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# Non-extractable Polyphenols from Cranberry: A Potential Anti-inflammation and Anti-colon Cancer Agent

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## ABSTRACT

Cranberry (*Vaccinium macrocarpon*) is abundant of polyphenols with various health benefits. Most studies focused on extractable polyphenols (EP), non-extractable polyphenols (NEP) were often neglected, although NEP may possess important biological functions. The objective of this work was to characterize EP and NEP fractions from whole cranberry and determine their potential in anti-inflammation and anti-colon cancer. Our results showed that among identified polyphenols, anthocyanins were major ones in cranberry EP fraction, while phenolic acids were most abundant in NEP fraction. The oxygen radical absorbance capacity (ORAC) of NEP was significantly higher than that of EP. EP and NEP showed anti-inflammatory effects in inhibiting LPS-induced production of nitric oxide in macrophages. At the concentrations tested, NEP showed significantly higher inhibition on production of nitric oxide in macrophages than EP, which was accompanied by decreased expression of iNOS and increased expression of HO-1. EP and NEP showed anti-cancer capacities in HCT116 cells. And NEP showed stronger inhibitory effects on viability and colony formation capacity of human colon cancer HCT116 cells than EP. Flow cytometry analysis demonstrated that NEP caused a cell cycle arrest at the G0/G1 phase and induced a significant cellular apoptosis in colon cancer cells. Overall, our results suggested that both of EP and NEP fractions from cranberry were bioactive, and importantly, NEP fraction showed a promising anti-inflammation and anti-colon cancer potential.


**KEYWORDS:** cranberry, non-extractable polyphenols, extractable polyphenols, colon inflammation, colon cancer

## INTRODUCTION

Polyphenols are a major class of dietary phytochemicals which are responsible for many health benefits. Current research on dietary polyphenols mainly focus on a fraction of polyphenols, known as extractable polyphenols (EP).[1, 2] EP can be extracted from food with aqueous-organic solvents (e.g. water, methanol, ethanol, acetone),[3, 4] and are often considered as “total phenolic content” for various biological studies. However, another fraction of polyphenols remains in the residues after solvent extraction, and they are known as non-extractable polyphenols (NEP).[2, 5] NEP are mainly polyphenols that bound to dietary fiber or other macromolecules through hydrophobic interaction, hydrogen bonding and covalent bonding.[1, 2] NEP is not significantly released from the food matrix during digestion in the stomach or small intestine, therefor they reach colon nearly intact.[6] In the colon, NEP may be released from food matrix by the action of microbiota and become bioavailable and bioactive. In contrast, EP tend to be absorbed and metabolized in the upper gastrointestinal tract, therefore, their biological fate is quite different from that of NEP. Only unabsorbed EP and their metabolites could reach the colon. The better colonic bioavailability of NEP than EP makes NEP promising candidates for promoting colon health.

Colon diseases such as inflammatory bowel diseases (ulcerative colitis and Crohn’s disease) and colon cancer have posed serious threats to the human health. Colon cancer is one of the leading causes of death in both of male and female in the United States, and colitis is a key risk factor for colon carcinogenesis.[9, 10] The prevention of colon cancer is the most logical method to controlling it, which is suggested by accumulating evidence.[11, 12] Berries and their components have been subject of many studies for the anti-inflammatory and anticancer effects. Cell culture studies have demonstrated the inhibitory effects on human colon cancer cells by the extracts of

various berries such as blackberry, black raspberry, red raspberry, blueberry, cowberry.[3] Berries (e.g. chokeberry, red raspberry, strawberry, cowberry) and their extracts have been shown to have anti-inflammatory and anticancer effects in the intestine of various animal models such as AOM-induced rat colon cancer model, Apc 1638+/- mouse and Muc2-/- mouse model, and DSS-induced colitis models. [13-15]

Cranberry is a native berry fruit of North America, and it is abundant of polyphenols including flavan 3-ols, proanthocyanidins, anthocyanins and phenolic acids.[16] Majority of studies on cranberry have been focused on its antibacterial actions.[17] To date, the inhibitory effects of cranberry on colonic inflammation and colon cancer have not been adequately studied. Cranberry extracts have been shown to inhibit colon cancer cell growth and suppress pro-inflammatory enzymes expression.[16] However, most published studies focused on EP of cranberry, and NEP has been neglected. The object of this study is to characterize EP and NEP fractions from cranberry and determine their potential in anti-inflammation and anti-colon cancer.

## MATERIALS AND METHODS

### Materials

Cranberry (Early Black variety) was harvested at State Bog, Wareham, MA (September 2011), and whole cranberry powder was obtained by freeze-drying from fresh cranberry fruits. The freeze-dried whole cranberry powder is deep red in color and free-flowing. procyanidin B2, procyanidin A2, cyanidin 3-arabinoside, peonidin 3-glucoside were from Shyuanye (Shanghai, China). Myricetrin, astragalin, myricetin, quercetin, ρ-coumaric acid, ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid, and benzoic acid were from Quality Phytochmicals, LLC (Ease Brunswick, NJ).

