Journal of Traditional and Complementary Medicine 13 (2023) 397–407

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Acacia nilotica stem bark extract ameliorates obesity, hyperlipidemia, and insulin resistance in a rat model of high fat diet-induced obesity

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ARTICLE INFO

Article history:
Received 10 October 2022
Received in revised form
21 February 2023
Accepted 6 March 2023
Available online 8 March 2023

Keywords:
A. nilotica
Obesity
Inflammatory cytokines
Oxidative stress
Insulin resistance

ABSTRACT

Background and aim: *Acacia nilotica* (*A. nilotica*) is an imperative plant with many medicinal uses. The current study aimed to investigate the protective effects of the stem bark of *A. nilotica* and its fractions in a high fat diet (HFD) rat model.
Experimental procedure: Seventy-two male albino rats were randomly divided into 9 groups, 8 rats per each. Group 1 was the normal control and received standard balanced diet. All the remaining groups were fed HFD for 8 weeks to induce obesity. Group 2 served as the HFD control group, group 3 received orlistat (5 mg/kg/day), groups 4 and 5 received total extract of *A. nilotica* stem bark (250 and 500 mg/kg). Groups 6 and 7 received *A. nilotica* ethyl acetate fraction (250 and 500 mg/kg), while groups 8 and 9 received butanol fraction (250 and 500 mg/kg).
Results and conclusion: Both doses of the ethyl acetate fraction of the stem bark of *A. nilotica* significantly decreased the body weight, blood glucose, lipid profile and improved insulin sensitivity. Levels of MDA, leptin and inflammatory cytokines were significantly decreased by the ethyl acetate fraction while adiponectin and HDL-C were significantly increased relative to the HFD control group. Both doses of the ethyl acetate fraction significantly abolished HDF induced oxidative stress and normalized the values of antioxidant enzymes. Furthermore, metabolic profiling of the ethyl acetate fraction was performed by UHPLC/Q-TOF-MS. In conclusion, the ethyl acetate fraction of *A. nilotica* stem bark possessed antioxidant, anti-inflammatory and insulin sensitizing properties in HFD rat model.
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1. Introduction

The incidence of obesity and overweight has increased globally.[1] Insulin resistance, chronic low-grade inflammation, and specific adipokines such as adiponectin, leptin, and resistin have been identified as essential contributors to the development and outcomes of obesity.[2]

The use of therapeutic plants and their bioactive constituents in the management and prevention of chronic diseases has gained more attention.[3] Numerous studies have reported the capability of medicinal plants to manage obesity; examples are lingonberry, ginger, omija fruits, aloe vera and red cabbages.[4] *Trigonella foenum-graecum* (Fabaceae) was claimed to have anti-diabetes, antioxidant, and anti-hyperlipidemic activities.[5] Wolfberry, the fruit of *Lycium barbarum* (Solanaceae) had protective effects against HFD induced liver oxidative stress injury.[6]

In addition, the anti-obesity effects of some plant-derived bioactive compounds; such as anthocyanin, dioscin, capsaicin, quercetin, and kaempferol; have been enormously reported.[7] Also, dihydromyricetin rich herbal mixture extracts were used in treatment of hepatitis, hypertension, and hyperglycemia, where they improved blood glucose, lipid profile, body fat deposition, and hepatic lipid accumulation in HFD rat.[8]

A. nilotica is a member of the *Fabaceae* family with many

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2023.03.005
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List of abbreviations

A. nilotica	*Acacia nilotica*
COX-2	Cyclooxygenase-2
FFA	Free fatty acids
GSH	Reduced glutathione
H&E	Hematoxylin and Eosin
HDL-C:	High density lipoprotein-cholesterol
HFD	High fat diet
HOMA-IR	Homoeostasis Model Assessment Insulin Resistance
HPLC	High performance liquid chromatography
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
LDL-C:	Low density lipoprotein-cholesterol
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NF-κB	Nuclear factor kappa B
PPARγ	Peroxisome proliferator-activated receptor γ
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
TNF	Tumor necrosis factor
UHPLC/Q-TOF-MS	Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
WHO	World Health Organization

therapeutic properties. It is widely distributed in tropical and subtropical regions, and contains a number of powerful chemicals that have antioxidant, anti-hypertensive, anti-inflammatory, anti-spasmodic, and anti-platelet aggregatory activities.[9]

The therapeutic value of the plant may change depending on the part of the plant. The stem barks from *A. nilotica* have been found to have more flavonoids and polyphenols when compared to roots and leaves. An *In vitro* study showed that the leaf extract of *A. nilotica* contains antioxidant chemicals that help to protect DNA from oxidative damage. Polyphenols which present in green pod extracts can chelate metals and scavenge free radicals.[10]

It is thought that the bark, pods and leaves of *A. nilotica* are effective against cancer, fever, diarrhea, and menstruation issues.[11] The bark of the plant has been used to treat viral, bacterial, amoeboid, fungal, bleeding piles, and leucodermal disorders because it contains a lot of tannins, catechin, epicatechin, and epigallocatechin gallate. Previous studies have shown that the bark of *A. nilotica* has high contents of kaempferol, umbelliferon, gallic acid, ellagic acid, which have strong antioxidant, antimutagenic and cytotoxic properties.[9]

The purpose of the present research was to investigate the potential anti-obesity, antioxidant, and anti-inflammatory effects of the total extract of the stem bark of *A. nilotica* and its ethyl acetate (EA) and butanol fractions in a high fat diet (HFD) rat model, and to use ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) analysis technique for chemical profiling of the most active fraction of the plant.

2. Materials and methods

2.1. Plant collection and preparation

A. nilotica stem bark was collected in February 2018 from El Dakhla Oasis, New Valley Governorate, Egypt. The plant sample was authenticated by Botany Department, Faculty of Science, Cairo University, Egypt.

2.2. Preparation of plant extracts

The stem bark of air-dried *A. nilotica* plant was turned into a fine powder. At room temperature, the powder (2 kg) was extracted by percolation with 70% ethanol (3 × 6L EtOH, each 48 h). The combined extracts were concentrated using rotary evaporator at 45 °C to afford the total dried extract (123 g). This extract was chromatographically fractionated by dissolving in approximately 600 mL distilled water (H_2O) in a separating funnel and then successively extracted with ethyl acetate and *n*-butanol by liquid-liquid fractionation. The resulted fractions were concentrated by evaporation till dryness using rotary evaporator to give 45, 40 and 38 g of ethyl acetate, *n*-butanol and remaining aqueous (H_2O) fractions, respectively. The extracts were stored at 4 °C for biological investigation. For the chemical profile of the active fraction, a solution (100 μg/mL) of ethyl acetate stem bark fraction of *A. nilotica* was prepared in high performance liquid chromatography (HPLC) analytical grade solvent of methanol, filtered using a membrane disc filter (0.2 μm) then subjected to UPLC-qTOF-MS analysis.

2.3. Experimental animals and study design

The present study was carried out on a total number of 72 healthy male Albino rats, weighing 150–200 g, purchased from the Animal House, Research Institute of Ophthalmology. Rats were kept at the animal House, Research Institute of Ophthalmology (Cairo, Egypt). They were housed in separate metal cages under controlled room temperature (22 ± 2 °C) and humidity (55 ± 5%). Rats were allowed one week for acclimatization and handled in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The Ethics committee of the Faculty of Pharmacy, Suez Canal University approved the research protocol (Code 201804MA1).

