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Traditional Herbal Drugs against Liver Diseases – Experimented *in vitro* using HepG2 Cells for Induced Steatosis

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ABSTRACT: *Salvia fruticosa* leaves, *Malva sylvestris* flowers, *Taraxacum officinale* aerial parts, *Plantago ovata* seeds, *Tanacetum parthenium* aerial parts, and *Allium sativum* bulbs are documented for traditional use against hepatic disorders and different liver diseases. To evaluate herbal drug material for potential use against liver diseases, at molecular level for the efficacy linked to ethnobotanical documented data.Different herbal extracts were prepared and standardized by HPLC, according to European Pharmacopoeia. Initially 0.25 mg/mL each standardized extract was applied to oleic+palmitic acid induced fatty liver using a HepG2 cell culture model. ALT, AST, GSH, and MDA levels were comparatively analyzed, in addition to cell Nil Red staining. The highest activity for MDA reduction was observed for the *A. sativum* extract at 48.2% level, followed by 36.4% for *M. sylvestris*, and *S. fruticosa* extracts with 27.3% reduction, respectively. Glutathione levels increased to 59.1% when *A. sativum* extract was applied. *M. sylvestris* extract increased the glutathione levels in the medium by 49.7%; *S. fruticosa* extract decreased ALT levels by 53.5% and *M. sylvestris* extract by 38.5%, whereas the standard resveratrol reduced ALT level by 30.9%, respectively. The AST levels for *M. sylvestris* extract was 46.5%, compared to resveratrol by 93%. *A. sativum, M. sylvestris,* and *S. fruticosa* standard extracts showed relatively good correlation and activity where further *in vivo* studies should be performed.

KEYWORDS: Ethnobotany; HPLC; fatty liver; cell culture.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is observed due many reasons such as diet, pharmaceuticals, lifestyle etc., except alcohol consumption [1]. The prevalence of NAFLD is likely to increase with the increasing incidence of obesity and diabetes. A very small proportion of patients with NAFLD, especially those with diabetes, may have risks of different liver-related complications such as cirrhosis, and hepatocellular carcinoma. Currently, the treatment of this disease is limited only to exercise, weight control, and regulating metabolic risk factors [2]. Linked to the increasing obesity epidemics, it is classified as one of the most common causes of chronic liver diseases both in adults and children, furthermore, impacts society with pharmaco-economic burdens.

The plants selected in this study were determined from among the plants mentioned in the European Pharmacopoeia and ethnobotanically used in "liver disorders". According to traditional medicine documentation, the aerial parts, and leaves of *Salvia fruticosa* Mill. (Syn. *Salvia triloba* L. f., Lamiaceae) is used as antiphlogistic herbal tea against cold symptoms in the throat, against cough, and stomachache [3]. Also, the use of *S. fruticosa* leaf infusions and decoctions are ethnobotanically reported against liver diseases [3,4]. *Malva sylvestris* L. is another herbal drug, used against liver diseases [5]. *Allium sativum* L. is used as a liver protecting

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remedy, which is documented traditionally and experimentally in the literature [6,7]. *Plantago ovata* Forssk. seeds are also used traditionally against hepatitis, and liver toxicity, which was also demonstrated in previous studies [8,9]. *Taraxacum officinale* L. is a well-known herbal drug in different countries as a traditional medicinal resource against liver diseases [10]. The effects of *Taraxacum* extracts prepared from its leaves against hepatotoxicity were also reported [11]. *Tanacetum parthenium* L., is another herbal source documented traditionally against liver diseases [12], its hepatoprotective effects were also demonstrated by *in vivo* experiments [13].

In this present study, based on ethnobotanical data *S. fruticosa* leaves, *M. sylvestris* flowers, *T. officinale* aerial parts, *P. ovata* seeds, *T. parthenium* aerial parts, and *A. sativum* bulb standardized extracts according to European Pharmacopoeia were evaluated *in vitro* using a fatty liver model. To the best of our knowledge, this study is the first detailed work using herbal standardized extracts against fatty liver diseases.