**Extraction of EP**

100g freeze-dried cranberry powder was mixed with 70% (v/v) acetone aqueous (1% acetic acid). The mixture was sonicated for 30 min at room temperature, and the supernatant was collected after centrifuging at 4000 g for 5 min. The residues were extracted twice more, pooled the supernatants. The supernatant was concentrated and then dissolved in methanol. Hexane was used to remove fat and other highly lipophilic molecules in extracts, after which the supernatant was concentrated at 40 ℃ to dryness. EP fraction was collected and stored in -20 ℃ for further studies.

**Extraction of NEP**

The residues obtained after EP fractionation were treated with 2 M NaOH at 37 ℃ for 2 h after purging container with nitrogen to protect polyphenols from oxidation. After alkaline hydrolysis, mixture was adjusted to pH 2 with 6 M HCl, centrifuged at 4000 g for 10 min to obtain supernatant. The supernatant was extracted by ethyl acetate and diethyl ether (1:1, v/v) for three times. The Pooled supernatants were concentrated to dryness under vacuum. The NEP fraction was collected and stored in -20 ℃ for further studies.

**Determination of total phenolic contents (TPC), flavonoid contents (FC), tannin contents (TC), and ORAC**

TPC was measured using the Folin-Ciocalteu method with some modifications[18]. Sample was dissolved in 50% methanol at the final concentration 0.4 mg/mL. Twenty μL Folin-Ciocalteu reagent was added to a 96-well plate containing 20 μL sample and 20 μL distilled water. The plate was tapped gently on all sides and kept at room temperature for 10 min, followed by

addition of 140 µL 7% sodium carbonate. After 90 min at room temperature, the absorbance was measured at 760 nm (BioTek Instrument, Inc. Vermont, VT, USA). Results are expressed as microgram of gallic acid equivalents per gram.

FC was determined as previously described with some modifications.[19] Briefly, 20 µL 0.4 mg/mL sample was mixed with 100 µL distilled water and 10 µL 5% sodium nitrite in a 96-well plate. After 6 min, 20 µL 10% aluminum chloride was added in the mixture and allowed to react for 5 min. Subsequently, 50 µL 1 M sodium hydroxide was added in the mixture. Tap the plate gently on all sides for 2 min, and the absorbance was read at 510 nm (BioTek Instrument, Inc. Vermont, VT, USA). The results are expressed as microgram of catechin equivalents per gram.

TC was tested as previously described with modifications.[20] The vanillin- $H_2SO_4$ solution was made of 4% vanillin in methanol and 30% $H_2SO_4$ in methanol in the ratio of 1:1. One hundred eighty µL vanillin-$H_2SO_4$ solution was added in a 96-well plate containing 20 µL 0.4 mg/mL sample. The absorbance was determined at 510 nm (BioTek Instrument, Inc. Vermont, VT, USA), and results are expressed as microgram of catechin equivalents per gram.

The antioxidant capacity was determined by ORAC assay following the previously published method with modifications using microplate reader (BioTek Instrument, Inc. Vermont, VT, USA).[21]

**LC-MS analysis**

Identification and quantification of selected flavonols, flavanols, anthocyanins and phenolic acids were carried out by LC-MS as described preciously with some modification.[22] Chromatographic separation of anthocyanins was carried out on Zorbax SB-Aq C18 column (150 mm ×4.6 mm, 5µm, Agilent Technologies, USA). Separation of flavonols, flavan 3-ols and

phenolic acids were achieved on Kinetex XB-C18 column (100 mm ×4.6 mm, 2.6 μm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in 95% water mixed with 5% ACN (solvent A), and 0.1% formic acid in ACN (solvent B). The elution program processed as follows: 0-2 min, isocratic elution with 5% B; 2-37 min, 5-95% B; 37-40 min, 95-100% B; 40-46 min, 100-5% B; 46-52 min, isocratic elution with 5% B. The flow rate was 0.4 mL/min, and injection volume was 10 µL. Proanthocyanidins B2, proanthocyanidins A2, ECG, myricetrin, astragalin, myricetin, p-coumaric acid, ferulic acid, protocatechuic acid, chlorogenic acid, caffeic acid, benzoic acid, cyanidin 3-arabinoside, peonidin 3-glucoside were used as external standards.

