BMC Complementary Medicine and Therapies

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In vitro antioxidant effects and in vivo hepatoprotective effects of *Osbeckia octandra*, *Vernonia cinerea* and *Atalantia ceylanica* on a high fat diet induced metabolic dysfunction-associated steatotic liver disease mouse model

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Abstract

Background Metabolic dysfunction-associated steatotic liver disease (MASLD), which was formerly known as nonalcoholic fatty liver disease (NAFLD) has become a global epidemic that is predicted to affect more than 50% of the world population by 2040. However, except for Resmetiron, there are no approved drugs in conventional medicine to treat MASLD. The aim of this study was to explore the hepatoprotective effects of the aqueous extracts (AEs) of *Osbeckia octandra* (*O. octandra*), *Vernonia cinerea* (*V. cinerea*), and *Atalantia ceylanica* (*A. ceylanica*) which have been extensively used to treat hepatic diseases in the Sri Lankan traditional medicine.

Methods During initial in vitro experiments, aqueous extracts (AEs) of these three medicinal plants were prepared and evaluated for antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and Trolox Equivalent Antioxidant Capacity (TEAC) assay. Cytotoxicity was assessed using the MTT assay on Vero cells to determine non-toxic doses for in vivo studies. For in vivo experiments, a high-fat diet (HFD)-induced MASLD mouse model was used. After co-treating the mice with AEs of the tested medicinal plants, their body weight gain was evaluated. Serum ALT, cholesterol, and triglyceride levels were analyzed using standard chemical methods. Hepatic lipid aggregation was evaluated by histopathology. Expression of TNF-α, IL-6, and PPAR-α genes was quantified using qRT-PCR. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test or LSD test. The level of significance was ($p < 0.05$).

Results Initial in vitro experiments revealed that AEs of these medicinal plants are rich sources of natural antioxidants with low cytotoxic effects. Subsequent in vivo experiments on the HFD-induced MASLD mouse model also demonstrated that AEs of these medicinal plants can ameliorate HFD-induced weight gain, dyslipidemia, and hepatic

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lipid aggregation. Reversal of downregulated PPAR-α gene expression confirmed their positive impact on lipid metabolism. Attenuation of increased serum ALT concentration provides evidence for their protective role against hepatic injury. In addition, these extracts could suppress the upregulation of TNF-α and IL-6 genes, which are crucial for inducing steatohepatitis.

Conclusion Aqueous extracts of *O. octandra*, *V. cinerea*, and *A. ceylanica* are rich in antioxidants and have the ability to suppress HFD-induced weight gain, dyslipidemias, hepatic steatosis, and hepatic inflammation providing solid evidence for their beneficial effects against initiation and progression of MASLD. Among these extracts, *O. octandra* stands out with the highest antioxidant activity, lowest cytotoxicity, and most promising hepatoprotective effects.

Keywords Antioxidants, *Atalantia ceylanica*, Hepatoprotective, High-fat diet, Metabolic dysfunction-associated steatotic liver disease, Nonalcoholic fatty liver disease, *Osbeckia Octandra*, *Vernonia cinerea*

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), which was formerly known as Nonalcoholic fatty liver disease (NAFLD) is characterized by metabolic dysfunction-associated steatosis which subsequently progresses into steatohepatitis, cirrhosis, and hepatocellular carcinoma. Although genetic factors have a significant contribution in the pathogenesis of this disease, metabolic risk factors such as obesity, dyslipidemia, insulin resistance, and type II diabetes mellitus are strongly associated with the disease [1].

According to the latest epidemiological studies, the average global prevalence of MASLD in adults is 25–30% while that of children is 7.4%. Although the prevalence varies by country, the highest prevalence of MASLD is reported in the Middle East and South America, followed by Asia, North America, and Europe. The lowest prevalence is reported in Africa. More than half the world population (55.7%) is predicted to have MASLD by 2040, which is a 42.3% increase from its prevalence in 2020. Along with the rising prevalence, its economic burden and the negative impact on the quality of life of affected individuals are also expected to increase across the globe [2–4]. Therefore, there is an urgent need for preventive and curative strategies to overcome MASLD [5]. To date, lifestyle modifications, including enhanced physical activity and reduced energy intake, have been considered the most important strategies for managing MASLD [6]. Indeed, certain off-label pharmacological interventions such as antioxidants, insulin sensitizers, and lipid-lowering drugs like statins have been reported to have favorable effects in managing this condition [7–9]. However, except for Resmetirom which was approved by the FDA in 2024, there are no specific drugs approved for treating MASLD in western medicine [10, 11]. In contrast, many traditional medicine systems in the world have been using numerous herbal medicines to manage this disease [12, 13]. A number of randomized clinical trials (RCTs) conducted to test the efficacy of herbal medicines to manage this condition have demonstrated their additive effect on life style changes in reversing early stages of MASLD [14]. In Sri Lankan traditional medicine, more than forty different medicinal plants have been used for treatment of various hepatic disorders. Especially, the aqueous extracts (AEs) of *Osbeckia octandra* (*O. octandra*), *Vernonia cinerea* (*V. cinere*a), and *Atalantia ceylanica* (*A. ceylanica*) have been extensively used in preparation of medicinal porridges to prevent and cure hepatic disorders [15]. Indeed, numerous in vitro and in vivo studies have provided evidence for the potential of these medicinal plants to exert hepatoprotective effects. However, most of the research on hepatoprotective effect of these three medicinal plants have been mainly conducted as in vitro studies. The limited number of in vivo studies conducted so far, have only explored their protective effects against chemical-induced hepatotoxicity [16–21]. For example, the studies conducted by Thabrew et al. and Jayathilaka et al. have demonstrated that *O. octandra* extracts have a protective role against CCl_4, Paracetamol, 2,6-dimethyl N-acetyl p-quinoneimine, D-galactosamine and tert-butyl hydroperoxide (TBH) induced hepatotoxicity [17, 19]. Another study conducted by Bogahawatta et al. has concluded that the AE of *O. octandra* can exert hepatoprotective effects on thioacetamide-induced cirrhotic rats through anti-inflammatory, antifibrotic and antiangiogenic effects [20]. Later on, they have identified certain phytochemicals responsible for antifibrotic effects, such as pedunculagin, casuarinin, and gallic acid in *O. octandra* [22]. Other than that, a study done by Hettihewa et al. had found that phytoconstituents including phenolic compounds, flavonoids, tannins and diterpenes in *O. octandra* [23]. An abundance of polyphenol compounds were found in a study conducted by Perera et al. where it suggests the Antiglycation activity [24].

A comprehensive literature review indicates that *V. cinerea* is rich in diverse phytochemical compositions, with sesquiterpene-lactones being its predominant secondary metabolites. Vernolide-A and vernolide-B have been identified as cytotoxic sesquiterpene lactones from *V. cinerea* [25] and additionally, triterpenoids such as β-amyrin and lupeol have been isolated from this plant [26, 27]. Phytochemical screening of the plant extract

confirmed the presence of several bioactive compounds like glycosides, triterpenoids, and esters, which could be responsible for the versatile medicinal properties of this plant. NMR data indicated the presence of Lupeol, 12-oleanen-3-ol-3ß-acetate, Stigmasterol, ß-sitosterol in n-hexane portion [28]. Another study conducted by Ketsuwan et al. found that *V. cinerea* contains antioxidant compounds such as tannins, catechin, flavonoids, nitrate, nitrite, and nicotine, with its leaf extract exhibiting the highest concentration of these bioactive compounds and the strongest protective effect against 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress in human red blood cells (RBCs) compared to the stem and flower extracts [29]. Rajamurugan et al. had identified 27 phytoconstituents in *V. cinerea*, with HPTLC quantification revealing gallic acid as the predominant phenolic compound, followed by rutin, quercetin, caffeic acid, and ferulic acid [30].

Several studies have demonstrated the hepatoprotective effects of *V. cinerea* also. According to Leelaprakash et al., ethanolic extract of *V. cinerea* has hepatoprotective effects against CCl_4-induced hepatotoxicity in a rat model [21]. An in vivo study by Pratheeshkumar et al. investigating the anti-inflammatory effects of *V. cinerea* found that its methanolic extract scavenged free radicals, inhibited lipid peroxidation, reduced nitric oxide levels, increased antioxidant levels, and significantly reduced carrageenan-induced inflammation and pro-inflammatory cytokine levels, demonstrating its anti-inflammatory potential [31]. According to an in vivo study done by Gokilawin et al., both pre and post-treatment with *V. cinerea* could exert hepatoprotective effects against CCl_4 induced hepatotoxicity in rats [32]. According to Naowaboot et al. co-treatment with the AE of this plant can reduce hyperlipidemia and triglyceride storage in a HFD induced obesity mouse model [33].

A. ceylanica leaves contain several bioactive phytochemicals, including polyphenols, tannins, flavonoids, alkaloids, and coumarins [34–37]. TLC analyses of the leaf extract and oil revealed the presence of polyphenols, flavonoids, tannins, and coumarins [38]. The essential oil contains volatile compounds such as decanal, lauraldehyde, caryophyllene oxide, caryophyllene, and α-cardinol, with caryophyllene recognized for its strong antibacterial, antifungal, and antiviral properties [34]. Additionally, compounds like 2,4,5-trimethoxy-benzaldehyde, acridone alkaloids, and carpachromene, the first flavone isolated from Atalantia species, contribute to its medicinal potential [35, 36].

When the hepatoprotective effects of *A. ceylanica* is considered, Fernanado and Soyza have demonstrated that a decoction of *A. ceylanica* leaves is a good source of antioxidants with effective hepatoprotective activity against ethanol-induced toxicity in porcine liver slices. According to their results, co-treatment with the decoction of *A. ceylanica* leaves has significantly reduced the ethanol induced lipid peroxide formation in hepatocytes as well as the ethanol induced leakage of liver enzymes [37]. In addition, a study conducted by Ulpathkumbura has highlighted the hypolipidaemic effects of *A. ceylanica* [39]. Further, several studies have demonstrated that *A. ceylanica* is a good source of polyphenols, flavonoids, and numerous antioxidants, highlighting its contribution to combating oxidative stress, a key driver of MASLD [37–42].

