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Anti Obesity Activity At Extract Of Medicinal Plants With Anti Oxidant Effects

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ABSTRACT
The present study describes the screening of eight herbs namely Cosmos caudatus, Pluchea indica, Lawsonia inermis, Carica papaya, Piper betle, Andrographis paniculata, Pereskia bleo, and Melicope lunu based on their antiobesity and antioxidant activities. Out of all tested herbs, Cosmos caudatus demonstrated excellent anti-obesity and antioxidant potential with pancreatic lipase inhibitory effect (21.7 ± 1.3%) and DPPH radical scavenging activity (IC50 value of 31.98 ± 1.22 µg/mL). Cosmos caudatus was selected for further studies and extracting solvent composition with best anti-obesity and antioxidant potential was identified. Hundred percent Cosmos caudatus ethanolic extract was found to be the most effective and showed highest anti-obesity and antioxidant activities. Moreover, metabolite profiling of Cosmos caudatus extract was also carried out using UHPLC-MS/MS. The analysis depicted the presence of quercetin-3-rhamnoside, catechin, kaempherol, kaempherol glucoside, quercetin, quercetin-3-glucoside, quercetin-O-pentoside, quercetinrhamnosyl galactoside, quinic acid, 1-caffeyolquinic acid, monogalloyl glucose, and procyanidin B1. Results revealed Cosmos caudatus as promising medicinal plant for the development of new functional food with prodigious applications in obesity.

I. INTRODUCTION

Obesity is a prevalent condition that is often brought on by the interplay of elements from the environment, diet, and genetics. It is currently one of the most significant health concerns facing contemporary societies everywhere. [1] It frequently co-occurs with other conditions such osteoarthritis, arteriosclerosis, hypertension, cancer, and diabetes. [2,3] There are already 500 million obese individuals globally, and the prevalence of obesity is rising tremendously. [4] A crucial enzyme, pancreatic lipase (PL), which is released by the pancreas, breaks down 50–70% of fat into monogylcerides and free fatty acids for entherocyte absorption. Fat buildup in adipose tissue is often reduced by inhibition of fat digestion and absorption. [5] Consequently, one of the main objectives for anti-obesity medication Inhibition of PL is thus one of the main objectives for anti-obesity drugs. [6] However, the triglyceride-rich lipoproteins, chylomicrons, and very low-density lipoproteins (VLDL) are hydrolyzed by the rate-limiting enzyme lipoprotein lipase (LPL), which results in the release of monoacylglycerol and non-esterified fatty acids (NEFA). These monoacylglycerol aeither used by the muscles for metabolic energy or re-esterified into TG and stored as neutral lipids in adipose tissue. Any imbalance in LPL activity affects the distribution of TG between muscle and

adipose tissue and thus influences obesity.[1,7] Consequently, compounds, which can inhibit activity of these lipases, are supposed to function as anti-obesity agents.[8] There have been extensive claims on the beneficial effects of various plant extracts especially based on their antioxidant properties.[9–12] It is well established that polyphenols found ubiquitously in the plant kingdom are effective antioxidant. They exhibit ability to scavenge free radicals through inhibition of some enzymes or by chelation of trace metals involved in the production of free radicals

and are also able to protect the antioxidant defense system.[13] Escalation in oxidative damage and oxidative stress markers are associated with several diseases such as obesity, diabetis, Alzheimer's diseases, other neurodegenerative diseases, cancer, and atherosclerosis.[14–16] It is important to note that any actions that can reduce oxidative stress would be therapeutically beneficial.[17–19] It is also important to relate the anti-obesity and antioxidant properties of these commonly consumed herbs. Obesity is associated with a state of excessive oxidative stress, which plays an important role in thepathogenesis of many diseases.[20,21] The side effects of some anti-obesity drugs and syntheticantioxidants have prompted scientists to search for safe and effective

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natural bioactive compounds that can target both anomalies. The present study was therefore aimed to evaluate the antioxidant and anti-obesity effects of Cosmos caudatus, Pluchea indica, Lawsonia inermis, Carica papaya, Piper betle, Andrographis paniculata, Pereskia bleo, and Melicope lunu in vitro and to determine the bioactive compounds (in the most active extract) that may be responsible for the bioactivities measured. The obtained information may help in seeking potential herb with excellent anti-obesity and antioxidant attributes and fatty acids .

