Targeted metabolomics for NAFLD: early detection and therapeutic efficacy of Eclipta prostrata in rats


1Therapeutic Chemistry Department, National Research Centre, Cairo, Egypt
2Biochemistry Department, Faculty of Sciences, Ain-Shams University, Cairo, Egypt.
3Cognosy Department, National Research Centre, Cairo, Egypt
4Medicinal and Aromatic Plants Department, National Research Centre, Cairo, Egypt

*Correspondence: dr_nohasaid@yahoo.com Accepted: 18 Oct. 2018 Published online: 17 Dec. 2018

Non-invasive method for early detection and safe drug targeting for managing nonalcoholic fatty liver disease (NAFLD) represent great challenges nowadays. Abnormalities in lipid metabolites levels have a considerable attention in NAFLD. This study was concerned with urinary metabolic biomarkers for the early detection of NAFLD as well as estimating the therapeutic efficiency of the methanolic extract of Eclipta prostrata (E. prostrata L) based on GC–MS followed by multivariate and univariate data analysis. Toxicity assay of extract showed its safety up to 600 mg/Kg B.Wt. Phytochemical screening of E. prostrata extract was performed using HPLC to pinpoint that rutin, quercetin, gallic acid and wedelolactone were the main active constituents of this extract. Fatty liver was induced in Wistar albino rats using high-fat diet together with cholic acid. Methanolic extract of E. prostrata was used for treatment of fatty liver in different doses. Histopathological finding of liver and NAFLD activity score (NAS) showed significant markers for the diagnosis of the early stage of the disease. Lipidomic analysis based on GC-MS showed significant increases in the levels of palmitic, oleic, stearic, myristic, and palmitoleic acids, in addition to glycerol, 1-monooestearin and 1,3-dipalmitin in the urine of rats bearing NAFLD. Treatment with E. prostrata extract ameliorated the abnormalities in the levels of these metabolites. The results concluded that E. prostrata in high dose had the potential efficacy to alleviate NAFLD. Nevertheless, lipidomic biomarkers could be used for the early detection of NAFLD and in the assessment of the mode of action of herbal medications.

Keywords: Eclipta prostrata, GC–MS, Lipidomics, Metabolic biomarkers, NAFLD

INTRODUCTION

The permanent deposition of fat in the liver due to fatty diet and sedentary lifestyles is the most proper cause of NAFLD contributing to inflammation, fibrosis and cirrhosis leading to several complications as liver cancer (Augustin et al., 2017). Lipids have the ability to regulate oxidative stress, inflammation, key transcription factors and may have significant effects on the progression of NAFLD. The possible key element in the mechanism of disease progression toward nonalcoholic steatohepatitis (NASH) is demonstrated by several lipid mediators typically associated with lipotoxicity such as diacylglycerols (DAGs) and free fatty acids (FFAs). These lipids from lipotoxicity were associated with hyperlipidemia, metabolic syndrome, insulin resistance, and type 2 diabetes, which are frequent comorbidities associated with NAFLD (Gorden et al., 2015).

NAFLD is described as a silent disease because of the deficiency of the early diagnosis...
Lipidomics is a novel field in the study of lipids in biological systems. E. prostrata, a member of the Asteraceae family, has shown promise in lipidomic studies (Zhao et al., 2015a). Among those herbs is the presence of phytosterols, phospholipids and sterols excreted in urine. Recently, the use of the herbal natural product has gained more interest in the world population in that field due to its higher contents of saponins and free fatty acids in serum, liver and heart of the animals. The liver is the main organ involved in the synthesis and metabolism of lipids, E. prostrata can act on the liver by promoting the metabolism and accelerating the excretion of excess lipids leading to hypolipidemic effect.

The principal clinical standard for final diagnosis is liver biopsy in some cases, it leads to inflammation and the patient may suffer from a subsequent risk of fibrosis. So, advanced diagnostic approach to avoid the requirements of invasive procedures are demanded as a novel management strategies for the growing epidemic NAFLD (Gorden et al., 2015).

The emersion of lipidomic approach as a targeted metabolomics has empowered researchers to study in depth lipid metabolism in both physiological and cellular levels than was previously possible in various diseases as NAFLD (Rockwell et al., 2016). Lipidomics is a novel technique encompasses analytical approaches for identification and quantification of the complete set of lipids, defined as lipidome in a given cell, tissue or organism as well as their interactions with other molecules. Mass spectrometry has been proven as a powerful tool in system biology for lipidomics assay. Lipidomics can be analyzed in different body fluids.

Urine displays a non-invasive, 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 in the assessment of organ function. Urinary lipidomic analysis was used for assessment of different diseases as cardio metabolic disorders, inflammatory disorders etc. (Hyötyläinen et al., 2017).

