of glucose in the medium declined when 5 μM insulin was added, indicating that glucose uptake was higher in the medium with added insulin than in the medium with no added insulin. When 30 ng/ml TNF-α was added along with the insulin, glucose concentration remained high indicating that glucose uptake was clearly inhibited (Table 2).

When NCF (500, 1000, 2000 ppm) was added to medium that had been treated insulin and TNF-α, the concentration of glucose declined dose dependently (Table 2).

3.5. The glucose metabolism effect of NCF

Compared to those in control group, HepG2 cells from insulin group were found by Western blot analysis to have higher levels of IR, IRS-1, IRS-2, PI3K-α, Akt/PKB, GLUT-2, AMPK, GCK, pyruvate kinase, and PPAR-γ proteins induced by insulin. Comparing insulin group with TNF-α group, we found TNF-α obviously decreased insulin-induced expression of those proteins. NCF reversed the damage caused by TNF-α (Figs. 5–8).

3.6. The MAPKs inhibited effect of NCF

Compared with insulin group, TNF-α group had higher expression the proteins p38, JNK1/2, iNOS and COX-2. NCF reversed TNF-α induced increases in p38, JNK1/2, iNOS and COX-2 (Fig. 9), but not ERK1/2 and p65 (data not shown).

4. Discussion

In this study, we proved non-catechin flavonoids (NCF) from Camellia sinensis seed extract that had no saponins and catechins, ameliorated TNF-α induced insulin resistance in HepG2 cells. This finding is the first report to present the discovery.

According to the results of chemical analysis, we proved that NCF did not contain saponins (Fig. 1) and catechins (Fig. 2), with high resolution UPLC-Q-TOF-MS analysis, we founded many flavonoids in NCF, and 8 major flavonoids were naringenin derivatives (Fig. 3). Two complementary colorimetric methods were employed.

![Fig. 6. Effect of NCF (500, 1000, 2000 ppm) with insulin or/and TNF-α on phosphatidylinositol-3-kinase-α (PI3K-α), serine/threonine kinase PI3K-linked protein kinase B (Akt/PKB), glucose transporter-2 (GLUT-2) protein expression in HepG2 cell treating 2, 4 or 6 h. Each value is presented as mean ± S.E. (n = 8). *p < 0.05 vs. RD; †p < 0.05 vs. HFD.](image-url)
to determine the amount of flavonoids in NCF. We found the major flavonoids in NCF were belonging to flavanones, ex. quercetin and naringin. The total flavonoids were 40.09 ± 1.04 mg/g (Table 1). Meanwhile, NCF had no cytotoxicity in this study (Fig. 4).

The concentration of glucose in the medium declined when 5 μM insulin was added, indicating that the Hep G2 cells ingested more glucose than no added insulin group. When 30 ng/ml TNF-α was added along with the insulin, glucose concentration remained high indicating that glucose uptake ability was inhibited (Table 2). It demonstrated that TNF-α damaged insulin activity. When NCF (500, 1000, 2000 ppm) was added to medium with insulin and TNF-α, the concentration of glucose declined dose dependently. We proved NCF could ameliorate TNF-α induced insulin resistance (Table 2).

Compared to those in control group, HepG2 cells from insulin group were found by Western blot analysis to have higher levels of IR, IRS-1, IRS-2, PI3K-α, Akt/PKB, GLUT-2, AMPK, GCK, pyruvate kinase, and PPAR-γ proteins. Comparing insulin group with TNF-α group, we found TNF-α obviously decreased insulin-induced expression of those proteins. NCF reversed the damage caused by TNF-α (Figs. 5–8). Insulin binds to cell surface insulin receptor (IR) which triggered a cascade of intracellular phosphorylation. Insulin binding stimulated the tyrosine kinase, and the kinase induced the phosphorylation of nonreceptor proteins, including insulin receptor substrate (IRS). IRS stimulated phosphatidylinositol 3-kinase (PI 3-kinase), and serine/threonine kinase PI3K-linked protein kinase B (Akt/PKB) activity, and then activated the glucose transporter (GLUT) to uptake glucose into cell (Mokashi et al., 2017; Cordero-Herrera et al., 2014). GLUT-2 was the principal transporter for transfer of glucose between liver and blood (Wang et al., 2016). When in insulin resistance and/or inflammation, IR, IRS-1, IRS-2, PI3K-α, Akt/PKB and GLUT-2 expressions or activities were deteriorated that reduced glucose uptake into cell (Sun et al., 2017). In this study, we found TNF-α induced insulin resistance and decreased GLUT-2 expressions as previously described (Chen et al., 2018). Adding NCF significantly ameliorated IR, IRS-1, IRS-2, PI3K-α, Akt/PKB and GLUT-2 expressions. NCF recovered the glucose uptake might be related with its improving insulin signaling.