2. RESULTS

2.1. Extraction and HPLC analysis

Extract yields of the six herbal extracts were calculated based on dry drug % (w/w), which varied from 1-12%, overall. The yield of the extract obtained from *M. sylvestris* flowers with 70% ethanol was 12%. *A. sativum* extract using methanol was calculated as 5.5%. The yield of the extract obtained from the leaves of *S. fruticosa* with 99%ethanol was determined as 6.5%. The yield of the extract obtained from *P. ovata* seeds with methanol was calculated as 1%. The yield of the extract obtained from the aerial parts of *T. parthenium* with methanol was 9%. The extract yield of the aerial parts of *T. officinale* using methanol was calculated as 8%. The herbal drugs were extracted separately with the solvents specified using 99.9% ethanol, methanol and 70% ethanol, respectively as in the European Pharmacopeia [14]. In this present study, the *in vitro* effects of the selected herbal drugs were evaluated initially based on their ethnobotanical uses. Thus, in this line extraction solvents were selected considering the ethnobotanical use such as the aqueous-polar preparations, also the solvents used in the relevant monographs in the European Pharmacopoeia [4,15–17].

The allicin content in the *A. sativum* methanol extract was calculated as 1.49%, according to the HPLC experiments (Figure 1-2). Previous studies [18,19] on allicin extraction and yields were reported, however due to cultivars and collection site variations the pharmaceutical material was not comparable.

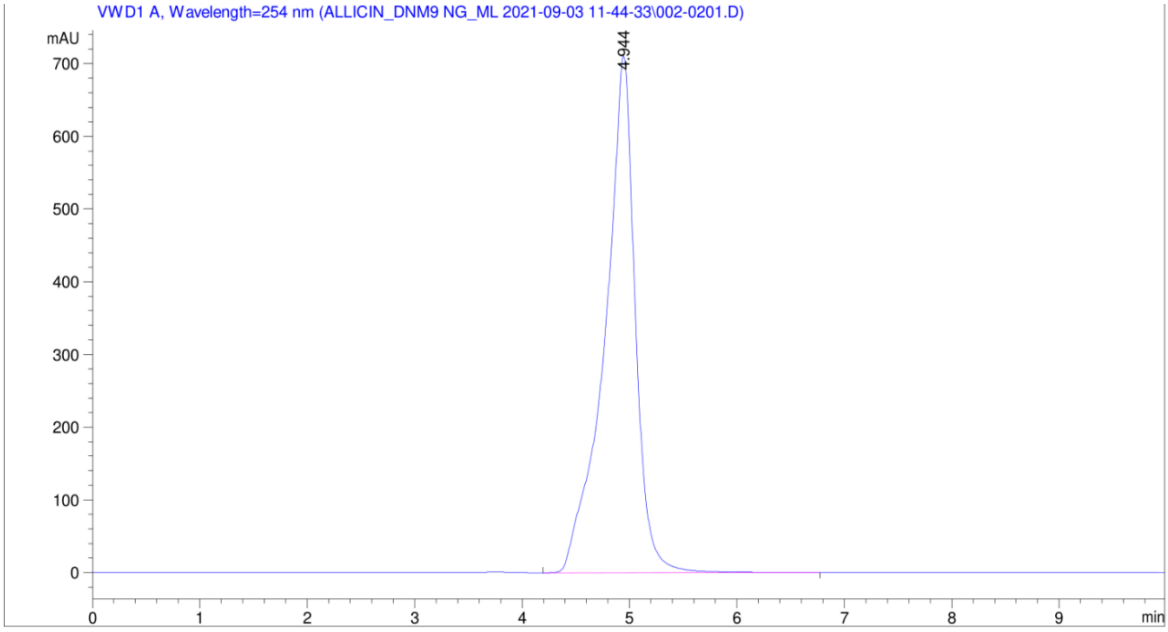

Figure 1: HPLC chromatogram of allicin (tR: 4.944).

