ORIGINAL ARTICLE

Hydroalcoholic *Moringa oleifera* L. Leaf Extracts Ameliorate Alcohol-induced Steatohepatitis in Zebrafish Larvae

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ABSTRACT

Introduction: Alcohol consumption for a prolonged duration induces fatty liver disease such as steatosis. This research aims to examine the bioactive compounds in hydroalcoholic *Moringa oleifera* (MO) leaf extracts using GC-MS and their effects on alcohol-induced liver steatosis in zebrafish larvae. **Methods:** The bioactive compounds in hydroalcoholic MO leaf extracts were examined using GC-MS. Zebrafish larvae at 120 hpf were exposed to 2% ethanol, and co-treated with different concentrations of hydroalcoholic extracts, for 32 h. The larvae were then stained with oil red O for fatty tissues in the liver and then measured by using ImageJ. **Results:** GC-MS revealed the presence of bioactive compounds 8,14-Seco-3,19-epoxyandrostane-8,14-di-one, 17-acetoxy-36-methoxy-4,4-dimethyl- (73.72%), 1,2,3-Propatriol, 1-indol-4-yl(ether) (4.43%), D-mannose (2.17%) and quinic acid (2.10%) in hydromethanolic extract. For hydroethanolic extract, 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (25.14%) and gamma-sitosterol (18.56%) are major constituents. Both hydromethanolic and hydroethanolic leaf extracts showed a reduction in the liver steatosis at the tested concentrations (0.1-0.3 mg/mL), with a significant reduction of 21-39.5% in liver injury as measured in fatty liver size. **Conclusion:** This finding indicates that hydroalcoholic MO leaf extracts reduce alcohol with a significant reduction of 21-39.5% in liver injury as measured in fatty liver size -induced liver steatosis, suggesting it could be a good supplement for alcohol-induced liver injury.

Keywords: GC-MS; *Moringa oleifera*; Hydroalcoholic; Steatohepatitis; Zebrafish larvae

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INTRODUCTION

Liver is one of the most important organs that regulate many physiological functions including synthesis, metabolism, and storage. The nature of the liver with cytochrome P450 enzymes for biotransformation increases the risk of liver injury, through reactive oxygen species generation and inflammation (1). Alcohol-induced liver toxicity is damage or injury that impairs liver function due to prolonged or excessive exposure to alcohol. Metabolism of alcohol in the liver primarily by the enzyme alcohol dehydrogenase and cytochrome P450 into acetaldehyde. During this process, nicotinamide adenine dinucleotide (NADH) will be produced as a by-product, which subsequently leads to the formation of fatty acids in the liver, resulting in fatty liver disease (1). Steatosis is the first stage of alcoholic liver disease, which involves excessive fat droplet accumulation in the hepatocytes, and if continues, leads to cirrhosis, which is the end stage of liver disease (1). The only way to reduce the progression of alcohol-induced liver injury is to stop alcohol intake. In addition, plant-based products that possess hepatoprotective activity have been reported (2).

Moringa oleifera is a form of indigenous Indian medicinal herb from the family of Moringaceae (3). Many nations, including India, Pakistan, the Philippines, Hawaii, and many parts of Africa, use the tree's leaves, flowers, and immature pods as the source of nutritious vegetables (4), as well as bioregulators to improve the growth of crop under drought stress (5). *M. oleifera* leaves, pods, seeds, gums, bark, and flowers are used to alleviate mineral and vitamin deficiencies, neutralize free radicals, enrich anemic blood, strengthen the immune system and act as a hypocholesterolemic agent. Previous research has been performed to evaluate the *M. oleifera* extracts and their healing

function in acetaminophen-induced hepatotoxicity due to oxidative damage (6). However, research to compare the anti-steatosis activity of hydroethanolic and hydromethanolic *M. oleifera* leaf extracts on the alcohol-induced liver injury on zebrafish embryos was not completely established. In this study, hydroalcoholic *M. oleifera* leaf extracts were used to examine its phytochemical constituents using GC-MS and their ability in reducing the alcoholic fatty liver disease in the zebrafish larvae model.