Weight loss via lifestyle changes or bariatric surgery is considered the only currently available therapeutic strategies for NAFLD and there is still no confirmed pharmacological option for its treatment (Augustin et al., 2017). Recently, the use of the herbal natural product has gained more interest in the world population in that field (Fakurazi et al., 2008). Among those herbs is Eclipta prostrata L. (Asteraceae), tender leaves and young shoots - cooked and used as a vegetable. The leaves are also used in chutneys and are also considered of high medical value. E. prostrata has both hypolipidemic and hepatoprotective effects via decreasing oxidative stress and controlling some enzymes involved in lipid metabolism (Sun et al., 2010). E. prostrata retracted the high levels of total lipids, total cholesterol, triacylglycerols, phospholipids and free fatty acids in serum, liver and heart of the animals due to its higher contents of saponins and phytosterol (Sun et al., 2010). E.prostrata prevented liver damage caused by CCl₄ in guinea-pigs and its powder was beneficial in the treatment of jaundice in children (Kumari et al., 2006). Many chemical constituents such as thiophenes, coumarins, triterpenoid, saponins, steroids and flavonoids have been isolated from E. prostrata (Yuan JC, Jiang YH, 2009). Wedelolactone is considered as the main component of E. prostrata that up-regulated protein level of adenosine monophosphate-activated protein kinase (AMPK)” the 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 (SREBP-1c) and hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), and improves liver steatosis” and PPARα as well as the gene expression of AMPK, PPARα, LPL, and LDLR(Zhao et al., 2015a). The liver is the main organ involved in the synthesis and metabolism of lipids, E. prostrata can act on the liver by promoting the metabolism and accelerating the excretion of excess lipids leading to hypolipidemic effect.

The present study was designed to study the effect of E. prostrata on NAFLD in rats in comparison to a well-known lipid-lowering agent (Lipanthyl®). Lipidomic analysis was used to detect a reproducible signature of lipid metabolites for early detection and characterization of NAFLD, in addition to disease management in urine.

**MATERIALS AND METHODS**

**Ethics and permissions**

The procedures of the study protocol were ethically reviewed and approved by the Ethics Review Committee of the National Research Centre (ERC-NRC) in Cairo (Approval no: 13002) and also 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 [CAIRC] under No. 997.

Experimental animal: Adult male Wistar albino rats (112) weighing 160-180 g and age ranged from 5-6 weeks were obtained from the animal house (NRC). The experimental animals were housed in wire cages and they were 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
The acclimatization period before doing the experiments. Rats were divided into two main groups, 70 in each. The first group was used to study the toxicity assay of *E. prostrata* (70 rats) and the second group was used for the therapeutic evaluation of methanolic extract of *E. prostrata* (42 rats).

**Chemicals**

MSTFA (N-methyl-N (trimethylsilyl) trifluoroacetamide) with 1% (vol/vol) trimethylchlorosilane, MTBE (Methyl-tert-butyl ether, Methanol (HPLC)), n-alkane C₆-C₄₀ standard and docosanol (an internal standard for relative quantification using (GC/MS) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Cholesterol powder was purchased from BIO BASIC CANADA INC. and cholic acid was purchased from LOBA Chemie. Commercially available assay kits for the determination of serum liver, lipid and kidney were purchased from Bio-Med (Cairo, Egypt). All other chemicals and solvents were of a high analytical grade. Standard diet was prepared according to the American Institute of Nutrition (Reeves et al., 1993).

**Experimental design**

**Preparation and phytochemical screening of *E. prostrata* methanolic extract**

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

Phytochemical screening of the dry methanolic 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 were estimated according to (Kim et al., 2003; Singleton and Rossi, 1965) respectively. 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 x 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B) at a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A); 0–5 min (80% A); 5-8 min (40% A); 8-12 min (50% A); 12-14 min (80% A) and 14-16 min (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. Thirteen standards including gallic acid, catechin, coffeic acid, syringic acid, rutin, coumaric acid, vanillin, ferulic acid, naringenin, quercetin, cinnamic acid, propyl gallate, dihydroxyisoflavon were purchased from Sigma, Aldrich (U.K) to be used as flavonoids and phenolic authentic references.

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/min as described by (Zhao et al., 2015b).

**Toxicity assay**

Methanolic extract of *E. Prostrata* (aerial part) was obtained by soxhlet, then dried using a vacuum rotary evaporator. Sub-acute toxicity of *E. prostrata* was estimated by using 70 male albino rats (160-180g), divided into 7 groups (10 each). All procedures involving toxicity on animals were performed in accordance with (*OECD 2001-guideline on acute oral toxicity (AOT)*). The animals were kept fasting for 3 hours prior to the experiment. The first group (G1) received normal food diet and tap water ad libitum to serve as control, while the other groups (G2 – G7) received dried methanolic extract of *E. prostrata* orally dissolved in water in doses 600, 400, 200, 100, 50, 20 mg/kg B.Wt., respectively (0.5 ml/rat) daily for two weeks. All animals were observed for mortality up to 48 h (short-term toxicity) and long-term toxicity (14 days). At the end of the experiment, the blood samples were collected from all groups after light anesthesia. 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 analysis.