II. MATERIALS AND METHODS

Plant materials and reparation of extracts Fresh leaves of Melicope lunu, Carica papaya, Pluchea indica, Lawsonia inermis, Pereskia bleo, Andrographis paniculata, Cosmos caudatus, and Piper betle were obtained from University

Agricultural Farm, identified by botanist from Faculty of Forestry, Universiti Putra Malaysia (UPM), Malaysia, and the voucher specimens were submitted in herbarium with vouture numbers H016, H017, H018, H019, H020, H021, H022, and H023, respectively. The leaf extracts of the plants were prepared using the modified method of Chang et al.[22] Fresh leaves were cut and washed under running tap water, followed by freeze drying for two days. The dried plant material was ground to powder and sieved for homogeneity. Absolute ethanol (100 mL) or aqueous ethanol with different ratios of ethanol and water (100:0, 80:20, 60:40, 50:50, and 40:60) was used as extracting solvent to

extract 10 grams of dried material for 24 hours at 40°C. The extracts were filtered, and solvent was evaporated off using rotary evaporator at 40°C. The resulting viscous extract was freeze-dried to ensure complete removal of water. Finally, the dried crude extract was diluted to required concentration for further experimental work.

In vitro pancreatic lipase (PL) inhibitory assay Plant extracts were prepared at different concentrations in 0.01 M Tris-HCL buffer. Porcine pancreatic lipase was dissolved in 0.01 M Tris-HCL buffer (25 units/mL). The substrate was prepared using the modified method of Fox and Stepaniak.[23] Briefly, olive oil (10% v/v) was mixed with Arabic gum mixture (10% w/v in 0.1 M Tris-HCL buffer, pH 8, 0.5 M NaCl, and 20 mM CaCl2) using a homogenizer. Inhibition of PL by plant extracts was determined using the method reported by Fukumoto et al. with some modifications.[24] Lipase solution (0.2 mL) was allowed to react with 0.5 mL of plant extract for 30 minutes at 4°C. Substrate emulsion (2 mL) was then added and incubated for 30 minutes at 37°C. One-mL acetone and ethanol (1:1) mixture was used to stop the reaction and titrated with 0.02 M NaOH until reached pH 9.4. Auto titrator

was used to perform the titrations (Metrohom, 785 DMP Titrino). The experiment was repeated thrice for each sample extract. The amount of free fatty acid (FFA) liberated was

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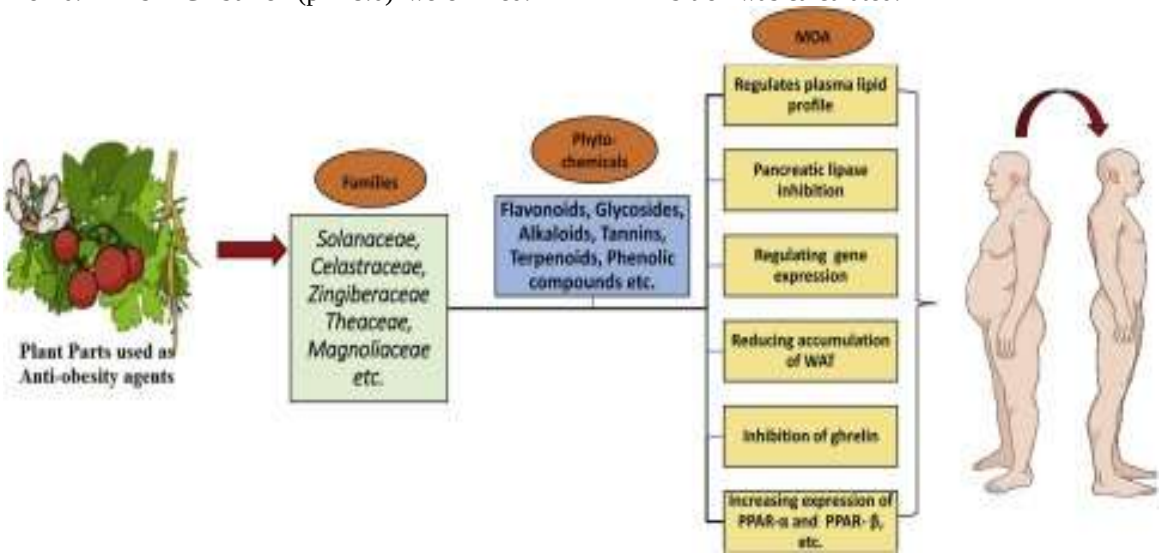
reflected by the amount of base required by the incubation mixture which is equivalent to PL activity. Control sample was equivalent to 100% enzyme activity. Percent inhibition was calculated based on the

following equation:
%inhibition ¼
100%___Vsample=Vcontrol__100_

where, Vsample is the amount of base added to sample, and Vcontrol is the amount of base added to
control.

