Hibiscus sabdariffa Linn. Extract Increase Arcuate Nucleus Leptin Receptor mRNA Expression and Promote White Fat Browning

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Abstract

Objective: This study aimed to test the hypothesis that Hibiscus sabdariffa Linn. Extract (HSE) would increase arcuate nucleus Lep-R, NPY, and white adipose tissue β3AR mRNA expression in DIO rats. This study also analyzed the potency of H. sabdariffa bioactive compounds as an activator of Lep-R and β3AR. Methods: Twenty-four male Sprague-Dawley rats were separated into four groups: Control (standard chow), DIO (HFD), DIO-Hib200 (HFD+HSE 200 mg/kg BW), and DIO-Hib400 (HFD+HSE400 mg/kg BW). HSE administration was administered orally for five weeks, once a day. Result: The administration of HSE significantly (P<0,05) increased the arcuate nucleus Lep-R expression, but not for the ARC NPY and WAT β3AR. The Lee index of DIO rats also significantly decrease (p<0,001 for a dose of 200 mg/kg BW and p<0,01 for a dose of 400 mg/kg BW) into the normal range ([?] 310). Among 39 bioactive compounds, 5-O-caffeoylshikimic acid has high free binding scores (-8,63) for Lep-R, and myricetin_3_arabinogalactoside has high free binding scores (-9,39) for β3AR. These binding predictions can activate Lep-R and β3AR. Conclusion: HSE increases leptin sensitivity and reduces obesity, and its bioactive compounds can activate the Lep-R and β3AR to regulate energy balance. HSE could be a potential therapeutic target for obesity.

INTRODUCTION

Globally, obesity affects more than 600 million of the total adult population. Obesity is caused by a chronic energy imbalance between food intake and energy expenditure that results in fat accumulation (1). Energy homoestasis is tightly regulated by the central nervous system, especially the hypothalamus's arcuate nucleus (ARC). ARC contains two opposing neuronal populations, each characterised by two specific neuropeptides. One of those populations is the appetite-suppressing proopiomelanocortin (POMC) neurons which provide a potent anorexigenic effect; secretion of the POMC neuropeptides from these neurons decreases food intake. By contrast, the second population is the appetite-stimulating agouti-related peptide neurons (AgRP) dan neuropeptide Y (NPY) neurons with a potent orexigenic effect; the release of AgRP/NPY increases food intake. Thus, in a situation of negative energy balance, the expression of AgRP/NPY is increased. On the contrary, AgRP/NPY levels are diminished during an energy surplus, and POMC levels are increased (2).

ARC is close to the median eminence (ME), with many fenestrated capillaries that create 'leaky' in the blood-brain barrier. The ME facilitates the transport of peripheral hormonal and nutritional metabolic signals from the circulation (3). The peripheral signal that is critical for energy homeostasis is leptin. Adipocytes produce leptin, and leptin receptors (Lep-R) are expressed in the ARC. Leptin inhibits food intake, promotes energy expenditure by acting on POMC, and inhibits NPY/AgRP neurons. Furthermore, leptin acts on ARC to promote non-shivering thermogenesis through a browning process to increase energy expenditure through increased sympathetic nerve activity (SNA) in adipose tissue (4). Increased sympathetic tone to adipose tissue increases the release of norepinephrine, which subsequently binds to and activates the β3-adrenergic receptor (β3AR), increasing expression of thermogenic protein uncoupling protein-1 (UCP-1) and its transcription factor (5).

Most people with obesity exhibit dramatically elevated circulating leptin levels resulting from decreased leptin sensitivity in the hypothalamus or leptin resistance. Therefore, the anorexigenic effect of leptin is agitated. One of the known mechanisms that cause leptin resistance is Lep-R down-regulation in the ARC (6). Leptin resistance also decreases sympathetic tone to adipose tissue, thus inhibiting the browning process (7). The uses of natural products for treating obesity are more considered due to the negative side effect of medical drugs. Moreover, the effect is short-term. *Hibiscus sabdariffa* Linn. (*H. sabdariffa*), commonly known as roselle, has been used for a long time as traditional medicine. *H. sabdariffa* has anti-obesity effects through its active compounds, such as flavonoids, quercetin, polyphenols, and anthocyanins. Studies have shown that some polyphenols can improve leptin resistance by decreasing leptin levels, increasing leptin transport across the blood-brain barrier, and increasing metabolic activity, including inducing the browning of white fat. Furthermore, quercetin can upregulate UCP1 in adipose tissue through sympathetic stimulation.

Our previous study has shown that *Hibiscus sabdariffa* Linn. extract (HSE) reduces fibroblast growth factor-21 (FGF21) resistance through the increase in FGF21 receptor (FGFR1) expression. Moreover, HSE induced browning of white tissue through the increase in expression of UCP1 and its transcription factor peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC1α) (8). This study aims to test the hypothesis that HSE would increase the arcuate nucleus Lep-R, white adipose tissue β3AR, and decrease the arcuate nucleus NPY mRNA expression in obese rats. Rats have long served as the preferred species for biomedical research animal models due to their anatomical, physiological, and genetic similarity to humans (9). A secondary purpose was to analyse the potency of *H. sabdariffa* bioactive compounds as an activator of Lep-R and β3AR.

METHODS

Experimental design

This study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental study (10). This animal study was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia (number:1381/UN2.F1/ETIK/2020). The sample of this study was obtained from the previous study by Kartinah et al. (8). The experimental procedure was done in the Animal Research Facility, Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia. Twenty-four (24) male *Sprague-Dawley*rats from the Animal Facility of Health Research Development, Ministry of Health, aged 6-10 weeks, weight 110-160 g, with a 12:12-hour dark-light cycle, were housed in three rats per cage and given free access to food and water.

Rats were first randomised into control and diet-induced obesity (DIO) groups. The control rats were given a standard diet (6,43% of fat, 23,60% of protein), while the DIO rats were given to high-fat diet (19,09% of fat, 24% of protein). The standard diet and high-fat diet were administered for 17 weeks. Rats were categorised as DIO if their Lee index value was [?] 310. The Lee index was measured after 17 weeks using the following formula (11):

$$\text{Lee index} = \frac{\sqrt[3]{\text{berat badan}}}{\text{panjang badan}} \; x \; 1000$$

The DIO rats were then randomised into three groups: DIO, DIO rats administered with HSE 200 mg/kg BW (DIO-Hib200), and DIO rats administered with HSE 400 mg/kg BW (DIOHib400). The standard diet for the control group and the high-fat diet for the DIO group were maintained until the study's end. Thus, the group was four: Control (C), DIO, DIO-Hib200, and DIO-Hib400. The total number of each group was six. The sample size was measured by the Federer formula.

