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BETALAINS AS ADJUVANT FOR THE TREATMENT OF OBESITY AND ASSOCIATED DISEASES AND A SIMPLE METHOD TO ISOLATE THEM FROM BEET ROOTS

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Abstract: Obesity is one of the most important public health problems and involves a multifarious process including environmental and genetic factors. Studies have shown a close relationship between a high body mass index (BMI), low-grade chronic inflammation and oxidative stress in humans. Natural pigments known as betalains have significant antioxidant properties through the direct elimination of free radicals and a notable role in the restoration of the balance of redox processes in the body. This review reflects the effect of betalains in the signaling pathways of obesity and associated diseases as type 2 diabetes mellitus (T2DM) and dyslipidemia. All studies on animal models and humans were critically analyzed. Results discussed here contributed to the fact that different betalain sources have become not only the raw material of food colorants but also therapeutic agents that may play an important role in a multimodal approach to treat the negative effects of obesity and associated comorbidities. A few-steps method to prepare a betalain supplement is also presented in this work.
Keywords: Beetroot; betalains; obesity; antioxidants; inflammation; oxidative stress.

OBESITY, CHRONIC INFLAMMATION AND OXIDATIVE STRESS

Genetic, environmental, socio-economic and dietary factors are among the causes of obesity that negatively affects the health status increasing the risk of cardiometabolic diseases, osteoarthritis, dementia, depression and some types of cancers[1]. According to World Health Organization (WHO), individual's choice of healthy foods is one of the most accessible and affordable measures to prevent obesity[2]. In a previous work, we underline the role of betanin in the industry as a functional food colorant with antioxidant and anti-inflammatory properties[3]. Other authors have indicated the potential use of betalains as functional ingredients that promote health and prevent diseases[4,5,6]. In this work, we point out the effect of betalains in the signaling pathways related to obesity, insulin resistance, and dyslipidemia (hypertriglyceridemia, elevated non-esterified fatty acids, and decreased high-density lipoprotein cholesterol).

Oxidative stress and inflammation are involved in the development of obesity. Oxidative stress is the result of an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and the neutralizing capacity of antioxidant agents in a living organism. ROS and RNS can be classified into radicals and non-radicals. Anion superoxide ($O^{2\cdot-}$), hydroxyl ($OH\cdot$), alkoxyradical ($RO\cdot$), peroxyl radical ($ROO\cdot$), nitrogen monoxide ($\cdot NO$) and nitrogen dioxide ($\cdot NO^2$) are examples among radicals. The non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$) and peroxynitrite ($ONOO^-$) among others[7,8]. Chronic low-grade inflammation is the most accepted mechanism to explain insulin resistance associated with obesity[9]. However, oxidative stress has emerged as an inducer of this pathological condition which arises from the inability of insulin to act normally in regulating nutrient metabolism in peripheral tissues. ROS/RNS are capable of attacking lipids, nuclear acids and proteins, resulting in oxidative damage to cells Furukawa *et al.* (2004) suggested that obesity may induce systemic oxidative stress[10]. Authors showed that ROS production was increased in 3T3-L1 adipocytes and the production of ROS increased selectively in adipose tissue of obese mice with an augmented NADPH oxidase expression and decreased expression of antioxidative enzymes including superoxide dismutase (SOD), glutathione

peroxidase (GPx), and catalase. These results are in accordance with other studies in the literature[11,12]. NADPH oxidase complex is a major source of ROS in various cells. Furukawa *et al*. also found that ROS increased the expression of monocyte chemotactic protein–1 (MCP-1), a chemoattractant for monocytes and macrophages in adipocytes.

Obesity (especially abdominal obesity) and insulin resistance are closely and reciprocally interrelated. These two parameters are the major causes of the metabolic syndrome (MS)[13] and oxidative stress was proposed as a mediator between obesity and MS[14]. Among the characteristics of the metabolic syndrome are atherogenic dyslipidaemia and a pro-inflammatory state, and each of these risk factors has several components (Fig. 1). The potential mechanisms of obesity-associated insulin resistance were reviewed recently[15,16]. Besides serving as a storage depot for lipid energy, adipose tissue is a highly active metabolic and endocrine organ[17]. Adipocytes produce a variety of biologically active molecules known as adipocytokines. The dysregulated production of adipocytokines, including plasminogen activator inhibitor–1 (PAI-1), tumor necrosis factor-alpha (TNFα), resistin, leptin, and adiponectin, participates in the pathogenesis of obesity-associated metabolic syndrome.