In Vitro Lipoprotein Lipase (LPL) Inhibitory Assay :

A modified method by Schotz et al.[25] was used for the preparation of substrate. An activator consisted of Apo C-II from human plasma and diluted to 1 µg/mL with 0.002 M Tris HCl (pH 8.0) was prepared. In the preparation of substrate, 0.6 mL triolein, 24 mL apo C-II, 3.6 mL of 1% BSA solution, 3.6 mL of 1% triton X-100, and 28.8 mL of 0.2 Tris HCl buffer (pH 8.0) weremixed. The mixture was then sonicated in ice for 3 minutes. Enzyme LPL from bovine milk was prepared by diluting with 0.02 M Tris HCl (pH 8.0) to a concentration of 25 units/mL. The LPL activity was then determined using a method reported by Chung and Scanu.[26] Briefly, 0.5 mL of previously diluted LPL was added to 0.5 mL of extracts and epicatechin (100 ppm) in test tubes followed by pre-incubation at 4°C for 30 minutes. Then, 1 mL of substrate emulsion was added to the mixture of enzyme and extracts, followed by incubation in waterbath at 37°C, to initiatehydrolysis. The reaction was stopped with the addition of 1 mL of 1 M NaCl. Control samples were consisted of mixture of enzyme and substrate only. The liberated free fatty acids (FFA) were titrated with 0.01 M NaOH until pH 9.4 using autotitrator (Metrohom, 785 DMP Titrino). Themamount of liberated free fatty acid (FFA) was reflected by the amount of base required by the incubation mixture which is equivalent to LPL activity. Control sample was equivalent to 100% enzyme activity. The experiment was repeated thrice for each sample extract, and percent inhibition was calculated.



Total Phenolic Content (TPC) :

Total phenolic content of plant extracts was determined according to the method of Verza et al.[27] Folin Ciocalteu reagent (0.5 mL), plant extracts (0.5 mL), and 7% sodium carbonate (10 mL) were allowed to react at room temperature for 1 hour. Absorbance of the resulting blue color complex was noted at 725 nm (Shimadzu UV Visible Spectrophotometer, UV-1650 PC, Japan). The concentration levels used were in the range from 0.02 to 0.1 mg/mL. A standardcurve was plotted with gallic acid standard, and the phenolic content was expressed as mg gallic acid equivalent (GAE)/g extract.

Total Flavonoid Content (TFC) :

Total flavonoid content of plant extracts was determined spectrophotometrically according to the method adapted from Quettier-Deleu et al.[28] The method was based on the formation of a flavonoid–aluminum complex having the absorbance at 430 nm. Rutin was used as standard

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for calibration curve. Diluted samples (1 mL) were separately mixed with 1 mL of 2% methanolic aluminum chloride solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm with UV–Vis spectrophotometer and total flavonoids content was expressed as mg rutin equivalent (RE)/g extract. DPPH (2,2-diphenyl-2-picrylhydrazyl) radical scavenging assay Scavenging activity of samples was assessed according to the method described by Brand-Antioxidant activity was expressed as IC50 (defined by the concentration of samples required to scavenge 50% of the free radicals). All the experiments were performed in triplicate using ascorbic acid, BHA, and α-tocopherol as positive controls. Plant extract (250 µL) at different concentrations was added to 1.75 mL of 25 ppm DPPH in methanol. All test samples were prepared in 24-well plates. The mixture was left to stand for 30 minutes at room temperaturein the dark. Absorbance was then noted using spectrophotometer (Biotek EL800 MicroplateReader) at 517 nm. The readings were compared with those of the blanks, and the percent radical scavenging activity of samples was then calculated using the following equation followed by the
measurement of IC50 values:Radical Scavenging Activity
ð%Þ¼__ABlank_ASample_=ABlank__100

B-Carotene–Linoleate Bleaching Assay :

