Protective efficiency of *Eclipta prostrata* on diet-induced nonalcoholic fatty liver disease in rats using the urinary lipidomics approach

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Background
Nonalcoholic fatty liver disease (NAFLD) is currently one of the leading causes of chronic liver disease in western countries and is characterized by excessive fat deposition in the liver. It affects 15–30% of the general population worldwide.
Objectives
This study aims to detect and evaluate a reproducible signature of lipid metabolome for early detection of NAFLD as well as protective efficiency of *Eclipta prostrata* based on targeted metabolomics analysis of urine.
Results and conclusion
A discrete divergence in levels of glycerol, myristic acid, cholesterol, 1,3-dipalmitin, and oleic acid was recorded in urine of rats bearing NAFLD in contrast to healthy ones. These metabolites were considered as diagnostic metabolic biomarkers for NAFLD. This study showed that these metabolites were less affected in rats given *E. prostrata* as a protective agent. It is concluded from this research that the lipidomic approach could be used for early diagnosis of NAFLD in urine as well as assessment of the stratification of protection by herbal medication.

Keywords:
biomarkers, *Eclipta prostrata*, lipidomics, nonalcoholic fatty liver disease

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Introduction

The permanent deposition of fat in liver owing to fatty diet and sedentary lifestyles is the most probable cause of nonalcoholic fatty liver disease (NAFLD), leading to fibrosis and cirrhosis [1]. Because lipids have the ability to regulate oxidative stress, inflammation, and key transcription factors, lipids appear more tangible and may have significant effects on the progression of NAFLD. The possible key elements in the mechanism of disease progression toward nonalcoholic steatohepatitis (NASH) are demonstrated by several lipid mediators typically associated with lipotoxicity such as diacylglycerols and free fatty acids (FFAs). These lipids from lipotoxicity are associated with hyperlipidemia, metabolic syndrome, insulin resistance, and type 2 diabetes, which are frequent comorbidities associated with NAFLD [2]. Because of insufficient methods for early diagnosis of NAFLD, it is described as a silent disease. The main and principal clinical standard for final diagnosis is liver biopsy, but in some cases, it leads to inflammation at the time of biopsy and the patient experiences a subsequent risk of clinically significant fibrosis. So, advanced diagnostic approaches to avoid the requirements of invasive procedures are demanded for novel management strategies for the growing epidemic of NAFLD [2].

The emersion of lipidomic approach has empowered researchers to study in depth lipid metabolism in both physiological and cellular levels than was previously possible in various diseases such as NAFLD [3]. Lipidomics is a novel technique that encompasses analytical approaches for identification and quantification of the complete set of lipids, defined as lipidome in a given cell or organism as well as their interactions with other molecules. Gas chromatography-mass spectrometry (GC-MS) has been proven as a powerful tool in system biology, and lipidome can be analyzed in different body fluids based on GC-MS. Urine displays a noninvasive, unique estimation of a body's ability to process or release structural metabolites and excrete bioactive signals. So, it can be used as a matrix for metabolic biomarker discovery and assessment of organ function [4]. In this study, urinary lipidomic analysis is used for assessment of protective efficiency of *E*clipta *prostrata* against NAFLD.

Recently, the use of herbal natural products has gained more interest among the world population [5]. Among

those herbs is *E. prostrata* L. (Asteraceae), which has a high potential medical value. *E. prostrata* has hypolipidemic and hepatoprotective effects by decreasing oxidative stress and controlling of genes involved in lipid metabolism [6]. *E. prostrata* reduced the high levels of total lipids, total cholesterol, triacylglycerols, phospholipids, and FFAs in serum, liver, and heart of the animals owing to its higher contents of saponins and phytosterol [6]. *E. prostrata* prevented liver damage caused by CCl_4 in guinea pigs, and its powder drug was detected to be beneficial in the treatment of jaundice in children [7]. Many chemical constituents such as thiophenes, coumarins, triterpenoid, saponins, steroids, and flavonoids have been isolated from *E. prostrate* [8]. Wedelolactone, which is considered as the main component of *E. prostrata*, upregulated protein levels of adenosine monophosphate activated protein kinase (AMPK), a key regulator of lipid metabolism. AMPK phosphorylation suppresses the activity of the key proteins involved in lipogenesis, such as sterol regulatory element-binding protein-1c and hydroxy-3-methyl-glutaryl-CoA reductase and improves liver steatosis and peroxisome proliferator-activated receptor alpha (PPARα) as well as the gene expression of AMPK, (PPARα, lipoprotein lipase (LPL), and low-density lipoprotein lipase receptor (LDLR) [9].

As liver is the main organ in the synthesis and metabolism of lipids, *E. prostrata* can act on the liver by promoting metabolism and accelerating the excretion of excess lipids, thereby producing hypolipidemic conditions.

Some people in Africa and Asia use *E. prostrata* powder twice a day before meals for liver diseases or jaundice. To assert this phenomena and based on our previous finding [10], the present study was designed to study the protective effect of *E. prostrata* against NAFLD in rats in comparison with a well-known lipid-lowering agent (Lipanthyl) using lipidomic analysis.

Materials and methods

Ethics and permissions

The procedures of study protocol was ethically reviewed and approved by Ethics Review Committee of the National Research Centre (ERC-NRC) in Cairo (approval no: 13002) and also according to the Declaration of Helsinki [11].

Biological materials

E. 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 (CAIRC), under No. 997.

Experimental animals: adult male Wistar albino rats (140) weighing 160–180 g and aged 5–6 weeks were obtained from the animal house at National Research Centre. The experimental animals were housed in wire cages and maintained under standard conditions (temperature, 22±5°C, humidity, 55±5%, and a 12 h light/dark cycle). The animals had access to standard laboratory feed and water *ad libitum* for 7 days as an acclimatization period before doing the experiments. The rats were divided into two main groups, with 70 rats each. The first group was used to study biochemical parameters to explore any adverse effects or toxicity using *E. prostrata* as a protective agent, whereas the second group was administrated NAFLD diet and used for the hepatoprotective evaluation of methanolic extract of *E. prostrate* based on lipidomic analysis.

Chemicals

N-methyl-N (trimethylsilyl) trifluoroacetamide with 1% (vol/vol) trimethylchlorosilane, methyl-tert-butyl ether, methanol (HPLC), and n-alkane C8–C40 standard were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Cholesterol powder was purchased from Bio Basic Canada Inc. (20 Konrad Crescent, Markham, ON L3R 8T4) and cholic acid was purchased from LOBA Chemie (Jehangir Villa, 107, Wodehouse Road, Colaba, Mumbai, India). All commercially available assay kits for the determination of serum aspartate aminotransferase, alanine aminotransferase, triacylglycerols, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein–cholesterol (HDL-C), urea, and creatinine levels were purchased from Bio-Med (Cairo, Egypt). All other chemicals and solvents were of high analytical grade. A standard diet was prepared according to the American Institute of Nutrition [12].