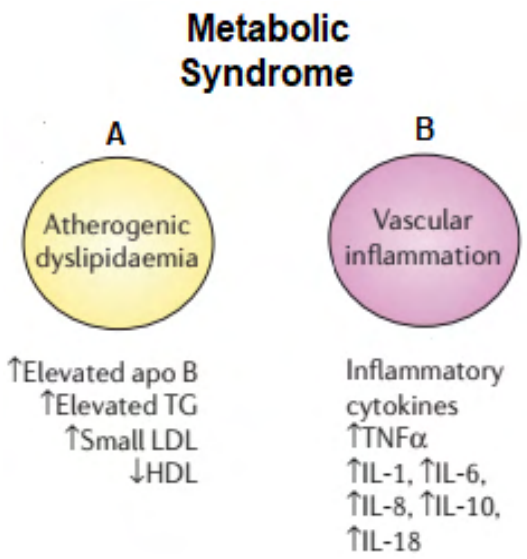


Figure 1: Characteristics of the metabolic syndrome. A-) Elevations in serum triglycerides (TGs), apolipoprotein B (apo B), small low-density lipoprotein (LDL) particles, and low levels of high-density lipoproteins (HDLs). B-) Elevations in pro-inflammatory cytokines.

During obesity, adipocytes increase in their size; therefore, adipose tissues become larger and dysfunctional, recruit macrophages[18] and other immune cells, promote systemic inflammation and uphold ectopic fat accumulation. Upon activation, many immune cells generate free radicals and, conversely, the synthesis of ROS/RNS species promotes an inflammatory state. Other evidences have shown an imbalance in the ratio of M1/M2 macrophages, with M1 "pro-inflammatory" macrophages being enhanced compared with M2 "anti-inflammatory" macrophages being down-regulated[19]. In this scenario, increased cytokine levels released by M1 macrophages and/or reduced anti-inflammatory signals from the M2 promote adipose tissue dysfunction and impairs glucose tolerance.[20]

EFFECTS OF BETALAINS IN OBESITY SIGNALING PATHWAYS

Betalains are natural pigments found in

approximately 17 families of vegetables of the *Caryophyllales* order. Many studies have shown their significant antioxidant properties through the direct elimination of free radicals and in the restoration of the balance of redox processes in the body. Betanin (betanidin 5-O-β-D-glucoside) is the main component of red-violet betalains (betacyanins) in beets and universally permitted as a food ingredient. Other source of betalains that have gained attention is pitaya, commonly known as dragon fruit, because of its economic value but also its health benefits[21]. Pitaya is the source of a relatively new identified betacyanin called hylocerenin, but it also contains betanin, phyllocactin, and their iso-forms[22]. Oxidative stress and inflammation are involved in the development of obesity. Altered circulating levels of inflammatory cytokines have been reported in obese adults. TNFα is secreted in high amounts in adipose tissue of obese rodents and is a potent negative regulator of insulin signaling[23] able to modulate peripheral insulin action[24] preventing the propagation of its signal. In addition, TNFα increases lipolysis in adipocytes, with consequent increase of circulating fatty acids[25], which can trigger insulin resistance in various tissues, such as liver and muscle[26].

Another well-known pro-inflammatory cytokine with increased levels in obese individuals is interleukin-1β (IL-1β). Collins *et al.* (2018) demonstrated a significant increase in IL-1β level in obese animals induced by a high-fat and high-sugar diet[27]. Recently, Dror *et al.* (2017) showed that a postprandial rise in glucose leads to acute elevation of macrophage-derived IL-1β, which contributes to postprandial insulin secretion. Insulin stimulates macrophages to produce IL-1β via glucose metabolism, subsequent mitochondrial ROS production reinforces a pro-inflammatory state[28]. Altogether, IL-1β and insulin, a key hormone in glucose metabolism, promote each other. Interleukin-6 (IL-6) also plays an important role in insulin resistance in human and, furthermore, it may act in concert with other cytokines that also are up-regulated in adipose cells in insulin resistance[29]. Human adipose tissue is a major site of IL-6 secretion[30], whose concentration levels have been correlated with risk for developing type 2 diabetes irrespective of the amount of body fat[31].