The rats were randomly divided into 9 groups, each containing 8 rats. Group 1 served as the normal group, in which rats were fed standard balanced diet for throughout the total period of the study (12 weeks). The standard chow diet consisted of carbohydrate 48.8%, protein 21%, and fat 3%, calcium 0.8%, phosphorus 0.4%, fiber 5%, moisture 13%, and ash 8%.[12] All the remaining groups were fed HFD for 8 weeks to induce obesity. The HFD was composed of 87.7% standard diet, 10% pork fat, 0.3% bile salts (w/w) and 2% cholesterol.[13] The HFD was freshly prepared every day by warming the components of the diet together with the basal diet. After induction of obesity, group 2 served as the HFD control group, while groups (3–9) were treated for 4 weeks as follows:

Group 3: received orlistat (Sigma-Aldrich, Egypt) (5 mg/kg/day) as a standard drug for 4 weeks.

Groups 4 and 5: received total 70% ethanol extract of *A. nilotica* stem bark at doses of 250 mg/kg and 500 mg/kg, respectively.

Groups 6 and 7: received ethyl acetate fraction of *A. nilotica* stem bark at doses of 250 mg/kg and 500 mg/kg, respectively.

Groups 8 and 9: received butanol fraction of *A. nilotica* stem bark at doses of 250 mg/kg and 500 mg/kg, respectively.

All treatments were given daily per oral for a total treatment period of 4 weeks. The weight of rats after induction of obesity and

before the start of treatment was recorded, and their weight at the end of the treatment period was also recorded. Percentage weight reduction was calculated for each rat according to the formula:

[Weight before treatment (g) − weight after treatment (g)] * 100

2.4. Collection of samples

At the end of the treatment period, overnight fasting rats were anesthetized with thiopental sodium (50 mg/kg) and blood samples were withdrawn from the orbital sinus. Blood was obtained in part using EDTA anticoagulant tubes to separate plasma by centrifugation at 4000 rpm for 10 min at 4 °C. Plasma was aspirated, and the erythrocytes were washed four times with 0.9% NaCl solution. Each wash was followed by centrifugation at 4000 rpm for 10 min. After the last wash, the washed centrifuged erythrocytes were made up to 2 mL with cold redistilled water, mixed and left to stand at 4°C for 15 min. The other blood portion was collected in plain tubes to separate serum by centrifugation at 3000 rpm for 15 min at 4 °C. Plasma, serum and erythrocyte lysate samples were stored at −80 °C.

Anesthetized rats were killed by cervical dislocation. White adipose tissue was dissected from rats, washed with ice cold phosphate buffered saline (pH 7.4), and fixed in 10% formalin for further histopathological examination.

2.5. Assessment of the biochemical markers

Fasting plasma glucose was determined by enzymatic colorimetric kit (Cat. No. GL 1320) (Biodiagnostic, Egypt). Fasting plasma insulin was detected by Rat Inulin ELISA Kit (Cat. No. MBS724709) (MyBioSource, USA), according to the manufacturer's instructions. Homoeostasis Model Assessment Insulin Resistance (HOMA-IR) was calculated for each rat by the equation: [fasting plasma glucose (mg/dL) * fasting plasma insulin (μIU/mL)]/405.[14]

Lipid profile was assessed in plasma of the experimental rats. Levels of total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG) were determined by colorimetric kits (Cat. No. TR2030, CH1202, CH1231, and CH1230, respectively) (Biodiagnostic, Egypt).

Serum levels of malondialdehyde (MDA) and reduced glutathione (GSH) were determined by colorimetric kits (Cat. No. MD2529 and GR2511, respectively) (Biodiagnostic, Egypt). Activities of catalase and superoxide dismutase (SOD) antioxidant enzymes in erythrocyte lysates were assessed by colorimetric kits (Cat. No. CA2517 and SD2521, respectively) (Biodiagnostic, Egypt).

Serum levels of the inflammatory cytokines interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) were determined by ELISA kits (Cat. No. MBS825017, and MBS269892, respectively) (MyBioSource, USA). Similarly, serum levels of the adipocytokines leptin and adiponectin were determined by ELISA kits (Cat. No. MBS730855, and MBS068220, respectively) (MyBioSource, USA), following the manufacturer's instructions.

2.6. Histopathological examination of the adipose tissue

Paraffin slices from adipose tissue were routinely stained with hematoxylin and eosin (H&E). The diameter of 100 adipocyte spaces in two random microscopic fields was determined using the image analysis system [ImageJ 1.45F] (National Institute of Health, USA) following the previously described procedure.[15] The mean diameter for each slide was then calculated.

2.7. UPLC-qTOF-MS analysis of the most active fraction

Based on the finding of the current *in vivo* study, the bioactive ethyl acetate extract fractionated from 70% ethanol extract of *A. nilotica* stem bark was chosen for further chemical profiling using UPLC-qTOF-MS.[16] Chromatographic separation was carried out using Sciex Exion LC coupled with TripleTOF 5600+ equipped with a Xbridge C18 column (3.5 μm, 2.1 × 50 mm; Waters). The elution binary gradient was applied at a flow rate of 0.3 mL min^{-1}: 0–1 min, isocratic 90% A (5 mM ammonium formate buffer pH = 8 containing 1% methanol [v/v]), 10% B (100% acetonitrile); 1–21 min, linear from 10 to 90% B; 21–25 min, isocratic 90% B; 25–28 min, isocratic 10% B. The injection volume from ethyl acetate extract was 10 μL. Eluted compounds from UPLC were detected using Micro-TOFQ hybrid quadrupole time-of-flight mass spectrometer equipped with an Apollo-II electrospray ion source in negative ion mode from *m/z* 100 to 1000 and the instrument settings were: dry gas, nitrogen, 190 °C; nebulizer gas, nitrogen, 1.6 bar; end plate offset, 500 V; capillary, 5500 V (+4000 V); funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp. For MS/MS fragmentation collision-induced dissociation energy 30 eV was used. Wiff file conversion was processed by Reifycs Abf (Analysis Base File) Converter software and Data analysis using MS-DIAL (RIKEN) software. Peaks and spectra were tentatively recognized by comparing mass spectra, retention times, and their fragmentation pattern with data the phytochemical dictionary of natural products (DNP) database as well as the information published in the literature.

2.8. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the statistical package for social science (SPSS Inc, Chicago, USA), version 19. One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was used to test the significance of the difference between quantitative variables in different groups. $P < 0.05$ was considered to be statistically significant.

3. Results

Weight gain was developed in the rats that received HFD, where the mean ± SD of the body weight of the rats in all groups (except the normal group) before treatment was 340 ± 22 g. Increased weight was accompanied with insulin resistance where values of fasting plasma glucose, fasting plasma insulin and HOMA-IR were significantly increased in the HFD control group in comparison to the normal group (Table 1). Insulin sensitivity and glucose homeostasis were significantly improved by treatment in the groups that received orlistat, butanol extract of the stem bark of *A. nilotica* (500 mg/kg), and both doses of the ethyl acetate fraction (250 and 500 mg/kg). The body weight of rats in all treated groups was decreased. The percentage of weight loss after treatment in all treated groups was significantly higher than that in the HFD control group. It was noticed that ethyl acetate fraction was the most active in decreasing body weight and diminishing insulin resistance. No significant difference was detected between both groups treated with the ethyl acetate fraction (250 and 500 mg/kg) and the group the received orlistat. Moreover, the group that received ethyl

Table 1
Effect of *A. nilotica* extracts on body weight, fasting plasma glucose, fasting plasma insulin, and HOMA-IR index and in the experimental rats.

