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Comparative antitumor and anti-inflammatory effects of flavonoids, saponins, polysaccharides, essential oil, coumarin and alkaloids from *Cirsium japonicum* DC

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ABSTRACT

Cirsium japonicum DC (Asteraceae) is a perennial thistle widely distributed in Asia, it is also consumed as functional food and herb in China. To analyze the health effects of *C. japonicum*, flavonoids, saponins, polysaccharides, essential oil, coumarin and alkaloids were extracted from *C. japonicum*, and their cytotoxicity to normal cells, anti-inflammatory effect against LPS-induced RAW 264.7 macrophages, antiproliferative effects against human lung adenocarcinoma cell A549 and anti-atherosclerosis activities in ox-LDL-stimulated RAW 264.7 cell were investigated. Results showed that coumarins exhibited strongest cell toxicity (IC_{50} = 162.7 μg/ml), and alkaloids showed slightly cytotoxicity at high concentration. Saponin could significantly inhibit cancer cell proliferation, especially for A549 cell and the inhibition rate reached to 47.0% at concentration of 200 μg/ml, which might result from the promotion of ROS generation in cancer cell. Saponin, essential oil and flavonoids could dose-dependently inhibit NO production in LPS-induced RAW 264.7 macrophages, whose inhibition rates were 65.4%, 73.0% and 80.4% at concentration of 50 μg/ml, respectively. Besides, saponin, essential oil and flavonoids also decreased lipid accumulation in ox-LDL-induced RAW 264.7 cell, which might be beneficial for cardiovascular health. These results indicated that different components from *C. japonicum* exhibited different bioactivities, providing useful information to better use thistle resources.

1. Introduction

Cirsium japonicum DC (Asteraceae) is a perennial thistle widely distributed in Asia area. In China, it is a widely used functional food and herb. The roots or whole plants of *C. japonicum* DC have long been used in the treatment of hepatitis, traumatic hemorrhage and hypertension, as well as tumor such as uterine cancer, liver cancer and leukaemia in folk (Liu et al., 2006). The *Cirsium* species also have been reported to exhibit many other bioactivities including hepatoprotective (E. H. Kim and Chung, 2013; Wan et al., 2014) and vasorelaxant (E. Y. Kim et al., 2008). *C. japonicum* water extracts could significantly reduce total cholesterol, triglycerides in serum in Ovariectomized Rats (Kwon et al., 2008), and inhibit human breast cancer cell MCF-7 proliferation and induce cancer cell apoptosis (D. Y. Kim et al., 2010). *C. japonicum* flavonoids promoted adipocyte differentiation and glucose uptake through activating PPARγ, which could benefit diabetes patients (Liao et al., 2012). *C. japonicum* ethanol extracts could also reduce hepatic triglyceride and cholesterol accumulation (Wan et al., 2014). We also reported previously that *C. japonicum* flavonoids protected human liver cells from chemical injury. *C. setidens* extracts could inhibit NO production in LPS-induced RAW 264.7 cell (Lee et al., 2009).

Secondary metabolites are organic compounds produced by plants, which exhibit beneficial health effects and may prove to be advantageous in the treatment of diseases like cancer, cardiovascular diseases and diabetes. The greatest feature of secondary metabolites is the large structural diversity and natural products continue to play an extremely important role in drug discovery and development process (Rungsung et al., 2015). Numerous compounds including flavonoids, phenolic acids, lignans, polyacetylenes, polysaccharide, sterols, triterpenes, sesquiterpene lactones, and alkaloids have been found in these species (Jeong et al., 2013; Jordon-Thaden and Louda, 2003; Khramova et al., 2011; Lai et al., 2014; Yim et al., 2003; Yoon et al., 2011). However, there are few reports about the bioactivities of the major components.

To better understand the relationship between main components

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and their bioactivities of *C. japonicum* DC, six components, including flavonoids, saponins, polysaccharides essential oil, coumarin and alkaloids were extracted from *C. japonicum* DC, and their cytotoxic, anti-inflammation, anti-cancer and anti-atherosclerosis activities were systemically evaluated and compared. It is hoped that the investigation will be beneficial for better using the thistle resource.

2. Materials and methods

2.1. Materials and reagents

The dried above-ground portion of *C. japonicum* DC, was purchased from Anhui take-off pharmaceutical co., LTD.

Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS) and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA). (3–4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), oil red O were obtained from Sigma Aldrich (St. Louis, MO, USA). Dil-ox-LDL and ox-LDL were obtained from Yiyuan Biotechnologies (Guangzhou, China). TriPure Isolation Reagent and FastStart DNA Master SYBR Green I kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). The water used in all assays was ultrapure by a Milli-Qwater purification system from Millipore. All other chemicals and solvents used in this study were of analytical grade.

2.2. Extraction and determination

For the total flavonoids and saponins, 100 g dried power of *C. japonicum* DC was extracted with 70% ethanol for 2h. After evaporation, crude extract was partitioned in water and extracted by petroleum ether, ethyl acetate, and n-butanol in sequence, which yielded petroleum ether fraction, ethyl acetate fraction, *n*-butanol fraction, and water fraction, respectively. The ethyl acetate fraction was taken as the total flavonoids (3.38g). The *n*-butanol fraction was separated by D101 macroporous resin eluted with a gradient of ethanol and water (0:100, 30:70, 50:50, 70:30, 5:95, v/v). The 50:50 fraction was obtained as total saponins (0.17g).

