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Research Article

A compatibility assay by antioxidant power and anti-proliferative properties in hepatocarcinoma cells with ursolic acid and foodborne microbial exopolysaccharides

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Abstract

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent, which produces harmful free radicals. However, natural antioxidants possess antioxidative properties and exogenous antioxidants are normally capable of inhibiting the oxidation in an organism. The synergy of different antioxidants would reverse the oxidation to a greater extent compared with a single antioxidant. In this research ursolic acid (UA) was analyzed, together with foodborne viili exopolysaccharides (EPS) for antioxidant activities and anti-proliferation properties of cancer cells. The strong antioxidant capacity of UA was observed at concentrations of 100 μg/ml in ferric reducing antioxidant power (FRAP) test, where mild synergy with viili EPS was visible. Interestingly, viili EPS had as strong antioxidative power as UA for the free radical scavenging activity in Fenton assay. By MTT assay with hepatoma HepG2 cells, the mild, but significant anti-proliferation by UA and EPS was seen at concentration of 200 μg/ml, a synergic inhibition was particularly seen at 12.5 μg/ml with a ratio of 3:2 EPS and UA. The senescence of HepG2 cancer cells after the treatment of UA and viili EPS were observed. These data indicated that UA is compatible with North European diets viili, together with potential antioxidative and anti-proliferation modulating activities, even though the mechanisms remain unknown.

Keywords: exopolysaccharide (EPS), antiinflammation, antioxidants, MTT assay, synergy, China.

Introduction

Reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism or host defense mechanism. Excessive free radical species attack cellular components that cause

damage to lipids, proteins, and DNA, which may initiate a chain of events resulting in the onset of a variety of diseases [1]. However, living organisms have developed complex antioxidant systems to counteract ROS and reduce their damage. One of the antioxidative signaling pathways is Nrf2 controlled antioxidative responsive elements (ARE) [2], and these antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, macromolecules such as albumin, ceruloplasmin and ferritin; and small molecules. The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system. The cooperation among different antioxidants provides greater protection against attack by ROS or nitrogen species, than any single compound alone [3].

Viili is a traditional yogurt originating in the Nordic countries, particularly popular in Finland [4, 5]. Viili with sticky texture and unique flavour contains different kinds of microbes: mesophilic lactobacillus, yeast and filamentous fungi; its unique stickiness is due to phosphate- containing exopolysaccharides (EPS) produced by the slime-forming LAB cremoris. The basic structure of viili EPS is mainly composed of D-glucose, D-galactose, L-rhamnose, and phosphate, the average molecular weight of viili EPS is about 2000KDa with an repeating unit of "→ 4-β- Glcp-(1→ 4)-β-D-Galp (1→4)-β-D-Glcp-(1→", and groups of α-L-Rhap and α-D-Galp-1-p attached to each side of Galp [6-8] and it has been claimed to have various functional benefits including its antioxidation, antiinflammation, anticancer, anti-ageing and natural immunity [9,10]. Viili has a sour taste resulting from microbial action of lactic acid bacteria and a surface-growing fungus *Geotrichum candidum*, which forms a velvet-like surface. In addition, viili also contains yeast: *Kluveromyces marxianus* and *Pichia fermentans*, all the microorganism form a unique symbiosis system. Single EPS producing strain has been isolated and structures are studied [11], the foodborne EPS was also shown to be able to induce inflammatory factors [12-15].

Ursolic acid (UA), a pentacyclic triterpene acid, is present in many plants, but mainly in *Plantago major*, a plant native to Asian and Europe [16]. UA has been shown to exhibit anti-carcinogenic property, particularly hindering proliferation of various types of cancer cells by inhibiting the STAT3 activation pathway and human fibrosarcoma cells by reducing the expression of matrix metalloproteinase-9 through the glucocorticoid receptor [17-19]. UA has been long used in herbal medicine and cosmetics, apparently UA is one of the most potent naturally derived COX-2 inhibitors that confers the anti-inflammatory and anti-ageing function [20].

The aim of this study was to examine the antioxidant activities and cell proliferation of the crude extracts of EPS from foodborne viili and traditional Chinese herbs, UA, and their interaction for any synergistic or antagonist activity. Here we compared the antioxidative and free radical scavenging activities of natural products of Asian and Europe, and their anti-proliferation by MTT assay to further understands viili EPS' roles as one of important group of non digestive ingredients in food that contribute to human health.