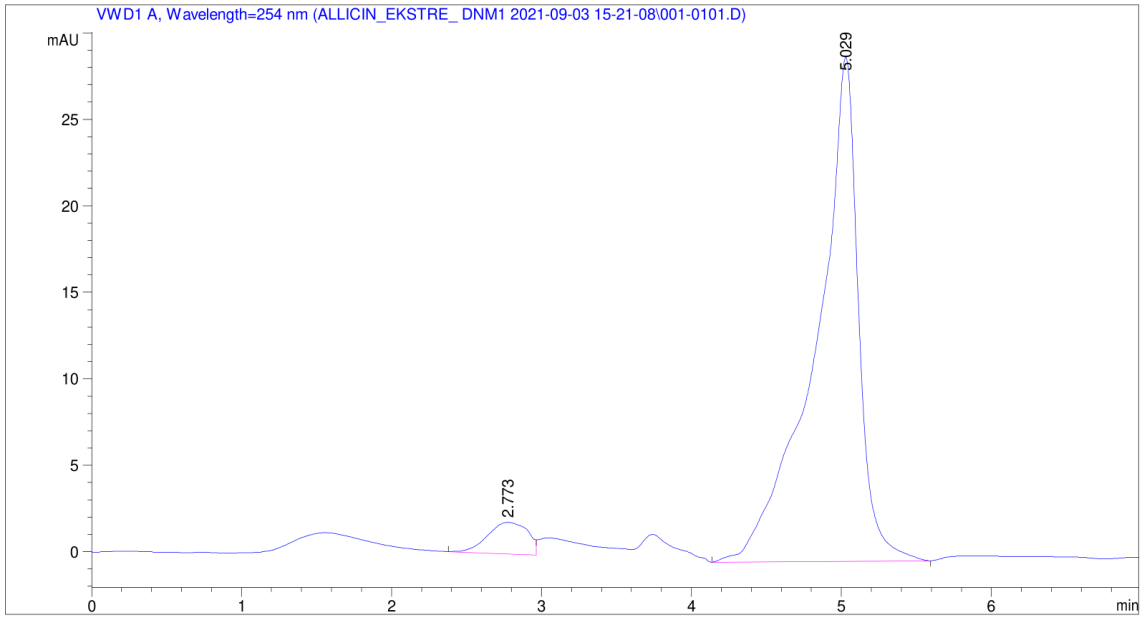

Figure 2: HPLC chromatogram of *A. sativum* ethanol extract.

The percentage amount of rosmarinic acid and salvigenin in the *S. fruticosa* ethanol extract was also calculated, where rosmarinic acid and salvigenin were 1.96% and 3.68% (w/w), respectively (Figure 3-5). As *S. fruticosa* polar extract contains rosmarinic acid and salvigenin as characteristic components [20]; salvigenin was analyzed together with rosmarinic acid by HPLC system. Salvigenin was extracted with n-hexane and dichloromethane from *S. fruticosa* aerial parts in previous studies [20,21] However, in this present study, *S. fruticosa* extract was standardized by two different flavone compounds, namely by both rosmarinic acid, and salvigenin for the first time, respectively.