Zielinska-Przyjemska *et al.* (2009) investigated the ability of red beetroot juice and chips to protect in vitro neutrophils from obese and non-obese individuals against oxidative damage[32]. Polymorphonuclear neutrophils (PMN) are the most abundant circulating immune cells and represent the first line of immune defense against infection. Neutrophils are among the cells responsible for the excessive production of reactive oxygen species observed in obesity[33]. Despite the absence of isolated betalains, authors observed that neutrophils from obese individuals had a significantly higher ROS production compared with the controls and beetroot products inhibited neutrophil oxidative metabolism in a concentration-dependent manner[32]. Several studies have shown that betalains prevent biological molecules oxidation. Betanin scavenged hypochlorous acid (HOCl) produced by human neutrophils[34] and partly prevented DNA damage in cultured HT-29 cells treated with H_2O_2[35]. In other study, betanin attached to LDL particles and showed be highly effective in preventing copper-induced lipid oxidation, likely due to its lipoperoxyl radical-scavenger effect[36].

Other results underline that betanin inhibits the production of lipid hydroperoxides in human LDL submitted to MPO/nitrite-induced oxidation protecting them from oxidative damage[37]. As the betalains are cationized compounds, their affinity for

membranes is improved by binding to the polar head of fatty acids or polar residues of apo B-100[36,38], a great beneficial attribute for antioxidants. High oxidized LDL (ox-LDL) levels are also associated with insulin resistance[39], which is tightly linked to the pathogenesis of metabolic syndrome[40]. Electron spin resonance spectroscopy (ESR) combined with spin trapping showed that betanin was able to dose-dependently scavenged DPPH- > galvinoxyl- > superoxide- > and hydroxyl-free radicals[35]. Ahmadi *et al*. (2020) showed that betanin (500 μM) attenuates neuroinflammatory effects decreasing the production of ROS, reactive nitrogen species, TNF-α, IL-1β, and IL-6 on LPS (lipopolysaccharide)-activated microglial cells *in vitro*[41]. In this scenario, there are increasing evidences that betalain supplementation may play an important role alone or associated with other antioxidants in a multimodal approach along with diet changes and exercise to treat the negative effects of obesity and associated comorbidities[42].

In accordance with results above, Song *et al*. (2016) observed that dietary of pitaya peel betacyanins (PPBNs) significantly reduced diet-induced body weight gain and ameliorated adipose tissue hypertrophy, hepatic steatosis, glucose intolerance, and insulin resistance of male C57BL/6J mice[43]. Authors observed that the body weight gain started to significantly decrease after 1 week in high-fat diet mice fed with supplementation of betacyanins (50, 100, and 200 mg of PPBNs /kg were tested). A significant decrease of serum levels of triglyceride (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) were also observed and histological analysis of liver demonstrated that administration of PPBNs effectively attenuated hepatic lipid accumulation in obese mice. Song *et al*. identified a total of 14 betacyanins in pitaya peel, including betanin, isobetanin, phyllocactin, and isophyllocactin, however, the authors do not know if all of them were the bioactive agents[43]. Khalili *et al*. (2009) have also observed the decrease of plasma TG, TC, and LDL-C levels and increase of high-density lipoprotein cholesterol (HDL-C) level in high-fat diet rats fed with red pitaya supplementation[44]. In this study, red pitaya was blended, homogenized, frozen at -80º C for two days, and freeze-dried for 3 days. The supplementation used in this study were 0.5%, 0.83%, and 1.17% red pitaya per daily diet (30 g) per day.

Betalain (betanin) lipid profile modulation was also investigated by Yahaghi *et al*. (2020) in a nonalcoholic steatohepatitis (NASH) model. Levels of HDL-C considerably increased and TG, TL (total lipid), LDL-C as well VLDL (very low-density lipoprotein) significantly lowered in mice that received a high-fat regime (HFR) for 4 weeks and shifted to a normal rodent diet with 5, 10 and 20 mg/kg doses of betanin by intra-peritoneum injection for 3 weeks[45]. At the highest dose used, betanin significantly increased adiponectin and decreased leptin as well. This study is a rare case where betanin was administered by intra-peritoneum injection. However, conflicting results were observed by Lugo-Radillo *et al*. (2020) wherein the consumption of isolated betanin from fresh red-purple pitaya fruits (*Hylocereus ocamponis*) produced no significant differences in body weight, increased blood serum total cholesterol levels (TC), and showed no relevant variations in TG and HDL-C levels in mice fed a high-fat diet[46]. Authors, by the other hand, showed that betanin significantly reduced epididymal fat pad weight and inhibited the inflammatory infiltration of the liver and the necrosis of hepatocytes in the steatosis and inflammatory infiltration of liver produced