H. sabdariffa plant was obtained from the centre of the biopharma studies, Bogor Agriculture University, and the extraction was carried out by the methanol maceration method. After the maceration, evaporation, and freeze-drying were carried out to evaporate the solvent and remove the vaporised methanol to prevent its toxicity. The extract used in this study is from the red calyx in the form of a paste. The administered HSE was given once a day orally using a syringe cannula injection for five weeks, and the control group was given 2 mL of distilled water. After five weeks, the Lee index was remeasured. Long-term use of methanol extract

does not have side effects. The methanol extract was found safe to the no-observed-adverse-effect-level (NOAEL) for rats' single-dose and repeated-dose toxicity tests (12).

Tissue collection

At the end of the study, rats were anaesthetised using the combination of 0,01 mL/kg BW xylazine hydrochloride and 0,05 mL/kg BW ketamine by intraperitoneal injection. Subcutaneous white adipose tissue (WAT) and brain were dissected, weighed, and frozen at -80°C until analysed. The arcuate nucleus was isolated with a reference based on research by Salehi et al. (13).

Gene expression analysis

Arcuate nucleus Lep-R and WAT β3AR mRNA expression were measured using quantitative real-time polymerase chain reaction (qPCR). RNA was extracted from frozen tissue using Zymo Research Quick-RNA Miniprep Plus Kit, reverse transcribed using Toyobo ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit, and quantified by quantitative polymerase chain reaction (PCR) using Bioline SensiFAST SYBR Hi-ROX Kit and measured on an Applied Biosystem 7500. Results were quantified using the 2-Ct method and shown as arbitrary units relative to the control group.

Enzyme-linked immunosorbent assay analysis

The arcuate nucleus NPY concentration was measured by Rat NPY ELISA Kit (Bioenzy) with the sandwich method according to the manufacturer's protocol. ELISA reader measured the colour intensity at 450 nm wavelength.

Molecular docking studies

The docking studies were carried out in AMD Ryzen 7 4800U installed in Lenovo Ideapad Slim 5 workstation. Molecular docking was performed for thirty-nine *H. sabdariffa* bioactive compounds from a study by Herranz-Lopez et al. (14) in the active site of Lep-R (PDB ID: 3V6O) and β3AR using AutodockTools-1.5.6 software. Each compound's simplified molecular-input line-entry system (SMILES) was identified from PubChem, National Library of Medicine, USA. The crystalline structure of β3AR was constructed by homology model using CPHmodels with protein sequence downloaded from Uniprot (P13945) and validated using Ramachandran Plot. The two and three-dimensional (2D and 3D) visualisation was performed using Discovery Studio and Molecular Operating Environment (MOE). The interaction of ligan-protein binding was analysed according to the score of free binding energy (ΔG), inhibition constanta (Ki), and the number of hydrogen bonds.

Statistical methods

Data are presented as the mean (SD). SPSS version 22 and GraphPrism version 8 were used for analyses. Shapiro Wilk and Levene tests were used for normality and homogeneity test. P >0,05 was considered normal and homogenous. One-way ANOVA and post-hoc LSD test were used to compare arcuate nucleus Lep-R and NPY mRNA expression among the four groups, Kruskal-Wallis and post-hoc Mann-Whitney U test were used to compare WAT β3AR mRNA expression among the four groups, and Two-way ANOVA was used for Lee index before versus after HSE treatment.P <0,05 was considered significant.

RESULTS

HSE treatment increases the expression of arcuate nucleus Lep-R mRNA levels in obese rats

In order to test the hypothesis that the administration of HSE would increase the arcuate nucleus Lep-R levels, we treated the DIO male Sprague-Dawley rats with HFD and HSE for five weeks. After five weeks, the arcuate nucleus Lep-R mRNA levels were determined among all four groups (Figure 1). The Lep-R mRNA expression in the DIO group was lower than in the control group. The administration of HSE at a dose of 200 did not significantly increase Lep-R mRNA expression. However, the administration of HSE at a dose of 400 mg/kg BW significantly increased the arcuate nucleus Lep-R mRNA expression 0,5-fold compared to the DIO group (P <0,05).

No υπρεγυλατιον οφ αρςυατε νυςλευς NΠΨ ανδ ΩAT β3AP μPNA ιν οβεσιτψ ρατς

After five weeks of HSE treatment, the arcuate nucleus NPY and WAT β3AR mRNA levels were determined (Figure 2 and Figure 3, respectively). The arcuate nucleus NPY and β3AR mRNA expression in the DIO group was lower than in the control group. There are no significant differences in arcuate nucleus NPY and WAT β3AR mRNA expression among the four groups after HSE administration. However, there is an increase of β3AR mRNA expression in the DIO-Hib400 group, almost close to the control group.

HSE treatment ameliorates the Lee index to the normal range

The mean baseline of the rat's body weigtht is described in the supplementary file Table 1. The induction of a high-fat diet has significantly made rats obese. It has been proven that mean of the Lee index is [?]310 (Figure 4). After five weeks of the HSE treatment, the mean of the Lee index of the DIO-Hib 200 and DIO-Hib 400 group was significantly decreased (p <0,001 and$p$ <0,01 respectively) into the normal range [?]310 compared to the DIO group (Figure 5). These results mean that the rats are no longer obese.

H. sabdariffa βιοαςτιε ςομπουνδς πρεδιςτεδ το αςτ ας αν αςτιατορ ον Λεπ-P ανδ β3AP

The *in silico* prediction was analysed by molecular docking. The Ramachandran Plot validation for β3AR crystalline structure is shown in Figure 5. Model validation highlighted 92,901% residues in the highly preferred observation region (green), 5,247% residues in the preferred observation region (orange), and 1,852% residues in the questionable observation region (red). The docking results consisting of ΔG score, inhibition constanta, and hydrogen bond results are described in Table 2 for Lep-R and Table 3 for β3AR. The ΔG scores provide a docking energy complex. Among screened 39 *H. sabdariffa* bioactive compounds,*5-O-caffeoyl shikimic acid* has the highest ΔG scores (-8,63) for Lep-R, and *myricetin_3_arabinogalactoside* has the highest ΔG score (-9,39) for β3AR. The 2D visualisation of *5-O-caffeoyl shikimic acid* and Lep-R, and *myricetin_3_-arabinogalactoside*and β3AR docking complex to analyse the amino acid and hydrogen bond interaction in the receptor active site shown in figure 6 and figure 7, respectively. The National Centre of Biotechnology Information (NCBI) database was used to analyse the amino acid residues and hydrogen bond interaction. The result is that the amino acid residues and hydrogen bond interaction are in the transmembrane region. This interaction may lead to the formation of aggregates that can alter and influence the biological activity of receptors. Thus, these ligand-receptor interactions can activate and induce the receptor signalling pathway. The 3D visualisation is shown in Figure 8.