Materials and Methods

Chemicals

Ursolic acid extract (40% purity) was supplied by Herbsoul Biotech (Xian, Shanxi, China). The EPS of viili was extracted by NaOH and chloroform/n-butanol (Sevag method), or trichloroacetic acid (TCA) precipitated by ethanol [21]. 2,4,6-tripyridyl-S-triazine (TPTZ) and other reagents for antioxidant assay were purchased from Soliba (Tianjin, China). MTT (3-(4,5-Dimethyl thiazol-2-yl)- 2,5-diphen yltetrazoliumbromide), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Hepatocarcinoma HepG2 cells were a gift from Prof. Xiao Yang's lab in Beijing Insitute of Biotechnology. RPMI1640 medium with L-glutamine (GIBCO, Invitrogen, USA), 10% fetal bovine serum (GIBCO, Invitrogen, USA), and 100 U/ml penicillin, 100mg/ml streptomycin was applied.

Antioxidant activity

Ferric to ferrous ion reduction at low pH causes the formation of a coloured ferrous-tripyridyltriazine complex, so ferric reducing antioxidant power (FRAP) can be measured at 593nm [22]. In short, FRAP working solution was prepared in 3 reagents, a: 300 mM acetate buffer, pH 3.6; b: 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) in 40 mM HCl; c: 20 mM FeCl36H2O, the working solution of FRAP was mixed in a ratio, a:b:c = 10:1:1 at the time of use, pre-warmed at 37ºC, then the aliquot of each sample (50 μl) was added to 1.5mL of FRAP reagent. The reaction was mixed by vortex and the absorbance at 593 nm was read against a reagent (blanked at a pre-determined time which was decided by sample-reagent mixing). In synergy assay, 0.5mg/ml of stock solution was used, respectively, to an equal concentration. A different final concentration of 0, 10, 20, 40, 50, 100 μg/mL filled with distilled water to 1mL and blank control group with only FRAP reagent and distilled water. The value or power of FRAP antioxidant was calculated according to change in absorbance of sample from 0 to 4 min / change in absorbance of standard from 0 to 4 min X FRAP's O.D. value.

Free radical scavenging activity: Fenton method

Fenton reaction of Fe^{2+} and H_2O_2 that produce a high reactivity of OH^-, where sodium salicylate can react with OH^-, and produce a dark substance that gives absorbance at 510 nm. However, if any materials that can scavenge OH^- will compete with sodium salicylate, thereby reducing the O.D value. The depletion of free radical OH^- was measured by preparing the same reagents in test group and control group except antioxidants in test, that is, 1ml of 9mM salicylic acid-ethanol solution, 1ml of 9mM $FeSO_4$, 1ml of 8.8mM H_2O_2, then filled with distilled water to 5 ml, the O.D was measured at 510 nm. The depletion of free radical OH^- was measured by a formula: Scavenging capacity (%) = (A0 - Ai)/A0× 100%, A0 was the O.D without EPS/UA; Ai was the O.D with EPS/UA.

Cell viability and antiproliferatoin by MTT assay and their morphology

In order to test possible cytotoxicity of concentrated viili EPS and synergistic activities with polysaccharide of UA, HepG2 cells, a human hepatocarcinoma were cultured in RPMI-1640 medium with 10% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37ºC, in 5% CO2 incubator. The cells were passaging twice before assay. We designated these preserved cells as passage 2 (P2), and used P2 cells for all experiments described here to reduce variation. As routine 80% confluent cells were collected and 10,000 cells were seeded in each well of a 96-well plate (200 μL/well) for MTT [3-(4, 5-dimethyl –thiazole-2-yl)-2, 5-diphenyl tetrazolium Bromide] assay. The medium was removed after 24 hours, and replaced with the medium containing different concentration of EPS at 12.5, 25, 50, 100, 200 μg/ml, separately, where control only with medium, and incubated another 48 hours. Subsequently medium was removed, and replaced with fresh medium containing a final concentration of 0.5 mg/mL MTT (stock solution in PBS). The wells are incubated for 4 hrs at 37°C. Later 100 μL of DMSO is added to solubilize the formazan crystals. The absorbance was taken at 570 nm. Six duplicates for each concentration and the cell proliferation rate was calculated as formula: test groups/control group X 100%. The image was recorded with inverse microscope optec BDS200-PH (Chongqing, China). All images were edited using Adobe PhotoShop 6.0 without adding artefacts and without loss of original resolution.

Statistical methods

Experimental results were presented as the mean ± standard deviation (SD) of 4–6 three parallel measurements. Each value is the mean ± S.D. The level of statistical significance employed in all cases was $p < 0.05$. The statistical calculations were performed using Statistica Software (StatSoft, Tulsa, OK, USA).