Biological Effect	Betalains Source	Model	Reference
Neutrophil oxidative metabolism ↓	Red beetroot juice and chips	Human neutrophils	Zielinska-Przyjemska *et al.* (2009)
HOCl ↓	Betanin from prickly pear fruits	Human myeloperoxidase (MPO)	ALLEGRA, M. *et al.* (2005)
DNA damage ↓	Commercial red beet extract	HT-29 cells	ESATBEYOGLU, T. *et al.* (2014)
Copper-induced lipid oxidation ↓	Betalains from prickly pear fruits	Human LDL particles	TESORIERE, L. *et al.* (2003)
LDL oxidation ↓	Betanin from cactus pear fruits	Human LDL particles	ALLEGRA, M. *et al.* (2007)
ROS ↓, RNS ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓	Betanin from red beetroots	Rat microglia cells	AHMADI, H. *et al.* (2020)
Body weight gain ↓, TG ↓, TC ↓, LDL-C ↓, hepatic lipid accumulation ↓	Pitaya peel betacyanins	Mice	SONG, H. *et al.* (2016)
TG ↓, TC ↓, LDL-C ↓, HDL-C ↓	Red pitaya	Rat	KHALILI, R. M. A. *et al.* (2009)
HDL-C ↓, TG ↓, TL ↓, LDL-C ↓, VLDL ↓, leptin ↓, adiponectin ↓	Betanin	Mice	YAHAGHI, L. *et al.* (2020)
Epididymal fat pad weight↓, TC ↓	Betanin from red pitaya	Mice	Lugo-Radillo *et al.* (2020)
NF-κB ↓, TC ↓, TG ↓, LDL-C ↓, HDL-C ↑	Commercial betanin	Rat	Abedimanesh *et al.* (2021)
Glucose ↓, TG ↓, TC ↓, LDL-C ↓, Hcy ↓	Betalain-rich extracts from red beetroot	Human	Rahimi *et al.* (2019)

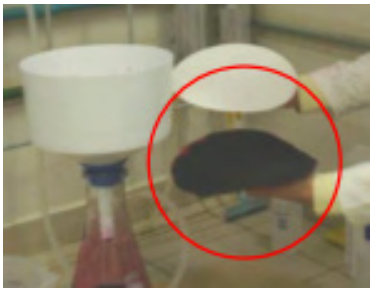
* Hypochlorous acid (HOCl), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), total lipid (TL), very low-density lipoprotein (VLDL), homocysteine (Hcy).

TABLE 1: *In vivo/in vitro* effects observed from different sources of betalains in biological models.

1-) The beet roots are grinded with ethanol (enough for grinding) for 10 min and the liquid phase is separated using nylon fabric (25 μm).

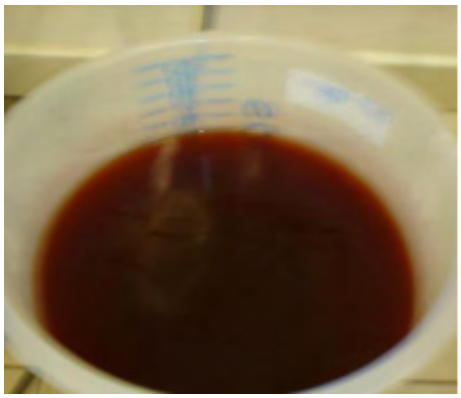
2-) Another amount of ethanol is added to the liquid phase (1:½, v/v) and stirred for 20 min (120 rpm). A black solid dispersed in the liquid phase is observed in this step.

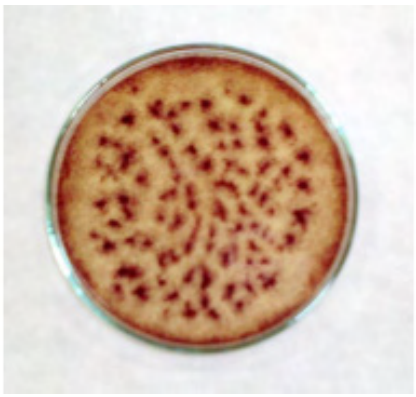
3-) A vacuum filtration is used to separate the black solid in the liquid phase.



