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The hypolipidemic properties study of total triterpenic acids from *Polyporus umbellatus*

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ABSTRACT

In the present study, we have provided a facile and feasible procedure for total triterpenic acids extraction from the traditional Chinese medicine Polyporus umbellatus as well as appraisal of their antihyperlipidemic activity in hyperlipidemic rats model. To optimize the extraction procedure, solvents were evaluated by mono-factor test, the other conditions including liquid/solid ratio, the size of sample, extraction temperature and extraction time were optimized by means of an orthogonal design $L_9(3^4)$. Additionally, the content of total triterpenic acids was measured based on the UV spectral data with oleanolic acid as standard agent. Finally, the hypolipidemic effect of Polyporus umbellatus total triterpenic acids(QPUTA) were tested in Triton WR-1339 induced hyperlipidemic rats. Specifically, high dosage QPUTA showed potent antihyperlipidemic activity and was found to decrease the plasma triglyceride levels (TG) by 77.97%, total cholesterol (TC) by 19.67%, accompanied by an increase in HDL-C/TC ratio by 54.13% to a greater degree than the reference fibrate. Therefore, QPUTA could be used to treat hyperlipidemic patients according to the in vivo hypolipidemic effects.

Keywords: *Polyporus umbellatus*; Triterpenic Acids; Traditional Chinese Medicine; Orthogonal Test; Hypolipidemic Activity.

INTRODUCTION

The east-west Qin Mountains (simplified Qiling) range in southern Shaanxi province are a natural boundary between the North and South of China. Qiling provide a huge variety of plant and wildlife, some of which are found nowhere else on earth, such as critically endangered giantpandas and Chinese crested ibis, NipponiaNippon[1-3]. *Polyporus Umbellatus* (**Figure1**) is an edible species of mushroom, found growing densely in the soil humus among forests of Qinling but very rare. The fruit body of *Polyporus Umbellatus* is also a traditional Chinese medicine sources contains a wide range of bioactive compounds with immune stimulating, anticancer, anti-inflammatory, and hepatoprotective properties[4,5].Especially, several polysaccharides and polyporenic acids found in *Polyporus Umbellatus* have been investigated as major pharmacologically active ingredients[6].

Figure 1. *PolyporusUmbellatus*

Moreover, some triterpenoids and triterpene derivatives are well-known for their diverse pharmacological effects including antiretroviral, antimalarial, and anti-inflammatory, anticancer properties, as well as a more recently discovered potential as a hypolipidemic agent, which can be listed as, Betulinic acid (a naturally occurring pentacyclictriterpenoid), Araloside A, Astragaloside IV, bacoside A, Cucurbitacin, Eleutheroside A (daucosterol) **(Figure 2)**[7-11].