**Determination of cell viability, colony formation, cell cycle and apoptosis**

Assays for cell viability, colony formation, cell cycle and apoptosis were performed as we previously described.[23-26] In brief, HCT116 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HCT116 (2,500 cells/well) were cultured in RPMI-1640 media supplemented with 5% heat-inactivated FBS, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Mediatech, Herndon, VA) at 37 ℃ with 5% $CO_2$ and 95% relative water humidity. After 24 hours of attachment, cells were treated with serial concentrations of EP and NEP for 24 h, then cell viability was determined by MTT assay.[23]

HCT116 (400 cells/well) were seeded in 6-well plates. After 24 hours attachment, cells were subjected to the treatment of serial concentration of EP and NEP. The media in 6-well plate were refreshed every 2 days. After 14 days, the determination of colony formation was conducted as we described previously.[26]

HCT116 ($4 \times 10^4$ cells/well) were seeded in 6-well plates for cell cycle and apoptosis analysis. After 24 hours of incubation and attachment, cells were treated with 16 μg (GAE)/mL NEP for 24 h, and then cells were collected by brief trypsinization (0.25% trypsin-EDTA; Mediatech)for analysis by flow cytometry to determine cell cycle or apoptosis.[23, 26]

**Nitric oxide assay and immunoblotting**

RAW 264.7 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). RAW 264.7 cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FBS (Medistech, Herndon, VA, USA), 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C with 5% $CO_2$ and 95% air. Griess reaction was used to determine the concentration of nitrite in the culture media, which is an indicator of NO production.[27] RAW264.7 cells ($5 \times 10^5$) were seeded in 96-well plates. After 24 hours, cells were treated with different concentrations of EP or NEP for 24 hours. After 24 hours treatment, the media were mixed with an equal volume of Griess reagent mixture (reagent A: 1% sulfanilamide in 5% phosphoric acid, reagent B: 0.1% naphthylethylenediamine dihydrocholoride aqueous). Then the production of NO was determined at 540nm with a plate reader.

Whole cell lysate from macrophages was extracted as we reported previously.[23-25, 28] Briefly, RAW 264.7 cells ($2.5 \times 10^5$ cells/mL) were cultured in cell culture dishes. After 24 h attachment, the media were replaced by 1μg/mL lipopolysaccharides (LPS) with or without serial concentration of NEP. After 24 h, cell lysate was collected and subject to western blot analysis as we did previously.[28]

**Data Analysis**

All results were presented as mean ±SD. Analysis of variance (ANOVA) was used to compare the differences among two or more groups. $\rho < 0.05$ was considered to be statistical significance.

## RESULTS AND DISCUSSION

### EP and NEP in cranberry

Acetone and acetone aqueous are commonly used to extract polyphenols from fruits and vegetables. It was reported that acidic acetone was more effective in extracting polyphenols such as anthocyanins, flavan-3-ols and phenolic acids, than other solvents.[4] In order to increase the extraction rate of phenolic conjugates (e.g. β-glycosides), 70% acetone aqueous with 1% acetic acid was used to extract EP from cranberry (the yoeld of EP is 25,610 mg/100g dry powder).[29] Alkaline hydrolysis was used to release NEP because it could release more NEP from plant matrix than acid hydrolysis or enzyme hydrolysis (the yield of NEP is 3,559.5 mg/100g dry powder).[30] TPC, FC and TC of EP and NEP were shown in Figure 1. In general, EP had higher TPC, FC and TC than NEP. NEP content of cereals was found to be higher than that of EP,[5, 31] while fruits had higher contents of EP than NEP.[32] The relative abundance of NEP vs. EP in cranberry was in consistence with the findings of previous studies about NEP from different fruits including apple, orange, banana and grape.[2, 33] Both EP and NEP were more abundant in tannins than flavonoids (Figure 1).

### Chemical profiles of EP and NEP

The chemical profiles of EP and NEP were determined by HPLC-MS, results were presented in Table 1. Majority of the phenolic compounds identified by comparing their retention times and fragmentation patterns with those of standards occurred in both EP and NEP, for example procyanidin A2, myricitrin, astragalin, p-coumaric acid, protocatechuic acid, chlorogenic acid

and benzoic acid. In EP fraction, the main constituents were anthocyanins, including cyanidin-3-arabinoside and peonidin-3-glucoside, whereas anthocyanins were not found in NEP fraction. The major components in NEP were phenolic acids, including chlorogenic acid, p-coumaric acid, ferulic acid, caffeic acid, protocatechuic acid and benzoic acid, and the total content of phenolic acids was more than 10 times higher than that of EP. Meanwhile, all the phenolic acids described above were found in NEP of cranberry for the first time, although some of them have been previously found in EP fraction of cranberry.[34] Previous studies reported that cranberry was abundant of proanthocyanidins that contributed to various health benefits, especially those with unusual A-type linkages.[35, 36] Importantly, proanthocyanidins were also found in NEP fraction, and their abundance was higher than that in EP. Although the major polyphenols in NEP were phenolic acids, other kinds of flavonoids were also found in NEP, such as myricitrin, myricetin, and astragalin. Although alkaline hydrolysis has been frequently used to release phenolics from fiber, it also can break the esters and glycoside linkages of phenolic components after the release, which is a factor to be considered when interpreting the chemical profiles of NEP fraction. Proanthocyanidins or condensed tannins have been reported in EP from cranberry.[37] Interestingly, our results showed the existence of tannins in cranberry NEP and their abundance was much higher than EP, especially procyanidin A2 as shown in Table 1.

