

Article

Postbiotic Metabolites of Proanthocyanidins Reduce Adipogenesis In Vitro by Suppressing De Novo Lipogenesis

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Abstract

Proanthocyanidins (PACs) are a key group of bioactive phytochemicals known to provide health benefits. Most PACs are non-bioavailable polymeric molecules that need to be biotransformed by colonic microbes into simple metabolites to exert their pharmacological effects. In this study, six previously unexamined PAC metabolites from *Saccharomyces cerevisiae*, 3-aminophenol (3-AMP), 3-aminosalicylic acid, 2,4-dihydroxy-6-methylbenzaldehyde, 4-hydroxyphenylacetamide (4-HPA), 3-phenyllactic acid, and 2,4,6-trihydroxyacetophenone, were tested for their antiadipogenic activity using an insulin-dependent 3T3-L1 preadipocyte differentiation model. Lipid accumulation in differentiating preadipocytes was visualized and measured with the Oil Red O assay. Only 3-AMP and 4-HPA significantly reduced lipid accumulation at a concentration of 25 μ M. To understand the cellular mechanisms, protein levels of key regulators of adipogenesis and lipid metabolism were analyzed using Western blotting. 3-AMP and 4-HPA may attenuate lipid accumulation by suppressing de novo lipogenesis, with 3-AMP downregulating the peroxisome proliferator-activated receptor (PPAR)- γ /acetyl-CoA carboxylase (ACC)/fatty acid synthase (FAS) axis and 4-HPA primarily inhibiting ACC/FAS signaling. Molecular docking studies indicated that 3-AMP may downregulate PPAR- γ expression through competitive inhibition of insulin receptors. These preliminary findings suggest that 3-AMP and 4-HPA exhibit potential antiadipogenic effects, highlighting PAC-derived postbiotics as promising nutraceuticals for mitigating obesity risk.

Keywords: natural products; proanthocyanidin metabolites; antiadipogenic activity; obesity; postbiotics; insulin receptor inhibition



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1. Introduction

Overweight and obesity are global health crises that affect about 2 billion people worldwide [1]. Obesity is a precursor of many non-communicable diseases, including cardiovascular diseases, diabetes, and certain cancers, which lead to premature death. Although being a non-communicable disease, the WHO has declared obesity an epidemic due to its increasing prevalence at an alarming rate [2]. Obesity is considered a complex chronic disease with multiple etiological risk factors. Poor dietary habits characterized by excessive caloric intake, combined with insufficient physical activity to expend this excess energy, are major contributors to the pathogenesis of obesity [3]. Genetic predisposition, epigenetic modifications, disruption of gut microbiota, and psychological stress are among the top contributors to the pathogenesis of obesity [3,4]. During the pathogenesis of obesity, the adipose tissue expands in size by increasing the number of adipocytes and the size of

already existing adipocytes to accommodate excess calorie influx as lipids. An increase in adipocyte number (adipocyte hyperplasia) occurs through adipogenesis, a process that involves the proliferation of preadipocytes followed by their differentiation into mature adipocytes [5,6]. Adipogenesis is an essential process of the pathogenesis of obesity, and therefore, a therapeutic target for obesity risk mitigation [6].

Lifestyle changes promoting low-calorie diets and increased physical activity are vital for the management of obesity and the prevention of relapse. However, the physiological adaptation during weight loss, especially the effort to maintain energy homeostasis, imbalance of circulating appetite-related hormones, shifts in nutrient metabolism (preference to utilize carbohydrates vs. lipids), and subjective appetite can jeopardize efforts for obesity management. Therefore, interventions through medication and surgery can be beneficial in the long-term management of obesity [7]. Dietary interventions such as supplementation with phytochemical food bioactives [8,9], probiotics, prebiotics, synbiotics, and their postbiotics [10] have been proven to be beneficial for obesity management. A broad range of plant bioactive compounds, especially bioactive peptides [8], alkaloids, terpenoids, and polyphenols, can alleviate obesity by suppressing adipogenesis [11,12].

Polyphenols are the largest group of phytochemicals and can be categorized into phenolic acids, flavonoids, lignans, or stilbenes based on their chemical structure [13,14]. Proanthocyanidins (PACs), also known as condensed tannins, are the most abundant polyphenols in plants [15] and the human diet [16]. PACs are oligomers or polymers of the flavonoid subclass flavan-3-ols. (Epi)catechin, (epi)afzelechin, and (epi)gallocatechin are the most common flavan-3-ol monomers of PAC. Most PACs in the human diet are procyanidins, which are exclusively composed of catechin and epicatechin monomers [15]. PACs are excellent pharmacological candidates for alleviating diabetes, cancers, microbial infections, cardiovascular, neurodegenerative [17], kidney [18], lung, and liver diseases [19]. Most of these pharmacological benefits of PAC are attributable to its antioxidant activity, anti-inflammatory activity, and effects on energy metabolism [17]. The potential of PAC to mitigate obesity is evident in multiple studies that reveal PAC-mediated mechanisms regulating appetite, nutrient absorption, adipokines, insulin sensitivity, adipogenesis, and gut microbiota [20]. However, the pharmacological effects of PAC can be significantly hindered by its low bioavailability [21]. Most of the ingested PAC transits the human gut without being absorbed. Therefore, the biological effects of PAC largely depend on the conversion into bioavailable and bioactive metabolites by colonic microbiota. However, PAC biotransformation by colonic microbes is inefficient and demands synbiotic approaches to improve the therapeutic efficacy of PAC [22]. In a recent study [23], we evaluated the potential of *Saccharomyces cerevisiae* to biotransform grape seed PAC to bioavailable and bioactive metabolites. Biotransformation of PAC with *S. cerevisiae* generates many simple metabolites (Figure 1), including 3-aminophenol (3-AMP), 3-aminosalicylic acid (3-ASA), 2,4-dihydroxy-6-methylbenzaldehyde (2,4-DHMB), 4-hydroxyphenylacetamide (4-HPA), 3-phenyllactic acid (3-PLA), and 2,4,6-trihydroxyacetophenone (2,4,6-THAP) [23], of which the biological activities have not been thoroughly evaluated against obesity. In the current study, we evaluated the potential of these metabolites to alleviate obesity by suppressing the differentiation of preadipocytes into mature adipocytes using an insulin-dependent adipogenesis model of 3T3-L1 murine preadipocytes in vitro, with cellular lipid accumulation measured as an indirect indicator of preadipocyte differentiation [24]. The preliminary findings of this study can be beneficial in identifying drug candidates and their mode of action for obesity management and highlight the potential to design PAC-based functional foods and synbiotics for health benefits.

