



Targeted metabolomics reveals the therapeutic impact of *Eclipta prostrata* on diet-induced non-alcoholic fatty liver disease in rats

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a growing clinical problem worldwide. The current study aimed to evaluate the therapeutic effect of the methanol extract of *Eclipta prostrata* (*E. prostrata*) in rats using gas chromatography–mass spectrometry (GC–MS) followed by multivariate and univariate data analysis. Phytochemical study of *E. prostrata* extract was performed using chemical, colorimetric, and high-performance liquid chromatography (HPLC) analysis. Forty-two Wistar albino rats divided into seven groups (six in each) were used in this study. Fatty liver was induced in rats using a high-fat diet together with cholesterol and cholic acid, then they were treated with different doses of *E. prostrata*. The histopathological examination of liver and NAFLD activity score showed significant markers for the diagnosis of the early stage of the disease. Treatment with high doses of *E. prostrata* (300 and 200 mg/kg BW) extract exhibited significant improvement in liver function and lipid profile in rats bearing NAFLD. Lipidomic analysis showed a discrete variability in the levels of some metabolites, including cholest-5-en-3-ol (3 β)–acetate; cholesta-4, 6-dien-3-ol, (3 β); urea; 1,3 dipalmitin; glycerol; linoleic acid; and arachidonic acid in sera of rats bearing NAFLD in contrast to healthy ones. Treatment with *E. prostrata* extract showed noticeable amelioration in the levels of these metabolites. The present results concluded that *E. prostrata* in high doses (300 and 200 mg/kg BW) had the potential efficacy to ameliorate NAFLD. Nevertheless, lipidomics could be used for the early detection of NAFLD and in the assessment of the mechanism of herbal medications.

INTRODUCTION

Approximately 35% of the world population is affected by fatty liver disease (Haga *et al.*, 2015). The progression of non-alcoholic fatty liver disease (NAFLD) begins with steatosis, steatohepatitis, fibrosis, and finally cirrhosis (Bellentani *et al.*, 1994). Numerous habits cause an accumulation of fats on the liver, such as excess consumption of fats daily and/or increased lipolysis in adipose tissue under insulin-resistant conditions. This leads to the higher availability of circulating non-esterified fatty acids, inappropriate synthesis and export of very low-density lipoproteins,

and increased *de novo* fatty acid synthesis (Diraison *et al.*, 2003). Advanced diagnostic approach to avoid the requirements for invasive procedures is demanded for novel management strategies for the growing epidemic NAFLD (Gorden *et al.*, 2015).

Lipidomics produces large-scale lipid classes with distinctive molecular species in a biological sample (Puri *et al.*, 2007). Lipidome analysis was used to identify lipid classes and allocation of fatty acids within these classes in the liver (Wolf and Quinn, 2008). Zehethofer and Pinto (2008) found the significance of lipidome in the pathogenesis of cancer. Plasma lipids metabolites were conducted in NAFLD and the findings showed a significant increase in total plasma monounsaturated fatty acids driven by palmitoleic (16:1 n7) and oleic (18:1 n9) acids (Puri *et al.*, 2009).

Recently, the use of the herbal natural product as therapeutic agents has gained more interest in the world (Mushtaq *et al.*, 2018). Among those herbs is *E. prostrata* (Asteraceae)

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which has high medical value (Fakurazi *et al.*, 2008; Sun *et al.*, 2010). Many chemical constituents such as thiophenes, coumarins, triterpenoid, saponins, steroids, and flavonoids have been isolated from *E. prostrata* (Kumari *et al.*, 2006; Yuan *et al.*, 2009). As the liver is the main organ in the synthesis and metabolism of lipid, *E. prostrata* can act on the liver by promoting the metabolism and accelerating the excretion of excess lipids leading to the hypolipidemic condition (Prabu *et al.*, 2011).

So, the present study applied a lipidomic approach to detect a reproducible signature of lipid metabolites for early detection and characterization of NAFLD. In addition, to investigate the possible mechanisms of *E. prostrata* administration in improving NAFLD *via* amelioration of certain metabolites in comparison with a well-known lipid-lowering agent (Lipanthyl®).

MATERIALS AND METHODS

Ethics and permissions

The procedures of the study protocol were ethically approved by Ethics Review Committee of the National Research Centre in Cairo, Egypt, which provided that the animals will not suffer at any stage of the experiment (Approval no: 13002) according to ARRIVE guidelines (Kilkenny *et al.*, 2010).

Biological materials

Eclipta Prostrata (aerial part) was purchased from Horticultural Research Institute, Agriculture Museum. The voucher specimen was deposited in the Herbarium of National Research Centre, Cairo, Egypt under No. 997.

Experimental animals: Adult male Wistar albino rats (42) aged 5–6 weeks and weighing 160–180 g were obtained from the Animal House at National Research Centre, Egypt. The experimental animals were housed in wire cages and were maintained under standard conditions (temperature, 22°C ± 5°C, humidity, 55% ± 5%, and a 12 hour light/dark cycle). The animals had access to standard laboratory feed and water *ad libitum* for 7 days as an acclimatization period before starting the experiments.

Chemicals

N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% (vol/vol) trimethylchlorosilane (TMS), Methyl *tert*-butyl ether (MTBE), Methanol (HPLC), n-alkane (C8–C40) standard, and docosanol (an internal standard for relative quantification using GC/MS) were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol powder was purchased from Bio Basic Canada Inc. and cholic acid was purchased from Loba Chemie. All commercially available assay kits for the determination of serum liver function [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] and lipid profile were purchased from Bio Med (Cairo, Egypt). Standard diet was prepared according to the American Institute of Nutrition (Reeves *et al.*, 1993). Thirteen standards including gallic acid, catechin, caffeic acid, syringic acid, rutin, coumaric acid, vanillin, ferulic acid, naringenin, quercetin, cinnamic acid, propyl gallate, and dihydroxy isoflavone were purchased from Sigma, Aldrich (UK) to be used as flavonoids and phenolic authentic reference.

Experimental design

Preparation and phytochemical study of *E. prostrata* methanol extract

Dry *E. prostrata* aerial part (2 kg) was crushed and immersed in 70% aqueous methanol solution (6 l) for 15 days. The insoluble mass was filtered out and soluble extract was concentrated with a vacuum rotary evaporator (HeidolphHei-VAP, Germany).

Phytochemical study of the dry methanol extract was performed. The triterpenes content was determined using the vanillin-perchloric acid colorimetric method (Mroczek *et al.*, 2012). For tannins, ferric chloride reagent was used (Banso and Adeyemo, 2006) and alkaloids were measured using Mayer's reagent and Iodine/KI reagent (Abdullahi, 2013; Joshi *et al.*, 2013). Molisch's reagent was used for carbohydrates and glycosides determinations (Joshi *et al.*, 2013). Total phenolic and flavonoids contents were estimated according to Kim *et al.* (2003); Singleton and Rossi (1965), respectively. For the quantitative determination of certain flavonoids and polyphenols, HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using a C₁₈ column (4.6 mm × 250 mm id, 5 μm). The mobile phase consisted of water (A) and 0.02% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/minute. The mobile phase was programmed consecutively in a linear gradient as follows: 0 minute (80% A); 0–5 minutes (80% A); 5–8 minutes (40% A); 8–12 minutes (50% A); 12–14 minutes (80% A); and 14–16 minutes (80% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35°C.

Also, Wedelolactone, the major coumarin in the dry methanolic extract of *E. prostrata*, was analyzed using HPLC. The standard control was dissolved in HPLC-grade methanol. The chromatographic analysis was performed at room temperature with a Cosmosil 5 C18-MS-II (250 mm × 4.6 mm) column using 60% methanol as the eluent with a flow rate of 0.9 ml/minute as described by Zhao *et al.* (2015).

Therapeutic effect of *E. prostrata*

Forty-two rats were subsequently divided into three main groups as follows:

- Negative control group: Six healthy rats received standard chow (SC) and tap water *ad libitum*.

- Positive control group: Six normal rats were fed a high-fat diet containing SC supplemented with cholesterol (1%), cholic acid (0.2%), and lard (10%) for 8 weeks as described by Ney *et al.* (1988).

- Treated group: Thirty normal rats were fed a high-fat diet containing SC supplemented with cholesterol, cholic acid, and lard for 8 weeks. Rats in this group were divided into five subgroups (six in each) to be treated orally by a gavage for extra 4 weeks, with methanol extract of *E. prostrata* after dissolving in distal water at doses 300, 200, 100, and 50 mg/kg body weight as described by Dhandapani (2007); and lipanthyl® drug 300 mg at a dose 5.35 mg/200 g BW rat (Bhardwaj and Gupta, 2012), respectively.