However, to the best of our knowledge, there is a dearth of information on the hepatoprotective effects of these three medicinal plants on HFD-induced MASLD animal models, which closely simulate the MASLD in humans.

Therefore, in the first stage of this study, we analyzed the hepatoprotective effects of the AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* in an in vitro setup through analysis of their antioxidants, because exogenous antioxidants can complement the endogenous antioxidants to neutralize the cellular oxidative stress (OS), one of the key etiological agents in initiation and progression of MASLD. In the second stage of this study, we analyzed the hepatoprotective effects of these three extracts on a high-fat diet (HFD) induced mouse model by analyzing their potential to ameliorate obesity, dyslipidemias, hepatic steatosis, and hepatic inflammation.

Materials and methods

Plant material

The matured whole plant of *V. cinerea*, fresh leaves of *A. ceylanica*, and *O. octandra* were collected from the first author's home garden, Western province, Sri Lanka (latitude:7°15' 34.1" N, longitude: 80°02' 32.8" E) [43]. These samples were authenticated and deposited in the National Herbarium of Sri Lanka by Ms. S. Ranasinghe, Deputy Director of the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka (Voucher specimen numbers: *O. octandra*- PDA00120417, *V. cinerea*-PDA00120416, *A. ceylanica*- PDA00120415).

Preparation of aqueous extracts

The collected medicinal plants were cleaned with tap water first, then with distilled water, and finally allowed to air dry at room temperature. The dried materials were ground into a fine powder and sieved with a 0.5 mm sieve before extraction. To prepare hot water extracts, 30 g of each plant powder was soaked for 30 min in distilled water at 100 °C. After filtration with Whatman No. 1 filter paper, the filtrates were frozen at -80 °C for 24 h to ensure complete solidification. Subsequently, they were subjected to freeze-drying (ChristAlpha 1-4LD plus, Germany) under vacuum at low temperatures (typically – 50 °C to -60 °C) to remove water content via

sublimation, preserving the bioactive compounds. The freeze-dried AEs were stored at -20 °C until analysis.

A total of 63 g of plant powder was extracted using 630 mL of hot water, yielding 465 mL of filtrate after extraction. Following freeze-drying, 1 g of dry extract was obtained, resulting in a percentage yield of 1.59%.

DPPH radical scavenging assay

DPPH radical scavenging activity of all three AEs were measured as described by Paranagama et al. [44]. The freeze-dried extract was dissolved in distilled water to prepare stock solutions, and eight different concentrations (0, 25, 50, 75, 100, 150, 175, and 200 µg/ml) were tested. Briefly, 200 µl of the sample (2000 µg/ml) diluted in 70% methanol was added to 1.8 ml of 1 mM DPPH-methanol solution and incubated in the dark at 25 °C for 30 min. The absorbance was measured using a UV spectrophotometer (UV-1800 UV-Vis spectrophotometer, Shimadzu, Japan) at 517 nm wavelength. L-ascorbic acid was used as the standard antioxidant. DPPH radical scavenging activity was expressed as a percentage inhibition of absorbance, which was calculated using the following equation. The assays were performed in triplicates.

$$\text{Percentage inhibition} = \left[\frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of the control}}\right] \times 100$$

The free radical scavenging activity was expressed as the 50% inhibition capacity (IC_{50}), which represents the amount of antioxidant required to neutralize 50% of the initial DPPH radical concentration. To determine the IC_{50} value, the percentage inhibition of absorbance was calculated and plotted against the sample concentrations to generate a dose-response curve. Regression equations were then used to calculate the IC_{50} values for each extract.

Although the DPPH assay results are commonly expressed as IC_{50} values, recent findings suggest that these values can be influenced by the initial DPPH concentration used in the assay. Therefore, it is recommended to express antioxidant activity in terms of equivalents of a well-known antioxidant standard, such as vitamin C, for more accurate comparisons [45]. To achieve this, the DPPH assay results were also expressed as vitamin C equivalents per milliliter of extract. A calibration curve was established by plotting the percentage inhibition of absorbance against a series of vitamin C concentrations (0–10 µg/ml). The regression equation obtained for the calibration curve was $y = -561774x^2 + 16528x$, $R^2 = 0.9981$, which was used to calculate the vitamin C equivalent antioxidant capacity of the tested extracts.

Trolox equivalent antioxidant capacity (TEAC)

TEAC of the AEs were analyzed as described by Re et al. [46]. In this assay, ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating the mixture in the dark at room temperature for 16 h. The $ABTS^{\bullet+}$ solution was then diluted with methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm.

For the assay, 10 µL (0.08 mg/mL) of each extract was mixed with 990 µL of the $ABTS^{\bullet+}$ reagent and incubated for 6 min at 30 °C. Trolox, a water-soluble analogue of vitamin E, was used as the standard. The antioxidant capacity of the extracts was expressed as Trolox equivalents per microliter (µg TE/µL) of extract.

To determine the IC_{50} values, the percentage inhibition of absorbance was calculated using the formula:

$$\text{Percentage inhibition} = \left(\frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of the control}}\right) \times 100$$

where absorbance of the control represents the absorbance of the $ABTS^{\bullet+}$ solution without the sample, and absorbance of the sample represents the absorbance in the presence of the extract. To determine the Trolox equivalent antioxidant capacity (TEAC) of the tested extracts, the percentage inhibition of absorbance was calculated and plotted against the concentrations of a serial dilution of the Trolox standard (ranging from 0.4 to 4.0 µmol/ml), resulting in a calibration curve. The resulting regression for the calibration curve was $y = -27.795x^2 + 203.33x - 0.5241$, R^2 0.9979. This equation was subsequently used to calculate the TEAC values of the extracts. The values of the extracts were statistically compared with those of Trolox (standard antioxidant) using one-way ANOVA followed by Tukey's post hoc test to determine significant differences ($p < 0.05$).

MTT assay

To identify the optimum concentration of AEs of the tested herbs to be used in the in vivo study, they were initially screened for cytotoxic effects using MTT assay as described by Paranagama et al. [44]. For this purpose, Vero cells (African green monkey kidney cells; ATCC CRL-1587) were cultured in DMEM, supplemented with, 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/mL) at a density of 2×10^5 cells/well in 96 well cell culture plates (Corning) and incubated in a humidified, 5% CO_2 incubator at 37 °C. Afterward, cells were treated with the plant extract serially diluted in DMEM for 48 h. Cells without any treatment served as the negative control and cells treated with 99% ethanol served as the positive control. Wells containing only the medium without any cells served as the blank. After treatment, the cells were rinsed three times with phosphate-buffered saline (PBS),

pH 7.4, and the MTT assay was performed as described by Plumb et al. [47]. The absorbance was measured at 570 nm and 620 nm in a Multiskan Ex plate reader from Thermoscientific, USA and the data were normalized to the negative control as shown in the equation.

$$\text{Cell viability} = \left(\frac{\text{Mean Absorbance of the (treatment} - \text{blank)}}{\text{Mean Absorbance of the (negative control} - \text{blank)}}\right) \times 100$$

Experimental animals

Thirty (30), 6-week-old, male ICR mice (average body weight 25 g) were obtained from the Medical Research Institute Animal Center, Colombo. The animals were acclimatized under standard environmental conditions of light/dark cycles (12 h/12 h) and temperature (26 ± 1 °C) for one week. They had *ad libitum* access to water and a commercial diet. The experimental protocol was approved by the Ethics Review Committee of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya (Id: VERC-20-08). All animal experiments were conducted according to the "Guide for the Care and Use of Laboratory Animals" [48].

Preparation of the High-fat diet

The HFD (Table 1) was made using ordinary chow pellets (650 g) together with lard (250 g), milk powder (50 g), and butter (50 g), according to the formulation suggested by Panda et al. [49]. The experimental animals were given the resulting mixture after it had been formed into pellets.

Experimental design and treatments

Mice were randomly assigned to six equal groups ($n = 5$ per group) and provided with feed and water ad libitum throughout the study. One group received a standard diet and served as the control group. The remaining five groups were fed a high-fat diet (HFD) *ad libitum* for five weeks to induce fatty liver. After this induction period, the mice were further divided into treatment groups. One group received atorvastatin (5 mg/kg body weight, orally, twice a week) as a reference drug. The other groups were treated with aqueous extracts (AEs) of *Osbeckia octandra*, *Vernonia cinerea*, and *Atalantia ceylanica* (each at 500 mg/kg body weight, orally, twice a week) while continuing the HFD regimen. Atorvastatin was selected as the control drug for our study due to its proven efficacy in managing MASLD by suppressing weight gain, improving dyslipidemia, reducing liver inflammation, and offering additional protective effects. Its safety profile, especially at lower dosages over extended periods, aligns with our study objectives, making it an ideal choice for comparison in MASLD management [50].

The control group, which received a normal diet, and the disease control group, which received the HFD, were both given an equivalent amount of distilled water (orally, twice a week) as a placebo. HFD was continued during the treatment period. The doses were calculated according to previous studies [51, 52].

Body weights were measured once a week. After 10 weeks all mice were euthanized by CO_2 asphyxiation [52]. Mice were placed in a chamber where CO_2 was introduced at a controlled flow rate of 20% of chamber volume per minute, ensuring unconsciousness before euthanasia. Anesthesia was not required, as CO_2 asphyxiation induces unconsciousness before death. Blood was collected from cardiac puncture and serum was separated and stored at -20 °C. Liver samples were extracted and fixed in 10% neutral buffered formalin (10% NBF) for histopathology and RNAlater for qRT-PCR [53].

