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## GENERAL DISCUSSION

Edible mushrooms have also been used traditionally to maintain health and promote longevity. These mushrooms with unique flavour and texture are recognized as an important source of biologically active compounds (Dey et al., 2010). Generally, mushrooms are rich in dietary fibres, minerals and vitamins and low in fats. Over the past years many reports have shown that *Pleurotus* spp. is an important genus of edible mushroom and it is a good source of bioactive molecules including polysaccharides that can act as antiproliferative, antifungal, antioxidant and antiviral agents (Telles et al., 2011). Recently, *Pleurotus* spp. is gaining recognition because they are well-known for the high nutritional value, therapeutic properties, and several environmental and biotechnological applications.

Eventough there are many reports on bioactive compounds from mushrooms but these reports are mainly on isolation and identification of compounds in mushrooms from *G. lucidum*, *P. ostreatus*, *P. florida*, *P. eryngii* A. *bisporus* and *V. volvacea*. To date, there are very limited reports on the chemical constituents of *P. sajor-caju*. In this research, the fresh fruiting bodies of *P. sajor-caju* were extracted with water and ethanol and further partitioned with water and ethyl acetate to separate the high molecular weight and low molecular weight compounds. The aqueous extract was re-extracted with butanol in order to extract the phenolic compounds from the aqueous layer. The secondary metabolites from the ethyl acetate extract were isolated using the silica-column chromatography and identified using various spectroscopic techniques such as GC-MS and NMR. Twenty-three compounds were identified from the ethyl acetate extracts whilst four compounds were identified from the butanol extract. The compounds identified in *P. sajor-caju* mainly consisted of mono/poly unsaturated or

saturated fatty acids, sterols, cinnamic acid, phenolic compounds namely 4-hydroxybenzaldehyde and nicotinamide.

The imbalance between the production of free radicals and their neutralization by antioxidant compound can cause the development of oxidative stress and it is well established that the occurrence of oxidative stress in our body leads to the progression of various life-style related diseases such as obesity, atherosclerosis, hyperlipidemia (Shimokawa et al., 2006), diabetes mellitus and inflammation (Heleno et al., 2010) It is also pertinent to note that human antioxidant defence systems may only partially prevent oxidative damage. Hence, there is currently great interest in using dietary supplements containing antioxidants with a view to protect the human body from oxidative damage. Thus the compounds identified in *P. sajor-caju* may also play an important role as dietary antioxidants in preventing various diseases. The most commonly used synthetic antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone but these antioxidants have restricted use in foods as they are suspected to be carcinogenic and are known to cause liver damage (Jayakumar et al., 2011). To investigate the potential antioxidant activity in *P. sajor-caju*, the compounds identified in *P. sajor-caju* were subjected to various antioxidant assays namely  $\beta$ -carotene bleaching, ferric reducing antioxidant power, trolox equivalent antioxidant capacity and lipid peroxidation assays. The antioxidant activities tested in this study differ from each other in terms of substrates probes, reaction conditions and quantitation methods. The  $\beta$ -carotene bleaching and lipid peroxidation assays are based on the inhibition of auto-oxidation of hydrocarbons however the chemistry for  $\beta$ -carotene bleaching is referred to measurement of the rate of oxygen uptake or conjugated-diene peroxides formation whereas lipid peroxidation assay is referred to artificially induce auto-oxidation of lipid

by  $FE^{2+}$ . On the other hand, FRAP and TEAC assays are based on electron transfer (ET) –based assay but FRAP is based on reducing activity meanwhile TEAC is based on radical scavenging capacity (Huang et al., 2005). The preliminary evaluation of antioxidant activity of *P. sajor-caju* extracts in Chapter Four indicated that the aqueous extract (AE) showed a remarkable antioxidant activity compared to the other extracts tested (BE EE, EAE, EP2, EP3, EP4 and EP5) in the antioxidant assays.