	Weight loss (%)	Glucose (mg/dL)	Insulin (μIU/mL)	HOMA-IR
Normal	2.7 ± 2.2	62 ± 11	2.9 ± 0.7	0.4 ± 0.2
HFD Control	1.4 ± 1.1	105 ± 18*	13.3 ± 2.3*	3.5 ± 1.1*
Orlistat	28 ± 2$^{*\#}$	68 ± 14$^{\#}$	4.9 ± 1.0$^{*\#}$	0.8 ± 0.3$^{*\#}$
Total (250 mg/kg)	4.2 ± 1.5$^{*\#\wedge}$	103 ± 14$^{*\wedge}$	12.1 ± 2.5$^{*\wedge}$	3.1 ± 1$^{*\wedge}$
Total (500 mg/kg)	10 ± 3.3$^{*\#\wedge}$	92 ± 10$^{*\wedge}$	11.6 ± 2.3$^{*\wedge}$	2.7 ± 0.8$^{*\wedge}$
EA (250 mg/kg)	27.8 ± 2.1$^{*\#}$	72 ± 7$^{\#}$	4.5 ± 0.6$^{*\#}$	0.8 ± 0.2$^{*\#}$
EA (500 mg/kg)	31.6 ± 2.3$^{*\#}$	64 ± 7$^{\#}$	3.4 ± 0.6$^{\#}$	0.5 ± 0.1$^{\#}$
Bu (250 mg/kg)	14.3 ± 1.9$^{*\#\wedge}$	92 ± 12$^{*\wedge}$	10.6 ± 1.6$^{*\wedge}$	2.4 ± 0.6$^{*\wedge}$
Bu (500 mg/kg)	16 ± 2.6$^{*\#\wedge}$	82 ± 11$^{*\#\wedge}$	9.1 ± 1.3$^{*\#\wedge}$	1.9 ± 0.5$^{*\#\wedge}$

EA, ethyl acetate fraction; Bu, butanol fraction. Data are represented as mean ± SD and analyzed by ANOVA followed by Tuckey's post hoc test. * significantly different compared to the normal group; $^{\#}$ significantly different compared to the HFD control group; $^{\wedge}$ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$.

acetate fraction at the dose of 500 mg/kg showed no significant difference in comparison to the normal group in terms of fasting plasma glucose, fasting plasma insulin and HOMA-IR values (Table 1).

As expected, the plasma lipid profile of the rats in the HFD control group showed significantly higher TG, TC, and LDL-C, and significantly lower HDL-C levels compared to the normal rats (Table 2). Levels of TG and LDL-C were significantly lowered in all treated groups except the groups that received 250 mg/kg of the total extract of the stem bark of *A. nilotica*. Levels of TC were significantly decreased in the groups that received orlistat, both doses of the ethyl acetate fraction and the high dose (500 mg/kg) of the butanol fraction, but only orlistat and the ethyl acetate fraction at both doses resulted in a significant increase in the levels of HDL-C (Table 2).

Markers of oxidative stress and antioxidant enzymes were determined in the study groups. Serum levels of MDA were significantly increased in the HFD control rats compared to the normal group. Serum levels of GSH, along with the levels of catalase and SOD in erythrocyte lysate, were significantly decreased in the HFD control rats relative to the normal group (Fig. 1). Levels of MDA were significantly decreased in serum in the groups that received orlistat, *A. nilotica* butanol fraction (500 mg/kg), and *A. nilotica* ethyl acetate fraction (250 and 500 mg/kg) (Fig. 1A). Serum levels of GSH as well as the activity of catalase and SOD enzymes in erythrocyte lysate were significantly increased by treatment with orlistat and both doses (250 and 500 mg/kg) of the butanol and ethyl acetate fractions of the stem bark of *A. nilotica* (Fig. 1B–D). Values of MDA, GSH, catalase and SOD in rats treated with both ethyl acetate fraction doses (250 and 500 mg/kg) were comparable to their values in the orlistat treated group. The higher dose of the ethyl acetate fraction (500 mg/kg) of *A. nilotica* abolished HDF induced oxidative stress and normalized the values of those markers (Fig. 1).

Development of oxidative stress in HFD rats was accompanied by significant increase in the serum levels of the inflammatory cytokines IL-1β and IL-6 (Fig. 2). Both markers were significantly decreased in all treated groups except the 250 mg/kg total extract receiving group. Serum levels of IL-1β and IL-6 in the group that received the higher dose (500 mg/kg) of ethyl acetate fraction of *A. nilotica* were not significantly different than their levels in the normal group (Fig. 2).

Serum levels of leptin were significantly increased, while serum levels of adiponectin were significantly decreased in the HFD control group compared to the normal group (Fig. 3). Leptin levels were significantly decreased in all treated groups except the group that received 250 mg/kg of the total ethanaolic extract of *A. nilotica* stem bark, while levels of adiponectin were significantly increased in the groups that were treated with orlistat, higher dose (500 mg/kg) of the butanol fraction, and both doses (250 and 500 mg/kg) of ethyl acetate fraction of *A. nilotica*. Levels of both leptin and adiponectin in the ethyl acetate fraction treated rats at both doses were not significantly different compared to the orlistat treated group. Interestingly, serum levels of leptin in the ethyl acetate fraction (500 mg/kg) group and serum levels of adiponectin in both 250 and 500 mg/kg ethyl acetate fraction receiving rats were not significantly different compared to the normal group (Fig. 3).

Histopathological examination of adipose tissue showed small regular adipocytes with thin cell membranes and small regular peripheral nuclei in the normal rats, while the adipocytes appeared distorted with irregular shapes, increased size and scattered congested vessels in the HFD control rats. Adipocyte diameter was significantly increased in the HFD control rats compared to the normal rats. The features of adipocytes were enhanced in all treated groups, and adipocyte diameter was significantly decreased in all treated groups, especially the groups that received ethyl acetate fraction of *A. nilotica* stem bark at both doses (250 and 500 mg/kg) and the group that received butanol fraction at 500 mg/kg dose, where adipocyte diameters in the three groups were not

Table 2
Effect of *A. nilotica* extracts on lipid profile in the experimental rats.