Table 1 Macronutrient composition of the High-Fat diet (HFD

Ingredient	Macronutrient Type	Component Name	Percentage of Final Diet
Chow pellets	Protein	Soy protein, wheat gluten, casein	7.6%
	Carbohydrate	Starch, fiber, lactose	10.4%
Milk powder	Protein	Casein, whey proteins	3.0%
	Carbohydrate	Lactose	1.5%
Lard	Fat	Palmitic acid, oleic acid, stearic acid	25.0%
Butter	Fat	Butyric acid, palmitic acid, oleic acid	8.3%

Body weight gain

Body weight gain was calculated as the difference between final and initial body weight and expressed as the percentage weight gain (%), which was calculated using the following equation.

$$\text{Percentage weight gain (\%)} = \left(\frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}}\right) \times 100$$

Serum biochemical parameters

Serum alanine aminotransferase (ALT), serum cholesterol, and serum triglyceride levels were measured using a biochemical analyzer (Erba Mannheim-CHEM7) based on spectrophotometry, following the manufacturer's instructions (Linear Chemicals, Spain).

Liver histopathology

Formalin-fixed liver tissues were routinely processed using an automatic tissue processor, undergoing dehydration through a series of alcohol and xylene baths before embedding them in paraffin blocks. The paraffin-embedded tissues were then sectioned at 3 µm using a semi-automatic microtome and mounted on glass slides. The tissue sections were stained with Haematoxylin and

Eosin (H&E). The histological sections were examined under a light microscope for degenerative changes and lipid accumulation in hepatocytes. Histological lesions were assessed semi-quantitatively following a blinded evaluation.

Gene expression analysis

RNA extraction

Liver samples from the median lobe were promptly preserved in RNAlater and stored at -80 °C. Tissue sections were crushed with liquid nitrogen and RNA extraction was performed using a RNeasy mini kit (Qiagen, Hilden, Germany). The quantity and quality of extracted RNA were measured using the NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Complementary DNA (cDNA) was synthesized from extracted RNA using a commercial kit (All in one 5X RT Master mix, applied Biological Materials Inc., Canada) and stored at -20 °C. Gene expression was semi-quantitatively assessed using the QuantStudio 6Flex qPCR machine. qRT-PCR was performed using Power SYBRTM Green PCR Master mix (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For one reaction, 5 µl of Power SYBRTM Green master mix, 0.3 µl of each forward and reverse primer (9 µM), 2 µl of template cDNA, and 2.4 µl of nuclease-free water in 10 µl of total reaction volume were used. Thermocycling conditions included initial incubation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 10 s, and extension at 72 °C for 15 s. The mRNA expression of selected target genes Tumor Necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), and Peroxisome Proliferator-Activated Receptor alpha (PPAR-α) was determined using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control as described elsewhere [54, 55]. All samples were run in triplicate. Gene expression was expressed as the relative fold change using the $2-\Delta\Delta CT$ method. The primer sequences used are listed in Table 2.

Table 2 Primers used for qRT-PCR

Primer	Forward	Reverse
TNF-α	5′-CCCAGGGACCTCTCTCTAATA-3′	5′-ATGGGCTACAG-GCTTGTCACT-3′
IL-6	5′-TGATGGATGCTTCCAAACTG-3′	5′-GAGCATTG-GAAGTTGGGGTA-3′
PPAR-α	5′-ACGCTGGGTCCTCTGGTT-3′	5′-GTCTTGGCTC-GCCTCTAA-3′
GAPDH	5′-TCGGAGTCAACGGATTTGGT-3′	5′-TTCCCGTTCT-CAGCCTTGAC-3′

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD) or standard error of mean (SEM). A one-way analysis of variance (ANOVA) followed by *Dunnett's* multiple comparison test, *Post-hoc* Tuckey's test, and Least Significant Difference (LSD) test were applied for analysis of data. The level of significance was set at $p < 0.05$ and 0.001.

Results

Antioxidant potential of *O. octandra*, *V. cinerea* and *A. ceylanica*

Among the AEs of the three medicinal herbs tested in this study, AE of *O. octandra* exhibited the lowest IC50 (IC50: 31.1 ± 0.6 µg/mL) while *V. cinerea* exhibited an intermediate IC50 (IC50: 237 ± 8.9 µg/mL) and *A. ceylanica* displayed the highest IC50 (1557 ± 0 µg/mL) in the DPPH assay. The IC50 of *O. octandra* was significantly lower than that of *V. cinerea* and *A. ceylanica* ($P < 0.05$) but higher than that of Ascorbic acid (3.51 ± 0.149 µg/mL), which was used as the positive control (Fig. 1(a)). These findings indicate that AE of *O. octandra* has the highest DPPH radical scavenging activity followed by *V. cinerea* and *A. ceylanica.* Similarly, the TEAC of *O. octandra* (110 ± 9 µg/mL) was significantly higher than that of *V. cinerea* and *A. ceylanica* which displayed similar TEAC values (40 ± 2 µg/mL) ($p < 0.05$) (Fig. 1(b)).

Cytotoxicity assessment of *O. octandra*, *V. cinerea*, and *A. ceylanica*

Since a relatively non-cytotoxic dose of the medicinal plant extracts must be used in in vivo studies, next, their cytotoxicity was analyzed on Vero cells using the MTT assay. As shown in Fig. 2, the concentration of the AEs required to cause 50% inhibition of the cell viability was 9.9 ± 0.3 mg/mL for *O. octandra*, 4.33 ± 0.47 mg/mL for *A. ceylanica* and 1.57 ± 0.39 mg/mL for *V. cinerea*. Among the three AEs, the IC50 of *O. octandra* was significantly higher than that of the other two AEs ($p < 0.05$) indicating its least cytotoxic effects on Vero cells.

A concentration-dependent decrease (Fig. 3) in cell viability was observed for all three extracts, with *O. octandra* showing the lowest cytotoxicity, maintaining over 50% viability even at the highest concentration of 10 mg/mL.

Effect of AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* on an experimental model of MASLD in mice

Effect on body weight gain

Throughout the 10-week study period, the weight of the mice were measured weekly (Fig. 4). The mean weight gain of mice at the end of the experiment was calculated as a percentage of their initial mean body weights. As shown in Table 3, the mice were fed on an HFD for

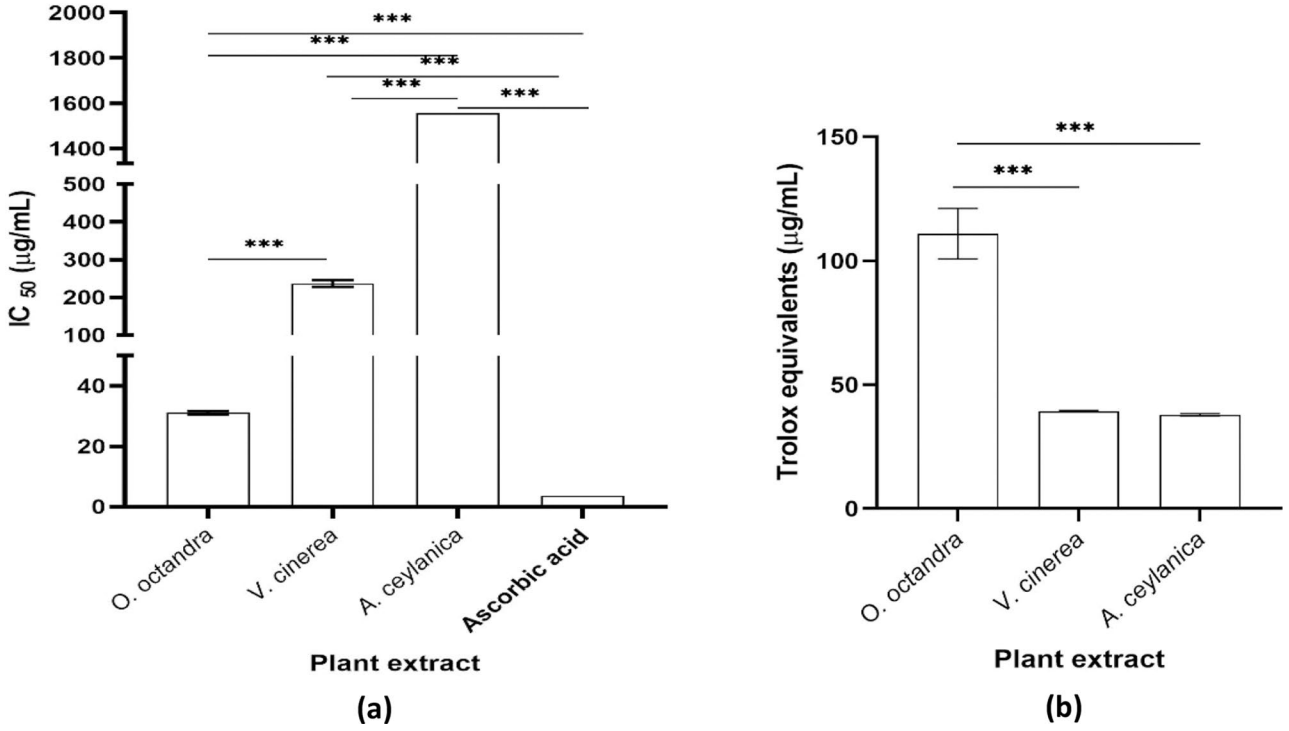

Fig. 1 Antioxidant capacity of AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* (**a**) Comparison of IC50 values of DPPH assay (**b**) Comparison of TEAC (µg/mL). Values are expressed as mean ± SD ($n = 3$), with error bars representing standard deviation. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. * denotes a significant difference from each other ($p < 0.05$). *Significance level: ***$p < 0.001$

10 weeks showed a marked increase in their weight gain compared to that of the negative control group ($p < 0.0001$). The percentage mean weight gain difference between the medicinal plant extracts treated groups and the atorvastatin-treated group was significantly lower compared to the HFD-fed group ($p < 0.0001$). Intriguingly, among the three medicinal plant extract treated groups, only the *O. octandra*-treated group showed a significantly lower weight gain compared to that of the atorvastatin-treated group ($p < 0.0001$).

