Introduction and Aim of Work

Chronic liver diseases (CLDs) constitute a major neglected global health problem and a heavy burden on the world economy. Its worldwide estimations is undervalued showing that 544 million people having CLDs with a rate of mortality of two million deaths each year ***(Marcellin and Kutala, 2018)***.

Non-alcoholic fatty liver disease (NAFLD) is considered nowadays as the most common causative factor for CLDs in developed nations as well as in developing countries. It is growing rapidly at an alarming rate ***(Perumpail et al., 2017)***. The prevalence of NAFLD accounts for 10-15% in Egypt ***(Kamal et al., 2018)*** .

The term fatty liver refers to a state of abnormal fat deposition and accumulation in more than 5% of hepatocytes in the form of triacylglycerols which occurs in the absence of alcohol abuse (NAFLD) ***(Cristina L. et al., 2015).***

Non-alcoholic fatty liver disease encompasses a wide range of liver diseases. It starts with simple hepatic steatosis, which is a reversible condition, passing through the more potentially aggressive form of inflammatory liver disease known as non-alcoholic steatohepatitis "NASH" with ballooning and with or without fibrosis ***(Ahmed et al., 2017)***.

The latter may proceeds to liver cirrhosis with a high risk for liver cancer or hepatocellular carcinoma (HCC) and then death due to hepatic failure ***(Oliveira et al., 2016)***.

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 ***(Feng et al., 2018)***.

As pointed out by ***Alisi et al., (2017)*** lipidomics analysis of plasma and urine became a potential candidate for metabolic biomarkers in several diseases.

The use of the herbal natural product has gained interest more recently among the world population. Among those herbs are the *Eclipta prostrata (E. prostrata) (Asteraceae)* and *Bauhinia retusa (B. retusa) (Caesalpiniaceae)* which have high potential medical value **(*Fakurazi et al., 2008)*** **.**

E. prostrata has hepatoprotection and lipid-lowering effects due to the high amount of saponins and phytosterol ***(Sun et al., 2010).*** Many chemical constituents such as thiophenes, coumarins, triterpenoid, saponins, steroids and flavonoids have been isolated from *E. prostrata* ***(Yuan JC and Jiang YH, 2009).***

Earlier studies on this plant showed its effectiveness in preventing CCl_4-induced liver damage in guinea-pigs and in the clinical studies, the powdered drug was found to be useful in the treatment of jaundice in children***(Kumari et al., 2006)***.

Bauhinia variegata and *Bauhinia purpurea* are genus of shrubs or tree. Many useful products such as tannins, fiber, gum and oil are obtained from *Bauhinia species*. Antioxidant and hepatoprotective activities of *Bauhinia spp* have been reported ***(Kuo et al., 1998)***. ***Lakshmi et al., (2011)*** reported that *Bauhinia spp* has hypolipidemic activity and a significant reduction in total cholesterol "TC", triacylglycerols "TAGs", low density lipoprotein- cholesterol "LDL-C", very low density lipoprotein – cholesterol "VLDL-C" levels but also increases the high density lipoprotein– cholesterol "HDL-C" level due to the presence of polyphenolic compounds flavonoids, tannins and proanthocyanidins in the ethanol extracts, which reduce oxidation of LDL-C.

Bauhinia retusa is another species that will be used in the present study to evaluate its hypolipidemic activity and hepatoprotective effect on experimental animals as this is not previously reported.

Aim of work

The main goals of this study are:

- To identify metabolic biomarkers for diagnosis and prognosis of nonalcoholic fatty liver diseases (NAFLD) based on lipidomic approach.
- To evaluate the therapeutic efficacy of natural products compounds on NAFLD compared to the lipid-lowering agent (lipanthyl) *via* lipidome analysis.
- To evaluate the protective effects of the selected natural product compunds on NAFLD *via* lipidome analysis.
- To assert the feasibility and suitability of targeting metabolomics techniques “lipidomic analysis” in management of NAFLD using herbal medication.

