Chapter 7
Assessment of the antidiabetic potential of red rice and rice-based products

7.1 Introduction

Diabetes mellitus is a multifarious metabolic disorder where concentration of fasting plasma glucose (FPG) is higher than 126 mg/dL, or in which blood glucose levels are above 200 mg/dL at any time of day. [2, 55] India is reported to have highest incidence of diabetics in the world with 3.8% of the rural population and 11.8% of urban population diagonised with having the disease. [52] There are two types of Diabetes mellitus namely type 1 and type 2 [27, 1] Type 2 diabetes mellitus (T2DM) accounts for approximately 90 % cases of diabetes cases and will soon declared a severe global epidemic of the twenty-first century.[26] According to King et al.[29] countries with the highest incidence of T2DM will India, China and United States. Dixit and Pokala[11] estimated that diabetes will effect over 100 million people within the Indian population by the year 2030.

Dipeptidyl peptidase-4 (DPP4) inhibitors are a novel type of oral glucose-lowering agents that regulate fasting plasma glucose, postprandial glucose, and HbA1c levels in the body. They act to halt the physiological breakdown of the incretin hormones glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), decreasing the inactivation of endogenous incretins to trigger the release of insulin in a glucose dependent manner.[12,46] Pharmacological involvement and clinically approved dipeptidyl peptidase-4 (DPP4) inhibitors are of vital importance in treating the diabetes epidemic.[15,21,22,34] Moreover, GLP-1 mimetics and GLP-1 secretagogues have proved effective in managing the disease. Glucagon-like peptide-1 (7–36)-amide is a 30-amino acid polypeptide which is secreted from gut endocrine L-cells and a potential therapeutic agent for the treatment of T2DM. GLP-1 acts as a formidable incretin to modulating postprandial blood glucose levels, stimulating glucose-dependent insulin secretion and inhibiting glucagon secretion and gastric emptying.[53,12] GLP-1 also regulates other diverse cellular processes to include increasing beta-cell mass by stimulating cell proliferation, inhibiting beta-cell apoptosis, aiding in the differentiation of primary neuronal (PC12) cells through stimulating cyclic AMP production and has suggested neuro and cardio protective effects.[32,45,51,7]

Pigmented red rice is an underutilized crop in North-eastern part of India. Over 400 plants are reported to have glucose lowering effects.[14] Lee et al.[33] reported that whole grains and cereals are recommended for diabetes to control blood glucose.This present study will investigate antidiabetic effects of *Oryza sativa* L. particularly its ability to inhibit contains DPP-4 and enhance GLP-1 secretion, to determine whether it can be

incorporated into a healthy cereal product. The functional foods concept gaining much publicity amongst the health conscious population and these foods are becoming well known for their ability to prevent and manage chronic diseases such as diabetes, obesity and hypertension [44,5] Rice is an important cereal and staple food crop for half of the world's population.[42,48] Bhonsle and Sellappan[6] illustrated that some rice varieties have antidiabetic properties. This suggest that these staple food product could be demonstrated as functional food and exploited as a preventive or management therapy for diabetes.

Rice bran, a by-product of rice milling, contains many bioactive compounds such as lipophilic components including γ-oryzanol, tocotrienols, and tocopherols and phenolic compounds such as ferulic acid, sinapic acid, and protocatechuic acid.[16,47] Bioactive compounds in rice bran exhibit tremendous health benefits to combat various disease such as cardiovascular health [50] inhibition of cancer,[40] and improvements in glucose homeostasis.[31] Pigmented or colored rice bran contains both lipophilic and phenolic compounds with antioxidant effects[41,39] cancer inhibiting effects [40,10] and can inhibit α-glucosidase activity.[54] It has also been reported that rice bran may prevent the development of fructose-induced insulin resistance in rats.[23]

Extrusion cooking technology is extensively used for the development of new products in a short time period. The raw material (feed) undergoes physicochemical changes mostly starch gelatinization, protein denaturation, amylose-lipid complex formation and degradation of heat sensitive components such as vitamins, antioxidants, and pigments.[25] Using cereal and pulses as raw material various studies have reported that the extrusion cooking process results in low fat, high fibre and protein rich extrudates.[3,17, 28,35] Various parameters are responsible for the outcome of extrudates to include types of extruder, feed material, material moisture content, barrel temperature, and screw speed.[30] It has been well established that diet contributes to the development of diabetes, therefore producing a nutritionally rich extrudate with anti-diabetic properties could provide a strategy for the management of disease. Various studies reported the association between the consumption of certain foods, their constituents, and the incidence of diabetes. Some studies have identified peptides and phenolic compounds which can control the level of blood glucose.[36]