**Antioxidant capacity of EP and NEP**

ORAC was used to determine the antioxidant capacity of EP and NEP. EP and NEP showed ORAC values of 984.17 μmol TE/g(extract) and 1258.53 μmol TE/g(extract), respectively (Fig.2). The abundance of anthocyanins, flavanols, flavonols and phenolic acids in EP fraction might contribute to the antioxidant capacity, especially anthocyanins (e.g., cyanidin 3-arabinoside and peonidin 3-glucoside) which have been reported for their antioxidant capacity

(Table 1).[38] Notably, phenolic acids were the dominant phenolic constituents in NEP (Table 1), and they may responsible for the antioxidant capacity of NEP.[39] Importantly, the major compounds reported in urine after the consumption of berries were phenolic acids,[40, 41] increasing numbers of studies have been focused on the phenolic acids produced from the metabolism of food components and their contribution to the human health.

**Anti-inflammatory effects of EP and NEP in RAW264.7 macrophages**

To determine the anti-inflammatory effects of EP and NEP, their effects on the growth of RAW264.7 macrophages were firstly determined by cell viability assay. Neither EP nor NEP showed cellular toxicity at the concentration range of 2 -16 μg gallic acid equivalent (GAE)/mL (data not shown). After establishing the nontoxic concentration range, the effects of EP and NEP on nitric oxide (NO) production induced by LPS (1μg/mL) in macrophages were determined. NO is an important inflammation mediator whose production can be induced by LPS. As shown in Figure 3, at 4 and 8 μg GAE/mL, EP treatment showed 15% inhibition of the production of NO in comparison with the LPS-treated positive control group. Although NEP caused similar suppression as EP on NO production at 4 μg GAE/mL, NEP markedly lowered the production of NO by 32% at 8 μg GAE/mL, when compared to the positive control group. At 16 μg GAE/mL, NEP caused 53% inhibition on NO production, while EP at the same concentration inhibited the production of NO by only 12%, when compared to the positive control. The difference between EP and NEP in terms of the suppression of NO production is expected to be due to their different chemical profiles. For example, higher levels of phenolic acids including chlorogenic and p-coumaric acids in NEP may contribute to the higher anti-inflammatory effects of NEP in comparison with EP.[42, 43]

Since NEP had much stronger inhibitory effects than EP on NO production in RAW 264.7 macrophages, we further investigated molecular mechanism by which NEP inhibited NO production. Inducible nitric oxide synthase (iNOS) is one of the important pro-inflammatory proteins.[28] The expression of iNOS was very low without LPS treatment, while LPS treatment significantly increased iNOS expression by 22.7-fold (Figure 4A). NEP showed a dose dependent inhibition on the expression of iNOS, and NEP at 8 and 12 μg GAE/mL significantly suppressed expression levels of iNOS by 68% and 84%, respectively (Figure 4A). We examined expression levels of nuclear p50 and p-IκBα, both of which play critical role in the mediation of inflammation. p50 is the functional subunit of NF-κB pathway, and phosphorylation of IκBα results in dissociation of IκBα from NF-κB, and activated NF-κB initiates pro-inflammatory responses.[28] The results demonstrated a dose dependent inhibition on the expression of nuclear p50 by NEP, and especially, NEP at 8 and 12 μg GAE/mL decreased the levels of nuclear p50 by more than 80% when compared to that of LPS-stimulated positive control cells (Figure 4B). Also, expression levels of p-IκBα were suppressed by NEP treatment at 8 and 12 μg GAE/mL. We further determined the effects of NEP on the expression levels of HO-1, an antioxidant enzyme, which involves in the inflammation. HO-1 expression contributes to the adaptive increase of cellular anti-oxidant capacity, and it is important targets for anti-inflammation remedy.[27] Results indicated that NEP treatment caused a dose-dependent increase in the expression of HO-1, especially, at 8 and 12 μg GAE/mL NEP increased HO-1 expression by 4.2-fold and 6.7-fold compared to the LPS-treated positive control cells, respectively (Figure 4C). The expression levels of Nrf2, a transcription factor of *HO-1* gene, which involves in the induction of HO-1,[29] was significantly increased by NEP treatment at 8 and 12 μg GAE/mL.