**Therapeutic effect of *E. prostrata***

The second main group (42 rats) was subsequently divided into three subgroups

Healthy control group (-ve control): six
healthy selected rats were received standard chow (SC) and tap water ad libitum.

Control group: six normal rats were fed a high-fat diet containing standard chow supplemented with cholesterol and cholic acid for eight weeks as described by (Ney et al., 1988) to serve as +ve control.

Treated group:
30 normal rats were left to feed a high-fat diet containing standard chow supplemented with cholesterol and cholic acid for eight weeks. Rats in this group were divided into 5 subgroups (6 in each) to be treated orally for extra four weeks, with methanolic extract of *E. prostrata* 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 /200g, respectively. 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., 2017). Animals were placed in individual cages and urine was collected over a 24 hr. period in tubes containing 1% sodium azide and centrifuged to remove all particulate matter then stored at −80 °C until further analysis after that animals were decapitated and livers were removed for histopathological examination.

Biochemical analysis
Liver functions (AST and ALT), kidney functions (Urea and Creatinine) and lipid profile (TC, T.G, HDL-CH) were analyzed in sera samples as described by Castelli et al.; Fossati & Prencipe and Reitman & Frankel(Castelli et al., 1977; Fossati and Prencipe, 1982; REITMAN and FRANKEL, 1957) respectively while, LDL-CH was calculated according to (Friedewald et al., 1972).

Histo-pathological examination
Left lobe liver sections from all rats in different groups were isolated and their preparation was carried out as demonstrated by (Smith A, 1978) for histopathological examination. They used to confirm the induction of NAFLD in rats and the therapeutic effect of the methanolic 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 micrometers were stained with hematoxylin & eosin (H&E) and Masson’s Trichrome. The sections were examined under light microscope followed by calculation of Non-alcoholic fatty liver disease 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

Urine lipidome
For GC–MS analysis, according to (Matyash et al., 2008), 200 μL of urine from studied groups or quality control sample (prepared by equal volumes of sera of all studied groups) was mixed with 20μl of urease suspension and incubated 1hr. at 37°C, then 1.7 ml of methanol (98%) were added to each tube containing 5 μL docosanol (1 mg/mL, internal standard) and vortex for 5 min., samples were centrifuged for 10 min. at 10.000g and 4 °C. 5 ml of MTBE was added to each tube, and samples were shaken for one hour at room temp. Subsequently, 1.25 ml of MS-grade H2O was added to each tube, mixed and allowed to stand for 10 min. at room temp., then centrifugation was done at 1000xg for 10 min. to separate two phases. The upper phase was transferred into a separate tube and dried by nitrogen gas. For metabolites derivatization, a volume of100 μL MSTFA containing 1% TMS was added to the mixture then incubated at 70°C for 30min. (Ammar et al., 2017).

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 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, the injection volume of 1 μL at a splitless 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 °C and 300 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 25-550 and solvent delay time 3.7 min. For metabolites identification, de convolution 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 acids mixture (Sigma, Aldrich) were injected for confirming peak
assignments. Retention indices (RI) were calculated relative to n-alkanes (C₈-C₄₀) 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 sample (QC). Data transformation is done using log transformation especially "Pareto" scaling option prior to multivariate data analysis. After that, both unsupervised Principle component analysis (PCA) and supervised Partial Least Square-Discriminant analysis (PLS-DA) was performed using this program (Wu et al., 2009).

**Statistical analysis**

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

**RESULTS**

**Phytochemical Screening of *E.prostrata***

The yield extract 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 *W*-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 gallic acid which represented 2.12 mg/g dry weight of the total extract. Wedelolactone is the characteristic chemical coumarin 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 min as shown in Fig. (1a, b).

**Toxicity assay of *E.prostrata***

The results showed that methanolic extract of *E. prostrata* (aerial part) had no toxic effect on liver and kidney functions as well as lipid profile compared to control group. Also, no mortality rate was recorded within treated rats by the different doses of this extract indicating the safety of using this plant up to 600 mg/kg as shown in Table (2).

**Urine metabolites profiling**

A total of 28 urine metabolites (free fatty acids, glycerolipids, sterols), its retention times (rt), RI and the mass-to-charge ratio (m/z) were represented in Suppl. Table S1. PCA was first applied to the GC/MS dataset with score plot (Suppl.Fig.S1) showing somewhat not clear segregation of sample groups. The first two components (PC1 and PC2) explained 42.3% and 28.3% 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.2a) showed better and more clear discrimination among sample groups with R² value (0.63) and Q² value (0.60).