In plant, various mineral elements are present and accumulate in different proportion after absorption from soil.[18] Arsenic (As) is a category 1 carcinogen by the IARC Monographs and is considered as a toxic element to humans, animals and plants. It is a consequence of different contaminants to include industrial production of pesticides,

herbicides, wood preservatives and mining, and can severely compromise human health [8,38] Several species (As) are reported in rice and include arsenite (As (III)), arsenate (As (V)), dimethyl arsenic acid (DMA) and monomethyl arsonic acid (MMA).[13,43] Arsenite and arsenate are inorganic arsenic (i-As) species and are carcinogenic in nature,[37] whereas organic As species MMA and DMA are less toxic but can be cancer promoters. [24]

In this study, the potential antidiabetic effects of pigmented red rice will be investigated. Moreover, the red rice will undergo an extrusion process to determine whether this can enhance antidiabetic effects. The safety of the rice and extruded product will be assessed for their arsenic content to ensure it is within the recommended levels. Finally, rice and extrudates will undergo solvent fractionation to determine the optimal extract for the use as a therapeutic agent or incorporation as a functional food.

7.2 Material and methods

7.2.1 Materials

Red rice (*Oryza sativa* L.) locally known as *Umling ame* (UA), and white rice *Pungpo ame* (WR) were collected from Manigong Circle, Arunachal Pradesh (India). Rice bran (RB) was obtained from UA by using polisher (Satake, Japan). The removed bran rice was also collected and named as polished red rice (PRR). Extruded products: control (C) extrudate (solely red rice: UA) and optimized (O) extruded products (UA incorporated with 11.25% passion fruit powder) were prepared using a twin extruder (Model FUE-1F,Flytech Engineering, Chennai, India. Before the extrusion process, the experiments were design by using central composite design (CCD) of total 30 runs. Optimization and validation was done in previous Chapter 5(A) .