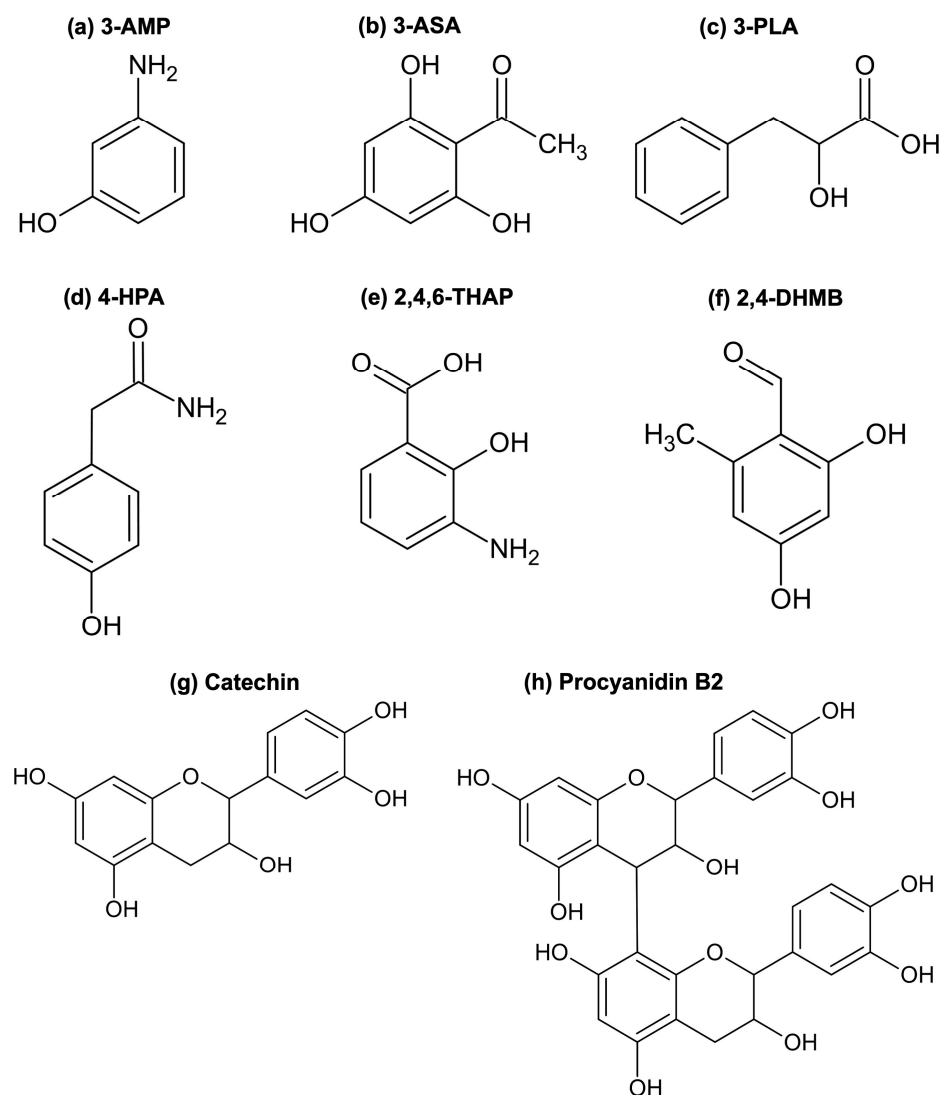


Figure 1. Chemical structures of the tested metabolites of *Saccharomyces cerevisiae*-mediated bio-transformation of proanthocyanidins. 2,4,6-THAP, 2,4,6-trihydroxyacetophenone; 2,4-DHMB, 2,4-dihydroxy-6-methylbenzaldehyde; 3-AMP, 3-aminophenol; 3-ASA, 3-aminosalicylic acid; 3-PLA, 3-phenyllactic acid; 4-HPA, 4-hydroxyphenylacetamide.

2. Results

2.1. Toxicity of the Selected PAC Metabolites in 3T3-L1 Preadipocytes

According to the International Organization for Standardization (ISO) 10993-5:2009 method of biological evaluation of medical devices, a substance is considered cytotoxic if it reduces cell viability to less than 70% compared with untreated control cells [25]. The tested metabolites 3-AMP, 3-ASA, 3-PLA, 4-HPA, and 2,4,6-THAP did not exhibit significant cytotoxicity toward 3T3-L1 preadipocytes at concentrations up to 250 μ M (Figure 2a–e), as cell viability remained above 70%. Only 2,4-DHMB depicted toxicity in the preadipocytes (Figure 2f), at a concentration of 250 μ M. Catechin and procyanidin B2 were also included in the study for comparison of the biological activities. Catechin was significantly toxic to the preadipocytes at a 250 μ M concentration (Figure 2g). Procyanidin B2 was not cytotoxic to the preadipocytes even at the highest tested concentration of 250 μ M (Figure 2h). Although 3-PLA and procyanidin B2 reduced cell viability in a dose-dependent manner from 75 μ M and 50 μ M onward, respectively, cell viability remained above 70% at all tested concentrations, including 250 μ M. Based on the MTS assay, 25 μ M and 100 μ M were chosen

for further experiments to ensure non-cytotoxic conditions while capturing dose-dependent biological responses.

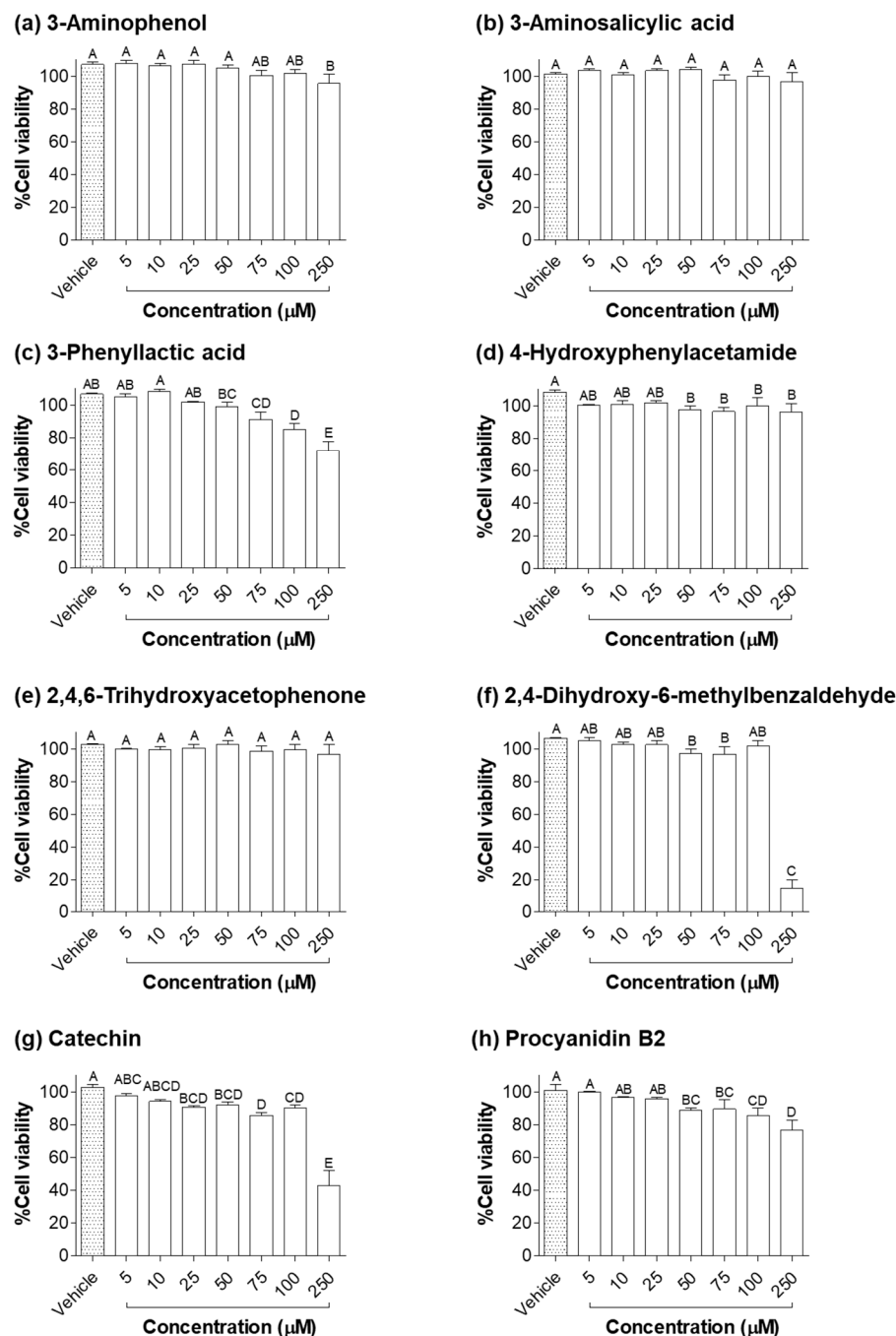


Figure 2. Toxicity of selected metabolites of *Saccharomyces cerevisiae*-mediated biotransformation of proanthocyanidins in 3T3-L1 preadipocytes. The preadipocytes were treated with the selected metabolites at 5–250 μM concentrations for 48 h. The toxicity of PAC metabolites in the preadipocytes was assessed by the MTS cell viability assay. The results were expressed as % cell viability compared to the negative control (preadipocytes not treated with the PAC metabolites). The results presented in the graphs are the mean $\pm$ standard deviation of three independent studies. Results were statistically analyzed using one-way analysis of variance (ANOVA), and means were compared by Tukey's mean separation method at a confidence level of 95%. Means that do not share a similar letter are significantly different at the 95% confidence level. 2,4,6-THAP, 2,4,6-trihydroxyacetophenone; 2,4-DHMB, 2,4-dihydroxy-6-methylbenzaldehyde; 3-AMP, 3-aminophenol; 3-ASA, 3-aminosalicylic acid; 3-PLA, 3-phenyllactic acid; 4-HPA, 4-hydroxyphenylacetamide.