Results

Mild synergy of antioxidation between viili EPS and UA

UA alone was reaffirmed as potent antioxidant by ferric reducing antioxidant power (FRAP) assay, while viili EPS alone was a weak antioxidant, but became stronger as concentration increased. Particularly viili EPS remained a maxium antioxidant power of UA at 100 μg/ml, which indicate UA has a foodborne compatibility with viili EPS, a different cultural origin (Figure 1).

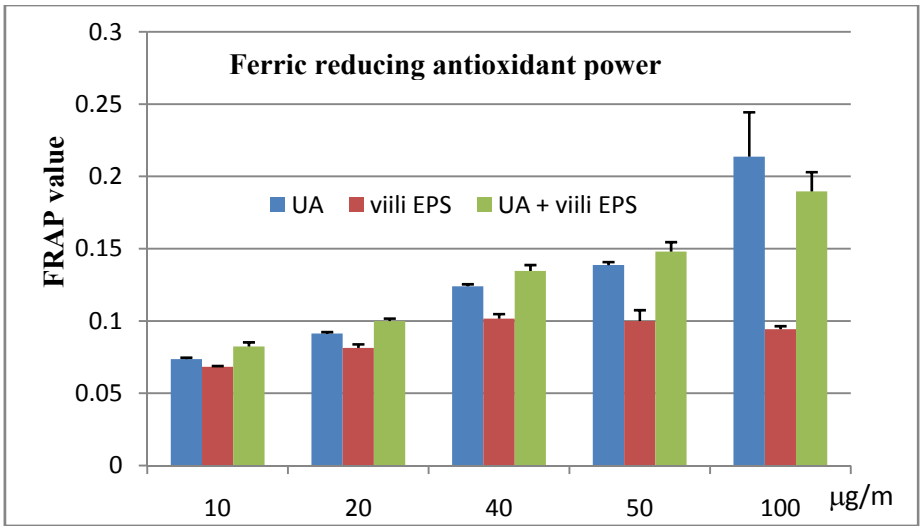

Figure 1. The ferric reducing antioxidant power (FRAP) assay with UA and viili EPS(s) were compared with control. For the possible synergy assay, the equal amount UA and viili EPS in each test concentration were applied.

Superior free radical scavenging activity (OH^-) of UA and viili EPS

An equal free radical scavenging activity of both viili EPS and UA was measured. Increase of the concentration of UA and viili EPS did not change the activity from 10 to 100 μg/mL (Figure 2), this was possible that Fenton's reagent regenerated activated carbons in UA and viili EPS, led to saturation.

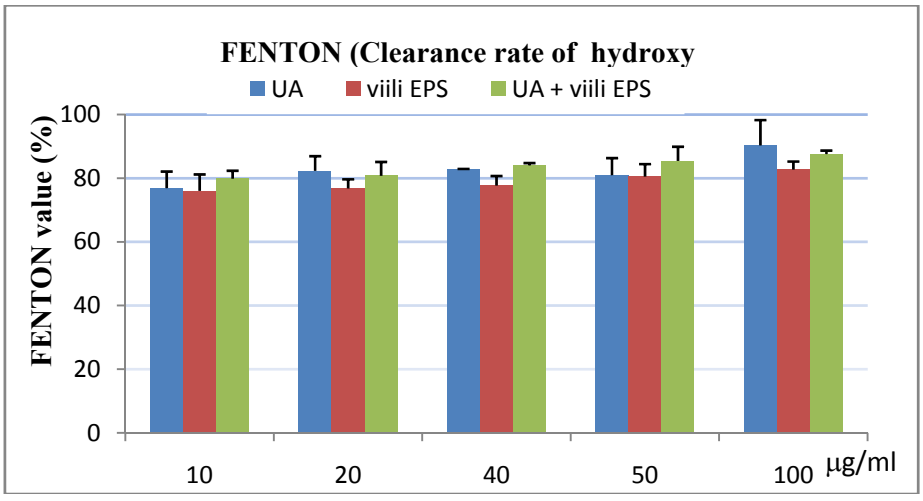

Figure 2. Free radical scavenging activity (Fenton assay) with UA and EPS(s) were compared with control. For the possible synergy assay, the equal amount UA and viili EPS in the test concentration were applied.

Ursolic acid and EPS(s) modulate cell proliferation

A mild anti-proliferation of UA and viili EPS was observed. The significance of the difference in 12.5μg/mL (viili EPS:UA=3:2), 50μg/mL (viili EPS:UA =1:1) and 200 μg/mL of all tests was seen ($p<0.05$), which indicated that anti-proliferation of HepG2 cells by viili EPS and UA was stable and apparently acted as a manner of modulation (Figure 3). The anti-proliferation could also indicate their anti-tumor and anti-inflammatory roles that could be used for combination in health food processing.

