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Effects of vinegar–egg on growth inhibition, differentiation human leukemic U937 cells and its immunomodulatory activity

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ABSTRACT

Vinegar and eggs have rich nutrients. In this study, the mixed form of both derived products, vinegar–egg solution and its products (vinegar–egg concentrate and vinegar–egg condensate) were chosen for an assessment of their biological activity. To further our understanding regarding the anticancer and immunomodulatory effects of vinegar–egg, we investigated its effects on the proliferation and differentiation of U937 cells. Vinegar–egg was treated using spray drying, freeze drying and vacuum concentration and used to stimulate human mononuclear cells. The conditioned media obtained from these cultures by filtration were used to treat U937 cells. Three conditioned media inhibited U937 cell growth by 22.1–67.25% more effectively than PHA-treated control (22.53%). CD11b and CD14 expression on the treated U937 cells were 29.1–45.4% and 31.6–47.2%, respectively. High levels of cytokines IL-1β, IFN-γ and TNF-α were detected in the three conditioned media. Vinegar–egg stimulates human mononuclear cells to secrete cytokines, which inhibit the growth of U937 cells and induce their differentiation.

1. Introduction

The egg is one of the few foods that is shared worldwide. Eggs have rich nutrients, high protein content and amino acid compositions that are similar to human proteins. Eggs have high quality (complete protein) protein and contain a large number of biologically active components. Many studies show that eggs have many biological activities, such as antifungal, antihypertensive, antitumor, antioxidant and immune regulation [1–5]. Vinegar is also an important condiment internationally. In the early stage, people used vinegar only as a condiment. As vinegar research increased, the use of vinegar has diversified from a condiment to many food types, such as meal, beverages and various healthcare products. Many studies have reported that vinegar has a variety of nutritional and healthcare functions and medical value; for example, vinegar contains more than 2% protein, at least 18 kinds of

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amino acids and 8 essential amino acids. Vinegar contains many energetic sugars, such as glucose, fructose and maltose. In addition, vinegar contains vitamins B1, B2 and C, and mineral salts, such as K, Ca, Fe, Zn, Cu and P, which are the essential for human body development, reproductive and metabolic processes [6–11]. Leukemia is the most common hematological malignancy, particularly in children. There are five major approaches to the treatment of leukemia include cytotoxic chemotherapy, interferon therapy, radiation therapy, the induction of differentiation and stem cell transplantation (SCT) [12]. Several studies were also show that there are less toxicity and more safety by using inducing leukemic cell differentiation [13,14]. Therefore, attempting to activate human mononuclear cells to induce U937 cells differentiation through cytokine secretion might be a natural and alternative way to treat leukemia [15]. Several studies have pointed out that dietary black soybean [16], bovine colostrums and their protein hydrolyzates [12,17] mushroom [18] and rice [15] are able to inhibit the human leukemic U937 cells growth and induced their differentiation via stimulation of cytokine secretion by human mononuclear cells. In the human diet, the egg and vinegar are common, and their nutrition has also been widely discussed and studied. But the egg treated with vinegar is seldom discussed. Based on "natural" and "cost" considerations, this study used the acidity of vinegar to replace commercial enzymes and mineral acids to hydrolyze the egg protein and to evaluate.

2. Materials and methods

2.1. Preparation of vinegar–egg

Fresh eggs and 9° rice vinegar were obtained from the traditional market. The eggs were washed with water, disinfected with 95% ethanol and then air dried. We added 9° rice vinegar to the egg sample (the ratio of eggs/vinegar were 1 g:3 mL) and placed them at room temperature for 72 h. After the egg shell was dissolved, the solution was filtered with sterile gauze. The filtrate was again incubated at room temperature for 72 h. The final vinegar–egg solutions were treated in three manners; spray drying (SVE) (Spray dryer system (CR22E, Eyela, Co. Ltd., Tokyo, Japan) was used at 50–55 °C for inlet temperature; 60–65 °C for outlet temperature and the flow was 1 mL/min), freeze drying (FVE) (Freeze-drier (EYELA FDV-2100, Rikakikai Co. Ltd., Tokyo, Japan) was used at −53 °C, 553 Pa for 72 h to dry) and vacuum concentration (VVE). The obtained samples were stored at −20 °C until use.

2.2. Antioxidation activities

2.2.1. Reducing power

A method developed by Liao et al. [19] for a reducing power test was used. In brief, sample solutions (the concentration of vinegar–egg concentrate was 0.1–2.0 mg/mL; the vinegar–egg solution and vinegar–egg condensate were 10–100%), α-tocopherol, and butylated hydroxyanisole methanolic solutions (positive control group) were spiked with 2.5 mL of phosphate buffer and 2.5 mL of 1% potassium ferricyanide. Mixtures were kept in a 50 °C water bath for 20 min, cooled by placing it in 20 °C water bath for 5 min, spiked with 2.5 mL of 10% trichloroacetic acid, and then centrifuged at 800 × *g* for 10 min. The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance at 700 nm was then detected with a spectrophotometer after reaction for 10 min; higher absorbance (A_{700}) represents stronger reducing power.

2.2.2. DPPH free radical scavenging activity

The scavenging effect on DPPH free radical was measured following the method described by Shimada et al. [20] with some modifications. Sample solutions (5 mL) and α-tocopherolmethanolic solutions (positive control group) were added to 1 mL of 1 mM DPPH in methanolic solution. The mixture was shaken and left to stand for 30 min at room temperature. The absorbance rates of the resulting solution and the positive control group was measured at 517 nm. The DPPH scavenging activity percentage was expressed as [1 − (Abs sample/Abs blank)] × 100%.

2.2.3. Ferrous ion chelating ability

The method described by Decker and Welch [21] was adopted. Five milliliters of the test solutions, including sample and EDTA solutions, was spiked with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. After reaction for 10 min, the absorbance at 562 nm of the resulting solutions was recorded. The higher ferrous ion chelating ability of the test sample gave a lower absorbance (A_{562}). The ferrous ion chelating ability percentage was expressed as [1 − (Abs sample/Abs blank)] × 100%.