At the end of the experimental period, blood samples were collected from the eyes of all rats. Samples were placed in sterilized tubes for serum separation by centrifugation at 5,000 rpm for 15 minutes. The obtained sera samples were then stored at -80°C for biochemical and lipidomic analysis. Animals were then decapitated and livers were removed for histopathological examination.

Biochemical analyses

Liver function enzymes (AST and ALT) were analyzed according to Bergmeyer *et al.* (1976) and lipid profile [total cholesterol (TC), triacylglycerides (TAGs) and high-density lipoprotein cholesterol (HDL-CH)] were analyzed in sera samples as described by Castelli *et al.* (1977); Fossati and Prencipe (1982); Reitman and Frankel (1957), respectively, while low-density lipoprotein cholesterol (LDL-CH) was calculated according to Friedewald *et al.* (1972).

Histopathological examination

Left lobe liver sections from all rats in different groups were isolated and their preparation were carried out as demonstrated by Smith and Bruton (1978) for histopathological examination to confirm the induction of NAFLD in rats and the therapeutic effect of the methanol extract of *E. prostrata* in comparison with lipanthyl. Liver sections were rapidly washed with ice-cooled saline, then placed in 10% formalin saline for 24 hours, and processed routinely for embedding in paraffin. Sections of 4 μm were stained with Hematoxylin & Eosin (H&E) and Masson's Trichrome. These sections were examined under light microscope followed by the calculation of NAFLD activity score (NAS) based on the individual scores of steatosis (0–3 points), lobular inflammation (0–3 points), and hepatocyte ballooning (0–2 points) to assess the severity of NAFLD (Straub and Schirmacher, 2010). A score of <4 was considered as steatosis

Lipidome analysis using GC–MS

Serum lipidome

Lipid was extracted from sera samples according to the method described by Matyash *et al.* (2008). In brief, 200 μl of serum from studied groups or quality control sample (prepared by equal volumes of sera from all studied groups) mixed with 1.5 ml of methanol (98%) containing 5 μl docosanol (1 mg/ml, internal standard) and vortex for 5 minutes. Samples were centrifuged for 10 minutes at 5,000 rpm and 4°C , then 5 ml of MTBE was added to each tube, and samples were shaken for 1 hour at room temperature. Subsequently, 1.25 ml of MS-grade H_2O was added to each tube, mixed, and allowed to stand for 10 minutes at room temperature, then centrifugation was done at 5,000 rpm for 10 minutes to separate two phases. The upper phase was transferred into a separate tube and dried by nitrogen gas. For lipid metabolites derivatization, a volume of 100 μl MSTFA containing 1% TMS was added to the dried lipid pellets and incubated at 70°C for 30 minutes (Ammar *et al.*, 2017). Agilent Technologies (GC–MS system) was equipped with a gas chromatograph (7890B) and mass spectrometer detector (5977A) with HP-5MS column

(30 m \times 0.25 mm internal diameter and 0.25 μm film thickness) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The carrier gas was helium which was used to perform analyses at a flow rate of 1.0 ml/minute, the injection volume was 1 μl at a splitless mode with temperature program: 80°C for 2 minutes; rising at $5^{\circ}\text{C}/\text{minutes}$ to 300°C and held for 5 minutes. The injector and detector were held at 280°C and 300°C , respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of 25–550 m/z and solvent delay time 3.7 minutes.

For metabolites identification: Deconvolution of the detected mass spectra was performed using automated mass spectral deconvolution and identification system (AMDIS 2.64) software and national institute of standards and technology (NIST) library for identification of measured peaks *via* measuring the matching against reference compounds already registered. Standard fatty acids mixture (Sigma, Aldrich) was injected for confirming peak assignments. Retention indices (RI) were calculated relative to n-alkanes ($\text{C}_8\text{--C}_{40}$) standards.

Data processing for multivariate analysis

The multivariate analysis for identified metabolites was performed using MetaboAnalyst—a comprehensive tool suite for metabolic data analysis—online software (<http://www.metaboanalyst.ca>) (Xia *et al.*, 2009). Metabolites MS signals were normalized using the reference sample quality control. Data transformation is done using log transformation, especially “Pareto” scaling option prior to multivariate data analysis. After that, both unsupervised principal component analysis (PCA) and supervised partial least square-discriminant analysis (PLS-DA) were performed using this program.

Statistical analysis

Biochemical data analysis was performed *via* GraphPad Prism ver. 6 using the one-way analysis of variance (ANOVA). Nonparametric (Mann–Whitney test) and Kruskal–Wallis test *via* SPSS version 17 were used for analysis of lipidomic metabolites.

RESULTS

Phytochemical study of *E. prostrata*

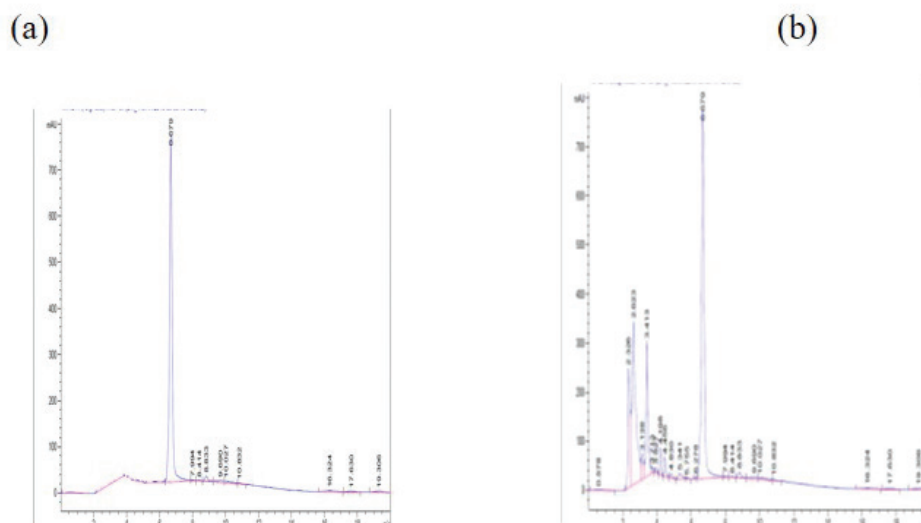
The extract yield was 28.5% (w/w) in terms of dry starting material and the phytochemical study revealed that the air dried powdered of *E. prostrata* (aerial part) is rich in glycosides, triterpenes, tannins, alkaloids, and coumarins. Also, its total content of polyphenols and flavonoids was 187.75 mg/g gallic extract and 144.04 mg/g rutin extract, respectively.

Data analysis by Agilent software showed the constituents of the extract as listed in Table 1. The major flavonoids were rutin and quercetin which represented 4.78 and 1.93 mg/g dry weight, respectively and the major phenolic compound was a gallic acid which represented 2.12 mg/g dry weight of the total extract.

Wedelolactone is the characteristic chemical coumarins constituent of *E. prostrata* with average content 5.00 mg/g dry weight. HPLC analysis of the wedelolactone standard showed a single peak at 350 nm with a retention time of approximately 7 minutes, as shown in Figure 1a and b.