For the alkaloids, dried power (1000g) of *C. japonicum* DC was extracted with 95% ethanol for 2h. After remove the ethanol by evaporation, the concentrate was dissolved in 2% hydrochloric acid and then filtered to remove the insoluble. Then the PH of water solution was adjusted to 10–11, and chloroform was used to extract the alkaloids in the water. After evaporation to remove to chloroform, total alkaloids were obtained (0.56g).

For the coumarin, dried power (200g) of *C. japonicum* DC was extracted with 95% ethanol for 3h. After remove the ethanol by evaporation, the concentrate was separated by AB-8 macroporous eluted with water and 70% ethanol, which yielded water fraction and 70% ethanol fraction. 70% ethanol was extracted with chloroform to obtain the coumarin (0.97g).

Essential oil was extracted by traditional method-steam distillation, as previously reported (Qin Ma et al., 2015). Dried power (100g) of *C. japonicum* DC was soaked in water for 12h and then extracted for 7h at 100 °C by an essential oil extractor. Finally, essential oil (70 mg) in the extractor was collected after dehydration.

Polysaccharides was extracted by a water extraction and alcohol precipitation method. Specifically, dried power (100g) of *C. japonicum* DC was extracted by water for 3h at 100 °C three times. The extract was then decolored with D354FD resin and deproteinated using sevag method, and then crude polysaccharides was obtained (8.1g).

The extraction and separation procedures of total flavonoids, saponins, polysaccharides, coumarin and alkaloids from *C. japonicum* DC are shown in Fig. 1.

2.3. Anti-tumor activity

2.3.1. Cell culture

The cell lines used in this study were human hepatic L02 cells, hepatocellular carcinoma cell line hepG2 and human lung cancer cell A549 were cultured in DMEM (Gibco BRL) containing 10% fetal bovine serum (FBS), 1% penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 °C in humidified incubator with 5% CO_2. The medium was changed every 1–2 days.

2.3.2. Cell cytotoxicity

The cells (5×10^4 cells/mL, 100μL/well) were seeded in 96-well plates overnight, and then samples of various concentrations were added after the supernatant was removed. After incubation for 24h, cell viability was tested by MTT assay.

2.3.3. Cell proliferation analysis

The effects of samples on the proliferation of HepG2 cell and A549 cell were determined by MTT assay. Cancer cells in exponential growth phase were plated into 96-well culture plates (5×10^4 cells/mL, 100μL/well) and incubated overnight. After cells were attached to the plates, samples dissolved in fresh medium of different concentration were added and incubated for 24h. Then the supernatant was removed, and cells were washed with PBS for three times. 200 μL of fresh media containing 0.5 mg/mL MTT was added to each well followed by incubation for 4 h at 37 °C. After this period, the supernatant was discarded and 150 μL of DMSO was added to dissolve formazan crystals. The absorbance was measured at 490 nm after the plates were shaken for 8 min in an ELISA reader.

2.3.4. Measurement of intracellular ROS

DCFH-DA, a fluorescent probe, was used to measure the intracellular ROS level in cells. After treated, A549 cells were collected, washed by PBS twice and incubated with 10 μM DCFH-DA for 20 min at 37 °C. The fluorescence intensity was determined at 485 nm for excitation and 530 nm for emission using a TECAN infinite M1000Pro Multilabel Microplate Reader (Tecan Group Ltd., Switzerland). Relative DCF fluorescence intensity of treated cells was expressed as percentage of control (as 100%). Fluorescent images were also taken by an Olympus BX53 Microscope (Olympus Corporation, Tokyo, Japan).

2.4. Anti-inflammation activity

2.4.1. Nitric oxide (NO) and IL-6 measurement

RAW 264.7 cells were seeded in 24-well plate at 2×10^5 cells/mL After 24 h, cell was treated with LPS (1 μg/mL) in the absence or presence of various concentrations of samples. After incubation for 24 h, the nitrite concentration of the supernatants (100 μL/well) was mixed with 100 μL of Griess reagent, and the absorbance was measured at 540 nm. Concentrations of IL-6 and TNF-α in cell culture media were also quantified by ELISA kits according to the manufacturer's instructions.

2.4.2. Quantitative real-time PCR analysis

For the analysis of mRNA expression, RAW264.7 cells were collected, and total RNA was extracted using TriPure Isolation Reagent and then reverse transcribed into cDNA using a Roche reverse transcription-PCR Kit according to the manufacturer's protocol. qPCR was performed on a Real-Time PCR System 7500 (Applied Biosystems) with the FastStart DNA Master SYBR Green I kit. The nucleotide sequences of primers are as reported before (Qin Ma et al., 2018). The relative quantities of mRNA were normalized with GAPDH.