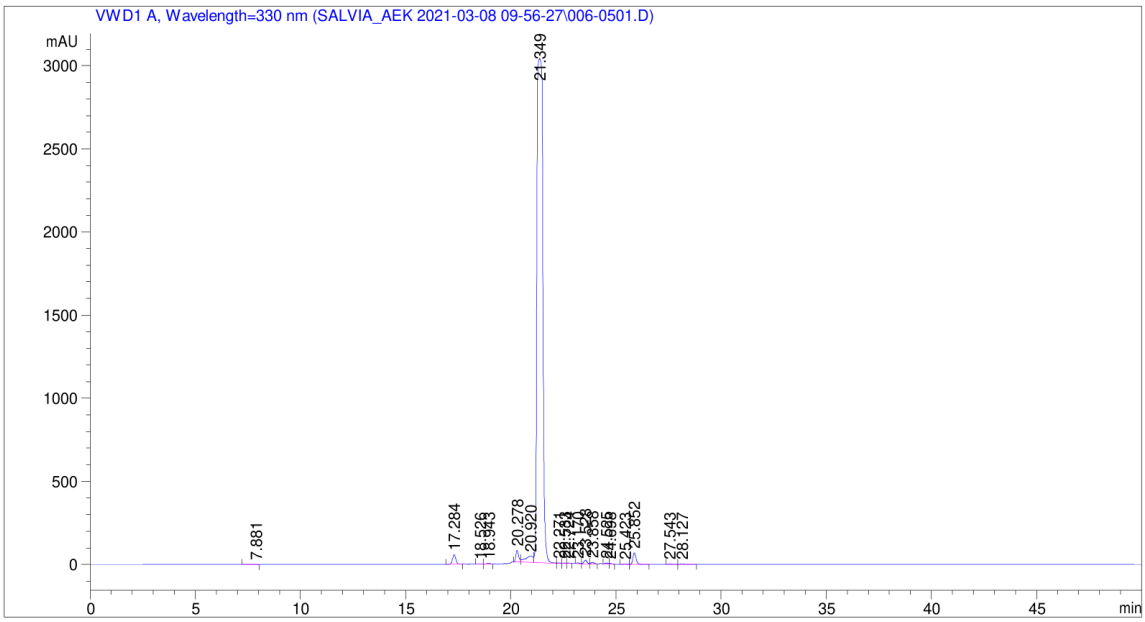

Figure 3: HPLC chromatogram of rosmarinic acid (tR: 21.349).

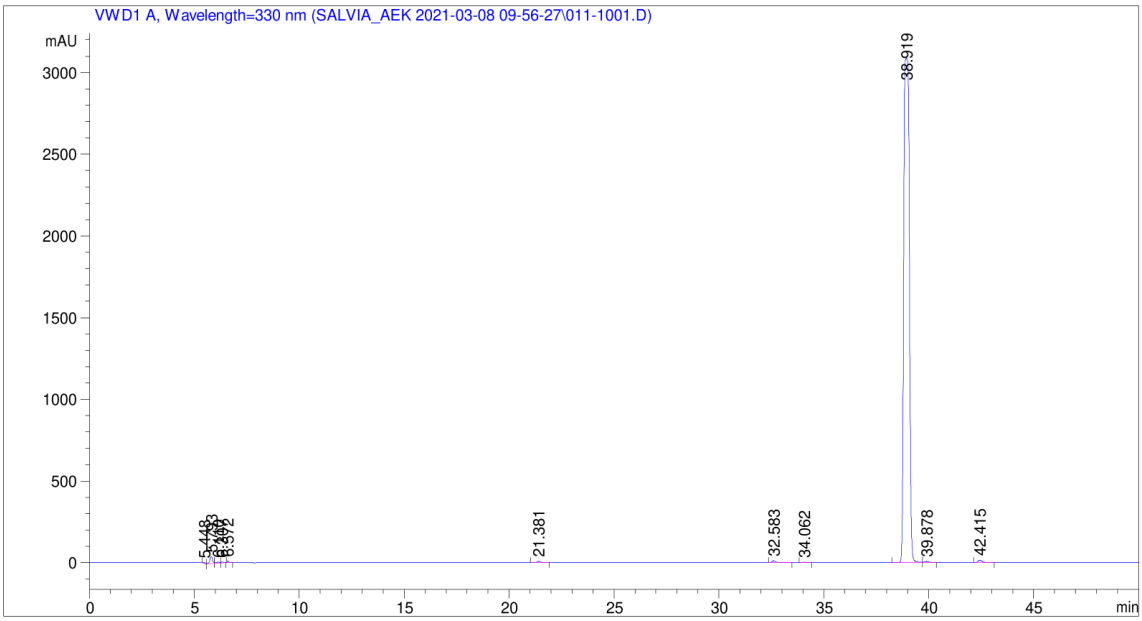

Figure 4: HPLC chromatogram of salvigenin (tR: 38.870).