Experimental design

Biological assay

Methanolic extract of *E. prostrata* (aerial part) was obtained by Soxhlet and then dried using a vacuum rotary evaporator. Subacute toxicity of *E. prostrata* was estimated using 70 male albino rats (160–180 g), divided into seven groups (10 each). All procedures involving animals were performed in accordance with the OECD guideline 425 [13]. The animals were kept fasting for 3 h before the experiment. The first group (G1) received normal food diet and tap water *ad libitum* to serve as control, whereas the other groups (G2–G7) received dried methanolic extract of *E. prostrata* orally

dissolved in water in doses 600, 400, 200, 100, 50, and 20 mg/kg, respectively (0.5 ml/rat) daily for 2 weeks. All animals were observed for mortality up to 48 h (short-term toxicity) and long-term toxicity (14 days), and LD_{50} was calculated. At the end of experiments, blood samples were collected from all groups. The samples were placed in sterilized tubes for serum separation by centrifugation at 5000 rpm for 15 min. The obtained sera samples were then stored at −80°C for biochemical analyses.

Protective evaluation of Eclipta prostrata

The protective group (70 rats) was subsequently divided into four subgroups:

(1) Healthy control group (negative control): 10 healthy selected rats received standard chow and tap water *ad libitum*.
(2) Control group (positive control): 10 normal rats were fed high-fat diet containing standard chow supplemented with cholesterol and cholic acid for 8 weeks as describe by Ney *et al.* [14].
(3) Protected group: 40 normal rats were left to feed high-fat diet containing standard chow supplemented with cholesterol and cholic acid. Rats were divided into four subgroups (10 in each). At the same time, rats of these subgroups were treated orally with methanolic extract of *E. prostrata* (aerial part) at doses 300, 200, 100, and 50 mg/kg body weight for 8 weeks, respectively, as described by Dhandapani [15].
(4) Standard reference group: 10 normal rats received high-fat diet containing standard chow supplemented with cholesterol and cholic acid followed by the lipid-lowering agent Lipanthyl drug 300 mg at a dose of 5.35 mg/200 g rat for 8 weeks.

At the end of the experimental period, urine samples were collected from all groups using experimental animal urine collectors as described by Ammar *et al.* [16]. Animals were placed in individual cages, and urine was collected over a 24-h period in tubes containing 1% sodium azide and centrifuged to remove all particulate matter and then stored at −80°C until further lipidomic analysis.

Histopathological examination

To confirm the induction of NAFLD in rats, the left lobe of liver was isolated from rats, rapidly washed with ice-cooled saline, and then placed in 10% formalin saline for 24 h and processed routinely by embedding in paraffin. Sections of 4 μm were stained with two different stains [hematoxylin and eosin (H&E) and Masson Trichome] and examined under a light microscope for histopathological examination as demonstrated by Smith [17].

Lipidome analysis using gas chromatography-mass spectrometry

Urine lipidome

The GC-MS system (Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA, United States) was equipped with a GC (7890B) and mass spectrometer detector (5977 A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m×0.25 mm internal diameter and 0.25 μm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 ml/min, injection volume of 1 μl at a split-less mode, and the following temperature program: 80°C for 2 min, rising at 5°C/min to 300°C, and held for 5 min. The injector and detector were held at 280 and 300°C, respectively. Mass spectra were obtained by electron ionization at 70 eV, using a spectral range of mass-to-charge ratios 25–550 and solvent delay time of 3.7 min.

For GC-MS analysis, 200 μl of urine was mixed with 20 μl of urease suspension and incubated 1 h at 37°C, then 1.7 ml of methanol (98%) was added to each tube and vortex for 5 min, then samples were centrifuged for 10 min at 10 000 g and 4°C, then 5 ml of methyl-tert-butyl ether was added to each tube, and aside then samples were shaken for 1 h at room temperature. Subsequently, 1.25 ml of MS-grade H_2O was added to each tube, mixed and allowed to stand for 10 min at room temperature, and then centrifugation was done at 1000*g* for 10 min to separate two phases. The upper phase was transferred into a separate tube and dried by nitrogen gas according to the method described by Matyash *et al.* [18]. For metabolites derivatization, a volume of 100 μl N-methyl-N (trimethylsilyl) trifluoroacetamide containing 1% trimethylsilyl (TMS) was added to the mixture and then incubated at 70°C for 30 min [16].

Metabolite identification

Deconvolution of the detected mass spectra was performed using AMDIS 2.64 software and NIST library for identification of measured peaks via measuring the matching against reference compounds already registered. Standard fatty acid mixture (Sigma, Aldrich, Missouri, PO Box 14508, St. Louis, MO, United States) was injected for confirming peak assignments. Retention indices were calculated relative to n-alkanes (C8–C40) standards.

Data processing for multivariate analysis

The multivariate data analysis for identified metabolites was processed by R package supported

by MetaboAnalyst R ver. 4–a comprehensive tool suitable for metabolic data analysis – online software (http://www.metaboanalyst.ca) [19]. Metabolites' MS signals were normalized using a reference sample (PQN) option, 'the control with less missing values.' Data transformation was done using log transformation, especially 'Pareto' scaling option, before multivariate data analysis. After that, both unsupervised principal component analysis and supervised partial least-squares discriminant analysis (PLS-DA) were performed using this program.

Statistical analysis

The univariate data analysis was done using nonparametric (Mann–Whitney test) and Kruskal–Wallis test via SPSS, version 17 (SPSS, Inc., Chicago, IL, USA) for lipidomic metabolites.

Results

Toxicity assay

The results showed that the methanolic extract of *E. prostrata* (aerial part) had no toxic effects on rats. No mortality was recorded among rats treated with different doses of this extract, indicating the safety of using this plant extract up to 600 mg/kg.

Urine metabolite profiling

Data listed in Table 1 represent the identity of 30 urine metabolites in all studied groups, their retention times (rt), retention indices, and mass-to-charge ratios. GC-MS chromatogram offers the average peaks from healthy control and fatty liver diseased rat, as shown in Fig. 1. Samples were scattered along PC1 with even biological replicates within the same sample failing to group together (Fig. 2). Consequently, supervised multivariate data analysis as PLS-DA was adopted for sample classification to help identify possible metabolite biomarkers for each experiment groups. PLS-DA score plot (Fig. 3) showed better discrimination between sample groups with fatty liver group clustering separately from all other protected groups, with R^2 value of 0.68 and Q^2 value of 0.65.