Review of Literature

The liver is the largest organ in the body weights 1200–1500 and comprises one-fiftieth of the total adult body weight. It is the major organ involved in the metabolism, detoxification and excretion of various exogenous substances such as xenobiotic ***(Osadebe et al., 2012)***. It has been estimated that the liver is responsible for 1,500 essential processes on a biochemical level. The failure of any of these biochemical functions could lead to death. One of the main functions is the proper handling of carbohydrates (sugars, starches), lipids (fat, cholesterol, bile acids), and proteins. Some of the important proteins produced by the liver include blood clotting factors and albumin. Essential vitamins and minerals are produced, stored, or altered by the liver for proper use in the body***(Kuntz and Kuntz, 2006a).***

Also, liver aids in the function of an immune system, the endocrine system, and in maintaining healthy blood cells. Also, it acts as the first line of defense against invading bacteria from the digestive tract ***(Williams, 2006).***

1.1. Liver disorders

There are a lot of liver disorders and they can be divided into infectious hepatic disorders (viral, bacterial and parasitic infection) and non-infectious hepatic disorders (mycotoxins,

aflatoxins, aging, non-alcoholic fatty liver (NAFLD), inheritable metabolic disorders and hepatocellular carcinoma (HCC). Also, environmental agents (radiation and heavy metals "lead, iron, arsenic, mercury and copper"), drugs (acetaminophen, erythromycin and estradiol), chemicals [carbon tetrachloride ($CC1_4$), paracetamol and thioacetamide (TA)], smoking and alcohol contribute to developing liver disorders***(Kuntz and Kuntz, 2006b)***

1.2. Fatty liver disease

Fatty liver is a reversible condition where large vacuoles of triacylglycerols fat accumulate in liver cells *via* the process of steatosis (i.e., abnormal retention of lipids within a cell) ***(Reddy and Rao, 2006)***. Fatty liver can be classified according to abuse of alcohol into alcoholic liver disease (ALD) and NAFLD ***(Bertola, 2018).***

1.2.1. Nonalcoholic fatty liver disease

NAFLD is one of the most common conditions in hepatology, mainly due to the current increase in the prevalence of obesity, diabetes, type of lifestyle and non-healthy diet ***(Farrell and Larter, 2006)***. It is characterized by an increase in intra-hepatocellular triacylglycerols, which is not due to alcohol or other known causes.

Hyperlipidemia, obesity and type 2 (non-insulin-dependent) diabetes mellitus are coexisting conditions frequently associated with NAFLD. In the fatty liver, the ability of insulin to inhibit the production of both glucose and VLDL-C is impaired. This results in hyperglycemia, hyperinsulinemia, and hypertriglyc- eidemia, which in turn lead to lower HDL-C concentration ***(Byrne et al., 2009)***.

1.2.1.a. Prevalence of NAFLD

NAFLD affects about 20–30% of the general population ***(Borai et al., 2018)*** .The highest prevalence of NAFLD is reported from the Middle East (31.79%) and South America (30.45%) whereas the lowest prevalence rate is reported from Africa (13.48%) ***(Chalasani et al., 2018)***. It may affect persons of any age and it has been described in most racial groups. In most series, the typical patient with NAFLD is a middle-aged woman ***(Rashid and Roberts, 2000)*** but some have found a higher prevalence of NAFLD in males than in females***(Luyckx et al., 1998)***.

1.2.1.b. Stages of nonalcoholic fatty liver

NAFLD includes a spectrum ranging from simple steatosis over steatohepatitis to cirrhosis and hepatocellular carcinoma (HCC) ***(Lazo and Clark, 2008)*** as shown in Figure (1).

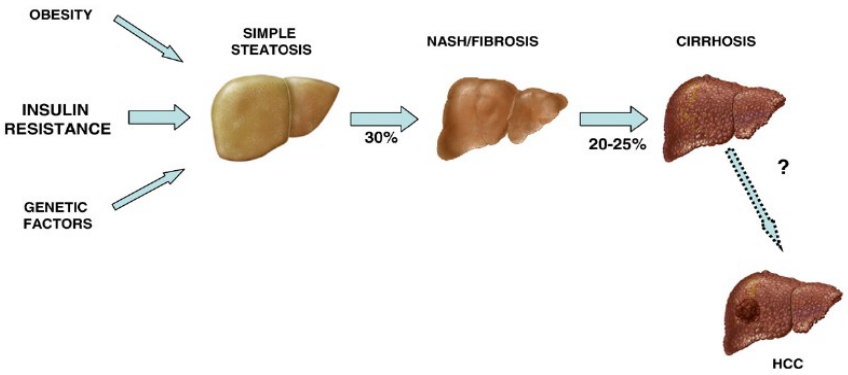

Fig. (1): A spectrum of non-alcoholic fatty liver disease ***(Trauner et-al., 2010).***

1.2.1.c. Mechanisms of NAFLD progression

NAFLD has a multifactorial etiology. Acombination of environmental, sedentary life style, metabolic factors, ethnicity, gender, genetic, predisposition, increased consumption of both high fat foods and beverages which is high in fructose concentration, participate in NAFLD ***(Misra et al., 2009; Zaki et al., 2014).*** The pathophysiology of NAFLD is complicated because of participating of many physiological factors in fat deposition in the liver. These factors are endogenous, such as excessive fatty acid influx from fat depots or *de novo* hepatic lipogenesis from non-lipid precursors, and exogenous factors, including excessive carbohydrate and fat intake ***(Antonucci et al., 2017) .***