Discussion

Leptin is found in adipocytes and controls appetite and body weight (15). The role of leptin in controlling energy balance is evidenced by leptin replacement which results in decreased appetite and increased energy expenditure in leptin deficiency subjects who experience hyperphagia and obesity (16). Leptin signalling is directly targeted in the ARC and mediated by Lep-R (15). Depolarisation of POMC neurons in ARC by Lep-R activation sends projections that reach the nucleus tract solitary (NTS) in the brainstem to decrease food intake and inhibit GABAergic tone to the rostral raphe pallidus (rRPa) to increase energy expenditure (16). However, leptin deficiency is rare in obesity; instead, most people with obesity exhibit hyperleptinemia due to the inability of Lep-R-expressing neurons in the hypothalamus to detect leptin. This ability can occur due to the downregulation of Lep-R expression in the ARC (17,18).

By real-time PCR, we have found that Lep-R expression was lower in the DIO group than in the control group (Figure 1). It is consistent with a study by Martin et al. that showed a decrease in Lep-R expression was found in experimental animal models with hyperleptinemia and obesity. Administration of high doses of leptin to mice resulted in the downregulation of Lep-R expression in the hypothalamus (17). Another study by Liu et al. (19) and Zhai et al. (20) also showed that high-fat DIO decreased Lep-R expression. The administration of HSE at 400 mg/kg BW in this study showed a significant increase in Lep-R expression than in the DIO group (Figure 1). In the normal state, Lep-R expression increases in the fasting state and goes down in the feeding state. However, in obesity, this increase is inhibited (20).

Decreased Lep-R expression in high-fat DIO has been reported to result from the activation of matrix

metalloproteinase-2 (MMP-2) and subsequent cleavage of the extracellular domain of the Lep-R. MMP is an endopeptidase involved in the proteolysis of affinity proteins, whose activity disrupts various biological functions. Activation of this protease is reported to be due to systemic or central inflammation in the hypothalamus in obesity. Activation of inflammation mediators has been reported to increase MMP-2 expression. Mazor et al. showed that rats fed a high-fat diet experienced increased hypothalamic MMP-2 activity. This activation of MMP-2 induces cleavage of the extracellular domain of the leptin receptor, thereby inhibiting the anorexigenic effect of leptin. Deleting hypothalamic MMP-2 has been shown to restore Lep-R expression and reduce circulating leptin levels in animal models of obesity (21).

A study by Kumar et al. (22) reported that several classes of active compounds were known to directly inhibit MMP or reduce MMP expression. One of the most common groups is flavonoids and polyphenols (23). Quercetin and amentoflavone were found to inhibit MMP or reduce MMP expression (22) directly. Consistent with that study, *H. sabdariffa* in modulating the increase in Lep-R expression is thought to occur through inhibition of MMP-2 by high flavonoid and polyphenol content, i.e., quercetin and anthocyanin, especially in the calyx (24). Further study is needed regarding *H. sabdariffa* on MMP-2 expression in the ARC.

Molecular docking is an approach used to predict the interaction between a small compound/ligand with a protein using computational methods, thus possibly characterising the ligand's properties at the binding site of the target protein. The molecular docking process consists of 2 main steps; predicting the conformation of the ligand and its position and direction on the protein's active site and assessing the binding affinity formed between the ligand and protein. The higher the value(G), the weaker the interaction. The low binding value (negative) makes the receptor-compound interaction stable because of the small free energy of the complex (25). According to the docking result, the highest affinity prediction for Lep-R is shown by *5-O-Caffeoylshikimc acid* (ΔG -8,63). Based on the 2D visualisation (Figure 6), the binding conformation forms hydrogen bond interaction in the Lep-R active site at the amino acid residues Ile482, His467, and Arg468. According to the NCBI database, this amino acid residue is at transmembrane regions. These results indicate that this compound can act as a Lep-R activator.*H. sabdariffa* is thought to activate the leptin receptor signalling pathway through this binding.

The study of adaptive thermogenesis is widely developed to increase energy expenditure; one of them is through the browning process. Only the brown adipose tissue (BAT) has thermogenic activity in average conditions. However, in adults, the number of BAT is decreased. The browning process changes the WAT phenotype to a beige adipocyte's BAT-like phenotype. The beige adipocyte has a high thermogenic capacity, thus increasing energy expenditure. The browning process is stimulated by sympathetic stimulation (26). In addition to decreasing appetite, Lep-R signalling in the ARC increases sympathetic stimulation to WAT (27). Increased sympathetic tone to WAT induces increased mobilisation of fatty acids, contributes to the development of beige adipocytes, and significantly increases the concentration of norepinephrine (NE) which then binds and activates β3AR. It is consistent with the study by Dodd et al. (28) that showed sympathetic denervation of WAT decreased the browning process.

This study showed that compared to the control group, the DIO group had decreased mRNA expression of the β3AR. It is consistent with the study by Collins et al. (29), Rayner (30), and Valentine et al. (31), which showed that in high-fat DIO, there is a downregulation of β3AR in WAT and decreased responsiveness to sympathetic stimulation. The decrease in β3AR expression was due to increased inflammatory cytokines in adipocytes in obesity. Acute inflammation can be catabolic and is associated with increased lipolysis. However, long-term high-fat diet-induced chronic inflammation is associated with decreased expression of genes involved in thermogenic pathways such as β3AR (31). Decreased expression and sensitivity of β3AR in obesity have also been reported due to decreased fatty acid mobilisation and decreased leptin sensitivity to increase sympathetic stimulation (32).

The administration of HSE did not significantly increase the expression of β3AR among all the groups. Consistent with this, a study by Jasper et al. (33) shows that cold induction in β3AR KO mice did not increase the expression of β3AR mRNA in white adipocytes, though the thermogenic protein UCP1 is

increased. This result indicates that the β3AR signalling pathway for the browning of white fat can be dispensable. Several non-adrenergic signalling molecules such as adenosine, cardiac natriuretic peptides, irisin (34), and FGF21 (8) have been reported can induce the browning of white fat (33). However, this compound can act as a Lep-R activator. *H. sabdariffa* is thought to activate the β3AR signalling pathway through this binding.