Pramanik et al., (2007) have shown that the bioactive constituent of the aqueous extract of *P. sajor-caju* was polysaccharides which mainly comprised of  $\beta$ -glucans and hetero-glucans. Thus, the polysaccharides (GE) was extracted and purified from *P. sajor-caju* using the method described by Roy et al., (2008). The GE was then used to measure the inhibition of lipid peroxidation and storage effect in heated cooking oil. GE significantly inhibited the lipid peroxidation and showed better activity than BHT. Furthermore after storing the GE treated cooking oil for one month, the peroxidation value of oil with addition of GE significantly decreased compared to the untreated oil. Hence, the polysaccharide from the aqueous extract of *P. sajor-caju* may play an important role for use in the food industry to retard lipid oxidative rancidity and also as potential natural preservatives. Furthermore, since this mushroom is easily available, it could readily be incorporated into the diet as a rich source of antioxidants and it could also conceivably be developed into a food supplement or pharmaceutical agent to treat oxidative stress-induced diseases such as diabetes.

Diabetes and obesity (diabesity) caused by a modern lifestyle is recognised as a serious metabolic disorder worldwide which are caused by elevated levels of free radicals (ROS/RNS). Since *P. sajor-caju* extracts have shown promising *in-vitro* antioxidant properties thus, the investigation was continued to study the potential of *P. sajor-caju* extracts in preventing diabetes and obesity using the *in-vitro* and *in-vivo*

models. In Chapter Five, 3T3-L1 cell line (Poulos et al., 2010) was used to study the effects of *P. sajor-caju* extracts (AE, BE, EE, EAE and GE) on lipogenesis, lipolysis and oxidative stress. The isolated fractions from the EAE extract of *P. sajor-caju* (EP2, EP3, EP4 and EP5) were not used in this study because these extracts showed poor antioxidant activities in the previous chapter.

In the cellular model, all *P. sajor-caju* extracts did not exhibit any cytotoxic effects on 3T3-L1 preadipocytes where AE, BE and GE stimulated the highest proliferation of preadipocytes. Besides that, these extracts also stimulated lipogenesis and lipolysis. At lower concentrations (0.1-1 µg/ml), all *P. sajor-caju* extracts stimulated lipogenesis and the activity was similar to insulin (positive control). Similarly, all *P. sajor-caju* extracts stimulated lipolysis and the activity was similar to isoproterenol (positive control). GE treated adipocytes stimulated lipolysis by up-regulating the expression of HSL and ATGL genes. Besides that, GE also activated the expression of GLUT-4 and adiponectin genes which makes GE a good agent in preventing hyperglycemia and may serve as potential insulin sensitizers without increasing the body weight gain. In addition to that, the up-regulation of leptin in GE treated adipocytes also indicates that GE may play an important role in regulating the body weight changes by controlling the appetite. Finally, during lipogenesis and lipolysis process, the adipocytes are highly sensitive to oxidative stress and this can alter the production of adipokines that plays an important role in maintaining the function of adipocytes and preventing diseases associated with oxidative stress (Soares et al., 2005). GE was able to significantly attenuate the oxidative indices (protein carbonyl and lipid hydroperoxides levels) that occurred in 3T3-L1 adipocytes, hence may prevent adipocytes injury or death. Based on the expression of genes investigated in the cellular model, this research may confirm that the polysaccharide extract (GE) of

*P. sajor-caju* have potential in treating obesity and diabetes, thus the investigation was continued by evaluating the efficacy of GE in lipid metabolism and glucose homeostasis using the C57BL/6J (*ob/ob*) mice model.