2.2.4. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of vinegar–egg solution and its products based on the method described by Liu et al. [22] Superoxide radicals are generated in PMS–NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiments, the superoxide radicals were generated in 3 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 mM) solution, 1 mL NADH (78 mM) solution and sample solution in water were mixed. The reaction was started by adding 1 mL of phenazine methosulphate (PMS) solution (10 mM) to the mixture. After reaction for 5 min at 25 °C, the absorbance at 560 nm of the resulting solutions was recorded. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was expressed as [1 − (Abs sample/Abs blank)] × 100%.

2.3. In vitro assay of ACE inhibition

The ACE inhibitory activity was measured using the spectrophotometric method and modification by Muguruma et al. [23]. The reaction mixture contained 5 mM Hip-His-Leu as a substrate, 300 mM NaCl and 8 mU enzymes in 100 mM sodium borate buffer (pH 8.3). A sample (150 μL, 150 mg/mL) was added to the reaction mixture referred to previously (150 μL) and mixed with 150 μL of 15 mM Hip-His-Leu containing 1 M NaCl. After incubation at 37 °C for 60 min, the reaction was stopped by adding 0.5 mL of 1 N HCl. The resultant hippuric acid was

extracted by the addition of 1.5 mL ethyl acetate and centrifuged (800 × *g*, 15 min). One milliliters (1 mL) of the upper layer was transferred into a glass tube and evaporated in a vacuum (Firstek DB-101, Firstek Co., Ltd, China) at room temperature for 2 h. The hippuric acid was redissolved in 1.0 mL of distilled water and absorbance at 228 nm was measured using a spectrophotometer (Unikon 930, Kontron Instruments, Italy). The inhibition was calculated as: Inhibition (%) = [1 − (Abs sample/Abs blank)] × 100%.

2.4. Amino acid, GABA and mineral analysis

All of the samples (100 mg for vinegar–egg concentrate; 3 mL for Vinegar–egg solution and Vinegar–egg condensate) were hydrolyzed with 10 mL of 6 M hydrogen chloride at 110 °C for 22 h under a nitrogen atmosphere to obtain the total amino acids. The amino acid composition analysis used the Hitachi L-8900 Amino acid analyzer (Hitachi Co. Ltd., Tokyo, Japan). Mineral analyses were performed as described by Liu et al. [24].The samples (1 g) were placed in a porcelain crucible and ashed in a muffle furnace at 500 °C for 24 h. After cooling, the material was digested in 2 mL of concentrated HNO3 in a microwave and then diluted with distilled water to 25 mL. This solution was filtered before storage. A blank digest was carried out in the same way. The concentrations of Fe, Zn, K, Ca, Mn, Cu, Cr and Mg were determined via flame atomic absorption spectrometry with a SpectrAA 220Z (Varian, USA) spectrometer. The concentrations of Pb, As and Cd were determined via graphite furnace atomic absorption spectrometry with SpectrAA 220Z (Varian,USA) spectrometer.

2.5. Preparation of conditioned medium

Human mononuclear cells isolated from peripheral blood were obtained from healthy adult volunteers between the ages of 22–25 years. Mononuclear cells (MNCs) were recovered by adding the blood to Ficoll–Hypaque solution (1.077 g/mL; Sigma Chemical Co.) and centrifuging at 400 × *g* for 30 min. A suspension of 1.0×10^7 cells/mL in an RPMI 1640 medium was prepared. The different VE powders were dissolved in phosphate-buffered saline (PBS; 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl dissolved 1 L ddH_2O, pH 7.0) and added to the aforementioned cell suspension in an amount between 100 and 1000 mg solid/mL medium. The media containing cells and vinegar–egg were incubated at 37 °C in a humidified 5% CO_2 atmosphere incubator for 24 and 72 h, and filtered through a 0.22 mm membrane to collect the cell-free fithroug. These VE-stimulated human peripheral blood mononuclear cell-conditioned media (MNC–CMs) were stored at −80 °C until used.

2.6. Cultivation of U937 cells

U937 cells obtained from the Bioresource Collection and Research Center (BCRC) at Hsinchu, Taiwan were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL), HEPES (100 μg/mL), sodium pyruvate and sodium bicarbonate. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Immediately before use the cell suspension was incubated in 12-well Petri dishes at an initial concentration of 1×10^5 cells/mL with or without (control group) the addition of different VE–MNC–CMs (20, 40, 100, 160 and 200 μg/mL) for 120 h, respectively, at 37 °C in 5% CO_2 (cell/sample ratio = 4/1, v/v). The control was treated with both the medium and PBS–MNC–CNs. The positive control was treated with PHA–MNC–CMs (5 μg/mL). The number of dead cells was counted using a trypan blue dye exclusion test [17]. Growth inhibition (%) was determined using the equation: Growth inhibition (%) = [1 − (cell number in sample group/cell number in control group)] × 100.

2.7. Evaluation of cell surface marker expression and U937 cell differentiation

U937 cells were incubated with each MNC–CM for 5 days. The cells were harvested and suspended in RPMI 1640 medium at 1×10^5 cells/mL. To evaluate cell differentiation, each cell suspension (1 mL) was mixed with 20 μL of fluorescein isothiocyanate (FITC)-labeled anti-human CD11b, CD14, IgG1, or IgG2a (BioLegend, San Diego, CA, USA). To evaluate cell surface marker expression, each cell suspension (1 mL) was mixed with 20 μL of fluorescein isothiocyanate (FITC)-labeled anti-human CD3, CD4, CD8, CD19, CD56, IgG1, or IgG2a (BioLegend, San Diego, CA, USA). Antibody-treated cells were allowed to rest for 30 min on ice in the dark, washed with PBS and resuspended in 0.5 mL of PBS. The percentage of positive cells was evaluated using a FACScan flow cytometer (Becton Dickinson, NJ).

2.8. Evaluation for phagocytic activity

The phagocytic activity of U937 cells was measured according to the method of Ma et al. [12]. Briefly, yeasts were suspended in PBS. They U937 cells collected from the day 3 cultures were washed, resuspended (1×10^6/mL) in FCS-containing RPMI 1640 medium and incubated (4×10^6/mL) with the yeast suspension at 37 °C for 30 min. After incubation the mixture of cells was displayed on a glass slide and observed under a light microscope. The percentage of yeast-ingesting U937 cells was recorded.