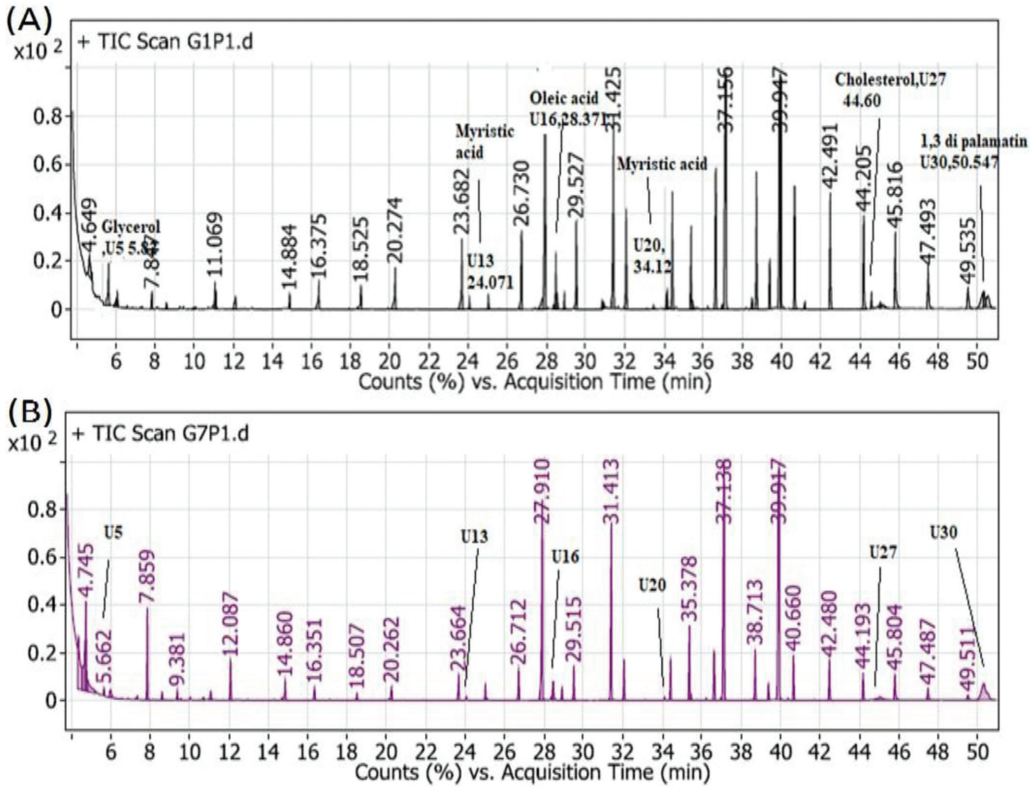
Metabolites contributing for group segregation revealed from VIP more than 1 score plot (Table 2)

Table 1 Gas chromatography-mass spectrometry assignments of metabolites identified in rat urine via gas chromatography-mass spectrometry after silylation

Peak	Metabolites	RT	KI	*m/z* value
1	Carbodiimide, bis(trimethylsilyl)-	4.344	720.2	171
2	Boric acid 3TMS	4.619	736.1	170
3	Tris(trimethylsilyl)borate	4.775	744.6	224
4	Tetrasiloxane, decamethyl-	5.680	795.6	207
5	Glycerol di-TMS	5.829	804.3	151
6	Acetamide, 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)-(CAS)	6.015	814.4	199
7	L-(+)-Lactic acid, trimethylsilyl ether, trimethylsilyl ester	6.093	818.9	147
8	Unknown1	9.327	1000.5	254
9	Unknown2	9.44	1007.6	229
10	Phosphoric acid, tris-TMS	11.069	1099.4	299
11	Unknown3	14.884	1313.2	229
12	cis-4,5-bis(trimethylsiloxy)-3,4,5,6-tetrahydro-1,2-dithiane	18.525	1518.9	147
13	Myristic acid, trimethylsilyl ester	24.071	1832.2	117
14	Pentadecanenitrile TMS	25.035	1886.5	126
15	Palmitic acid, TMS	27.934	2049.9	313
16	9-Octadecenenitrile TMS	28.371	2074.7	95
17	Oleanitrile TMS	28.478	2081.4	126
18	Octadecanenitrile TMS	28.916	2105.8	97
19	Stearic acid, TMS	31.425	2249.6	341
20	Myristic acid, 2,3-bis(trimethylsiloxy)propyl ester	34.126	2413.3	343
21	Oleamide, N-trimethylsilyl	34.174	2416.2	75
22	trans-13-Docosenamide	35.389	2492.6	55
23	Hexadecenenitrile TMS	35.461	2498.1	262
24	Palmitoleic acid trimethylsilyl ether	37.132	2610.3	145
25	2-Monostearin trimethylsilyl ether	39.40	2767.1	129
26	1-Monostearin-di-TMS	39.923	2803.5	339
27	Cholesterol TMS	44.60	3163.2	329
28	Unknown4	45.049	3199.1	134
29	Silane, diethylhexadecyloxy(2-hexyloxy)-	50.307	3566.3	98
30	1,3-dipalmitin trimethylsilyl ether	50.547	3579.6	371

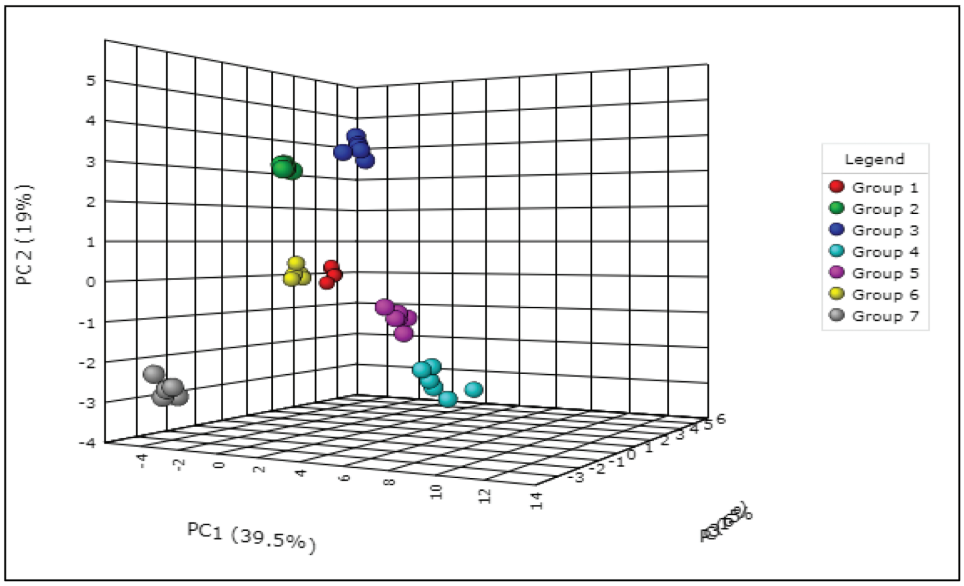
TMS, trimethylsilyl.