Zebrafish, Danio rerio is the ideal model for hepatotoxicity research due to the similarity in physiological processes, high homology, and metabolism to humans (7). Moreover, zebrafish larva permits easy observation to monitor their development due to their transparency and is a good alternative model for biomedical research (8).

MATERIALS AND METHODS

Leaf collection and extraction

The MO leaves were collected from Bangsar, Kuala Lumpur, Malaysia and sent for identification and authenticated by a botanist from Institute of Bioscience, Universiti Putra Malaysia (SK3282/18). The leaves were extracted following the procedures previously described (9). In brief, the dried leaves were pulverised into powder, and approximately 20g of leaves powder was extracted using Soxhlet apparatus with 220mL solvent of aqueous methanol (methanol:water, 80:20 v/v) and aqueous ethanol (ethanol:water, 80:20 v/v) at the temperature of 60-80 °C for 12-16 h. The yield for hydroethanolic and hydromethanolic extract is 14.85% and 13.07%, respectively.

GC-MS analysis

GC-MS analysis of the two hydroalcoholic extracts of *M. oleifera* was performed using a Perkin ELmer Clarus SQ8T, fitted with an Elite-5MS capillary column (30 m x 0.25 mm inner diameter, 0.25 µm film thickness) (10, 11). Ultra-high purity helium (99.99 %) was used as carrier gas at a constant flow rate of 1.0 mL/min. The temperature of the injection, transfer line and ion source were 240 °C, 150 °C and 230 °C, respectively. The ionization energy was 70 eV. The oven temperature was programmed from 70 °C (hold for 3 mins) to 290 °C at a rate of 10 °C/min (hold for 9 mins) (12, 13). The crude samples were diluted with methanol (1/100, v/v) and filtered. The filtered extracts (1 uL) were injected at a split ratio of 10:1. The data were obtained by collecting the full scan mass spectra within the scan range of 40-550 amu. The identification and characterization of chemical compounds of the extracts were done by matching with those standards available in the NIST mass spectrum libraries.

Zebrafish husbandry

The zebrafish were purchased and maintained in the tank with a temperature of 25oC for about 11 hours of fixed photoperiod on a daily routine, following OECD guidelines (14). The mating procedure and experimentation were conducted based on the protocol established in the laboratory (15).

Alcohol exposure and treatment in larvae

Zebrafish larvae at 120 h post-fertilization (hpf) were chosen and ten larvae were placed on each well of 24-well plates and exposed to 2% ethanol in E3 media at a final volume of 1 mL, for 32 hours to induce liver toxicity as described (16), in the presence or absence of hydromethanolic *M. oleifera* extract at concentrations of 0.1 – 0.3 mg/mL. The groups were (I) control untreated, (II) control with 2% ethanol, (III) 2% ethanol with 0.3 mg/mL extract, (IV) 2% ethanol with 0.2 mg/mL extract, and (V) 2% ethanol with 0.1 mg/mL extract. The same procedure was repeated for hydroethanolic extract.

Oil red O staining and liver measurement

Exposure of zebrafish larvae to 2% ethanol did not induce death in the larvae. The alive larvae post ethanol exposure were recollected and washed twice with phosphate-buffered saline (PBS). The whole larvae were then fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Then, the larvae were washed twice with PBS, followed by consecutively infiltrated with 20%, 40%, 80%, and 100% of propylene glycol for 15 minutes each, at room temperature. The larvae were then stained with 0.5% Oil Red O in 100% propylene glycol at the temperature of 65°C in the dark for 1 hour. The stained larvae were then infiltrated with 100%, 80%, 40% and 20% of propylene glycol for 20 minutes at room temperature, followed by PBS. The stained larvae were stored in 70% glycerol at 4°C (15). For analysis, the larvae were examined and photographed under an inverted microscope, the liver size was measured, and the area was quantified using Image-J software.

Statistical analysis

All the data were compared and analyzed using non-parametric Mann-Whitney U test (IBM SPSS version 21). A p-value of less than 0.05 ($p<0.05$) is considered statistically significant.