Table 1. Polyphenols and flavonoids content of methanolic extract of *E. prostrata* using HPLC.

| | Polyphenols | | | | | Flavonoids | | | | |
|--------------|-------------|--------------|---------------|----------|---------------|----------------|-------|------------|-----------|---------------------|
| | Gallic Acid | Coffeic Acid | Syringic Acid | Vanillin | Cinnamic Acid | Propyl Gallate | Rutin | Naringenin | Quercetin | DihydroxyisoFlavone |
| R.T (minute) | 3.18 | 4.81 | 5.16 | 8.22 | 11.1 | 10.17 | 5.61 | 9.12 | 10.59 | 10.50 |
| Conc. (mg/g) | 2.12 | 0.24 | 0.17 | 0.58 | 0.09 | 0.09 | 4.78 | 0.73 | 1.93 | 0.10 |
| % | 64.43 | 7.3 | 5.2 | 17.63 | 2.74 | 2.74 | 63.40 | 9.68 | 25.6 | 1.33 |

**Figure 1.** HPLC result of wedelolactone in standard (a) and *E. Prostrata* methanol extract (b).**Table 2.** Biochemical analysis of blood samples of healthy, NAFLD, and treated groups.

| Parameters Groups | Liver function | | Lipid profile | | | | |
|---------------------------|---------------------------|---------------------------|----------------------------|----------------------------|---------------------------|----------------------------|---------------------------|
| | AST (U/l) | ALT (U/l) | TC (mg/dl) | TAG (mg/dl) | HDL-C (mg/dl) | LDL-C (mg/dl) | HDL-C/LDL-C ratio |
| Negative control | 31.16 ^a ± 0.42 | 20.20 ^a ± 0.67 | 96.00 ^a ± 0.83 | 62.45 ^a ± 0.93 | 46.69 ^a ± 0.38 | 36.82 ^a ± 1.28 | 1.26 ^a ± 0.04 |
| Positive control | 95.46 ^b ± 0.47 | 43.14 ^b ± 0.61 | 200.18 ^b ± 1.05 | 185.22 ^b ± 0.62 | 23.11 ^b ± 0.48 | 140.07 ^b ± 0.96 | 0.16 ^b ± 0.003 |
| (%Change) | 206.35 | 113.54 | 108.53 | 196.59 | -50.50 | 280.4 | -87.30 |
| Treated Group | | | | | | | |
| Lipanthyl | 52.13 ^c ± 0.66 | 26.28 ^c ± 0.53 | 125.11 ^c ± 0.56 | 85.00 ^c ± 0.53 | 37.86 ^c ± 0.49 | 70.14 ^c ± 0.93 | 0.54 ^c ± 0.03 |
| (%Change) | 67.29 | 30.10 | 30.32 | 36.11 | -18.91 | 90.58 | -57.14 |
| <i>E. prostrata</i> (300) | 54.02 ^c ± 0.57 | 28.34 ^c ± 0.46 | 139.20 ^d ± 0.51 | 94.19 ^d ± 0.57 | 33.08 ^d ± 0.46 | 87.28 ^d ± 0.76 | 0.37 ^d ± 0.02 |
| | 73.36 | 40.30 | 45.00 | 50.82 | -29.15 | 137.05 | -70.63 |
| <i>E. prostrata</i> (200) | 73.07 ^d ± 0.56 | 32.37 ^d ± 0.83 | 158.34 ^e ± 0.46 | 102.38 ^e ± 0.69 | 30.85 ^d ± 0.38 | 107.01 ^e ± 0.54 | 0.29 ^e ± 0.01 |
| | 134.50 | 60.25 | 64.94 | 63.94 | -33.93 | 190.63 | -76.98 |
| <i>E. prostrata</i> (100) | 80.64 ^e ± 0.39 | 36.24 ^e ± 0.61 | 170.22 ^f ± 0.52 | 128.26 ^f ± 0.56 | 28.14 ^e ± 0.58 | 116.21 ^f ± 0.33 | 0.24 ^f ± 0.01 |
| | 158.79 | 79.41 | 77.31 | 105.38 | -39.73 | 215.62 | -80.95 |
| <i>E. prostrata</i> (50) | 88.38 ^f ± 0.54 | 39.29 ^f ± 0.47 | 185.70 ^g ± 0.88 | 140.33 ^g ± 0.40 | 26.23 ^e ± 0.27 | 131.40 ^g ± 0.79 | 0.20 ^g ± 0.003 |
| | 183.63 | 94.50 | 93.44 | 124.71 | -43.82 | 256.87 | -84.13 |

Data are represented in this table as Mean ± SE, different letters are significant at $p \leq 0.05$.

Biochemical assay

Toxicity assay of *E. prostrata* is carried out and the results reported its safety up to 600 mg/kg BW. Data listed in Table 2 showed a significant elevation in the levels of ALT, AST, TC, triacylglycerides (TAGs), and LDL-C. The results also showed depletion in the level of HDL-C and HDL/LDL-C ratio in the serum of rats bearing NAFLD. It is also noticed that liver enzymes and lipid profile were markedly ameliorated in treated animals with high and moderate doses of *E. prostrata* compared with the standard drug lipanthyl.

Serum metabolites profiling

A total of 35 serum metabolites including [free fatty acids (FFAs), glycerolipids, sterols, oxysterols, cholesteryl ester, and sugars], its retention times (Rt), RI, and the mass-to-charge ratio (m/z) were represented in Supplementary Table 1. The percentage of different lipid types in normal healthy and NAFLD groups were represented in Figure 2. PCA was first applied to the GC/MS dataset with score plot (Supplementary Figure 1) showing somewhat not clear segregation of sample groups. The first two components (PC1 and PC2) explained 26.2% and 20.2% of the total

variance, respectively. Consequently, a supervised data analysis method was adopted to derive better samples classification. PLS-DA was applied. PLS-DA score plot (Fig. 3a) showed better and more clear discrimination among sample groups with R^2 value (0.69) and Q^2 value (0.56).

Metabolites contributing for groups segregation revealed from variable importance in projection (VIP) score plot (>1) (Fig. 3b) include cholest-5-en-3-ol (3 β)- acetate, urea, cholesta-4,6-dien-3-ol, (3 β)-, myristic acid 1, 1,3-dipalmitin, glycerol, palmitic acid, 2-monostearin, myristic acid, and stearic acid. The levels of these metabolites were different in all studied groups.

For further separation among groups, the Hierarchical Clustering/Dendrograms model was displayed as shown in Figure 3c. This Dendrograms model showed that fatty liver group was located far from the normal group, while treated groups were found in between and suggested that the dose 300 mg/kg had the most ameliorative effect against NAFLD after lipanthyl group. PLS-DA model and its derived VIP score plot were further employed to identify metabolites markers related to NAFLD by modeling healthy control versus non-alcoholic fatty liver rats (Fig. 4a and b). The PLS-DA model exhibits R^2 value (0.98) and Q^2 value (0.96), with control and NAFLD groups being clearly discriminated from each other. Cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β)-, urea, glycerol, and 1,3-dipalmitin were most elevated in NAFLD group, whereas arachidonic acid and linoleic acid were detected at lower levels as compared to the healthy control one.

PLS-DA model encompassing the three groups: normal control, NAFL untreated, and lipanthyl drug-treated groups were attempted (Fig. 5a and b). Score plot showed discrimination between NAFL group and normal control, with lipanthyl treated animal group clustering closer to normal control. The corresponding VIP score plot (Fig. 5b) showed decreased levels of cholest-5-en-3-ol (3 β)-acetate, cholesta-4,6-dien-3-ol, (3 β)-, glycerol, urea, 1,3-dipalmitin, and 2-monostearin concurrent with increasing in linoleic acid levels in the serum lipanthyl drug group compared with NAFL group. On the other hand, treated groups of *E. prostrata* at doses 300, 200, 100, and 50 mg/kg BW were found also the ones that clustered most closely to the control group in the PLS-DA plot. Consequently, another PLS-DA plots were constructed encompassing untreated

NAFLD group, normal control with different doses of *E. prostrata* (300, 200, 100, 50 mg/Kg BW) separately as shown in [(Suppl. Fig.S2a), (Suppl.Fig.S3a), (Suppl.Fig.S4a) and (Suppl.Fig.S5a), respectively]. Metabolites observed from VIP score plot in treated group administered *E. prostrata* at dose 300 mg/kg BW model revealed a decrease in cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β)-, glycerol, urea, 2-monostearin, and an increase in arachidonic and linoleic acids (Supplementary Figure 2b) as compared with NAFLD group. While, metabolites observed from VIP score plots in treated groups administered *E. prostrata* at doses 200,100, and 50 mg/kg BW model revealed a decrease in cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β)-, urea, glycerol, 1,3-dipalmitin, 2-monostearin, and an increase in arachidonic acid and linoleic acid levels (Supplementary Figures 3b, 4b, and 5b), respectively as compared with NAFLD group. Fold change of these metabolites was performed using univariate data analysis of Metabo-Analyst (Table 3).

