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Strawberry, loquat, mulberry, and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation using murine peritoneal macrophages

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Abstract

We hypothesized the juices from strawberry, loquat, mulberry and bitter melon exhibit anti-inflammatory activities using lipopolysaccharide (LPS)-stimulated murine peritoneal macrophage cultures. Selected juices were administered as a prophylactic, postmortem or concurrent event relative to LPS stimulation to clarify the effective mechanisms. Selected fruits and vegetable juices were administered to macrophage cultures for 24 h prior to LPS stimulation (model A). Selected samples were administered to cell cultures at 24 h following LPS treatment (model B). Selected fruits and vegetable juices and LPS were simultaneously co-cultured with macrophages for 24 h (model C). The LPS-induced secretions of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α and anti-inflammatory cytokine IL-10 were determined. The results showed that strawberry, loquat, mulberry and bitter melon administration increased IL-10 production by LPS-stimulated peritoneal macrophages in dose-dependent manners in experimental model A. Simultaneously, loquat, mulberry, and bitter melon administrations significantly ($P < 0.05$) decreased the levels of IL-1β, IL-6 and/or TNF-α. Administration with loquat and bitter melon to experimental model C significantly increased IL-10 production. This study suggests that strawberry, loquat, mulberry, and bitter melon juices exhibit a prophylactic effect on LPS-induced inflammation of peritoneal macrophages via increasing anti-inflammatory cytokine and/or decreasing pro-inflammatory cytokines secretions.
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Key words: Strawberry; Loquat; Mulberry; Bitter melon; Prophylactic effects on LPS-induced inflammation; Murine peritoneal macrophages

1. Introduction

Inflammation is a complicated immune process that can be defined by the sequential release of mediators such as pro-inflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF), interferon (IFN)-γ, IL-6, IL-12, IL-18, and the granulocyte-macrophage colony-stimulating factor. Inflammation is settled by anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IFN-α, and the transforming growth factor (TGF)-β (Hanada & Yoshimura, 2002). Although inflammation is overall a protective response against xenobiotics, chronic and uncontrolled inflammation is detrimental to tissues (Gil, 2002), which may cause chronic inflammation-derived diseases, such as cardiovascular diseases (Frostegard et al., 1999), autoimmune rheumatoid arthritis (RA) (Jara, Medina, Vera-Lastra, & Amigo, 2006), systemic lupus erythematosus (SLE), cancers (Karin, Lawrence, & Nizet, 2006), and aging-associated diseases, such as Alzheimer's or Parkinson's disease (Sarkar & Fisher, 2006). Thus, inhibition of the overproduction of inflammatory mediators, especially pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, may prevent or suppress a variety of inflammatory diseases (Kim et al., 2003). Much evidence has accumulated over the past several years implicating the anti-inflammatory

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effects of IL-10. Li, Guo, and Yang (2005) hypothesized that enhancing anti-inflammatory cytokine IL-10 may be a promising approach for acute coronary disease therapy.

A negative correlation between the consumption of diets rich in fruits, and vegetables and the risks for chronic angiogenic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers exists (Chen et al., 2006; Prior, 2003; Saleem, Husheem, Harkonen, & Pihlaja, 2002; Zhang, Vareed, & Nair, 2005). Fruits and vegetables, especially some deep-coloured varieties, are good sources of phenolics (Cieslik, Greda, & Adamus, 2006), including flavonoids (Qian, Liu, & Huang, 2004) and anthocyanins, as well as carotenoids (Sass-Kiss, Kiss, Milotay, Kerek, & Toth-Markus, 2005; Trappey, Bawadi, Bansode, & Losso, 2005). Phenolic compounds have been reported to have a strong antioxidant activity (Johnson, 2005) and exhibit a wide range of biological and pharmacological activities both *in vitro* and *in vivo*, like the inhibition of cyclooxygenase (COX), induction of CD95 signaling-dependent apoptosis, effects on cell division cycle and the modulation of nuclear transcription factor (NF)-κB activation (Falchetti, Fuggetta, Lanzilli, Tricarico, & Ravagnan, 2001). Suppressing NF-κB activation may further inhibit *in vitro* and *in vivo* inflammatory mediator releases (Kim et al., 2003). Therefore, dietary fruit and vegetable intervention is suggested to have immuno-modulatory effects such as anti-inflammation and reduce the risk for asthma and atopic disease (Devereux & Seaton, 2005; Sanchez-Moreno et al., 2006).