	TG (mg/dL)	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
Normal	99 ± 11	144 ± 22	79 ± 14	45 ± 6
HFD Control	171 ± 32*	210 ± 34*	149 ± 19*	27 ± 9*
Orlistat	112 ± 14$^{\#}$	172 ± 28$^{*\#}$	114 ± 19$^{*\#}$	36 ± 6$^{*\#}$
Total (250 mg/kg)	155 ± 24$^{*\wedge}$	190 ± 30*	131 ± 15$^{*\wedge}$	28 ± 10$^{*\wedge}$
Total (500 mg/kg)	151 ± 21$^{*\#\wedge}$	182 ± 28*	123 ± 14$^{*\#}$	29 ± 10$^{*\wedge}$
EA (250 mg/kg)	120 ± 12$^{*\#}$	167 ± 18$^{*\#}$	104 ± 8$^{*\#}$	39 ± 8$^{*\#}$
EA (500 mg/kg)	118 ± 12$^{*\#}$	165 ± 13$^{*\#}$	101 ± 7$^{*\#}$	40 ± 8$^{\#}$
Bu (250 mg/kg)	150 ± 22$^{*\#\wedge}$	183 ± 25*	122 ± 16$^{*\#}$	31 ± 5*
Bu (500 mg/kg)	143 ± 17$^{*\#\wedge}$	179 ± 18$^{*\#}$	120 ± 9$^{*\#}$	30 ± 6*

EA, ethyl acetate fraction; Bu, butanol fraction; TG, triglycerides; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol, HDL-C, high density lipoprotein-cholesterol. Data are represented as mean ± SD and analyzed by ANOVA followed by Tuckey's post hoc test. * significantly different compared to the normal group; $^{\#}$ significantly different compared to the HFD control group; $^{\wedge}$ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$.

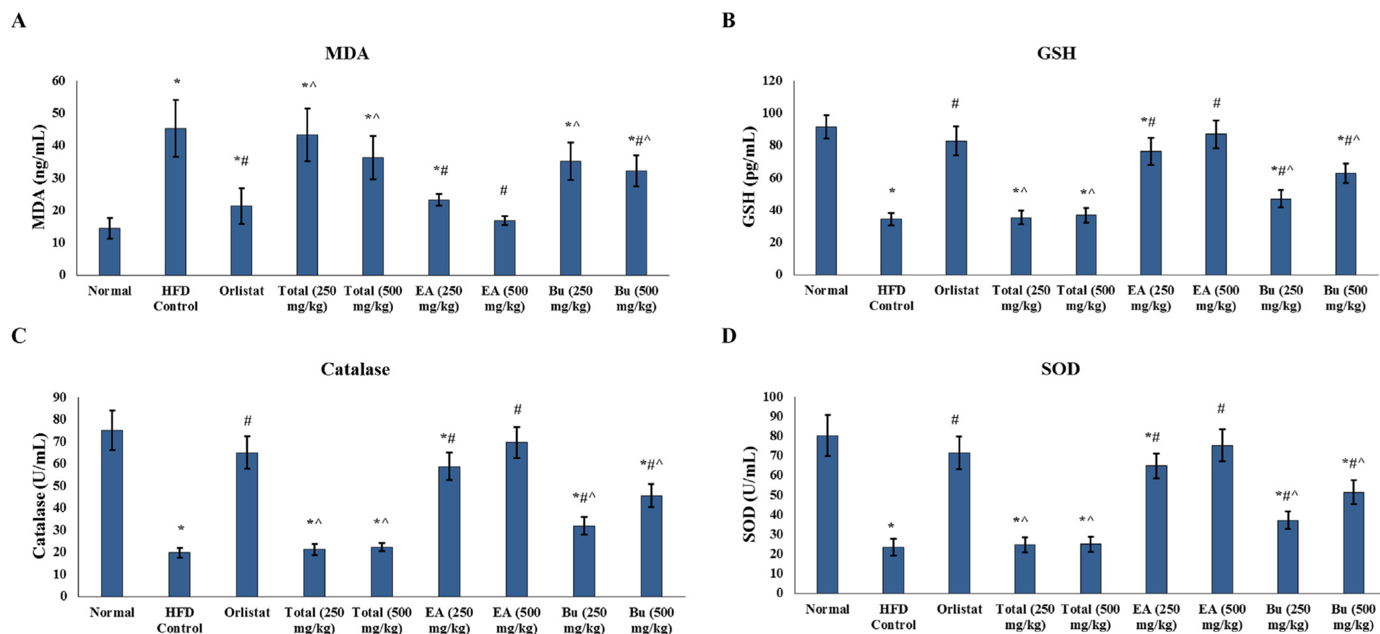


Fig. 1. Antioxidant effects of *A. nilotica* stem bark extracts in the experimental rats. Serum levels of **(A)** MDA, **(B)** GSH in the study groups. Activity of **(C)** catalase, and **(D)** SOD in erythrocyte lysates of the experimental rats. EA, ethyl acetate fraction; Bu, butanol fraction; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase. Data are represented as mean ± SD and analyzed by ANOVA and Bonferroni's post-hoc test. * significantly different compared to the normal group; # significantly different compared to the HFD control group; ^ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$.

significantly different compared to the orlistat treated rats (Fig. 4).

Metabolic profiling of the bioactive ethyl acetate extract fractionated from 70% ethanol extract of *A. nilotica* stem bark was carried out using UPLC-qTOF-MS in negative ionization mode using DNP databases as well as by comparing with the reported data in the literature. LC-MS of ethyl acetate extract revealed the annotation of 31 secondary metabolites (Suppl. Table 1 and Suppl. Fig. 1) according to their molecular weights and mass fragmentation patterns. These annotated phytochemicals were divided into 30 flavonoids and one phenolic acid. The basic structures of all the tentative identified compounds are listed in Suppl. Table 1 and illustrated in Suppl. Fig 2. In the studied extract, just one phenolic acid was characterized as caffeic acid (**12**).[17] Meanwhile, the flavonoid compounds provided the presence of diverse flavonoid (flavonoids and/or flavonoid glycosides) subclasses including, twelve flavonols (**4**–**6**, **8**, **15**, **21**–**23**, **25**–**26** and **30**–**31**),[10,15,18–21] five flavones (**1**, **9**, **11** and **19**–**20**),[10,15,18,22] five flavanones (**3**, **10**, **18** and **27**–**28**),[23–25] two flavanols (**16**–**17**),[10,24] a biflavanol (**13**),[24] a flavanonol (**2**),[10] an isoflavone (**7**),[26] a dihydrochaclcone (**14**),[17] and two stilbenes (**24** and **29**).[25,27]

In MS analysis, the type of the sugar substructure of the tentatively identified plant flavonoid glycosides were determined by the neutral loss of 146, 162, and 176 amu corresponding to deoxyhexose (rhamnose), hexose (glucose) and hexouronic acid (glucuronic acid) moieties, respectively as shown in compounds **1**, **6**–**7**, **9**–**10**, **14**–**15**, **18**, **20**–**21**, **23**–**26** and **30**–**31**. Also, the *p*-coumaroyl and *p*-coumaroylhexoside moieties which present in compound **25** could be observed by a neutral loss of 146 and 308 amu, respectively.[21] Numerous of these annotated phenolic compounds in the ethyl acetate fraction of *A. nilotica* stem bark (e.g. taxifolin, quercetin, myricetin, catechin, epicatechin, naringenin, naringenin-7-*O*-glucopyranoside, kaempferol-7-*O*-neohesperidoside and caffeic acid) have been previously reported in this plant.[10,28]