Effect on serum biochemical parameters

Since serum ALT is a marker for evaluation of hepatic injury, next we compared the mean serum ALT concentration among the treatment and control groups of mice. As shown in Figure 5, the serum ALT concentration after feeding with the HFD for ten weeks was elevated to 32.6 ± 2.3 U/L which was significantly higher compared to the control group (19.87 ± 4.8 U/L) that was treated with the standard diet for a similar period (p <0.05). This finding indicates the marked hepatocellular injury induced by the HFD in the tested mice. However, all the treatment groups (*O. octandra* -18.91 ± 0.9 U/L; *V. cinerea* - 22.52 ± 1. 2 U/L; *A. ceylanica* - 23.65 ± 1.0 U/L) showed a significantly lower mean serum ALT concentration compared to the HFD fed group (32.6 ± 2.3 U/L) (p <0.05) demonstrating their protective role against the HFD induced hepatocellular injury in mice. Furthermore, the ALT levels in the *O. octandra* treated group were lower than those observed in the atorvastatin treated group (21.20 ± 1.7 U/L) and were comparable to the control mice, further supporting its potential hepatoprotective effects.

Since dyslipidemia is a common feature of MASLD, we next compared the serum triglyceride and cholesterol levels among different treatment and control groups. As shown in Fig. 6, the HFD-fed group exhibited the highest mean serum cholesterol levels (259.42 ± 37.2 mg/dL) and mean serum triglyceride levels (319.46 ± 73.9 mg/dL), which were significantly higher than those of the control group (cholesterol 81.43 ± 5.8 mg/dL; triglycerides 115.36 ± 9.3 mg/dL) ($p < 0.05$). This finding emphasizes the pronounced negative impact of the HFD on serum cholesterol and triglyceride levels. Intriguingly all treatment groups showed a significant reduction in the mean serum cholesterol levels (*O. octandra* – 173.42 ± 13 mg/dL; *V. cinerea* – 186.32 ± 15.14 mg/dL; *A. ceylanica* – 191.42 ± 9.5 mg/dL; atorvastatin – 185.50 ± 23.7 mg/dL) compared to the HFD fed group (259.42 ± 37.2 mg/dL) ($p < 0.05$). However, there was no statistically significant difference among these treatment groups ($p > 0.05$), indicating the equally effective cholesterol-lowering effect of the tested medicinal plant extracts which was comparable to that of atorvastatin. In contrast, only atorvastatin (189.50 ± 26.1 mg/dL) and *O. octandra* (183.46 ± 17.6 mg/

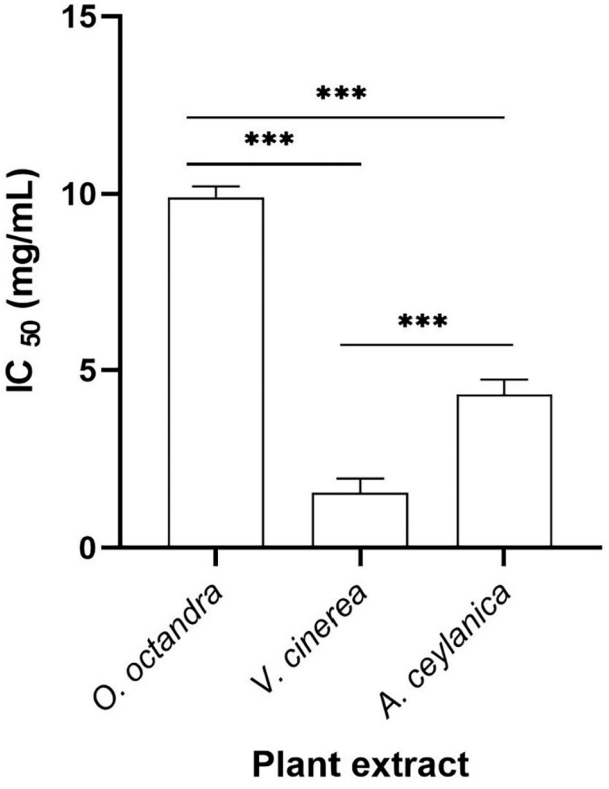

Fig. 2 Comparison of IC 50 values of the AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* in the MTT assay. Values are expressed as mean ± SD ($n = 3$), with error bars representing standard deviation. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. * denotes a significant difference from each other ($p < 0.05$). *Significance levels: ***$p < 0.001$

dL) treated groups exhibited a significantly lower mean serum triglyceride level compared to the group fed with a HFD (319.46 ± 73.9 mg/dL) ($p < 0.05$). The absence of a statistically significant difference between the mean serum triglyceride levels in the atorvastatin and *O. octandra*-treated groups indicates that the triglyceride-lowering effect of *O. octandra* is comparable to that of atorvastatin in this MASLD model. Although the AEs *V. cinerea* and *A. ceylanica* could ameliorate the HFD-induced increase in serum triglyceride levels, it did not reach a statistically significant level.

Effect on expression of TNF-α (a) IL-6 (b) PPAR-α (c) genes

Since increased secretion of the pro-inflammatory cytokines; TNF-α and IL-6, and reduced secretion of PPAR-α from the liver are known to play a crucial role in the pathogenesis of MASLD, next we analyzed the effect of AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* on the relative expression of these genes in the liver cells of experimental animals. As shown in Fig. 7, the fold increment of TNF-α and IL-6 mRNA expression in the HFD group of mice (TNF-α: 0.471 ± 0.07and IL-6: 0.590 ± 0.19), were significantly higher compared to those of the control group (TNF-α: 0.016 ± 0.014 and IL-6: 0.106 ± 0.03) ($p < 0.05$). Compared to the group which received the HFD, the TNF-α mRNA expression was significantly decreased in the *O. octandra* (0.038 ± 0.034: $p = 0.008$), *V. cinerea* (0.149 ± 0.053: $p = 0.044$) and atorvastatin (0.130 ± 0.055: $p = 0.033$) treated groups, but the decrease showed no significant change in the *A. ceylanica* treated group (0.357 ± 0.195: $p = 0.623$). In contrast, IL-6 expression was significantly reduced in all treatment groups (*O. octandra*: 0.171 ± 0.078; $p = 0.028$, *V. cinerea*: 0.108 ± 0.002; $p = 0.021$, *A. ceylanica*: 0.113 ± 0.002; $p = 0.022$, atorvastatin: 0.142 ± 0.017; $p = 0.016$) when compared to the group which received the HFD only. However, differences between the fold change in mRNA expression of these genes in the groups co-treated with the tested medicinal plants and the group co-treated with the control drug atorvastatin were not significant ($p \leq 0.05$).

As shown in Fig. 7c, the PPAR-α expression in the control group was 4.379 ± 0.89-fold. It was increased in the group fed only with the HFD (2.664 ± 0.127; $p = 0.251$) without reaching a significant level compared to the control group. A statistically notable upsurge of PPAR-α expression compared to the group fed only with HFD was observed in the *O. octandra* (5.607 ± 0.978; $p = 0.05$) and the atorvastatin (5.843 ± 0.093; $p = 0.027$) treated groups. However, the PPAR-α expression exhibited higher levels in the *A. ceylanica* treated group despite not reaching statistical significance (3.406 ± 2.406; $p = 0.610$) when compared to the group fed only with HFD whereas the *V. cinerea*-treated group exhibited a statistically no significant change in declining of PPAR-α expression (2.376 ± 0.031; $p = 0.842$). On the other hand, when compared with the group treated with atorvastatin, the group treated with *V. cinerea* showed a significant difference ($p = 0.04$).

Effect on liver histology

Since HFD-induced MASLD leads to lipid accumulation in hepatocytes, hepatocyte degeneration, and disruption of hepatic architecture, we analyzed liver histopathology in all experimental groups to evaluate the extent of liver damage and assess the protective effects of the treatments (Fig. 8) (Table 4). As expected, the livers of the control group showed normal hepatic architecture with intact hepatocytes (Fig. 8A) while the group fed only with the HFD exhibited severe lipid aggregates (arrows) and cloudy degeneration in the cytoplasm of many hepatocytes (Fig. 8B). In contrast, only a few hepatocytes displayed lipid aggregates (arrowheads) and mild cytoplasmic changes in the cytoplasm in atorvastatin treated group (Fig. 8C) and *O. octandra* treated group (Fig. 8D). In comparison to the control group, many hepatocytes displayed lipid aggregates (arrows) and moderate