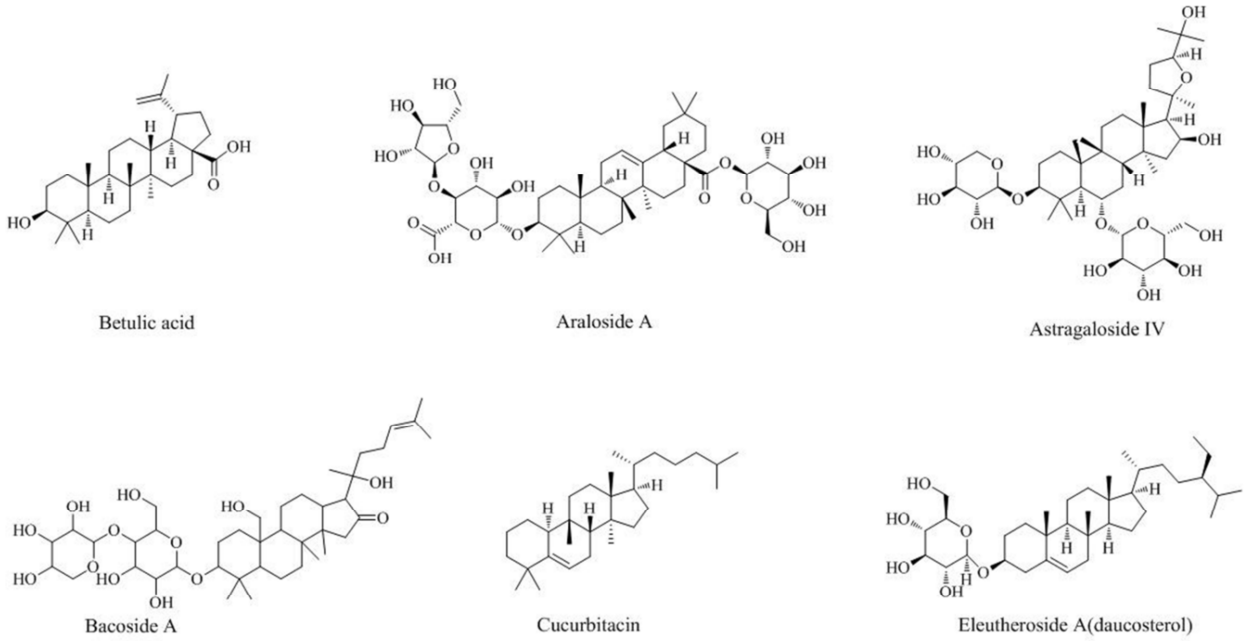

Figure 2. Naturally Occurring Pentacyclic Triterpenoids

Recently, the increase in total cholesterol is known as the abnormal index oflipid metabolism in the body, such as coronary artery disease or problems in lipid metabolism. In general, LDL-C is said to be a risk factor for atherosclerosis and cardiovascular diseases due to its action on the accumulation of cholesterol in the arterial wall, causing the hardening of the artery. HDL (high-density lipoprotein) in the blood is also deemed to play the role of reverse cholesterol transport (RCT), transporting cholesterol from extra-hepatic tissues to the liver cholesterol, thus lowering the risk of cardiovascular disease.

Although studies have shown the potential role of triterpenoids as hypolipidemic agents, to the best of our knowledge, triterpenoids from *Polyporus Umbellatus*, especially *Polyporus Umbellatus* triterpenicacids (QPUTA), have not been investigated as potential lipid-lowering agents. Thus, the potential hypolipidemic activities of the QPUTA were firstly investigated using Triton WR-1339 induced hyperlipidemic rats as a model. Before the hypolipidemic study, mono-factor test and an orthogonal design $L_9(3^4)$ were applied for the optimization of QPUTA extraction conditions for the first time.

EXPERIMENTAL SECTION

Polyporus Umbellatus were purchased from Zhengzhou medicine market and identified as the *Polyporus Umbellatus* by pharmacognosia experts. The roots of *Polyporus* were cutted while the stem and corona were retained, dried, crushed, over 20 mesh sieve. Standard oleanolic acid for the use of content determination were purchased from National Institute of Standard Pharmaceutical and Biological Products, Beijing, China. Distilled water, glacial acetic acid, chloroform, ethanol, perchloric acid, ethyl acetate and petroleum ether, etc. were supplied by commercial chemical companies such as Sinopharm Reagents and Sigma-Aldrich (analytical pure). Shimadzu UV-2550 UV-visible spectrophotometer (supplied by Shanghai International Trade Co. Dan Ding) SZFJ herbal grinder (Guangzhou Xu Long Machinery Co., Ltd.) R210 rotary evaporator (Switzerland BUCHI Company) DK-S26 electric heated water bath (Shanghai Electronic Technology Co., Ltd. Kai ago).

3.1. Extraction of the triterpenoids from Polyporus Umbellatus

20 grams of Polyporus Umbellatus powder were accurately put into a round-bottom flask with 400 mL alcohol solution. The alcohol solution was optimized by a mono-factor test and the influential factors such as particle size, extraction temperature, extraction time and ratio of liquid to solid were optimized by means of an orthogonal design $L_9(3^4)$. The mixture was refluxed, filtrated and extracted for several times respectively. After filtration, the total

filtrate were concentrated to extract under decompression, dried at 80^{o}C, appropriate amount of distilled water was added to wash products, then standing and centrifuged 5 min at the speed of 2500r/min, the supernatant was discarded, the precipitate was dried at 80^{o}C. Continuously,3 times amount of petroleum ether was added to the precipitate, after ultrasonic extraction and filtration, the extract was retained in the petroleum layer, which was distilled off under reduced pressure to obtain relatively total triterpenoids (QPUTA).