Among fruits, berries such as bilberries, blackberries, blueberries, cranberries, elderberries, raspberries and strawberries contain diverse anthocyanins and exert differential inhibition effects on COXs activity (Seeram, Momin, Nair, & Bourquin, 2001). Among these berries, strawberries contain abundant amounts of phenolic compounds (Hakkinen & Torronen, 2000; Versari, Biesenbruch, Barbanti, Farnell, & Galassi, 1998) and have demonstrated anticancer activity in several different experimental systems (Hannum, 2004). Mulberry fruit water extract inhibits the development of atherosclerosis in cholesterol-fed rabbits (Chen et al., 2005). Furthermore, mulberry anthocyanins extracted from fruits decrease the *in vitro* invasiveness of cancer cells (Chen et al., 2006). Bitter melon (*Momordica charantia*) fruit, commonly known as bitter gourd or karela, is used as a vegetable in South Asia, South America, and Asian countries and may be the most widely used traditional natural treatment for diabetes (McCarty, 2004; Raza et al., 1996). The methanol extract of bitter melon also exhibits a potent liver triacylglycerol-lowering activity in rats (Senanayake et al., 2004). In our preliminary study, we found that bitter melon showed a significantly ($P < 0.05$) negative correlation with splenocyte proliferation (Lin & Tang, 2007). We also found that some deep-coloured fruits and vegetables are phenolic-rich, especially flavonoid-rich (Lin & Tang, 2007), and hypothesized they have potential in immuno-modulation, such as anti-inflammation.

Inflammatory cells such as monocytes and macrophages can produce a large repertoire of cytokines and participate in the pathogenesis of granulomatous diseases (Peracoli et al., 2003), hepatitis (Sass et al., 2002), and damaging neuro-inflammatory insults such as hypoxia-ischemia (Kremlev & Palmer, 2005). Lipopolysaccharide (LPS), a component of the gram negative cell wall, was used to stimulate peritoneal macrophages and induce the production of pro-inflammatory or anti-inflammatory cytokines via the stimulation of Toll-like receptors in macrophages (Pengal et al., 2006). In this study three kinds of fruits, strawberry, loquat, and mulberry, and one kind of vegetable, bitter melon, were selected to test their *in vitro* anti-inflammatory activities using LPS-stimulated primary peritoneal macrophage cultures. The selected fruits and vegetables were administrated to peritoneal macrophage cultures as a prophylactic, postmortem, and concurrent event relative to endotoxin shock by the addition of LPS (Kruzel, Harari, Mailman, Actor, & Zimecki, 2002). Here, we report on the administration effects of juices from selected fruits and vegetables on the production of pro- (IL-1β, IL-6, and TNF-α) and anti-inflammatory (IL-10) cytokines by LPS-stimulated peritoneal macrophages.

2. Materials and methods

2.1. Collection and preparation of strawberry, loquat, mulberry, and bitter melon samples

Three deep-coloured fruit species including one variety of *Fragaria ananassa* (strawberry), one variety of *Eriobotrya japonica* (loquat), and one variety of *Morus alba* (mulberry), were selected in December, 2002. One vegetable variety of *M. charantia* L. (bitter melon) fruit was collected for this study in November, 2002. Selected fruits and vegetables were purchased from a supermarket in Taichung, Taiwan. The fresh samples were purchased and immediately, without storage, squeezed to juices. The edible portions of deep-coloured fruits and vegetables were weighed, washed and chopped, respectively, to squeeze fruit and vegetable juices using a manual stainless screw squeezer (Vegetable and Fruit Grinder, manual type, Mei-Er-Then Co., Ltd., Taipei, Taiwan, ROC). Juices were centrifuged at 10,000*g* (4 °C) for 30 min, and then the supernatants were collected using suction filtration through filter papers (Toyo No. 5B, Toyo Roshi Co., LTD., Tokyo, Japan). The filtrates were measured, lyophilized, and stored at −20 °C for future use (Lin & Tang, 2007). Total phenolic and flavonoid contents of the lyophilized samples were determined by the Folin-Ciocalteu method and aluminum chloride colorimeteric method, respectively. The detail protocol on total phenolic and flavonoid contents are described in Lin and Tang (2007). The data were expressed as milligram (mg) gallic acid equivalents (GAE)/g lyophilized powder and mg quercetin equivalents (QE)/g lyophilized powder, respectively. The moisture content of lyophilized powder from the selected sample juices was

determined according to the AOAC method (AOAC, 1984).

2.2. Primary peritoneal macrophages source

Female BALB/cByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC, and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). The animal room was kept on a 12-hr-light and 12-hr-dark cycle. Constant temperature (25 ± 2 °C) and relative humidity (50–60%) were maintained. After the mice were acclimatized for four weeks, they were sacrificed to obtain peritoneal macrophages. All mice of BALB/c strain weighing 20–25 g were used throughout the experiment. The animal use protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC.