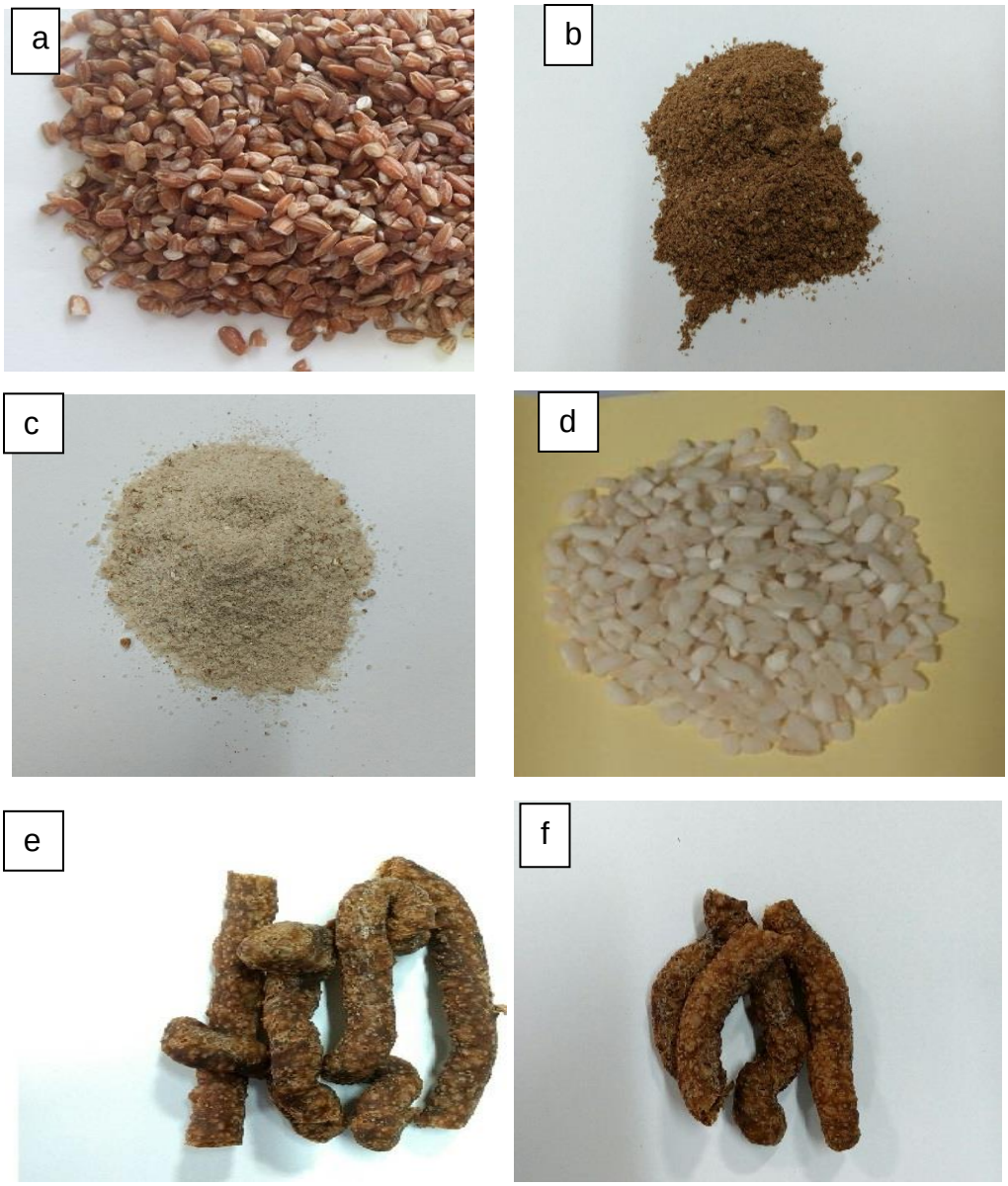

Fig 7.1. Photographs of six samples tested. (a) red rice locally known as umling ame (UA), (b) red rice bran (RB), (c) white rice locally known as pungpo ame (WR), (d) Red rice polished (PRR), (e) extruded red rice (C: control) and (f) extruded red rice incorporated with passion fruit powder (O :optimized)

7.2.2 Extraction

Bioactivity guided fractionation was carried out on rice samples (10g) by immersing the crude powder in 50 ml of solvents of different polarity *n*-hexane, 50:50 ethanol: water and water. They were kept on a roller mixer overnight at room temperature. Supernatants were obtained by filtration before allowing the solvents to evaporate on a heat block at 50˚C. Dried extracts were stored at -20 ˚C prior to use in assay.

7.2.3 Determination of DPP-4 inhibition activity

DPP-4 inhibition of rice extracts was carried out in a 96-well plate as previously described by Fujiwara and Tsuru. [20] Sample extracts were dissolved in HEPES buffer (p H 7.4) at a concentration of 2mg/ml and assessed fluorometrically using Gly-Pro-aminomethylcoumarin (1mmol/l; BaChem Ltd, Switzerland) and purified porcine DPP-4 (1U/ml: Merk Chemical, UK) Berberine (1mg/ml: Flourochem) was used as a positive control as it has already shown potent DPP-4 inhibitory activity.[2] After addition of all solution (n=3) incubate at 37˚C for 1 h with agitation. Later, 100 µL of 5mM acetic acid was added to stop the reaction. The plate was read using a fluorescent microplate reader (Tecan Saffire II, Reading,UK) at excitation and emission wavelength of 351 and 430nm,respectively.

7.2.4 STC-1 pGIP/Neo cell culture studies

STC-1 cells transfected with a plasmid (pGIP/Neo) encoding neomycin phosphotransferase were obtained from Dr. B. Wice (Washington University of St.Louis) with permission from Dr D. Hanahan (University of California, San Francisco, CA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l D-glucose with L-glutamine, without sodium pyruvate (Gibco, Paisley, UK) and supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 400 µg/ml geneticin (G418 disulfate salt; Sigma, UK). Cells were incubated in a 5% CO2 humidified atmosphere at 37˚C and used between passage numbers 20 - 50 when 70 – 90 % confluence had been reached. STC-1 pGIP/Neo cells were seeded in 12 well plates at a density of 2 million cells per well with 1 ml DMEM and incubated overnight at 37˚C in a 5% CO2 humidified atmosphere to allow attachment. Media was removed and cells were washed twice with HEPES buffer (pH 7.4) and pre-incubated in HEPES for 1 h. After removal of buffer, samples at 2 mg/ml were reconstituted in HEPES and added to cells in

triplicate for 3 h. After the incubation period supernatant was removed, centrifuged at 1000 g for 10 min to remove cellular debris and stored at -20˚C prior to analysis.