RESULTS

Compound identification using GCMS

Hydromethanolic and hydroethanolic of *M. oleifera* leaf extracts were analysed for their bioactive compounds using GC-MS by relating the retention time (min), peak area (%) and mass spectral fragmentation patterns of peaks in chromatogram to known compounds described by the NIST library. A total of 7 peaks corresponding to the phytochemical

constituents were identified in the hydroalcoholic extracts of *M. oleifera* leaf (Supplementary Figure 1 and 2).

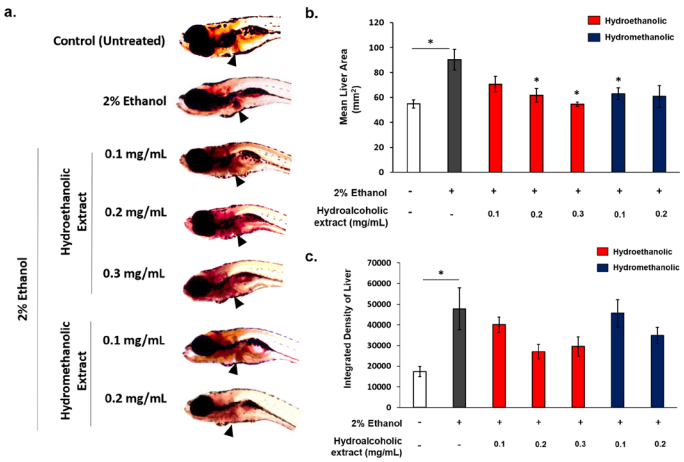

Figure 1 : Anti-steatosis activity of hydroalcoholic M. oleifera leaf extracts. (a) Liver morphology and staining intensity (arrow) in zebrafish larvae treated with 2% ethanol and hydroalcoholic extracts at different concentrations. The pictures shown are representative of each group with a similar pattern. (b) Mean liver area (mm2) of zebrafish larvae at different concentrations of M. oleifera leaf extracts as indicated. (c) Integrated density of the red oil O dye in the liver. Data represent the mean ± SEM of five larvae. * p<0.05 for control vs 2% ethanol, and 2% ethanol vs treatment groups using Mann-Whitney U test.

The phytochemical constituents found in the hydromethanolic extract of *M. oleifera* leaf were 8,14-Seco-3,19-epoxyandrostane-8,14-dione,17-acetoxy-3beta-methoxy-4,4-dimethyl- (73.72%), 1,2,3-Propatriol, 1-indol-4-yl(ether) (4.43%), beta-l-Rhamnofuranoside, 5-O-acetyl-thio-octyl- (2.45%), d-Mannose (2.17%), quinic acid (2.10%), and hexanedioic acid, bis(2-ethylhexyl)ester (0.04-0.11%) (Table I). Meanwhile, the major phytochemical constituents present in hydroethanolic extract of *M. oleifera* leaf were 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (25.14%), gamma-sitosterol (18.56%), 1-S-heptyl-1-thio-d-Glucitol (3.60%), 2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine (2.55%), 1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)- (1.71%), beta.-l-Rhamnofuranoside, 5-O-acetyl-thio-octyl- (1.17%) and hexadecenoic acid, l-(hydroxymethyl)-1,2-ethanediylester (0.07%) (Table II).

Anti-steatosis activity of hydroalcoholic *M. oleifera* leaf extracts in alcohol-induced liver injury

Acute alcoholic liver steatosis can be observed in the zebrafish larvae liver after oil red O staining. In this study, zebrafish larvae were exposed to 2% ethanol at 120 hpf for 32 hours to induce alcoholic liver disease and treated with hydroalcoholic MO leaf extracts. Red oil O dye used in this study is to stain triglycerides and lipids. As shown in figure 1a, the liver of the larvae in the 2% ethanol exposure group was stained positive in red oil O dye, which indicates alcohol-induced liver steatosis. The liver size in 2% ethanol-treated larvae was relatively larger, with an area of 90.2 ± 8.2 mm^2 compared to the untreated control (54.82 ± 3.3 mm^2). However, hydroalcoholic extracts treated larvae reduced the ethanol-induced increase in liver size. For instance, hydroethanolic leaf extract at concentrations of 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL dose-dependently reduced the liver size, which are 70.7 ± 6.2 mm2, 61.6 ± 5.4 mm^2 and 54.6 ± 1.7 mm^2, respectively. The study was conducted at a concentration up to 0.2 mg/mL, as the LC_{50} is approximately 0.3 mg/mL as reported in the previous study (9). Hydromethanolic extract has similar anti-steatosis activity in ethanol-induced liver injury, where the average liver size is 63.0 ± 4.6 mm^2 and 60.7 ± 8.7 mm^2, respectively for 0.1 mg/mL and 0.2 mg/mL (Figure 1b).