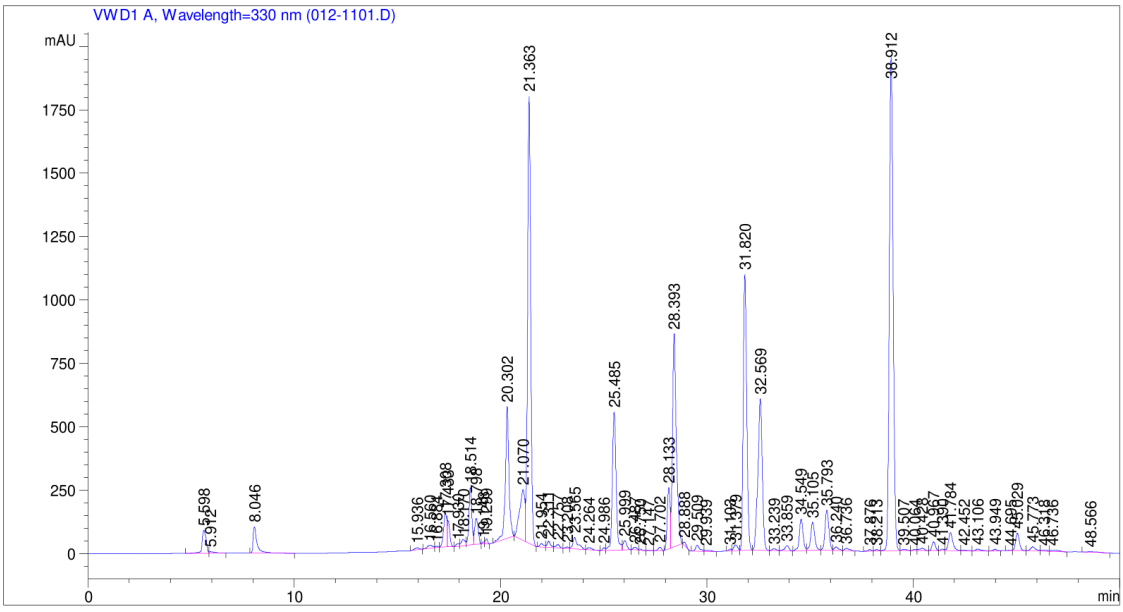

Figure 5: HPLC chromatogram of *S. fruticosa* extract.

The β-sitosterol content of *P. ovata* methanol extract was calculated as 0.33% (w/w) quantitatively (Figure 6-7). According to previous work, *Plantago* seed extract contained β-sitosterol, which was confirmed in a standardized extract [22].

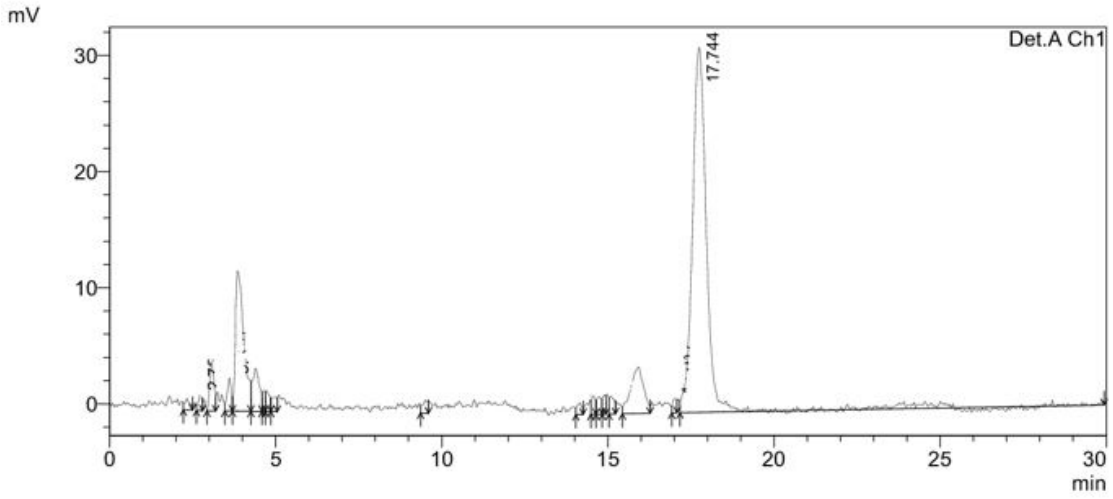

Figure 6: HPLC chromatogram of β-sitosterol (tR: 17.786).