It is known that, healthy liver contains about 5% as total lipid, of which one fourth as trigacylglycerols. There are several factors that regulate the fat content of liver. Normal

lipid content in the liver is maintained by the balance between the factors that increase liver fat (either endogenous or exogenous as previously mentioned) and the factors that decrease liver fat (mobilization of fats from the liver into blood stream to fat depots and degradation of fatty acids in the liver). Any relative decrease or increase in the rate of any of these factors results in abnormal fat accumulation in the liver forming the fatty liver or the condition that is called steatosis.

There are several factors that participate in the progression of NAFLD from normal steatosis into steatohepatitis (NASH). The theory of the "two-hits" was considered one of the most important hypothesis in this respect ***(Day and James, 1998)***. According to this theory, accumulation of lipids in hepatocytes, is the "first hit" for establishment of NAFLD mainly in the form of triacylglycerols that results from an imbalance between the synthesis and uptake of fatty acids by hepatocytes on one side and the fatty acids, oxidation and export on the other side ***(Angulo, 2002 & Mehta et al., 2002).***

Then, oxidative stress is the "second hit" that is responsible for the progression of NAFLD from simple steatosis to steatosis associated with necro-inflammatory activity or NASH. The oxidative stress may cause steatohepatitis by many mechanisms among which lipid

peroxidation and cytokine induction such as tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-ß) and interleukin (IL-8) with subsequent cell death ***(Mehta et al., 2002 & Rolo et al., 2012).***

This old traditional view of the "two-hits" hypnosis becomes obsolete and another more complex "multi parallel-hit hypothesis" has developed which includes a wide range of parallel hits, such as oxidative stress, insulin resistance, environmental elements, genetic and epigenetic mechanisms, cytokines and microbial changes ***(Del Ben et al., 2017)***.

The "multiple hit hypothesis" for NAFLD development and progression into NASH and fibrosis stated that environmental and dietary factors, in addition to obesity, lead to increases in serum levels of total cholesterol (TC) and free fatty acids (FFAs), insulin resistance development, proliferation of adipocyte and intestinal microbiome dysfunction and changes.

Insulin resistance acts on adipose tissue leading to dysfunction of adipocytes, increase in lipolysis and adipokines release as well as release of pro-inflammatory cytokines e.g.: tumor necrosis factor- α "TNF- α" & interleukin-6 "IL-6". Thus maintaining the state of insulin resistance. In the liver, *de novo* lipogenesis is amplified by insulin resistance. The increased flux of hepatic FFAs derived from the above-

mentioned processes and from gut microbiome altered activity leads to the following situations: TAGs synthesis and accumulation the 'toxic' levels of free cholesterol, fatty acids and other lipid metabolites that causing mitochondrial dysfunction by oxidative stress and reactive oxygen species "ROS" production as well as increased stress of endoplasmic reticulum (ER), all these factors leading to hepatic inflammation. Also, it enhanced small bowel permeability occurs with consequent increased circulating levels of some molecules that contribute to increase the inflammation and ER stress, such as lipopolysa- ccharides (LPS), and to pro-inflammatory cytokines release ***(Buzzetti et al., 2016).***

1.2.1.d. Clinical features of NAFLD

Most subjects with NAFLD are clinically silent and asymptomatic but can manifest with non-specific symptoms such as right upper quadrant discomfort or fatigue. Diagnosis of NAFLD may be performed through different stages:

Clinical chemistry showed that, NAFLD is the main causes of persistent elevation of both alanine amino transferase "ALT" and gamma-glutamyl transferase "γGT". The level of aspartate amino transferase "AST" may be elevated too. It is reported that, the levels of ALT in serum not associated with the degree of steatosis or fibrosis. Lipid profile could reveal

hypertriglyceridemia as nonalcoholic fatty liver related diseases ***(Croke and Sampson, 2012).***

Magnetic resonance imaging (MRI) is used to detect fat, even when central obesity is the result in poor ultrasound findings (***Croke and Sampson, 2012) .***

Liver biopsy remains the standard diagnosis of NAFLD through the evaluation of the degree of steatosis, inflammation and fibrosis, beside its ability to differentiate simple steatosis from cirrhosis ***(Gariani et al., 2013)***.

Therefore, more advanced diagnostic technique such as lipidome analysis is demanded to avoid the invasive procedures.