The antioxidant activity of plant extracts was also determined based on β-carotene bleaching method developed by] BHT was used as the standard, and all the tests were performed in triplicate. β-carotene (0.2 mg in 1 mL chloroform), linoleic acid (0.02 mL), and Tween 20 (0.2 mL) were transferred into a round-bottomed flask, and the mixture was added to 0.2 mL of plant extracts or standard or ethanol (as control). Chloroform was removed using a rotary evaporator. Following evaporation, 50 mL of distilled water was added to the mixture and shaked vigorously to form emulsion. Two-mL aliquots of the emulsion were pipetted into the test tubes and immediately placed in water bath at 50°C. The absorbance measurements were then taken at 20-minute intervals for 2 hours at 470 nm. Degradation rates (DR) were calculated according to first-order kinetics, using the
following equation: ln a=b ð Þ_l=t ¼ DRsample or DRstandardwhere a is the initial absorbance (470 nm) at time 0, b is the absorbance (470 nm) at 20, 40, 60, 80, 100, or 120 minutes, t is the time. Antioxidant activity (AA) was expressed as percent of inhibition

Relative to the control, using the following formula: AA ¼ ðDR control_DR sample or standard DR control_100

High-Performance Liquid Chromatography (HPLC) Analysis

The HPLC analysis of plant extract was carried out according to the protocol developed by Crozier et al. with some modifications.[31] The samples were prepared by dissolving 10 mg of crude ethanolic plant extracts in 1 mL of methanol. The resulting solution was then filtered prior to analysis. The standards were prepared by dissolving 1 mg of catechin, epicatechin, rutin, quercetin-3-rhamnoside, quercetin, myricetin, fisetin, hesperitin, naringin, and genistein in 1 mL of HPLC grade methanol. The sample and standards were analyzed using HPLC system (Waters Delta 600 with 600 Controller) with photodiode array detector (Waters 996). A Phenomenex-Luna (5 µm) PFP-2 (4.6 mm i.d. × 250 mm) column was used, and for elution of the constituents, two solvents denoted as A and B were employed. Solvent A was 0.1% formic acid in deionized water, and solvent B was acetonitrile. Gradient elution was performed as followed for solvent A: 0 min (95%), 12–20 min (75%), 22–30 min (85%), 35 min (95%). The flow rate used was 1 mL/min, and the injection volume was 10 µL. The detector was set at the range of 210–366 nm. The retention times, peak areas, and UV spectra of the major peaks were analyzed. Rutin, catechin, and quercetin-3-rhamnoside standards (20–140 µg/mL) were further used for quantification based on standard curve.

Liquid chromatography–mass spectroscopy (LC-MS/MS) analysis : Crude ethanolic plant extracts (5 mg) were suspended in 1 mL methanol and then filtered through a PTFE filter (pore size 0.22 µm). The analysis was carried out on a Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer (AB Sciex 3200QTrap LCMS/MS with Perkin Elmer FX 15 UHPLC system). LCMS analysis was carried out according to internal protocol developed by instrumentsmanufacturer (AB Sciex, Canada).Scan ranges from 100 to 1200 m/z for full scan and 50–1200 m/z for MS/MS scan. The chromatographic separation was performed on a Zorbax C18 column