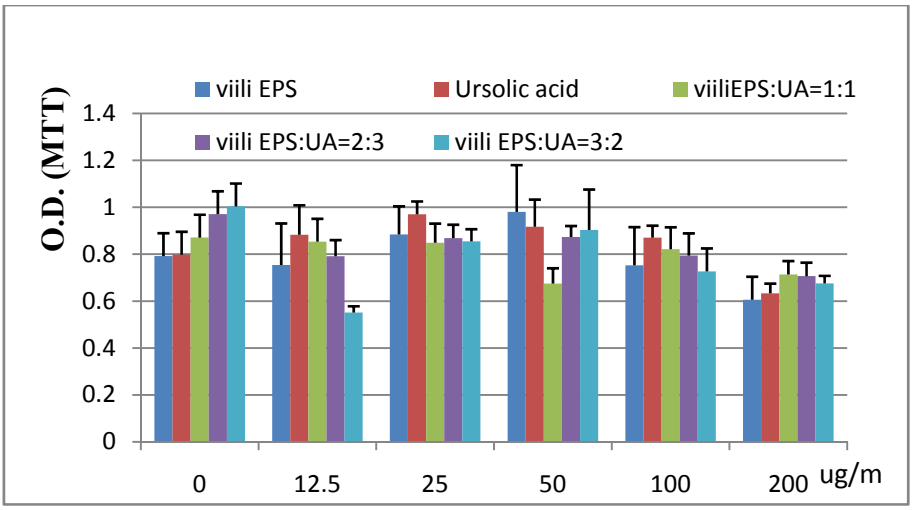

Figure 3. Cell proliferation of HepG2 under different concentration of UA and viili EPS by the O.D. of MTT.

The P-value for the difference between the 0 and 200μg/mL of all 5 tests and 12.5 μg/ml with a ratio of 3:2 EPS and UA was <0.05.

HepG2 cell morphology

Inhibition of cell proliferation was seen by MTT assay, but no obvious apoptosis were observed to HepG2 cells with viili EPS and UA at concentration of 12.5 μg/mL. However, the cell senescences from simple morphology were observed, both viili EPS and UA are able to stimulate cancer cell ageing (Figure 4).

Discussion

The steady synergy of FRAP between UA and viili EPS at concentration 10-50 μg/mL indicates a compatible cell environment. The antioxidants of different sources play important roles in ageing and anti-ageing even though the mechanism is comlex, the antioxidant power of viili EPS in a modulation manner was vital. UA gave strongest antioxidant power at concentration of 100μg/mL, also in superoxide anion radical ($O2^-$) scavenging activity assay (data not shown). The polysaccharides extracted with NaOH and CHCl3/Butanol (5:1) were directly applied in antioxidant assays and MTT assay. The complex of proteoglycans and glycoproteins is believed to give a stronger bioactivity *in vivo* and *in vitro* [23,24]. The bioactive function of polysaccharides depends on molecular weight, side chains, sulfate, or phosphate, or amino group density. The biological properties of polysaccharides are closely related to the chemical structure and molecular weight [25,26]. Polysaccharides with high molecular weight (MW) seem to have relatively high biological activities [27]. It is possible that high MW of viili EPS give the potent ROS scavenging power regardless of concentration in test. Traditional Chinese diets and medicine contain large amount of polysaccharides including different kinds of mushrooms, herbs, bone, which have been widely regarded as antioxidants, anti-inflammation and anti-ageing agents. Introduction of viili EPS to