Figure 1



GC-MS peaks of urine samples from healthy (b) and diet-induced fatty liver in rats (a). GC-MS, gas chromatography-mass spectrometry.

Figure 2

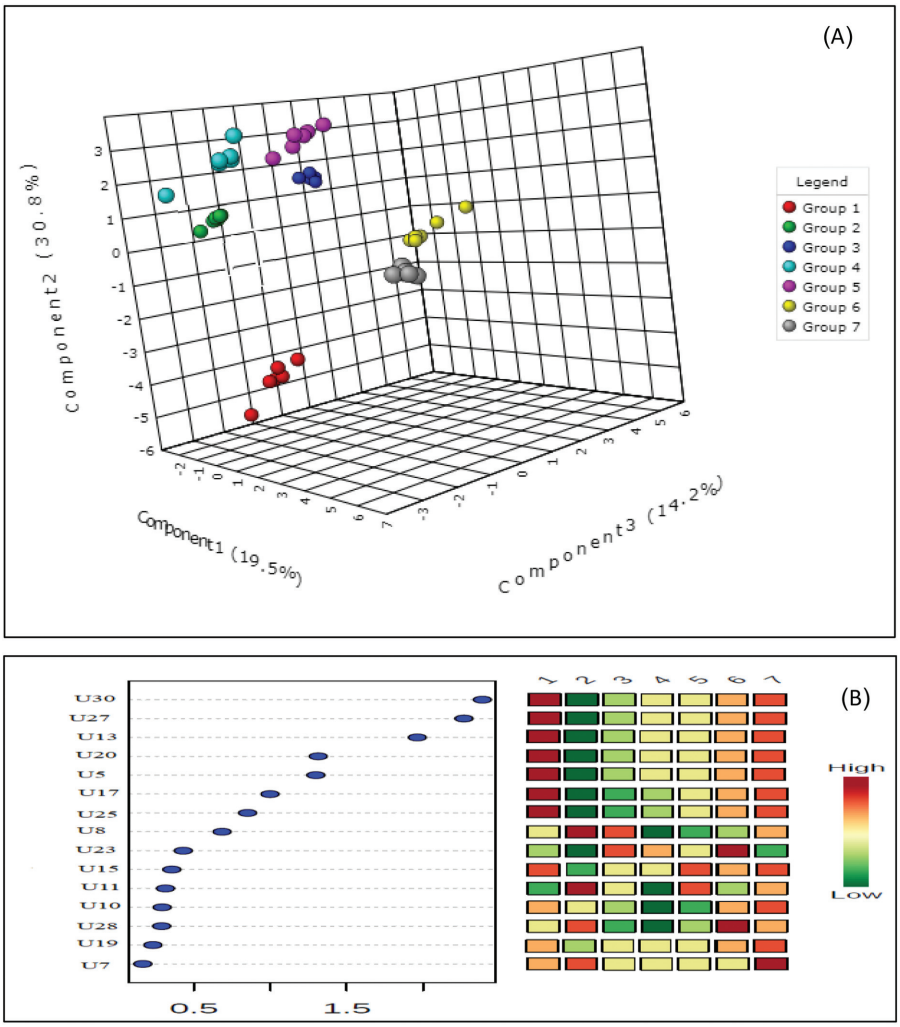


Principal component analysis (PCA) score plots obtained from modeling urine metabolites in different rat groups. Score plot showing a separation between healthy control rats, protective, and fatty liver diseased rats compared with the standard reference group. Sample codes are presented as follows: (a) fatty liver group, (b) healthy control, (c) standard drug, (d) extract dose 300 mg/kg, (e) extract dose 200 mg/kg, (f) extract dose 100 mg/kg, and (g) extract dose 50 mg/kg.

include 1,3-dipalmitin, cholesterol, myristic acid, glycerol, oleic acid, and 2-monostearin.

For further separation between all groups and determination of their arrangement according to their efficacy of these metabolites, the hierarchical clustering/dendrogram model of all groups is displayed in Fig. 4. This dendrogram model show that the presence of fatty liver group is the most distant from normal group, and all protected groups

Figure 3



PLS-DA score plots obtained from modeling rats' urine lipidomic metabolites in the different protective groups. (a) The score plot showing a separation of healthy control animals, NAFLD rats, and protected groups. (b) VIP score plot for determination of important metabolites (VIP score ≥1). U30: 1,3-dipalmitin, U27: cholesterol, U13: myristic acid 1, U20: myristic acid 2, U5: glycerol, U17: oleic acid, and U25: 2-monostearin. The numbering of peaks follows that listed in table 8 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. Sample numbers are presented as follows: (a) fatty liver group, (b) healthy control, (c) standard drug lipanthyl, and extract doses: 300 mg/kg body weight (d), 200 mg/kg body weight (e), 100 mg/kg body weight (f), and 50 mg/kg body weight (g). GC-MS, gas chromatography-mass spectrometry; NAFLD, nonalcoholic fatty liver disease; PLS-DA, partial least-squares discriminant analysis.

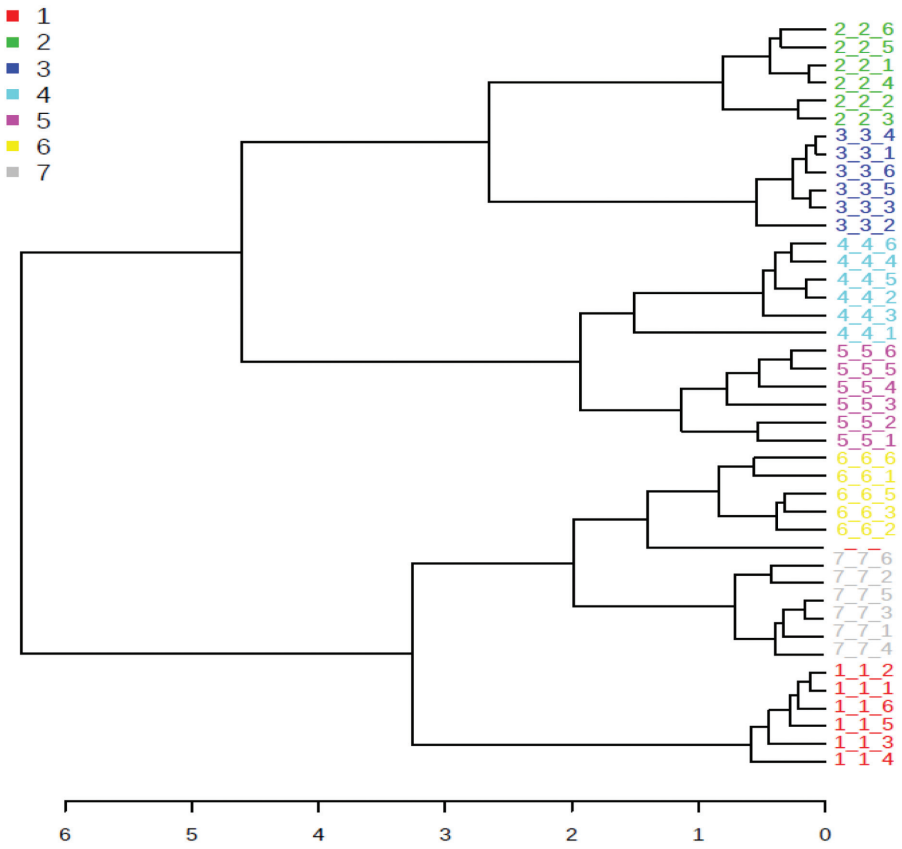
Table 2 Random forest classification performance