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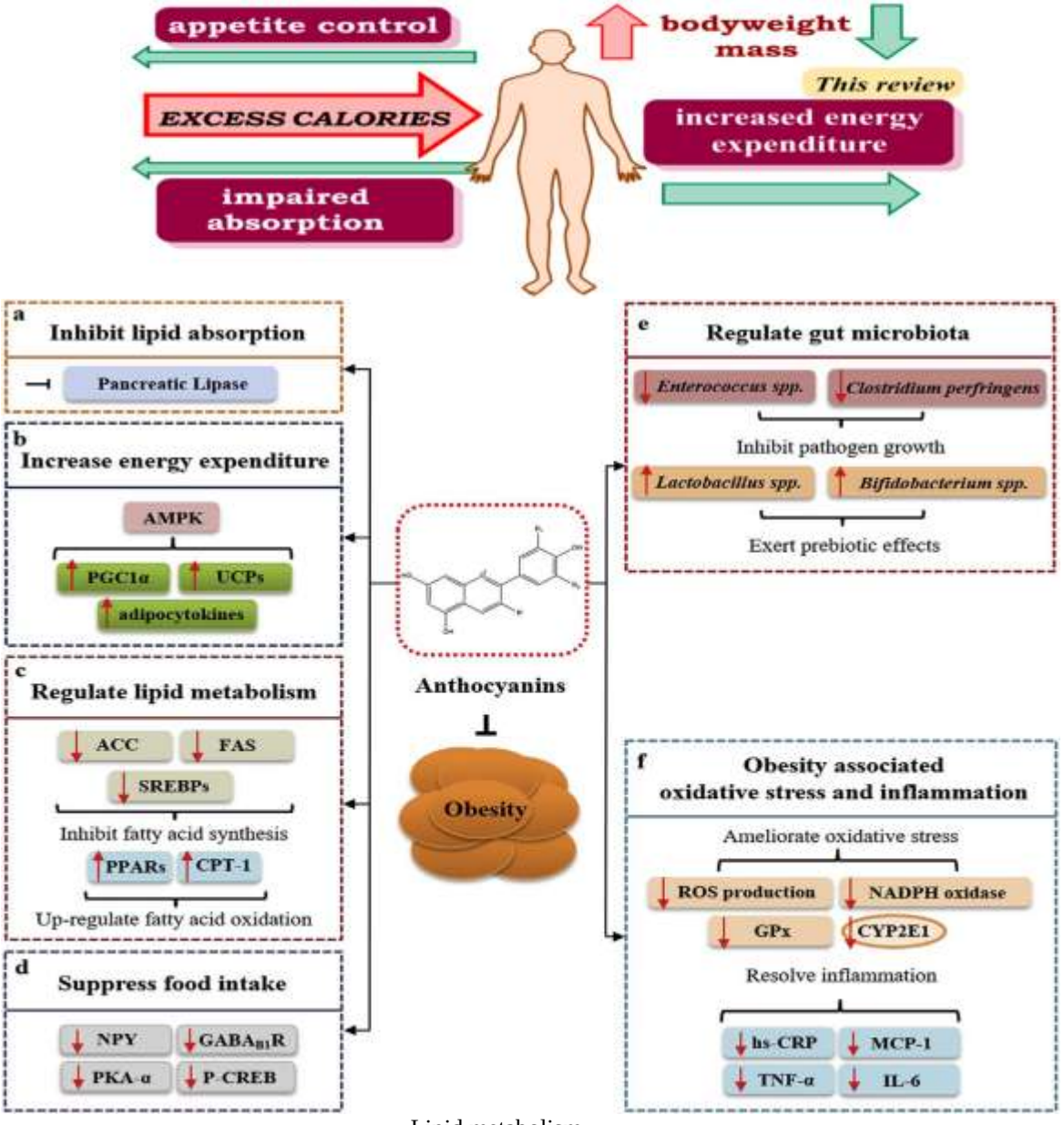
(150 mm × 4.6 mm × 5 Um) with a gradient mobile phase-comprising water (solvent A) and acetonitrile (solvent B) (each with 0.1% formic acid and 5 mM ammonium formate). The ionization mode used was negative. The gradient program started from 10% B to 90% B for a period of 0.01 min to 8.0 min, hold for 2 min and back to 10% B in 0.1min and re-equilibrated for 3 minutes with a flow rate of 800 µl/minute, and an injection volume of 20 µL was used. The UHPLCMS/MS system was equipped with Analyst 1.5.2 (a mass spectrometric software) and an ACD spectral library (ACD Labs, Toronto, ON, Canada). The resolved peaks were identified based on their accurate masses, molecular ion peaks, mass fragmentation patterns, comparison with ACD mass spectral library, literature data.

Statistical analysis

All the experimental data were expressed as mean ± standard deviation. Data were analyzed for oneway ANOVA using SPSS 20.0. Duncan's multiple-range test was used to assess difference between means. A significant difference was considered at the level of $p < 0.05$.



Lipid metabolism

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Fig. 1. Molecular Mechanisms Of The Anti-Obesity Effects Of Anthocyanins.A. Various Anthocyanins-Rich Extracts Are Potent PancreaticLipase Inhibitors. B. Anthocyanins Up-Regulate AMPK Expression. AMPK Activation Increase Expression Of PGC1α, Up-Regulated ExpressionOf Ucps And Increase Excretion Of Adipocytokines. C.Anthocyanins Inhibit Fatty Acid Synthesis Through Down-RegulatingExpression Of ACC, FAS And Srebps, As Well As Up-Regulating FattyAcid Oxidation Through Increasing Expression Of Ppars And CPT-1.D. Anthocyanins Reduce The Expression Of NPY, GABAB1R And DecreaseExpression Of PKA-A And P-CREB. E. Anthocyanins InhibitPathogen Growth, Such As Enterococcus Spp. And Clostridium Perfringens,As Well As Exerting Prebiotic Effects, Such As EnhancingGrowth Of Lactobacillus Spp. And Bifidobacterium Spp. F.