3.2. Qualitative analysis of QPUTA

For the qualitative identification of triterpenoids, appropriate amount of QPUTA obtained by ethanol extraction was reacted with different solutions respectively which can be listed as follows: anhydride-sulfuric acid (20:1,v/v), trichloroacetic acid, glacial acetic acid-ethyl chloride and chloroform-concentrated sulfuric acid. All the phenomena observed were in consistent with the references [12-14].

3.3. Quantification analysis of QPUTA by chromogenic method

In this paper, the determination of the total content of *QPUTA* were directly determined by chromogenic method. Oleanolic acid (10 mg) was accurately added into a 10mL volumetric flask, then diluted with ethyl acetate to the marked line to afford a concentration of 1.0mg/mL reference solution. *QPUTA* sample (10 mg) was placed in a 10mL volumetric flask, diluted with ethyl acetate to afford the sample solution.

The color developing agent applied in this experiment was prepared by the procedure as follows, 5% vanillin-acetic acid solution mixed with 2mL of perchloric acid were heated at 65^{o}C for 20min, then the tubes were taken out and cooled in ice water and warmed up to room temperature after being shaken for 2 min. Then ethyl acetate was added in order to make the total volume being 10 mL. Subsequently, the absorbance was scanned using a Double beam UV/Vis spectrophotometer in the range of 200–700 nm with a blank solution as reference.

Different concentrations of the oleanolic acid standard solution were taken and colored according to the chromogenic method, tested in the UV-visible region of spectrophotometer. The scanning results indicated that the maximum absorption was at 240nm(**Figure 3**), thus the absorbance A at Vis240nm was selected as the determination wavelength with a glass cell of 1cm. The contents of QPUTA were determined by reading the values from the standard curve.

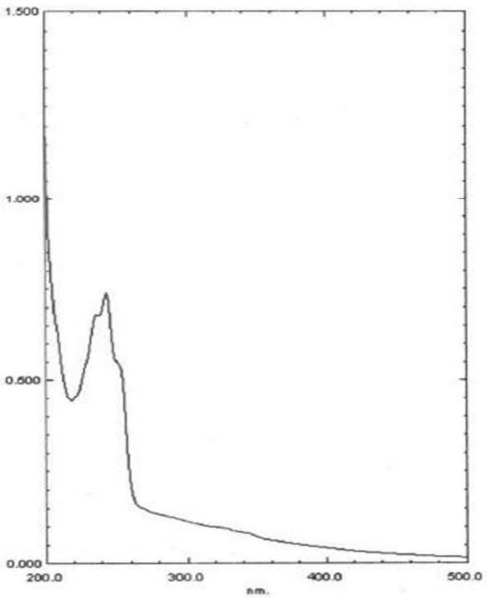

Figure 3. UV Spectrum of QPUTA

The standard curve which was used as the benchmark for the yield determination was obtained as follows. A mixed stock solution consisting of oieanolic acid(1.0 mg/mL) was prepared. The different volumes of the stock solution with 0.0,0.2,0.4,0.8,1.2,1.6,2.0mLoleanolic acid standard solution were precisely transferred into 10 mL test tubes with ethyl acetate to volume marked line, respectively. The mixture was then shaken, colored according to the chromogenic method. Specifically, after the solvent was heated to evaporation in a water-bath, 0.2 mL new mixed 5% (w/v) vanillin–acetic acid solution and 1.2 mL perchloric acid were added, mixed and incubated at 70^{o}C for 15 min. Then, absorbance (A) was taken as the abscissa, the concentration (C) as the vertical axis, the standard curve (**Figure 4**)was based on the measurement results, the regression equation was: C=0.1517A-0.0441, correlation

coefficient R=0.9971, which indicated the good relationship of triterpenoids within the linear range of 0.0046 ~ 0.0075mg/mL .