2.4.3. Western blot

Cells were lysed in RIPA buffer with protein inhibitors on ice. After denaturation, protein was loaded on SDS-PAGE gels, and then

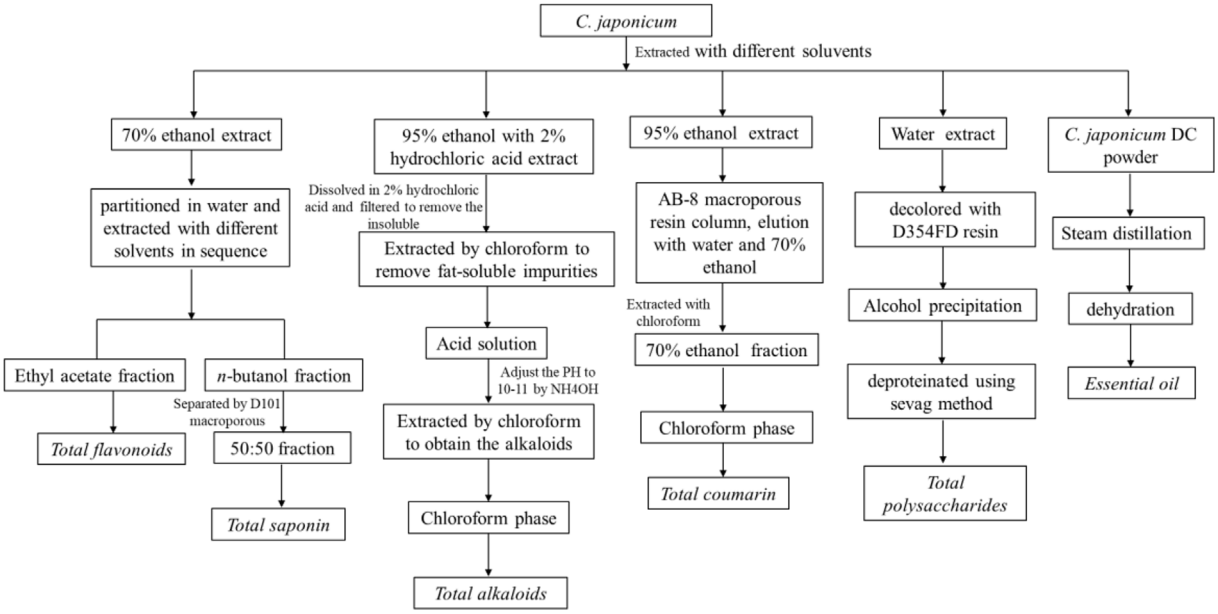

Fig. 1. The extraction and separation procedure of total flavonoids, saponins, polysaccharides, coumarin and alkaloids from *C. japonicum*.

transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After probed by antibodies, membranes were detected by Enhanced Chemiluminescent. Image J software was used for quantification.

2.5. Determination of lipid accumulation in ox-LDL induced RAW264.7 cell with oil red O staining

To observe lipid droplets in RAW 264.7 cell, cells were cultured with 80 μg/ml ox-LDL for 24 h in the presence or absence of main components from *C. japonicum*. After fixed with 4% paraformaldehyde, cells were stained in oil red O solution (0.5% w/v in 60% isopropanol) for 30 min. Images were taken by an Olympus microscope with cellSens software (Olympus Corporation, Tokyo, Japan).

2.6. Statistical analysis

The experiments were performed in three replications. The data are expressed as mean ± standard deviation. The significant differences between the means of parameters were calculated by Dunnett's multiple comparisons test ($P < 0.05$) using the SPSS 17.0.

3. Results and discussion

3.1. Antitumor activity

The MTT assay was first performed to assess the samples' cytotoxic effects on L02 cell. results are shown in Fig. 2A, coumarin showed significant cytotoxicity on L02 cell when the concentration increased to 100 μg/ml. Alkaloids also inhibited cell growth at a dose of 200 μg/ml. Saponin, essential oil, flavonoids, and polysaccharides didn't show cytotoxicity to L02 cell, and essential oil even promoted cell proliferation. The anti-proliferative activities of main compounds on HepG2 and A549 cell line were also measured by MTT assay. The cell proliferation inhibition rates of samples on HepG2 and A549 are shown in Fig. 2B and C. The coumarin also significantly inhibited the cancer cells growth, which might result from its cytotoxicity. Saponin and alkaloids exhibited anti-proliferative effect on cancer cells, followed by flavonoids. Polysaccharides showed little effect on cancer cell proliferation and essential oil could even promote the proliferation of cancer cells, especially for the A549 cell. Saponin could significantly inhibit cancer proliferation, especially for A549 cell with an inhibition rate of 47.0% at the concentration of 0.2 mg/mL. Excessive levels of Reactive oxygen species (ROS) are toxic to cells, which could kill the cells (Raj et al., 2011; Trachootham et al., 2009). So, we use DCFH-DA probe to investigate the ROS levels in A549 cell treated with or without samples. As we can see in Fig. 2D and E, saponin and alkaloids could significantly increase the fluorescence in A549, which indicated the increased ROS levels in cancer cell.

3.2. Anti-inflammation activity of main components from C. japonicum DC

3.2.1. Effect of main compounds on NO production in LPS-induced RAW 264.7 cell

To investigate the anti-inflammation activity of separated components from *C. japonicum* DC, their cytotoxicity was first evaluated by MTT assay. As we can see in Fig. 3A, saponin and flavonoids showed no cytotoxicity to RAW264.7 cell, and polysaccharides promoted the cell growth at all concentrations (25–200 μg/ml). Coumarin could significantly inhibit cell growth, especially at high concentration; the cell viability rate was only 9.4% at the concentration of 200 μg/ml. Alkaloids also showed some cytotoxicity to RAW 264.7 cell, the cell viability dropped from 88.25% to 52.4% when the concentration of alkaloids increased from 25 μg/ml to 200 μg/ml.