Metabolites contributing for groups segregation revealed from VIP score plot (> 1) (Fig.2b) include palmitic acid, oleic acid, glycerol,1,3 dipalmitin, palm italic acid, cholesterol, citric acid, urea, myristic 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 Fig. (2c).This Dendrograms model showed that fatty liver group located far from the normal group while treated groups were found in between and suggested that a dose 300mg/kg B.Wt. has the most treated 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 modelling healthy control versus nonalcoholic fatty liver rats (Fig.3a&b). The PLS-DA model exhibits R² value (0.98) and Q² value (0.97), with control and NAFLD groups being clearly discriminated from each other. palmitic acid, oleic acid,1,3 dipalmitin, citric acid, urea, glycerol, palmitoleic acid, 1-monostearin, cholesterol, myristic acid and stearic acid were increased in the urine of NAFLD group as compared to healthy control. PLS-DA model encompassing the 3 groups: normal control, NAFLD and lipanthyl drug-treated groups were attempted (Fig.4a&b). Score plot showed discrimination between NAFLD group and normal control, with lipanthyl treated animal group clustering closer to the normal control.
Figure. (1). HPLC result of wedelolactone in standard (a) and *E. Prostrata* methanolic extract (b)
Table 1: polyphenols and flavonoids content of methanolic extract of *E. prostrata* using HPLC

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Flavonoids</th>
<th>Dihydroxy-isoflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc. (mg/g)</strong></td>
<td><strong>R.T (min.)</strong></td>
<td><strong>%</strong></td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>Coffei Acid</td>
<td>Syringic Acid</td>
</tr>
<tr>
<td>2.12</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td>3.18</td>
<td>4.81</td>
<td>5.16</td>
</tr>
<tr>
<td>66.25</td>
<td>7.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table (2): Biochemical characterization of blood samples from rats treated with different doses of *E. prostrata*.

<table>
<thead>
<tr>
<th>Doses</th>
<th>Liver functions (Mean ±S.E)</th>
<th>Kidney functions (Mean ±S.E)</th>
<th>Lipid profile (Mean ±S.E)</th>
<th>HDL/LDL ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST (U/l)</td>
<td>ALT (U/l)</td>
<td>Urea (mg/dl)</td>
<td>Creatinine (mg/dl)</td>
</tr>
<tr>
<td>0 (control)</td>
<td>30.65±0.79</td>
<td>21.31±0.90</td>
<td>37.90±1.15</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>26.62±1.56</td>
<td>23.57±0.79</td>
<td>38.04±1.15</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>26.29±0.87</td>
<td>19.99 ±0.46</td>
<td>37.06±1.23</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>25.49±0.61</td>
<td>20.95±0.54</td>
<td>36.87±0.72</td>
<td>0.62±0.03</td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>28.16±0.87</td>
<td>18.02±0.90</td>
<td>37.59±0.51</td>
<td>0.64±0.04</td>
</tr>
<tr>
<td>400 mg/kg</td>
<td>29.49±0.76</td>
<td>19.36±0.49</td>
<td>37.04±0.64</td>
<td>0.59±0.03</td>
</tr>
<tr>
<td>600 mg/kg</td>
<td>27.27±0.74</td>
<td>18.89±0.31</td>
<td>37.63±0.67</td>
<td>0.59±0.04</td>
</tr>
</tbody>
</table>

Data are represented as mean ±S.E
Suppl.Figure. (S1): 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 B.Wt. (5): extract dose 200mg/kg, B.Wt. (6): extract dose 100mg/kg B.Wt. (7): extract dose 50mg/kg B.Wt.
Figure. (2): PLS-DA score plots obtained from modelling urine 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). U15: Palmitic acid, U16: Oleic acid, U11: Glycerol2, U28:1, 3 dipalmitin, U24: Palmitoleic acid, U27: Cholesterol,
U12: Citric acid, U9: Urea, U22: Myristic acid, U4: Glycerol1. Peaks numbering follow that listed in Suppl. Table (S1) 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 urine samples. Sample codes are presented as follows: (1) fatty liver group, (2) healthy control, (3) standard drug lipanthyl, and extract doses: 300mg/kg B.Wt.(4), 200mg/kg B.Wt. (5), 100mg/kg B.Wt. (6), 50mg/kg B.Wt. (7).