The *myricetin_3_arabinogalactoside* is a flavonoid compound (14). Kuppusamy and Das (35) show that flavonoids can act synergically with NE on adipose tissue β3AR to induce lipolysis. Flavonoids have also been known to induce browning by increasing the expression of the thermogenic gene UCP1, increasing energy expenditure, and inhibiting the development of high-fat DIO, which is expected to reduce body weight (36). Faria et al. (37) also proved that flavonoids could cross the blood-brain barrier (BBB) and access the central nervous system. Thus, although it does not have the potency to increase the β3AR expression,*H. sabdariffa* is thought to have the potency to bind and activate β3AR to induce the browning of white fat.

The results of increased expression and signalling of Lep-R in the ARC and activation of WAT β3AR by *H. sabdariffa* are expected to promote weight loss. All the possible mechanism is shown in Figure 8. This study proved that administration of HSE for five weeks on high-fat DIO rats was able to significantly reduce the means of Lee index at a dose of 200 mg/kg BW (P <0,001) and also at a dose of 400 mg/kg BW (P <0,01). This decrease is related to the control of leptin resistance to inhibit food intake and increased sympathetic stimulation to WAT. At the end of the study, the obese rats are no longer obese. It is consistent with the study by Marhuenda et al. (38), Herranz-Lopez et al. (14), Boix-Castejon et al. (39), and Chang et al. (40) that showed the administration of *H. sabdariffa* was able to reduce body weight, body mass index, and fat mass.

Conclusion

Our *in vivo* and *in silico* study identifies the potency of H. *sabdariffa* to regulate energy balance in obesity by increasing leptin sensitivity in the ARC through increased Lep-R mRNA expression and activating Lep-R signalling. Moreover, H. *sabdariffa*bioactive compound synergises with WAT β3AR. Together, these two effects are expected to reduce food intake, increase sympathetic tone to WAT, and induce the browning of white fat to increase energy expenditure in high-fat DIO rats. We acknowledge this studies are limited as the mRNA expression and limitation of the H. *sabdariffa* bioactive compound database could be more. The ARC Lep-R protein expression and the increase in blood norepinephrine need to be identified to explore further.

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Conflict of Interest: The authors declare that no competing financial interest or personal relationship could have appeared to influence the work reported in this paper.

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List of tables:

Table 1. Mean baseline of rat's body weight

Group	**Mean body weight (g)**
Control	**276.2**
DIO	**363.4**
DIO-Hib200	**303.6**
DIO-Hib400	**340.6**

Table 2. Docking score of *H. sabdariffa* and Lep-R

Compound	**ΔΓ (κcαλ/μoλ)**	**pK_i**
1-O-caffeoylquinic acid	-6.61	14.22 uM
Methyl digallate-meta	-7.95	1.5 uM
Chlorogenic acid	-7.24	4.9 uM
Chlorogenic acid quinone	-8.17	1.02 uM
Ethylchlorogenate	-8.25	889.76 nM
5-O-Caffeoylshikimic acid	**-8.63**	**472.71 nM**
Cryptochlorogenic acid	-4.8	303.12 uM
Coumaroylquinic acid	-6.41	19.93 uM
Caffeoylglucose	-3.32	3.7 mM
N-Feruloyltyramine	-6.11	33.15 uM
Neochlorogenic acid	-7.97	1.44 uM
Hibiscus acid dimethylester	-4.78	314.34 uM
2-O-trans-Feruloyl hydroxicitric acid	-8.44	652.15 nM
Hibiscus acid	-3.78	1.7 mM
Hibiscus acid hydroxyethylesther	-5.74	62.5 uM
Hydroxycitric acid	-3.75	1.78 mM
2-O-caffeoylhydroxycitric acid	-7.74	2.14 uM
Myricetin-3-arabinogalactose	-7.79	1.94 uM
Kaempferol-3-O-rutinoside	-4.84	281.66 uM
Cyanidin-3-sambuboside	-3.37	3.41 mM
Kaempferol-3-O-sambuboside	-3.93	1.31 mM
Prodelphinidin-B3	-4.48	522.5 uM
Delphinidin-3-sambubioside	-3.91	1.35 mM
Quercetin-3-rutinoside	-3.91	1.37
Delphinidin-3-O-beta-D-sambubioside	-4.11	965.55 uM
Quercetin-3-glucoside	-5.18	160.87 uM
Quercetin	-6.63	13.78 uM
Methyl-epigallocatechin	-7.34	4.19 uM
Myricetin-3-glucoside	-4.59	429.46 uM
Leucoside	-2.92	7.26 mM
Quercetin 3-O-glucuronide	-6.41	19.93 uM
3-Methylquercetin	-7.64	2.51 uM
Myricetin	-6.64	13.63 uM
Quercetin-3-sambubioside	-3.67	2.05 mM
Kaempferol-3-glucuronide	-5.86	50.5 uM
Kaempferol	-6.65	13.35 uM
Tetra-O-methyljeediflavanone	-7.2	5.26 uM
Quercetin 3.7-diglucuronide	-4.51	491.23 uM
Methyl digallate-para	-6.91	8.6 uM
Setmelanotide (positive control)	-3.52	2.61 mM