7.2.5 Measurement of cell viability

Trypan blue was used to assess cell viability. Trypsin (1 ml) was added to each well and incubated at 37˚C for 1 min to detach cells. Following this, 1 ml of DMEM was added to neutralise trypsin and resulting solution was centrifuged at 1000 g for 5 min. The supernatant was discarded and cells were re-suspended in DMEM. The cell suspension was added to trypan blue (1:1) and cell viability measured using a Countess Automated Cell Counter (Invitrogen, Life Technologies Ltd, UK).

7.2.6 Determining GLP-1 secretion from STC-1 pGIP/Neo cells

For determining hormone secretion from STC-1 pGIP/Neo cells, GLP-1 levels were measured by means of ELISA (Millipore, UK) in accordance to manufacturer's instructions. For total GLP-1 determination, assay buffer, standards and samples were incubated in the pre-coated 96 well plate overnight at 4˚C. Plates were washed 5 times with wash buffer and detection conjugate was added to each well and incubated for a further 2 h. The plate was again washed 3 times prior to 20 min incubation with substrate. Stop solution was added to each well and the fluorescence measured on a fluorescence plate reader (Tecan Saffire II; Reading, UK) at excitation and emission wavelengths of 355 nm and 460 nm, respectively.

7.2.7 Inductively coupled plasma mass spectrometry (ICP-MS) of samples

7.2.7.1 Sample preparation for arsenic (As) speciation

Sample preparation for As speciation was described by using Signes-Pastor et al. [49] method. Samples were dried and milled for 3 min at 500 rpm with a 1 min rotation and a reverse rotation using a Retch PM100 rotary ceramic ball mill. The samples (0.1g) were weighed accurately to a weight of 0.1 g into 50 ml polypropylene centrifuge tubes to which 10 ml of 1% conc. Aristar nitric acid was added and allowed to sit overnight. Batches were prepared with a blank rice CRM (NIST 1568b Rice flour) which has the As species As_i, and dimethylarsinic acid (DMA) concentrations certified. Later, microwave digestion was

done in an CEM MARS 6 instrument for 30 min at 95˚C using a 3 stage slow heating program: to 55 ˚C in 5 min held for 10 min., to 75 ˚C in 5 min., held for 10 min. to 95 ˚C in 5 min., held for 30 min. A 1 ml aliquot was transferred to a 2 ml polypropylene vial and then, 10 µl of analytical grade hydrogen peroxide was added to convert any arsenite to arsenate to facilitate subsequent chromatographic detection.

7.2.7.2 Chemical analysis

Sample were digested in 1% nitric acid and digested sample solutions were run on a Thermo Scientific IC5000 Ion Chromatography (IC) system, with an Thermo AS7,2 × 250 mm column (and a Thermo AG7, 2 ×50 mm guard column) and a gradient mobile phase (A: 20 mM Ammonium Carbonate, B 200 mM Ammonium Carbonate – Starting at 100% A, changing to 100% B, in a linear gradient over 15 min.) interfaced with a Thermo ICAP Q ICP-MS that monitored m/z+ 75, using He gas in collision cell mode. The chromatograms obtained were compared with that for authentic standards; DMA and As_i. As present under each chromatographic peak was calibrated using a DMA concentration series.

7.2.8 Statistical analysis

Statistical analysis was conducted using Graph Pad Prism version 5 software (Graph Pad Software, USA). All experiments were carried out in triplicates. Data are expressed as mean± SEM and analysis by one -way ANOVA with Tukey's multiple comparison test (*$p<0.05$,**$p<0.01$,***$p<0.001$).

7.3 Results and discussion

Sequential extraction was carried out on each sample. The yield obtained from the different solvents varied greatly, ranging from 0.21 – 1.91 % (Table 1). The most effective extractant was hexane, resulting in yields of ≥ 0.82 %. Extruded products produced the largest yields when compared to the raw rice materials. On extraction with hexane, C (control) and O (optimized) produced 1.44 % and 1.04 % yields, respectively. With ethanol: water as an extractant, C (control) and O (optimized) produced a yield of 1.02 % and 1.49 %, respectively, and with the use of water, C(control) and O (optimized) produced yields of 1.18 % and 1.91 %, respectively. This demonstrates that when red rice

products are extruded, more compounds, or a larger amount of specific compounds, can be extracted from the material, potentially giving greater scope for the use of this product as a functional food considering the scale up processes needed to ensure the cost-benefit and feasibility of production.