2.3. Peritoneal macrophages isolation

The primary peritoneal macrophages from mice were collected according to the method described by Lin, Wu, Liu, and Lai (2006). Briefly, the adult female BALB/c mice were anesthetized with diethyl ether, bled using a retro-orbital venous plexus puncture to collect blood and immediately euthanized by CO_2 inhalation. Peritoneal cells were prepared by lavaging the peritoneal cavity with 2 aliquots of 5 ml sterile Hank's balanced salts solution (HBSS) ((50 ml of 10× HBSS (Biochrom AGL 2025), 2.5 ml of antibiotic-antimycotic solution (100×) containing 10,000 units/ml of penicillin, 10,000 μg/ml of streptomycin, and 25 μg/ml of amphotericin B in 0.85% saline (Atlanta biologicals-B22110), 20 ml of 3% bovine serum albumin (BSA, Sigma-Aldrich Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2–7.4, 0.2 μm filtered), 2.5 ml of 7.5% $NaHCO_3$ (Biochrom KG-L1713), 425 ml sterile water)) for a total of 10 ml through peritoneum. The peritoneal lavage fluid was centrifuged at 200*g* for 10 min at 4 °C. The cell pellets were collected and resuspended in TCM medium (2% TCM (Celox Laboratories Inc., St. Paul, Minnesota, USA), and 0.5% Antibiotic-Antimycotic solution (100×) (Atlanta biologicals-B22110) in endotoxin-free RPMI 1640 (M30115 Atlanta)). The peritoneal adherent cells (>90% of monocytes/macrophages) from each animal were adjusted to 2×10^6 cells/ml in TCM medium with a hemocytometer using the trypan blue dye exclusion method. The primary peritoneal cells were cultured in 48-well plates at 37 °C in 5% CO_2/95% air for 3 h. Cells were washed twice with fresh medium to remove non-adherent cells. Peritoneal adherent cells were re-immersed in TCM medium and immediately conducted to one of the following three LPS-induced inflammation cell culture models.

2.4. Peritoneal macrophages cultures

Three LPS-induced inflammation cell culture experimental models were designed according to the addition time of endotoxin LPS and test samples. The selected fruits and vegetable samples were administrated to peritoneal macrophage cultures as a prophylactic, postmortem, and concurrent event relative to endotoxin shock by the addition of LPS (Kruzel et al., 2002). Experimental model A was designed to be an inflammation-prophylactic cell culture model. The peritoneal macrophages (2×10^6 cells/ml in TCM medium, 0.25 ml/well) were administrated with different samples of selected fruits and vegetables at a final concentration of 0, 10, and 500 μg/ml in TCM medium (0.25 ml/well), respectively. The 48-well plates were then incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for up to 24 h. Cells were washed twice, re-immersed in 0.25 ml/well fresh medium and immediately stimulated with 0.25 ml/well stimulus at a final concentration of 10 μg/ml LPS (Sigma-Aldrich Co., L-2654). The plates were incubated for another 24 h. To obtain the supernatants, the plates were centrifuged at 200*g* for 10 min. The supernatants in cell cultures were collected and stored at −80 °C for cytokine assays. Experimental model B was designed to evaluate the effects of juice administrations to cell cultures on the clearance of cytokine production following an acute exposure to LPS. The peritoneal macrophages were stimulated with 10 μg/ml LPS for 24 h incubation. Cells were washed twice, re-immersed in fresh medium and immediately treated with different samples of selected fruits and vegetable at a final concentration of 0, 10, and 500 μg/ml, respectively. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for up to 24 h. The plates were then centrifuged at 200*g* for 10 min to obtain the supernatants. The supernatants in the cell cultures were collected and stored at −80 °C for cytokine assays. Experimental model C was designed to be an inflammation-concurrent cell culture model. The peritoneal macrophages in the presence of stimulus (LPS at the final concentration of 10 μg/ml, 0.25 ml/well) and different administration concentrations (0, 10, 500 μg/ml, 0.25 ml/well) of selected fruits and vegetables were co-plated in 48-well plates. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for up to 24 h. Then, the plates were centrifuged at 200*g* for 10 min to obtain the cell culture supernatants. The supernatants in cell cultures were collected and stored at −80 °C for cytokine assays.

2.5. Determination of cell viability (MTT assay)

To evaluate the cytotoxicity of the test samples, cell viability at the end of each experiment was determined by

3-(4,5-dimethylthiazol-2,5-diphenyl)-tetrazolium bromide (MTT, Sigma-Aldrich Co., M-5655) assay (Chen et al., 2006; Lin & Tang, 2007). Briefly, after incubation of peritoneal macrophages cultured in the plate, aliquots of 10 μl (5 mg/ml) MTT in PBS were added to each well. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for another 4 h. After incubation, the plates were centrifuged at 200*g* for 10 min. The culture medium was then discarded. The plates were further carefully washed with PBS buffer three times to avoid possible interference with the conversion of MTT into formazan crystals due to juices. Aliquots of 100 μl dimethyl sulfoxide (DMSO, Hanawa 040-01605) were added to each well and oscillated for 30 min to lyse the cell membrane and release formed insoluble formazan from cells. The absorbance was measured at 550 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Eugendorf, Austria). The cell viability was described as survival rate (%) compared to the mean control absorbency.

2.6. Determination of cytokines secretion by an ELISA

The cell culture supernatants collected from different LPS-induced inflammation experimental models were conducted to determine the levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), and anti-inflammatory cytokine (IL-10). Cytokine (IL-1β, IL-6, IL-10, and TNF-α) levels were determined using sandwich ELISA kits, respectively. The IL-1β, IL-6, IL-10, and TNF-α concentrations were assayed according to the cytokine ELISA protocol of manufacturer's instructions (mouse DuoSet ELISA Development system (R&D Systems). The sensitivity of these cytokine assays used in this study was <15.6 pg/ml.