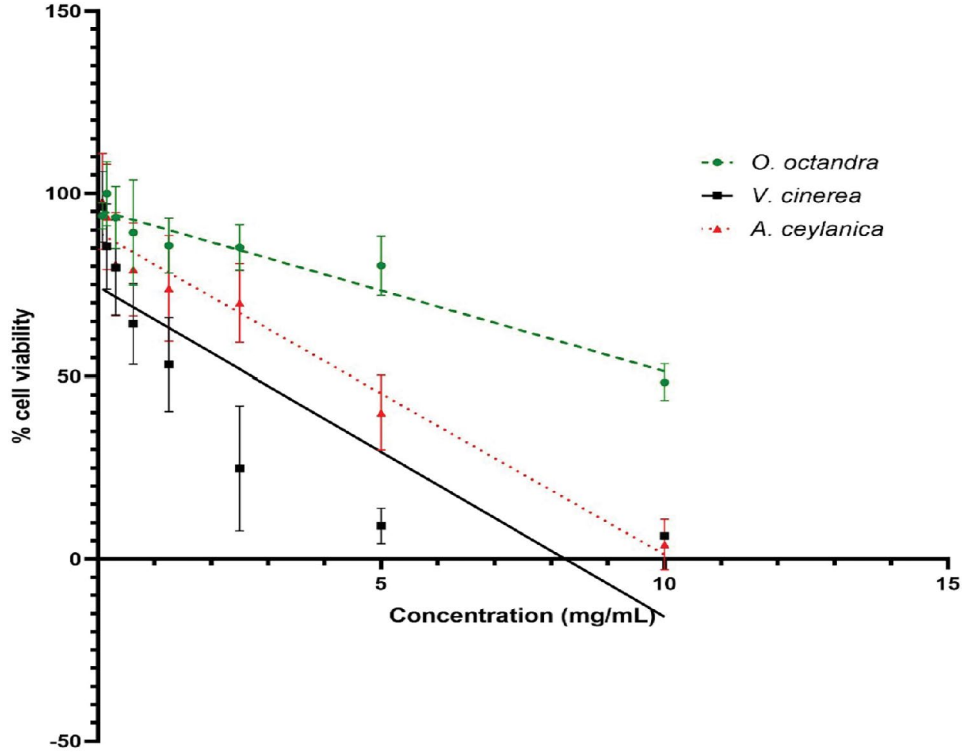

Fig. 3 The percentage of cell viability after 48 h treatment of varying concentrations (0–10 mg/mL) of AEs from *O. octandra* (green dashed line), *V. cinerea* (black solid line), and *A. ceylanica* (red dotted line) serially diluted in DMEM. Data points represent mean ± standard deviation (SD)

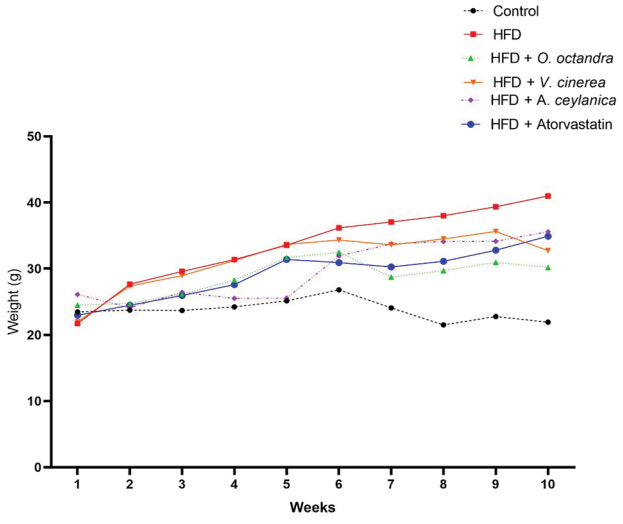

Fig. 4 Body weight changes over 10 weeks in experimental groups: Control, HFD, HFD + *Osbeckia octandra*, HFD + *Vernonia cinerea*, HFD + *Atalantia ceylanica*, and HFD + Atorvastatin. The first five weeks involved only HFD administration, followed by five weeks of HFD combined with respective treatments. Data are represented as mean ± SD

degenerative changes in the cytoplasms in groups co-treated with *V. cinerea* (Fig. 8E) and *A. ceylanica* (Fig. 8F). However, compared to the HFD fed group, all treatment groups demonstrated lower levels of lipid aggregates (Fig. 8C, D, E and F).

Other than the steatosis pattern, distinct structural changes were also observed in the hepatocytes across treatment groups. The HFD-fed group showed clear signs of hepatocellular swelling with loss of normal cell alignment and the narrowing of sinusoidal spaces. In the atorvastatin-treated group (Fig. 8C), hepatocytes appeared more uniformly arranged with well-defined cell borders and open, regular sinusoids, indicating tissue recovery. The *O. octandra* treated group (Fig. 8D) similarly showed improved cellular organization with minimal distortion of liver tissue. In contrast, livers from the *V. cinerea* (Fig. 8E) and *A. ceylanica* (Fig. 8F) treated groups displayed mild hepatocyte enlargement and sinusoidal dilatation, suggesting only partial restoration of normal tissue structure.

Effect of treatments on hepatic steatosis: semi-quantitative histological analysis

Liver histology was evaluated using a semi-quantitative scoring method based on the extent of macrovesicular steatosis observed in Hematoxylin and Eosin stained sections. Steatosis was graded on a 0–3 scale according to the estimated percentage of hepatocytes containing lipid vacuoles, as per the NAS criteria [56]. The HFD-only group exhibited severe steatosis (Score 3), while treatment groups showed varying degrees of improvement. The *O. octandra* and *V. cinerea* groups showed

Table 3 Weight gain of experimental groups during the 10 weeks study period

Variables	Control (Normal diet)	HFD only	HFD + O. octandra	HFD + V. cinerea	HFD + A. ceylanica	HFD + Atorvastatin
Initial BW (g) (Mean ± SD)	23.47 ± 1.6	21.72 ± 1.8	24.50 ± 3.1	21.99 ± 1.19	24.14 ± 5.0	26.12 ± 5.02
Final BW (g) (Mean ± SD)	27.40 ± 0.77	40.99 ± 8.2	30.20 ± 2.1	32.73 ± 8.8	35.59 ± 3.15	34.91 ± 7.8
Mean Weight gain difference (%)	14.34%$^{*\wedge}$	47.01%$^{\#\wedge}$	18.87%$^{*\#\wedge}$	32.81%$^{*\#\wedge}$	32.17%$^{*\#\wedge}$	25.18%$^{*\#}$

[3] Data are expressed as mean ± standard deviation (SD). Initial and final body weight (BW) measurements were taken at the start and end of the study, respectively. The percentage of mean weight gain difference was calculated by comparing the final BW to the initial BW for each group. The groups include: HFD only, Control (normal diet), HFD + *O. octandra*, HFD + *V. cinerea*, and HFD + *A. ceylanica* ($n = 5$). Significance difference was assessed using one-way ANOVA and Tukey's post hoc test, #$p < 0.05$ compared to the control group, *$p < 0.05$ compared to the HFD group, ^$p < 0.05$ compared to the atorvastatin-treated group

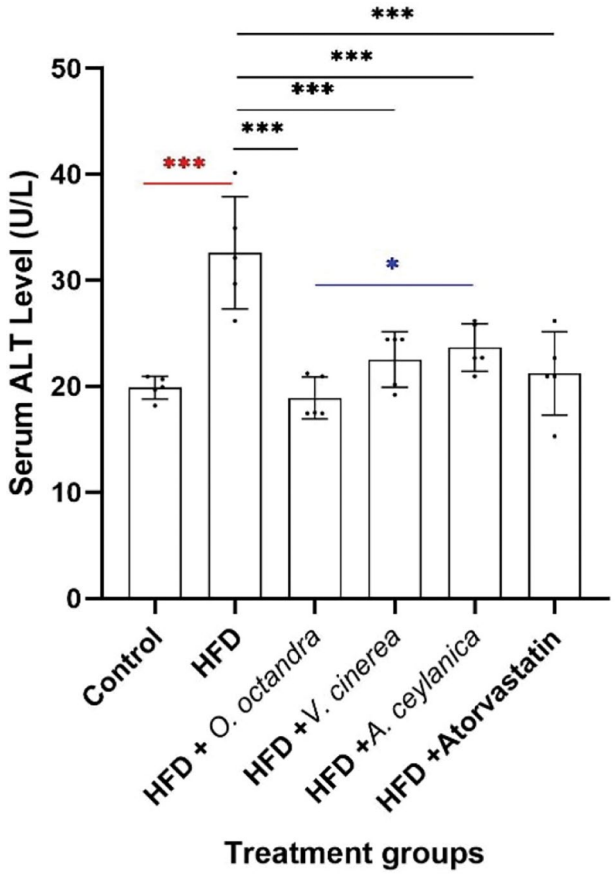

Fig. 5 Graphical illustration of the serum test results for ALT (U/L) in experimental groups. Values are expressed as mean ± SEM ($n = 5$), with error bars representing SEM. Statistical significance was determined using one-way ANOVA followed LSD test. * denotes a significant difference from each other ($p < 0.05$). *Significance levels: *$p < 0.05$, ***$p < 0.001$

mild steatosis (Score 1), whereas the atorvastatin and *A. ceylanica* groups showed moderate reductions in lipid accumulation (Score 2). No steatosis was observed in the normal diet control group (Score 0). These findings are summarized in Table 4.

Discussion

MASLD is the fastest growing liver disease in the world which parallels the increasing prevalence of overweight and obesity [57]. However, only a limited number of drugs are available yet to control the MASLD associated early pathologies such as metabolic dysfunction and hepatic inflammation as well as hepatic fibrosis and hepatocellular carcinoma occurring towards more advanced stages of the disease [58]. In this study, we attempted to identify HFD-induced MASLD mitigating effects of the AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica*, which have been used in the preparation of medicinal porridges to prevent and treat liver diseases in the Sri Lankan traditional medicine system [59, 60].

The in vitro antioxidant potential observed in the tested aqueous extracts (AEs) highlights their relevance in targeting oxidative stress (OS), a key pathological factor in the progression of MASLD. Among the tested plants, *O. octandra* exhibited the most prominent free radical scavenging capacity, suggesting a stronger potential to mitigate oxidative damage in hepatocytes. The relatively moderate antioxidant activity of *V. cinerea* also indicates a promising, though less pronounced, capacity to support hepatic protection, while the comparatively low activity observed in *A. ceylanica* may reflect a limited role in combating OS.

The therapeutic relevance of these findings lies in the well-established link between oxidative stress and hepatic lipid accumulation, inflammation, and fibrosis. Persistent oxidative damage not only promotes lipid peroxidation but also activates pro-inflammatory signaling and fibrogenic pathways, all of which contribute to MASLD pathogenesis. Therefore, the antioxidant properties of these plant extracts, particularly *O. octandra*, could serve as a foundational mechanism for their hepatoprotective effects observed in vivo. This is consistent with previous studies demonstrating the capacity of natural antioxidants to stabilize hepatic redox balance, reduce cellular injury, and prevent disease progression in steatotic liver models. The ability of these extracts to modulate OS may thus provide a mechanistic basis for their potential therapeutic use in managing MASLD [17, 61, 62].