Bold: The lowest ligand-protein binding energy

Table 3. Docking score of *H. sabdariffa* and β3AR

Compound	**ΔΓ (κcαλ/μoλ)**	**pK_i**
Tetra_O_methyljeediflavanone	-8.78	368.72 nM
Kaempferol_3_O_sambubioside	-4.5	500.89 uM
5_O_Caffeoylshikimic acid	-5.73	62.9 uM
Quercetin 3_sambubioside	-4.82	293.06 uM
Quercetin 3_rutinoside	-4.09	1.0 mM
Quercetin 3_O_glucuronide	-6.31	23.81 uM
Quercetin 3_7_diglucuronide	-5.38	113.11 um
Prodelphinidin B3	-6.85	9.57 Um
Neochlorogenic acid	-5.97	41.78 Um
Myricetin_3_glucoside	-6.37	21.5 uM
Myricetin_3_arabinogalactoside	**-9.39**	**137.84 uM**
Myricetin	-6.95	8.03 uM
Moupinamide	-6.58	15.05 uM
methyl_epigallocatechin	-8.34	774.71 nM
Methyl digallate-meta	-7.95	1.49 uM
Methyl digallate-para	-8.21	966.53 nM
Leucoside	-5.03	204.89 uM
Kaempferol-3-O-rutinoside	-6.68	12.69 uM
Caffeoylglucose	-3.87	1.45 mM
1-O-Caffeoylquinic acid	-6.25	26.02 uM
Chlorogenic acid	-5.96	42.69 uM
Chlorogenic acid quinone	-5.9	47.32 uM
Cryptochlorogenic acid	-5.39	111.97 uM
Cyanidine 3_sambubioside	-4.68	373.44 uM
Delphinidin 3_O_beta_D_samubioside	-4.22	800.54 uM
Delphinidine 3_sambubioside	-4.77	317.66 uM
Ethylchlorogenate	-8.9	300.47 nM
Hibiscus acid	-3.27	4.02 mM
Hibiscus acid dimethylester	-3.98	1.21 mM
Hydroxycitric acid	-3.18	4.65 mM
Kaempferol	-7.05	6.78 uM
Kaempferol_3_glucuronide	-6.87	9.23 uM
Quercetin_3_glucoside	-6.67	12.85 uM
Coumaroylquinic acid	-6.4	20.34 uM
3_Methylquercetin	-6.43	19.19 uM
2-O-trans-Feruloylhydroxitric acid	-6.62	13.95 uM
2-O-caffeoylhydroxycitric acid	-6.79	9.48 uM
Hibiscus acid hydroxyethylesther	-4.86	275.48 uM
Quercetin	-6.43	19.36 uM
Mirabegron (positive control)	-7.22	5.06 uM

Bold: The lowest ligand-protein binding energy

List of figure:

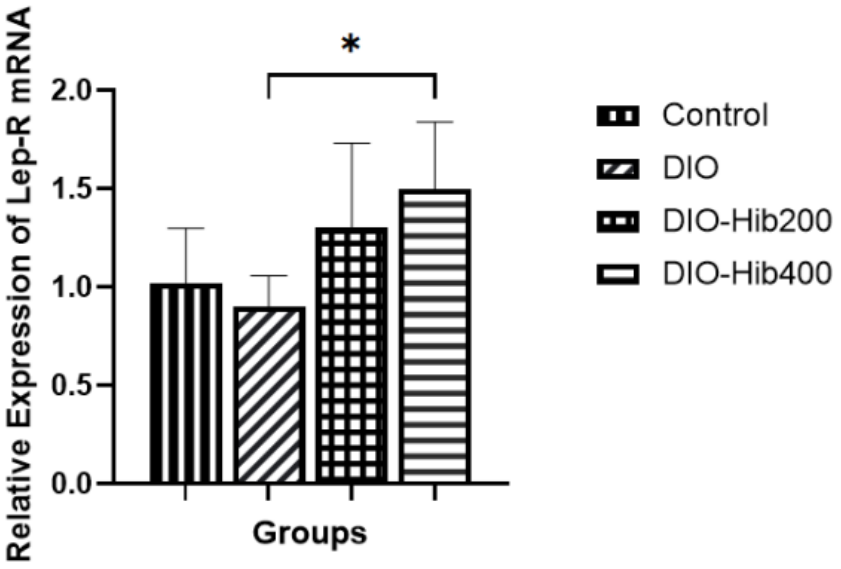


Figure 1. Average Lep-R expression. Data are depicted as mean ± SD. Significant differences are shown as $*p<0.05$.

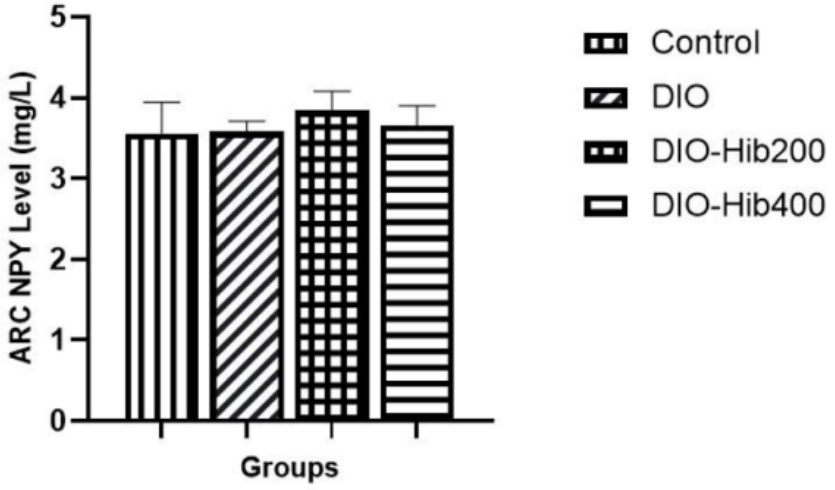


Figure 2. Average levels of NPY (mg/L). Data are depicted as mean ± SD. No significant differences are shown ($p>0.05$).

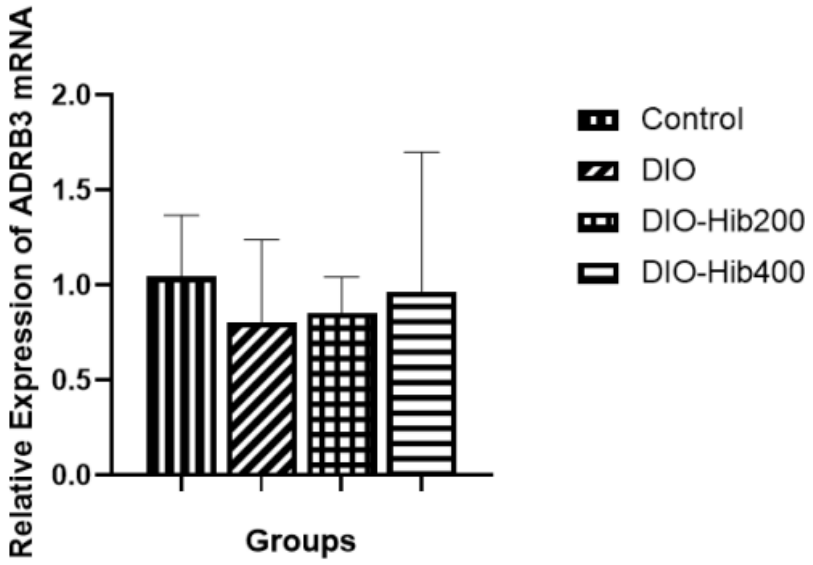


Figure 3. Average β3AR expression. Data are depicted as mean ± SD. No significant differences are shown ($p>0.05$).