Table 7.1. Percentage yield obtained from each sample when extracted using different solvents

Solvent used	Sample	Yield (%)
n-hexane	UA	0.82
	PRR	0.82
	WR	0.99
	RB	0.91
	C	1.44
	O	1.04
Ethanol:water (50:50)	UA	0.49
	PRR	1.01
	WR	0.37
	RB	1.22
	C	1.02
	O	1.49
Water	UA	0.26
	PRR	0.21
	WR	0.60
	RB	0.80
	C	1.18
	O	1.91

7.3.1 Inhibition of DPP-4 activity

Several researchers have demonstrated the ability of foods and food products to inhibit DPP-4 (refs). Here, hexane extracts of red rice and extrudates were unable to inhibit the activity of the enzyme (Fig. 2(a)). However, all ethanol:water extracts of rice samples inhibited DPP-4 activity significantly ($p < 0.001$; Fig 7. 2(b)). Specifically, RB was the most potent at inhibiting DPP-4 activity by 70.48±1.06 %, followed by UA (42.55±0.84 %), PRR (35.91±1.27 %), WR (29.14±1.23 %), O (25.49±1.86 %), then C(13.55±3.97 %). Furthermore, WR when extracted with water was able to significantly inhibit DPP-4 activity by 8.78±4.84 % when compared to a buffer control ($p < 0.001$; Fig 7. 2(c)). DPP-

4 inhibitory activity was found to be retained in the ethanol: water extracts of both extruded products C (control) and O(optimized), albeit at reduced levels. Extracts from O (optimized) containing passion fruit powder, had greater inhibitory activity than the control extrudate, suggesting that passion fruit may possess its own antidiabetic activities.