	1	2	3	4	5	6	7	Class error
1	6.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	6.00	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	6.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	6.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	6.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00	6.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00	0.00	6.00	0.00

are found between them. Among all studied groups, it is noticed that the standard drug group was close to the control group in the dendrogram model, whereas the protective dosage groups 300, 200, 100, and 50 mg/kg were between the normal and positive groups. The closer to the normal is group 300 mg/kg followed by 200 and 100 mg/kg, suggesting that the highest dose of *E. prostrata* was the most influential dose.

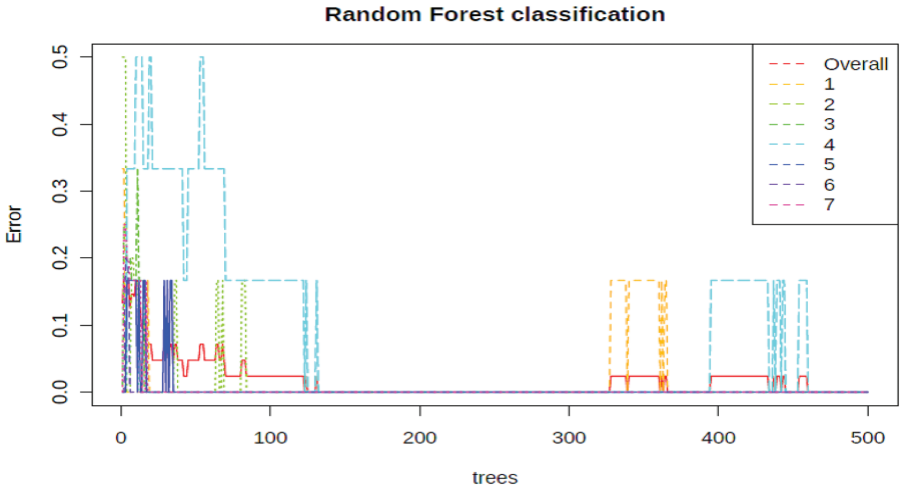
Random forest (RF) is a supervised learning algorithm suitable for high-dimensional data analysis. It is used as an ensemble of classification trees. RF also provides

Figure 4



Hierarchical clustering/dendrograms of the six rat groups with fatty liver group showing separation from the normal control group and protective groups in urine samples. Sample codes are presented as follows: (a) fatty liver group, (b) healthy control, (c) standard drug, and extract doses: 300 mg/kg (d), 200 mg/kg (e), 100 mg/kg (f), and 50 mg/kg (g).

Figure 5



Cumulative error rates by random forest classification. The overall error rate is shown as the black line; the red and green lines represent the error rates for each class.

other useful information such as out-of-bag error, variable importance measure, and outlier measures. RF analysis is performed using the RF package in R. Table 2 shows the confusion matrix of the RG. Figure 5 shows the cumulative error rates of RF analysis for given parameters. Figure 6 shows the important features ranked by RF. Figure 7 shows the outlier measures of all samples for the given parameters. The out-of-bag error is 0.

PLS-DA score plot and its derived VIP score plot analysis are listed in Table 2 to determine whether

biomarkers for the NAFLD status in rats could be revealed from urine analysis. The PLS-DA score plot explained 67% of the total variance (R^2=0.95) with the prediction goodness parameter Q^2=0.93. As compared with the control group, cholesterol, myristic acid, glycerol, and oleic acid were increased in urine of the NAFLD group.

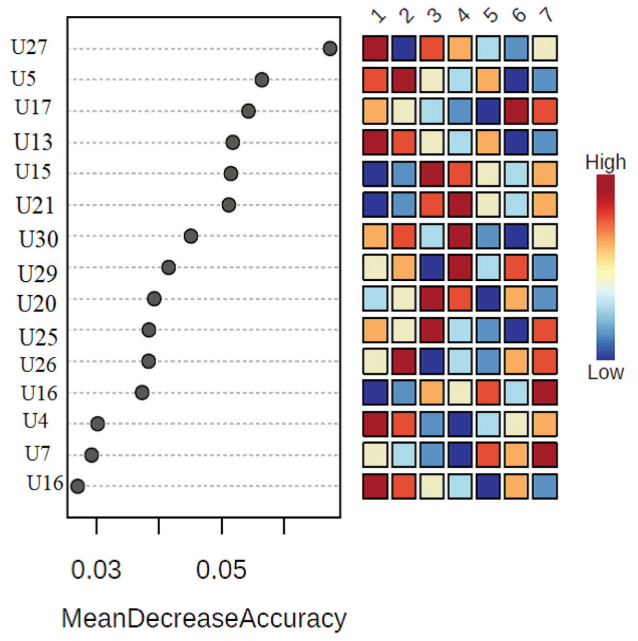
To affirm the results of metabolic biomarkers obtained from multivariate data analysis, fold change of metabolites was attempted and further subjected to statistical analysis (univariate data analysis 'Kruskal–Wallis and Mann–Whitney tests'), as shown in Table 3.

Fold change was performed by using MetaboAnalyst ver. 4. Statistical comparisons using equal nonparametric Mann–Whitney test was performed. Results were considered only significant if *P* value less than 0.05 using Kruskal–Wallis test analysis ([a]denotes common metabolites showing significance using Kruskal–Wallis test and PLS-DA analysis, and * denotes urine metabolites showing significant difference among urine protected groups). Sample numbers are presented as normal healthy (N), drug (D), fatty liver (F), and 300, 200, 100, and 50 (different dosages of *E. prostrata* methanolic extract).