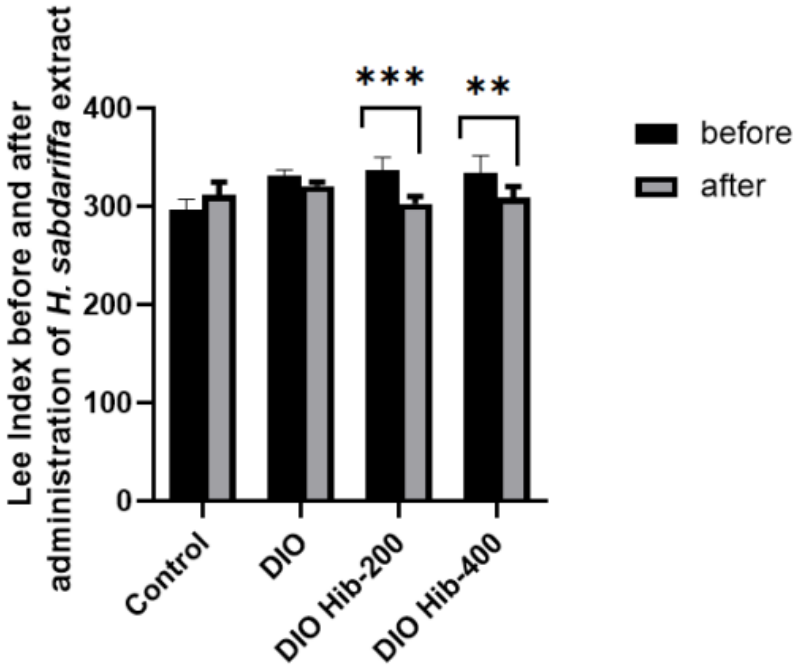


Figure 4. Average Lee index score pre- and post- HSE administration. Data are depicted as mean ± SD.

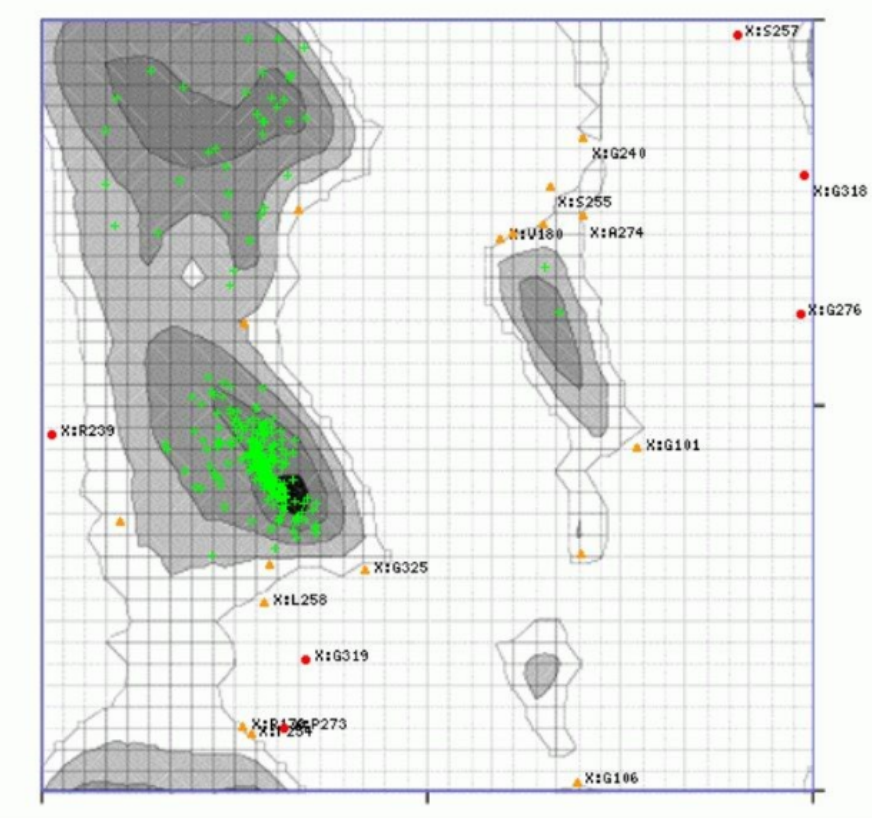


Figure 5. Ramachandran plot of modeled human β3AR. The Ramachandran plot statistics – Model validation highlighted 92.9% (301) residues in most favored regions (green), 5.3% residues in additional allowed regions (orange), and 1.9% residues in disallowed regions (red). The Ramchandran plot is used for model validation.

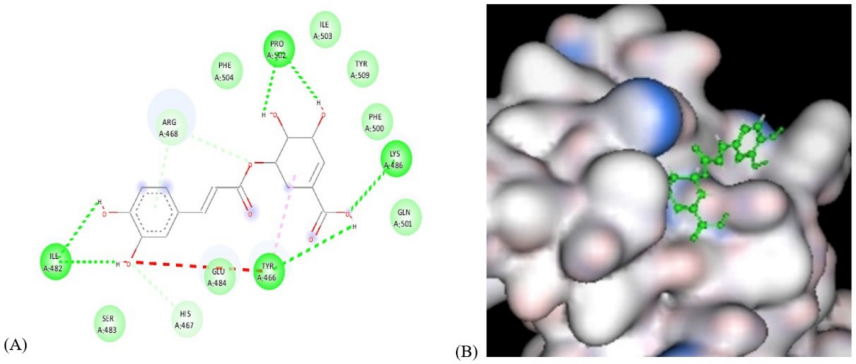


Figure 6. Docking complex of *5-O-Caffeoylshikimic acid* on crystal structure of Lep-R. The figure shows predicted docking site of*5-O-Caffeoylshikimic acid* on Lep-R. (A) 2D docking pattern. (B) 3D view.

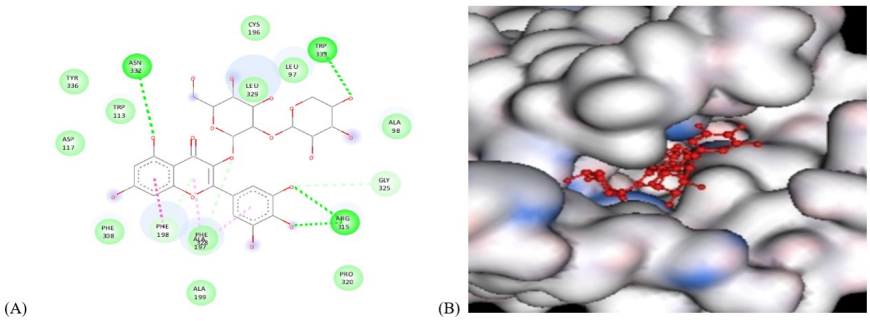


Figure 7. Docking complex of *Myricetin_3_arabinogalactoside* on crystal structure of β3AR. The figure shows predicted docking site of*Myricetin_3_arabinogalactoside* on β3AR. (A) 2D docking pattern. (B) 3D view.