**Anti-colon cancer effects of EP and NEP on human colon cancer cells**

Accumulating studies suggest that various polyphenols derived from fruits and vegetables possess anticancer effects in various models. As a rich resource of polyphenols, EP and NEP from cranberry potentially also have anticancer capacity. NEP cannot be released from the food matrix in the stomach or small intestine, they reach colon nearly intact,[6] where they may show biological benefits after being released from matrix by gut microbiota-mediated fermentation. Therefore, we determined the anti-colon cancer potential of cranberry EP and NEP. Cell viability assay revealed that both EP and NEP showed inhibition on the growth of cancer cells. NEP had stronger inhibitory effects on the growth of human colon cancer HCT116 cells than EP at the concentration range of 2-16 μg GAE/mL, and NEP treatment showed a dose-dependent inhibition (Figure 5A). At the concentration of 16 μg GAE/mL, NEP and EP caused inhibition on cell viability by 20% and 60%, respectively. In consistence with the cell viability assay, colony formation assay indicated that EP and NEP showed suppression on the colony formation of colon cancer cell. And NEP had stronger inhibitory effects on colony formation of colon cancer cells, for example, at 6 μg GAE/mL, NEP and EP inhibited the colony formation of HCT116 cells by 64% and 35%, respectively (Figure 5B).

Since NEP showed stronger inhibitory effects on the growth of the colon cancer cells in comparison with EP, we further investigated the mechanism by which NEP inhibit the colon cancer cell viability and colony formation. We determined the effects of NEP on cell cycle progression and cellular apoptosis of HCT116 cancer cells by flow cytometry as we described previously.[26] Based on results obtained from cell viability and colony formation assay, we selected a NEP dose of 16 μg GAE/mL to determine its effects on cell cycle progression and apoptosis. The results revealed that NEP-treatment significantly increased the cell population in G0/G1 phase and decreased the cell population in S phase in comparison with the control,

suggesting a cell cycle arrest at the G0/G1 phase (Figure 6A). After treatment with NEP, early and late apoptotic cell populations were increased by 3.8-fold and 6-fold, respectively, compared to the control group (Figure 6B).

In summary, this study provided an understanding of EP and NEP from cranberry in terms of their chemical properties and biological functions. Chemical profile of cranberry NEP was firstly determined, which showed that NEP possessed higher contents of phenolic acids than EP. Meanwhile, NEP showed higher anti-oxidant capacity than EP (Fig.2). EP fraction in fruits have been extensively studied, such as their anti-oxidant, anti-inflammatory, anti-cancer, and antibacterial effects. However, reports about NEP have been scarce. NEP could bind to fiber and they may reach colon intact,[1] so that the biological effects of NEP have been considered important for colon health. Our results indicated that both EP and NEP from cranberry had various biological effects. Importantly, NEP showed the promising anti-inflammatory and anti-cancer capacities. The high bioavailability of NEP in the colon facilitate the realization of these activities in the colnic tissues. Moreover, the potential interaction between gut microbiota and NEP could also play an important role in colon health. NEP could be biotransformed by by gut microbiota to produce bioactive metabolites that may contribute to the promotion of colon health as well.[7] Therefore, it is important to investigate the interaction of gut microbiota and NEP in the colon in terms of production of bioactive metabolites. Meanwhile, composition and functions of gut microbiota can be altered by the presence of polyphenols in the colon, which in turn could affect colon health.[8] Unfortunately, the mode of interplay between NEP and gut microbiota in the colon is largely unknown, and much more efforts are needed to explore this promising research area. The knowledge obtained from our study is helpful for a better understanding of polyphenols in berries, especially the potential biological effects of NEP in the colon. Overall,

this study provided the first line of evidence to support the notion that NEP could be a promising beneficial agent for colon health. Certainly, further investigation is warranted to elucidate the detailed model of actions of NEP in the colon, especially in relationship with gut microbiota.

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Figure Legends

**Figure 1.** Total phenolic content (TPC), flavonoid content (FC), and tannin content (TC) of EP and NEP fractions of cranberry (GAE: gallic acid equivalents; CE: (+)-catechin equivalents). Data represent mean ± SD (n=3), * indicates $p<0.05$, ** indicates $p<0.01$.

**Figure 2.** Oxygen radical absorbance capacity (ORAC) of EP and NEP (TE: Trolox equivalents). Data represent mean ± SD (n=6), ** indicates $p<0.01$.

**Figure 3.** Effects of EP and NEP on LPS-induced nitric oxide production in RAW 264.7 macrophages. The cells were treated with or without 1 μg/mL LPS or LPS plus different concentrations of EP and NEP fractions for 24 hours. Then the media was collected and determined the NO production as described in Materials and Methods. Results were expressed as mean ± SD (n=6). *$p< 0.05$ and **$p<0.01$ indicate statistical significance, when compared to LPS treatment group.

**Figure 4.** Inhibitory effects of NEP on LPS-induced proteins expression of iNOS (A), p-50, p-IκBα (B), HO-1 and Nrf2 (C) in RAW 264.7 macrophages. RAW 264.7 cells were seeded in culture plates for 24 h, and then treated with different concentrations of NEP. After 24 h, cells were collected for Western blotting analysis. The numbers underneath the blots represent band intensity that was normalized by β-actin (cytosolic fraction) or PARP (nuclear fraction). *$p<0.05$ and **$p<0.01$ indicate statistical significance in comparison with LPS-treated group (n=3). The standard deviations (all within ±15% of the means) were not shown.