2.2. The Potential of Selected PAC Metabolites to Suppress Preadipocyte Differentiation

Cellular lipid accumulation was evaluated by Oil Red O (ORO) assay to measure 3T3-L1 preadipocyte differentiation (Figure 3). Cellular lipid accumulation is a biomarker of preadipocyte differentiation into mature adipocytes [24]. Incubation of preadipocytes with induction and insulin-rich media (the adipogenesis model) increased the cellular ORO retention (Figure 3c), hence cellular lipid accumulation, by 84% (Figure 3t). The cellular lipid accumulation was significantly lower in the preadipocytes treated with 3-AMP (Figure 3d,e) and 4-HPA (Figure 3j,k) at 25 μ M and 100 μ M levels. This reduction in cellular lipid accumulation was concentration-dependent. The highest reduction in cellular lipid accumulation was observed in the preadipocytes treated with 4-HPA, which limited cellular lipid accumulation to 45% and 34% (vs. 84% of adipogenesis model) at 25 μ M and 100 μ M levels, respectively. Lipid accumulation in the preadipocytes treated with 3-AMP was increased only by 61% and 48% at 25 μ M and 100 μ M levels, respectively. Other tested PAC metabolites, 3-ASA, 3-PLA, 2,4-DHMB, catechin, and procyanidin B2 could significantly suppress cellular lipid accumulation only at 100 μ M concentration (54–66% cellular lipid accumulation vs. 84% of adipogenesis model). Interestingly, 2,4,6-THAP did not significantly reduce lipid accumulation in the preadipocytes at both 25 μ M and 100 μ M levels. Out of the tested PAC metabolites, 4-HPA and 3-AMP were substantially more capable of reducing the lipid accumulation in the preadipocytes, hence, differentiation of the preadipocytes into mature adipocytes.

2.3. Cellular Mechanisms of the Selected PAC Metabolites in Reducing Adipogenesis

The cellular mechanisms mediated by 3-AMP and 4-HPA to reduce adipogenesis were studied by measuring the expressions of peroxisome proliferator-activated receptor (PPAR)- γ , acetyl-CoA carboxylase (ACC)/phosphorylated (*p*)-ACC ratio, and fatty acid synthase (FAS), which are key regulators of cellular lipid metabolism and adipogenesis (Figure 4). Catechin, procyanidin B2, and 5-tetradecyloxy-2-furoic acid (TOFA) were also tested for comparison. TOFA is a known allosteric inhibitor of ACC [26]. Incubation of the 3T3-L1 preadipocytes in the induction and insulin-rich media for differentiation into mature adipocytes (the adipogenesis model) significantly increased the cellular protein levels of PPAR- γ , ACC/*p*-ACC ratio, and FAS (Figure 4). Procyanidin B2 and 3-AMP effectively reduced the cellular PPAR- γ protein level at 100 μ M concentration. TOFA and catechin (at both 25 μ M and 100 μ M concentrations) significantly reduced the cellular PPAR- γ protein level (Figure 4b). However, treatment with 4-HPA was ineffective in mitigating the increase in PPAR- γ protein level in the differentiating preadipocytes. The ACC inhibitor TOFA could suppress the ACC/*p*-ACC protein level ratio (activation of ACC) in differentiating preadipocytes to a level comparable to that of undifferentiated preadipocytes. Catechin, procyanidin B2, and 3-AMP reduced the activation of ACC at the 100 μ M concentration, and these ACC/*p*-ACC protein level ratios were comparable with the undifferentiated preadipocytes (Figure 4c). Interestingly, 4-HPA reduced the ACC/*p*-ACC protein level ratios at 25 μ M and 100 μ M concentrations to levels comparable with the undifferentiated preadipocytes. The cellular FAS level was increased by about 2.5-fold in the differentiating preadipocytes. Catechin and procyanidin B2 reduced the cellular FAS protein level at 100 μ M concentration. TOFA at 10 μ M concentration, 3-AMP, and 4-HPA at both 25 μ M and 100 μ M concentrations reduced the cellular FAS protein to levels comparable with undifferentiated preadipocytes (Figure 4d). These results indicate that the antiadipogenic activity of 3-AMP and 4-HPA is mediated through regulation of the expression of key regulators of adipogenesis and cellular lipid metabolism.

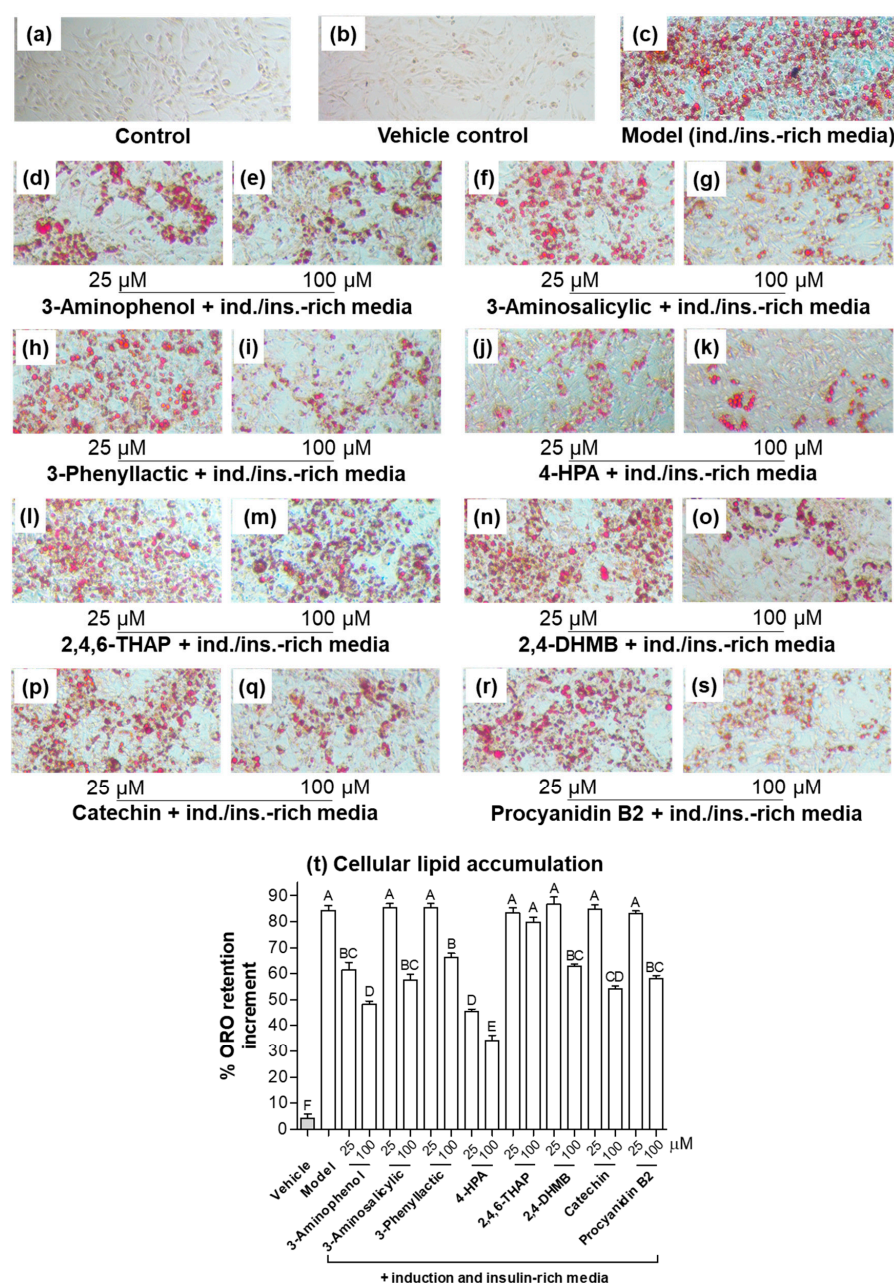


Figure 3. The potential of selected metabolites of *Saccharomyces cerevisiae*-mediated biotransformation of proanthocyanidins to mitigate lipid accumulation in the differentiating 3T3-L1 preadipocytes. The preadipocytes were differentiated into mature adipocytes by exposing them to an induction and insulin-rich medium for seven days (model, ind./ins.-rich media). The differentiating preadipocytes were treated with the selected metabolites at 25 μ M and 100 μ M concentrations, and lipid accumulation was visualized under the microscope (a–s) and quantified by the Oil Red O assay (t). The cellular lipid accumulation was measured by quantifying the % increment of Oil Red O stain retention in preadipocytes compared to the control, not induced for lipid accumulation by exposure to the induction and insulin-rich media. The results were presented as mean $\pm$ standard deviation of three individual experiments. Results were statistically analyzed using one-way analysis of variance (ANOVA), and means were compared by Tukey's mean separation method at a confidence level of 95%. Means that do not share a similar letter are significantly different at the 95% confidence level. 2,4,6-THAP, 2,4,6-trihydroxyacetophenone; 2,4-DHMB, 2,4-dihydroxy-6-methylbenzaldehyde; 4-HPA, 4-hydroxyphenylacetamide.