2.7. Statistical analysis

Data are expressed as mean ± S.E. and analyzed statistically using ANOVA and Duncan's New Multiple Range test or Dunnett's multiple comparison test. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. Total phenolic and flavonoid contents in the lyophilized powder from strawberry, loquat, mulberry, and bitter melon juices and their appearances

The samples selected in this study were three species of deep-coloured fruits and one species of vegetables. Therefore, the lyophilized powder from strawberry, loquat, mulberry, and bitter melon juices, respectively reflected reddish, yellowish, blackish, and yellowish colours. The lyophilized powder from strawberry, loquat, mulberry, and bitter melon juices also showed a viscous appearance with high moisture contents (37.1, 37.8, 32.2, 33.7%, respectively) (Table 1). Furthermore, the results showed that the total phenolic and flavonoid contents in the lyophilized powder from strawberry, loquat, mulberry, and bitter melon juices varied considerably. Total phenolic contents from strawberry, loquat, mulberry, and bitter melon juices were 20.9 ± 1.4, 13.8 ± 1.3, 58.8 ± 0.3, 19.0 ± 1.6 mg GAE/g lyophilized powder (Table 1). Total flavonoid contents from strawberry, loquat, mulberry, and bitter melon juices were 0.84 ± 0.29, 0.98 ± 0.11, 9.70 ± 3.11, 1.99 ± 0.55 mg QE/g lyophilized powder (Table 1).

Table 1
Total phenolic and flavonoid contents of the lyophilized powders from strawberry, loquat, mulberry, and bitter melon juices

Materials	Total phenolic content (mg GAE/g lyophilized powder)	Total flavonoid content (mg QE/g lyophilized powder)
Strawberry	20.9 ± 1.0^{b}	0.84 ± 0.17^{c}
Loquat	13.8 ± 0.9^{c}	0.98 ± 0.06^{c}
Mulberry	58.8 ± 0.2^{a}	9.70 ± 1.79^{a}
Bitter melon	19.0 ± 1.1^{b}	1.99 ± 0.32^{b}

Data are expressed as mean ± SE in 2–3 separated experiments. Superscript letters with different letters in the same column indicate significant difference ($P < 0.05$) analyzed by Duncan's multiple range test. Means of moisture content in the lyophilized powder from strawberry, loquat, mulberry, and bitter melon juices were 37.1, 37.8, 32.2, and 33.7%, respectively.

3.2. The effects of strawberry, loquat, mulberry, and bitter melon juice administrations on cell viability in peritoneal macrophages under different LPS-stimulated experimental conditions

In this study, we first determined the cytotoxicity of juices from strawberry, loquat, mulberry, and bitter melon by treating primary peritoneal macrophages with these test samples of various concentrations (10–500 μg/ml) for 24 h followed by a MTT assay. The remaining cell viability was determined to be >92% by the MTT assay (Table 2). Compared to the controls, the test samples did not significantly affect the cell viability of peritoneal macrophages. Under the three LPS-induced inflammatory experimental conditions, the cell viability of macrophages was determined to

Table 2
Effects of strawberry, loquat, mulberry, and bitter melon juice administration on the cell viability of peritoneal macrophages *in vitro*

Administration samples	Concentrations (μg/ml)	Cell viability of macrophages (% of control)
Strawberry	10	130 ± 10
	500	127 ± 14
Loquat	10	132 ± 8
	500	123 ± 16
Mulberry	10	124 ± 7
	500	127 ± 15
Bitter melon	10	115 ± 5
	500	92 ± 10

Data are expressed as mean ± SE in three separated experiments.

Table 3
Effects of strawberry, loquat, mulberry, and bitter melon juice administration on the cell viability of LPS-stimulated peritoneal macrophages under the three inflammation experimental models *in vitro*

Experimental models	Samples	Concentrations (μg/ml)	Cell viability of LPS-stimulated peritoneal macrophages (% of control)
A	Strawberry	10	99 ± 3
		500	93 ± 7
	Loquat	10	99 ± 3
		500	109 ± 5
	Mulberry	10	106 ± 5
		500	106 ± 6
	Bitter melon	10	109 ± 4
		500	85 ± 6
B	Strawberry	10	107 ± 8
		500	121 ± 11
	Loquat	10	104 ± 10
		500	117 ± 13
	Mulberry	10	109 ± 16
		500	133 ± 16
	Bitter melon	10	87 ± 11
		500	79 ± 6
C	Strawberry	10	123 ± 18
		500	117 ± 24
	Loquat	10	128 ± 16
		500	136 ± 21
	Mulberry	10	124 ± 16
		500	123 ± 22
	Bitter melon	10	124 ± 17
		500	102 ± 22

Data are expressed as mean ± SE in three separated experiments.

be >85% (model A), >79% (model B), and >102% (model C), respectively (Table 3). The results showed that only a high dose (500 μg/ml) administration with bitter melon juice slightly, but not significantly, affected the cell viability of peritoneal macrophages. On the other hand, most selected fruits and vegetable juices which administrated to different experimental models slightly, although not significantly, increased cell viability of macrophages (Tables 2 and 3). Thus, the concentrations of strawberry, loquat, mulberry, and bitter melon juices from 10 to 500 μg/ml were used for the following experiments.