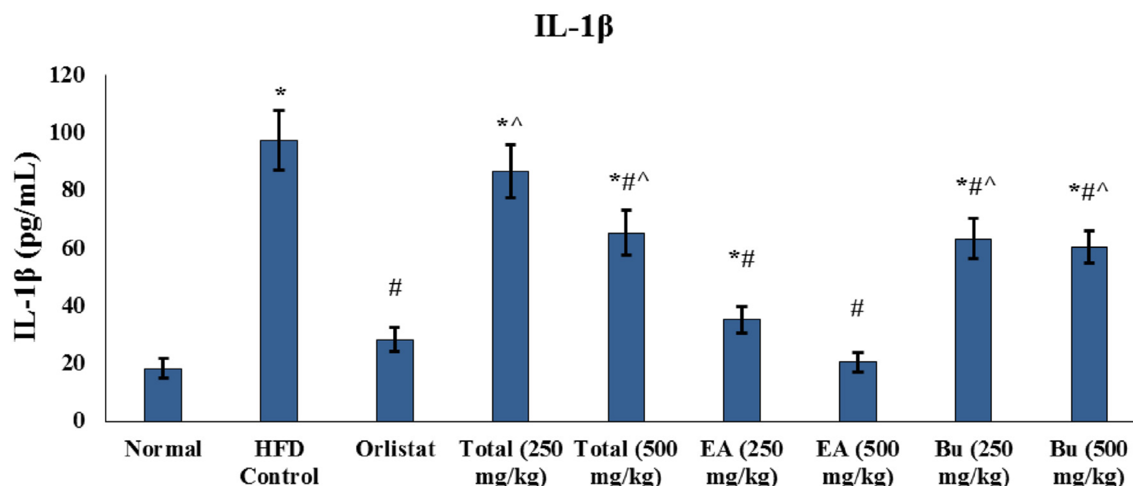
4. Discussion

A. nilotica, a member of the *Fabaceae* family, is a well-known medicinal plant. The main types of phytochemical components in this plant are alkaloids, flavonoids, tannins, fatty acids and polysaccharides.[28] The antibacterial, antioxidant and anticancer activities of *A. nilotica* leaf extracts have been previously established.[29,30] Also, the therapeutic value of *A. nilotica* pods extract on diabetic nephropathy induced by streptozotocin in rats[31] and the protective impact of the polyphenolic extract of *A. nilotica* on alloxan monohydrate-induced diabetes[32] were previously investigated.

Despite its extensive use in traditional medicine and in management of a variety of metabolic problems for decades, the mechanisms contributing to the therapeutic impact of *A. nilotica* stem bark in preventing body weight gain, hyperlipidemia, and insulin resistance were not fully discussed. So, the present work aimed to provide an updated study of the anti-obesity, antioxidant, and anti-inflammatory effects of the stem bark extracts of *A. nilotica* in a HFD rat model.

A. nilotica contains a lot of phenolic chemicals especially flavonoids that act as potent antioxidants and have a variety of biological outcomes, such as anti-inflammatory, anti-cancer, anti-diabetic, and anti-aging effects.[10,28] Accordingly, one of the main targets in the current research was to study the poly-phenolic (flavonoid) rich fractions of *A. nilotica* stem bark in a bio-assay guided identification. Thus, we have initially extracted the total extract of this plant stem bark using 70% ethanol. After that, the total extract was successively fractionated by liquid-liquid partition chromatography using ethyl acetate, and then n-butanol. We have particularly used these two solvents because ethyl acetate and n-butanol are considered as two of the favorable and suitable solvents usually used for successively extract and/or fractionate both of less and more polar flavonoids.

Long-term consumption of HFD causes hyperlipidemia, hyperglycemia, and insulin resistance in experimental animals.[33] Additionally, the activity of autophagy is generally reduced in HFD models. Autophagy is important in the process of adipocyte



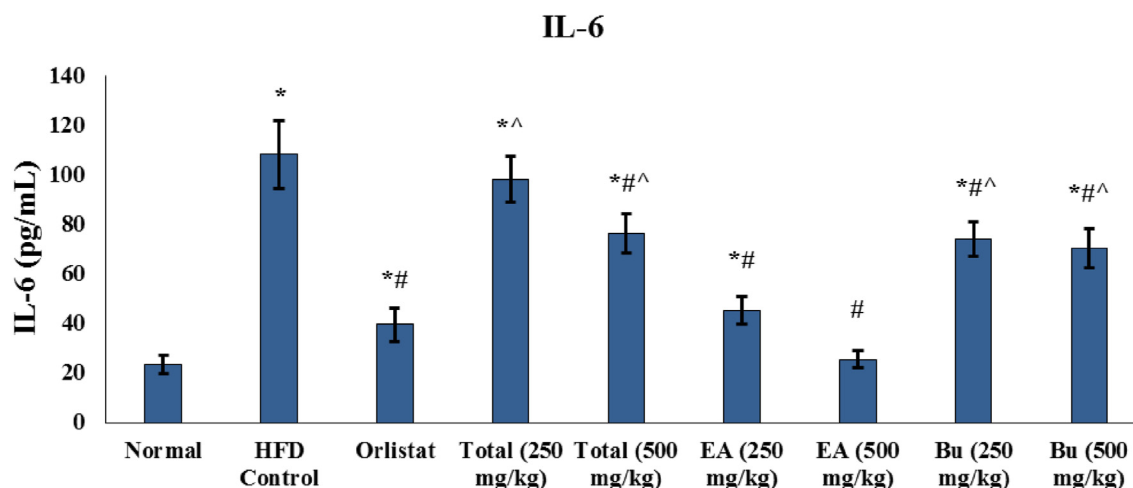


Fig. 2. Effect of *A. nilotica* stem bark extracts on the serum levels of adipocytokines in the experimental rats. **(A)** Leptin, and **(B)** adiponectin. EA, ethyl acetate fraction; Bu, butanol fraction. Data are represented as mean ± SD and analyzed by ANOVA and Bonferroni's post-hoc test. * significantly different compared to the normal group; # significantly different compared to the HFD control group; ^ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$.

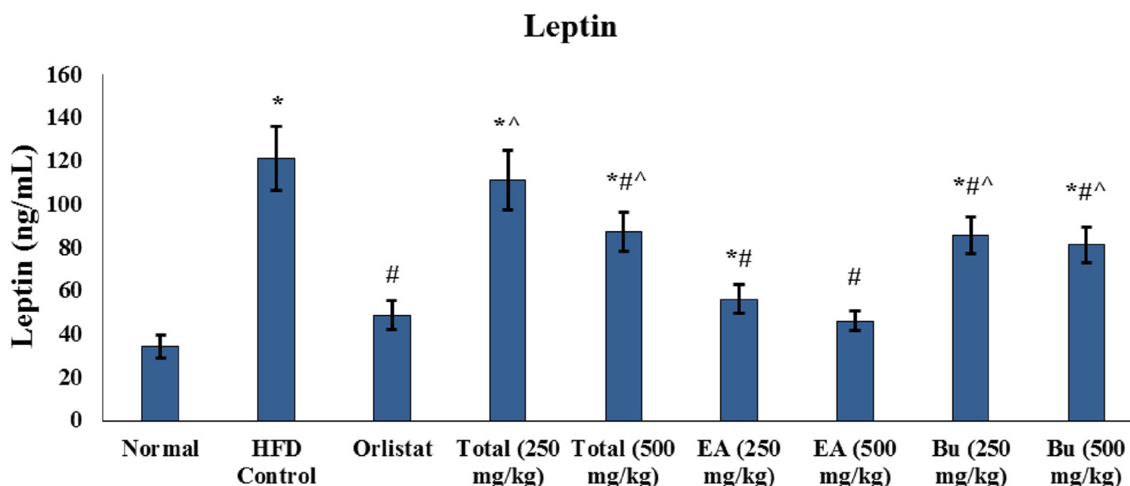
differentiation. Lipophagy, the autophagy mediated disposal of lipid droplets, plays an important role in lipid homeostasis and its stimulation by natural polyphenols prevents abnormal lipid accumulation in the tissues.[34,35] Insulin resistance in obese mice is increased by defective autophagy, which increases endoplasmic reticulum stress.[36] Also, Autophagy affects many cellular processes including oxidative stress, inflammation, and innate and acquired immune response, autophagy modifies the inflammation status of adipose tissue by regulating the synthesis of proinflammatory cytokines including IL-6, IL-8, and IL-1β. The adipose tissue secretes more proinflammatory cytokines when autophagy is inhibited.[37]