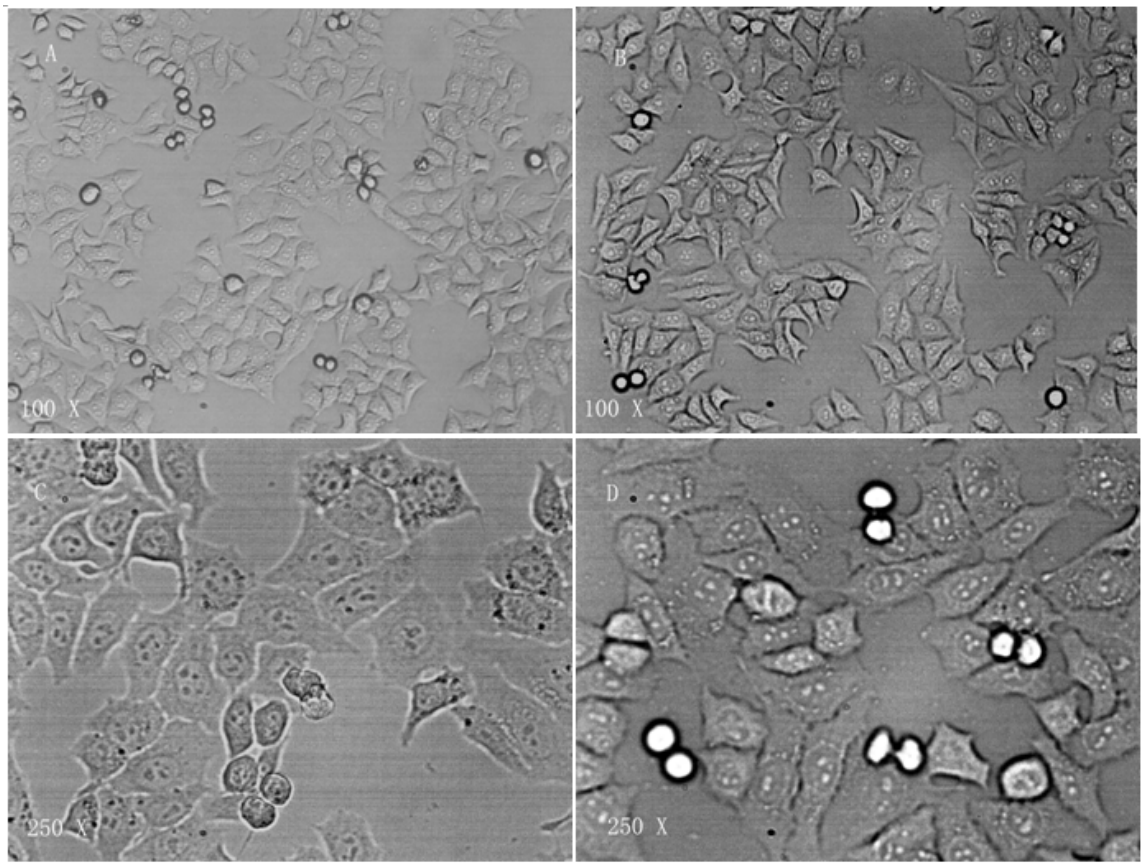

Figure 4. Representive morphology of HepG2 without and with treatment of UA and viili EPS.

(A) HepG2 without addition of UA and viili EPS, 100X, (B) HepG2 with treatment of UA and viili EPS (12.5μg/mL) 100X. (C) HepG2 without addition of UA and viili EPS, 250X. (D) HepG2 with treatment of UA and viili EPS (12.5μg/mL), 250X.

Asian diets, or health products would be of benefit for Asians since the compatibility we found here. These internal antioxidations guarantee the cells anti-senescence and normal function with, or without external antioxidants. However, the external antioxidation does activate, or maintain the internal ARE genes even thought they are also highly expressed in cancers [28].

Typically mushroom EPS carries amino acid groups, viili EPS, or other lactobacillus EPS carries phosphate groups, where hyaluronic acid (MW < 1000K) carries sulphate groups. Because of the β-glycosidic bonds most EPS(s) have no nutrition function, but play very important roles to maintain the immunity and antioxidative capacity. However, as foodborne, the weak antioxidant power of viili EPS is reasonable [29], but this does not exclude the antioxidant power in gastric intestine *in vivo*, for instance viili EPS showed a consistently scavenging activity from low to high concentration (Fenton assay). Our results indicate that EPS from viili is safe, in the meantime also possible to stimulate cell proliferation in a lower concentration. No toxicity, or apoptosis was observed this is probably that polysaccharides only loosely bound to the cell surface. But the senescence of HepG2 cancer cells after the treatment of UA and viili EPS indicates that they are of a capacity of anti-tumor. However, this does not indicate the role of any endogenous or exogeneous antioxidants contribution [30, 31], so it is still far to understand the specific functions of UA and viili EPS, particularly on their interaction in cell and cytosolic levels. So further study on the stimulation of the inflammatory factors and antioxidative signaling, particularly on COX-2/Nrf2/ARE are needed.

Conclusion

The antioxidant capacity of UA increased with concentration, while viili EPS had much mild antioxidant power, but a mild synergic interaction was seen. The free radicals scavenger potency of viili EPS and UA was not dose dependent. Significant anti-proliferation of HepG2 cells with UA and viili EPS at concentration of 200μg/mL by MTT assay, and the cell senescences at 12.5μg/mL indicated a modulation manner. However, all these tests have shown a compatibility of herbal UA and foodborne viili EPS, which indicates they can be processed together in health or functional food.

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