2.9. Evaluation of cytokine production in SVE, FVE and VVE–MNC–CM

Human cytokine ELISA kits were purchased from Bender (San Diego, CA). MNCs were stimulated with different Vinegar–egg and incubated for 24 and 72 h. Each cell suspension was filtered to obtain the different VE–MNC–CMs. The levels of cytokines IL-1β, IFN-γ and TNF-α were determined using commercial enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (Bender, San Diego, CA). The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm.

2.10. Measurement of nitrite production as an assay of NO release

Nitric oxide (NO) production by MNCs was assayed using the Griess reaction system. After a 72-h incubation, each

VE–MNC–CM (100 μL) was mixed with an equal volume of Griess reaction mixture (1% sulfanilamide/0.1% naphthylethylenediamine/2.5% H_3PO_4) in a 96-well flat-bottomed microtiter plate and incubated at room temperature for 15 min. The absorbance was read at 540 nm using a microplate reader (mQuant, from BIO-TEKInstruments, Inc.,Winooski, VT). The nitrite concentration was determined through comparison with a sodium nitrite standard curve.

2.11. Growth of human MNCs

The growth of human MNCs was assessed using an MTT assay as follows. Human MNCs were incubated with SVE, FVE and VVE at 37 °C in 5% CO_2 for 24 and 72 h (cell/sample = 4/1, v/v). Then, 25 μL of MTT reagent was added and the cells were incubated for another 4 h at 37 °C in 5% CO_2. An aliquot (100 μL) of MTT lysis buffer (25 mL N,N-dimethyl formamide and sodium dodecyl sulfate 10 g/mL were mixed) was added, and the cells were incubated for 16–18 h at 37 °C in 5% CO_2. The absorbance was measured at 570 nm.The growth index was calculated by applying the following equation. Growth index = (MTT value of sample stimulated cells/MTT value of medium control cells).

2.12. Statistical analysis

Results are reported as the mean ± standard deviation of three separate experiments. Data were analyzed using one-way analysis of variance and Duncan's multiple range test, which were performed using statistical software (SAS Institute 2001). Values of $p < 0.05$ were regarded as statistically significant.

3. Results

3.1. Antioxidant activities of the vinegar–egg solution, vinegar–egg concentrate and vinegar–egg condensate

The vinegar–egg solutions were shown to have the greatest superoxide anion scavenging (SOD) relative to other vinegar–egg products (vinegar–egg concentrate and vinegar–egg condensate) (Table 1). The superoxide anion radical scavenging ability of vinegar–egg concentrate (2.0 mg/mL) was 55.68%; vinegar–egg solution was up to 50.26% (at 10%) and 83.52% (at 100%). As for the reducing power (RP) of the vinegar–egg solution, absorbance at 700 nm ranged between 0.12 and 0.55 at samples concentration of 10–100%. The highest activity was in vinegar–egg solution 100% (0.55), and the lowest was in Vinegar egg condensate 10% (0.12; Table 1). The Vinegar–egg concentrate (2 mg/mL) showed the highest activity (0.33). The DPPH free radical scavenging activities ranged between 5.63% and 50.28%. The ferrous ion chelating ability (FICA) ranged between 13.08% and 35.39%. Meanwhile, the superoxide anion scavenging ranged between 28.9% and 83.52%. The antioxidant ability of DPPH free radical scavenging activity, ferrous ion chelating ability and reducing power were, in order, vinegar egg solution > vinegar egg concentrate > vinegar egg condensate.

Table 1 – The antioxidant activities of the vinegar–egg solution, vinegar–egg concentrate and vinegar–egg condensate.

Antioxidant activity	Sample											
	Vinegar–egg concentrate (mg/mL)				Vinegar egg solution (%)				Vinegar egg condensate (%)			
	0.1	0.5	1.0	2.0	10*	50*	100		10	50	100	
SOD (%)	28.9 ± 0.32^{e}	32.74 ± 0.64^{d}	42.47 ± 2.72^{c}	55.68 ± 0.43^{b}	50.26 ± 1.75^{d}	73.62 ± 0.99^{b}	83.52 ± 0.54^{a}		31.33 ± 1.88^{e}	45.74 ± 3.60^{d}	52.18 ± 0.60^{c}	
DPPH (%)	5.63 ± 1.83^{f}	14.92 ± 1.10^{e}	24.01 ± 3.05^{d}	34.04 ± 1.18^{c}	35.57 ± 1.42^{c}	41.44 ± 1.65^{b}	50.28 ± 1.52^{a}		35.03 ± 2.01^{c}	33.87 ± 2.84^{c}	37.62 ± 2.48^{c}	
FICA (%)	18.23 ± 1.40^{d}	19.31 ± 1.97^{d}	21.05 ± 0.48^{d}	23.86 ± 0.90^{c}	19.13 ± 0.27^{d}	27.97 ± 0.87^{b}	35.39 ± 2.25^{a}		17.99 ± 0.43^{d}	13.08 ± 0.87^{e}	18.79 ± 1.83^{d}	
RP (Abs 700 nm)	0.14 ± 0.00	0.17 ± 0.00	0.22 ± 0.00	0.33 ± 0.01	0.15 ± 0.00	0.26 ± 0.00	0.55 ± 0.05		0.12 ± 0.01	0.13 ± 0.00	0.13 ± 0.00	

Results from three separate experiments are expressed as mean ± SD; *10 and 50% based on vinegar–egg solution and vinegar–egg condensate as 100%, diluted with water; a–c: Data bearing with identical letter in the same row are not significantly different ($p < 0.05$).

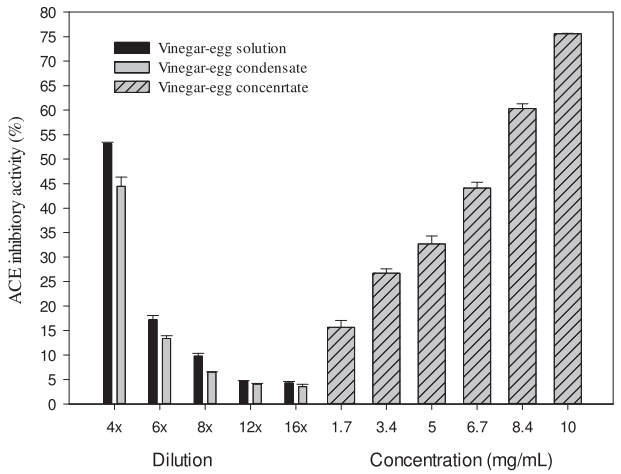

Fig. 1 – The ACE inhibitory activities of vinegar–eggs solution, vinegar–eggs concentrate and vinegar–egg condensate.