Because of the cytotoxicity of coumarin, only saponin, flavonoids, polysaccharides and essential oil were chosen for the NO production measurement (Fig. 3B). Among them, polysaccharides and alkaloids showed weak inhibition effect on the NO production induced by LPS. Flavonoids, saponin and essential oil showed excellent inhibition effects on NO production, the inhibition rates of which were 94.2%, 87.5% and 95.7% at the concentration of 100 μg/ml respectively. Cell morphology also showed that LPS could change cell to irregular form with pseudopodia, which was inhibited by the flavonoids, saponin and essential oil (Fig. 3C).

3.2.2. Effect of Saponin, essential oil and flavonoids on IL-6 and TNF-α secretions in LPS-induced RAW264.7 cell

We further investigated the effects of saponin, essential oil and flavonoids on pro-inflammatory cytokine secretion in LPS-induced RAW264.7 cell. LPS could significantly promote the proinflammatory cytokine IL-6 and TNF-α secretion (Fig. 4). Saponin, essential oil and flavonoids could significantly decrease the concentration of IL-6 in cell cultural medium (Fig. 4A) and also inhibit TNF-α (Fig. 4B) secretion.

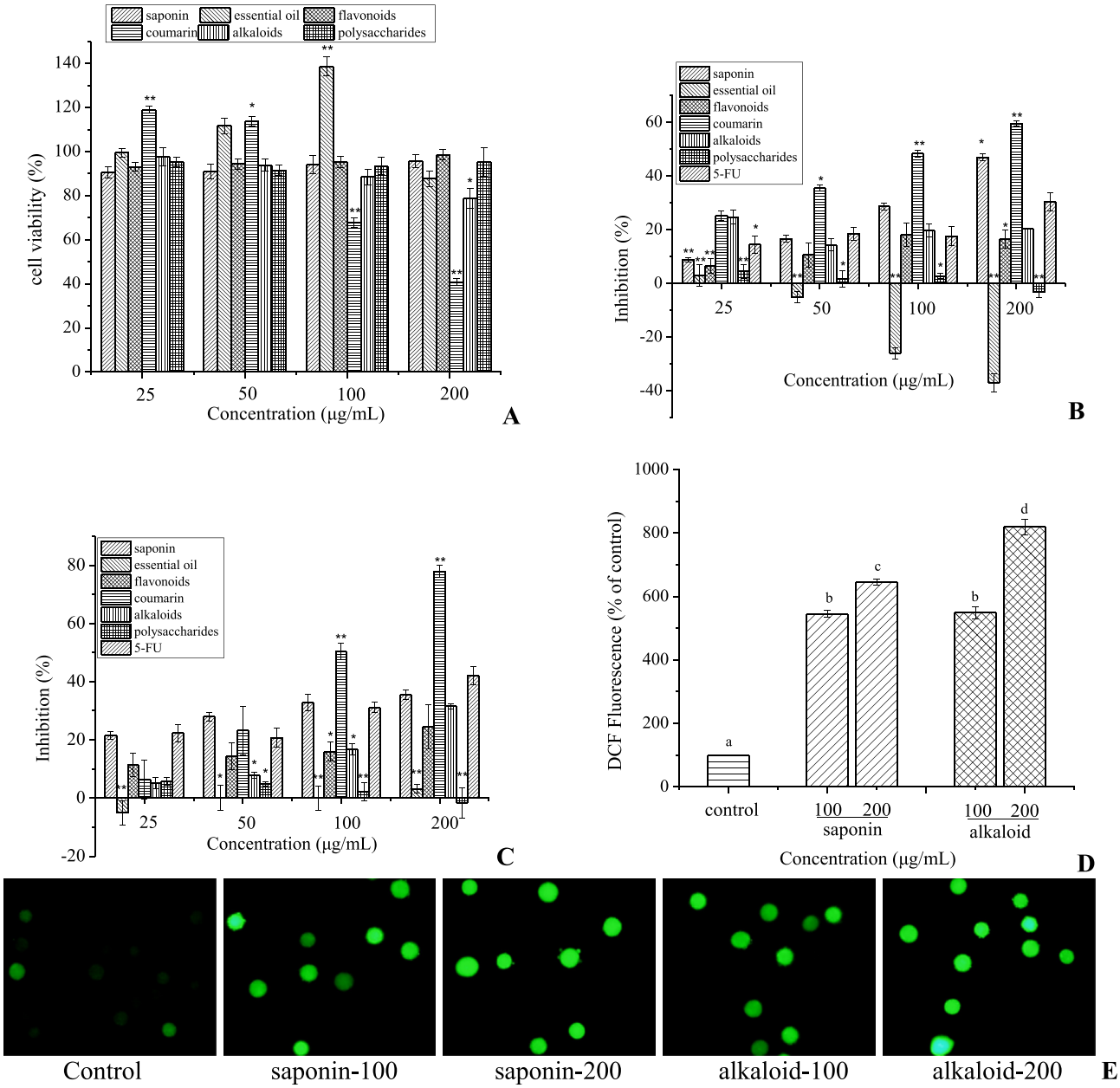

Fig. 2. Inhibition effects of main components on cancer cells proliferation. Inhibition effect of main components on human normal liver cell L02 (A), cancer cell A549 (B) and HepG2 (C). Effect of saponin and alkaloids on ROS generation in A549 (D) and cell fluorescent images (F). *P < 0.05, **P < 0.01, statistically significant in comparison with control or 5-FU. Letters refer to significant differences (p < 0.05).