3.3. The effects of strawberry juice administrations on the cytokine secretions by peritoneal macrophages under different LPS-stimulated experimental conditions

To examine the anti-inflammatory potential and effective mechanism of the selected fruits and vegetables against inflammation *in vitro*, different fruits and vegetable samples were administrated to different LPS-stimulated macrophage models. Table 3 shows the effects of strawberry administrations on pro- and anti-inflammatory cytokine secretions by primary peritoneal macrophages under different LPS-stimulated experimental conditions. Strawberry juice (10–500 μg/ml) administration before LPS stimulation (Table 4, model A) did not significantly ($P > 0.05$) affect pro-inflammatory cytokines IL-1β, IL-6, and TNF-α secretions by LPS-stimulated peritoneal macrophages. However, strawberry juice administration markedly ($P < 0.05$) increased anti-inflammatory cytokine IL-10 secretion in a dose-dependent manner. The results suggested that strawberry juice administration may exhibit a prophylactic effect against LPS-induced inflammation of primary peritoneal macrophages via increasing the secretion of anti-inflammatory cytokine IL-10.

Strawberry juice (10–500 μg/ml) administration after LPS stimulation (Table 4, model B) did not significantly affect pro-inflammatory cytokines IL-1β, and TNF-α secretions by LPS-stimulated peritoneal macrophages. However, strawberry juice administration significantly ($P < 0.05$) increased the secretions of pro-inflammatory cytokine IL-6, and anti-inflammatory cytokine IL-10 in a dose-dependent manner. The results exhibited that strawberry juice administration could not fully inhibit the inflammation status of peritoneal macrophages stimulated by LPS.

Table 4
Effects of strawberry juice administration on pro- and anti-inflammatory cytokine secretions by LPS-stimulated peritoneal macrophages *in vitro* under different experimental models

Experimental model	Sample concentrations (μg/ml)	Pro-inflammatory cytokines			Anti-inflammatory cytokine
		IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10 (pg/ml)
A	0	242 ± 48	3056 ± 316	1564 ± 160	16 ± 16
	10	220 ± 27	2979 ± 246	1666 ± 235	31 ± 19
	500	150 ± 27	2403 ± 362	1721 ± 275	114 ± 47*
B	0	147 ± 37	270 ± 91	44 ± 3	n.d.
	10	143 ± 48	434 ± 75	44 ± 6	34 ± 17*
	500	129 ± 44	650 ± 109*	48 ± 5	65 ± 40
C	0	254 ± 58	5204 ± 1199	956 ± 89	199 ± 51
	10	230 ± 61	7097 ± 201	1403 ± 163*	286 ± 68
	500	258 ± 62	6173 ± 538	1260 ± 175	390 ± 110

Data are expressed as mean ± SE in five separated experiments. Asterisk (*) means significantly different ($P < 0.05$) from the control (0 μg/ml) under same experimental model within same column assayed with Dunnett's multiple comparison test. n.d.: not detectable.

Strawberry juice (10–500 μg/ml) concurrent administration with LPS to macrophages (Table 4, model C) did not significantly affect pro-inflammatory cytokines IL-1β, and IL-6 secretions by macrophages. Low dose (10 μg/ml) strawberry juice administration significantly ($P < 0.05$) increased the secretion of pro-inflammatory cytokine TNF-α., while strawberry juice administration increased, but not statistical increase, the secretion of anti-inflammatory cytokine IL-10 in a dose-dependent manner. The results exhibited that strawberry juice administration could not fully inhibit the inflammation status of macrophages together with LPS stimulation.

3.4. The effects of loquat juice administrations on the cytokine secretions by peritoneal macrophages under different LPS-stimulated experimental conditions

Table 5 shows that the effects of loquat administrations on pro- and anti-inflammatory cytokine secretions by primary peritoneal macrophages under different LPS-stimulated experimental conditions. Loquat juice (10–500 μg/ml) administration before LPS stimulation (Table 5, model A) did not significantly affect pro-inflammatory cytokine IL-1β secretion by LPS-stimulated peritoneal macrophages. However, low dose (10 μg/ml) of loquat juice administration significantly ($P < 0.05$) inhibited pro-inflammatory cytokine IL-6 secretion. High dose (500 μg/ml) administration with loquat juice significantly ($P < 0.05$) inhibited pro-inflammatory cytokine TNF-α secretion in a dose-dependent manner. In the meantime, loquat juice administration increased, but not statistical increase, the secretion of anti-inflammatory cytokine IL-10 by LPS-stimulated peritoneal macrophages in a dose-dependent manner. The results suggested that loquat juice administrations exhibited a prophylactic effect against LPS-induced inflammation of primary peritoneal macrophages.