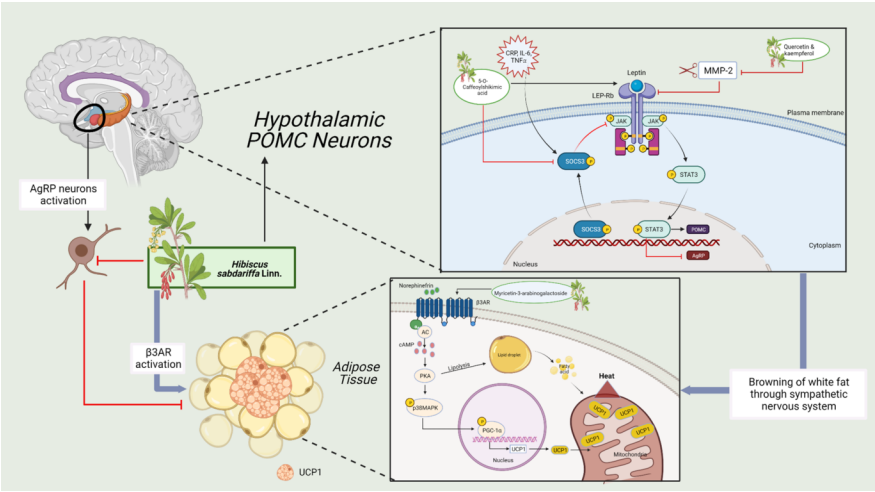
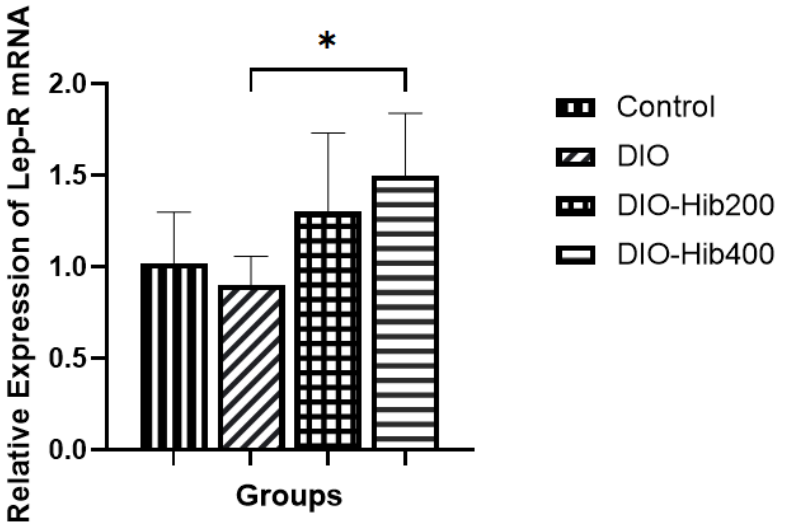
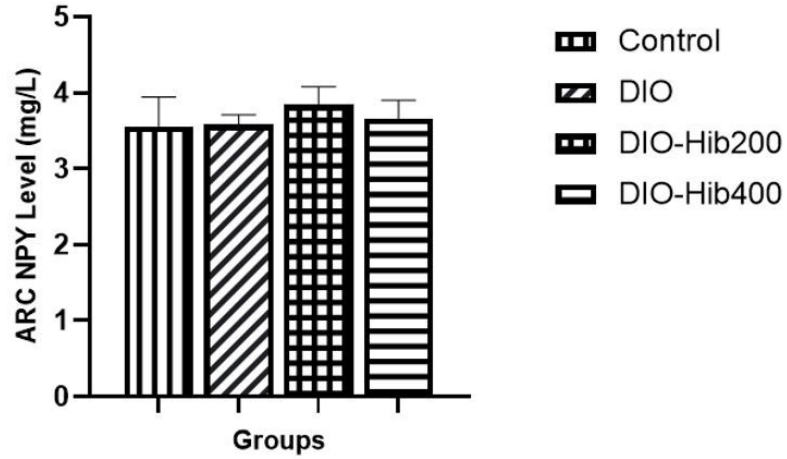
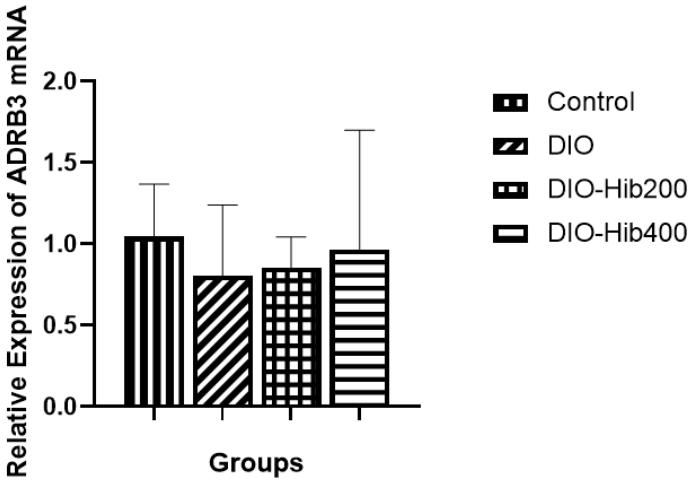
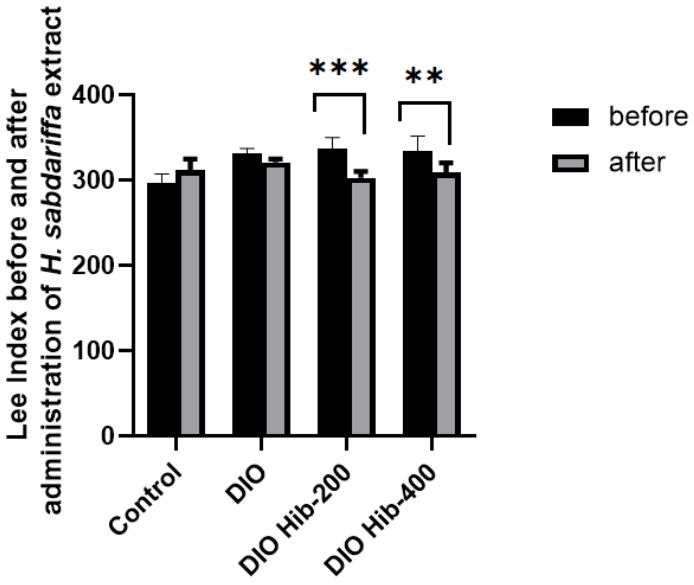