**Figure 5.** (A) Inhibitory effects of EP and NEP fractions on the viability of HCT116 human colon cancer cells in MTT assay. HCT116 cells (2500 cells/well) were seeded in 96-well plates for 24 h, and then treated with serial concentrations of EP and NEP. After 24 h, cells were subjected to MTT assay. Data represent mean ± SD (n=6). (B) Inhibitory effects of NEP and EP fractions on the colony formation capacity of HCT116 cells. HCT116 (400 cells/well) were seeded in 6-well plates. After 24 hours, cells were subjected to the treatment of different concentrations of EP and NEP. The media in 6-well plate were refreshed every 2 days. After 14 days, the determination of colony formation was conducted. Data represent mean ± SD (n=3).

(C)Images of colony formation of HCT116 cells under different treatments. *$p<0.05$ and **$p<0.01$ indicate statistical significance in comparison with control group.

**Figure 6.** Effects of NEP fraction from cranberry on cell cycle progression (A) and apoptosis (B) of HCT116 human colon cancer cells. HCT116 were seeded in 6-well plates for 24 h, then treated with or without 16 μg GAE/mL NEP. After 24 h treatment, cells were collected and subjected to cell cycle or apoptosis analysis with help of flow cytometry as described in Materials and Methods. * $p<0.05$ and **$p<0.01$ represent statistical significance in comparison with control group (n=6).

| compound | Retention Time | MS (m/z) | NEP (mg/g extract) | EP (mg/g extract) |
|---|---|---|---|---|
| procyanidin B2 | 8.99 | 579[M+H]$^+$ | N | 0.09 ± 0.02 |
| procyanidin A2 | 10.15 | 577[M+H]$^+$ | 1.12 ± 0.23 | 0.40 ± 0.00 |
| ECG | 9.98 | 443[M+H]$^+$ | 0.18 ± 0.06 | 0.13 ± 0.00 |
| myricetrin | 9.60 | 463[M+H]$^+$ | 1.09 ± 0.20 | 0.81 ± 0.02 |
| astragalin | 10.10 | 447[M+H]$^+$ | 0.58 ± 0.12 | 0.20 ± 0.01 |
| myricetin | 11.50 | 319[M+H]$^+$ | 0.15 ± 0.02 | N |
| quercetin | 22.72 | 303[M+H]$^+$ | 0.16 ± 0.01 | 0.18 ± 0.06 |
| total flavonols and flavanols | | | 3.28 | 1.81 |
| | | | | |
| cyanidin 3-arabinoside | 26.01 | 420[M+H]$^+$ | N | 1.79 ± 0.07 |
| cyanidin 3-galactoside | 30.86 | 450[M+H]$^+$ | N | 2.10 ± 0.04 |
| peonidin 3-galactoside | 22.99 | 464[M+H]$^+$ | N | 5.43 ± 0.12 |
| peonidin 3-glucoside | 26.74 | 464[M+H]$^+$ | N | 1.95 ± 0.18 |
| total anthocyanins | | | | 11.27 |
| | | | | |
| p-coumaric acid | 27.02 | 165[M+H]$^+$ | 4.52 ± 0.11 | 0.31 ± 0.12 |
| ferulic acid | 27.30 | 195[M+H]$^+$ | 0.33 ± 0.01 | N |
| protocatechuic acid | 21.20 | 153[M-H]$^-$ | 0.46 ± 0.02 | 0.16 ± 0.04 |
| chlorogenic acid | 21.53 | 355[M+H]$^+$ | 10.54 ± 0.29 | 0.33 ± 0.13 |
| caffeic acid | 24.30 | 181[M+H]$^+$ | 0.37 ± 0.04 | N |
| benzoic acid | 7.16 | 123[M+H]$^+$ | 0.10 ± 0.03 | 0.66 ± 0.11 |
| total phenolic acids | | | 16.31 | 1.46 |

**Table 1** Phenolic Compounds Identified by LC-MS in EP and NEP fractions from cranberry.

Results are indicated as meat ± SD; N, not detected.

Figure 1.

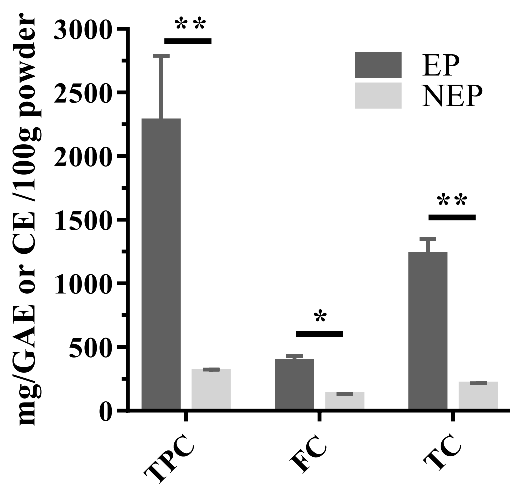

Figure 2.