1.3. Lipidomic analysis

Lipidomics is a novel technique encompasses analytical approaches for identification and quantific- ation the complete set of lipids, defined as lipidome in a given cell, tissue or organism as well as their interactions with other molecules. 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 as NAFLD ***(Sethi et al., 2017)***.

Recently, lipidomics is used, largely due to the development of mass spectrometry (MS). It can be defined as

the large-scale study of lipid species and their related networks and metabolic pathways that exist in cells or any other biologic system***(Sethi and Brietzke, 2017)***.

Lipidomics is the identification and characterization of all lipid species and their biological functions in protein expression involved in lipid metabolism.

Sethi et al., (2017) reported that lipids are the core to the regulation and control of cellular function and disease. Therefore, lipidomics approach becomes an emerging field of basic and translational research associated with many diseases***(Sethi and Brietzke, 2017).***

1.3.1. Lipidomics strategies

There are two main strategies: targeted and non-targeted lipid analyses. The first one is used for the quantitative analysis of the specific known lipids. While, non-targeted lipid analysis is used to identify all lipid species concurrently ***(Sethi and Brietzke, 2017)***.

1.3.2. Lipidomic profile

Many analytical methods have been used for lipids analysis such as thin-layer chromatography (TLC), gas chromatography (GC), and massspectrometry (MS).

Taking into consideration, lipid analysis needs aconsecutive methods and technologies, such as lipid

extraction, MS-based analytical techniques and bioinformatics technology. A flowchart of lipidomics technology is shown in Figure (2).

Lipidomics technology provides clear insights into the main functions of lipid species in health and disease. It is also used to identify potential metabolic biomarkers for establishing preventive or therapeutic programs for NAFLD ***(Sethi and Brietzke, 2017)***

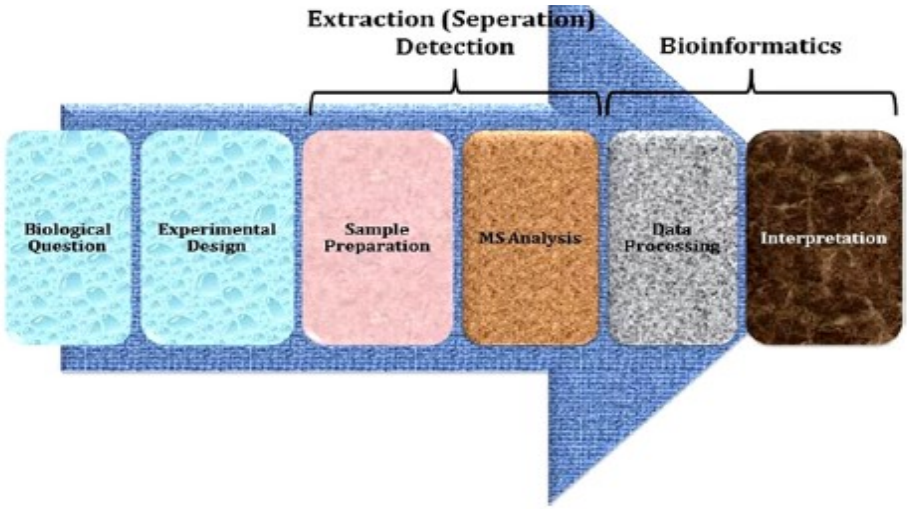

Fig. (2): Workflow summarizing the different steps in lipidomics analysis ***(Sethi and Brietzke, 2017).***

1.3.3. Sample preparation and lipid extraction

Liquid extraction is the most common method for lipid extraction. Numerous different solvents have been used for lipid extraction, but presently the most common methods

for lipidomic profiling are based on modified Folch extraction, i.e., extraction with a chloroform-methanol mixture in ratio of 2:1 (v/v), or extraction with methyl tert-butyl ether (MTBE)-methanol-water or MTBE methanol -chloroform ***(Sethi et al., 2017).***

Sample evaporation as another major concern for the handling of lipid extracts in low volumes of volatile chloroform and methanol. On the other hand, the methyl-tertbutyl ether (MTBE): MeOH protocol, has a high sample-to-solvent ratio, making it more challenging to use***(Schuhmann et al., 2012).***

1.3.4. Multiple lipidomic platforms

Lipidomics is the high-throughput molecular technique to identify and quantify the utmost number of molecular lipid species highlighting the full lipidome of cells upon genetic or external perturbations.

Approximately 104 different structures of lipids have been stored in the most comprehensive lipid structure database (LIPID MAPS, http://www. Lipid- maps.org).

As pointed out by ***Jung et al., (2011),*** MS-based lipidomic approachs have been applied to measure the metabolic networks of certain lipid classes. However, single MS methods is still non capable of outputting the complete lipidome. Therefore, multiple MS platforms are argently