Pathway-based analysis: we identified statistically enriched KEGG pathways in metabolites data sets using a hypergeometric distribution-based approach through the software Metaboanalyst R 4, as shown in Table 4.

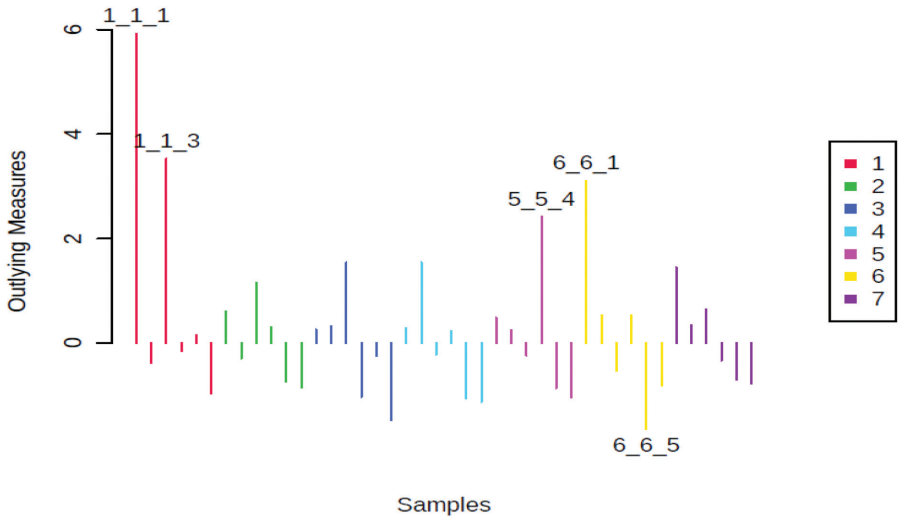
The table below shows the detailed results from the pathway analysis. Since we are testing many pathways at the same time, the statistical *P* values from enrichment analysis are further adjusted for multiple testings. In particular, the total is the total number of compounds in the pathway, the Hits is the actually matched number from the user uploaded data, the Raw p is the original p value calculated from the enrichment analysis, the FDR p is the *P* value adjusted using false discovery rate, and the impact

Figure 6



Significant features identified by random forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. The numbers are as used in Table 1.

Figure 7



Potential outliers identified by random forest. Only the top five are labeled. 1_1_3: indicates fatty liver sample 3; 5_5_4: group 5 administrated dose 200 mg/kg sample 4; and 6_6_1 and 5: group 6 administrated dose 100 mg/kg samples 1 and 5.

Table 3 The common metabolite level alterations according to partial least-squares discriminant analysis score (VIP>1) and fold change in all experimental groups

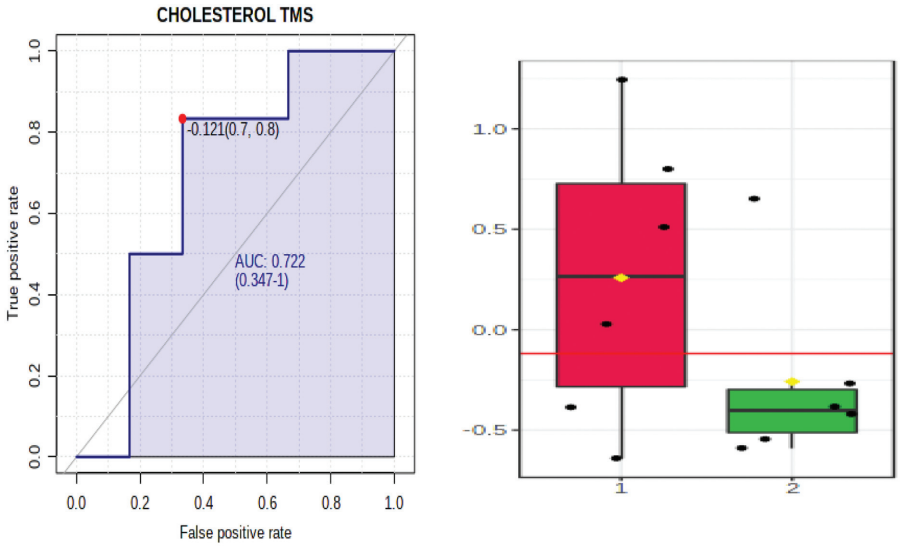
Metabolites	VIP score	Fold change							Trend
		F/N	Trend	D/F	300/F	200/F	100/F	50/F	
[a]Glycerol, U5	1.3	*3.63	↑	*0.56	*0.63	*0.65	*0.74	*0.83	↓
[a]Myristic acid, U20	1.3	*3.23	↑	*0.42	*0.55	*0.71	*0.82	*0.89	↓
[a]Cholesterol, U27	2.3	*7.10	↑	*0.18	*0.23	*0.41	*0.60	*0.80	↓
[a]1,3-dipalmitin, U30	2.4	*2.12	↑	*0.57	*0.70	*0.78	*0.88	0.96	↓
[a]Oleic acid, U16	1.0	*2.13	↑	*0.59	*0.73	*0.79	*0.86	*0.90	↓

[a]Denotes for common metabolites showing significant using Kruskal-Wallis test and PLS-DA analysis. *Denotes for urine metabolites showing significant difference among urine protected groups . Sample numbers are presented as: normal healthy (N), drug (D), fatty liver (F), 300, 200, 100, and 50 (different dosage of *E. prostrata* methanolic extract).

Table 4 KEGG pathway with fold enrichment of metabolites

KEGG term	Total	Expected	Hits	Raw *P*	-log10(*P*)	FDR	Impact
Glycerol, C00116							
Glycerolipid metabolism	16	0.04	1	4.18E−02	1.38E+00	1.00E+00	0.24
Oleic acid, C00712							
Biosynthesis of unsaturated fatty acids	36	0.10	1	9.22E−02	1.04E+00	1.00E+00	0.00
Cholesterol, C00187							
Steroid biosynthesis	42	0.11	1	1.07E−01	9.71E−01	1.00E+00	0.03
Myristic acid, C06424							
Fatty acid biosynthesis	47	0.12	1	1.19E−01	9.24E−01	1.00E+00	0.00

Figure 8



ROC curve analysis of potential urine protective biomarkers to differentiate the control group from NAFLD group. NAFLD, nonalcoholic fatty liver disease; ROC, receiver operating characteristic.