Anthocyanins Ameliorate Oxidative Stress By Reducing ROS Production,Inhibiting Expression Of NADPH Oxidase And Gpx, As Well . As Reducing Expression Of CYP2E1. Anthocyanins Could Also ResolveInflammation Through Decreasing Levels Of Hs-CRP, MCP-1, TNF-A.

Various researches have shown that anthocyanins could directlyaffect lipid metabolism. 150 mg Black soybean (BS) extract containing 10 mg cyanidin 3-glucoside per gram was administrated to high-fat fed (HFF) rats daily for 6 weeks. Compared with HFF rats, BS affected fatty acid composition in subcutaneous fat, such as several saturated, monounsaturated and n-6 polyunsaturated fatty acid. Such reduction in fatty acid contents may have implications in suppressing inflammation (Sato et al., 2015). Adzuki beans were suggested to be a health-beneficial food that contained anthocyanin, adzukisaponin and catechin. Intake of 10% and 20% adzuki bean with high-fat diet (HCD) for 10 weeks was reported to ameliorate serum and hepatic triglyceride levels in C57BL/6 male rice (Kim, Hong, Jeon, & Kim, 2016). Anthocyaninrich Aronia melanocarpa (AM) extract was reported to inhibit hepatic lipid accumulation through down-regulating PPARγ2 expression both in vitro and in vivo. C57BL/6N mice given high fat diet (HFD) and FL83 cells under treatment of free fatty acid (FFA) were used in the study (Park et al., 2017). Strawberry fraction enriched with anthocyanins was shown to have larger impact on decreasing LDL-cholesterol and triglycerides contents than untreated extract in human hepatocellular carcinoma (HepG2) cells (Forbes-Hernández et al., 2017). There are also adequate researches that report anthocyanins to play essential roles in regulating lipid metabolism instead of direct effects. BCAO decreased hepatic triglyceride, up-regulated gene expression in fatty acid oxidation, and down-regulated mRNA expression of FAS and SREBP-1 genes associated with fatty acid synthesis (Park et al., 2015). Rutgers scarlet lettuce extract (RSLE) contains 43.0 mg/g cyanidin 3-glucoside equivalents. RSLE treated groups at 100 or 300 mg/kg had a lower ratio of liver weight to body weight as well as decreased total liver lipids compared to control group after 28 days of treatment (Cheng et al., 2014). One study supplemented mice with purified cyaniding 3-glucoside or Queen Garnet plum juice (QG) that contains up to 275 mg/100 g fresh fruit (200 mg/100 mL cyanidin 3-glucoside, 30 mg/100 mL cyanidin 3-rutinoside). The result showed that cyanidin 3- glucoside and QG both reversed metabolic signs induced by high-fat diet (Bhaswant et al., 2015). Drinking LFWE decreased sizes of livers as well as perirenal and epididymal adipose tissues, and cell sizes of epididymal adipose tissues in test subjects (Wu et al., 2013a). Consumption of CBE at doses of 100 and 200 mg/kg body weight per day reduced epididymal fat and triacylglycerol. CBE also regulated gene expression in pathways involved in adipogenesis. Expression of genes Fabp4, Fas and Lpl were inhibited (Qin& Anderson, 2012). Cyanidin 3- glucoside is shown to regulate lipid metabolism in in vivo and in vitro studies. Cyanidin 3-glucoside rich purple corn color (PCC) consumed in 2 g/kg diet prevented body weight gain and ameliorated diet-induced adipocytes hypertrophy in mice. PCC blocked lipid accumulation, suppressed the expression of genes involved in fatty acid and triacylglycerol synthesis and lowered SREBP-1 mRNA level (Tsuda, Horio, Uchida, Aoki, & Osawa, 2003). Cyanidin 3-glucoside rich honeysuckle anthocyanins (HA) supplemented at 100 or 200 mg/kg suppressed body weight gain. HA reduced serum and liver lipid profiles (Wu et al., 2013b). Anthocyanins purified from various species are also reported to regulate lipid metabolism. Cornelian cherry anthocyanins (CA) consist of cyanidin 3-galactoside, pelargonidin 3-galactoside and delphinidin 3-galactoside. CA supplemented at 1 g/kg of diet induced 24% decrease ofbody weight gain and decreased liver lipid and triacylglycerol accumulation in mice (Jayaprakasam, Olson, Schutzki, Tai, & Nair, 2006). Sweet cherry anthocyanins (CACN) consist of cyanidin 3-(2G-

