RGC Ref. No.:
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(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR
THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines:
1. *Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
2. *Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

The synergistic effect of using pioglitazone in combination with piceatannol in treating nonalcoholic fatty liver disease in rats

2. Investigator(s) and Academic Department(s) / Unit(s) Involved

Research Team	Name / Post	Unit / Department / Institution
Principal Investigator	Dr. CHAN Shun-wan / Associate Professor	Department of Food and Health Sciences, Technological and Higher Education Institute of Hong Kong
Co-Investigator(s)	Dr. MOK Daniel Kam-wah / Associate Professor	Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University

3. Project Duration

	Original	Revised	Date of RGC / Institution Approval *(must be quoted)*
Project Start Date	1st Jan 2017	N/A	N/A
Project Completion Date	31st Dec 2019	31st Dec 2020	18th June 2020 by RGC
Duration *(in month)*	36	48	18th June 2020 by RGC
Deadline for Submission of Completion Report	31st Dec 2020	31st Dec 2021	18th June 2020 by RGC

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. To evaluate the synergistic effect of using pioglitazone in combination with piceatannol in treating high-fat diet (HFD)-induced NAFLD in rats

2. To investigate whether the beneficial effects of using pioglitazone in combination with piceatannol in treating HFD-induced NAFLD in rats is via hepatic autophagic enhancing pathway

3. To use metabolomics approach for the comparison of serum metabolic changes in normal rats, NAFLD rats and NAFLD rats treated with pioglitazone in combination with piceatannol

5.2 Revised objectives

Date of approval from the RGC: N/A

Reasons for the change:

1.

2.

3. ....

5.3 Realisation of the objectives

(Maximum 1 page; please state how and to what extent the project objectives have been achieved; give reasons for under-achievements and outline attempts to overcome problems, if any)

Objective 1: To evaluate the synergistic effect of using pioglitazone in combination with piceatannol in treating HFD-induced NAFLD in rats

To achieve this objective, two *in vivo* studies were performed, and HFD (standard rat chow supplemented with 1% cholic acid, 2% cholesterol and 5.5% oil) was fed for 4 weeks to induce NAFLD in rats. In the first *in vivo* study, the dose of monotherapy (either pioglitazone or piceatannol) that could elicit about 50% reduction (ED_{50}) of the increased total liver lipid content was identified. Based on the first *in vivo* study, the ED_{50} of pioglitazone (8 mg/kg/day) and piceatannol (30 mg/kg/day) were chosen for the second *in vivo* study evaluating the synergistic effect of combination drug therapy of pioglitazone and piceatannol to treat NAFLD in the rat model, and the experimental grouping is listed as follows:

- Group 1: Control group
- Group 2: HFD group (4 weeks HFD, then 4 weeks normal diet)
- Group 3: PG group (4 weeks HFD, then 4 weeks normal diet plus ED_{50} of pioglitazone, 8 mg/kg/day, *p.o.*)
- Group 4: PIC group (4 weeks HFD, then 4 weeks normal diet plus ED_{50} of piceatannol, 30 mg/kg/day, *p.o.*)
- Group 5: PG+PIC-L group (4 weeks HFD, then 4 weeks normal diet plus a combination drug therapy with pioglitazone (2 mg/kg/day, *p.o.*) and piceatannol (7.5 mg/kg/day, *p.o.*))
- Group 6: PG+PIC-M group (4 weeks HFD, then 4 weeks normal diet plus a combination drug therapy with pioglitazone (4 mg/kg/day, *p.o.*) and piceatannol (15 mg/kg/day, *p.o.*))
- Group 7: PG+PIC-H group (4 weeks HFD, then 4 weeks normal diet plus a combination drug therapy with pioglitazone (8 mg/kg/day, *p.o.*) and piceatannol (30 mg/kg/day, *p.o.*))

The levels of serum biomarkers including triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, aspartate aminotransaminase and alanine aminotransaminase of rats in various treatment groups were analyzed. Liver samples were also collected for further analyses, such as total liver lipid content, liver sectioning and the expressions of autophagy related proteins.

Objective 2: To investigate whether the beneficial effects of using pioglitazone in combination with piceatannol in treating HFD-induced NAFLD in rats is via hepatic autophagic enhancing pathway

To achieve this objective, the expressions of autophagy enhancing pathway related proteins, such as LC3-II, p62, Atg 3, Atg 7, Atg 5 and Atg 5-Atg 12 complex, in the livers from various treatment groups from the *in vivo* studies were evaluated using Western blot. The other potential intracellular signaling pathways, such as lipid metabolism related pathways, were also investigated.