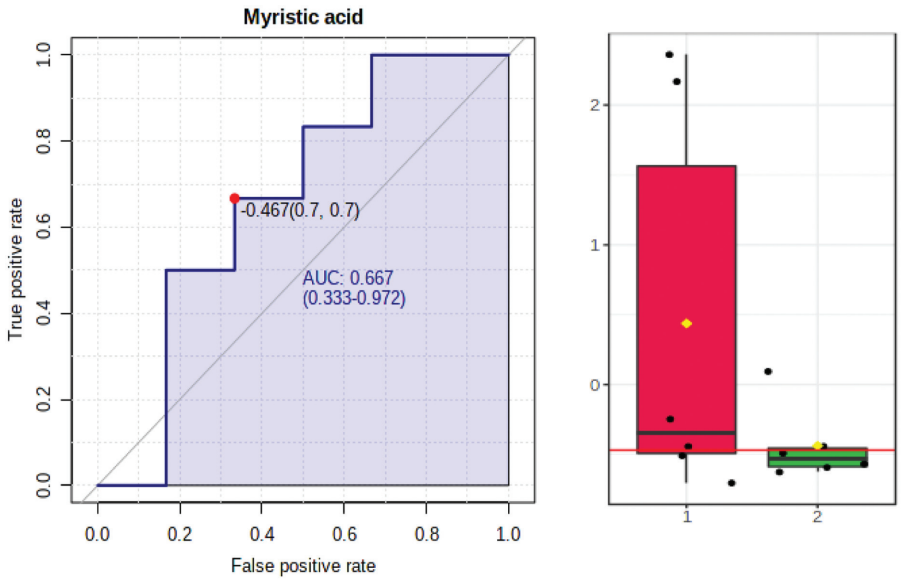
is the pathway impact value calculated from pathway topology analysis.

Moreover, we performed a receiver operating characteristic curve analysis for those common metabolites between NAFLD rats and normal controls, as shown in Figs 8 and 9. The highest area under the curve values near to 1 were represented in two components. Cholesterol has area under the curve value of 0.72 (0.7, 0.8) and myristic acid has area under the curve value of 0.67 (0.7, 0.7). As shown from these results, cholesterol and myristic acid provided the best discriminative power in urine protective groups.

Histopathology results

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 body weight. The liver

Figure 9



ROC curve analysis of potential urine protective biomarkers to differentiate the control group from NAFLD group. NAFLD, nonalcoholic fatty liver disease; ROC, receiver operating characteristic.

sections in the control group showed hepatic tissue with preserved (intact) lobular hepatic architecture and normal morphological appearance (H&E, ×400, and Masson Trichrome, ×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, ×400, and Masson Trichrome, ×400), as shown in Fig. 10a 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 Fig. 10c, owing to decreasing hepatocyte ballooning and micro and macrovesicular steatosis. NAS calculation is shown in Fig. 10c. Masson trichrome staining showed no evidence of fibrosis. These results report that the current study was performed during the early stages of NAFLD, where NAS equals 3.5, and progression of the disease can be stopped through administering *E. prostrata* methanolic extract.

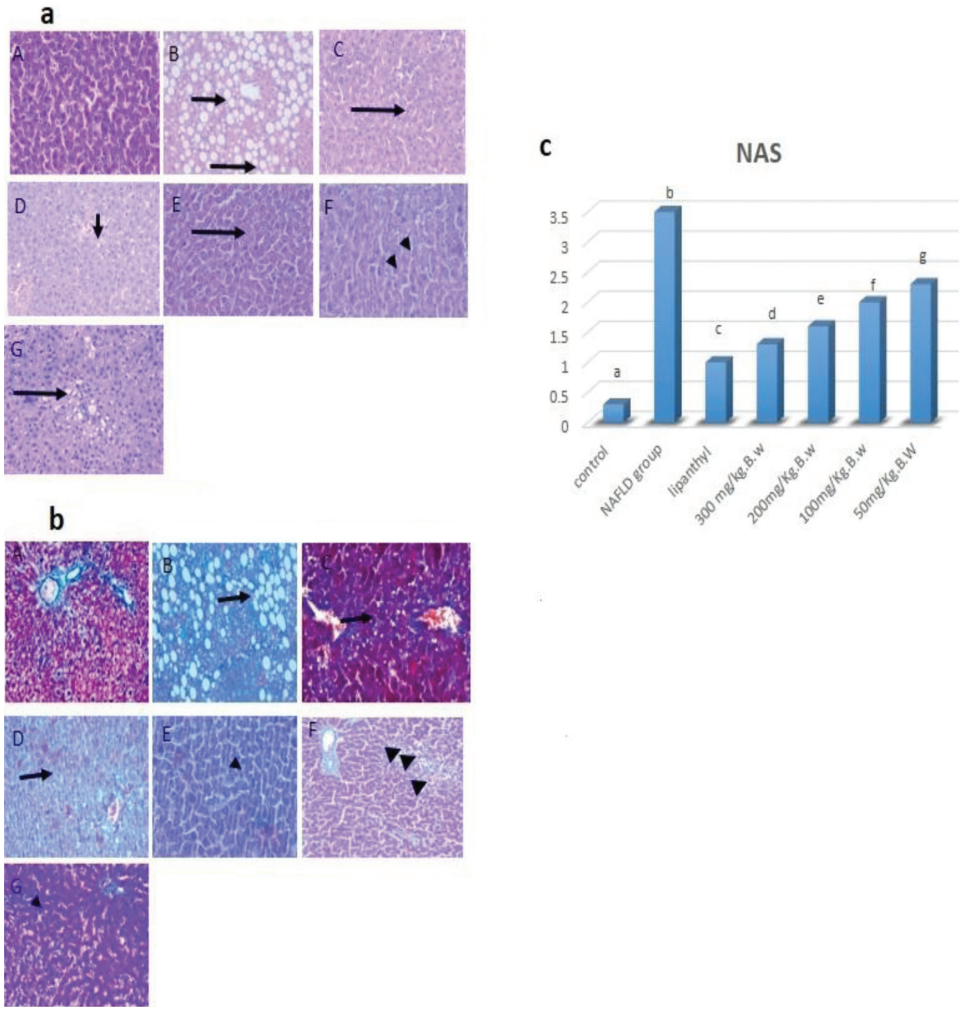
Discussion

The most common cause of liver-related morbidity and mortality worldwide is NAFLD due to excessive fat aggregation in the liver. There are different stages of this disease depending on the degree of fat accumulation ranging from simple fat accumulation in more than 5% of the hepatocytes (hepatic steatosis) to NASH with necroinflammation and sometimes fibrosis and ultimately cirrhosis. Altered lipid homeostasis, that is, fatty acids, triacylglycerol, and cholesterol metabolism, is associated with NAFLD and its progression [20]. In our study, the integration of cholesterol and fatty acids in the high-lipid diet resulted in steatosis, cellular ballooning, and inflammation, as declared by Serviddio *et al.* [21].