The black solid isolated in the filter paper.

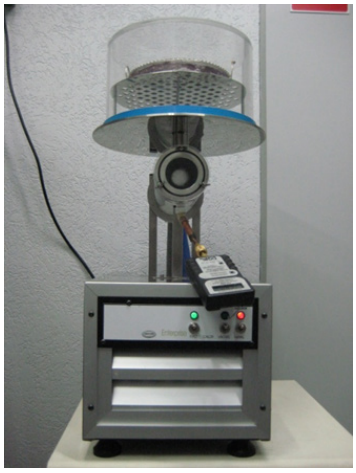
4-) A third amount of ethanol is added to the liquid phase (1:4, v/v) and stirred for 40 min (90 rpm).





A red solid (beet colorant) dispersed in the liquid phase is observed after step 4.

5-) The liquid phase is easily poured after the precipitation of red colorant.



6-) Beet colorant is dried under vacuum until constant weigh.

Beet colorant dried.

7-) Beet colorant grinded.

Figure 2: Production of beet colorant via precipitation with anhydrous ethanol[50].

in mice chronically fed a high-fat diet. Neither inflammatory infiltrate nor necrotic hepatocytes were found in livers from the betanin group. All key results from the studies mentioned are presented in Table 1.

Other potential targets for the development of therapies to prevent and reduce the incidence of metabolic disease complications are adenosine 5'-monophosphate-activated protein kinase (AMPK) and sirtuin-1 (SIRT1). Studies have shown that the activation of AMPK signaling suppresses the expression of the nuclear factor-kappa B (NF-κB) by increasing the expression of SIRT1[47]. NF-κB acts on genes for pro-inflammatory cytokines, chemokines, immunoreceptors, and adhesion molecules. Several research groups have explored the modulation action of new bioactive compounds on the transcription factor NF-κB in the control of inflammatory process[48]. The effect of betanin on AMPK, SIRT1, and NF-κB gene expression by real-time PCR in the whole blood and liver of the diabetic rats was recently investigated by Abedimanesh *et al.* (2021)[49]. Authors used commercial betanin purchased from TCI (Japan) and their findings reveled the antidiabetic, antihyperlipidemic, and hepatoprotective properties related to this betacyanin. The results demonstrated that treatment of diabetic rats with betanin (10 and 20 mg/kg.b.w/day) significantly upregulated the mRNA expression of AMPK and SIRT1 and downregulated the expression of NF-κB in comparison with diabetic control rats. Furthermore, the effect of betanin on serum lipid profile was in accordance with other studies described above. Betanin significantly lowered TC levels at the dose of 20 mg/kg and LDL-C and TG at the doses of 10 and 20 mg/kg as well increase HDL-C serum levels compared to the diabetic control rats.

Despite their beneficial biological effects, betalain supplements are commercially rare. In this scenario, a method that precipitates betalains using anhydrous ethanol brings a new option for supplement industries[50] Fig. 2 illustrates the steps involved in the production of beet colorant via precipitation with ethano[l]. Products obtained from this method show 6% (m/m) betalain content, are nitrate free, and have shown significative anti-inflammatory and analgesic properties[51,52].

Regarding biological effects of betalains observed in clinical trials, Rahimi *et al.* (2019) highlighted protective effects in individuals with coronary artery disease (CAD), Table 1. This study indicated that consumption of betalain-/betacyanin-rich extracts significantly reduced homocysteine (Hcy) concentration in CAD patients. Elevated levels of homocysteine can induce the ROS formation and inflammation[53].

CONCLUSION

Obesity represents a major health challenge. Results discussed here reinforce betalains as functional ingredients since they show a large variety of effects aligned on the improvement of lipid profile, inhibit neutrophil oxidative metabolism, prevent biological molecules oxidation as observed in LDL particles, decrease the production of ROS, reactive nitrogen species, TNF-α, IL-1β, and IL-6, and reduce diet-induced body weight gain and ameliorated adipose tissue hypertrophy. Even though the majority of these studies have been performed with extracts, there are a significant proof of the extraordinary potential of these compounds. More research is needed to ensure the efficacy of betalains working alone or associated with other antioxidants and with current pharmacotherapy in preventing/ reverting associated comorbidities of obesity.

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