Objective 3: To use metabolomics approach for the comparison of serum metabolic changes in normal rats, NAFLD rats and NAFLD rats treated with pioglitazone in combination with piceatannol

To achieve this objective, serum metabolites in various experimental groups will be extracted and isolated for ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) analysis. The serum metabolite changes in various treatment groups were identified.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. To evaluate the synergistic effect of using pioglitazone in combination with piceatannol in treating HFD-induced NAFLD in rats	✓	100%
2. To investigate whether the beneficial effects of using pioglitazone in combination with piceatannol in treating HFD-induced NAFLD in rats is via hepatic autophagic enhancing pathway	✓	100%
3. To use metabolomics approach for the comparison of serum metabolic changes in normal rats, NAFLD rats and NAFLD rats treated with pioglitazone in combination with piceatannol	✓	100%

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

In this study, the synergistic effect of using pioglitazone in combination with piceatannol in treating HFD-induced nonalcoholic fatty liver disease (NAFLD) was investigated. The gross appearance of the liver (Annex, Figure 1) demonstrated that feeding the rats with HFD for 4 weeks could lead to NAFLD; pioglitazone and piceatannol combined treatments reduced the severity of hepatic steatosis. Pioglitazone and piceatannol combined treatments were found to significantly reduce the total liver lipid content in the rat model in a dose-dependent manner ($p < 0.05$) when compared with the HFD group (Annex, Figure 2). The results showed that PG+PIC-M and PG+PIC-H had a higher degree of liver lipid reduction effect than either the pioglitazone or the piceatannol mono-therapeutic treatments, suggesting that pioglitazone and piceatannol combinations exert a synergistic effect against lipid accumulation in liver. Pioglitazone and piceatannol combined treatments were also shown to reduce serum triglyceride and total cholesterol levels (Annex, Figure 3). It confirms pioglitazone and piceatannol combined treatment could provide synergistic effect in treating HFD-induced NAFLD in rats (addressed objective 1 of the current study).

According to the results of Western blot (Annex, Figure 4), the protein expression of LC3-II in liver samples from the PG+PIC-H group was significantly increased when compared to that from the HFD group ($p < 0.05$). The protein expressions of Atg 3, Atg 7 and Atg 5-Atg 12 complex in liver samples were increased in the PG+PIC-H group. Results also showed that there is no accumulation of p62. These data suggested that pioglitazone and piceatannol combined treatments promote autophagy in the liver. Besides, the protein expression of PPAR-α in liver samples from the PG+PIC-M and PG+PIC-H groups was significantly up-regulated when compared to that from the HFD group ($p < 0.05$ and $p < 0.001$, respectively), suggesting that pioglitazone and piceatannol combined treatments promote fatty acid β-oxidation. These results showed that pioglitazone in combination with piceatannol provides a synergistic treatment effect on NAFLD rats via the promotion of hepatic autophagy as well as the modulation of lipid metabolism in the liver (addressing objective 2 of the current study).

The serum metabolites in various experimental groups were analyzed and compared. It was found that the metabolomics profile of pioglitazone and piceatannol combination drug therapy group was significantly different from that of the HFD group but it was getting closer to that of the control group (Annex, Figure 5). The distribution of 12 identified metabolites in various treatment groups acquired in UPLC-Orbitrap-MS were shown in a heat map (Annex, Figure 6). Our results also found that serum levels of isoleucine, methionine, lithocholic acid, glycocholic acid, taurodeoxycholic acid, deoxycholic acid, DHA, PGE1, and (±)5-HETrE were significantly increased in the PG+PIC-M group compared with the HFD group; while serum level of trptophan was significantly decreased in the PG+PIC-M group compared with the HFD group (Annex, Figure 7). These serum metabolites were found as potential biomarkers indicating that pioglitazone and piceatannol combination drug therapy could effectively modulate the lipid metabolism and result in improvements in NAFLD (addressing objective 3 of the current study).

6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

We will continue to investigate the possibility of using pioglitazone in combination with piceatannol to treat NAFLD. Metabolomics study will also be continued to determine different serum biomarkers of NAFLD so as to clarify the pathogenesis of NAFLD and identify potential therapeutic targets and/or diagnostic markers for NAFLD. In this study we confirmed that using pioglitazone in combination with piceatannol is an effective approach to handle NAFLD. In the metabolomics study, 12 metabolites was found to increase significantly in NAFLD animal treated with pioglitazone in combination with piceatannol. Some of the metabolites are secondary bile acids. It is anticipated that gut microbiota may play a role in the observed beneficial effects of our proposed combination therapy. In fact, gut microbiota dysbiosis has been observed in patients with metabolic diseases, such as NAFLD, obesity, and diabetes mellitus. It has been suggested that gut microbiota play an important role in the development of these metabolic diseases. Investigations on the gut microbiota structure may help to clarify the relationships between gut microbiota and the pathogenesis of NAFLD as well as unveil the mechanistic pathway of using pioglitazone in combination with piceatannol to treat NAFLD. Data may provide a new direction on the therapy of NAFLD. Thus, further studies on comparing the gut microbiota structure between NAFLD animals and NAFLD animal treated with pioglitazone in combination with piceatannol are proposed so as to confirm the role of gut microbiota on the beneficial effects of using pioglitazone in combination with piceatannol to treat NAFLD.