E. prostrata is an herbal medicine that contains wedelolactone, a major coumarin isolate, which has different biological effects, such as antihepatotoxicity and liver protection, besides its ability to reduce blood lipids in hyperlipidemic rats; however, the mechanism of its effect is poorly understood [9]. So, the present study was designed to explain the mechanism based on the metabolomics approach.

In this study, targeted urine lipidomics from rats with NAFLD was compared with normal rats using a GC-MS based lipidomics approach. Our results reported that administration of high-fat diet is associated with elevation in levels of two FFAs, myristic (14.0) and oleic acid (18:1n9), in addition to glycerolipids (glycerol, 1,3-dipalmitin) and sterol lipids (cholesterol). These metabolites may be considered promising markers for early detection of NAFLD. Moreover, they could be used as biomarkers for stratification of treatment by methanolic extract of *E. prostrata* during its restoring to relatively normal status. This study is considered complementary to our study [10] in serum.

Figure 10



Protected effect of lipanthyl and different doses of *E*clipta *prostrata* methanolic extract on liver histology and NAS. (a) H&E staining (×400) and (b) Masson Trichome (×400). (c) NAS. Data are expressed as mean±SE, n=6 per group. Groups with different letters are significantly different at P value less than 0.05. Statistical analyses one-way analysis of variance with LSD post-hoc test. Sample codes are presented as follows: (a) healthy control, (b) fatty liver group, (c) standard drug lipanthyl, and extract doses: 300 mg/kg body weight (d), 200 mg/kg body weight (e), 100 mg/kg body weight (f), and 50 mg/kg body weight (g).

The high levels of saturated and monounsaturated fatty acids (MUFA) may be owing to two reasons. The first one was the use of lard in diet in this study, which is rich in saturated fatty acid and MUFA, and the second one was the *de novo* lipogenesis of these fatty acids as an important factor that can lead to an increase in these fatty acids in serum and urine.

Myristic acid is a long-chain saturated fatty acid, predominantly produced from the fatty acid synthase pathway. The last step in fatty acid biosynthesis is catalyzed by fatty acid synthase, and thus, it is believed to be a major determinant of the maximal hepatic capacity to generate fatty acids by *de novo* lipogenesis. Under normal conditions, excess carbohydrates are converted into fatty acid, followed by esterification to triacylglycerols. In NAFLD condition, the synthesis of FFA was increased, leading to progression of the disease. In NAFLD, FFAs are the major source of fat accumulation in the liver. This may be a result of *de novo* lipogenesis, dietary fatty acids, and/or insufficient fatty acid utilization [22].

Dairy fat – a rich source of myristic acid has been reported to acutely raise LDL levels. In this context, Fernando *et al.* [22] reported that the administration of dietary saturated fatty acids (14.0 and 18.0) leads to acute elevation of LDL and ApoB levels with decreased HDL level. Myristic acid can retard endothelial cell growth and produce proinflammatory responses [23]. Moreover, its serum concentration is useful in the prediction of serum total cholesterol [24]. It can also be useful for distinguishing between NASH and simple steatosis [25].

The increase in oleic acid (18 : 1n9) owing to the increase in the activity of 9 stearoyl-CoA, a sterol regulatory element-binding protein-1c target as pointed out by Puri *et al.* [26], oleic acid is associated with high levels of hepatic cholesterol, triacylglycerols, and low level of hepatic HDL-c. Moreover, it causes decreases in the expression of PPAR-α, superoxide dismutase-1, cell proliferation, and increases in lipid peroxide production. In the high-fat group, stearic acid is highly converted to oleic acid, and this enhanced stearoyl-CoA-1 activity responsible for the synthesis of MUFA. These MUFA entered into the formation of hepatic triacylglycerols and cholesterol [27].

Lipid metabolism abnormalities and insulin resistance present in NAFLD lead to increase in glycerolipids (glycerol and sequentially accumulation of diacylglycerol and triacylglycerol), which are formed mainly from the esterification of FFA with glycerol-3-phosphate. Circulating high levels of triacylglycerols have been associated with NAFLD in experimental animals and humans, which may be owing to diminishing of fatty acids oxidation and/or decreasing clearance of triacylglycerols from blood [22].

The increase of cholesterol in our study matches with Puri *et al.* [28], who reported that the noted increase in cholesterol level in NAFLD was owing to numerous aberrations that included deterioration of adenosine triphosphate synthesis which increases 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and mitochondrial abnormalities.

In this study, these metabolites were relatively restored to their normal levels using the methanolic extract of *E. prostrata* (300 mg/dl). This may be as declared by Zhang *et al.* [29], who mentioned that the lipid-lowering activity of this plant was linked with the downregulation of gene involved in lipogenesis. This gene is HMGR, 'a key enzyme in cholesterol biosynthesis.' The upregulation of genes involved in lipid metabolism and fatty acid oxidation in the liver are as follows: (a) PPARα 'regulates the expression of genes encoding proteins involved in lipid metabolism and fatty acid oxidation such as LPL;' LPL is the rate-limiting enzyme for the hydrolysis of the triglycerides core of circulating triglyceride-rich lipoproteins.

(b) LDLR plays a significant role in lipid metabolism because LDL-C in circulation is primarily cleared by LDLR in the liver, such that when the LDLR gene is upregulated, clearance of LDL-C is increased, which leads to a reduction in the serum LDL-C content [30].

(c) Lecithin-cholesterol transferase is 'responsible for the conversion of cholesterol into cholesteryl esters and may play a core role in reverse cholesterol transport' [31]; D-scavenger receptor class B type 1 receptor is a well-known HDL receptor [32] and is also an important positive regulator of reverse cholesterol transport' [29].

The extract of *E. prostrata* improved oxidative stress in the liver by enhancing antioxidant enzymes and therefore had a hepatoprotective effect. This hypothesis is supported by the finding of Zhao *et al.* [9], who reported that administration of the *E. prostrata* extract significantly decreased the body weight gain and reduced serum lipid levels in a hamster model of hyperlipidemia. Zhang and Guo [33] pointed out that phytochemical evaluation of *E. prostrata* revealed the presence wedelolactone, demethylwedelolactone, isodemethylewedelolactone, and strycholactone, which have antihyperlipidemic effects.

Conclusion

E. prostrata can protect from NAFLD and may prevent its subsequent stages.

Comparing these results with those of the previous serum [10] study, we deduced that 75% of identified metabolites in serum were detected in urine samples. Approximately 42% of the detected metabolites in serum were affected by high fat diet-induced NAFLD versus alteration in five metabolites only in urine samples. Glycerol, myristic acid, oleic acid, and cholesterol were found to be common altered metabolites in both serum and urine samples. Therefore, urine can be used as a convenient sample for diagnosis and evaluating the protective effect of *E. prostrata*.

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Conflicts of interest

There are no conflicts of interest.

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