AMP-activated protein kinase (AMPK) played a role to regulate cellular energy homeostasis (Chao et al., 2016). GCK was a key enzyme in glucose consumption (Lu et al., 2012). Pyruvate kinase was the enzyme that catalyzed the step of glycolysis (Bhuvaneswari and Anuradha, 2012). Peroxisome proliferator-activated receptors (PPARs) had been found to regulate insulin sensitivity, adipocyte differentiation, lipid metabolism, and glucose homeostasis (Polvani et al., 2016). Many studies had demonstrated that insulin resistance and/or inflammation impaired the interac-

![Fig. 7.](image-url)
The inflammatory state usually accompanied the metabolic syndrome (Odegard et al., 2016). In chronic inflammatory state, the insulin sensitivity of skeletal muscle, liver and adipocyte were impaired by accumulation of macrophages, and then contributed to the development of insulin resistance (Kuroda and Sakaue, 2017; Liu et al., 2016). These macrophage released amount of proinflammatory cytokines, such as TNF-α, IL-1β or MCP-1, which increased to account for 40% of total cells in adipose tissue (Kuroda and Sakaue, 2017; Liu et al., 2016; Budluang et al., 2016). In addition, TNF-α blocked tyrosine phosphorylation of the insulin receptor, interfered with insulin signal transduction, and activated the mitogen-activated protein kinases (MAPKs) and NF-κB pathway (Li et al., 2017; Santos et al., 2013). Activated MAPKs and NF-κB also inhibit insulin receptor phosphorylation (Vazquez-Prieto et al., 2012). As we known, TNF-α induced insulin resistance and impaired glucose uptake that was via insulin signaling damage. However, adding NCF significantly reversed TNF-α induced insulin resistance, and ameliorated the glucose uptake might be related with its improving insulin signaling.

The MAPK family (JNK, ERK, and p38) and NF-κB played essential roles in inflammation and innate immunity which could induce more serious damage. High levels of inflammatory cytokines induced by metabolic syndrome were capable of activating MAPKs and NF-κB (Kuroda and Sakaue, 2017; Liu et al., 2016; Budluang et al., 2016; Li et al., 2017; Santos et al., 2013; Vazquez-Prieto et al., 2012). Activated JNK (Priyanka et al., 2017), ERK (Lu et al., 2013), p38 (Huang et al., 2015) or NF-κB (Tsai et al., 2014) was detected in insulin resistance, and they had been proved that inhibiting expressions of those proteins expressions could improve its’ related disease (Priyanka et al., 2017; Lu et al., 2013; Huang et al., 2015; Tsai et al., 2014). Meanwhile, the downstream proteins of MAPKs and NF-κB, such as iNOS and COX-2, also played important roles to damage the insulin signaling. iNOS induced oxidative stress that had emerged as a potent modulator of the insulin-signaling pathway in obesity (Tsuzuki et al., 2015). The increased expression of COX-2 induced inflammation that contributed the hepatic insulin resistance (Chuang et al., 2011). Compared with insulin group, TNF-α group had higher expression the proteins p38, JNK1/2, iNOS and COX-2. NCF inhibited TNF-α induced inflammation that contributed the hepatic insulin resistance (Chuang et al., 2011). Compared with insulin group, TNF-α group had higher expression the proteins p38, JNK1/2, iNOS and COX-2. NCF inhibited TNF-α induced increases in p38, JNK1/2, iNOS and COX-2 (Fig. 9), but not ERK1/2 and p65 (data not shown). In this study, we confirmed that TNF-α inhibited the glucose uptake effect of insulin and induced insulin resistance, and it was related with TNF-α stimulated p65, JNK1/2, ERK1/2 and p38 that caused insulin resistance and abnormal glucose metabolism. We also found the proteins expressions of iNOS and COX-2 were induced significantly by TNF-α. Adding NCF significantly reduced p38, JNK1/2, iNOS and COX-2 expressions. NCF improved insulin resistance might come about through its inhibition of p38 and JNK1/2 pathways.

5. Conclusion

NCF inhibited TNF-α-induced p38 and JNK1/2 pathways, iNOS and COX-2 expressions which attenuating insulin-activated IR, IRS-1, IRS-2, PI3K-α, Akt/PI3K and GLUT-2 proteins and glucose uptake. Moreover, NCF ameliorated TNF-α-impaired AMPK, GCK, pyruvate kinase, and PPAR-γ and glucose metabolism. Based on these findings, NCF protected insulin receptor levels, enhanced glucose transport into cell and glucose consumption from inflammation. Excluded saponins and catechins from Camellia sinensis (L.) O. Kuntze seed, this finding is the first report to present the discovery. We suggest that NCF might potentially be used to control metabolic syndrome-induced insulin resistance.
Acknowledgments

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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