3.2. *The ACE inhibitor activities of vinegar–eggs solution, vinegar–eggs concentrate and vinegar–egg condensate*

The results of angiotensin-converting enzyme (ACE) inhibition ability showed that the ACE inhibition of vinegar–egg concentrates (at 6.7, 8.4 and 10 mg/mL), were 44.9, 61.0 and 75.6%, respectively (Fig. 1). ACE inhibition of vinegar–egg solution and vinegar egg condensate (at 25%; 4-fold dilution, 4×) were 53.17 and 44.46%, respectively. The results showed that vinegar–egg solution and their products have strong ACE inhibition capacity.

3.3. *Amino acid, GABA and mineral analysis*

The free amino acid compositions of the vinegar–eggs solution and vinegar–egg products were shown in Table 2. The results show that the vinegar–egg solution contains the necessary amino acids, including: The basic amino acid (BAA): His (2.05 nmol/mL), Arg (2.34 nmol/mL), Lys (1.54 nmol/mL); the branched chain amino acid (BCAA): Val (0.08 nmol/mL), Leu (0.90 nmol/mL), Ile (0.32 nmol/mL); the aromatic amino acids, such as Tyr (1.50 nmol/mL), Phe (1.07 nmol/mL) and other amino acids that are beneficial to the human body, such as Asic acid: Asp (3.12 nmol/mL), Glu (2.47 nmol/mL); Thio group (–SH): Cys (1.25 nmol/mL), Met (10.69 nmol/mL); Hydroxy group (–OH): Ser(14.48 nmol/mL), Thr (0.63 nmol/mL) and Pro (6.24 nmol/mL). Table 2 shows that the total content of free amino acids in vinegar–egg solution were 91.83 nmol/mL; among these, 9 kinds of essential amino acids accounted for 22.37% of the total free amino acids. In addition, vinegar–egg also contains GABA, 40.78 μg/mL. As shown in Table 3, the vinegar–egg solution contains rich large minerals such as Ca (3.2 ppm), Mg (4.2 ppm) and K (153.1 ppm), and contains trace elements such as Fe (3.4 ppm), Zn (0.7 ppm), Cu (0.64 ppm), Mn (0.13 ppm) and Cr (0.99 ppm).

3.4. *Inhibition effects of vinegar–egg on U937 cell growth by MNC–CMs*

The results are shown in Table 4. The MNC–CMs prepared with VVE inhibited the growth of U937 cells more effectively than those prepared with SVE and FVE or the PHA-treated control. The sample concentration and MNC–CM incubation time were significantly related to U937 cell growth inhibition.

Table 2 – The contents of free amino acids and GABA of vinegar–egg concentration, vinegar–egg solution and vinegar egg condensate.

Amino acid (nmol/mL)	vinegar egg solution	vinegar egg concentrate	vinegar egg condensate
Aspartic acid	$3.12 \pm 0.15^{b*}$	4.93 ± 0.34^{a}	1.71 ± 0.01^{c}
Threonine	0.63 ± 0.01^{b}	7.29 ± 0.04^{a}	0.46 ± 0.0^{b}
Serine	14.48 ± 0.13^{b}	83.45 ± 3.58^{a}	14.73 ± 1.25^{b}
Glutamic acid	2.47 ± 0.08^{b}	38.23 ± 1.07^{a}	1.78 ± 0.24^{b}
Glycine	36.00 ± 1.47^{b}	132.68 ± 9.73^{a}	11.03 ± 2.21^{b}
Alanine	7.15 ± 0.84^{b}	244.55 ± 7.68^{a}	5.92 ± 0.37^{b}
Cysteine	1.25 ± 0.19^{b}	48.68 ± 2.57^{a}	0.76 ± 0.01^{c}
Valine	0.08 ± 0.0^{b}	9.83 ± 0.89^{a}	0.19 ± 0.0^{b}
Methionine	10.69 ± 1.02^{b}	22.47 ± 0.54^{a}	3.87 ± 0.62^{c}
Isoleucine	0.32 ± 0.01^{b}	6.76 ± 0.29^{a}	0.36 ± 0.04^{b}
Leucine	0.90 ± 0.00^{b}	11.30 ± 0.31^{a}	0.75 ± 0.07^{b}
Tyrosine	1.50 ± 0.31^{a}	1.20 ± 0.04^{a}	0.29 ± 0.0^{b}
Phenylalanine	1.07 ± 0.05^{b}	5.86 ± 0.01^{a}	1.83 ± 0.16^{b}
Lysine	1.54 ± 0.08^{b}	178.97 ± 11.34^{a}	2.49 ± 0.53^{b}
Histidine	2.05 ± 0.67^{b}	5.49 ± 0.48^{a}	0.56 ± 0.01^{c}
Arginine	2.34 ± 0.43^{b}	4.27 ± 0.08^{a}	0.86 ± 0.01^{c}
Proline	6.24 ± 0.88^{b}	20.82 ± 1.06^{a}	1.69 ± 0.34^{c}
Total	91.83	826.78	49.28
GABA (μg/mL)	40.78	303.75	10.83

Results from three separate experiments are expressed as mean ± SD.
*a–d: Data with identical letters in the same row are not significantly different ($p < 0.05$).

Table 3 – The content of heavy metals and trace elements in vinegar eggs-solution, vinegar–egg concentrate and vinegar–egg condensate.