Suppl.Figure. (S2): PLS-DA score plots obtained from modelling NAFLD (1) healthy control groups (2) and (4) *E.prostrata* dose (300mg/Kg.B.Wt.) groups. (a) Score plot of PC1 and PC2.(b) VIP score plot for determination of important metabolites (VIP score >1). U15: Palmitic acid, U24:Palmitoleic acid, U16:Oleic acid, U27:Cholesterol, U12:Citric acid, U28:1,3 dipalmatin, U22:Myristic acid2, U9:Urea, U4: Glycerol1, U21: Stearic acid, U26: 1-monostearin. Peaks numbering follow that listed in Suppl.Table (S1) for metabolite identification using GC-Ms. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.
Figure (3): PLS-DA score plots obtained from GC-MS data by modelling 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). U15: Palmitic acid, U16: Oleic acid, U28: 1,3 dipalmitin, U12: Citric acid, U9: Urea, U4: Glycerol1, U24: Palmitoleic acid, U26: 1-monostearin, U27: Cholesterol, U22: Myristic acid, U21: Stearic acid, U11: Glycerol2. Peaks numbering follow that listed in Suppl. Table (S1) for metabolite identification using GC-Ms. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.
Suppl. Figure. (S3): PLS-DA score plots obtained from modelling NAFLD (1) healthy control groups (2) and (5) *E.prostrata* dose (200mg/Kg.B.Wt.) groups.(a) Score plot of PC1 and PC2,(b) VIP score plot for determination of important metabolites (VIP score >1). U15: Palmitic acid, U11:Glycerol2, U9: Urea, U12: Citric acid, U4: Glycerol1, U24: Palmitoleic acid, U26: 1-monostearin, , U27: Cholesterol, U21: Stearic acid, U22: Myristic acid2, U21: Stearic acid, U11: Glycerol2. Peaks numbering follow that listed in Suppl.Table (S1) for metabolite identification using GC-MS. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.

Figure.(4): PLS-DA score plots obtained from modelling NAFLD (1) healthy control groups (2) and (3) lipanthyl drug groups.(a) Score plot of PC1 and PC2,(b) VIP score plot for determination of important metabolites (VIP score >1). U15: Palmitic acid, U16: Oleic acid, U28: 1,3 dipalmatin, U4: Glycerol1, U7: Citric acid, U9: Urea, , U24: Palmitoleic acid, U26: 1-monostearin, U27: Cholesterol, U22: Myristic acid2, U21: Stearic acid, U11: Glycerol2. Peaks numbering follow that listed in Suppl.
Table (S1) for metabolite identification using GC-Ms. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.

Suppl.Figure. (S4): PLS-DA score plots obtained from modelling NAFLD (1) healthy control groups (2) and (6) E.prostrata dose (100mg/Kg.B.Wt.) groups. (A) Score plot of PC1 and PC2. (B) VIP score plot for determination of important metabolites (VIP score >1). U15: Palmitic acid, U12: Citric acid, U9: Urea, U28:1, 3 dipalmatin, U16: Oleic acid, U4: Glycerol1, U24: Palmitoleic acid, U26: 1-monostearin, U21: Stearic acid, U27: Cholesterol, U22: Myristic acid. Peaks numbering follow that listed in Suppl.Table (S1) for metabolite identification using GC-Ms. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.

The corresponding VIP score plot (Fig.4b) showed decreased levels of palmitic acid, oleic acid, 1, 3dipalmatin, glycerol, citric acid, urea, palmitoleic acid, 1-monostearin, cholesterol, myristic acid and stearic acid in the urine lipanthyl drug group compared with NAFLD group.

On the other hand, treated groups of E.prostrata at doses 300,200,100 and 50 mg/Kg.B.Wt. were found also the ones that clustered most closely to the control group in the PLS-DA plot. Consequently, another 4 PLS-DA models were constructed encompassing untreated NAFL group, normal control and with E.prostrata at doses 300 (Suppl.Fig. S2a), 200 (Suppl.Fig.S3a), 100 (Suppl.Fig.S4a) and 50 (Suppl.Fig.S5a) mg/Kg.B.Wt. respectively.

Metabolites observed from VIP score plot in treated group administered E.prostrata at dose 300 mg/Kg.B.Wt. model revealed for a decrease in palmitic acid, palmitoleic acid, oleic acid, cholesterol, citric acid, 1,3 dipalmatin, myristic acid, urea, glycerol, steearic acid and 1-monostearin levels (Suppl.Fig.S2b) as compared to NAFLD group. Metabolites observed from VIP score plot in the treated group administered E.prostrata at dose 200 mg/Kg.B. Wt. model revealed for a decrease in palmitic acid, glycerol, urea, citric acid, palmitoleic acid, 1-monostearin, cholesterol, steearic acid, myristic acid and 1,3 dipalmatin levels (Suppl.Fig.S3b) as compared to NAFLD group. Metabolites observed from VIP score plot in the treated group administered E.prostrata at dose 100 mg/Kg.B.Wt. model revealed for a decrease in palmitic acid, citric acid, urea, glycerol, cholesterol, 1-monostearin, oleic acid, palmitoleic acid, 1-monostearin, stearic acid, cholesterol and myristic acid (Suppl.Fig.S4b) as compared to the NAFLD group. Metabolites observed from VIP score plot in the treated group administered E.prostrata at dose 50 mg/Kg B.Wt. model revealed for a decrease in palmitic acid,
citric acid, cholesterol, oleic acid, 1,3 dipalmitin, glycerol, myristic acid, palmitoleic acid and 1-monostearin, (Suppl.Fig.S5b) as compared to NAFLD group.