Table I : The phytochemical constituents identified in the hydromethanolic extract of M. oleifera leave using GC-MS

No.	PubChem CID	Compound Name	Molecular formula	Molecular weight	Retention time (min)	Peak Area (%)
1	40468165	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	370.6	2.38	0.11
2					2.47	0.04
3	18950	d-Mannose	$C_6H_{12}O_6$	180.2	12.84	2.17
4	6508	Quinic acid	$C_7H_{12}O_6$	192.2	15.01	2.10
5	583972	1,2,3-Propatriol, 1-indol-4-yl(ether)	$C_{11}H_{13}NO_3$	207.2	23.61	4.43
6	537841	beta-l-Rhamnofuranoside, 5-*O*-acetyl-thio-octyl-	$C_{16}H_{30}O_5S$	334.5	24.19	2.45
7	550132	8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-36-methoxy-4,4-dimethyl-	$C_{24}H_{36}O_6$	420.5	28.89	73.72

Table II : The phytochemical constituents identified in the hydroethanolic extract of M.oleifera leave using GCMS

No.	PubChem CID	Compound Name	Molecular formula	Molecular weight	Retention time (min)	Peak Area (%)
1	5319879	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568.5	2.09	0.07
2	541564	2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine	$C_{11}H_{12}N_4O_6$	296.2	12.87	2.55
3	22213798	1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)-	$C_6H_{12}O_4$	148.2	15.01	1.71
4	545763	1-*S*-heptyl-1-thio-d-Glucitol	$C_{13}H_{28}O_5S$	296.4	23.20	3.60
5	537841	beta.-*l*-Rhamnofuranoside, 5-*O*-acetyl-thio-octyl-	$C_{16}H_{30}O_5S$	334.5	23.81	1.17
6	457801	gamma-sitosterol	$C_{29}H_{50}O$	414.7	32.10	18.56
7	5319879	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{40}O_4$	356.5	37.35	25.14

The integrated density by quantifying the amount of dye in the liver was analyzed. As shown in figure 1c, the hydroalcoholic-treated larvae have lower stains in the liver compared to ethanol-treated larvae. The lower the stain in the liver, the lower the amount of lipids in the liver. Altogether, this suggests that hydroalcoholic extracts have comparable activity in reducing alcohol-induced liver steatosis in zebrafish larvae.

DISCUSSION

The yields and the types of phytochemicals depend on the parts of a plant, the solvent used, and the methods of extraction. This study aimed to examine the phytochemical constituents of *M. oleifera* leaf extracts from the Soxhlet extraction method, using GC-MS and its ability to reduce alcohol-induced steatosis. As shown in Table-I and Table-II, the phytochemical constituents found are different from those produced from successive extraction methods using ethanol, followed by methanol (17) and petroleum ether and dichloromethane of *M. oleifera* root extracts (18). This might be due to the different methods used in the extraction, and hydroalcoholic solvent may facilitate the exploitation of phytochemical constituents that may be soluble in either organic solvent and/or water.