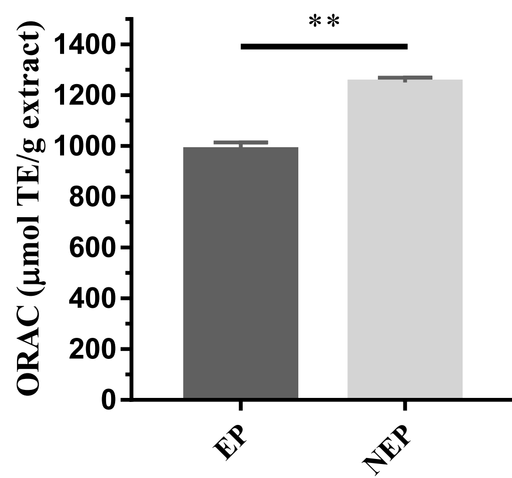

Figure 3.

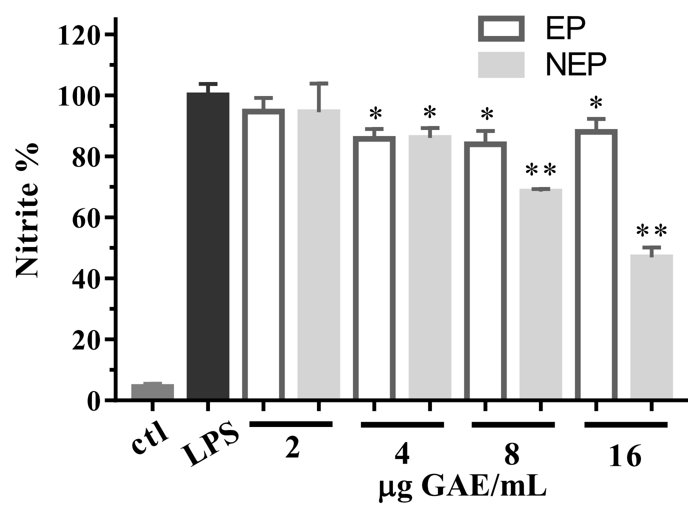

Figure 4.