Our findings showed that HFD group become significantly larger during the trial period when compared to their starting body weights and the normal group. Additionally, a considerable drop was noticed in body weight after treatment with ethyl acetate fraction of *A. nilotica*.

The potent physiological impact of the ethyl acetate fraction that was observed in the current study might be due to the existence of predominant bioactive flavonoids such as taxifolin, quercetin, myricetin, catechin, epicatechin, naringenin, naringenin-7-*O*-glucopyranoside, kaempferol-7-*O*-neohesperidoside in *A. nilotica* as described previously[10,28] as well as other identified known compounds like gossypin and baicalin.

The positive effects of flavonoids, particularly epicatechin against obesity are supported by a lot of evidence. It interacts with digestive enzymes and nutrients in the gastrointestinal tract; this gives epicatechin the chance to reduce obesity by decreasing the absorption and digestion of calorie-rich nutrients.[38] Furthermore, it increases fat oxidation, prevents fat synthesis, and stimulates energy wasting.[39]

Increased fasting blood sugar and serum insulin levels in our study clearly indicate that insulin's ability to control blood sugar was impaired in HFD-fed control rats. According to the HOMA-IR index, the level of insulin resistance was high. Treatment of HFD-



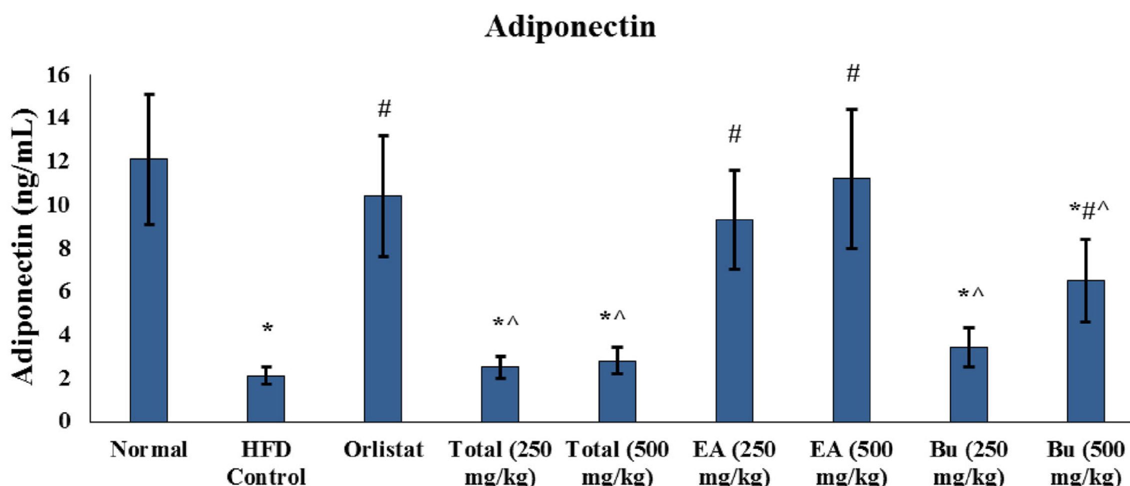


Fig. 3. Effect of *A. nilotica* stem bark extracts on the serum levels of inflammatory cytokines in the experimental rats. **(A)** IL-1β, and **(B)** IL-6. EA, ethyl acetate fraction; Bu, butanol fraction; IL-1β, interleukin-1 beta; IL-6, interleukin-6. Data are represented as mean ± SD and analyzed by ANOVA and Bonferroni's post-hoc test. * significantly different compared to the normal group; # significantly different compared to the HFD control group; ^ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$.

fed rats with the ethyl acetate fraction of *A. nilotica* (either 250 or 500 mg/kg) resulted in lowering fasting blood sugar, serum insulin, and the HOMA-IR index. These findings are consistent with many studies suggesting that *A. nilotica* might contribute to the treatment of diabetes and insulin resistance,[40,41] which can be linked to its active ingredients that were revealed by the metabolic profiling that was conducted in the current work (Suppl. Table 1).

Epicatechin and epicatechin-rich foods have a potent effect in improving glucose homeostasis and insulin sensitivity in humans and animal models of obesity and T2DM.[42] The favorable effects of epicatechin on glucose metabolism are further supported by *in vitro* investigations in hepatocytes and adipocytes; these studies found that epicatechin can directly affect glucose homeostasis in cells by increasing insulin receptor substrate-1, activating tyrosine phosphorylation and insulin receptor in response to insulin stimulation, reducing NADPH oxidase upregulation and inhibiting gluconeogenesis through the phosphatidylinositol-3-kinase and 5′-AMP-activated protein kinase pathways.[43] Also, Baicalin was suggested to reduce gluconeogenic activity and hepatic insulin resistance *via* inhibition of p38 mitogen-activated protein kinases/peroxisome proliferator-activated receptor gamma pathway.[44]

Numerous studies have demonstrated that the flavonoid taxifolin had anti-hyperglycemic action through modulating gastric enzymes; taxifolin exhibited significant inhibiting action against pancreatic lipase, α-glucosidase and α-amylase, furthermore taxifolin has anti-inflammatory and antioxidant activities in alloxan diabetic rat model.[45,46] Kaempferol is another *A. nilotica* flavonoid that exhibited anti-obesity benefits through decreasing adipogenesis and boosting lipolysis in 3T3-L1 cells.[7] Furthermore, it ameliorated dyslipidemia and hyperglycemia in HFD induced obesity through altering imbalance of intestinal microbiota and intestinal inflammation, and counteracting Toll-like receptor 4/nuclear factor-kappa B (NF-κB) pathway activation response.[47]

Dyslipidemia, characterized by elevated levels of TG and LDL-C with decreased HDL-C levels, is a critical risk element for non-alcoholic fatty liver, cardiovascular disease and insulin