Unit (ppm)	Vinegar egg solution	Vinegar egg concentrate	Vinegar egg condensate
Pb	ND	ND	ND
As	ND	ND	ND
Cu	$0.64 \pm 0.01^{c*}$	5.25 ± 0.08^{a}	1.06 ± 0.03^{b}
Cd	0.05 ± 0.00^{b}	2.45 ± 0.01^{a}	ND
Cr	0.99 ± 0.04^{b}	3.70 ± 0.11^{a}	0.81 ± 0.01^{b}
Mn	0.13 ± 0.01^{b}	4.42 ± 0.04^{a}	0.06 ± 0.00^{c}
Ca	3.2 ± 0.32^{b}	21.4 ± 0.91^{a}	ND
Mg	4.2 ± 0.95^{b}	38.8 ± 2.79^{a}	ND
Zn	0.7 ± 0.06^{b}	58.6 ± 1.34^{a}	ND
Fe	3.4 ± 0.62^{b}	64.6 ± 2.82^{a}	0.7 ± 0.03^{c}
K	153.1 ± 16.28^{b}	62108.3 ± 104.85^{a}	169.90 ± 10.79^{b}

Results from three separate experiments are expressed as mean ± SD.
*a–d: Data with identical letters in the same row are not significantly different ($p < 0.05$).

3.5. *Evaluation of U937 cell differentiation*

Fig. 2(A) depicts the CD11b levels on U937 cells treated with SVE, FVE and VVE–MNC–CM at 200 μg/mL. Each result was significantly higher than that for the medium and the PBS group but lower than the PHA group. U937 cells treated with VVE–MNC–CM at 200 μg/mL produced the highest percentages of CD11b expression. As seen in Fig. 1(B), the CD14 expression in the treated groups was also significantly higher than the control groups (medium and the PBS groups) but lower than PHA group.

3.6. *Cell surface marker expression in U937 cells*

The proportions of different immune cell types and the effects of different vinegar–egg products can be determined by assessing U937 cell surface marker expressions. As seen in Table 5, the percentage of cells expressing CD3 (T cells), CD4

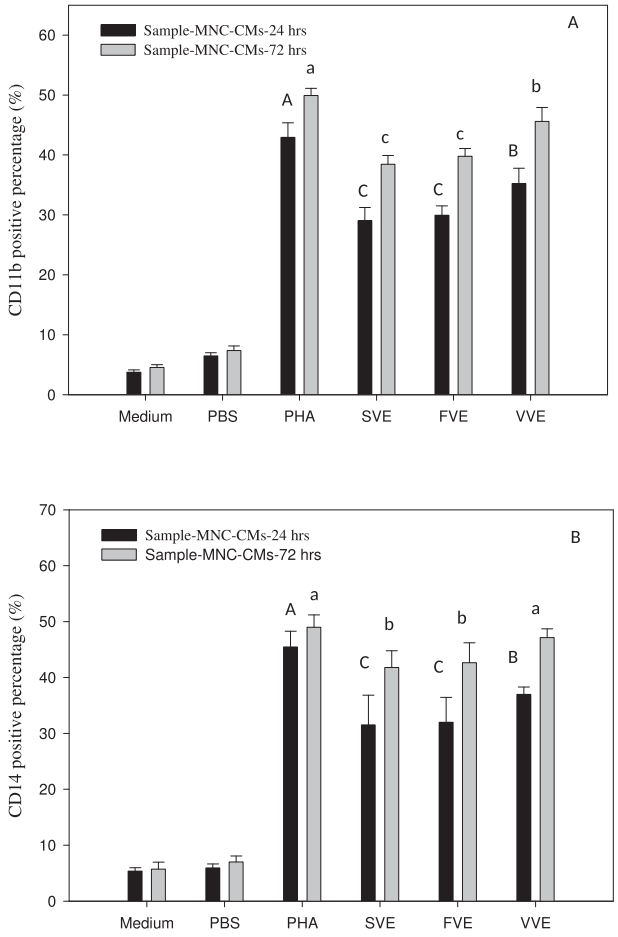

Fig. 2 – CD11b and CD14 expression in U937 cells incubated for 120 h in the presence of 20% MNC–CMs prepared with 200 μg/mL of SVE, FVE and VVE for 24 and 72 h. Results from three separate experiments are expressed as mean ± SD; different letters indicate significant differences among samples tested ($p < 0.05$). PHA: MNC–CM stimulated with PHA (5 μg/mL); PBS: MNC–CM stimulated with PBS; Medium: MNC–CM stimulated with RPMI 1640.

Table 4 – Inhibition of U937 cell growth by MNC–CMs.

Growth inhibition (%) Vinegar–egg concentration in PBS (μg/mL) for incubating MNCs					
Samples	20	40	100	160	200
SVE-24 h	22.16 ± 1.25^{cC}	26.24 ± 1.34^{cB}	28.85 ± 1.52^{cB}	32.45 ± 1.05^{dA}	33.42 ± 1.06^{eA}
FVE-24 h	25.26 ± 1.41^{cC}	28.15 ± 1.88^{cC}	28.64 ± 1.31^{cC}	32.47 ± 3.47^{dB}	40.77 ± 3.56^{dA}
VVE-24 h	32.67 ± 1.16^{bC}	36.56 ± 2.86^{bC}	40.01 ± 1.53^{bB}	43.78 ± 0.23^{cB}	50.34 ± 3.44^{cA}
SVE-72 h	25.39 ± 2.54^{cD}	38.17 ± 2.40^{bC}	43.93 ± 1.96^{bB}	48.26 ± 3.66^{bAB}	51.09 ± 2.92^{cA}
FVE-72 h	25.76 ± 2.05^{cE}	37.94 ± 4.64^{bD}	47.01 ± 1.88^{abC}	53.48 ± 1.55^{abB}	58.04 ± 2.28^{bA}
VVE-72 h	41.10 ± 4.21^{aD}	46.37 ± 3.23^{aD}	51.67 ± 1.36^{aC}	59.44 ± 3.70^{aB}	67.25 ± 3.41^{aA}
Medium	6.84 ± 1.52				
PBS	12.59 ± 1.46				
PHA	22.53 ± 2.57				

1) U937 cells were incubated in the presence of MNC–CMs for 120 h before counting.
2) MNC–CMs were prepared by incubation of MNCs with different vinegar–egg samples for 24 and 72 h.
3) Results from three separate experiments are expressed as mean ± SD. A–C: Data with identical letters in the same row are not significantly different ($p < 0.05$). a-d: Data with identical letters in the same column are not significantly different ($p < 0.05$).
4) PHA, MNC–CM stimulated with PHA (5 μg/mL); PBS, MNC–CM stimulated with PBS; Medium, MNC–CM stimulated with RPMI 1640.

