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Oleaginous Yeast *Rhodosporidium toruloides* as a Tool for Rapid Evaluation of Anti-Obesity Candidates: Inhibitory Effect of Persimmon Leaf Fermentate on Lipid Accumulation S

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The aim of this study was to examine the efficiency of *Rhodosporidium toruloides* as a new tool to evaluate the triglyceride (TG) reduction effects of anti-obesity candidate materials. Unfermented and fermented persimmon leaf hot water extracts (UFPLE and FPLE) were used as anti-obesity agents. The content of TG in *R. toruloides* treated with FPLE was less than those with UFPLE by about 11% ($p < 0.05$) relative to the control (*R. toruloides* incubated in YPD medium without the agents). Fat reduction in 3T3-L1 cells achieved by FPLE was about 13% higher than that achieved by UFPLE.

Keywords: Persimmon leaf, fermentation, lactic acid bacteria, triglyceride, lipid accumulation, *Rhodosporidium toruloides*

Plants contain various substances of physiological significance; however, their contents are low. Bioconversion, or fermentation, can increase the concentrations of the bioactive compounds in plants [1, 2]. Previous studies have shown that the concentrations of flavonols, quercetin, and kaempferol in mulberry leaf extract and saponin and platycodon D in *Platycodon grandiflorum* root extract increase after fermentation [3, 4]. The antioxidant activity of flavonol-fortified fermented mulberry leaf extract was approximately twice that of unfermented mulberry leaf extract [3].

Diospyros kaki Thunb. (persimmon) is a deciduous fruit tree belonging to the Ebenaceae family and grows in warm regions [5]. It is mainly cultivated in East Asia, including Korea, Japan, and China. Persimmon fruits are commonly eaten raw, dried, cooked, or fermented (persimmon vinegar); its leaves are most frequently consumed as tea for beneficial effects on various diseases, including diabetes mellitus, atherosclerosis, ischemia, and hypotension [5–7], owing to their high content of tannins and flavonoids [5, 8]. Some in vivo studies have suggested that persimmon leaves ameliorated hyperglycemia, dyslipidemia, and fat accumulation [9–11].

In addition, some studies showed that fermented persimmon and persimmon vinegar exhibited antioxidant [12] and protective effects against alcohol-induced fat deposition [13] and hepatic injury [14]. Persimmon fruit juice fermented with lactic acid bacteria isolated from kimchi (a Korean traditional fermented vegetable) has been reported [15], and persimmon leaves have been used in the manufacture of fermented functional foods, such as bread and beverages [16–18].

Quercetin-3-*O*-(2′′-*O*-galloyl-β-D-glucopyranoside) and kaempferol-3-*O*-(2′′-*O*-galloyl-β-D-glucopyranoside) are the main polyphenols in persimmon leaves [19]. In addition, the aglycones have shown anti-obesity and antidiabetic effects in both in vitro and in vivo models [20]. In this study, a lactic acid bacterium was selected to produce a persimmon leaf fermentate containing high concentrations of quercetin and kaempferol to enhance its anti-obesity activity. To investigate the inhibitory effect of the persimmon leaf fermentate on triglyceride (TG) and lipid accumulation, the inhibitory effect of the persimmon leaf fermentate on

TG accumulation in *Rhodosporidium toruloides* was evaluated, and the fat reduction effect was confirmed in 3T3-L1 cells.

The persimmon leaf-fermenting lactic acid bacterium *Pediococcus pentosaceus* was isolated from nuruks, which is a traditional Korean starter culture. *R. toruloides* (KCTC No. 7134, Type Strain) was purchased as a freeze-dried culture from the Korean Collection for Type Cultures (KCTC, Korea). The dried persimmon leaves were extracted with distilled water at 100°C for 9 h. The extract was centrifuged at 12,000 ×*g* for 15 min, and dried using a freeze dryer (PVTFD100R; ilShinBioBase, Korea). The powder (5 g) was dissolved in distilled water (500 ml) and fermented at 37°C for 72 h using *P. pentosaceus*. To determine the quercetin and kaempferol contents of the fermentate, high-performance liquid chromatography-diode array detection (Waters Corp., USA) was performed according to the method described by Lee *et al*. [3].

The freeze-dried unfermented persimmon leaf hot water extract (UFPLE) and fermented persimmon leaf hot water extract (FPLE) were added to a yeast extract-peptone-dextrose (YPD) broth (Becton, Dickinson and Company, USA) at a final concentration of 1%. Subsequently, 2 µl of precultured *R. toruloides* was inoculated in 20 ml of the YPD broth and incubated at 25°C for 72 h with shaking at 180 rpm. Lipid extraction was performed according to the method described by Sha [21]. The concentration of TGs in the yeast culture was determined using a serum TG determination kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. To determine the inhibitory effects of UFPLE and FPLE on lipid accumulation in 3T3-L1 cells, differentiation of 3T3-L1 preadipocytes was induced using the method described by Lee *et al*. [22]. All experiments were performed in triplicate; data were analyzed using the SPSS software package (SPSS, USA). Multiple comparisons were performed among all data using Duncan's multiple range test, and $p < 0.05$ was considered statistically significant.