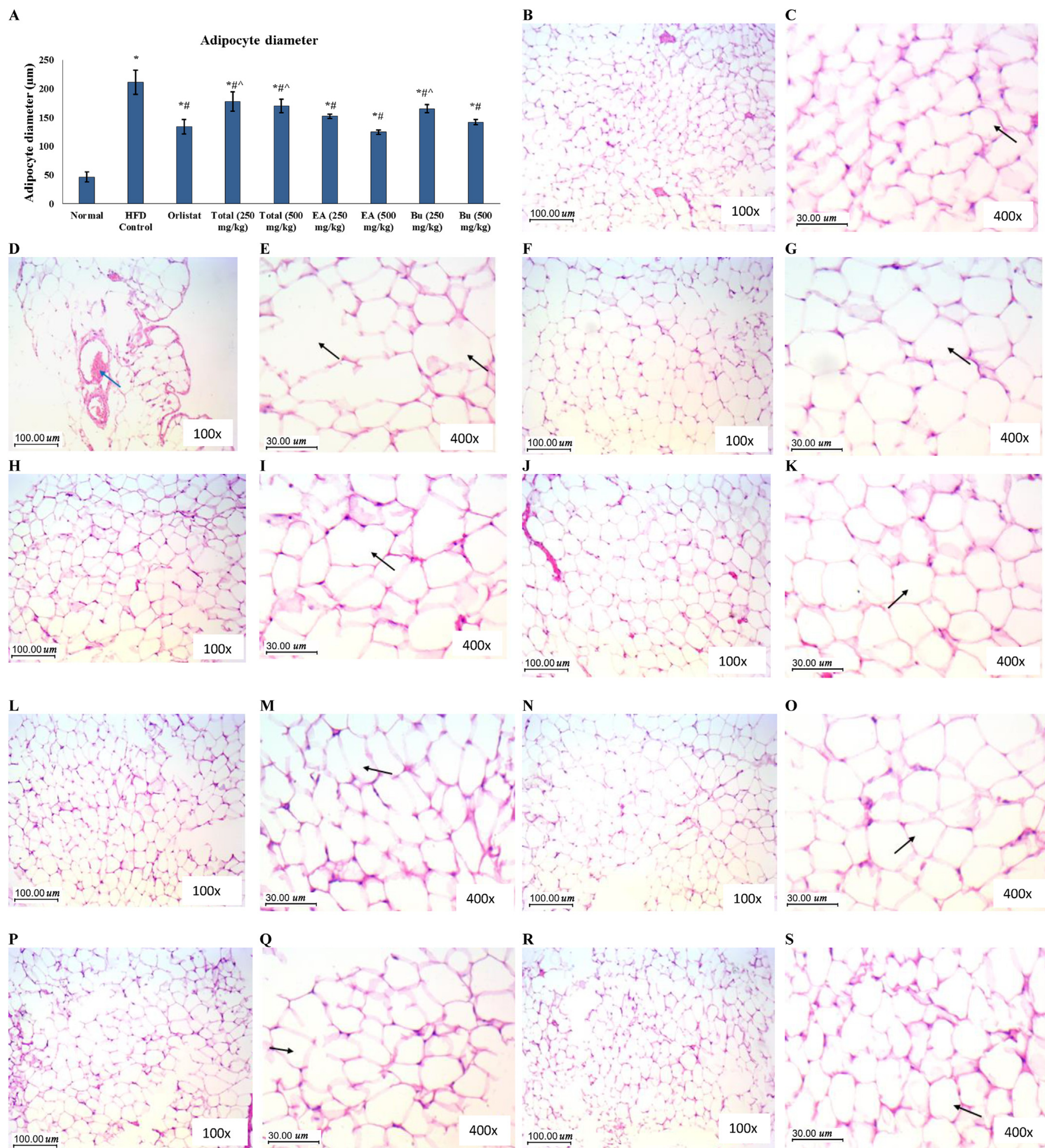


Fig. 4. Histopathological examination of the adipose tissue of the experimental rats. **(A)** Adipocyte diameter represented as mean ± SD and analyzed by ANOVA and Bonferroni's post-hoc test. * significantly different compared to the normal group; # significantly different compared to the HFD control group; ^ significantly different compared to the orlistat treated group. Differences were considered significantly different at $P < 0.05$. **(B)** & **(C)** Normal group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral nuclei. Adipocytes are of small average size with regular arrangement. **(D)** & **(E)** HFD Control group (100*x* and 400*x*, respectively); section in adipose tissue showing adipocytes (black arrow) with distorted irregular shapes and increased size and irregular contours, with scattered congested vessels (blue arrow). **(F)** & **(G)** Orlistat group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral nuclei. Adipocytes are of smaller size than obese group. **(H)** & **(I)** Total extract group (250 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and slight distortion of cells contours and increased cell size. **(J)** & **(K)** Total extract group (500 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes with thin cell membranes and small regular peripheral nuclei. Adipocytes are predominantly regularly arranged with few cells showing distortion and increased size (arrow). **(L)** & **(M)** Ethyl acetate group (250 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral nuclei. Adipocytes are arranged more regularly with average size. **(N)** & **(O)** Ethyl acetate group (500 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral

resistance.[48] In our study, Four-week administration of ethyl acetate fractions of *A. nilotica* or (250 and 500 mg/kg) considerably enhanced lipid profiles in HFD-fed rats as evidenced by decreased TG, TC, LDL levels, and raised serum HDL levels, toward a healthy index.

It has been previously reported that *A. nilotica* leaf extract decreased levels of blood lipids and blood sugar levels in alloxan-induced diabetic rats.[40] Epicatechin, the major *A. nilotica* constituent, decreases absorption of lipids by suppressing the activity of pancreatic lipase in the gastrointestinal lumen, in addition to controlling lipid absorption, epicatechin also regulates the transcription factors that expressed during cholesterol and TG synthesis, such as peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element-binding protein.[49] Furthermore, epicatechin was able to reduce circulating free fatty acids (FFA) by inhibiting white adipose tissue inflammation and, as a result, lipolysis.[50] Epicatechin may also target miRNAs which have been implicated as lipid metabolic regulators in adipose tissue and the liver.[51]

Baicalin therapy reduced TG content and lipid accumulation in human hepatoma cells treated with palmitic acid. Increased expression of solute carrier family 2 member 1 and the down-regulation of NF-κB subunit, sterol regulatory element-binding transcription factor 1, tumor necrosis factor (TNF), and PPARγ were probably the mechanisms responsible for the baicalin-induced decrease in lipid and TG accumulation.[52] Taxifolin was also reported to have anti-hyperlipidemic properties through inhibiting cellular cholesterol, TG, and phospholipid synthesis from esterification. Furthermore, taxifolin inhibited hepatic lipid production by altering apolipoprotein B and apolipoprotein A-I secretion.[53]

The level of oxidative stress in the obese population is another established factor that influences the impact of obesity on insulin resistance and the cardiovascular system.[54] Consumption of HFD has been linked to a pronounced rise in oxidative stress.[55] In the current study, the ethyl acetate fraction of *A. nilotica* demonstrated considerable reduction in MDA serum levels and increase in SOD, GSH and catalase activity. The ethyl acetate extract of the stem bark of *A. nilotica* has considerable hydroxyl free radical scavenging potential due to existence of polyphenolic chemicals in the extract. The observed results confirm the *in vitro* antioxidant activity of *A. nilotica* bark that was previously reported.[56,57]