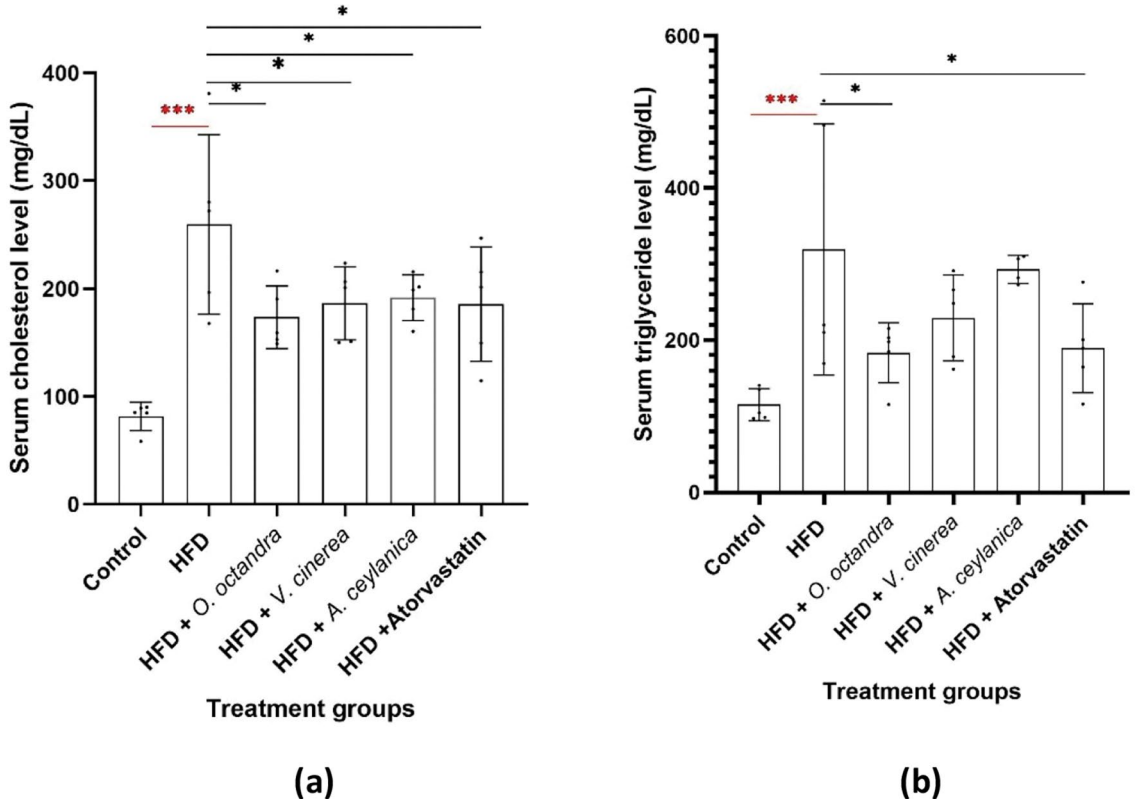

Fig. 6 Graphical illustration of the (**a**) serum cholesterol levels (mg/dL) and (**b**) serum triglyceride levels (mg/dL) in experimental groups. Values are expressed as mean ± SEM ($n = 5$), with error bars representing SEM. Statistical significance was determined using one-way ANOVA followed LSD test. * denotes a significant difference from each other ($p < 0.05$). *Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

In our second stage of experiments using in vivo assays, we could successfully establish a HFD induced MASLD mouse model with an increase in weight gain, increase in serum cholesterol, triglyceride and serum ALT levels, upregulation of IL-6 and TNF-α genes with a concomitant down-regulation of PPAR-α gene to statistically significant levels, along with histopathological evidence for destruction of hepatic architecture and development of steatohepatitis, compared to the control. Co-treatment with AEs of all tested plants could ameliorate these pathologies to different extents.

A plethora of previous research has identified obesity as one of the primary causes of MASLD [62]. Therefore, the ability to inhibit weight gain will be a promising characteristic of any drug targeted at ameliorating the initiation and progression of MASLD [63]. According to our results, co-treatment with AEs of all tested plants could significantly inhibit HFD-induced obesity in mice. It has been well established that the liver is the principal coordinator of lipid metabolism in the body, which is modulated by the level of PPAR-α gene expression in hepatocytes. Deregulation of hepatic lipid metabolism is associated with hepatic steatosis with concomitant hyperlipidemia [64]. Based on our findings, HFD-induced hypercholesterolemia and hypertriglyceridemia could be notably attenuated by co-treatment with *O. octandra* and *V. cinerea* indicating their potential to restore the HFD-induced hyperlipidemia.

The upregulation of PPAR-α mRNA by *O. octandra* suggests a potential mechanism for its hypolipidemic effect, while the effects of *V. cinerea* and *A. ceylanica* may involve alternative pathways. However, confirmation at the protein and functional levels is necessary to validate this mechanism. Additionally, the aqueous extracts of all three plants demonstrated a protective role against HFD-induced hepatic inflammation by significantly downregulating IL-6 and TNF-α mRNA expression. While all extracts were similarly effective in reducing IL-6 gene expression, *O. octandra* showed the strongest inhibitory effect on TNF-α upregulation. Future investigations should assess corresponding protein expression and inflammatory markers to further substantiate these findings.

In hepatic steatosis, lipids become toxic to hepatocytes. Especially, when hepatic mitochondrial oxidative machineries are overwhelmed with the excessive influx of free fatty acids, mitochondrial uncoupling can cause an overproduction of free radicals. The resulting Oxidative stress can create a pro-inflammatory environment in the liver leading to the steatohepatitis stage of

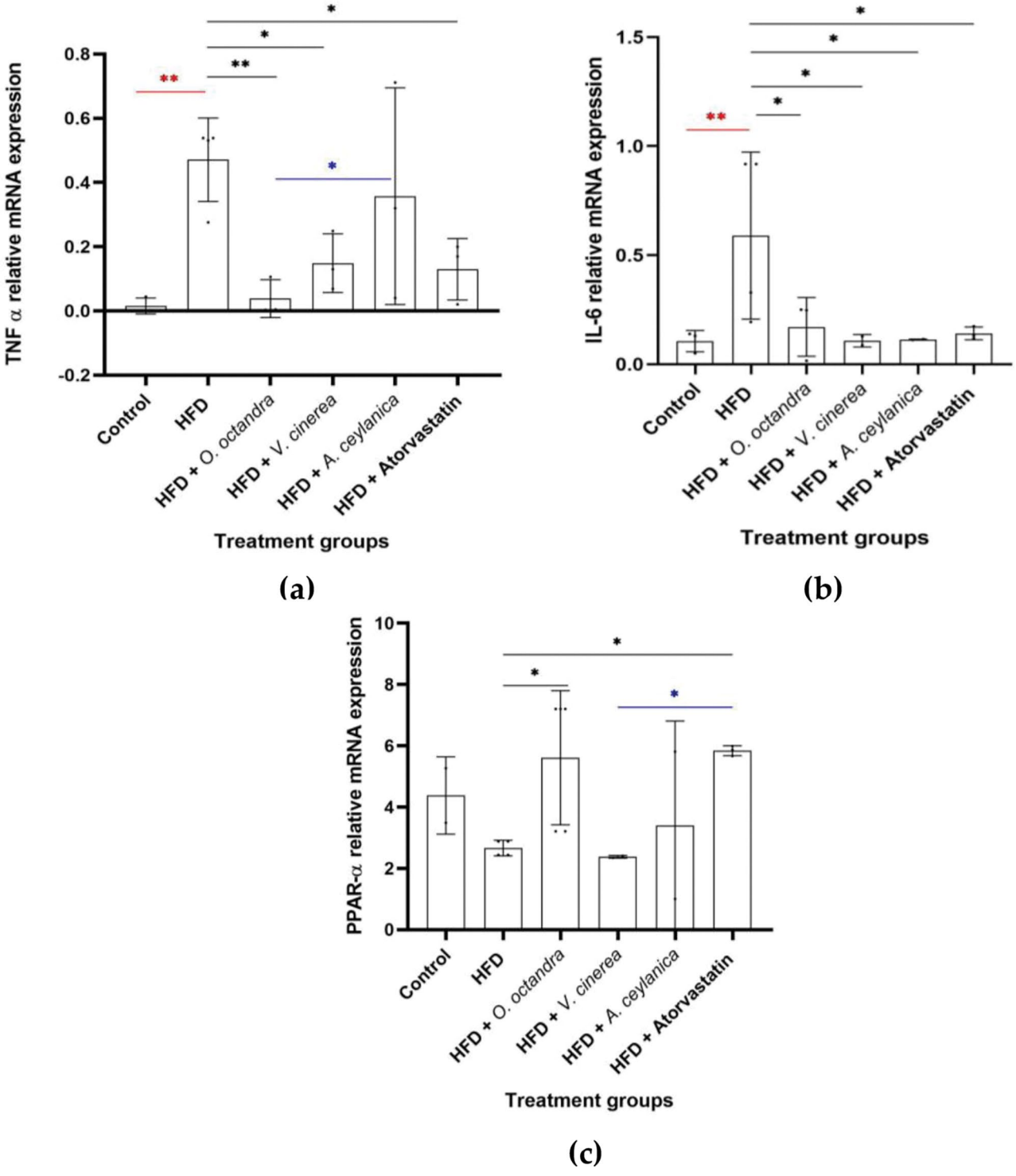

Fig. 7 Relative mRNA expression of (**a**) TNF-α (**b**) IL-6 (**c**) PPAR-α in experimental groups. Values are expressed as mean ± SEM (n=5), with error bars representing SEM. Statistical significance was determined using one-way ANOVA followed LSD test. * denotes a significant difference from each other ($p < 0.05$). *Significance levels: *$p < 0.05$, **$p < 0.01$, n=5