Table 5 – Cell surface marker expression in U937 cells.

$CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ expressed percentages (%)					
Samples (200 μg/mL)	$CD3^+$ (T cell)	$CD4^+$ (Th cell)	$CD8^+$ (Tc cell)	$CD19^+$ (B cell)	$CD56^+$ (NK cell)
SVE	25.96 ± 2.39^{C}	28.60 ± 1.57^{C}	24.87 ± 2.01^{C}	15.70 ± 1.04^{B}	25.82 ± 1.97^{C}
FVE	25.45 ± 1.36^{C}	33.67 ± 1.45^{B}	24.57 ± 1.35^{C}	16.70 ± 1.69^{B}	29.03 ± 1.35^{AB}
VVE	35.09 ± 2.11^{A}	39.58 ± 2.10^{A}	31.03 ± 1.98^{A}	22.42 ± 2.52^{A}	33.92 ± 1.58^{A}
PBS	11.86 ± 2.40^{C}	14.63 ± 2.71^{D}	16.19 ± 1.25^{D}	11.47 ± 1.13^{C}	12.97 ± 2.23^{D}
PHA	30.49 ± 2.53^{B}	29.12 ± 1.06^{C}	28.36 ± 1.18^{B}	23.34 ± 3.29^{A}	28.39 ± 1.73^{B}

1) Results are expressed as the mean ± SD from three separate experiments; different letters indicate significant differences among samples tested ($p < 0.05$).
2) PHA, MNC–CM prepared with PHA (5 μg/mL); PBS, MNC–CM prepared with PBS.

(Th cells), CD8 (Tc cells), CD19 (B cells) and CD56 (NK cells) among those treated with SVE, FVE and VVE–MNC–CM at 200 μg/mL for 72 h were significantly higher than the percentages of these cells present in the medium and PBS groups. The percentages expressing CD4, CD8 and CD56 were even higher than in the positive control group (PHA). The percentage of U937 cells treated with VVE–MNC–CM expressing CD markers was more effective than those treated with other MNC–CMs; therefore, it can be concluded that vinegar–egg has immunomodulatory effects (see Table 5).

3.7. *Phagocytosis in U937 cells*

As the results in Fig. 3, U937 cells treated with VE–MNC–CM prepared with the SVE, FVE and VVE at 200 μg/mL showed significantly stronger phagocytic activity than the medium and the PBS-treated group did (Fig. 3), suggesting that certain mediators in VE–MNC–CM triggered the maturation of U937 cells into macrophages.

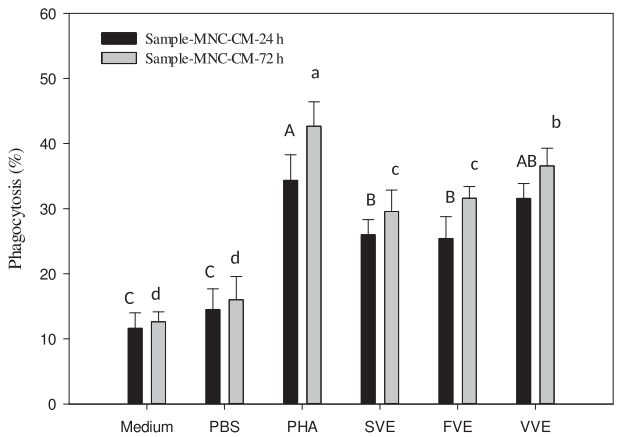

Fig. 3 – The phagocytes activity of U937 cells incubated for 120 h in the presence of 20% MNC–CMs prepared with 200 μg/mL of SVE, FVE and VVE. Results from three separate experiments are expressed as mean ± SD; different letters indicate significant differences among samples tested ($p < 0.05$). PHA: MNC–CM stimulated with PHA (5 μg/mL); PBS: MNC–CM stimulated with PBS; Medium: MNC–CM stimulated with RPMI 1640.

3.8. *Cytokine contents and NO production by SVE, FVE and VVE–MNC–CM*

The results are presented in Table 6. The IL-1β, IFN-γ and TNF-α content of the conditioned media stimulated by SVE, FVE and VVE–MNC–CM at 100 μg/mL for 72 h were significantly higher than those in the medium and PBS groups. The VVE treatment was more effective in stimulating MNC to secrete IL-1β, TNF-α and IFN-γ than treatment with the other extracts. Furthermore, it is evident that the cytokine content increased with increasing incubation time. From the results, it can be concluded that vinegar–egg is capable of stimulating MNC to secrete cytokines. As shown in Table 6, the NO contents (1.86 μM) of MNC–CM prepared by stimulating MNCs with 100 μg/mL VVE for 72 h were higher than those of MNC–CM prepared using SVE and FVE.

3.9. *The MNC growth affected by vinegar–egg*

Table 6 illustrates the effect of vinegar–egg on MNC growth. Treatment of MNCs with vinegar–egg had a greater effect on MNC growth than treatment with PBS. The greatest growth index was achieved by treatment of VVE at 100 μg/mL for 72 h. These results indicate that the vinegar–egg is able to stimulate MNC growth.

3.10. *Evaluation of the influence of lipopolysaccharides using polymyxin B*

The aforementioned growth inhibitory and differentiation cytokine secretion and NO production inducing effects of VE–MNC–CM were not blocked by the addition of 100 μg/mL polymyxin B during the preparation different VE–MNC–CM. As present in Fig. 4, polymyxin B had no significant effect on the secretion of treated IL-1β, IFN-γ and TNF-α. This indicates that there was no bacterial contamination in the samples.

4. Discussion

For functional peptides with a progressive hydrolysis reaction, the gradual decomposition of the protein into a variety of molecular weight of the multi-peptide: tri-peptides, di-peptides and amino acids, increase its nutrition and functional ability. The antioxidant ability of DPPH radical

Table 6 – The MNC growth index, the contents of cytokines and NO secreted by MNCs stimulated with different treatments of vinegar–egg.