3.2.3. Effect of Saponin, essential oil and flavonoids on iNOS, IL-6 and TNF-α mRNA expression in LPS-induced RAW264.7 cell

To investigate whether these components can regulate the expression of proinflammatory cytokines in transcriptional level, RAW264.7 cells were induced by LPS with or without the treatment of saponin, essential oil and flavonoids and the gene expressions were measured by qPCR. Compared to the control group, LPS markedly up-regulated iNOS, IL-6 and TNF-α gene expressions (Fig. 5). When cells were treated with components, the gene expressions displayed different changes. Saponin, essential oil and flavonoids could dose-dependently inhibit iNOS gene expression (Fig. 5A). Besides, the concentration of IL-6 in cell cultural medium (Fig. 5B) was also strongly decreased by saponin, followed by essential oil and then flavonoids. However, these three components showed no effects on TNF-α gene expression (Fig. 5C).

3.2.4. Saponin, essential oil and flavonoids inhibited p65 NF-κB and JNK MAPKs activation in LPS-induced RAW264.7

It is well known that NF-κB is important signaling pathways in regulating the LPS-induced proinflammatory cytokines expressions. We determined the effect of saponin, essential oil and flavonoids on p65 NF-κB activation. LPS could significantly promote p65 NF-κB phosphorylation (Fig. 6A), which were significantly inhibited by essential oil (Fig. 6B). Saponin also could decrease p65 NF-κB phosphorylation in some degree, and flavonoids showed no inhibitive effect.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that could regulate the synthesis of inflammation cytokines at the level of transcription and translation (Kaminska, 2005). We also investigated the effect of saponin, essential oil and flavonoids on MAPKs pathway. As we can see in Fig. 6A, LPS could activate c-Jun amino-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) (Fig. 6A). When cells were treated with

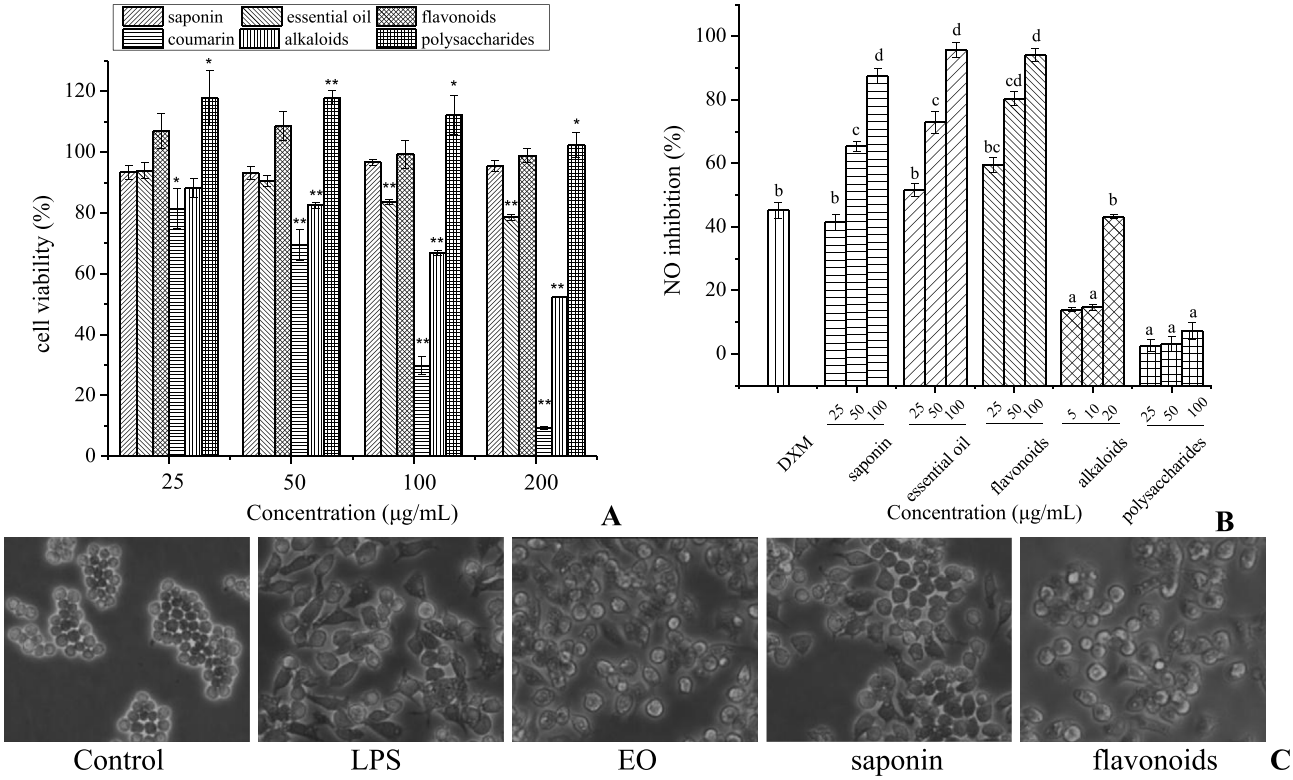

Fig. 3. The effect of main components from *C. japonicum* on LPS-induced NO production in RAW 264.7 cell. (A) Cell viability was determined using the MTT assay. (B) RAW 264.7 cells were treated with LPS (1.0 μg/ml) with or without the presence of samples at indicated concentrations, and Dexamethasone (DXM) was taken as a positive control. After 24 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. (C) Images of essential oil, saponin and flavonoids at concentration of 50 μg/ml treated cells were also taken. The data represent the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01, statistically significant in comparison with control. Letters refer to significant differences (p < 0.05).

main components from *C. japonicum*, the JNK activation was inhibited in some degree (Fig. 6C), though there was no significance. But no inhibition was observed in ERK phosphorylation (Fig. 6B). These results indicated that the anti-inflammation effect of components from *C. japonicum* was different and might be related to the suppression of p65 NF-κB and JNK MAPKs activations.