MASLD [65]. Steatohepatitis can induce severe hepatic injury which may progress into later stages of MASLD, namely cirrhosis and hepatocellular carcinoma [66]. ALT is a liver-specific enzyme that leaks into the bloodstream when there is a hepatic injury. Therefore, serum ALT concentration is used as a biomarker to evaluate liver function. Among the tested plants, *O. octandra* and *A. ceylanica* were equally effective in significantly reducing

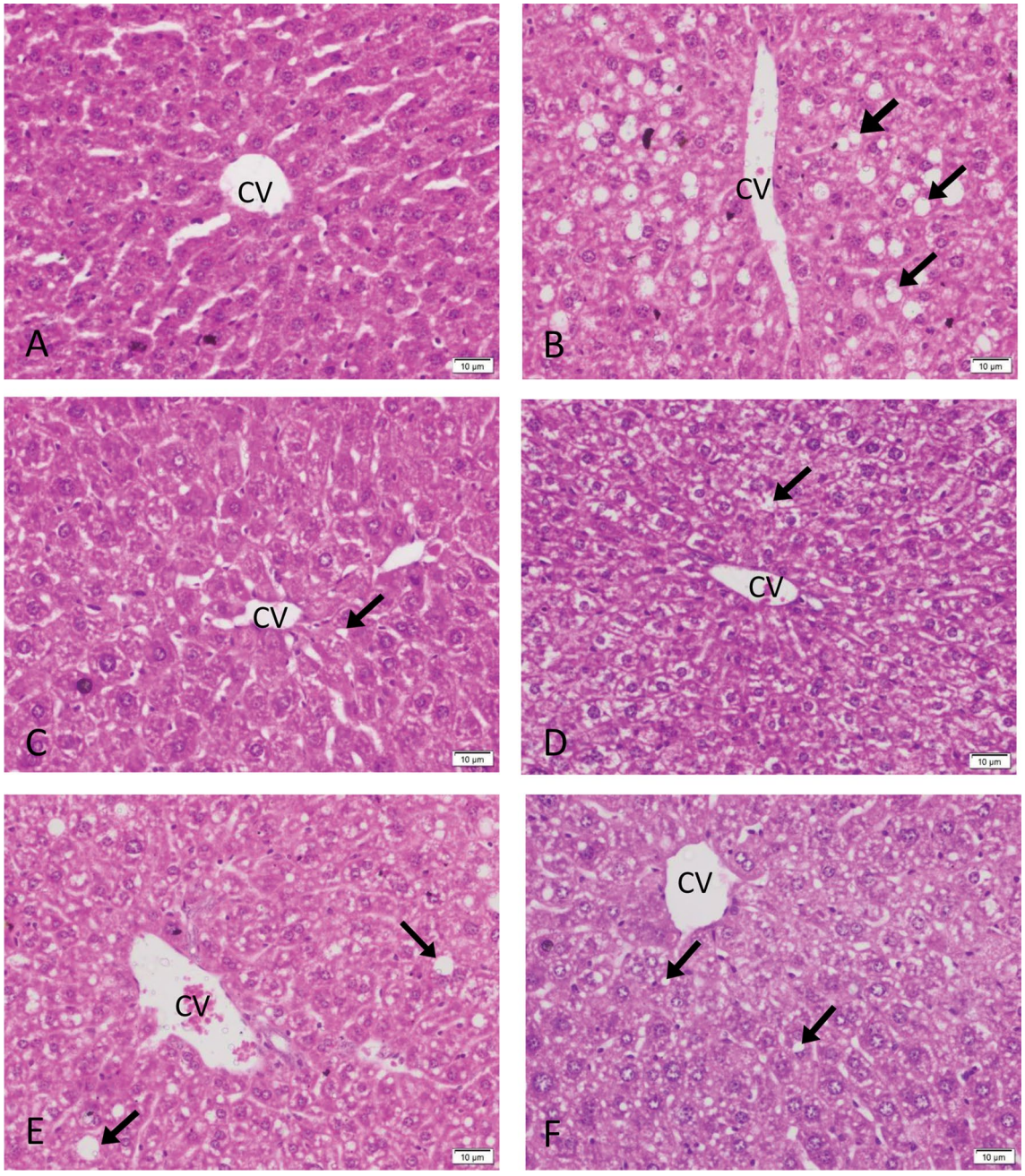

Fig. 8 Histopathology of livers of mice fed with normal diet (**A**) and livers of mice fed with HFD (**B**-**F**). Livers of the control group, fed with a normal diet show normal hepatic architecture (**A**). Many hepatocytes with large lipid vacuoles (arrows) are seen in the livers of the group fed only with HFD (**B**). Hepatocytes with fewer lipid aggregations (arrows) are seen in the livers of the atorvastatin-treated group (**C**) and *O. octandra*-treated group (**D**). Livers of the group treated with *V. cinerea* (**E**) and *A. ceylanica* treated group (**F**) showed some hepatocytes with lipid aggregates (arrows) in the cytoplasm. HE, Bar = 10 µm

the HFD-induced rise of plasma ALT concentration. Their plasma ALT-lowering effect was comparable to that of atorvastatin. Although *V. cinerea* also demonstrated a significant potential to inhibit the HDF-induced rise in plasma ALT concentration, its effectiveness was less than that of *O. octandra*.

The pathological examination of liver tissues across different groups revealed significant variations in hepatic architecture and cellular integrity, indicating differences in their impact on liver health. It is noteworthy that, the minimal hepatic alterations observed with *O. octandra* treatment suggest a potential hepatoprotective effect, which may be comparable to standard therapeutic

Table 4 Semi-Quantitative scoring of hepatic steatosis in experimental groups

Group	Treatment	Steatosis Score (0–3)	Steatosis Severity	Description
A	Normal Diet (Control)	0	None	Normal hepatic architecture, no visible lipid vacuoles
B	High-Fat Diet (HFD)	3	Severe	Extensive macrovesicular steatosis in > 66% of hepatocytes
C	HFD + Atorvastatin	2	Moderate	Noticeable lipid accumulation in ~ 34–40% of hepatocytes
D	HFD + *O. octandra*	1	Mild	Scattered hepatocytes with small lipid vacuoles (~ 10–20%)
E	HFD + *V. cinerea*	1	Mild	Fewer lipid droplets; less severe than atorvastatin group
F	HFD + *A. ceylanica*	2	Moderate	Moderate number of hepatocytes with lipid vacuoles (~ 30–35%)

interventions. The semi-quantitative histological scoring revealed the lower steatosis scores in treatment groups indicating protective effects against HFD-induced liver damage. *O. octandra* and *V. cinerea* showed mild steatosis (score 1), suggesting better efficacy, while atorvastatin and *A. ceylanica* showed moderate improvement (score 2). These findings highlight the ability of *O. octandra* to mitigate HFD-induced liver damage, emphasizing the need for further studies to explore its mechanisms and potential applications in liver disease management.

Although the present study did not directly investigate the molecular mechanisms underlying the hepatoprotective effects of the tested extracts, previous research provides valuable insights into potential pathways involved. Earlier discoveries of phytochemicals can explain the current results. These different outcomes are represented by various phytochemical contents, particularly phenols and flavonoids. Flavonoids may inhibit oxidative stress by regulating malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT). Flavonoids may ameliorate MASLD by regulating lipid metabolism, intestinal flora, and autophagy [67]. Researchers have concluded that theaflavins significantly reduce ROS production in steatotic hepatocytes and TNF-α production in Lipopolysaccharides (LPS) stimulated RAW264.7 cells [68]. Activating the farnesoid X receptor 1 (FXR1)/TGR5 signaling pathway, activating the PTEN-induced kinase 1 mediated mitochondrial phagocytosis, restoring the levels of superoxide dismutase, catalase, and glutathione, regulating bile acids, and upregulating FXR expression are the actions carried out by various types of flavonoids [67]. Several polyphenols may be able to stop steatosis and its development into NASH, according to current cell and animal research. Improvements in adipokine modulation, increased insulin sensitivity, a decrease in de novo lipogenesis (by SREBP-1c), and an increase in Fatty acid-oxidation activity, which would lower the liver's lipid burden, are likely the processes behind these discoveries. A unifying trigger for the regulation of all these molecular processes, according to recent findings, is the activation of the activated protein kinase/Sirtuin 1 axis [69]. Polyphenols may prevent hepatocyte injury associated with MASLD through enhancing antioxidant defense through the nuclear factor erythroid 2–related factor 2 pathway [70].

According to the previous studies the hepatoprotective activity which is aligning with the antioxidant capacity of *O. octandra*, *V. cinerea*, and *A. ceylanica* is linked to their rich phytochemical composition, with *O. octandra* containing phenols, flavonoids, tannins, steroids, and alkaloids [23, 71]; *V. cinerea* rich in phenols, sesquiterpenoids, tannins, steroids, catechins, and flavonoids [30, 72]; and *A. ceylanica* notable for its high levels of phenols and flavonoids, along with benzaldehyde derivatives and acridone alkaloids [36–38, 73]. Even though AEs of plants were rich in the same phytochemicals, the quantity variation can be the reason for the revealed results.