Quinic acid is part of the *M. oleifera* composition as reported in other studies using hydroalcoholic *M. oleifera* leaves (19) and flowers (20). The d-mannose (2.17%) detected in hydromethanolic leaf extract is also reported in the aqueous extract of *M. oleifera* leaf (21, 22). D-mannose was also detected in hydroalcoholic extract of other plants (11) using GC-MS, hence suggesting that hydroalcoholic solvent can extract sugar type of compounds. Other compounds detected in our study are similar to those previously reported in other extraction methods of *M. oleifera* including beta-1-rhamnofuranoside, 5-O-acetyl-thio-octyl, gamma-sitosterol, hexadecanoic acid, 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester in GC-MS (23, 24). There are few compounds were tentatively characterized for the first time in *M. oleifera* leaf extracts, with the highest probability and match value exceeding 550 are hexanedioic acid, bis(2-ethylhexyl) ester, 8,14-Seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3ϐ-methoxy-4,4-dimethyl- and 1,2,3-Propatriol, 1-indol-4-yl(ether) in hydromethanolic extract, 2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine, 1,2,3,5-Cyclohexanetetrol, (1.alpha.,2.beta.,3.alpha.,5.beta.)-, and 1-S-heptyl-1-thio-d-Glucitol in hydroethanolic extract. 1,2,3-Propatriol, 1-indol-4-yl(ether) is a compound that is found in another plant and detectable by GC-MS (25).

Zebrafish larvae treated with hydroalcoholic extracts were found to have a comparable liver size with the untreated control (Supplementary Figure 3), hence concluded hydroalcoholic extracts do not induce fatty liver in larvae. Taken together, the results obtained in this study are consistent with our previous findings, whereby the hydromethanolic extract of *M. oleifera* leaf possesses better antioxidant capacity (IC_{50} = 15.92 ± 3.62 mg/mL) than the ethanolic extract (IC_{50} = 25.28 ± 2.89 mg/mL) (9). The antioxidant capacity of an extract can be associated with providing therapeutic effects to the liver in reversing the harmful effects of alcoholic liver steatosis. The ROS and alcohol dehydrogenase enzymatic levels are not determined, which is the limitation of the study. However, according to the previous study by Fakurazi et al., the hepatoprotective property of ethanolic *M. oleifera* leaf extracts in acetaminophen-induced hepatotoxicity exerted their hepatoprotective properties by inhibiting oxidative stress due to the presence of phenolic compounds and its antioxidant nature (6). This suggests that the hydroalcoholic *M. oleifera* leaf extracts used in this study can reduce the ROS. Further in-depth studies on its mechanism in reversing alcohol-induced steatosis are warranted.

It has been also hypothesized that phenolic compounds are highly reactive toward peroxyl radicals via a formal hydrogen atom transfer, which provides the basis of their antioxidant activity. Quinic acid (2.10%), a phenolic compound present in hydromethanolic extract of *M. oleifera* leaf, although not the major constituent in the extract, may have contributed to the said antioxidant property to a certain extent. A recent study reported that quinic acid derivatives possess potent antioxidant activities with lower IC_{50} values than vitamin C, which contributed to its hepatoprotective effects in an H_2O_2-induced L02 cell oxidative injury model (26). In the hydroethanolic extract, the presence of 9-octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (25.14%) and gamma sitosterol (18.56%), are worth mentioning, as compounds containing this fatty acid ester and phytosterol have been also reported to have antioxidant activities (27, 28), and anti-inflammatory activity (24).

Apart from the antioxidant activities, the anti-steatosis property of hydromethanolic extract of *M. oleifera* leaf could be attributed to the major chemical constituent in the extract, 8,14-Seco-3,19-epoxyandrostane-8,14-dione,17-acetoxy-36-methoxy-4,4-dimethyl- (73.7%), which is a steroid (29), and postulated it might have anti-inflammatory properties. In addition, quinic acid which could form chlorogenic acid has been reported to possess liver protective activity (30, 31).

CONCLUSION

In conclusion, hydroalcoholic extracts of *M. oleifera* leaf were effective in reversing alcohol-induced hepatic steatosis in zebrafish larvae in a dose-dependent manner, with hydromethanolic extract exerting significant anti-steatosis property at a lower concentration than the hydroethanolic extract. However, the anti-steatosis mechanism of these extracts has not yet been determined, although the antioxidant and anti-inflammatory properties and the phytochemical constituents of the extracts may have played a significant role.

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