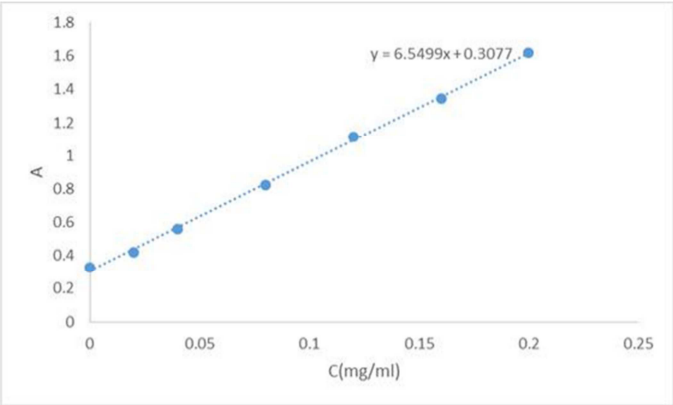

Figure 4. The Curve of Oleanolic Acid Standard Solution

3.4.Hypolipidemicactivityanalysis

In this study, the hypolipidemic activity of QPUTA was studied in the Triton induced hyperlipidemic male rats by intragastric administration of the QPUTA. 40 Albino Wistar male rats (150–200 g) were numbered individually and divided into 5 groups of 8 animals. They were kept in a room with controlled temperature (25–26^{o}C), humidity (60–80%) and 12/12h light/dark cycle under hygienic conditions. Animals were acclimatized for one week before starting the experiment with free access to the normal diet and water. Hyperlipidemia was developed by intra-peritoneal administration of Triton-WR1339 (Tyloxapol, Sigma-Aldrich, USA) at a dose of 400 mg/kg to all animals except the control. Simultaneously, the QPUTA were suspended in Tween 80 followed by an intragastric administration(25 mg/kg BW for LDG, 50 mg/kg BW for HDG). Animals of CG and HG groups without treatment with QPUTA were given vehicle only (**Figure 5**). Bezafibrate(100 mg/kg BW) was used as standard for the hypolipidemic activity.

After treatments (12 h), animals were anaesthetized briefly with diethyl ether and blood was taken from their tail vein using a heparinised capillary. The blood samples were immediately centrifuged (2500 rpm/10 min) and the plasma was used for lipid analysis, which included total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL). The concentrations were calculated and expressed in mg/dl using Friedwald formula, while the atherogenic index (AI) was calculated as (TC-HDL/HDL). Data obtained in the test were compared against the control group using the one-way analysis of variance method and followed by a post-hoc Dunnett test. Statistical analysis was performed using the SPSS 16.0 stat. software. Results were presented as mean ± standard error mean (mean ± SEM).

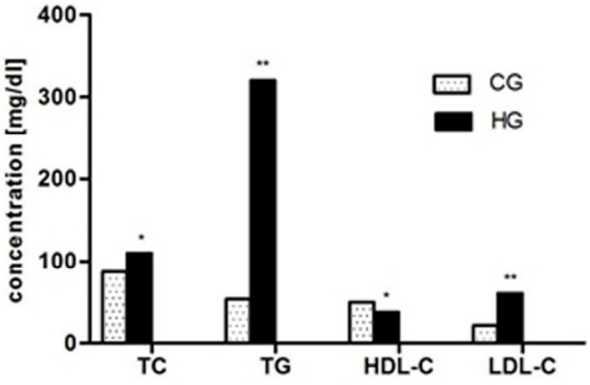

Figure 5. Effect of Triton-WR1339 on lipid profile after 12 h

*Values are means ±SEM from eight animals in each g roup. CG: control group; HG: hyperlipidemic control group; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol. HG is compared to CG. *p< 0.05, **p< 0.01.*

RESULTS

4.1. Optimization of QPUTA Extraction Conditions

4.1.1. Effect of Solvents On Extracting the *QPUTA* (n=3)

In this work, under the conventional heating reflux extraction conditions, solvents were evaluated by mono-factor test, in which the total mass of target compounds were extracted continuously for three times in different solvents. 20.0 g sample of 0.20–0.15 mm size was put into a round bottom flask with 100 mL organic solvent. The extraction temperature was controlled by heating in a water bath at solvent refluxing temperature for 40 min. After the extraction, the contents were filtered and evaporated to dryness. The procedure of heat reflux extraction of material was repeated twice in the same manner. The experimental results showed that the extraction yield of methanol, ethanol, acetone and acetonitrile were 0.35%, 0.72%, 0.61% and 0.68% respectively.