Also, non-parametric Kruskal–Wallis test pointed out that cholest-5-en-3-ol (3 β)- acetate, cholesta-4, 6-dien-3-ol, (3 β), urea, 1, 3-dipalmitin, glycerol, linoleic acid, arachidonic acid, and cholesterol at ($p \leq 0.05$) are the most impacted metabolites between all studied groups and suggested for discrimination between healthy and NAFLD ones. From these results, there are seven metabolites common in multivariate analysis using PLS-DA and univariate analysis using Kruskal–Wallis test that (cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β), urea, 1,3 dipalmitin, glycerol, linoleic acid and arachidonic acid). By following the PLS-DA VIP score plots derived results, fold change suggested a significant decrease ($P \leq 0.05$), using non-parametric Mann-Whitney test, in arachidonic and linoleic acids levels versus an increase in cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β), urea, 1,3 dipalmitin and glycerol amounts in NAFLD group as compared to healthy group. The group administered lipanthyl drug and all doses of *E. prostrata* methanol extract showed a marked increase in arachidonic and linoleic acids levels versus a decrease in cholest-5-en-3-ol (3 β)- acetate, cholesta-4,6-dien-3-ol, (3 β), urea, 1,3 dipalmitin, glycerol compared to NAFLD group gradually.

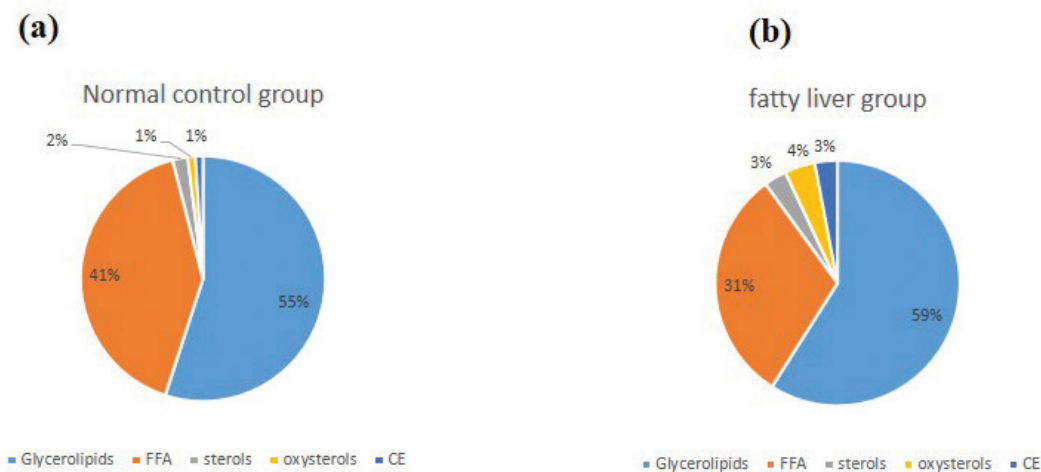


Figure 2. The percentages of different lipid types in normal healthy control group (a) and fatty liver group (b). FFA = free fatty acids; CE = cholesteryl ester.

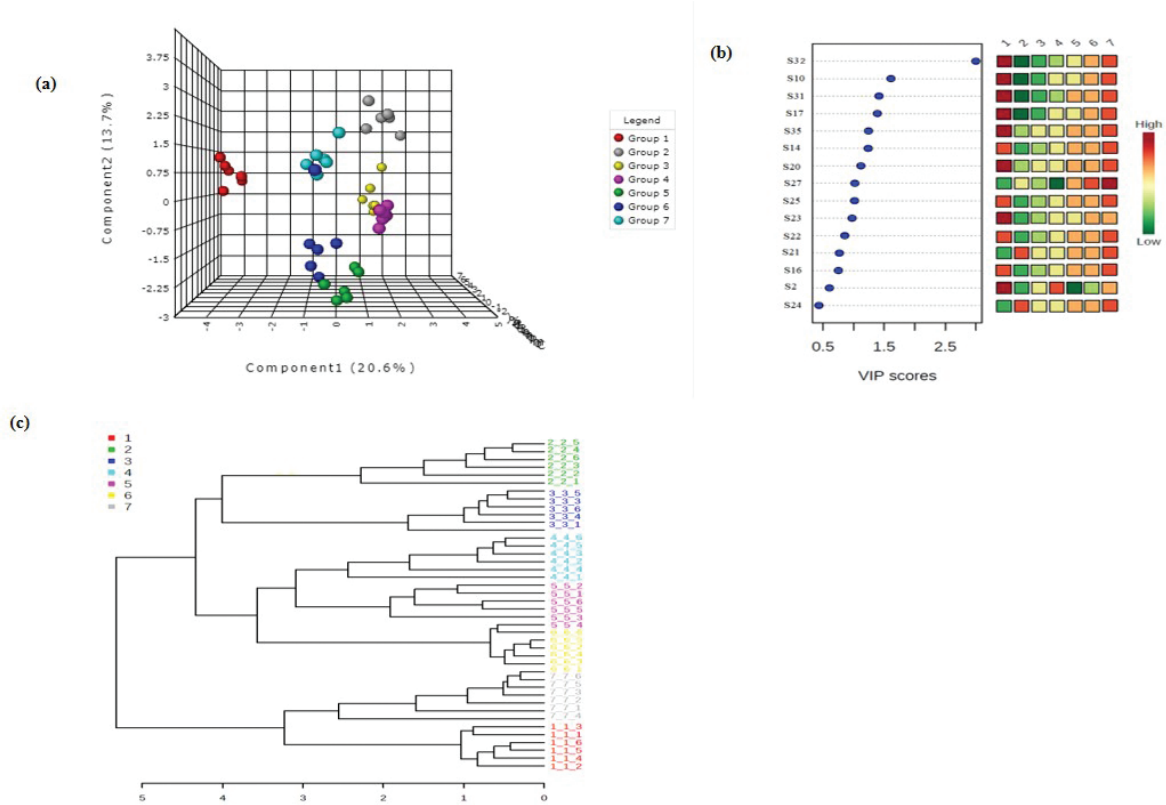


Figure 3. PLS-DA score plots obtained from modeling serum metabolites in the different groups. (a) The score plot showing a separation of healthy control animals, NAFLD rats, and treated groups. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S10: Urea, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S17: Myristic acid 1, S35: 1,3-Dipalmitin, S14: Glycerol, S20: Palmitic acid, S27: 2-Monostearin, S25: Myristic acid2, S23: Stearic acid. Peaks numbering follow that listed in [Supplementary Table 1](#) for metabolite identification using GC–Ms. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study. (c) Hierarchical Clustering/Dendrograms of the six groups with fatty liver group showing separation from the normal control group and treated groups in serum samples. Sample codes are presented as follows: (1) fatty liver group, (2) healthy control, (3) standard drug lipanthyl, and extract doses: 300 mg/kg BW (4), 200 mg/kg BW (5), 100 mg/kg BW (6), and 50 mg/kg BW (7).

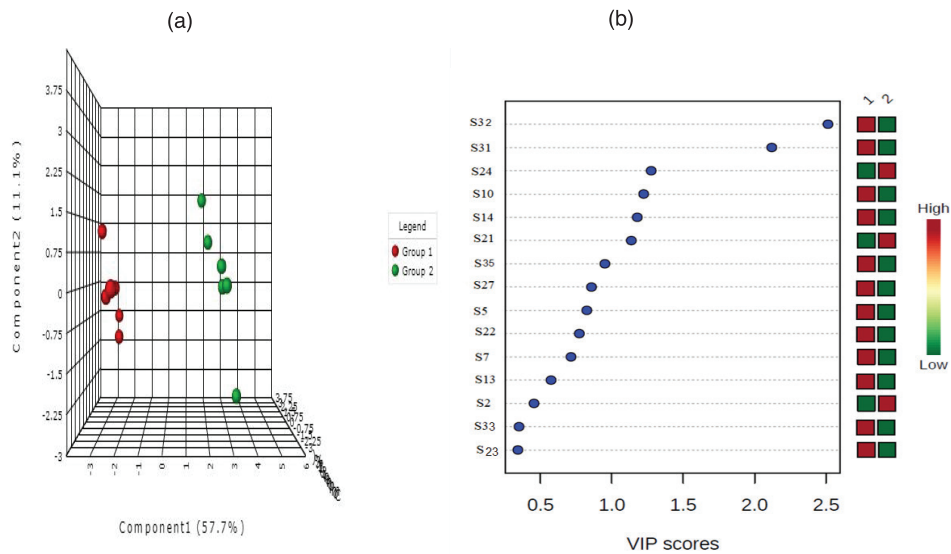


Figure 4. PLS-DA score plots obtained from GC–MS data by modeling NAFLD (1) versus healthy control groups (2) against each other. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S24: Arachidonic acid, S10: Urea, S14: Glycerol, S21: Linoleic acid, S35: 1,3-dipalmitin. Peaks numbering follow that listed in [Supplementary Table 1](#) for metabolite identification using GC–Ms. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study.