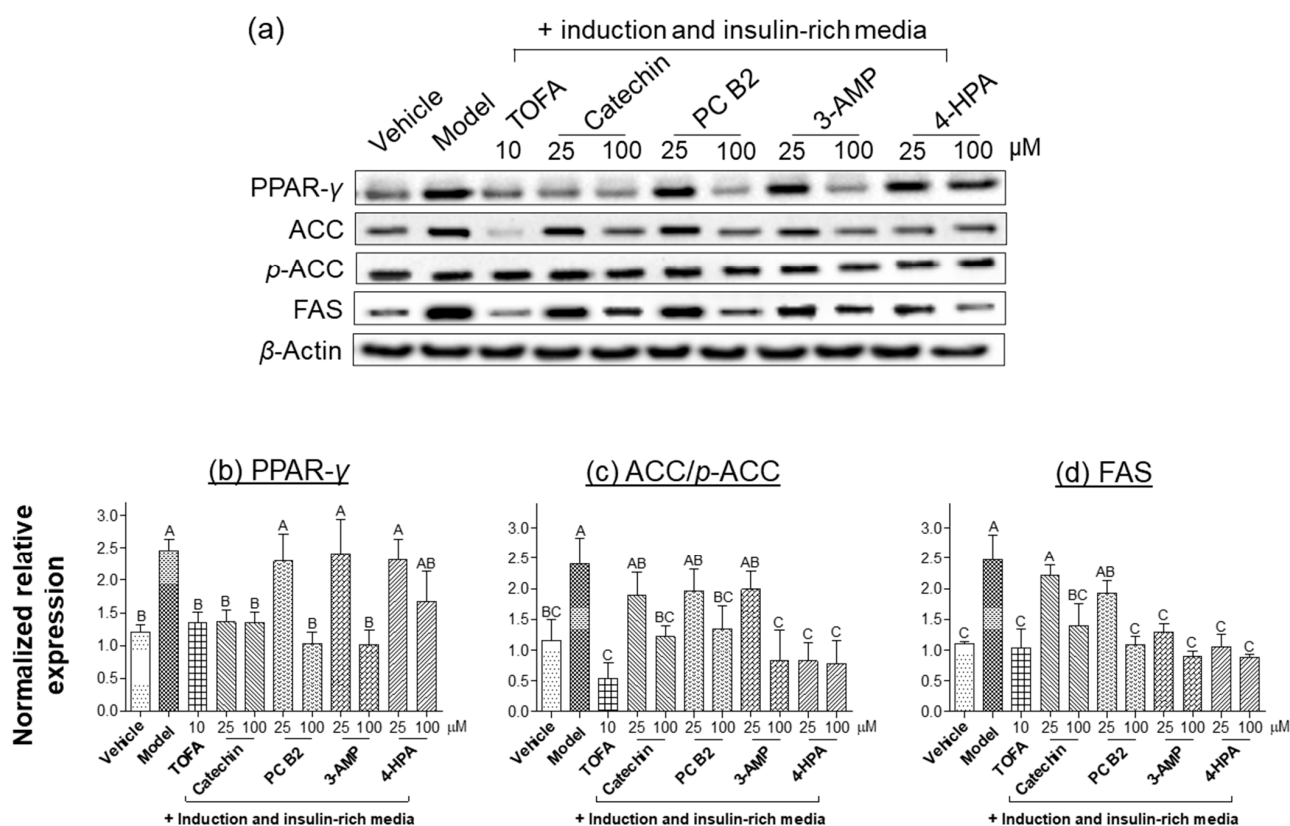


Figure 4. The effects of 3-AMP, 4-HPA, TOFA, catechin, and PC B2 on the protein levels of the key regulators of lipid metabolism in the differentiating 3T3-L1 preadipocytes. The preadipocytes were differentiated into mature adipocytes by exposing them to an induction and insulin-rich medium for seven days (model). The differentiating preadipocytes were exposed to the selected treatments, and the cellular protein levels of key regulators of lipid metabolism were measured by the Western blotting technique. All the measured cellular protein levels were normalized by comparing them with the β -actin levels. The results were expressed as normalized relative expression compared to the negative control (undifferentiated preadipocytes). The results presented in the graphs are the mean $\pm$ standard deviation of three independent studies. Results were statistically analyzed using one-way analysis of variance (ANOVA), and means were compared by Tukey's mean separation method at a confidence level of 95%. Means that do not share a similar letter are significantly different at the 95% confidence level. (a) The expression of protein levels of key regulatory proteins of lipid metabolism was determined by western blotting. 3-AMP, 3-aminophenol; 4-HPA, 4-hydroxyphenylacetamide; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; PC B2, procyanidin B2; *p*-ACC, phospho-acetyl-CoA carboxylase; PPAR- γ , peroxisome proliferator-activated receptor- γ ; TOFA, 5-tetradecyloxy-2-furoic acid.

2.4. Insulin Receptor Tyrosine Kinase (IRTK)-Ligand Interactions at the Insulin Binding Site

In an insulin-rich medium, cellular PPAR- γ expression is induced through activation of the insulin receptor (IR) signaling [27]. Therefore, we analyzed the potential of 3-AMP and 4-HPA in the activation of IR signaling through molecular docking analysis. Molecular docking analysis revealed moderate binding affinities of 3-AMP and 4-HPA to the insulin receptor (IR) ectodomain, with binding affinities of -4.51 ± 0.03 kcal/mol and -5.24 ± 0.09 kcal/mol, respectively. Three primary binding sites were identified, including insulin-binding site 1, site A and B (Figure 5a,b). Both ligands interacted within insulin-binding site 1, sharing key residues within a 5 Å cutoff distance from the insulin-binding pocket (Table 1). Along with many other residues at the binding site 1, 3-AMP formed strong hydrogen bonds with key amino acids Arg252 (2.3 Å), Arg702 (2.48 Å), and Glu706 (2.11 Å) at the L-1 and α CT' domains. 4-HPA interacted with Arg702 through Pi-cation

bond (3.74 Å) at the α CT' domain. Site A located in proximity to the residues Gly5, Val7, and Arg252 at the L1 and cysteine-rich (CR) domains, may serve as a potential allosteric binding site on the IR. Site B was located at the L2–L2' junction, a region where the IR forms a V-shaped dent, and is distant from the well-known insulin binding sites. Specifically, 3-HPA interacted with the Fn3-1' and α CT', while 4-HPA formed hydrogen bonds with L1, L2, Fn3-1', and α CT', engaging both monomers at site 1 (Table 1). When comparing the binding interactions and distance to key residues at the insulin binding domain, 3-AMP exhibited potentially higher inhibition of IRTK compared to 4-HPA.