A. nilotica possesses important anti-free radical capabilities due to a high number of antioxidants like curcumin, flavonoids, terpenoids, phenolics and tannins. They have the capacity to scavenge oxygen nitrogen-derived free radicals by giving an electron or hydrogen atoms, chelating metal catalysts, inhibiting oxidases and activating antioxidant enzymes. As a result, they can lessen the interaction of oxidants and other toxic compounds.[58]

It has been reported that epicatechin is one of the most potent antioxidants[59,60]; mainly due to the presence of ortho-hydroxyl groups that are accountable and necessary for their direct detoxifying effects when they react with hydrogen peroxide and superoxide.[61] Epicatechin also enhances antioxidant defenses by inhibiting enzymes involved in reactive oxygen species (ROS) production such as mitochondrial succinoxidase, NADH oxidase and microsomal monooxygenase.[62]

The *A. nilotica* flavone gossypin had an antioxidant effect in mice with femur fracture; where in gossypin-treated groups, higher levels of SOD, GSH, and low MDA levels were detected and this is consistent with our results. Free radical scavenging properties and specific signals by genes of antioxidant and anti-inflammatory compounds are two aspects that contribute to gossypin's antioxidant effect.[63] Taxifolin, another flavonoid detected in the ethyl acetate fraction of *A. nilotica*, has been found to prevent stress-induced apoptosis, particularly oxidative stress, and endoplasmic reticulum stress through the phosphatidylinositol-3-kinase/Protein kinase B pathway.[64]

Leptin is a peptide hormone that controls appetite, body weight, proinflammatory immunological responses, angiogenesis, and lipolysis. The pleiotropic actions of leptin are important in regulating the body mass through a negative feedback process between the hypothalamus and the adipose tissue.[65] In the current study, levels of leptin in the both groups that received the ethyl acetate fraction at 250 and 500 mg/kg were considerably lower than the obese control group; this is in line with prior research that found a link between body weight loss and a lower leptin level.[66]

Interestingly, the reduction of leptin levels was accompanied with the reversal of insulin resistance. This effect might result from the suppression of cytokines that cause leptin resistance; this is backed up by other studies that demonstrated that hypothalamic oxidative stress causes leptin resistance which triggers the development of insulin resistance.[67] Epicatechin therapies were reported to reduce serum leptin levels, which lessens the risk of body weight gain and adipose tissue accumulation.[68]

Conversely, our results revealed that low levels of adiponectin in HFD induced obesity were significantly increased by treatment with both doses of *A. nilotica* stem bark ethyl acetate fraction. Adiponectin has anti-inflammatory, antioxidant, and cardiovascular regulating properties in addition to its insulin-sensitizing properties.[69] The *A. nilotica* active compound Kaempferol had a protective effect aganist obesity and T2DM in HFD mice, it also boosted adiponectin expression while decreasing leptin expression.[70]

Since obesity is the main contributor to chronic low-grade inflammation, adipose tissue can upregulate pro-inflammatory interleukins by accumulating excess lipids. These interleukins may facilitate immune cell infiltration into adipose tissue, leading to low-grade inflammation and abnormal adipocyte function.[12] The primary source of local and systemic inflammatory mediators including IL-6, IL-1β, and TNF-α is thought to be adipose tissue macrophages. These cytokines produce insulin resistance *via* triggering cytokine suppressors signaling proteins.[71]

IL-1 is a master cytokine because of its involvement in local and systemic inflammation. Overexpression of IL-1β in adipose tissues leads to infiltrating of immune cells which results in low-grade inflammation[12] Circulating levels of IL-6 could be an indicator of how severe the systemic and ongoing chronic inflammation that results from severe obesity. Pro-inflammatory cytokine IL-6 suppresses lipoprotein lipase activity and increases endothelium lipase's lipolytic activity, both have been linked to low HDL-C values during acute or chronic inflammatory conditions.[72] In the current study, high levels of IL-6 and IL-1β in obese rats were significantly reduced upon treatment with either *A. nilotica* stem barks extracts, especially the ethyl acetate fractions. The anti-inflammatory effect was supported by improvements in the histopathological findings among the groups that received treatment. In a mouse model of obesity-induced by HFD, epicatechin supplementation reduced the activation of pro-inflammatory signaling pathways such as c-Jun N-terminal kinase, NF-κB, as well as macrophage recruitment.[70]

nuclei. Adipocytes are arranged regularly with average size. **(P)** & **(Q)** Butanol group (250 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral nuclei. Adipocytes are arranged regularly with average size. **(R)** & **(S)** Butanol group (500 mg/kg) group (100*x* and 400*x*, respectively); section in adipose tissue showing many adipocytes (arrow) with thin cell membranes and small regular peripheral nuclei. Adipocytes are arranged regularly with average size.

Epicatechin reduced cypermethrin-induced inflammation in rats by significantly lowering plasma TNF-α and IL-6 levels, possibly through inactivating the NF-κB and mitogen-activated protein kinase (MAPK) pathways.[59,73,74]

Gossypin is another *A. nilotica* flavonoid with potent immunomodulatory and anti-inflammatory effects; increased IL-1, IL-6 and TNF levels were lowered after gossypin administration.[75] Gossypin suppresses the inflammatory pathway by decreasing NF-κB immunopositivity.[76,77] It was found to inhibit the production of IL-6, and expression of cyclooxygenase-2 COX-2 in bone marrow-derived mast cells, supporting taxifolin's anti-inflammatory properties.[13]

Kaempferol was suggested to reduce chronic inflammation and oxidative stress, according to previous studies.[78] Furthermore, kaempferol significantly reduced neutrophil and macrophage infiltration, this was followed by a decline in cytokines such as IL-6 and TNF-α indicating an anti-inflammatory effect.[47]

5. Conclusion

In conclusion, this study emphasizes the potent anti-obesity, antioxidant, and anti-inflammatory effects of the ethyl acetate fraction of the stem bark of *A. nilotica* in a HFD rat model. The current work provided evidence that the ethyl acetate fraction of *A. nilotica* was effective in preventing and decreasing obesity, insulin resistance and hyperlipidemia in diet-induced obesity in rats. These effects may be due to reduced cytokine production, inhibition of leptin resistance and boosting adiponectin, which functions as an anti-adipogenic and anti-obesity agent. However, more investigation is needed to examine the clinical safety and efficacy data with a variety of doses to detect its efficacy in the treatment of obesity and insulin resistance. Also, further qualitative and quantitative verification of the definite pure constituents of the promising *A. nilotica* stem bark ethyl acetate fraction is strongly recommended.

CRediT authorship contribution statement

ARH and MKE designed the study. OAS, ARH and MKE performed the laboratory experiments. OAS, SSK and ETM analyzed the data. OAS, SSK and ETM wrote the original manuscript. SSK and ETM revised the manuscript and prepared the final version. All authors read and approved the final manuscript.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and material

The research data is available upon request from the corresponding author.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

We acknowledge the support of Prof. Azza El Hadidy, Professor of Plant Taxonomy and Flora, Faculty of Science, Cairo University, for the excellent plant handling and maintenance. We also are grateful to Prof. Dina Abo-elmatty, Professor of Biochemistry, Faculty of Pharmacy, Suez Canal University, and Prof. Noha Mesbah, Professor of Biochemistry, Faculty of Pharmacy, Suez Canal University, for their kind guidance and support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2023.03.005.

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