The extraction yield of target compound was defined as follows: Yield (%)=(Mass of target compound in extraction solution/Mass of sample)×100%

Table 1. Effects of Solvent on Extraction Rate of QPUTA

Solvent	A	Content（%）
methanol	0.167	0.35
ethanol	0.194	0.72
acetone	0.178	0.61
acetonitrile	0.189	0.68

4.1.2. Orthogonal Experiment for Extracting Optimization

An orthogonal design $L_9(3^4)$ was applied on the optimization of the other main extraction parameters, which can be listed as follows, the liquid/solid ratio (A), extraction temperature (B), size of sample (C) and extraction time (D). The factors and the corresponding levels used in the orthogonal design are shown in Table 2. Nine experimental trials were investigated according to the orthogonal design and the results are demonstrated in Table 3. The variance analysis of orthogonal experiment were also calculated and listed in Table 4.

Table 2. Factors and Levels of Orthogonal Experiment

Levels	Factors			
	Liquid/solid ratio A	Extraction temperature B /ºC	Size of sample C /mm	Extraction time D /h
1	5:1	40	0.15-.030	1
2	10:1	60	0.30-0.50	2
3	20:1	80	0.50-1.00	3

Table 3. Results and Analysis of Orthogonal Test $L_9(3^4)$

Test No.	Factors				QUPTA yield/%
	A	B	C	D	
1	1	1	1	1	0.178
2	1	2	2	2	0.332
3	1	3	3	3	0.453
4	2	1	2	3	
5	2	2	3	1	0.783
6	2	3	1	2	0.191
7	3	1	3	2	0.812
8	3	2	1	3	0.701
9	3	3	2	1	0.560
Ⅰ	0.918	1.862	1.950	1.026	0.657
Ⅱ	2.386	1.083	1.972	1.996	
Ⅲ	1.963	2.322	1.345	2.245	
R	0.902	1.205	0.338	1.453	

Table 4. Variance Analysis of Orthogonal Experiment

Factors	Sum of squared deviations	DOF	F value	P
A	0.050	2	1.000	>0.05
B	0.263	2	19.875	<0.05
C	1.061	2	2.571	<0.05
D	0.685	2	12.498	<0.05

Note: $F_{0.1}(2,2) = 9.00$； $F_{0.05}(2,2) = 19.00$； $F_{0.01}(2,2) = 99.00$

In Table 3, all of four factors had an influence on the extraction yields of the target triterpenoids. Comparing the R values, the influence of factors on the mean extraction yield of Polyporus Umbellatus decreased in the order: extraction time, extraction temperature, liquid/solid ratio and size of sample (D>B>A>C). The analysis of variance of the extraction yields also indicated that the extraction time and the extraction temperature had obvious influence on extraction yields of the target compounds, while extraction time and solid-liquid ratio had no significant impact. The reasons were that ethanol were viscous liquid which had worse diffusion ability, and high extraction temperature could decrease the viscosity of ethanol. Moreover, it was difficult for ethanol to permeate through the larger size of sample, so that target triterpenoids in sample could not be transferred into the solutions quickly or completely. According to the largest donating rule, the largest value which affects the extraction yields should be the selected value. According to Table 3, the average yields under every level can be concluded as, $A_2>A_3>A_1$,$B_3>B_1>B_2$,$C_2>C_1>C_3$,$D_3>D_2>D_1$,respectively. Therefore, considering time and solvent saving, the optimum condition of QPUTA extraction was $A_2B_3C_2D_3$ as follows: the ratio of liquid/solid was 10:1, the extraction temperature was 80 ^oC, the size of sample was 0.30-0.50 mm, and the extraction time was 3 hours. This indicated that the extraction yield could be enhanced using a combination of those factors at different levels in the preparation process

Under the optimized conditions, Qinling *Polyporus* powder were extracted using ethanol with high polarity, the filtrate were concentrated and mixed with distilled water. Subsequently, the mixture were subjected to centrifugalization, the precipitate was washed with petroleum ether and extracted with chloroform respectively to afford the triterpenoids. The amount of triterpenoids contents, expressed as g of oleanolic acid equivalents per 100 g of Polyporus Umbellatus was 0.82 g/100 g.