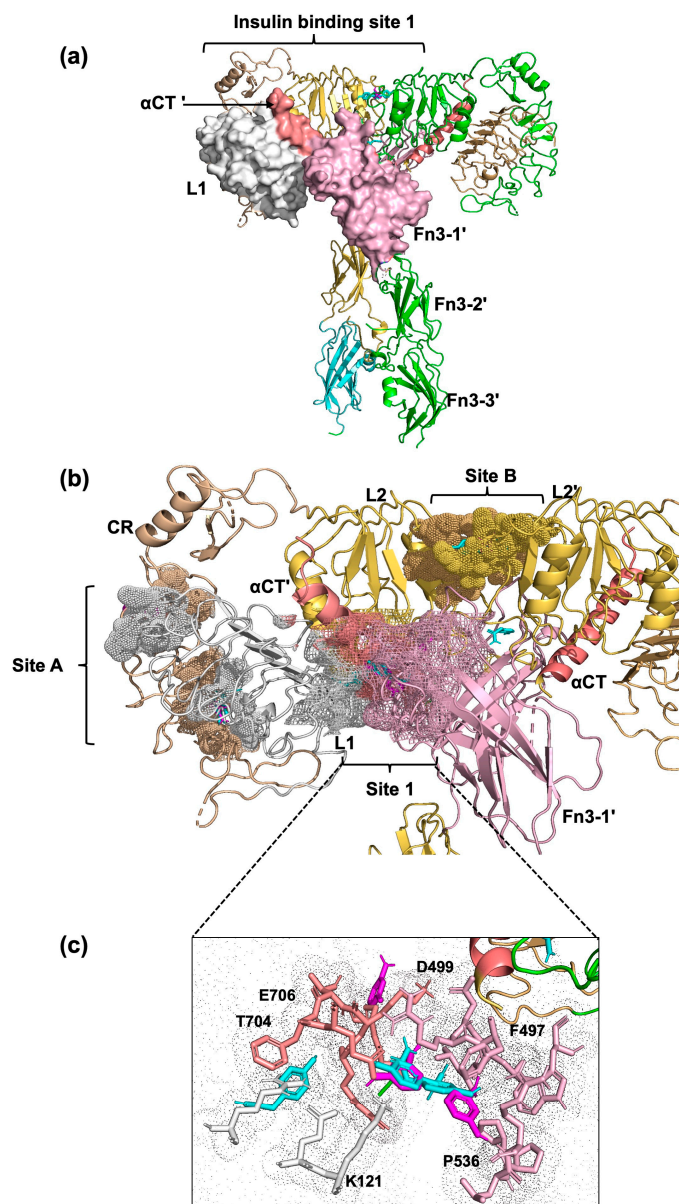


Figure 5. The orientation and binding site of 3-AMP (pink) and 4-HPA (cyan) on the insulin receptor (IR) ectodomain. (a) Insulin binding site 1 and functional domains of the 2α – 2β insulin receptor homodimer, the critical regions involved in ligand binding. (b) The three primary (site 1, A and B) on the tandem repeat (TR) ectodomain with proposed 3-AMP and 4-HPA interactive regions. (c) The orientation of 3-AMP and 4-HPA at site 1, highlighting their special arrangement and potential interactions with key receptor residues. 3-AMP, 3-aminophenol; 4-HPA, 4-hydroxyphenylacetamide; CR, cysteine-rich domain, Fn3-1', fibronectin type 3 repeat domain-1'; L1, L2, and L2', leucine-rich repeats large domains 1, 2, and 2'; α CT', α -chain of carboxy terminal'.

Table 1. The key interacting amino acids of insulin receptor tyrosine kinase (IRTK) ectodomain with 3-AMP and 4-HPA.

Binding Site	Molecule Name	Amino Acid Residue
Insulin Binding Ectodomain		
Site A (L1 and CR)	3-AMP (3 poses out of 9)	Tyr3, Gly5, Val7, Val28, Arg252, Met110, Arg135, Lys190, Cys192, Cys196, Lys197
	4-HPA (2 poses out of 9)	Met110, Arg135, Ile136, Lys190, Cys192, Cys196, Lys197, Lys198, Gly214
Site B (L2 and L2')	3-AMP (2 poses out of 9)	Arg454, Asn455, Asp456
	4-HPA (2 poses out of 9)	Tyr398, Asn455, Arg454, Asp456
Site 1 (L1, Fn3-1', and α CT')	3-AMP (4 poses out of 9)	Asp404, Phe497, Arg498, Leu693, Leu696, Leu500, Pro537, Arg539, Ser699, Arg702, Glu706
	4-HPA (3 poses out of 9)	Try144, Asn348, Glu141, Asn143, Phe497, Arg498, Leu500, Pro537, Phe701, Arg702

3-AMP, 3-aminophenol; 4-HPA, 4-hydroxyphenylacetamide; CR, cysteine-rich domain, Fn3-1', fibronectin type 3 repeat domain-1'; L1, L2, and L2', leucine-rich repeats large domains 1, 2, and 2'; α CT', α -chain of carboxy terminal'.

3. Discussion

PAC can alleviate obesity and obesity-related diseases through multiple mechanisms [20]. However, the pharmacological effects of PAC largely depend on the biotransformation into bioactive metabolites by the colonic microbiota [21,22]. Thus, in a recent study [23], we explored the potential for developing PAC-based synbiotics to biotransform highly polymeric PAC from grape seeds into bioactive and bioavailable metabolites by incubating PAC with *S. cerevisiae*. The mixture of metabolites from this biotransformation could effectively alleviate lipid accumulation in a non-alcoholic fatty liver disease model of AML12 mouse hepatocytes exposed to palmitic acid. Several metabolites detected in this study (Figure 1), such as 3-AMP, 3-ASA, 2,4-DHMB, 4-HPA, 3-PLA, and 2,4,6-THAP, have not been previously reported as metabolites of PAC biotransformation nor thoroughly evaluated for pharmacological effects to alleviate obesity [23]. Therefore, the bioactivities of these metabolites were assessed in an adipogenesis model of 3T3-L1 preadipocytes.

The cytotoxicity of the selected PAC metabolites in preadipocytes was assessed using the MTS assay. Among the tested metabolites, only 2,4-DHMB significantly reduced preadipocyte viability, with cell viability falling below 70% at higher concentrations (>100 μ M). The 2,4-DHMB, also known as *o*-orsellinaldehyde, has been investigated as a bioactive metabolite produced by edible mushrooms [28,29]. Although information on the cytotoxicity of 2,4-DHMB in preadipocytes is scarce, its selective toxicity in human carcinoma cells Hep3B and MRC-5 has been established [28]. Studies evaluating the antiadipogenic effects of 3-AMP, 3-ASA, 3-PLA, 4-HPA, and 2,4,6-THAP by restricting the proliferation and differentiation of preadipocytes are limited. Among catechin and procyanidin B2, only catechin significantly reduced preadipocyte viability to below 70% at a concentration of 250 μ M. Consistent with our findings, studies evaluating the cytotoxicity of dietary flavonoids have demonstrated that catechin [30] and procyanidin B2 [31] do not affect the viability, hence proliferation, of the 3T3-L1 preadipocytes at concentrations of 100 μ M and lower. Experiments based on the absolute cell numbers can be recommended for further evaluating the antiproliferative activities of the tested PAC metabolites in 3T3-L1 preadipocytes [32].

The potential of the tested PAC metabolites to reduce the differentiation of preadipocytes to mature adipocytes was indirectly evaluated by measuring cellular lipid accumulation. Cellular lipid accumulation is an excellent indication of the differentiation of preadipocytes into adipocytes and their maturation [24]. The tested metabolites, other than 2,4,6-THAP, could significantly reduce the accumulation of lipids in preadipocytes at 100 μ M concentration. Only 3-AMP and 4-HPA could effectively suppress the lipid accumulation at the low 25 μ M concentration. Therefore, the mechanisms of 3-AMP and 4-HPA in the reduction in cellular lipid accumulation were studied by measuring the expression of the major regulators of adipogenesis and lipid metabolism, namely, PPAR- γ , ACC, and FAS. The PPAR- γ is widely recognized as the master regulator of adipogenesis, as in fibroblasts, its ectopic expression alone is sufficient to induce adipogenesis. PPAR- γ is also a central regulator of lipogenesis [33,34]. Therefore, we assessed the effects of 3-AMP and 4-HPA on the cellular protein levels of PPAR- γ and its downstream targets, the lipogenic ACC and FAS enzymes [35]. ACC is the rate-limiting enzyme of fatty acid synthesis [36]. Catechin, procyanidin B2, and 3-AMP reduced the PPAR- γ protein level, leading to a subsequent reduction in ACC/p-ACC and FAS in the differentiating preadipocytes. Catechin was significantly more effective than 3-AMP and procyanidin B2 in reducing the PPAR- γ protein level. The potential of catechin to suppress adipocyte differentiation by downregulating PPAR- γ had been previously demonstrated in the 3T3-L1 preadipocytes [37]. Interestingly, 4-HPA was unable to reduce the PPAR- γ protein level, yet it downregulated the ACC/p-ACC and FAS levels in differentiating preadipocytes. Therefore, 4-HPA may have counteracted the upregulation of ACC/p-ACC and FAS through a PPAR- γ independent mechanism. Knowledge on the effects of 4-HPA on lipid metabolism remains limited, which warrants further evaluation of its potential inhibitory effects on ACC and its ability to reduce cellular lipid accumulation by promoting lipolysis. In future studies, the expression of PPAR- γ , ACC, and FAS could be evaluated at the mRNA level to investigate their transcriptional and post-transcriptional regulation by 3-AMP and 4-HPA. Mechanistic assays using PPAR- γ and ACC inhibitors, or knockdown cell models, can be conducted to further validate these preliminary results on the antiadipogenic effects of 3-AMP and 4-HPA. Moreover, assessing the effects of 3-AMP and 4-HPA on the activities of ACC and FAS enzymes, together with direct quantification of lipid synthesis using labeled precursors (e.g., ^{14} C-labeled glucose) [38], may further elucidate the antiadipogenic mechanisms of 3-AMP and 4-HPA.