The VIP-score plots for all studied groups showed that: palmitic acid, oleic acid, glycerol, cholesterol, citric acid, urea, myristic acid, palmitoleic acid, stearic acid, 1-monostearin and 1,3 dipalmitin may discriminate between healthy control and NAFLD groups and measure the treated effect of different doses of *E. prostrata* via restoring these metabolites levels to their normal. Fold change of these metabolites was performed using univariate data analysis of Metabo-Analyst-3 (Table 3). Also, non-parametric Kruskal-Wallis test pointed out that glycerol, palmitic acid, oleic acid, stearic acid, myristic acid, palmitoleic acid, 1-monostearin and 1,3-dipalmitin at (P≤0.05) are the most impacted metabolites between all studied groups and suggested for discrimination between healthy and NAFLD ones. The previous results showed that glycerol, palmitic acid, oleic acid, stearic acid, myristic acid, palmitoleic acid, 1-monostearin and 1,3-dipalmitin are common metabolites in multivariate analysis using PLS-DA and univariate analysis using Kruskal-Wallis test as shown in Fig. (5).

By following the PLS-DA VIP score plots derived results fold change suggested for a significant increase (P≤ 0.05), using non-parametric Mann-Whitney test, in glycerol, palmitic acid, oleic acid, stearic acid, myristic acid, palmitoleic acid, 1-monostearin and 1,3-dipalmitin amounts in NAFLD group as compared to the healthy group. Drug lipanthyl group showed a marked decrease in glycerol, palmitic acid, oleic acid, stearic acid, myristic acid, palmitoleic acid, 1-monostearin and 1,3-dipalmitin amounts compared to NAFLD group. The elevation of glycerol, palmitic acid, oleic acid, palmitoleic acid was reversed in all groups treated with all doses of *E. prostrata*. But, Doses 300, 200 and 100 affect only in stearic acid, myristic acid, 1-monostearin and 1,3-dipalmitin elevation compared to NAFLD group.

Figure 5: Venn diagram of important features found by univariate kruskal-wallis (K.W)(A) and multivariate tests PLS-DA(B), respectively. Univariate tests identified 8 features of significance while 11 features were found by multivariate statistics in urine samples.
Suppl. Figure. (S5): PLS-DA score plots obtained from modelling NAFLD (1) healthy control groups (2) and (7) E.prostrata dose (50mg/Kg.B.Wt.) groups. (a) Score plot of PC1 and PC2. (b) VIP score plot for determination of important metabolites (VIP score >1). U15: Palmitic acid, U12: Citric acid, U27: Cholesterol, U16: Oleic acid, U28: 1,3 dipalmatin, U4: Glycerol1, U13: Myristic acid1, U11: Glycerol2, U24: Palmitoleic acid, U26: 1-monostearin, U22: Myristic acid 2. Peaks numbering follow that listed in Suppl. Table (S1) for metabolite identification using GC-Ms. The colored boxes on the right indicated indicate the relative concentrations of the corresponding metabolite in each group under study.
Figure. (6): Therapeutic Effect of lipanthyl and methanolic extract of *E.prostrata* on liver histology and NAS. (a) H & E staining (400X) and (b) Masson Trichome (400X). (c) NAS. Data is expressed as mean ± SE, n = 6 per group. Different letters are significant different at p < 0.05. Statistical analyses way ANOVA 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: 300mg/kg B.Wt. (D), 200mg/kg B.Wt. (E), 100mg/kg B.Wt. (F), 50mg/kg B.Wt. (G).

Table (3): The common metabolite level alterations in urine according to PLS-DA score (VIP>1) and fold change in all treated experimental groups.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>N/F</th>
<th>D/F</th>
<th>300/F</th>
<th>200/F</th>
<th>100/F</th>
<th>50/F</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Glycerol, U11</em></td>
<td>3.69</td>
<td>3.08</td>
<td>2.52</td>
<td>1.43</td>
<td>0.57</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Palmitic acid, U15</em></td>
<td>4.51</td>
<td>3.64</td>
<td>2.15</td>
<td>1.61</td>
<td>0.62</td>
<td>0.26</td>
</tr>
<tr>
<td><em>Oleic acid, U16</em></td>
<td>2.46</td>
<td>1.83</td>
<td>1.43</td>
<td>0.78</td>
<td>0.28</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Stearic acid, U21</em></td>
<td>2.8</td>
<td>2.07</td>
<td>1.78</td>
<td>0.56</td>
<td>0.24</td>
<td>0.13</td>
</tr>
<tr>
<td><em>Myristic acid, U22</em></td>
<td>2.73</td>
<td>1.45</td>
<td>0.81</td>
<td>0.52</td>
<td>0.23</td>
<td>0.11</td>
</tr>
<tr>
<td><em>Palmitoleic acid, U24</em></td>
<td>3.12</td>
<td>2.52</td>
<td>2.11</td>
<td>1.53</td>
<td>1.23</td>
<td>0.42</td>
</tr>
<tr>
<td><em>1-Monostearin, U26</em></td>
<td>3.13</td>
<td>2.06</td>
<td>1.54</td>
<td>0.81</td>
<td>0.43</td>
<td>0.21</td>
</tr>
<tr>
<td><em>1,3-Dipalmitin, U28</em></td>
<td>4.21</td>
<td>2.34</td>
<td>1.81</td>
<td>0.84</td>
<td>0.31</td>
<td>0.09</td>
</tr>
<tr>
<td>Urea, U9</td>
<td>0.19</td>
<td>0.15</td>
<td>0.12</td>
<td>0.09</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Citric acid, U12</td>
<td>0.21</td>
<td>0.18</td>
<td>0.14</td>
<td>0.10</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>Cholesterol, U27</td>
<td>0.20</td>
<td>0.17</td>
<td>0.18</td>
<td>0.12</td>
<td>0.08</td>
<td>0.05</td>
</tr>
</tbody>
</table>