4.2. Effects of QPUTA on Rat Plasma Lipid Profiles

The plasma total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL-C) and low density lipoprotein (LDL-C) levels of QPUTA and bezafibrate (BF) treated rats 12 h after Triton WR-1339administration are shown in Table 5. In comparison with the normal control group (CG), Triton WR-1339 caused a significant increase in plasma cholesterol and triglyceride concentrations measured after 12h of Triton injection, the plasma total cholesterol was increased by 26.62% and triglycerides by more than six times. Meanwhile, Triton induced significantly reduction in HDL cholesterol levels of hyperlipidemic control (HG) in comparison with the CG. Importantly, the elevated plasma TG levels produced by Triton WR-1339 administration were significantly suppressed by bezafibrate (BF,70.04%), QPUTA high dosage (HDG, 77.97%) and QPUTA low dosage (LDG, 70.44%) with respect to the hyperlipidemic control. No significant difference in TG levels was observed with both high and low dosage of QPUTA, compared to HG-treated rats.

Table 5. Effect on Plasma Lipid Levels of QPUTA Treated Rats 12 h After Triton Administration

Groups	TC(mg/dL)	TG(mg/dL)	HDL-C(mg/dL)	LDL-C(mg/dL)	AI
CG	87.1±5.5	53.4±4.6	49.7±3.8	21.4±2.0	0.75±0.17
HG	110.3±6.3*	320.1±16.7**	37.5±2.0*	60.5±1.9**	1.94±0.34**
LDG	97.8±5.4^{a}	94.6±6.3^{a}	43.1±2.6^{a}	37.5±3.1^{c}	1.27±0.15$^{a\,c}$
HDG	88.6±5.7^{a}	89.5±5.9$^{a\,c}$	57.8±4.2^{c}	24.8±2.4^{a}	0.53±0.10^{b}
BF	116.5±6.4^{c}	95.9±5.0$^{a\,c}$	47.2±3.1^{a}	54.3±2.7^{c}	1.47±0.28^{c}

Values are means ±SD (n=8 per group). CG: normal control group; HG: hyper lipidemic + 4% DMSO control group; LDG: QPUTA (5mg/kg)+4% DMSO; HDG: QPUTA (10mg/kg)+4% DMSO; BF: bezafibrate(100 mg/kg) +4% DMSO; The atherogenic index (AI) was calculated as (TC-HDL/HDL). [a]A significant decrease at p< 0.05, when compared with HG values. [b]A significant decrease atp< 0.01, when compared with CG values. [c]A significant increase at p< 0.01, when compared with CG values.

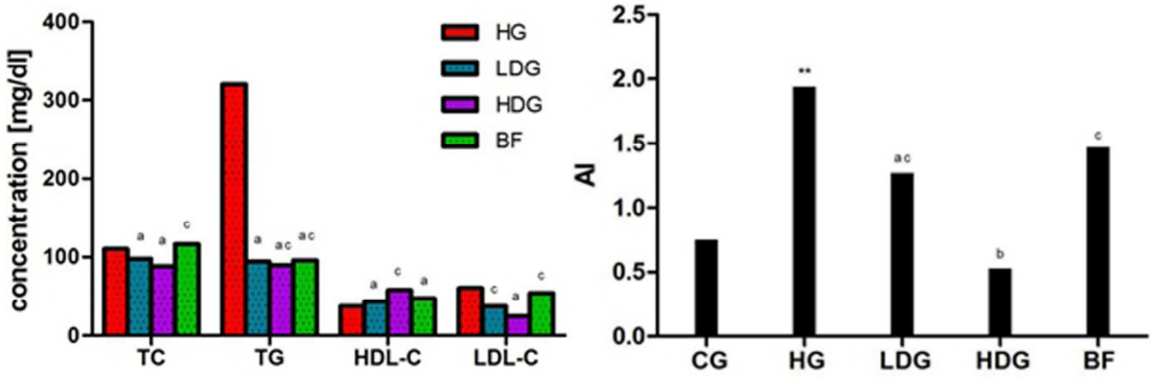

Figure 6.Comparison of Plasma Lipid Levels and Atherogenic Index

HDL-cholesterol levels were significantly increased 12 h after Triton administration in the QPUTA-treated (+14.93% of LDG group and +54.13% of HDG group) and BF-treated rats (+25.87%) compared to HG. Furthermore, there was significant decrease in TG levels after 12 h with -70.45% of LDG group and -71.72% of HDG group compared with group HG. Except BF, both the LDG and HDG demonstrated an obvious and significant reduction in plasma LDL-C levels. Specifically, -38.02% of LDG and -59.01% of HDG in respect to HG treated rats. After 12 h of treatment, it was observed that slightly down regulation in plasma TC levels between both QPUTA groups (-11.33% of LDG and -19.67% of HDG group) in comparison to HG treated rats (**Figure 6**).