In adipocytes, insulin regulates energy and lipid metabolism by promoting de novo lipogenesis and suppressing lipolysis [39]. In this study, 3T3-L1 preadipocytes were cultured in an insulin- and glucose-rich medium to accelerate adipogenesis. Insulin initiates intracellular signaling cascades by binding to IRTK, leading to the upregulation of PPAR- γ through the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin complex 1 (mTORC1) axis [27]. IRTK is a transmembrane glycoprotein composed of two α and two β subunits dimerized through disulfide bonds. Insulin binds to the extracellular domain of the IR at two distinct sites: site 1, which comprises the L1 domain of one IR chain and the α CT' and Fn3-1' domain of the opposing chain, and site 2, located at Fn3-1 of the α -chain, enabling the docking of up to four insulin molecules [40,41]. Some studies suggest an alternative model where IR contains two insulin-binding sites formed by the L1 domain of one α -chain and the α CT' domain of the other, while site 2 resides on Fn3-1 β sheets [42,43].

In the present study, molecular docking analysis predicted potential binding sites of 3-AMP and 4-HPA within the ectodomain of the IR, overlapping with insulin-binding residues at L1, α CT', and Fn3-1'. At the proposed site A, 3-AMP forms hydrogen bonds with insulin-binding residues Gly5 and Ser27 at the orthosteric site 1 [44], suggesting that 3-AMP may competitively bind and reduce/inhibit insulin docking at this site. Mutations

of Arg252 to Cys and Glu706 to Ala can reduce/inactivate insulin binding, reduce the rate of maturation, and insulin-induced receptor downregulation [45–47]. Ligand electrostatic interactions of certain residues of the L2 region (Arg454 and Glu453) and the Fn3-1 region (Arg498 and Asp496) are known to force conformational changes resulting in IR burial [46].

Site 1 residues Arg498 and Glu706, as well as site 2 residues Pro536 and Pro537 [46], were found within 5 Å of the 3-AMP and 4-HPA-binding site. In comparison to 4-HPA, 3-AMP, at site A, makes hydrogen bonds with Arg252 (2.23 and 2.11 Å) and Glu706 at site 1, suggesting that it could reduce/inhibit the insulin docking at its favorable site 1 in the IR. Even though the binding affinity is low between 3-AMP and IR, 3-AMP makes strong interactions with key residues that regulate the activity of IR at the active binding site that are likely to be transient and reversible. The observed reduction in lipogenesis in 3-AMP-treated 3T3-L1 cells may be attributable to the modulatory effect on insulin-IR ectodomain binding, which occurs through both direct inhibition at the orthosteric site and indirect inhibition via conformational changes in the binding site via allosteric inhibition.

The modulatory effect of IR prevents phospho-ligand binding and may partially attenuate the downstream cell signaling cascade, especially with 3-AMP. 3-AMP displays a weak receptor affinity and may not act as a highly potent kinase inhibitor. Its activity is more consistent with signal modulation at physiologically relevant concentrations than in comparison to a pharmacological-grade receptor barrier. Given that complete inhibition of the insulin receptor could disrupt systemic glucose homeostasis, the weak binding affinities observed in this study suggest that these compounds act as modulators of IR signaling, rather than as pharmacological inhibitors. Future studies incorporating selective IRTK inhibitors, in the presence and absence of 3-AMP, are warranted to determine whether these compounds exert functional effects on IRTK activity and de novo lipogenesis in 3T3-L1 adipocytes. In silico analyses could be further extended to computationally estimate the absorption, distribution, metabolism, and excretion (ADME) properties of the tested PAC-derived metabolites, particularly 3-AMP and 4-HPA. Approaches such as quantum mechanical calculations, molecular docking, and pharmacophore modeling, along with more advanced techniques including quantitative structure-activity relationship (QSAR) analysis, molecular dynamics simulations, and physiologically based pharmacokinetic (PBPK) modeling, could be employed to complement experimental findings [48]. Taken together, our preliminary results indicate that metabolites of grape seed PAC biotransformation by *S. cerevisiae* may possess pharmacological effects beneficial for the mitigation of obesity. Out of the tested metabolites, 3-AMP and 4-HPA could significantly suppress the differentiation of 3T3-L1 preadipocytes by mitigating lipid accumulation. This reduction in cellular lipid accumulation can be governed by the influence of 3-AMP and 4-HPA on the cellular protein levels of the key regulators of lipid metabolism (Figure 6).

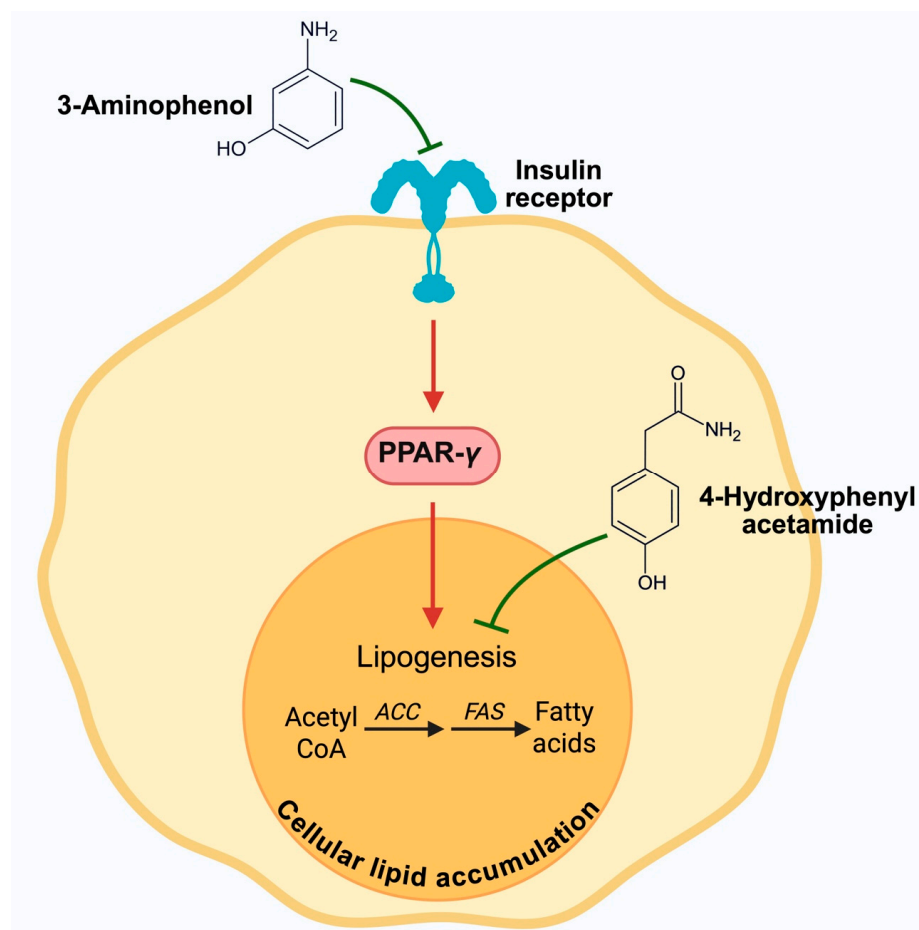


Figure 6. Proposed mechanisms of 3-aminophenol (3-AMP) and 4-hydroxyphenylacetamide (4-HPA)-mediated antiadipogenic activity in differentiating 3T3-L1 preadipocytes. In adipocytes, lipid accumulation primarily depends on de novo lipogenesis. During lipogenesis, ACC and FAS convert excessive glucose into fatty acids. As the master regulator of adipogenesis, PPAR- γ can promote lipogenesis by upregulating the expression of ACC and FAS. Under insulin-rich conditions, the insulin receptors (IRs) get stimulated and upregulate PPAR- γ , which subsequently promotes lipogenesis. 3-AMP may suppress lipogenesis in differentiating preadipocytes, thereby inhibiting adipogenesis, by downregulating the PPAR- γ /ACC/FAS axis, potentially through competitive inhibition of IRs. Similarly, 4-HPA may reduce lipogenesis by downregulating ACC/FAS, interestingly, through a PPAR- γ -independent mechanism. The proanthocyanidin metabolites, 3-AMP and 4-HPA, may potentially be developed as antiadipogenic agents to mitigate the risk of obesity. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; PPAR- γ , peroxisome proliferator-activated receptor- γ . (Created with BioRender.com, a license purchased, accessed on 5 November 2025).