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 to standard drug lipanthyl. Results pointed out that the most effective dose of *E.prostrata* extract was 300mg/Kg.B.Wt. The liver sections in the control group showed hepatic tissue with preserved (intact) lobular hepatic architecture and normal morphological appearance (H&E, x400) and (Masson Trichrome, x400). In contrast, the NAFLD group showed preserved (intact) lobular hepatic architecture, hepatocyte ballooning, severe micro and macro vesicular steatosis (black arrows) and moderate lobular inflammation (H&E,
x400(Masson Trichrome, x400) as shown in Fig. (6a,b) respectively leading to about 12 fold change (p<0.001) increase in NAS. This value was significantly decreased in all treated groups as compared to NAFLD group Fig (6c) due to decreasing hepatocyte ballooning and micro & macrovesicular steatosis. NAS calculation is shown in Fig (6c), Masson Trichome 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**

The most common cause of liver-related morbidity and mortality worldwide is NAFLD which occurs 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 hepatocytes (hepatic steatosis) to non-alcoholic steatohepatitis (NASH) with necro-inflammation and sometimes fibrosis and ultimately cirrhosis. Altered lipid homeostasis i.e. fatty acids, TAG and cholesterol metabolism is reported to be associated with NAFLD and its progression (Rajwal and McClean, 2017). Cholesterol and fatty acids in the high lipid diet resulted in steatosis, cellular ballooning and inflammation as declared by Serviddio et al., (2016). In this study, we investigate the metabolites related to early diagnosis of NAFLD integration due to the high-fat diet.

*E. prostrata* is a herbal medicine with different biological effects including anti-hepatotoxicity and liver protection. *E. prostrata* reduces the blood lipids in hyperlipidemic rats as well (Zhao et al., 2015a). The mechanism of its effect is poorly understood but may be attributed to its content of flavonoids, polyphenols and coumarins. In this study, we aimed to explain the major urinary metabolites which are affected by *E.prostrata* used to treat NAFLD in a rat model.

Our results showed that NAFLD was associated with an elevation in the levels of five free fatty acids. Three of them were saturated (palmitic acid 16.0, stearic acid 18.0 and myristic acid 14.0) and the rest were monounsaturated (oleic acid18:1n9 and palmitoleic acid 16:1n7). In addition, elevation in glycerolipids levels (glycerol, 1-monostearin and 1, 3 dipalmitin) was observed in urine. These lipid metabolites may be considered as promising markers to distinguish NAFLD rats from healthy animals using urine samples and to determine the therapeutic effectiveness of the methanolic extract of *E.prostrata*. The relationship between these metabolites was illustrated in diagram (1).

The high levels of saturated (SFA) and monounsaturated (MUFA) fatty acids may be due to two reasons. The first one might be the use of lard in the diet which is rich in SFA and MUFA, and the second one might be the denovo lipogenesis of these fatty acids.

Palmitic acid is the first fatty acid formed during fatty acids synthesis and is the originator of longer fatty acids. Palmitic acid can be converted to stearic acid via the action of isoforms 1 and 6 of fatty acid elongase (Elov-1 and Elov-6)(Gambino et al., 2016; Otsuki and Oku, 1995). Oleic and palmitic acids were reported to be the most plentiful circulating fatty acids in NAFLD subjects serum (Gambino et al., 2016). Our results suggested that palmitic acid elevation may be associated with steatosis in hepatocytes of rats and this is parallel to Moravcova et al., (2015) finding. They concluded that palmitic acid displays a dose-dependent cytotoxic effect in rats hepatocytes via reactive oxygen species (ROS) production in primary culture in addition to its ability to increase serum total cholesterol and LDL-cholesterol concentrations relative to stearic or oleic acid (Thijssen and Mensink, 2005).