Sample (100 μg/mL)	Content of cytokines (pg/mL)								Content of nitric oxide (NO, μM/mL)		MNC growth index
	IL-1β		IFN-γ		TNF-α						
	Incubation time (h)										
	24	72	24	72	24	72	24	72	24	72	
SVE	1562.17 ± 329.08^{aB}	2145.21 ± 14.29^{cA}	664.34 ± 73.11^{bA}	706.69 ± 84.25^{aA}	2496.53 ± 446.53^{aA}	2643.75 ± 169.18^{bA}	1.13 ± 0.11^{bB}	1.28 ± 0.10^{cA}	1.05 ± 0.09^{aB}	1.18 ± 0.18^{aA}
FVE	1720.27 ± 23.92^{aB}	2227.88 ± 18.92^{bA}	734.73 ± 64.54^{abB}	862.84 ± 70.83^{aA}	2820.54 ± 504.64^{aA}	2925.86 ± 275.81^{abA}	1.13 ± 0.07^{bB}	1.41 ± 0.14^{bA}	1.08 ± 0.08^{aB}	1.26 ± 0.21^{aA}
VVE	1896.18 ± 273.70^{aB}	2540.40 ± 97.56^{aA}	791.11 ± 84.58^{aB}	903.97 ± 61.56^{aA}	2715.50 ± 152.51^{aB}	3341.35 ± 189.96^{aA}	1.58 ± 0.10^{aB}	1.86 ± 0.12^{aA}	1.14 ± 0.08^{aB}	1.30 ± 0.22^{aA}
Medium	141.28 ± 35.27^{bA}	148.96 ± 13.34^{dA}	18.85 ± 0.00^{cA}	18.89 ± 0.00^{bA}	210.65 ± 19.50^{bA}	243.11 ± 16.34^{cA}	0.93 ± 0.10^{cB}	1.05 ± 0.01^{dA}	–	–
PBS	110.50 ± 0.00b^{bA}	118.18 ± 6.56^{dA}	39.82 ± 8.17^{cA}	42.89 ± 0.00^{bA}	222.34 ± 29.62^{bA}	268.43 ± 21.3^{cA}	0.81 ± 0.0^{cB}	1.02 ± 0.01^{dA}	1.03 ± 0.06^{aB}	1.12 ± 0.63^{bA}

1) Results are expressed as mean ± SD from three separate experiments.
2) A–B: Data with identical letter in the same row are not significantly different ($p < 0.05$).
3) a–d: Data with identical letter in the same column are not significantly different ($p < 0.05$).

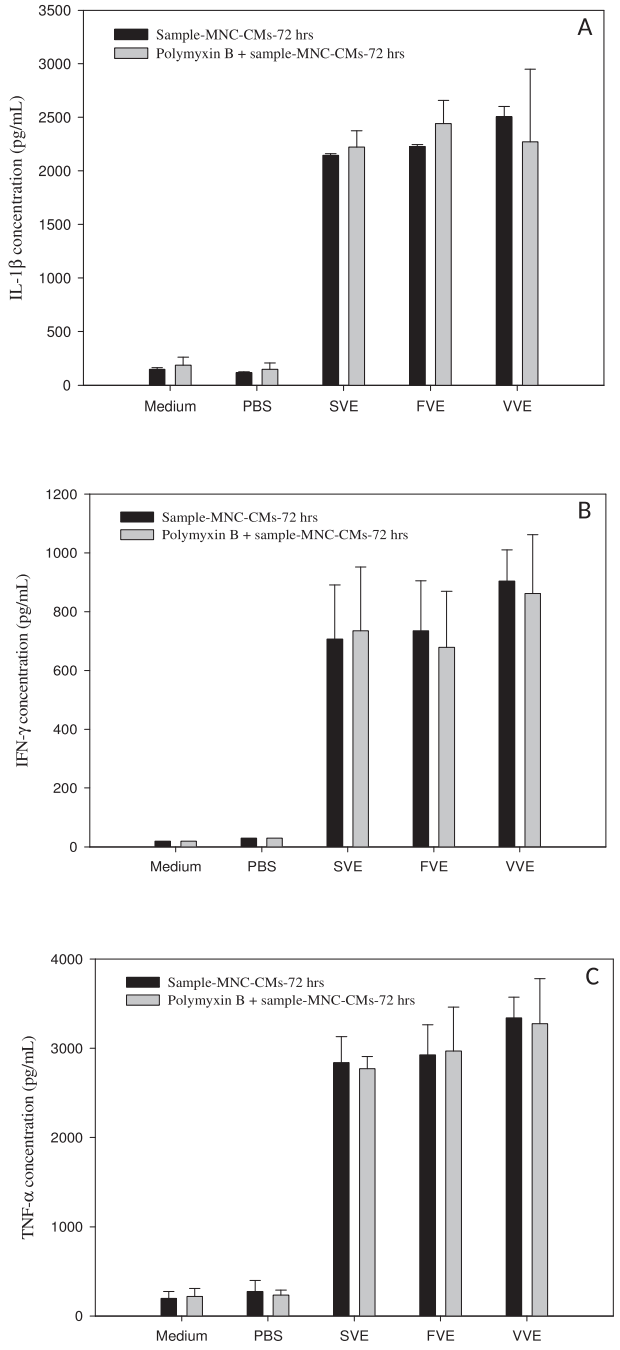

Fig. 4 – Comparison of IL-1β, TNF-α and IFN-γ content in MNC–CM prepared by 72 h stimulation with SVE, FVE, VVE (100 μg/mL) and polymyxin B (100 μg/mL). Cytokines were measured with ELISA kit. Results from three separate experiments are expressed as mean ± SD.

scavenging activity, ferrous ion chelating ability and reducing power were, in order, vinegar–egg solution > vinegar–egg concentrate > vinegar–egg condensate (Table 1). Yang et al. (2012) [26] showed that the addition of Pro and aromatic amino acids (Tyr, Phe and Trp) in vinegar could enhance DPPH free radical scavenging ability and reducing power, respectively. When adding Pro and aromatic amino acids, the DPPH free radical scavenging ability and reducing power were more

significant than with the individual addition. It can be concluded from Table 2 that the vinegar–egg is rich in free amino acids, and its antioxidant capacity is believed to be related to the amino acids contained in it. In addition, Table 3 show that vinegar–egg contains Zn, Cu and Fe elements; Zn and Cu are the cofactors of the superoxide dismutase, while iron is support factor of catalase. Superoxide dismutase and catalase both have good resistance to free radicals.