7. Layman's Summary

(Describe <u>in layman's language</u> the nature, significance and value of the research project, in no more than 200 words)

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition of emerging clinical importance and considered as the most common cause of liver dysfunction. It ranges from simple fatty liver to non-alcoholic steatohepatitis with different degrees of fibrosis that can progress to cirrhosis. Under the current population ageing and obesity epidemic, the risk of chronic metabolic diseases, including NAFLD, cardiovascular diseases and diabetes, is increasing. Currently, NAFLD is one of the most common liver disorders worldwide. It affects not only adults but also children. A recent prospective cross-sectional study reported that prevalence of NAFLD in the general population of Hong Kong was about 42%. The increase in NAFLD prevalence becomes an important public health concern and this imposes a huge social and economic burden to our society. Our study showed that using pioglitazone in combination with piceatannol provided synergistic effect in treating HFD-induced NAFLD in rat model, providing a foundation for the future pre-clinically and clinical studies using combination drug therapy, which help to improve existing strategies for NAFLD prevention and treatment. The development of better NAFLD treatment can in turn reduce the prevalence of NAFLD and relieve the social and economic burden.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project

(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and Journal / Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the Institutional Repository *(Yes or No)*
Year of Publication	**Year of Acceptance** *(For paper accepted but not yet published)*	**Under Review**	**Under Preparation** *(optional)*						
2017	**2017**	**N/A**	**N/A**	**Sham TT, Zhang H, Mok DKW, Chan SW*, Wu JH, Tang SG, Chan CO*.**	**Chemical Analysis of Astragali Complanati Semen and Its Hypocholesterolemic Effect Using Serum Metabolomics Based on Gas Chromatography-Mass Spectrometry. Antioxidants. 2017, 6(3):57.**	**No**	**Yes**	**Yes**	**Yes**
N/A	**N/A**	**N/A**	**Under Preparation**	**Kwok TK, Ng YF, Seto SW, Mok DKW, Chan SW***	**Water extract of Rhizoma Atractylodis Macrocephalae alleviates fat accumulation in L02 through AMPK/SIRT1 and AMPK/mTOR signaling pathway. Journal of Food Biochemistry.**	**No**	**Yes**	**Yes**	**N/A**
N/A	**N/A**	**N/A**	**Under Preparation**	**Zhang H, Ng YF, Mok DKW, Chan SW***	**Hepatoprotective effect of piceatannol against oxidative stress-induced liver injury in normal human liver cells. Antioxidants.**	**No**	**Yes**	**Yes**	**N/A**
N/A	**N/A**	**N/A**	**Under Preparation**	**Ng YF, Zhang H, Man KY, Chan CO, Mok DKW, Chan SW***	**The synergistic effect of using pioglitazone in combination with piceatannol in treating nonalcoholic fatty liver disease in rats. Journal of Functional Food**	**No**	**No**	**Yes**	**N/A**

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered

(Please attach a copy of each conference abstract)

Month / Year / Place	**Title**	**Conference Name**	**Submitted to RGC** *(indicate the year ending of the relevant progress report)*	**Attached to this Report** *(Yes or No)*	**Acknowledged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
June/2019 /The Hague	Metabolomics study on the effect of combination treatment between pioglitazone and piceatannol on Non-Alcoholic Fatty Liver Disease in Rats	Metabolomics 2019	Yes, in 2020	No	Yes	No

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning

(Please elaborate)

The research experience and new knowledge generated from the current project has been contributed to the development of BSc (Hons.) in Food Science and Safety's final year projects. The established research technique has been used in the practical sessions and teaching and learning materials of some BSc (Hons.) in Testing and Certification teaching module, such as Biochemical & DNA Technologies and Advanced Instrumentation Analysis.

11. Student(s) Trained

(Please attach a copy of the title page of the thesis)

Name	**Degree Registered for**	**Date of Registration**	**Date of Thesis Submission / Graduation**
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

12. Other Impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

This project further consolidates the collaboration between the Hong Kong Polytechnic University and the Technological and Higher Education Institute of Hong Kong (THEi). Additionally, new research collaborations on related area have been established between other higher education institutions, such as Tung Wah College. Recently, our team have secured another Faculty Development Scheme (FDS) project entitled, "A mechanistic study on the combined use of esculetin and probiotics in preventing Parkinson's disease in mice" (Ref. No.:

UGC/FDS25/M03/21). It is another project about using combination therapy to treat chronic disease.

13. Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (please specify)	
No. of outputs arising directly from this research project	4 (1 published and 3 under preparation)	1	0	0	Type	No.
					Undergraduate final year projects	2

14. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	Reasons
Nil	