Histopathology examination

Liver sections showed distinct differences between healthy control and fatty liver groups and how these changes were gradually eliminated after the administration of the methanolic extract of *E. prostrata* as compared with standard drug lipanthyl. Results pointed out that the most effective dose of *E. prostrata* extract was 300 mg/kg BW. The liver sections in the control group showed hepatic tissue with preserved (intact) lobular hepatic architecture and normal morphological appearance (H&E, $\times 400$) and (Masson Trichrome, $\times 400$). In contrast, the NAFLD group showed preserved (intact) lobular hepatic architecture, hepatocyte ballooning, severe micro and macrovesicular steatosis (black arrows), and moderate lobular inflammation [H&E, $\times 400$ (Masson Trichrome, $\times 400$)] as shown in Figure 6a and b, leading to about 12-fold change ($p < 0.001$) increase in NAS. This value was significantly decreased in all treated groups as compared with NAFLD group, as shown in Figure 6c, due to decreasing hepatocyte ballooning and micro and macrovesicular steatosis. NAS calculation is shown in Figure 6c. Masson Trichrome staining showed no evidence of fibrosis. These results reported that the current study was performed during the early stages of NAFLD where NAS equal 3.5 and it can be prevented from progression through administering *E. prostrata* methanolic extract.

DISCUSSION

In the present study, ALT and AST were measured as markers of liver injury and results showed that they could be used as surrogate indicators of NAFLD with lipid profile. These illustrations give some support to the findings of Sanyal *et al.* (2015) who pointed out that NAFLD was the most common cause

of abnormal liver biochemistry. ALT and AST showed significant amelioration in groups treated with *E. prostrata*. The therapeutic activity of *E. prostrata* could be due to its active constituents (flavonoids, alkaloids, tannins, triterpenes, and coumarins) which have free radicals scavenging properties and indirectly increase the antioxidants levels by decreasing the levels of lipid peroxidative products (Arun and Balasubramanian, 2011).

The study of Bravo *et al.* (2011) pointed out that feeding rats with a high-fat diet induce insulin resistance, hypertriglyceridemia, hepatic steatosis, and liver damage as characteristic features of NAFLD. In our study, the enhancement in the lipid profile of treated rats with *E. prostrata* might be attributed to the lipid-lowering activity of this plant which is linked with the down-regulation of “hydroxy-3-methylglutaryl-CoA reductase (HMGR),” a key enzyme in cholesterol biosynthesis and the up-regulation of peroxisome proliferator-activated receptor α (PPAR α) (Tammela *et al.*, 2005), low density lipoprotein receptor (Arun and Balasubramanian, 2011), lecithin-cholesterol transferase (Rader *et al.*, 2009), and scavenger receptor class B type I receptor SR-BI, which is a well-known HDL receptor (Kozarsky *et al.*, 1997).

In this study, NAFLD was associated with perturbations in lipid metabolic pathways in terms of FFAs and elevation in glycerolipids (1,3-dipalmitin and glycerol), oxysterols [cholesta-4,6-dien-3-ol, (3 β)], cholesteryl ester (cholest 5-en-3ol-3 acetate), and urea.

The present study indicated lower levels of linoleic acid (18:2 n6) and arachidonic acid (20:4 n6) in NAFLD group which may be due to their consumption for producing eicosanoids. These eicosanoids play a vital role in the progression of hepatotoxicity (Maciejewska *et al.*, 2015; Puri *et al.*, 2007).

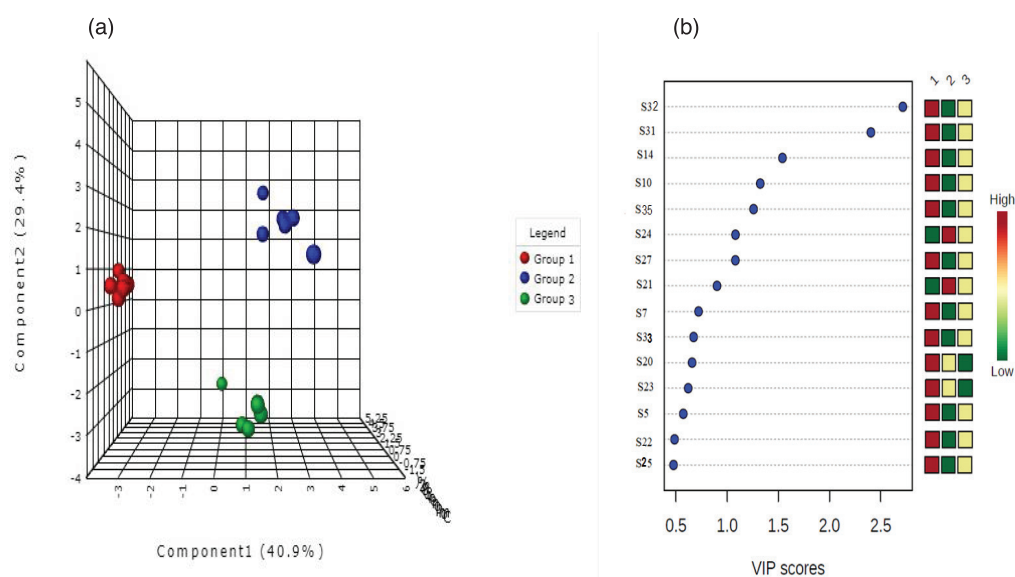


Figure 5. PLS-DA score plots obtained from modeling NAFLD (1) healthy control (2) and (3) lipanthyl drug-treated groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S14: Glycerol, S10: Urea, S35: 1,3-dipalmitin, S27: 2-Monostearin, S21: Linoleic acid. Peaks numbering follow that listed in Supplementary Table 1 for metabolite identification using GC–Ms. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

Table 3. The common metabolite level alterations according to PLS-DA score (VIP>1), Kruskal-Wallis test analysis and fold change in all treated experimental groups.

| Metabolites | Fold change | | | | | |
|---|-------------|-------|-------|-------|-------|-------|
| | F/N | D/F | 300/F | 200/F | 100/F | 50/F |
| ^a Urea, S10 | *6.39 | *0.28 | *0.42 | *0.53 | *0.74 | *0.87 |
| ^a Glycerol, S14 | *4.05 | *0.37 | *0.50 | *0.67 | *0.80 | *0.87 |
| ^a Linoleic acid, S21 | *0.12 | *5.43 | *4.51 | *2.97 | *1.89 | *1.44 |
| ^a Arachidonic acid, S24 | *0.15 | *4.18 | *3.28 | *2.58 | *1.80 | *1.43 |
| ^a Cholesta-4,6-dien-3-ol, (3 β)-, S31 | *4.75 | *0.45 | *0.53 | *0.62 | *0.69 | *0.79 |
| ^a Cholest 5 -en-3ol -3 β acetate, S32 | *6.42 | *0.32 | *0.47 | *0.63 | *0.77 | *0.88 |
| ^a 1,3-dipalmitin, S35 | *3.38 | *0.37 | *0.48 | *0.58 | *0.69 | *0.78 |

Fold change was performed by using Metabo-Analyst ver. 3; the Statistical comparisons equal non-parametric Mann-Whitney test was performed. Results were considered only significant if $P \leq 0.05$. ^a: denotes for metabolites showing significant using Kruskal-Wallis test analysis. *: denotes for metabolites showing significant difference among treated, normal and fatty liver groups. Sample codes are presented as follows normal healthy (N), drug (D), fatty liver (F), 300, 200, 100, and 50 mg/Kg BW (different dosage of *E. prostrata* extract).