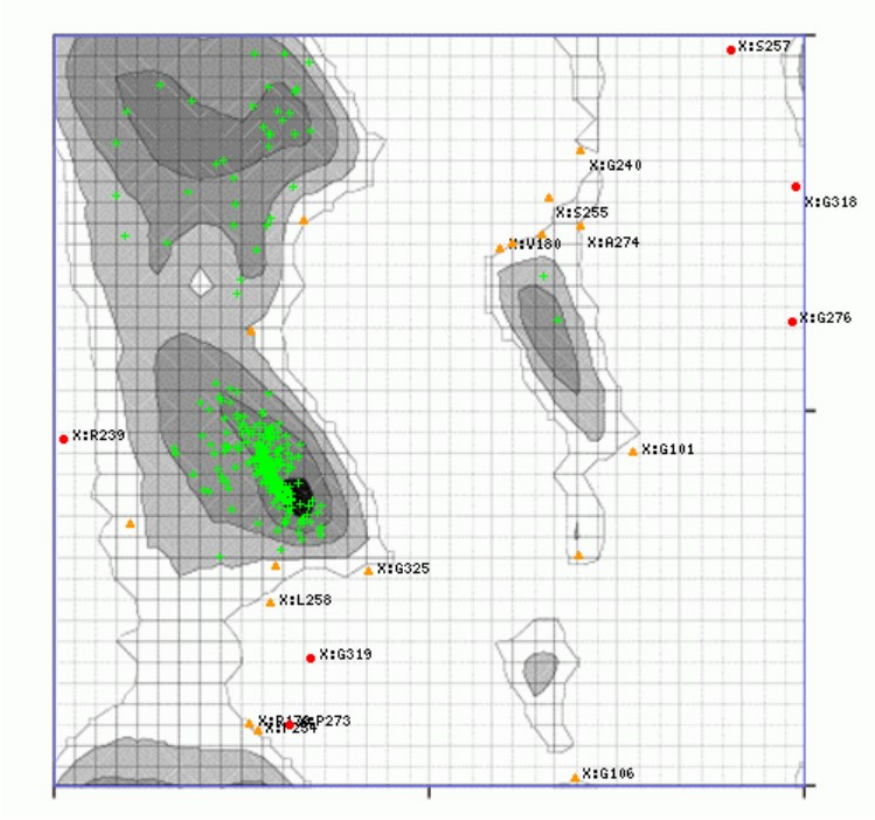
Figure 8. H. *sabdariffa* regulates energy balance through arcuate nucleus and promotes WAT browning. H. *sabdariffa* increases ARC Lep-R mRNA expression and enhances Lep-R activation, transcribing anorexigenic neuropeptides. Once leptin signaling has been increased in POMC neurons, the sympathetic nervous system increases the WAT browning process. Furthermore, *H. sabdariffa* is predicted can enhance β3AR activation to directly increase WAT browning process and scale up the energy expenditure. (Created with BioRender.com)

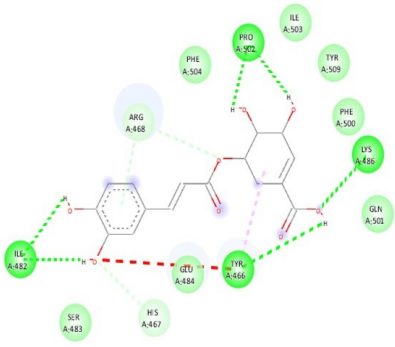












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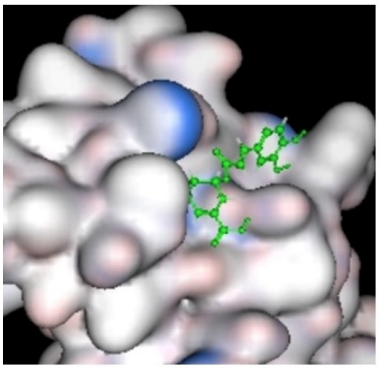

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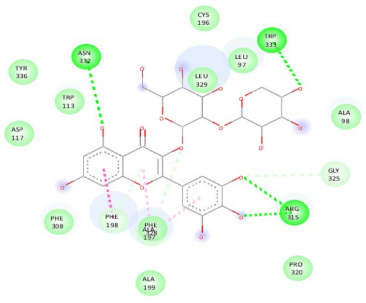

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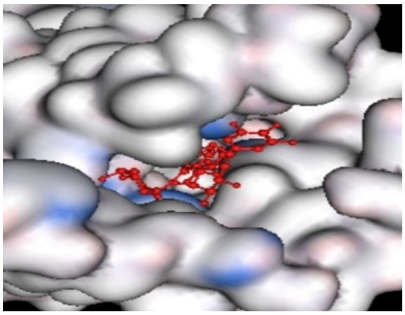

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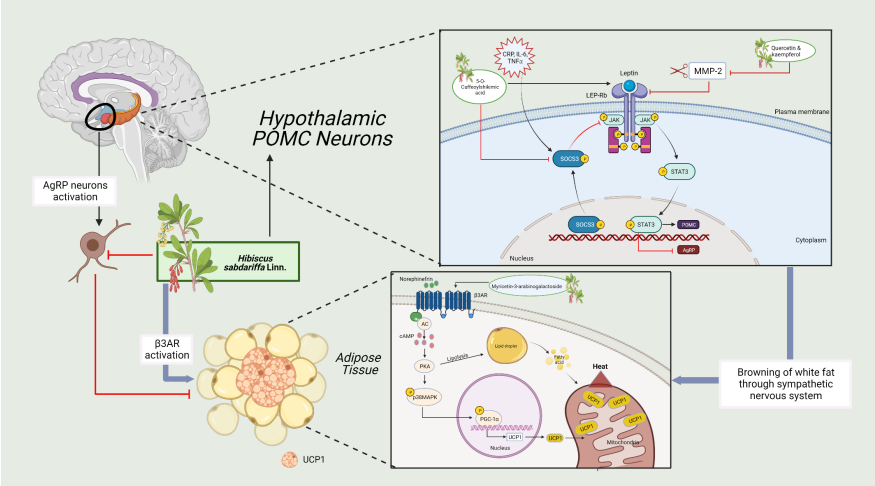

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