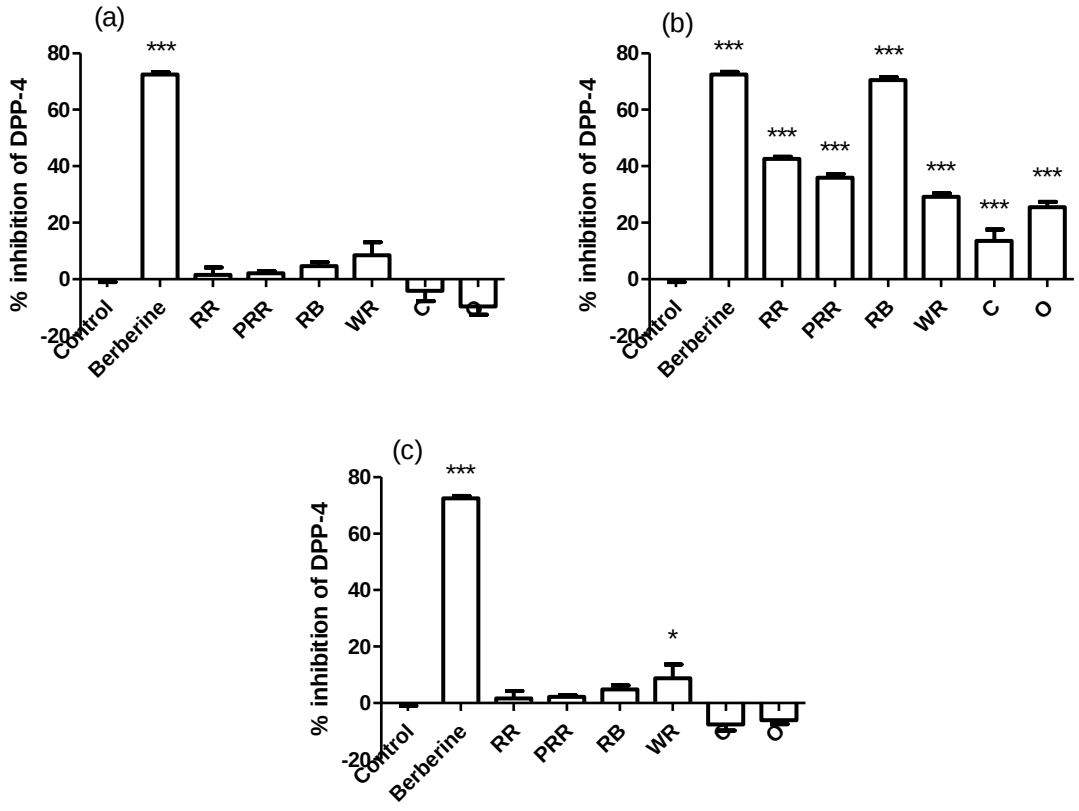

Fig 7.2. DPP-4 inhibition activity

Inhibition of DPP-4 by rice samples extracted by (a) hexane, (b) ethanol:water (50:50), and (c) water at 2 mg/ml. Bars represent mean ± SEM. Data analysed using one way ANOVA followed by Tukey's Multiple Comparisons Test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; n=3). Berberine at 1 mg/ml was used as a positive control. RR = red rice, PRR = red rice polished, RB = red rice bran, WR = white rice, C = control extrudate and O = optimized extrudate

7.3.2 GLP-1 secretion

The ability of rice extracts to enhance GLP-1 secretion is illustrated in Fig. 7.3. *n*-Hexane extracts were able to potently stimulate GLP-1 secretion. In particular, PRR, C(control)

and O(optimized) enhanced secretion of GLP-1 3.14-fold ($p<0.01$), 3.48-fold ($p<0.001$) and 6.06-fold ($p<0.001$), respectively when compared to buffer control (Fig. 3(a)). Ethanol: water extracts of WR and O(optimized) also significantly stimulated GLP-1 secretion 3.33-fold and 4.19-fold, respectively ($p<0.001$; Fig. 3(b)). None of the water extracts had any effect on incretin secretion (Fig. 7.3(c)).