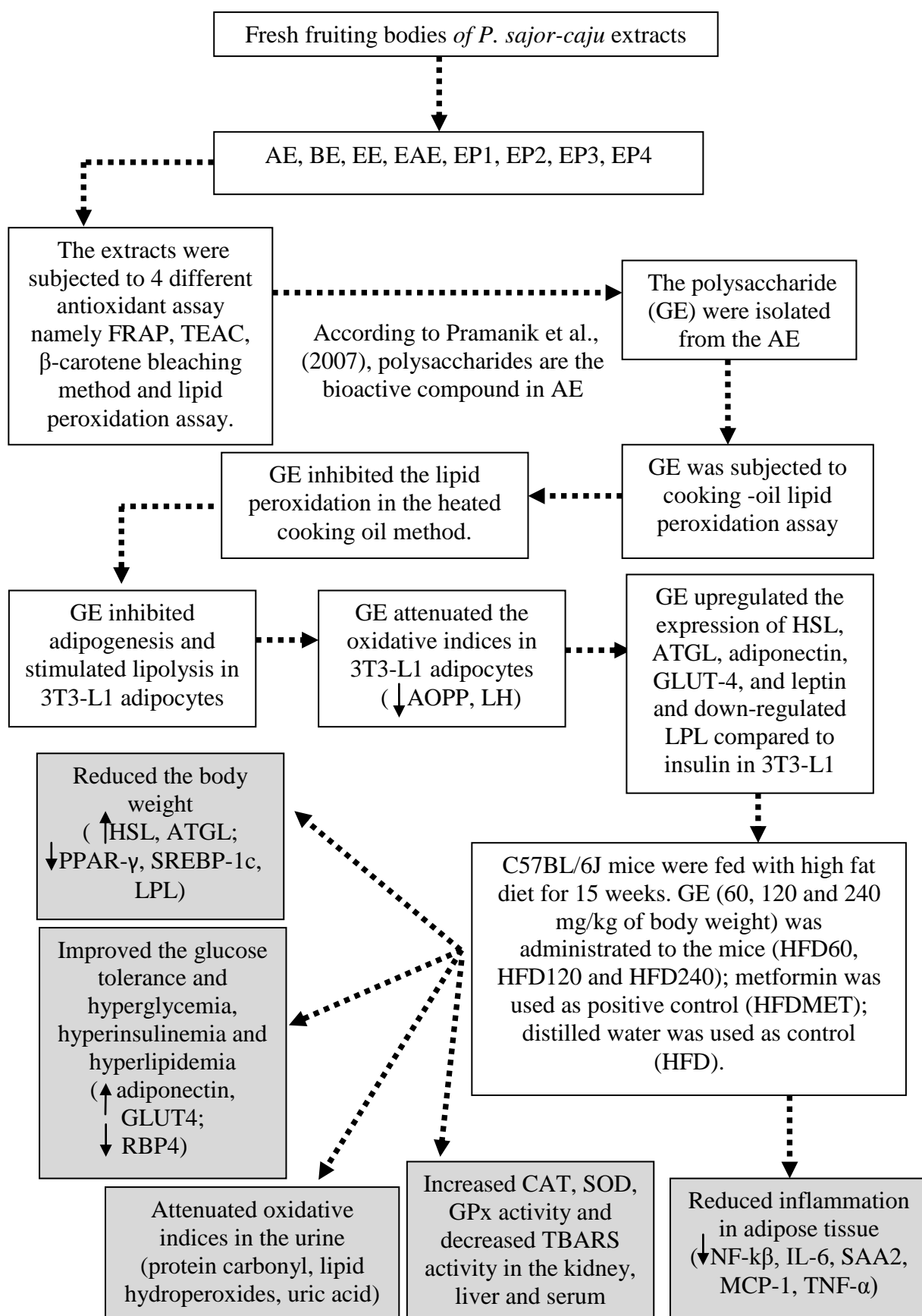
It is well reported that, abnormal lipid and glucose metabolisms in human may cause the development of dyslipidemia, abdominal obesity, hyperglycemia, insulin resistance and inflammation which then leads to diabetes mellitus. The results from Chapter Six showed that, GE significantly reduced the weight gain and lipid levels (TG, TC, LDL-c and HDL-c) in high-fat diet induced obese C57BL/6J mice. Thus, GE may be considered as potential hypolipidemic agent. Besides that, GE was also able to protect the mice fed a high-fat diet against hyperglycemia and hyperinsulinemia by improving the glucose tolerance level. Hence, when treated with GE, HOMA-insulin resistance index was significantly lower indicating the mice in GE treated groups were not insulin resistance.