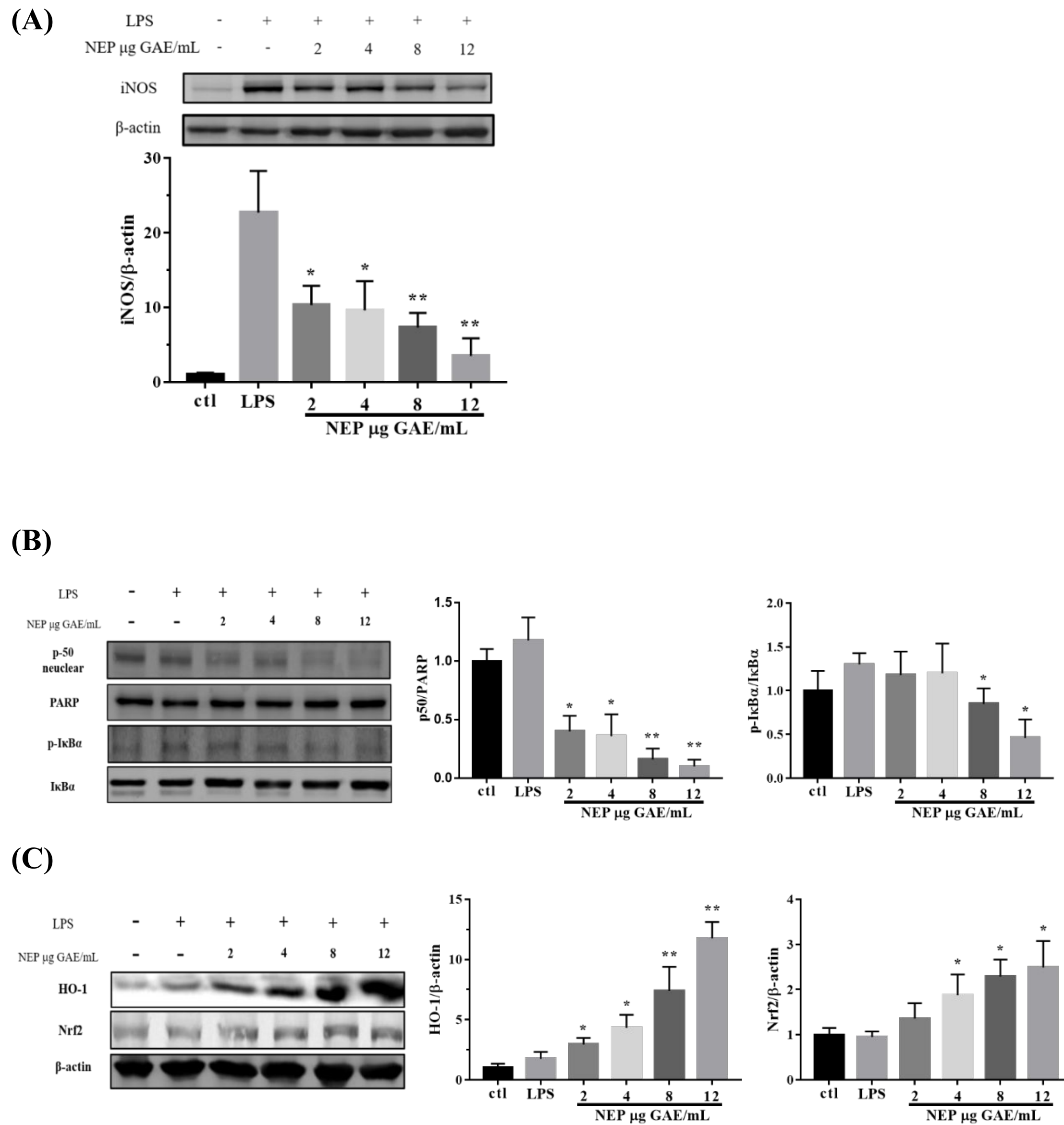

Figure 5.

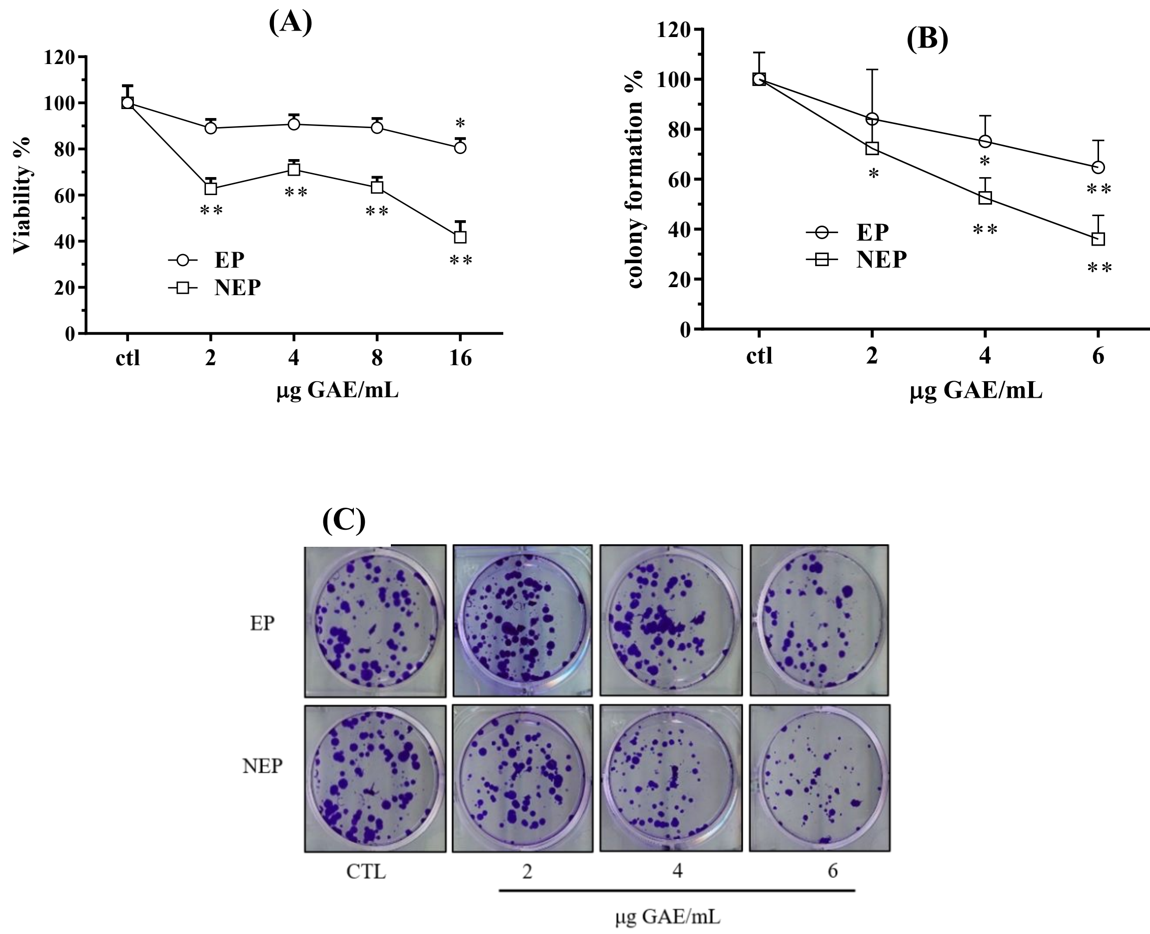

Figure 6.