4. Materials and Methods

4.1. Materials and Chemicals

The metabolites of *S. cerevisiae*-mediated biotransformation of grape seed PAC, 3-aminophenol (3-AMP, 100242), 3-aminosalicylic acid (3-ASA, 255300), 4-hydroxyphenylacetamide (4-HPA, 387738), 3-phenyllactic acid (3-PLA, P7251), and 2,4,6-trihydroxyacetophenone (2,4,6-THAP, T64602) were purchased from MilliporeSigma, Oakville, ON, Canada. The other metabolite, 2,4-dihydroxy-6-methylbenzaldehyde (2,4-DHMB, TRC-D480863) was purchased from Toronto Research Chemicals Inc., North York, ON, Canada. Dulbecco's Modified Eagle's Medium-high glucose (DMEM), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, fetal bovine serum (FBS), insulin solution, L-glutamine, and penicillin–streptomycin solution for cell culture were purchased from MilliporeSigma, Oakville, ON, Canada. Other chemicals and materials, 5-tetradecyloxy-

2-furoic acid (TOFA), bovine serum albumin (BSA), catechin (C1251), ethylenediaminetetraacetic acid (EDTA), isopropanol, Oil red O stain (ORO), paraformaldehyde, phenazine methosulphate (PMS), polyvinylidene difluoride (PVDF) membranes, procyanidin B2 (PHL89552), protease inhibitor cocktail powder (p2714), sodium deoxycholate, sodium fluoride, Tris-HCl, and Triton X-100 were also purchased from MilliporeSigma, Oakville, ON, Canada. All chemicals and reagents used in this study were of analytical grade or higher purity. CellTiter 96[®] aqueous MTS reagent powder (G1111) was purchased from Promega Corporation, Madison, WI, USA. The antibodies of the Western blotting technique, acetyl-CoA carboxylase (ACC, 3676S), phosphorylated (*p*)-ACC (11818S), anti-mouse secondary antibody (7076P2), anti-rabbit secondary antibody (7074S), fatty acid synthase (FAS, 3180S), peroxisome proliferator-activated receptor- γ (PPAR- γ , 2443S), and β -actin (12620S) were purchased from Cell Signaling Technology, Inc., Danvers, MA, USA.

4.2. Cell Line and Culture Conditions

The biological activity of the selected PAC metabolites in reducing adipogenesis was evaluated using 3T3-L1 murine preadipocytes. 3T3-L1 (CL-173TM) fibroblasts of mouse embryos were purchased from ATCC[®], Manassas, VA, USA. 3T3-L1 cells were cultured in the complete growth medium prepared by supplementing DMEM with 10% FBS, L-glutamine (4 mM), and penicillin (100 U/mL)–streptomycin (100 $\mu\text{g mL}^{-1}$). Cells were cultured at 37 °C in a humidified incubator with a 5% CO₂ atmosphere. Cell cultures were maintained by replenishing with fresh culture medium every two days and sub-culturing at a confluency of 80%.

The differentiation of 3T3-L1 preadipocytes into mature adipocytes was conducted by exposing the cells to an induction medium followed by an insulin-rich medium. The induction medium was prepared by adding dexamethasone (1 μM), IBMX (0.5 mM), and insulin (10 $\mu\text{g mL}^{-1}$) into the complete DMEM medium. The insulin medium was prepared by adding insulin (10 $\mu\text{g mL}^{-1}$) to the complete DMEM medium. Initially, the preadipocytes were allowed to reach full confluency. After two days of reaching full confluency, the preadipocytes were cultured with the induction medium for three days to initiate differentiation. Subsequently, these cells were cultured with the insulin-rich medium for four days to complete the differentiation into mature adipocytes. The insulin medium was replenished with fresh insulin medium after two days [12].

4.3. Toxicity of the PAC Metabolites in 3T3-L1 Preadipocytes

Toxicity of the selected PAC metabolites in 3T3-L1 preadipocytes was determined by the MTS cell viability assay. The toxicity of catechin and procyanidin B2 was also evaluated for comparison. Initially, cells were seeded in 96-well plates at a cell density of 1×10^4 cells/well. The cells were allowed to reach full confluency and treated with 5–250 μM concentrations of the PAC metabolites, catechin, and procyanidin B2 for 48 h. Subsequently, 20 μL of MTS/PMS (MTS, 333 $\mu\text{g mL}^{-1}$ and PMS, 25 μM of final concentration) solution was pipetted into each well, and the cells were incubated for 3 h at normal culture conditions. The absorbance for each well was measured at 490 nm wavelength by using a microplate reader [49]. The absorbance values of treatments were compared with the negative control (preadipocytes not treated with PAC metabolites), and the results of three independent experiments were expressed as % cell viability $\pm$ standard deviation (SD). Non-cytotoxic concentrations of the PAC metabolites, catechin, and procyanidin B2 were determined for further experimentation based on the results of the MTS assay.

4.4. Antidifferentiation Activity of the PAC Metabolites in 3T3-L1 Preadipocytes

The differentiation of preadipocytes to mature adipocytes was indirectly assessed by measuring the cellular lipid accumulation [12]. The cellular lipid accumulation was

visualized and quantified by the ORO assay. Initially, 3T3-L1 preadipocytes were seeded in 6-well plates at a density of 2×10^5 cells/well and allowed to reach full confluency. After two days of reaching full confluency, the cells were cultured in the induction medium for three days, followed by the insulin-rich medium for four days to induce differentiation. During this differentiation period, cells were treated with 25 μ M and 100 μ M concentrations of the selected metabolites. The antiadipogenic activities of catechin and procyanidin B2 (at 25 μ M and 100 μ M levels) were also evaluated for comparison with the PAC metabolites. A stock of ORO solution was prepared by dissolving 60 mg of ORO in 20 mL of 100% isopropanol. The working ORO solution was prepared by mixing the stock solution in deionized (DI) water (3:2 *v/v*) and filtering through 0.22 μ m syringe filters. After the differentiation and treatment period, the cells were gently washed twice with PBS and fixed by incubating in a 4% paraformaldehyde solution for 45 min at room temperature (RT). Then, cells were gently washed twice with DI water and permeabilized by incubating with 60% aqueous isopropanol for 5 min at RT. Cellular lipids were stained by incubating the cells with the ORO working solution for 15 min at RT. Subsequently, the cells were gently washed with DI water (3 times) to remove excess ORO solution. The cells were visualized under a brightfield microscope (at $\times 100$ magnification) while immersed in DI water. Cellular lipid accumulation was indirectly measured by quantifying the ORO stain retained in the cells. Initially, wells of the 6-well plate were gently washed with 60% aqueous isopropanol by placing them on a slow rocker (70 rpm) for 5 min $\times$ 3 times. ORO stain in cells was extracted by adding 1 mL of 100% isopropanol into each well and placing it on a slow rocker (70 rpm) for 30 min. The ORO extracts were pipetted (100 μ L) into a 96-well plate in triplicate, and absorbance values were measured at 492 nm wavelength. The absorbance values of the treatments were compared against the preadipocytes not induced for differentiation (negative control), and the results of three independent experiments were expressed as % ORO retention increment $\pm$ SD [23,49].