Loquat juice (10–500 μg/ml) administration after LPS stimulation (Table 5, model B) did not significantly affect pro-inflammatory cytokines IL-1β, and TNF-α secretions by LPS-stimulated peritoneal macrophages. However, loquat juice administration significantly ($P < 0.05$) increased the secretions of pro-inflammatory cytokine IL-6, and anti-inflammatory cytokine IL-10 by LPS-stimulated peritoneal macrophages in a dose-dependent manner. The results exhibited that loquat juice administrations could not fully inhibit macrophage inflammation stimulated by LPS.

Loquat juice (10–500 μg/ml) concurrent administration with LPS to macrophages (Table 5, model C) did not significantly affect pro-inflammatory cytokines IL-1β, IL-6, and TNF-α secretions by peritoneal macrophages. However, high dose (500 μg/ml) of loquat juice administration significantly ($P < 0.05$) increased the secretion of anti-inflammatory cytokine IL-10. The results suggested that loquat juice administration might inhibit the inflammation in LPS-stimulated macrophages via increasing the secretion of anti-inflammatory cytokine IL-10 under this concurrent experimental model.

3.5. The effects of mulberry juice administrations on the cytokine secretions by peritoneal macrophages under different LPS-stimulated experimental conditions

Table 6 shows that the effects of mulberry juice administration on pro- and anti-inflammatory cytokine secretions by primary peritoneal macrophages under different LPS-stimulated experimental conditions. Mulberry juice (10–500 μg/ml) administration before LPS stimulation (Table 6, model A) did not significantly affect pro-inflammatory cytokine IL-1β secretion by LPS-stimulated peritoneal macrophages. However, mulberry juice administration significantly ($P < 0.05$) inhibited pro-inflammatory cytokines IL-6, and TNF-α secretions by LPS-stimulated peritoneal macrophages in a dose-dependent manner. In the meantime, mulberry juice administration increased, but not statistically significant increase, the secretion of anti-inflammatory cytokine IL-10 in a dose-dependent manner.

Table 5
Effects of loquat juice administration on pro- and anti-inflammatory cytokine secretions by LPS-stimulated peritoneal macrophages *in vitro* under different experimental models

Experimental model	Sample concentrations (μg/ml)	Pro-inflammatory cytokines			Anti-inflammatory cytokine
		IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10(pg/ml)
A	0	242 ± 48	3056 ± 316	1564 ± 160	16 ± 16
	10	209 ± 18	2367 ± 132*	1357 ± 280	34 ± 13
	500	277 ± 68	2647 ± 399	682 ± 118*	70 ± 33
B	0	147 ± 37	270 ± 91	44 ± 3	n.d.
	10	165 ± 56	434 ± 81	42 ± 6	31 ± 16*
	500	168 ± 53	483 ± 70*	37 ± 11	85 ± 46*
C	0	254 ± 58	5204 ± 1199	956 ± 89	199 ± 51
	10	233 ± 66	6255 ± 324	1221 ± 193	190 ± 34
	500	198 ± 35	5786 ± 434	1165 ± 185	386 ± 74*

Data are expressed as mean ± SE in five separated experiments. Asterisk (*) means significantly different ($P < 0.05$) from the control (0 μg/ml) under same experimental model within same column assayed with Dunnett's multiple comparison test. n.d.: not detectable.

Table 6
Effects of mulberry juice administration on pro- and anti-inflammatory cytokine secretions by LPS-stimulated peritoneal macrophages *in vitro* under different experimental models

Experimental model	Sample concentrations (μg/ml)	Pro-inflammatory cytokines			Anti-inflammatory cytokine
		IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10(pg/ml)
A	0	242 ± 48	3056 ± 316	1564 ± 160	16 ± 16
	10	184 ± 27	2314 ± 168*	1532 ± 278	61 ± 37
	500	301 ± 113	1337 ± 263*	1010 ± 237*	62 ± 42
B	0	147 ± 37	270 ± 91	44 ± 3	n.d.
	10	172 ± 60	430 ± 83	44 ± 6	47 ± 23*
	500	190 ± 29	654 ± 89*	41 ± 6	106 ± 45*
C	0	254 ± 58	5204 ± 1199	956 ± 89	199 ± 51
	10	235 ± 74	5920 ± 232	1435 ± 209*	195 ± 39
	500	204 ± 44	5455 ± 342	893 ± 150	614 ± 108*

Data are expressed as mean ± SE in five separated experiments. Asterisk (*) means significantly different ($P < 0.05$) from the control (0 μg/ml) under same experimental model within same column assayed with Dunnett's multiple comparison test. n.d.: not detectable.

The results suggested that mulberry juice administration exhibited a prophylactic effect against LPS-induced inflammation in peritoneal macrophages.

Mulberry juice (10–500 μg/ml) administration after LPS stimulation (Table 6, model B) did not significantly affect pro-inflammatory cytokines IL-1β, and TNF-α secretions by LPS-stimulated peritoneal macrophages. However, mulberry juice administration significantly ($P < 0.05$) increased the secretion of pro-inflammatory cytokine IL-6, and anti-inflammatory cytokine IL-10 in a dose-dependent manner. The results suggested that mulberry juice administrations could not fully inhibit the inflammation in macrophages stimulated by LPS.