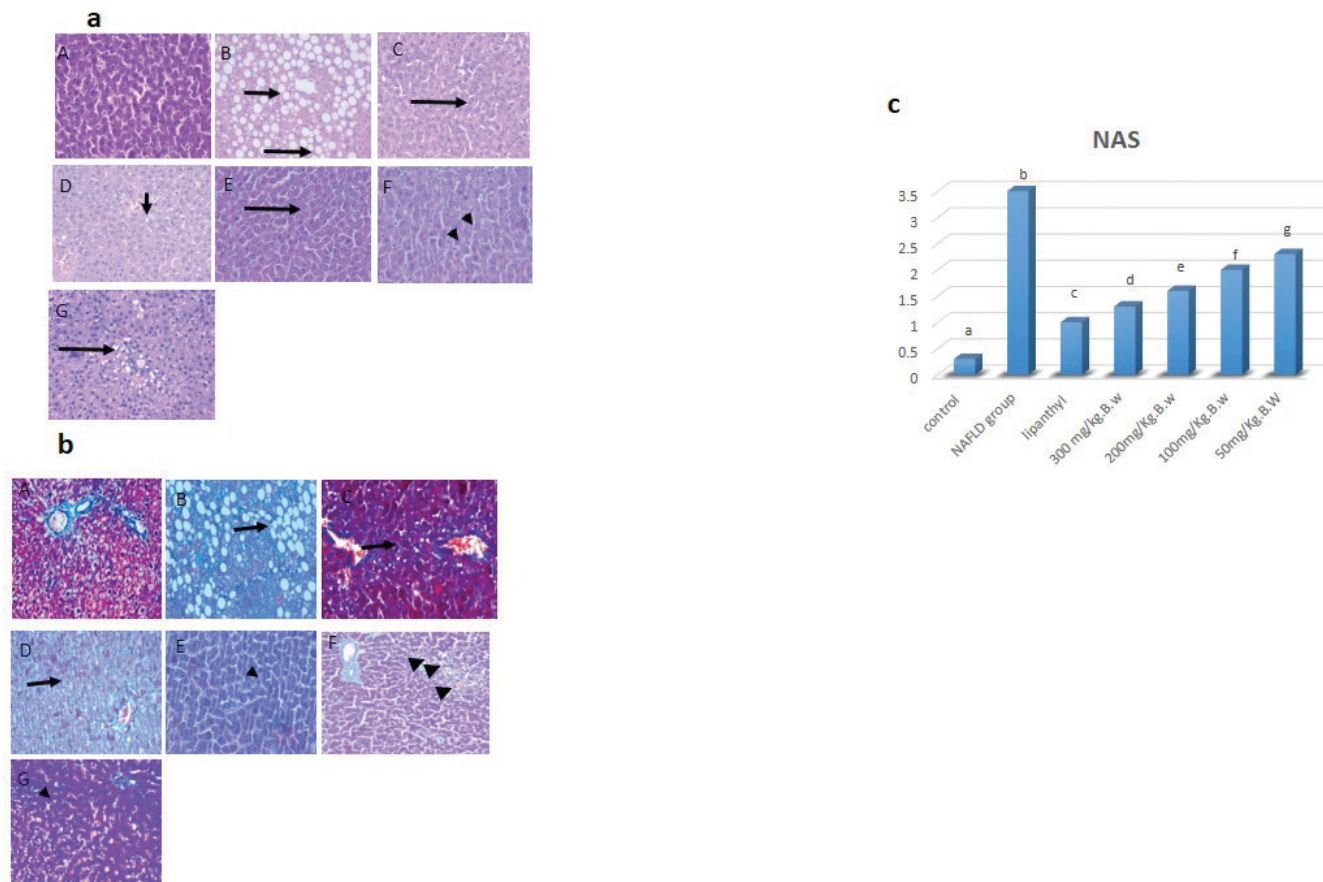


Figure 6. Therapeutic effect of lipanthyl and methanolic extract of *E. prostrata* on liver histology and NAS. (a) H&E staining (400 \times) and (b) Masson Trichrome (400 \times). (c) NAS. Data are expressed as mean \pm SE, $n = 6$ per group. Different letters are significantly different at $p < 0.05$. Statistical analyses one way ANOVA with LSD *post hoc* test was used. Sample codes are presented as follows: (A) healthy control (B) fatty liver group, (C) standard drug lipanthyl, and extract doses: 300 mg/kg BW (D), 200 mg/kg BW (E), 100 mg/kg BW (F), and 50 mg/kg BW (G).

Lipid metabolism abnormalities and insulin resistance in NAFLD increase the glycerolipids, as in the study, where glycerol and 1,3-dipalmitin levels were raised and sequentially accumulation of TAGs was observed which were produced through esterification of FFA with glycerol-3-phosphate (Rodríguez *et al.*, 2014).

Interestingly, three oxysterol compounds, cholesta-4, 6-dien-3-ol, cholesta-4, 6-dien-3-one, and cholesta-3, 5-dien-7-one are derived from the oxidation of cholesterol in the erythrocyte membrane by reactive oxygen species. In our study, we noticed the increased level of cholesta-4,6 dien-3-ol in the NAFLD group

as compared with healthy control group indicating the elevated cholesterol level in this group and its oxidation products (Liu and Shan, 2006) and these results agreed with Serviddio *et al.* (2016) who reported that oxysterols play a vital role in the pathogenesis of NAFLD. Puri *et al.* (2007) reported that the observed raised cholesterol level in NAFLD was due to numerous aberrations that included deterioration of adenosine triphosphate synthesis which increases the 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and mitochondrial abnormalities. In addition, cholest 5-en-3 α -ol-3 acetate which is present in all human fluids and organs is elevated in plasma in association with different lipid-related diseases as atherosclerosis and NAFLD due to the decreased level of high-density lipoprotein (Kuivenhoven *et al.*, 1998).

An increased urea level in NAFLD found in the present results is similar to the clinical report of Targher *et al.* (2011) in patients with NAFLD and higher blood urea nitrogen which could have a prognostic value.

The amelioration in the previous metabolites levels observed in *E. prostrata* extract treated animals may be due to the presence of main active constituents as flavonoids (rutin and quercetin), phenols [gallic acid (GA)], and coumarins (wedelolactone) which regulate lipid metabolism. Wu *et al.* (2011) reported that the flavonoid rutin can improve hepatic injury by promoting activities of some enzymes as adenosine 5-monophosphate (AMP)-activated protein kinase (AMPK), sterol regulatory element binding proteins-1, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, glycerol-3-phosphate acyltransferase, fatty acid synthase, and acetyl-coenzyme carboxylase which are involved in lipid synthesis and metabolism. Moreover, Porras and Nistal (2017) observed the reduction in insulin resistance and NAFLD activity score that accompanied quercetin administration *via* its reduction of lipid accumulation is due to its modulation of lipid metabolism. Chao *et al.* (2014) reported that the target of GA treatment is lipid metabolism and proved that GA can improve lipid homeostasis in high-fat-diet-induced NAFLD in mice. Wedelolactone may promote lipid metabolism by up-regulation of AMPK and PPAR α levels and thus was found to be beneficial against hepatic steatosis (Zhao *et al.*, 2015).

CONCLUSION

The current study represented the main altered lipid metabolites related to NAFLD in serum samples using a lipidomic approach to act as key points to control this disease in the early disease stage. This study also highlights the therapeutic effect of *E. prostrata* through its regulation to the progression of NAFLD and amelioration of these metabolite profiles and pointed that the highest dose used in the present study of methanol extract of *E. prostrata* has the maximum therapeutic effect compared with lipanthyl drug.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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ABBREVIATIONS

| | |
|----------------------------|--|
| ACC: | acetyl-coenzyme carboxylase acids |
| ALT: | alanine aminotransferase |
| AMPK: | adenosine monophosphate-activated protein kinase |
| AST: | aspartate aminotransferase |
| DAG: | diacylglycerol |
| FFA: | free fatty acid |
| GC–Ms: | gas chromatography–mass spectrometry |
| GPAT: | glycerol-3-phosphate acyltransferase |
| HDL-C: | high-density lipoprotein cholesterol |
| HMG CoA: | β -Hydroxy β -methylglutaryl-CoA |
| HMGCR: | hydroxy-3-methyl-glutaryl-CoA reductase |
| HPLC: | high-performance liquid chromatography |
| LCAT: | lecithin-cholesterol transferase |
| LDL-C: | low-density lipoprotein cholesterol |
| LDLR: | low-density lipoprotein receptor |
| MS-grade H ₂ O: | mass spectra high-grade water |
| NAFLD: | non-alcoholic fatty liver disease |
| NASH: | non-alcoholic steatohepatitis |
| PCA: | principal component analysis |
| PLS-DA: | partial least square discriminate analysis |
| PPAR α : | peroxisome proliferator-activated receptor |
| SR-BI: | scavenger receptor class B type I receptor |
| SREBP-1c: | sterol regulatory element binding protein-1c |
| SS: | simple steatosis |
| TAG: | triacylglycerides |
| TC: | total cholesterol |

AUTHOR CONTRIBUTIONS

Azza Salah Helmy and Naglaa M Sherif conceived and coordinated the study. Hassan Zaki Ghanem helped in performing experiments. Nabaweya Ali Ibrahim providing an extract *E. prostrata*. Abdel-Nasser G El-Gendy helped in identifications of metabolites and in statistical analysis. Noha Said Hussein performed experiments and prepared the manuscript. Abdel-Hamid Zaki Abdel-Hamid involved in designing the hypothesis and experiments, conceived and coordinated the study and made a revision to the manuscript. All authors read and approved the final version of the manuscript.