3.3. Anti-atherosclerosis activity of main components from C. japonicum DC

Macrophage plays major roles in the development of atherosclerosis. The lipid-loaded cells promote the formation of fatty streak in early stages of atherosclerosis (Moore and Tabas, 2011), which further results in thrombosis and vascular blockage. We investigated the effect of flavonoids, saponin, EO and polysaccharides on lipid accumulation in macrophage derived foam cells induced by ox-LDL. As we can see in Fig. 7, ox-LDL could significantly promote the lipid uptake in macrophages. When cells were pretreated with flavonoids, saponin and EO, we can see lipids decrease. However, polysaccharides can't inhibit the lipid accumulation in ox-LDL-induced RAW 264.7.

The beneficial properties of function foods and herbal drugs derived from these secondary metabolites produced by plants. Flavonoids, naturally exist in many fruits, vegetables and tea, are important dietary antioxidant. Quercetin was the major dietary flavonoid, followed by kaempferol, myricetin, luteolin and apigenin(Cook and Samman, 1996). It also reported that flavonoids were used to treat a variety of diseases, such as inflammation, atherosclerosis and liver damage (Pares et al., 1998). Flavonoids are the most abundant ingredients in *C. japonicum*. They could protect liver cell against chemical injury and scavenge free radicals *in vitro*.(Q. Ma et al., 2016). In this study, we also found that they can significantly inhibit NO production in LPS-induced macrophages and decrease the lipid accumulation in ox-LDL induced RAW 264.7 cell. Saponins are glycosides of steroids and triterpenoids that are reported to exhibit many bioactivities, such as sedative (Jiang et al., 2007) anticancer(Sun et al., 2010), and antiinflammation (Uemura et al., 2010). In this part, the saponin from *C. japonicum* showed best anti-proliferative effect against A549 cell, the inhibition effect of which was 47.0% at concentration of 0.2 mg/mL. Coumarin consists of an aromatic ring fused to a condensed lactone ring and has been shown to be hepatotoxic in animal studies (Ratanasavanh et al., 1996). Pyrrolizidine alkaloids, mainly occurring in species of the plant families Asteraceae, Fabaceae and Boraginaceae, are hepatotoxic and carcinogenic (Wiedenfeld, 2011). We found that coumarin showed greatest cytotoxicity, and alkaloids also inhibited human liver cell growth. In this research, we also investigated the anti-inflammatory mechanisms of *C. japonicum*, and flavonoids, saponins and essential oil showed different effects on MAPKs and NF-κB activation. All these three components showed weak effects on MAPKs phosphorylation, and only saponins could inhibit JNK activation in some degree. It is reported that protopanaxadiol saponin fraction from Korean Red Ginseng could exhibit anti-inflammatory effect through suppression of JNK activation (Yang et al., 2015). However, the effective targets of saponin from *C. japonicum* on MAPKs pathway need to be further investigated. Flavonoids exhibited little effects on MAPKs and NF-κB activation. Literature (Zhang and Tsao, 2016) reported that some dietary polyphenols can attenuate oxidative stress mediated inflammation, which also give us indications for its anti-inflammatory mechanism investigation.

Essential oils are aromatic and volatile oily liquids, which possess many bioactivities, such anti-inflammation, antimicrobial activities. We found that essential oil could inhibit NF-κB activation. Further studies were still needed to illuminate the regulatory mechanisms.