DISCUSSION

Triton WR-1339has been used to produce acute hyperlipidaemia in animal models in order to test QPUTA and to study cholesterol and triacylglycerol metabolism. It appears clear from these results that the Triton and QPUTA administration oppositely affected on plasma lipid levels and the cardiovascular risk marker (AI). The observation has been attributed to Triton WR-1339, a non-ionic detergent (oxyethylated tertiary octyl phenol formaldehyde polymer), induces hyperlipidemia in adult rats after parenteral administration due to the inhibition of lipoproteinlipase activity. The Triton-induced rat model gave a similar pattern of lipid profile changes 12 h after Triton WR-1339 administration(Figure6). Meanwhile, the down regulation of plasma HDL-C levels could be explained by understanding the mechanism through which Triton WR-1339 administration results mostly from a progressive displacement of the apo A-1 protein from the HDL surface, without loss of lipid[15,16].

As mentioned above, the results demonstrated the potential hypolipidemic effect of QPUTA in Triton WR-1339 induced hyperlipidemic rats. QPUTA at both doses of 25 mg/kg BW and 50 mg/kg BW significantly reduced plasma TG and increased HDL. Since the proportion of triglyceride in VLDL is many times higher than cholesterol, it is not surprising that the hypolipidemic activity of QPUTA was significantly higher for triglycerides than for cholesterol. Clearly, there is ample evidence to suggest that QPUTA are able to restore, at least partially, catabolism of B-lipoproteins as hypothesized by previous reports with other lipid-lowering agents. In addition, both high and low dosage of QPUTA increased HDL, which called “good cholesterol” plays an important role in facilitating the mobilization of triglycerides and cholesterol from plasma to liver where it undergoes catabolism and then eliminated in the form of bile acids.

Promisingly, both high and low dosage groups of QPUTA administration 12 h after Triton injection is more significant than the reduction induced by bezafibrate at a dose of 100 mg/kg body weight, which in this study has been used as standard reference hypolipidemic drug. In contrast, total cholesterol levels were not significantly changed which agrees with the mechanism of action of fibrates in that their total cholesterol-lowering activity is not strongly marked, but the triglycerides decreasing effect of them is very impressive especially by stimulation of the gene expression of lipoprotein lipase. Table 5also revealed the changes of atherogenic index (AI)in control and treated rats. QPUTA provided a beneficial action on rat lipid metabolism in regard to the reduction of AI.

In general, QPUTA treatment was able to improve serum lipid metabolites of Triton WR-1339 induced hyperlipidaemic rats, including decreasing the levels of triglyceride, total cholesterol, LDL cholesterol. In addition, QPUTA increased HDL levels, which are known for their preventive role against atherogenesis. The results of this study showed that QPTA could reverse the hyperlipidemia in experimental hyperlipidaemic rats, and thus may lead to a decrease in the risk might be directly or indirectly related with micro- and macro-vascular disease and related cardiovascular disease.

The present study evaluated the hypolipidemic effects of triterpenoids extracts from Qiling *Polyporus Umbellatus* in Triton-induced hyperlipidemic rats. It illustrated that after oral administration of QPUTA at a dose of 50 mg/kg for 12 hours, the serum total cholesterol (TC), triglycerides (TG), LDL and VLDL cholesterol levels in Triton-induced hyperlipidemic rats were significantly decreased and at a dose of 25 mg/kg, the serum TC, TG, LDL and VLDL cholesterol levels were also significantly reduced. Furthermore, QPUTA administration could also significantly decrease the atherogenic index, which is related with risk of micro- and macro-vascular disease and atherosclerosis.

As a conclusion, the present study confirmed for the first time the *in vivo* hypolipidemic effects of QPUTA. The observed pharmacological effects of QPUTA on hyperlipidemic rats extend our knowledge about the potential bioactivities and applications of triterpenoids extracts abundant in Qiling *Polyporus Umbellatus*. Impressively, it could be used by hyperlipidemic patients to decrease the complications of hyperlipidemia. Further studies are necessary to determine the exact nature of the active principles, the mechanism of action and to assess the safety of triterpenoids in Qiling *Polyporus Umbellatus*.

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