Since oxidative damage may also provoke the progression of metabolic disorders, thus the protein damage (protein carbonyl), lipid damage (lipid hydroperoxides), uric acid and DNA damage (8-OHdG) concentrations were evaluated in the urine. A potential advantage of measuring oxidation products in urine is that their levels may provide an integrated assessment of the rate of endogenous oxidative stress. Besides that, another advantage is the relatively high concentration of oxidized oxidation products such as lipid, proteins and nucleic acids in urine, which may facilitates their measurement accurately. Finally, measuring oxidative markers in urine samples could provide a non-invasive way to monitor the effectiveness of antioxidant therapy in mice and humans (Coate & Huggins, 2010). GE treated groups, decreased the concentrations of the protein carbonyl, lipid hydroperoxides and uric acid levels compared to HFD group, however there were no significant differences observed in

DNA damage level. Elevated levels of SOD, CAT and GPx concentrations was observed in the kidney, liver and serum of GE treated groups compared to HFD group. This shows that, GE treated groups reduced the oxidative damage products mainly by stimulating the enzymic antioxidants in the body.

To understand the pathway involved for the action of GE, gene expressions of several key molecular markers was investigated. GE down-regulated the expression of PPAR- $\gamma$ , LPL and SREBP-1c genes while up-regulated the expression of HSL and ATGL. This means, GE decreased adipogenesis by inhibiting the key adipose transcription factors while stimulated lipolysis via HSL and ATGL expressions. Besides that, down regulation of LPL proves that the mice in GE treated groups were hypolipidemic. Mice in GE treated groups did not develop hyperglycemia or hyperinsulinemia and showed good glucose tolerance compared to HFD group because these groups up-regulated the expression of adiponectin and GLUT-4 and down-regulated the expression of RBP-4. These genes play important roles in the modulation of glucose homeostasis and insulin sensitization. Finally, the inflammation associated genes (TNF- $\alpha$ , IL-6, MCP-1 and SAA-2) were also down-regulated in the GE treated groups thus, the oxidative stress level was also decreased in these groups. Besides that, the inhibition of NF- $\kappa$ B factor cascade also may lead to lower glucose levels and attenuation of insulin resistance in GE treated groups.

In summary, the antioxidant, anti-obesity, anti-diabetic and anti-inflammatory properties of GE isolated from *P. sajor-caju* have been substantiated in this study. The results provided here undoubtedly show that *P. sajor-caju* and the isolated polysaccharide (GE) have valuable medicinal potential which may be exploited for the benefit of mankind.



**Figure 7.1: Overview of important findings and implications from the present research**

## 7.1 Future Research

The inception of this study was centered on the isolation of the bioactive compounds and elucidating the biochemical and molecular events surrounding the activities demonstrated by *P. sajor-caju* extract. Our intention was to provide new scientific data to explain previous anecdotal reports of medicinal activities of this mushroom. Here, other possible aspects which are relevant are proposed for future investigation.

Firstly, the composition of polysaccharides in this study is based upon the investigation reported by Pramanik et al., (2005 and 2007). In this study, the polysaccharides from *P. sajor-caju* were isolated and purified, however, the identification and detailed structure of the polysaccharides was not carried out. The identification of these polysaccharides are important because parameters such as culture techniques, temperature, pH, water and air in different regions are not the same thus, this can influence the compounds present in *P. sajor-caju*.