Mulberry juice (10–500 μg/ml) concurrent administration with LPS to macrophages (Table 6, model C) did not significantly affect pro-inflammatory cytokines IL-1β, and IL-6 secretions by peritoneal macrophages. However, low (10 μg/ml) and high doses (500 μg/ml) of mulberry juice administrations significantly ($P < 0.05$) increased the secretions of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10, respectively. The results suggested that mulberry juice administrations could not fully inhibit the inflammation in macrophages together with LPS stimulation.

3.6. The effects of bitter melon juice administrations on the cytokine secretions by peritoneal macrophages under different LPS-stimulated experimental conditions

Table 7 shows that the effects of bitter melon juice administrations on pro- and anti-inflammatory cytokine secretions under different LPS-stimulated experimental conditions. Bitter melon juice (10–500 μg/ml) administration before LPS stimulation (Table 7, model A) significantly ($P < 0.05$) inhibited pro-inflammatory cytokines IL-1β, IL-6, and TNF-α secretions by LPS-stimulated peritoneal macrophages in a dose-dependent manner. In the meantime, bitter melon juice administration increased, but not statistical increase, the secretion of anti-inflammatory cytokine IL-10 in a dose-dependent manner. The results suggested that mulberry juice administration exhibited a prophylactic effect against LPS-induced inflammation in peritoneal macrophages.

Bitter melon juice (10–500 μg/ml) administration after LPS stimulation (Table 7, model B) did not significantly affect the secretion of pro-inflammatory cytokine TNF-α by LPS-stimulated peritoneal macrophages. However, bitter melon juice administration significantly ($P < 0.05$) increased the secretions of pro-inflammatory cytokines

Table 7
Effects of bitter melon juice administration on pro- and anti-inflammatory cytokine secretions by LPS-stimulated peritoneal macrophages *in vitro* under different experimental models

Experimental model	Sample concentrations (μg/ml)	Pro-inflammatory cytokines			Anti-inflammatory cytokine
		IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	IL-10(pg/ml)
A	0	242 ± 48	3056 ± 316	1564 ± 160	16 ± 16
	10	168 ± 59	1579 ± 627*	928 ± 447	53 ± 46
	500	91 ± 15*	422 ± 167*	84 ± 46*	78 ± 39
B	0	147 ± 37	270 ± 91	44 ± 3	n.d.
	10	188 ± 61	446 ± 82	49 ± 9	68 ± 44
	500	261 ± 49*	499 ± 78*	43 ± 8	260 ± 151
C	0	254 ± 58	5204 ± 1199	956 ± 89	199 ± 51
	10	286 ± 57	6618 ± 381	1043 ± 243	311 ± 61
	500	614 ± 70*	5823 ± 592	975 ± 168	886 ± 135*

Data are expressed as mean ± SE in five separated experiments. Asterisk (*) means significantly different ($P < 0.05$) from the control (0 μg/ml) under same experimental model within same column assayed with Dunnett's multiple comparison test. n.d.: not detectable.

IL-1β, and IL-6 in a dose-dependent manner. In the meantime, the anti-inflammatory cytokine IL-10 secreted by LPS-stimulated peritoneal macrophages also increased, although not significantly, in a dose-dependent manner. The results suggested that bitter melon juice administration could not fully inhibit the inflammation in macrophages stimulated by LPS under this experimental model.

Bitter melon juice (10–500 μg/ml) concurrent administration with LPS to macrophages (Table 7, model C) did not significantly affect pro-inflammatory cytokines IL-1β, IL-6, and TNF-α secretions by macrophages. However, high dose (500 μg/ml) of bitter melon juice administration significantly ($P < 0.05$) increased the secretion of anti-inflammatory cytokine IL-10 in a dose-dependent manner. The results suggested that bitter melon juice administrations might inhibit the inflammation in macrophages together with LPS stimulation via increasing the secretion of anti-inflammatory cytokine IL-10.

4. Discussion

Based on the secretions of pro- and/or anti-inflammatory cytokines in this *in vitro* study, we have established three anti-inflammation models and evaluated potentially anti-inflammatory food materials according to the administration order of test samples and/or endotoxin LPS to the peritoneal macrophage cultures. Administered fruits and vegetable juices and/or endotoxin LPS caused no significant effects on macrophage cell viability (Tables 2 and 3), but resulted in significant changes in pro- and anti-inflammatory secretions by peritoneal macrophages (Tables 4–7). This study suggests that the established anti-inflammation models are suitable for the evaluation of food materials and the juices from strawberry, loquat, mulberry, and bitter melon were first subjected to the established models.