The UFPLE and FPLE were extracted with methanol, and the quercetin and kaempferol contents in the extracts were analyzed using high-performance liquid chromatography [3]. The chromatograms of UFPLE and FPLE are shown in Fig. 1. Quercetin and kaempferol contents in FPLE powder were about 1.8- and 1.7-fold those in UFPLE powder, respectively (Table 1).

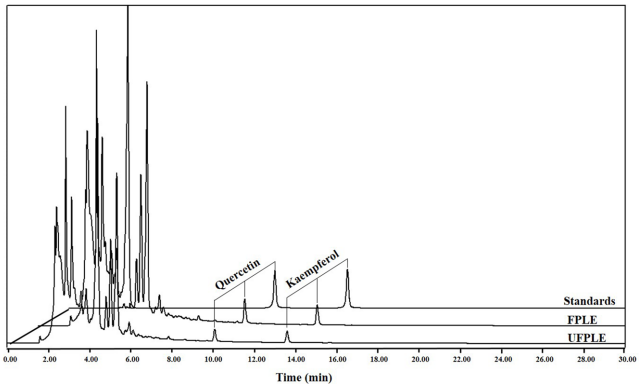

Fig. 1. Chromatogram of reversed-phase high-performance liquid chromatography of UFPLE and FPLE.
Standard quercetin and kaempferol were used as molecular references. UFPLE, unfermented persimmon leaf hot water extract; FPLE, fermented persimmon leaf hot water extract.

R. toruloides growth was not inhibited in media containing 1% UFPLE or FPLE (Table 2). The total lipid contents in *R. toruloides* cultured in media containing 1% UFPLE or FPLE were 184.63 ± 0.20 and 177.61 ± 0.37 µg/g wet cell mass, respectively, which were 12.0% and 15.4% less than that in the control *R. toruloides* incubated in YPD medium without UFPLE and FPLE (Table 2). The TG content in

Table 1. Quercetin and kaempferol contents in the UFPLE and FPLE powders.

Sample	Quercetin (mg/100 g)	Kaempferol (mg/100 g)
UFPLE	7.22 ± 0.33	6.75 ± 0.22
FPLE	13.14 ± 0.38	11.24 ± 0.22

UFPLE, unfermented persimmon leaf hot water extract powder; FPLE, fermented persimmon leaf hot water extract. Data are the means ± standard deviations from three independent experiments.

Table 2. Effects of UFPLE and FPLE on inhibition of triglyceride (TG) accumulation in *Rhodosporidium toruloides*.

Sample	Wet cell mass (mg)	Total lipid content (µg/g wet cell mass)	TG content (µg/mg total lipid)
Control	110.42 ± 0.24^{c}	209.85 ± 0.49^{a}	0.36 ± 0.00^{a}
UFPLE	125.64 ± 0.17^{a}	184.63 ± 0.20^{b}	0.23 ± 0.02^{b}
FPLE	123.40 ± 0.20^{b}	177.61 ± 0.37^{c}	0.19 ± 0.01^{c}

UFPLE, *R. toruloides* incubated with the unfermented persimmon leaf hot water extract powder; FPLE, *R. toruloides* incubated with the fermented persimmon leaf hot water extract; Control, *R. toruloides* incubated in YPD medium without UFPLE and FPLE. Data are the means ± standard deviations from three independent experiments. The superscript letters represent significant difference at $p < 0.05$ by Duncan's multiple range test.

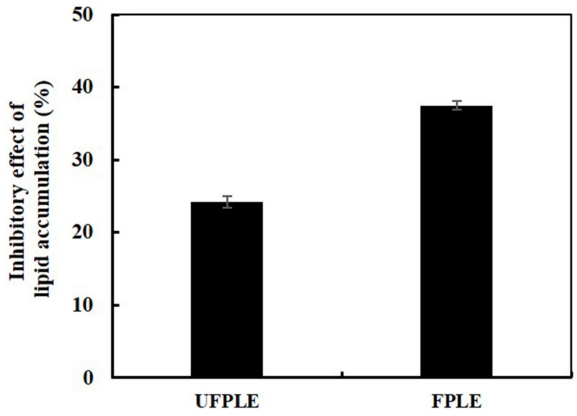

Fig. 2. Inhibition of lipid accumulation in 3T3-L1 cells by UFPLE and FPLE treatment.

UFPLE, unfermented persimmon leaf hot water extract; FPLE, fermented persimmon leaf hot water extract.