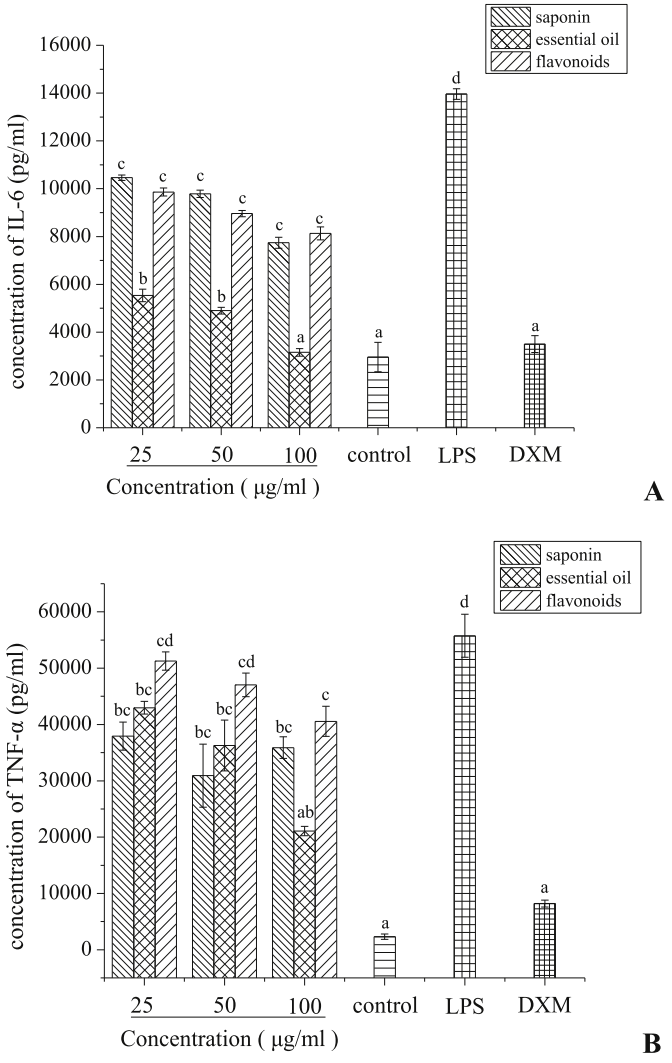

Fig. 4. Effect of essential oil, saponin and flavonoids from *C. japonicum* on pro-inflammatory cytokines production IL-6 (A) and TNF-α (B). Letters refer to significant differences ($p < 0.05$).

4. Conclusion

Coumarins from *C. japonicum* showed greatest cytotoxicity, followed by alkaloids. Saponins exhibited good anti-proliferative effect against cancer cells, especially A549 cells, which might result from the promotion of ROS generation. Essential oil, flavonoids and saponin significantly inhibited NO production and IL-6 secretion in LPS-induced RAW 264.7 cells. Besides, these three components could also inhibit lipid accumulation in RAW 264.7 cell stimulated by ox-LDL. These results suggest that different ingredients from *C. japonicum* exhibited different bioactivities, which provide useful information to better use the thistle resources.

Acknowledgements

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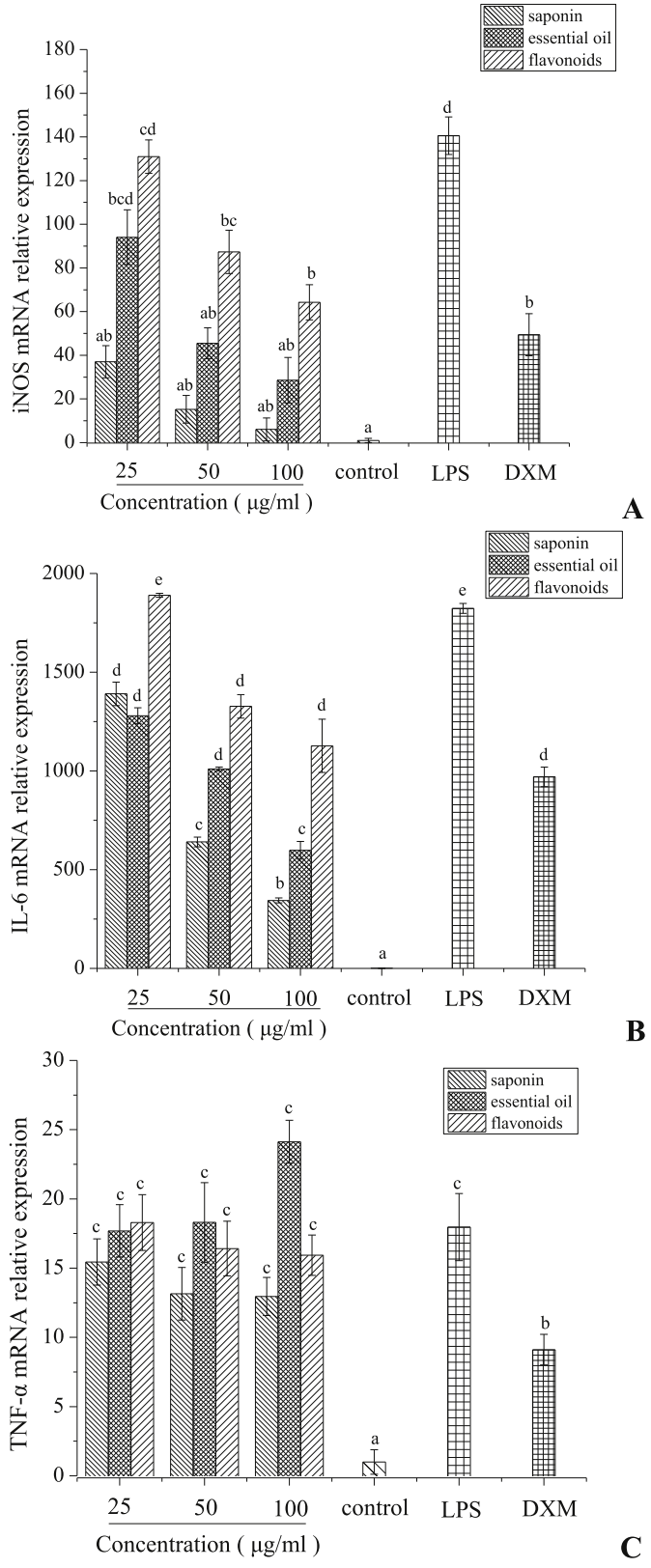

Fig. 5. Effect of essential oil, saponin and flavonoids from *C. japonicum* on iNOS (A), IL-6(B) and TNF-α (C) mRNA expression. Letters refer to significant differences ($p < 0.05$).