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glucosylrutinoside), cyanidin 3-rutinoside and pelargonidin 3-rutinoside. 40 and 200 mg/kg CACN were added to diet and induced 5.2% and 11.2% decrease in body weight gain in mice. CACN attenuated the size of epididymal adipocytes and reduced serum lipids. In 3T3-L1 cells, 200 μg/mL CACN reduced lipid accumulation by 30.7% (Wu, Tang, Yu, Gao, Hu, Chen, et al., 2014). Blueberry anthocyanins (BA) are composed of 9 different structures, such as 24.4% petunidin 3-arabinoside, 16.4% delphinidin 3-glucoside and 12.5% cyanidin 3-galactoside. Consumption of BA at 200 mg/kg reduced 19.4% body weight gain in mice. BA could effectively attenuate epididymal adipocytes, improve lipid profiles, and down-regulate the expression levels of FAS genes (Wu, Jiang, Yin, Long, & Zheng, 2016a). BCAO increased gene expressions of carnitine palmitoyltransferase I (CPT-1) and PPAR-α involved in fatty acid oxidation and decreased mRNA expressions of FAS and SREBP-1c associated with fatty acid synthesis (Park et al., 2015). Mulberry anthocyanins (MACN) consist of cyanidin 3-glucoside, cyaniding 3-rutinoside and pelargonidin 3-glucoside. Male C57BL/6 mice were divided into four groups and given free access to control diet, HFD with 45% calories from fat, HFD added with 40 mg/kg diet MACN and HFD added with 200 mg/kg diet MACN. MACN at 40 mg/kg and 200 mg/kg inhibited 11.8% and 21.4% body weight gain as well as attenuated lipid accumulation and lowered the size of adipocytes (Wu et al., 2013b).

2.4. Suppress food intake

The control center of appetite lies in part of the brain called hypothalamus. Neurons in hypothalamus detect and organize signals of body energy status to control food intake and energy expenditure. Proopiomelanocortin (POMC) and agouti-related peptide (AgRP) cells are the key cell types in this process, which generate peptides competitively binding to the melanocortin receptors MC3R and MC4R. Leptin is produced by adipose tissue and modulates the activity of these cells. POMC neurons secrete anorexic neuropeptides like cocaine-and-amphetamine- regulated transcript (CART) and POMC that reduces appetite while AgRP neurons are inhibited in response to leptin. However, leptin resistance and disorder in other adipocytokines, such as adiponectin and ghrelin, are quite common for obesity state (Saltiel, 2016). Thus, targeting leptin resistance may be a promising way to develop
anti-obesity therapies. Black bean seed coat anthocyanins contain cyanidin 3-glucoside, petunidin 3-glucoside and delphinidin 3-glucoside. 24 mg/kg daily intake of black bean seed coat anthocyanins reduced the expression of neuropeptide Y (NPY) and induced an increase of γ-amino butyric acid receptor (GABAB1R) in hypothalamus. Moreover, decreased expression of GABAB1R downstream signaling molecules, such as protein kinase A- α (PKA α) and phosphorylated cAMP-response element binding protein (p-CREB) in hypothalamus followed such effect (Badshah et al., 2013). Purified cyanidin 3-glucoside enhanced adipocytokines (leptin and adiponectin) secretion in isolated rat adipocytes (Tsuda et al., 2004). CACN, MACN, HA reduced leptin secretion in mice. HA and CBE also increased serum adiponectin levels (Qin & Anderson, 2012; Wu et al., 2013b; Wu et al., 2014; Wu, Yin, Zhang, Long, & Zheng, 2016b; Wu et al., 2013c).