Secondly, in Chapter Six, only high fat diet with different concentrations of fats was fed to the mice. To further investigate the effect of diet on development of obesity, high cholesterol diet and high sucrose/fructose diet should also be studied. For example, it is unclear whether diets (atherogenic diet) used to induce obesity may provoke a diabetic phenotype or whether diets used to induce diabetes (diabetogenic diet) may also provoke the development of atherosclerosis (Surwit et al. 1995). Thus, understanding how these diets influence glucose and lipid metabolism will contribute to establishing animal models for diabetes accelerated obesity. In addition to diet, genetic factors contribute to the susceptibility to obesity and diabetes should also be taken into consideration. It is now well established that inbred mouse strains differ in their susceptibility to high fat and high cholesterol diet-induced obesity with C57BL/6 mice



showing susceptibility and strains A/J, BALB/c and C3H/He showing resistance to this disorder. A/J mice are also resistant to the weight gain and insulin resistance experienced by C57BL/6 upon feeding a high fat and high sucrose diet (Schreyer et al., 1998). These and other observations show that profound interactions between diet and genetic factors influence lipid metabolism and glucose homeostasis.

It is most likely that the expression of the genes studied is associated with a protein-level activation/deactivation by phosphorylation. AMP-activated kinase (AMPK) activation has a direct relevance towards lipid and glucose metabolism by phosphorylating downstream enzymes involved in lipid biosynthesis and glucose uptake (Hwang et al., 2008). Thus further investigations are required to validate the possible involvement of GE in AMPK signals which may have implicit relevance to data presented in this study (see section 5.3.5 and 6.3.6). Besides that, another possible mechanism that plays an important role in provoking inflammation was not investigated in this study which is the toll-like receptors (TLRs) pathway. TLRs, which are essential for the development of innate immunity to pathogens, trigger the production of pro-inflammatory cytokines. For example, there are several evidences that proof involvement of TLR2 and TLR4 in metabolic functions and in innate immune responses in obesity. It has been suggested that TLR4 induces inflammation and insulin resistance in insulin-target tissues, such as adipose tissue and muscles of obese mice and human subjects. Besides that, reports have demonstrated that the activation of a TLR2 signalling pathway is related to the development of insulin resistance in adipocytes. The adipocytes and preadipocytes that were isolated from the adipose tissues of the *ob/ob* mice, which are leptin and leptin receptor deficient, respectively, were characterized by more significant up-regulation of TLR1 to TLR9 expression than with the wild-type mice (Kim et al., 2011).

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## ❖ CONCLUSION

The findings of current work showed that *P. sajor-caju* extracts may have potential medicinal benefits because:

- 1) The chemical composition of *P. sajor-caju* extracts reveals that it consists of different types of sterols, saturated or mono/polyunsaturated fatty acids, phenolic compounds and polysaccharide. Thus it is high in fibers and low in calories.
- 2) The polysaccharides extract obtained using the hot-water extraction method showed the highest antioxidant activities.

The administration of polysaccharide (GE) may enhance the lipid metabolism and glucose homeostasis in 3T3-L1 cells and C57BL/6J mice as follows:

- 1) The polysaccharides extract stimulated lipolysis and lipogenesis (at lower concentrations) in 3T3-L1 cell lines.
- 2) The activation of GLUT-4 and adiponectin genes in GE treated adipocytes proves that this extract may enhance glucose uptake.
- 3) The polysaccharide extract was able to protect the C57BL/6J mice against hyperlipidemia, hyperglycemia, hyperinsulinemia and oxidative damage by enhancing the glucose tolerance, insulin sensitivity and antioxidant activity.
- 4) The activation of HSL and ATGL genes proves that this extract stimulated lipolysis which may have decreased the adipose tissue differentiation in the mice.

- 5) The polysaccharide extract attenuated the inflammation caused by high-fat diet by inhibiting the NF- $\kappa$ B cascade signalling pathway.

The medicinal benefit of *P. sajor-caju* is not limited to what we have presented and therefore deserves further investigations so that its full potential could be harnessed for the benefit of mankind and there is great potential for development into viable medicinal products.