UFPLE- and FPLE-treated cells was 36.1% and 47.2% less than that in the control, respectively (Table 2). FPLE-treated cells showed 11.1% less TG accumulation than the UFPLE-treated cells did.

To confirm the effect of FPLE on lipid reduction in mammalian cells, 3T3-L1 preadipocytes were differentiated with rosiglitazone in the presence of FPLE (0.5 mg/ml, this concentration was selected because it did not affect cell viability) (data not shown). The UFPLE and FPLE reduced lipid accumulation in 3T3-L1 cells by 24.2% and 37.5%, respectively (Fig. 2). The reduction achieved by FPLE was 13.3% higher than that achieved by UFPLE. This higher lipid-reducing effect of FPLE may be attributed to the increase in its quercetin and kaempferol contents after fermentation. The lipid-reducing effects of quercetin and kaempferol on 3T3-L1 cells have previously been studied; the TG content in samples treated with 50 μM quercetin and kaempferol was 49% and 31% less than that in the untreated control, respectively [23].

Mice and their preadipocytes have been traditionally used as models for obesity research. Owing to its cost efficiency and convenience in generating adipocytes, the 3T3-L1 cell line, which is derived from mouse 3T3 preadipocytes, is widely used as an in vitro model for initial studies of obesity, particularly in the screening and evaluation of anti-obesity candidate materials. However, experiments using 3T3-L1 cells are challenging because of the low differentiation efficiency attributable to the changes that occur during cell passaging and storage [24].

Oleaginous microorganisms such as yeasts, filamentous fungi, and microalgae can accumulate lipids at more than 20% of their dry cell weight [25, 26]. For this reason, these microorganisms have been studied as the main lipid source for biodiesel production [25]. Yeasts are the major eukaryotic oleaginous microorganisms, including the genera *Rhodosporidium*, *Rhodotorula*, *Yarrowia*, *Candida*, *Cryptococcus*, *Trichosporon*, and *Lipomyces* [21, 27]. Among the oleaginous yeasts, *R. toruloides* is known as a carotenoid-producing, non-pathogenic basidiomycete [28]. This yeast is capable of accumulating lipids at over 70% of its dry cell biomass through carbohydrate catabolism under nitrogen limitation [28, 29]. Owing to its ability to accumulate fat, *R. toruloides* is a potentially useful tool for the screening and evaluation of anti-obesity candidate materials, and it can be a convenient alternative to overcome the problems of cellular in vitro model systems.

R. toruloides is a robust oleaginous yeast that produces TGs in lipid droplets (LDs) through the utilization of carbon substrates [28, 30]. Based on the results of previous in vitro and in vivo anti-obesity studies [31, 32] on *Garcinia cambogia* extract (GCE), which is currently used as a weight-loss supplement, the effect of GCE on inhibition of TG accumulation in *R. toruloides* was evaluated through a preliminary experiment (unpublished). Culturing in the presence of 2% GCE markedly decreased TG accumulation in *R. toruloides* without growth inhibition (Figs. S1A, S1B). In addition, to visually compare the LDs produced, the *R. toruloides* cells incubated in YPD medium with 2% GCE were stained with Nile red [33], which is used to localize and quantitate lipids, particularly neutral LDs within cells. It was confirmed that the amount of LDs (green spots) produced in the cells treated with 2% GCE was lower than that in the control (Fig. S2). The effect of 2% GCE on the suppression of TG accumulation in *R. toruloides* as an in vitro system appears to be consistent with the anti-obesity effect shown in rats fed a high-fat diet supplemented with 2% GCE [32].

The synthesis of TGs is one of the most important factors in the development of obesity. Glycerol 3-phosphate dehydrogenase (GPDH), fatty acid synthase (FAS), and ATP-citrate lyase (ACL) are involved in the synthesis of TGs in human adipocytes [34]. *R. toruloides* also contains genes encoding GPDH, FAS, and ACL [28, 35]. Therefore, besides TG accumulation, changes in the expression levels of the majority of TG synthesis-associated proteins can also be confirmed in *R. toruloides*.

Overall, using a new rapid and easy in vitro microorganism-based anti-obesity test system, the inhibitory effect of FPLE on TG accumulation was evaluated. The FPLE-treated *R. toruloides* showed about 11% reduction in TG accumulation.

In addition, the effect of FPLE in this in vitro system was consistent with the anti-obesity effects observed in mammalian 3T3-L1 cells. Based on these results, this microbial system can be potentially applied to the development of functional food materials beneficial for various diseases (diabetes, hepatic steatosis, and cardiovascular disease) caused by TG accumulation. This is the first study to provide a rapid and easy in vitro microorganism-based anti-obesity test system for food materials. However, before *R. toruloides* can be practically applied as a screening and evaluation tool for the anti-obesity effects of various materials, many studies are required to crosscheck the results obtained from the in vitro and in vivo (mouse) model systems used here, including evaluations of lipid accumulation and gene expression levels.

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