Indeed, previous research has also shown the hepatoprotective effects of crude extracts as well as isolated bioactive secondary metabolites in these plants. For example, both in vitro and in vivo studies conducted by Thabrew et al. and Jayathilaka et al. have provided evidence for the hepatoprotective effect of crude extracts of *O. octandra* against chemically induced hepatotoxicity [16–19]. Recently, Bogahawatta et al. have shown that casuarinin, pedunculagin, and gallic acid in the AEs of *O. octandra* have antifibrotic effects against chemically (thioacetamide) induced fibrosis of rat liver [20]. Similarly, Grayer et al. have demonstrated that gallic, protocatechuic, and ellagic acids in the AEs of *O. aspera*, which is closely related to *O. octandra*, have hepatoprotective activity against bromobenzene and 2,6-dimethyl-N.-acetyl p-quinoneimine toxicity in HepG2 liver cells [74]. Another study on the AE of *O. octandra* by Hettihewa et al. have shown that it is a good source of phenolic and flavonoid compounds [23]. Generally, the antioxidant potential of phenolics and flavonoids has been well established [75], therefore, the AE of *O. octandra* must be a good source of natural antioxidants owing to its high content of phenolic and flavonoids. When *V. cinere*a is considered, Leelaprakash et al. and Naowaboot et al. have demonstrated the hepatoprotective effects of its crude extracts on animal models [21, 33]. Further, as reviewed by Trang et al. and Fadlina *et al. V. cinere*a possesses numerous secondary metabolites with potential hepatoprotective effects [76, 77]. Specifically, hirsutinolide-sesquiterpene lactones, the most characteristic secondary metabolites of *V. cinerea* are reported to have strong anti-inflammatory properties [78]. As shown by Fernando and Soyza in 2014 in an in vitro study, AE of *A. ceylanica* is

also a rich source of phenolics and flavonoids with antioxidant and hepatoprotective effects [37].

O. octandra stands out with a significant positive impact on ameliorating HFD-induced MASLD, consistent with previous studies that have demonstrated its hepatoprotective effects against various hepatic toxins such as tert-butyl hydroperoxide, acetaminophen, carbon tetrachloride (CCl_4), and galactosamine [16–18, 20]. According to Thabrew et al. AE of *O. octandra* can reduce liver enzyme elevations and improve liver histopathology in carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats [79]. The same research group has demonstrated the potential of *O. octandra* extracts to mitigate liver injury from D-galactosamine and tert-butyl hydroperoxide in isolated rat hepatocytes, reducing protein synthesis inhibition and lactate dehydrogenase (LDH) release while lowering lipid peroxidation [17]. Additionally, *O. octandra* extract was shown to protect the liver against paracetamol-induced injury in mice, enhancing liver function and histopathology. Another study conducted by Thabrew et al. has shown the potential of the *O. octandra* extract to improve hepatocyte viability against 2,6-dimethyl N-acetyl p-quinoneimine induced damage [19]. Furthermore, Bogahawaththa et al. indicated that *O. octandra* extracts can exert hepatoprotective effects in thioacetamide-induced cirrhotic rats by inhibiting pro-inflammatory and pro-fibrotic cytokine secretion and angiogenesis [20]. However, this is the first study to demonstrate the hepatoprotective effects of *O. octandra* against HFD-induced hepatic injury.

As for *V. cinerea*, hepatoprotective effects have been shown in a HFD-induced MASLD mouse model by Naowaboot et al. [33]. Consistent with our findings, they have demonstrated that water extract of *V. cinerea* has the potential to ameliorate the HFD-induced weight gain, hypertriglyceridemia, steatosis and increase in TNF-α gene expression. In addition, they have also demonstrated its ability to ameliorate HFD-induced insulin resistance. Similarly, another study conducted by Leelaprakash et al. also demonstrated the hepatoprotective effects of an ethanolic extract of *V. cinerea* against CCl_4-mediated hepatotoxicity in a rat model. They have shown its ability to suppress the CCl_4-mediated increase of serum hepatic enzyme and hepatocellular lipid peroxide concentrations and enhance the CCl_4-mediated reduction in hepatocellular antioxidant concentration [21]. Further, as reviewed by Chang et al. in 2024, *V. cinerea* is a good source of antioxidants, which is consistent with our findings [76].

Conforming with our findings on the hepatoprotective effect of *A. ceylanica*, an in vitro study conducted by Fernando and Soyza has demonstrated a moderate antioxidant potential in the AE of *A. ceylanica* (DPPH radical scavenging activity: IC50 = 131.2 ± 36.1 µg/ml) which was almost two-fold higher than that reported in our study [37]. The same study has reported that co-treatment with AE of *A. ceylanica* at a concentration of 2 mg/ml has a hepatoprotective effect against ethanol-induced hepatotoxicity (5 M for 2 h) in porcine liver slices which was evident by a significant reduction in the leakage of liver enzymes (ALT, AST, and LDH) from liver slices and a significantly low concentration of lipid peroxides in the homogenized liver slices [37].

In this study, we have revealed its potential to ameliorate HFD-induced MASLD through numerous mechanisms. On one hand, it can neutralize free radicals and inhibit weight gain which are among the key contributing factors for the initiation of MASLD. On the other hand, it can attenuate the deregulation of lipid metabolism, aggregation of lipids, and development of a proinflammatory environment in hepatocytes, indicating its potential to ameliorate the progression of early stages of HFD-induced MASLD towards more advanced stages. Considering the fact that liver transplantation is the only effective intervention to address advanced stages of MASLD such as hepatic cirrhosis and hepatocellular carcinoma, which is limited by difficulty in finding donors and the high cost, AE extract of *O. octandra* is a promising candidate to be developed as an effective chemoprophylaxis agent to prevent initiation and progression of the disease.

However, our study has several limitations. We used crude extracts of the plants without isolating or identifying the specific active compounds responsible for the observed effects. As a result, the exact bioactive constituents contributing to the observed hepatoprotective effects remain unknown. Furthermore, when we analyzed the effect of AEs of the tested plants on gene expression, they were limited to mRNA expression levels. We also used a relatively small sample size per group, which may limit the statistical power and generalizability of our findings. Including a larger cohort in future studies would help validate the observed effects more robustly. In addition, only male mice were included in this study, which may not fully capture sex-based differences in disease progression and response to treatment. Including female animals in future research could provide a more balanced understanding of the hepatoprotective effects across sexes.

Moreover, the in vitro cytotoxicity evaluation was conducted using the Vero cell line, which, while commonly used, is derived from non-hepatic tissue and may not accurately reflect hepatocyte-specific responses to the plant extracts. Employing liver-derived cell lines or primary hepatocytes in future experiments would better mimic the hepatic environment and improve the translational relevance of cytotoxicity data. Addressing these gaps in future studies could provide a more

comprehensive understanding of the therapeutic potential and mechanisms of action of these plant extracts.

Conclusions

AEs of *O. octandra*, *V. cinerea*, and *A. ceylanica* are good sources of natural antioxidants with protective effects against a HFD-induced MASLD in a mouse model. Of the three, *O. octandra* stands out as the best source of antioxidant, anti-obesity, anti-hypolipidemic, and anti-inflammatory compounds to inhibit the initiation and progression of HFD-induced MASLD.

Abbreviations

ABTS	2,2-azinobis-3-ethylbenzothiazolin-6-sulfonic acid
AE	Aqueous extract
ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CCL_4	Carbon tetrachloride-4
cDNA	Complimentary DNA
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl hydroxyl
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FDA	Food and drug administration
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HFD	High Fat Diet
IC50	Half-maximal inhibitory concentration
ICR	Institute for Cancer Research
IL-6	Interleukin-6
LDH	Lactate Dehydrogenase
LSD	Least Significant difference
MASLD	Metabolic Dysfunction Associated Steatotic Liver Disease
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-etrazolium bromide
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-alcoholic steatohepatitis
NBF	Neutral Buffered Formalin
Nrf2	Nuclear factor erythroid 2 (NFE2)-related factor 2
OS	Oxidative stress
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPAR-α	Peroxisome Proliferator-Activated Receptor Alpha
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SD	Standard Deviation
EM	Standard Error of the Mean
SIRT-1	Sirtuin-1
SREBP-1c	Sterol regulatory element binding transcription factor 1
TEAC	Trolox Equivalent Antioxidant Capacity
TNF-α	Tumor Necrosis Factor-alpha
UV	Ultra violet
VERO	*Verda reno*
WHO	World Health Organization

Acknowledgements

The authors extend their heartfelt gratitude to Mrs. Waruni Jayaweera, Mr. N.S.I. Udagedara, Mrs. Shanika Rajawasam, Mr. K.M. Karunajeewa, Mr. D.P.G.S.P. Jayasinghe, and Mr. T.M.I.P.K. Bandara from the Division of Parasitology, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, as well as Mr. M.G.B. Gangadhara and Mr. R.M.A. Rathnakumara from the Department of Basic Sciences, Faculty of Dental Sciences, University of Peradeniya, for their invaluable technical assistance and support.

Author contributions

J.R. conceptualized the study. A.M.A.U.A. designed the methodology alongside M.P.P., K.K.W., and J.R. The extraction process was performed by A.M.A.U.A., and the antioxidant assay was carried out by A.M.A.U.A., M.P.P., and O.C.P. Cytotoxicity assays were conducted by A.M.A.U.A., while gene expression studies were undertaken by A.M.A.U.A. and T.A.N.M. Data analysis and curation, as well as manuscript preparation, were managed by A.M.A.U.A. The manuscript underwent critical review by M.P.P., K.K.W., T.A.N.M., and J.R. Visualization tasks were performed by A.M.A.U.A. The project was supervised by K.K.W., M.P.P., and J.R., with resources provided by J.R. and M.P.P. All authors have read and approved the final manuscript.

Funding

This research received no external funding.

Data availability

Findings of this study are available within the paper and any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The animal study protocol was approved by the Committee for Ethical Clearance on Animal Research of Faculty of Veterinary Medicine and Animal Science, University of Peradeniya. (protocol code: 20400, date of approval: 10.12.2020, Id: VERC-20-08)

Consent for publication

All authors consented for publication.

Competing interests

The authors declare no competing interests.

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Received: 1 December 2024 / Accepted: 12 May 2025
Published online: 23 May 2025

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