Strawberry administration in experimental model A resulted in a rather uniform inhibition of pro-inflammatory cytokine secretion; IL-1β by 38%, IL-6 by 21%. Simultaneously, the secretion of anti-inflammatory cytokine IL-10 significantly increased by 613% (Table 4, model A). Strawberry extracts exhibit anti-inflammatory potential via inhibiting COX-1 and COX-2 activity (Seeram et al., 2001). This study further suggests that strawberry juice exhibits prophylactic effects against inflammation via immuno-modulating the pro- and anti-inflammatory cytokine secretions. In strawberries, the most abundant bioactive compounds were ellgic acid, and certain flavonoids, such as anthocyanin, catechin, quercetin and kaempferol (Hannum, 2004). We also found that total phenolic content in strawberry juice (Table 1) were moderately higher than those in other selected fruits and vegetables (Lin & Tang, 2007). The present study further suggests that phenolics in strawberry juice might contribute to anti-inflammation.

Loquat administrations in experimental model A resulted in a significant inhibition of pro-inflammatory cytokine secretion; IL-6 by 23%, TNF-α by 56%. Simultaneously, the secretion of anti-inflammatory cytokine IL-10 increased by 338% in a dose-dependent manner (Table 5, model A). Furthermore, loquat administrations in experimental model C resulted in a significant increase of anti-inflammatory cytokine IL-10 secretion (Table 5, model C). This study first suggests that loquat juice exhibits prophylactic and concurrent anti-inflammation effects against LPS-induced inflammation *in vitro* via the mechanisms of immuno-modulating pro- and anti-inflammatory cytokine secretions. The report on bioactive compounds in loquat juice is still limited. We found that total phenolic and flavonoid contents in loquat juice (Table 1) were much lower than those in other selected fruits and vegetables. Thus, the main anti-inflammatory components in loquat might not be phenolics.

Mulberry administrations in experimental model A resulted in a significant inhibition of pro-inflammatory cytokine secretion; IL-6 by 56%, TNF-α by 35%. Simultaneously, the secretion of anti-inflammatory cytokine IL-10 increased by 288% in a dose-dependent manner (Table 6, model A). Mulberry juice administrations in experimental models B and C resulted in a simultaneously significant increase in pro- and anti-inflammatory cytokine secretions (Table 6, models B and C). The results suggest that the anti-inflammatory action mechanism for mulberry components acts as a prophylactic rather than a postmortem repairing agent. Chung et al. (2003) reported that oxyresveratrol from mulberry fruits exhibited a significant inhibition against LPS-induced inflammation *in vitro* and *in vivo* via inhibiting the translocation of NF-κB, and the activity of COX-2 and iNOS. The bioactive compounds in mulberry were identified as prenylflavonoids, coumarin, and stilbene (Oh et al., 2002). The phenolic compounds in mulberry fruits might down-regulate NF-κB binding and COX-2 activity and further result in a decrease of inflammatory mediators, such as secretions of IL-6, and TNF-α (Chung et al., 2003). This study further suggests that mulberry juice exhibits prophylactic effects against inflammation via immuno-modulating pro- and anti-inflammatory cytokine secretions. Total phenolic and flavonoid contents in mulberry juice (Table 1) were the highest among those selected in this study. Inevitably, phenolics in mulberry juice are suggested to be anti-inflammatory components.

Bitter melon administrations in experimental model A caused a significant inhibition of pro-inflammatory cytokine secretion; IL-1β by 62%, IL-6 by 86%, TNF-α by 95%. Simultaneously, the secretion of anti-inflammatory cytokine IL-10 increased by 388% in a dose-dependent manner (Table 7, model A). Bitter melon administration in experimental model C resulted in a significant increase in anti-inflammatory cytokine IL-10 secretion (Table 7, model C). These results suggest that the anti-inflammatory action mechanism for bitter melon components might act as both prophylactic and concurrent anti-inflammation agents rather than a postmortem repairing agent. Huang and Wu (2002) indicated that bitter melon extracts inhibited PGE_2 production by a macrophage cell line. This study

further suggests that bitter melon juice exhibits prophylactic and concurrent effects against inflammation via the immuno-modulating pro- and/or anti-inflammatory cytokine secretions in primary peritoneal macrophage culture model. The bioactive compounds in bitter melon, including charantin, momordin, polypeptide-P, vicine (Shane-McWhorter, 2001), and monoacylglycerol (Konishi et al., 2004), have been reported. A 30 kDa antiviral protein, MAP30, which isolated from seeds of bitter melon (*M. charantia*) exhibits potential of anti-inflammatory drugs (Bourinbaiar & Lee-Huang, 1995). Furthermore, total phenolic and flavonoid contents in bitter melon juice (Table 1) were moderately lower than those in other selected fruits. This study suggests that the mainly anti-inflammatory components in bitter melon fruits might not be phenolics.

The present study provides scientific supporting evidence for the use of fruits and vegetables juices as an anti-inflammatory agent against inflammed macrophages *in vitro*. To unravel potentially pharmacological actions of the selected fruits and vegetables, the bioactive compounds should be further clarified and conducted to animal studies.

5. Conclusion

This study showed that strawberry, loquat, mulberry, and bitter melon administration before endotoxin treatment *in vitro* alleviate LPS-induced inflammation of peritoneal macrophages via decreasing the secretion of pro-inflammatory cytokines (IL-1β, IL-6, or TNF-α) and/or increasing the secretion of anti-inflammatory cytokine (IL-10).

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