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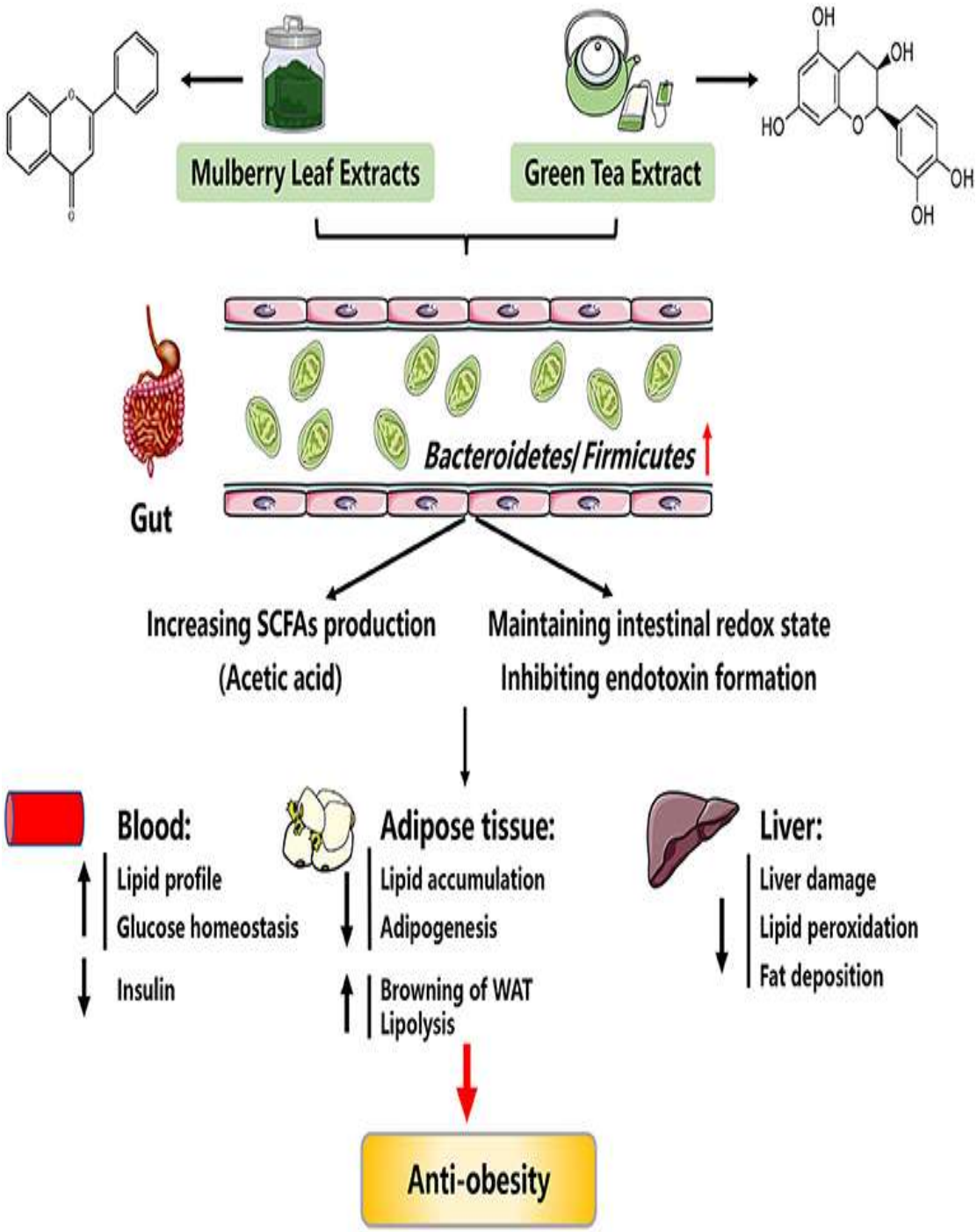

ANTI OBESITY ACT ON MEDICINAL TEA EXTRACT

III. CONCLUSION

The study revealed that some of the herbs such as Cosmos caudatus, Pluchea indica, and Lawsonia inermis exhibited high free radical scavanging activities, high total phenolic content, and potent pancreatic lipase inhibitory effects. The 100% Cosmos caudatus ethanolic extract was proven to be the most efficient extract enriched with complex mixture of phenolic and flavonoid compounds. Strong positive correlation between bioactive compounds and that of free radical scavenging and anti-lipase activity was observed. The identified phenolic and flavonoid compounds

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(quercetin-3-rhamnoside, catechin, rutin, 1-caffeyolquinic acid, kaempherol, kaempherol glucoside, quercetin-3-glucoside, quercetin-O-pentoside, and quercetin-rhamnosylgalactoside) may be responsible for the observed antioxidant and anti-obesity activities of Cosmos caudatus extract. This research may provide a basis for in vivo study and strong foundation for future development of standardized herbal medications or active ingredients with great applications in prevention and treatment of obesity.

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