4.5. Cellular Mechanisms of the Selected PAC Metabolites-Mediated Antiadipogenic Activity

The cellular mechanisms mediated by the selected PAC metabolites to suppress adipogenesis were studied by measuring the cellular protein levels of the key regulators of lipid metabolism. Synthesis and accumulation of triglycerides promote the differentiation of 3T3-L1 preadipocytes [50]. The cellular protein levels of ACC/p-ACC ratio, FAS, and PPAR- γ were measured as the key regulators of lipid metabolism using the Western blot technique. Initially, 3T3-L1 cells were seeded in T25 cell culture flasks at a density of 5×10^5 cells/flask and allowed to reach full confluency. Two days after reaching full confluency, cells were incubated with the induction medium for three days, followed by insulin-rich medium for four days to induce differentiation. During this period, cells were treated with 25 μ M and 100 μ M concentrations of 3-AMP and 4-HPA. Only 3-AMP and 4-HPA were selected to study the mechanisms of suppression of adipogenesis based on the results observed in the ORO assay. The antiadipogenic mechanisms of ACC inhibitor TOFA (10 μ M concentration), catechin and procyanidin B2 (25 μ M and 100 μ M concentrations) were also compared. Radio-immunoprecipitation assay (RIPA) buffer was prepared by mixing Tris-HCl (50 mM), NaCl (150 mM), Triton X-100 (1%, *v/v*), sodium deoxycholate (0.5%, *v/v*), EDTA (1 mM), and NaF (10 mM) in DI water. The RIPA buffer was mixed with the protease inhibitor cocktail (10 μ L in 1 mL of RIPA buffer) to prevent protein degradation during extraction. After the treatments, cells were harvested, and proteins were extracted using the RIPA buffer. Protein concentrations in the extracts were determined by the PierceTM Coomassie (Bradford) protein assay kit (ThermoScientific, Rockford, IL, USA). Then, the extracted proteins were denatured using the Blue Loading Buffer pack manufactured by New England BiolabsTM Inc. (Ipswich, MA, USA). Proteins were

loaded into a sodium dodecyl-sulfate polyacrylamide gel (20 µg/well) and subjected to gel electrophoresis (90 min at 80 V and 400 mA) for size-based separation. Proteins on the gel were transferred onto PVDF membranes by using the Bio-Rad Trans-Blot® Turbo™ system (Hercules, CA, USA). PVDF membranes were blocked in a blocking solution of 5% non-fat milk powder (*w/v*) in 1 × Tris-buffered saline (TBST) for 1 h at RT and probed with primary antibodies (1:1000 *v/v* in 5% BSA *w/v*) at 4 °C overnight. The PVDF membranes were washed with 1 × TBST solution (20 min × 3) and probed with secondary antibodies (1:5000 *v/v* in 5% non-fat milk powder *w/v*) for 1 h at RT. The PVDF membranes were washed with 1 × TBST solution (20 min × 3) and developed for imaging by using enhanced chemiluminescence (ECL) based Clarity™ and Clarity Max™ Western ECL substrates Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PVDF membranes were imaged using the signal accumulation mode of the Bio-Rad Chemidoc MP™ imaging system (Universal hood III, Hercules, CA, USA). The images were analyzed by ImageJ software (version 1.8.0_345). The protein levels were normalized by comparing with β-actin levels, and the results were expressed as relative protein levels compared to the preadipocytes not induced for differentiation (negative control) [19]. The results are presented as mean ± SD of three independent experiments.

4.6. Insulin Receptor Tyrosine Kinase (IRTK) Ligand Binding Analysis

In this study, 3T3-L1 cells were cultured in an insulin-rich medium to promote adipogenesis. To evaluate the inhibitory effects of 3-AMP on insulin-activated *de novo* lipogenesis, molecular docking studies were conducted against the IRTK ectodomain. The cryo-electron microscopy structure of the ectodomain and crystal structure of the kinase domain of the IRTK were obtained from the Protein Data Bank (PDB) (www.rcsb.org; accessed on 2 December 2025). Specifically, the dimeric insulin receptor ectodomain (PDB ID: 6PXV, resolution 3.20 Å), bound to four insulin molecules, was analyzed to determine the binding interactions of 3-AMP and 4-HPA [40,51].

To assess protein-ligand interactions, ligand-free proteins, 4-HPA and 3-AMP were analyzed using the PyRx Virtual Screening Tool, integrated with AutoDock Vina version 0.8 (PyRx-Python Prescription, The Scripps Research Institute, San Diego, CA, USA). Grid boxes were constructed to encompass all relevant residues. The protein-ligand interactions at the insulin-binding ectodomain were examined at insulin-binding site 1, which consists of the leucine-rich repeats large domain (L)-1, the α-chain of carboxy terminal' (αCT') domain, and the fibronectin type 3 repeat domain-1' (Fn3-1') [41]. The grid box parameters for the insulin-binding pocket (site 1) of the IRTK ectodomain were as follows:

Center coordinates: $x = 179.539$, $y = 135.009$, $z = 117.574$

Size dimensions: $x = 86.263$, $y = 63.486$, $z = 68.299$

For the full protein cytoplasmic tyrosine kinase domain, the grid box parameters were:

Center coordinates: $x = 13.824$, $y = 63.029$, $z = 17.509$

Size dimensions: $x = 53.491$, $y = 57.559$, $z = 50.653$

All docking and structural analyses were performed using Discovery Studio Visualizer v21.1.0.20298 (BIOVIA, San Diego, CA, USA) and the PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC, Mannheim, Germany). Interactions between the ligands and amino acid residues were examined, including polar and non-polar interactions, with all residues within a 5 Å cutoff distance from the ligand being monitored.

4.7. Statistical Analysis

The statistical data analysis was conducted using the Minitab® statistical software (version 21.3.1, State College, PA, USA). All the results were expressed as mean ± standard deviation of three independent experiments. Means were statistically compared by the one-

way analysis of variance (ANOVA) with Tukey's mean separation method at a confidence level of 95%.

5. Conclusions

In a previous study exploring the potential to develop grape seed PAC-based synbiotics, we demonstrated the potential of *S. cerevisiae* to biotransform highly polymeric PAC to simple metabolites. Several of these metabolites, namely, 3-AMP, 3-ASA, 2,4-DHMB, 4-HPA, 3-PLA, and 2,4,6-THPA, were evaluated for their pharmacological effects to mitigate adipogenesis using an insulin-dependent 3T3-L1 preadipocyte model. Tested PAC metabolites did not significantly reduce the viability of preadipocytes (>70% cell viability) at concentrations lower than 100 μ M. Cellular lipid accumulation was quantified as a biomarker of the differentiation of preadipocytes into mature adipocytes. Only 3-AMP and 4-HPA could significantly reduce lipid accumulation in the differentiating preadipocytes at a 25 μ M concentration. 3-AMP may suppress de novo lipogenesis by downregulating the PPAR- γ /ACC/FAS axis, whereas 4-HPA downregulates ACC/FAS signaling through a PPAR- γ -independent mechanism. 3-AMP may downregulate the PPAR- γ protein expression and subsequent activation of ACC and FAS through competitive inhibition of IRs. Although we did not measure lipolysis, the observed reduction in lipid content may result from both decreased lipogenesis and increased lipolysis, which warrants further investigation. It is critical to validate these preliminary results using human preadipocytes and in vivo experiments. Moreover, it is essential to establish whether 3-AMP and 4-HPA are generated in vivo by the human gut microbiota or through probiotic supplementation, and to determine their physiologically achievable concentrations. Future studies should also explore how interindividual variability in gut microbiota composition influences the formation of 3-AMP and 4-HPA from PAC. Our findings suggest that the PAC metabolites 3-AMP and 4-HPA exhibit antiadipogenic potential and may be promising candidates for the development of therapeutic strategies such as precision nutraceuticals to reduce obesity risk.

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Abbreviations

2,4,6-THAP, 2,4,6-trihydroxyacetophenone; 2,4-DHMB, 2,4-dihydroxy-6-methylbenzaldehyde; 3-AMP, 3-aminophenol; 3-ASA, 3-aminosalicylic acid; 3-PLA, 3-phenyllactic acid; 4-HPA, 4-hydroxyphenylacetamide; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ORO, Oil Red O;

PAC B2, procyanidin B2; PAC, proanthocyanidins; *p*-ACC, phospho-acetyl-CoA carboxylase; PPAR- γ , peroxisome proliferator-activated receptor- γ ; TOFA, 5-tetradecyloxy-2-furoic acid.

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