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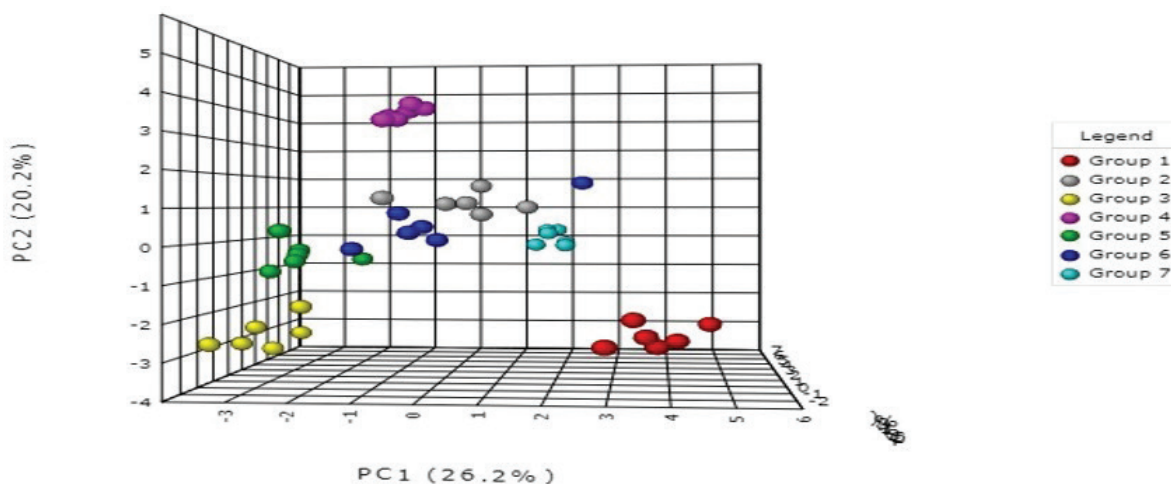
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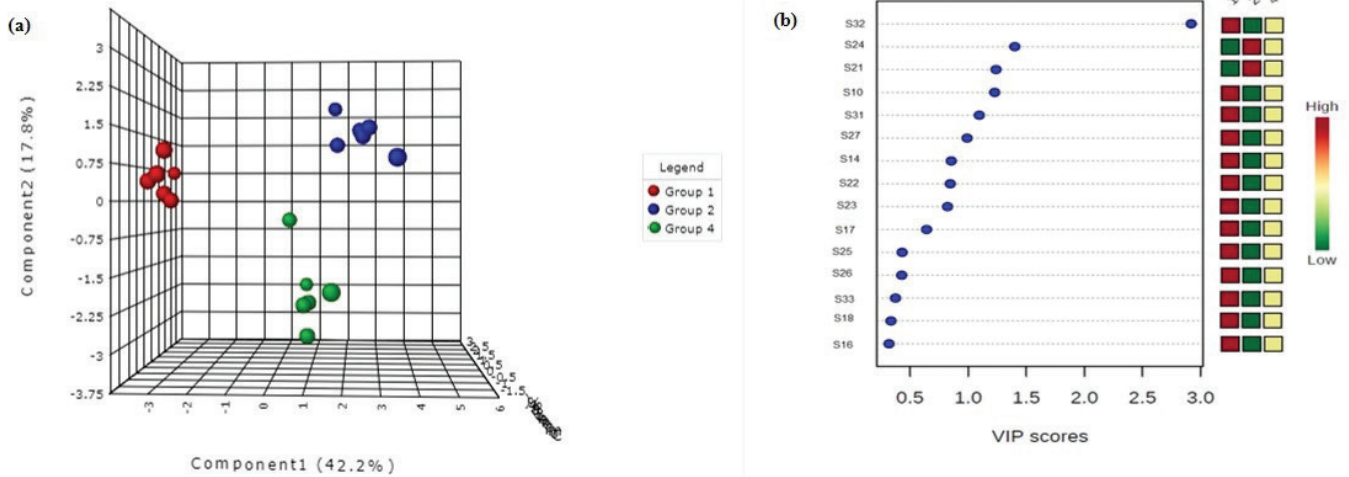
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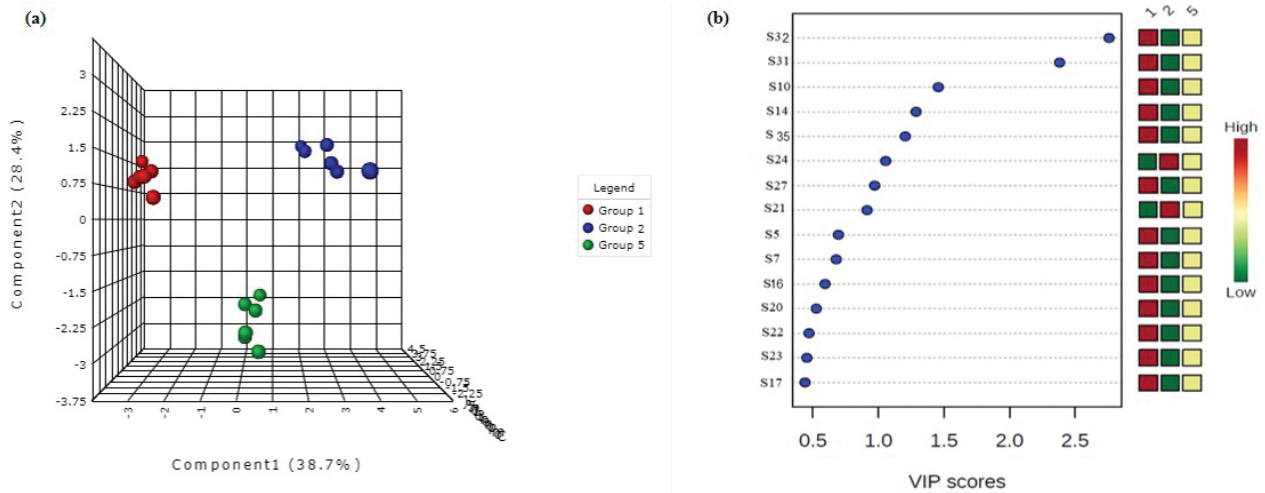
Supplementary Table 1. GC–MS assignments of targeted metabolites identified in treated rat serum after silylation.

| Peaks no. | Metabolites | RT | KI | m/z |
|-----------|---|--------|---------|-----|
| S1 | Bis(trimethylsilyl)carbodiimide | 4.248 | 714.8 | 172 |
| S2 | unknown1 | 4.53 | 730.7 | 204 |
| S3 | Tris(trimethylsilyl)borate | 4.649 | 737.4 | 184 |
| S4 | 2-endo-Hydroxy-4-thiahomoadamantane | 4.763 | 740.8 | 247 |
| S5 | Glycerol 1 di-TMS | 5.859 | 806.1 | 73 |
| S6 | Lactic acid, di-TMS | 6.015 | 813.5 | 150 |
| S7 | B-Hydroxybutyric acid (tms) | 8.141 | 935 | 160 |
| S8 | Unknown2 | 9.369 | 1,003.3 | 212 |
| S9 | Unknown3 | 9.464 | 1,006.6 | 184 |
| S10 | Urea, N,N'-bis(trimethylsilyl)- (CAS | 10.027 | 1,040.4 | 147 |
| S11 | Benzoic acid trimethylsilyl ester | 10.111 | 1,045.7 | 179 |
| S12 | L-Leucine, N-(trimethylsilyl)-, trimethylsilyl ester | 10.914 | 1,090.7 | 189 |
| S13 | Phosphoric acid, tris-TMS | 11.052 | 1,098.1 | 301 |
| S14 | Glycerol2-TRI-TMS ETHER | 11.105 | 1,100.2 | 135 |
| S15 | Unknown4 | 14.884 | 1,313.4 | 229 |
| S16 | Alpha-Glycerophosphate 4TMS | 22.831 | 1,782.8 | 317 |
| S17 | Myristic acid, trimethylsilyl ester | 24.077 | 1,832.3 | 285 |
| S18 | β -Galactopyranose, pentakis-TMS | 24.698 | 1,867.8 | 219 |
| S19 | Glucose 5TMS | 27.49 | 2,025.3 | 117 |
| S20 | Palmitic acid, trimethylsilyl ester | 27.94 | 2,050.8 | 57 |
| S21 | Linoleic acid trimethylsilyl ester | 30.844 | 2,214.8 | 337 |
| S22 | Oleic acid, trimethylsilyl ester | 30.928 | 2,220.4 | 72 |
| S23 | Stearic acid, trimethylsilyl ester | 31.443 | 2,249.3 | 303 |
| S24 | Arachidonic acid, trimethylsilyl ester | 33.473 | 2,372.5 | 161 |
| S25 | Myristic acid, 2,3-bis(trimethylsilyloxy)propyl ester | 34.132 | 2,413.3 | 343 |
| S26 | Palmitoleic acid trimethylsilyl ether | 37.174 | 2,609.1 | 371 |
| S27 | 2-Monostearin trimethylsilyl ether | 39.402 | 2,766.3 | 129 |
| S28 | Cholesta-3,5-diene | 39.731 | 2,789.6 | 430 |
| S29 | Bis(trimethylsilyl)monostearin | 39.941 | 2,803 | 128 |
| S30 | Cholesta-2,4-diene | 40.743 | 2,864 | 81 |
| S31 | Cholesta-4,6-dien-3-ol, (3 β)- | 40.905 | 2,876.2 | 366 |
| S32 | Cholest-5-en-3-ol (3 β)-, acetate | 41.205 | 2,897.2 | 213 |
| S33 | CHOLESTEROL TMS | 44.618 | 3,163.6 | 366 |
| S34 | Unknown5 | 50.277 | 3,566 | 431 |
| S35 | 1,3-Dipalmitin trimethylsilyl ether | 50.541 | 3,579.6 | 371 |