ACE inhibitory capacity changes of vinegar–egg solution, vinegar–egg condensate and vinegar–egg condensate are shown in Fig. 1. The results show that the ACE inhibitory rates of vinegar–egg solution and vinegar–egg condensate were 53.17% and 44.46%, respectively, while the ACE inhibition rate of vinegar–egg concentrate at concentration of 8.4 mg/mL exceeded 50%. At the concentration of 10 mg/mL, the inhibitory rate of ACE was 75.7%. ACE activity and its amino acid content, structure and composition are closely related. Cheung et al. (1980) [27] showed that the activity of ACEi was mainly dependent on the C-terminal amino acid, while the C-terminal amino acids were aromatic amino acids (Phe, Tyr and Trp), with Pro having higher ACE inhibitory activity. In addition, the C-terminal amino acids were basic amino acids (Lys, His and Arg); branched amino acids (BCAA: Val, Leu and Ile) had higher affinity to ACE and higher inhibitory activity. Kim et al. (2001) [28] reported that the higher the hydrophobic amino acids and acidic amino acids, the stronger the inhibitory effect of ACE.

There are two ways for natural products to act on leukemic U937 cells, either by direct inhibition of the proliferation of leukemic cells or by stimulating the secretion of differentiation-inducing factors from immune cells [29]. In this study the SEV, FEV and FFE obtained from vinegar–egg were used to inhibit the growth of human leukemic U937 cells in an indirect model. The results are shown in Table 4. VVE–MNC–CMs showed more effectiveness inhibition of U937 cells than others. In addition, the effectiveness of inhibition decreased with a reduction in samples concentration. Growth inhibitions of U937 cells treated with VVE–MNC–CMs at 200 μg/mL for 24 and 72 h were 50.34% and 64%, respectively (Table 4). MNC–CMs prepared with other substances have also been shown to inhibit U937 cell growth: the cold water extract of *Flammulina velutipes* at 800 μg/mL inhibited growth by 60% [18]; the water extract of rice (*Oryza sativa* L.) at 500 μg/mL inhibited growth by 35% [15]; bovine skimmed colostrums collected on the second day postpartum at 800 μg/mL inhibited growth by 50% [12]; bovine colostrums proteins and their hydrolyzates at 100 μg/mL inhibited growth by 57.45% [17] and the semi-purified polysaccharides of *Antrodia camphorate* and *Ganoderma lucidumat* 100 μg/mL inhibited growth by 55: and 58%, respectively [30,31].

Human leukemic U937 cells are immature monoblasts which can be induced by certain materials to differentiate into mature monocytes and macrophages expressing surface antigens CD11b and CD14, respectively [32]. Fig. 2(A) shows that CD11b expressions in the treated groups (28.9–45.1%) were significantly higher than in the medium (4.4%) and PBS group (7.5%). Fig. 2(B) shows that CD14 expressions in the treated groups (30.7–41.7%) were also significantly higher than in the medium (5.6%) and PBS group (7.2%). The detection of cell surface antigens proved that vinegar–egg is capable of inducing the differentiation of U937 cells. MNC–CMs prepared from stimulation by the polysaccharide fractions of P. *cocos, Cordyceps sinensis* and stimulation by the bovine colostrums and their protein hydrolyzates were found to promote the differentiation of about 67%, 50%, 42.6% and 57.8% of U937 cells into mature lymphocytes, respectively [12,17,33,34]. The present study found similar a immunomodulatory effect in vinegar–egg.

Cytokines are small protein molecules produced by many different cells and are involved in cell–cell communication, either systemic or localized. Cytokines are classified as immunomodulating proteins, but have many other functions as well. As immunologic cell–cell communication proteins, when cells are stimulated or injury will be released, thereby affecting cell growth, activation, inflammation, immunity, tissue repair or tissue fibrosis [29]. However, the mechanism for the induction of cytokine secretion in MNCs remains unclear. Further investigations using clinical or animal models would be worthwhile. To investigate the effects of vinegar–egg on cytokine-stimulating activities, human MNCs were treated with SVE, FVE and VVE at 100 μg/mL, and then the cytokine contents were determined (Table 6). The U937 cell growth inhibition observed in this study may be associated with the antitumor activity of TNF-α [12,17]. IL-1β may be involved in the mechanism of the human leukemic U937 cells growth inhibition because it is an enhancer of TNF-α activity [12,17]. IFN-γ are able to induce differentiation of U937 cells [17,34] and NO can induce cell death in U937 cells [17,35].

In this study the vinegar–egg products moderately inhibit the growth of human leukemic U937 cells and induce their differentiation into mature monocytes/macrophages. LPS, a kind of endotoxin in gram-negative bacteria, is often contaminated in the extracts of natural products and may over stimulate the immunopotentiation of these samples. We depleted the possible influence of LPS by using polymyxin B, a LPS blocking agent, and demonstrated that the activity of vinegar–egg products may not be due to LPS (Fig. 4).

5. Conclusion

In this study, we demonstrated that vinegar–egg not only has strong antioxidant activities and ACE inhibitory capacity, but also could moderately inhibit the growth of U937 cells while inducing U937 cell differentiation into mature monocytes/macrophages. According to the current results suggests that the antitumor activity induced by vinegar–egg is due to the stimulation of an immunomodulating response. The results of this study also show that vinegar–egg exhibits an immunomodulatory effect through the modulation of human MNCs, the secretion of cytokines (IL-1β, TNF-α and IFN-γ) and NO from MNC into MNC–CM. It may be stated from the said results that vinegar–egg not only inhibits the growth of U937 cells and exhibits their immunomodulatory activities, but also stimulates the growth of MNC. Further studies will examine the mechanism that induces cytokine secretion in MNC, and investigate the peptide compositions of hydrolyzates. Based on the results obtained in this study, we propose that vinegar–egg be investigated as a potential anticancer agent that operates through immunomodulatory activities.

Conflicts of interest

The authors declare no conflict of interest.

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