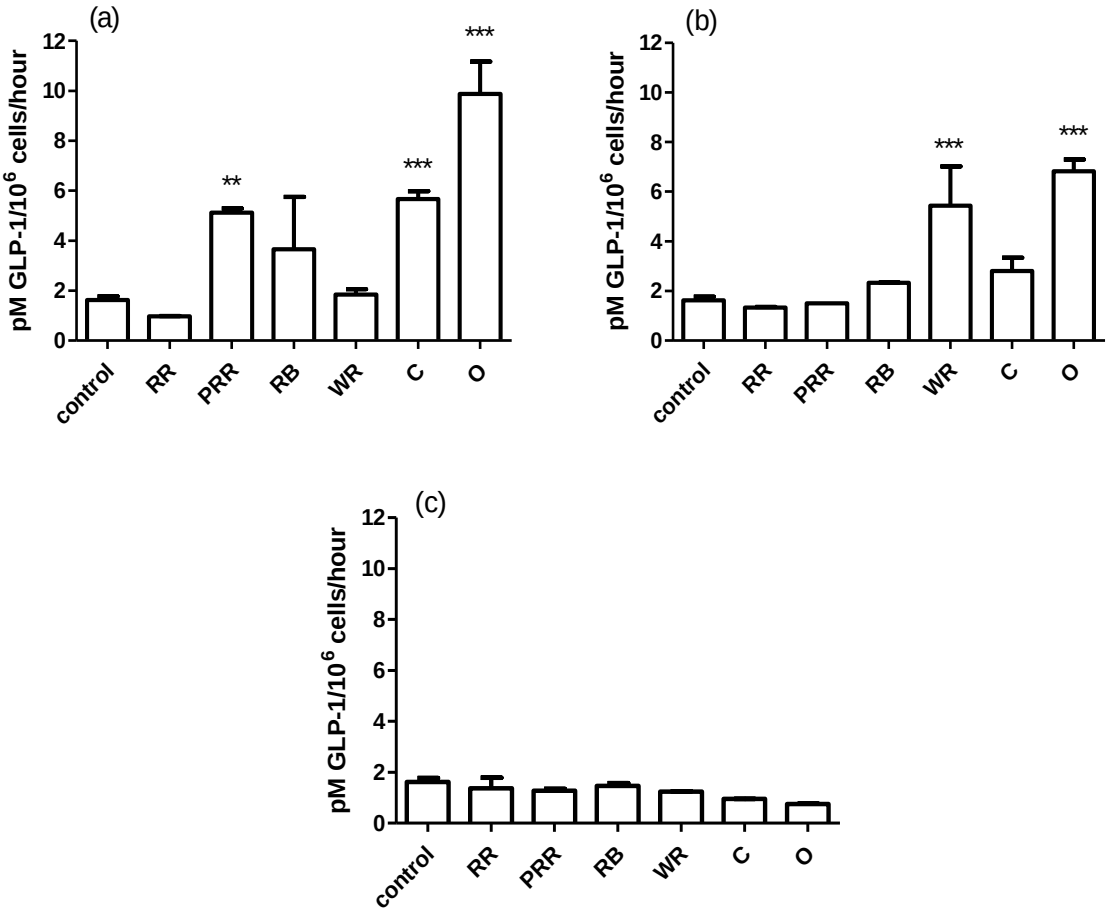

Fig.7.3 Effect of different rice sample extracts on GLP-1 secretion.

GLP-1 secretion stimulated from STC-1 pGIP/Neo cells by rice samples extracted by (a) hexane, (b) ethanol:water (50:50), and (c) water at 2 mg/ml. Bars represent mean ± SEM. Data analysed using one way ANOVA followed by Tukey's Multiple Comparisons Test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; n=3). UA = red rice, PRR = red rice polished, RB = red rice bran, WR = white rice, C = control extrudate and O = optimized extrudate.

7.3.3. Inductively coupled plasma mass spectrometry (ICP-MS) for elemental analysis

ICP-MS was conducted in the four rice raw materials (UA, WR, RB and PRR) samples and the two extrudate products (C and O) to determine levels of arsenic. As mentioned, arsenic is highly abundant in rice and is carcinogenic in nature [13,43] therefore arsenic was measured to determine whether these rice samples were 'safe' for consumption. As shown in Table 2, DMA content was highest in UA (0.010mg/kg) > WR (0.005mg/kg) > RB, PRR, O (0.003 mg/kg) > C (0.002mg/kg). As V (i-As) content was found in significantly higher proportions and was highest in RB > C >UA > O> WR > PRR, ranging from 0.026 – 0.176 mg/kg. Australian food standard established the maximum total As content for cereals as 1 mg/kg [19], suggesting that all samples tested here are well below the recommended limits. To date, the Codex Alimentarius Commission has not formulated any formal guidelines on the safe limits of heavy metals in either white or brown rice, however Asian countries like China, whose staple food crop is rice, has recommended that the safe content for As is limited to < 0.2 mg/kg. Choi et al. [19] reported that grain milling can decreased the content of As and distribute the accumulation of As closer to the surface rather than in the inner core. These results demonstrate that extrusion processing lowers the content of DMA as it was found in lower quantities in C and O when compared to the raw rice materials. This suggests a more in depth study on the effect of extrusion on As species should be carried out as it may be a promising process which can reduce As content in rice and cereals.

Table 7.2 Inductively coupled plasma mass spectrometry (ICP-MS) analysis for arsenic species

Sample	**DMA (Dimethylarsinic acid)**	**As V**	**Total (mg/Kg)**
UA	0.01	0.057	0.067
WR	0.005	0.055	0.06
RB	0.003	0.176	0.179
PRR	0.003	0.026	0.029
C	0.002	0.062	0.063
O	0.003	0.057	0.06

7.4 Conclusin

In total six sample, ethanol: water extracts of rice samples inhibited DPP-4 activity significantly. Specifically, RB was the most potent at inhibiting DPP-4 activity when compared with other samples. *n*-Hexane extracts were able to potently stimulate GLP-1 secretion. Ethanol: water extracts of WR and OE also significantly stimulated GLP-1 secretion but none of the water extracts had any effect on incretin secretion. The ICP-MS study revealed that DMA content was highest in UA (0.010mg/kg) > WR (0.005mg/kg) > RB, PRR, O (0.003 mg/kg) > C (0.002mg/kg). As V (i-As) content was found in significantly higher proportions and was highest in RB > C > UA > O > WR > PRR, ranging from 0.026 – 0.176 mg/kg. Between two extruded products C (control) and O (optimized), the optimized extruded product containing passion fruit powder, had greater inhibitory activity than the control, suggesting that passion fruit may possess its own anti-diabetic activities. Therefore, these samples can further investigate and contributes in pharmacological research area.

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