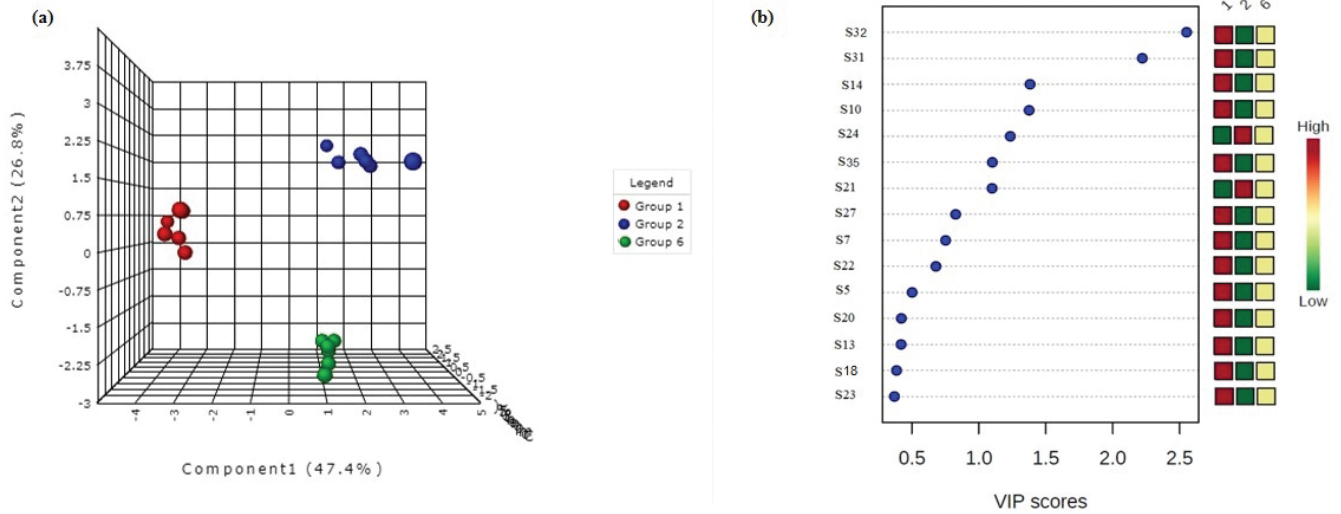
**Supplementary Figure 1.** The PCA scores plots of the seven studied groups derived from GC–MS. The number codes 1, 2, 3, 4, 5, 6, and 7 denote the following different groups: (1) fatty liver group, (2) healthy control, (3) standard drug, (4) extract dose 300 mg/kg BW, (5) extract dose 200 mg/kg BW, (6) extract dose 100 mg/kg BW, and (7) extract dose 50 mg/kg BW.



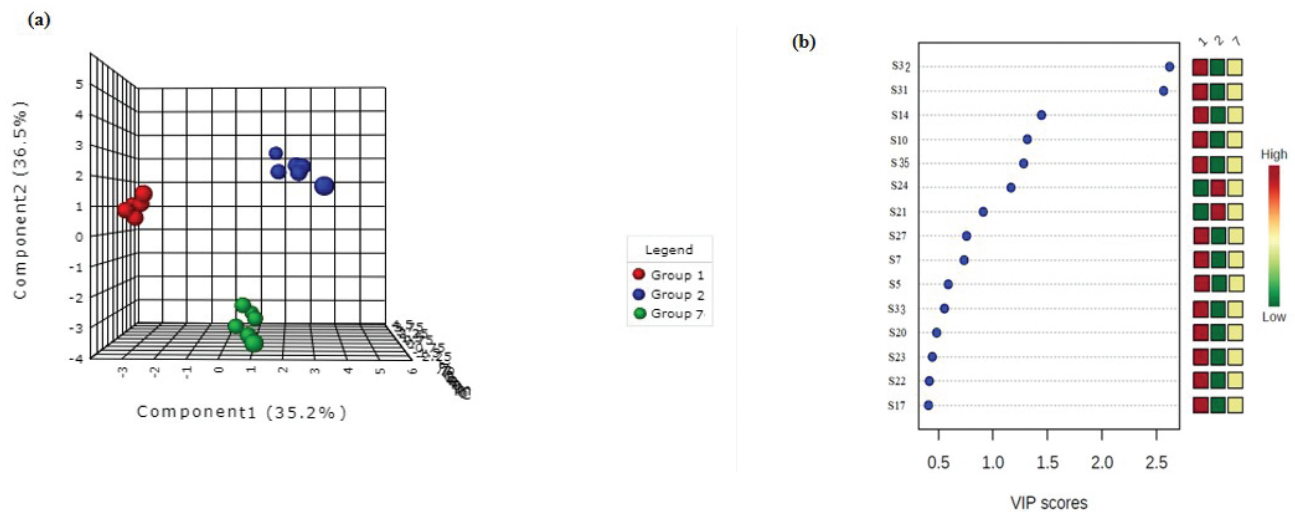
Supplementary Figure 2. PLS-DA score plots obtained from modeling NAFLD (1) healthy control groups 2 and 4 *E. prostrata* dose (300 mg/kg BW) groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S24: Arachidonic acid, S21: Linoleic acid, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S27: 2-monostearin, S14: Glycerol. Peaks follow that listed in [Supplementary Table 1](#) for metabolite identification using GC-MS. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study.



Supplementary Figure 3. PLS-DA score plots obtained from modeling NAFLD (1) healthy control groups 2 and 5 *E. prostrata* dose (200 mg/kg BW) groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S10: Urea, S14: Glycerol, S35: 1,3 dipalmitin, S27: 2-Monostearin, S24: Arachidonic acid, S21: Linoleic acid. Peaks follow that listed in [Supplementary Table 1](#) for metabolite identification using GC-MS. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study.



Supplementary Figure 4. PLS-DA score plots obtained from modeling NAFLD (1) healthy control groups 2 and 6 *E. prostrata* dose (100 mg/kg BW) groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S14: Glycerol, S10: Urea, S24: Arachidonic acid, S35: 1,3 dipalmitin, S21: Linoleic acid, S27: 2-Monostearin. Peaks follow that listed in [Supplementary Table 1](#) for metabolite identification using GC-MS. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study.



Supplementary Figure 5. PLS-DA score plots obtained from modeling NAFLD (1) healthy control groups 2 and 7 *E. prostrata* dose (50 mg/kg BW) groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score > 1). S32: Cholest-5-en-3-ol (3 β)- acetate, S31: Cholesta-4,6-dien-3-ol, (3 β)-, S14: Glycerol, S10: Urea, S35: 1,3 dipalmitin, S24: Arachidonic acid, S21: Linoleic acid, S27: 2-Monostearin. Peaks follow that listed in [Supplementary Table 1](#) for metabolite identification using GC-MS. The colored boxes on the right indicated the relative concentrations of the corresponding metabolite in each group under study.