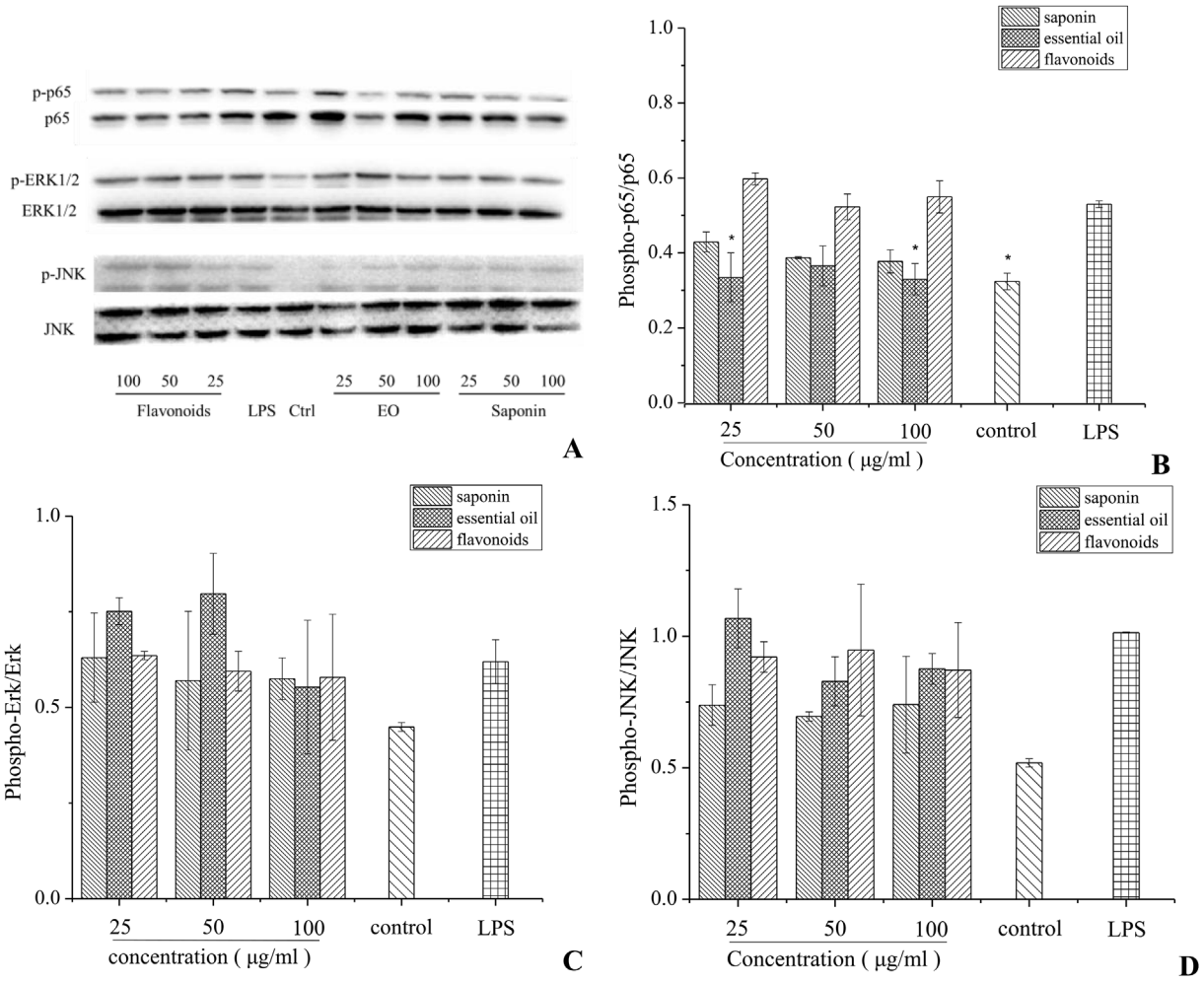

Fig. 6. Effect of saponin, essential oil and flavonoids on p-ERK1/2, p-JNK, and p-p65 NF-κB expression. RAW264.7 cells were pretreated with samples for 2 h, and then treated with LPS (1 μg/ml) for 1h. (A)The blots shown are representative of blots yielding similar results. Image J software was used for quantification of (B)p65, (C)ERK and (D)JNK phosphorylation. Band densities were determined using densitometric analysis and were normalized to the total protein level. *P < 0.05, **P < 0.01, statistically significant in comparison with LPS group.

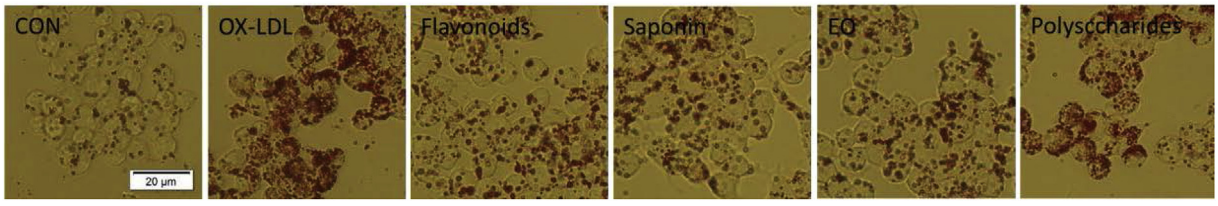

Fig. 7. The effect of main components (50 μg/ml) from *C. japonicum* on ox-LDL-induced lipid accumulation in RAW 264.7 cell.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.020.

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