Stearic acid, a unique long-chain SFA, is produced from carbohydrates via the fatty acid synthesis machinery. Stearic acid was found to be associated with lowered LDL-cholesterol in comparison with other saturated fatty acids.
In the other hand, it tended to raise LDL cholesterol, lower HDL cholesterol, and increase the ratio of total to HDL cholesterol in comparison with unsaturated fatty acids (Emken, 1994; Hunter et al., 2010). In contrast, Puri et al., (2009) reported that stearic acid (18:0) content was not significantly changed in NAFL and NASH. This opposed our finding where the stearic acid level was elevated in the fatty liver group and this was concurrent with Alkhouri et al., (2009) who pointed to stearic acid can induce hepatoma cell line through increase endoplasmic reticulum stress followed by rising apoptotic cell death.

Myristic acid is long chain saturated fatty acid, predominantly produced from fatty acid synthase (FAS) pathway which catalyzes the last step in fatty acids biosynthesis. Thus, it is believed to be a major determinant of the maximal hepatic capacity to generate fatty acids by de novo lipogenesis (Fernando et al., 2011). Diary fat; a rich source of myristic acid has been reported to acutely raise LDL levels. Feng et al., (2017) 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 levels. Myristic acid could retard endothelial cells growth and produce pro-inflammatory responses according to Harvey et al., (2010). Moreover, its serum concentration was used in the prediction of serum TC (Bradbury et al., 2010).Tomita et al., (2011) founded that myristic acid level was used to distinguish between non-alcoholic steatohepatitis and simple steatosis.

The increase in MSFA Palmitoleic acid (16:1 n7) and Oleic acid (18:1n9) levels may be due to
the increase in the activity of stearoyl CoA desaturase (Δ9 SCD), a sterol regulatory element binding protein-1 target, which catalyzes the conversion of stearic acid (18:0) to oleic acid (18:1 n9) as pointed out by Puri et al., (2009). Oleic acid was associated with high levels of hepatic cholesterol, triacylglycerols and low level of hepatic HDL-cholesterol. It was associated with a reduction in the expression of PPAR-α (peroxisome proliferator-activated receptors), superoxide dismutase-1, cell proliferation and increased lipid peroxide production. In the study of Jordão, (2012) stearic acid was supposed to highly convert to oleic acid in the high-fat group through enhancement of SCD-1 activity responsible for the synthesis of MUFA. These MUFA were used for the synthesis of hepatic triacylglycerols and cholesterol. The high level of serum 16:1 palmitoleic acid profile is strongly related to fatty liver disease (FLD), which is probably a reflection of stearoyl-CoA desaturase (SCD) activation and hepatic lipogenesis. Circulating 16:1 palmitoleic acid is highly associated with endogenous sources of fatty acid synthesis and TAG as illustrated by Feng et al., (2017). Thus, palmitoleic acid could be used as a diagnostic sign of triglyceridemia.

Lipid metabolism abnormalities and insulin resistance present in NAFLD leading to increasing of glycerolipids in our work where (glycerol "sequentially accumulation of DAG and TAG", 1-monostearin and 1,3-dipalmitin) were elevated in NAFLD group. They were formed mainly from the esterification of free fatty acids (FFA) with glycerol-3-phosphate. Circulating the high-level of TAGs have been associated with NAFLD in experimental animals and humans which were may be due to the diminishing of fatty acids oxidation and/or decreasing the clearance of TAGs from blood (Fernando et al., 2011).

The amelioration in 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 and oxidative stress in liver (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 (SREBP-1), 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase (HMGCR), glycerol-3-phosphate acyl transferase (GPAT), fatty acid synthase (FAS), and acetyl-coenzyme carboxylase (ACC) which 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 AMP-activated protein kinase (AMPK) and PPARα levels and thus was found to be beneficial against hepatic steatosis (Chao et al., 2014). Also, the extract of E. prostrata improved oxidative stress in the liver by enhancing anti-oxidant enzymes and therefore had a hepatoprotective effect. This hypothesis is supported by the finding of Zhao et al., (2015a) who reported that administration of E. prostrata extract significantly decreased the body weight gain and reduced serum lipid levels in a hamster model of hyperlipidemia. Zhang and Guo, (2001) pointed out that the phytochemical evaluation of E. prostrata revealed the presence of wedelolactone, which have an anti-hyperlipidemic effect.

CONCLUSION

The current study represented the main altered lipid metabolites related to early detection of NAFLD in urine samples using a lipidomic approach to act as keys points to control this disease. This study also highlighted to the therapeutic effect of E. prostrata through its regulation to the progression of NAFLD and amelioration of this metabolite profile and pointed that the high dose of methanol extract of E. prostrata has the maximum therapeutic effect compared to the lipanthyl drug.

ABBREVIATIONS
CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

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Ethical approval and consent to participate
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or the national research committee.

AUTHOR CONTRIBUTIONS
All authors approve the manuscript for publication.

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165.


Noha et al., Targeted metabolomics for NAFLD