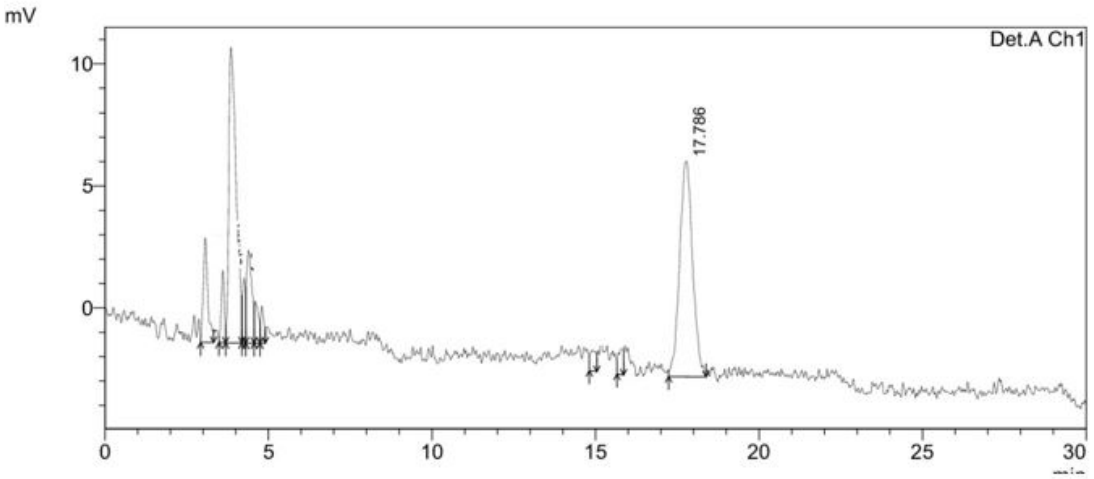

Figure 7: HPLC chromatogram of *P. ovata* extract.

The characteristic anthocyanin malvin was 0.19% according to the HPLC analyses of *M. sylvestris* flower aqueous ethanol extract (Figure 8-9). Malvin quantification within *M. sylvestris* flowers was by TLC, in the European Pharmacopoeia monograph. The analytical results showed that *M. sylvestris* extract followed the Pharmacopoeia.

Figure 8: HPLC chromatogram of malvin (tR: : 6.941).

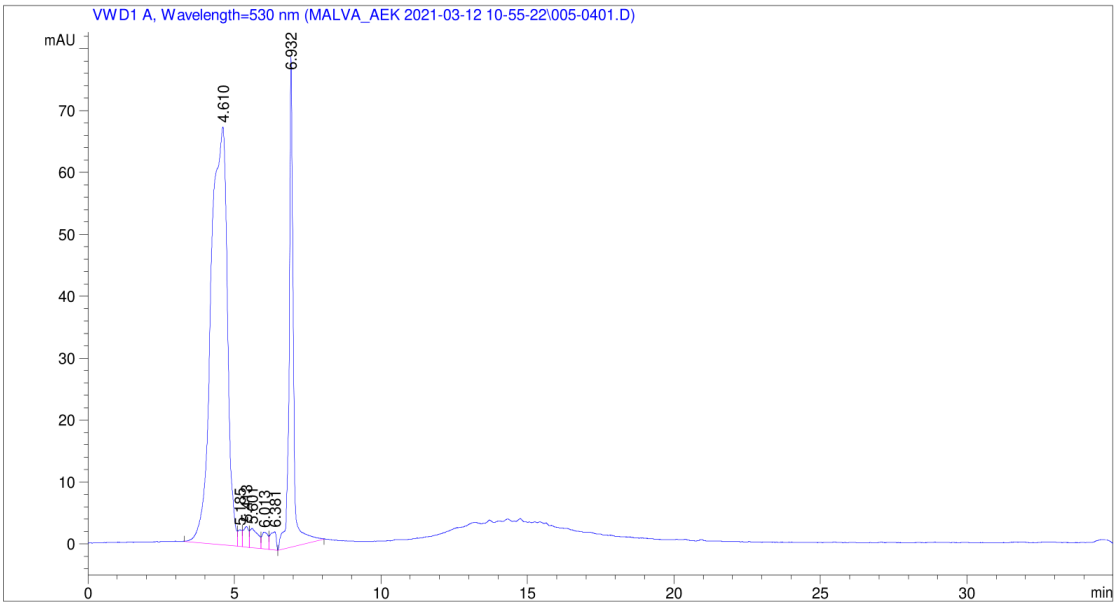

Figure 9: HPLC chromatogram of *M. sylvestris* extract.

The methanol extract from the aerial parts of *T. parthenium* was studied using the HPLC method for the parthenolide content, where an amount of 0.5% (w/w) was calculated, as *T. parthenium* extracts, were previously standardized to parthenolide [23,24].

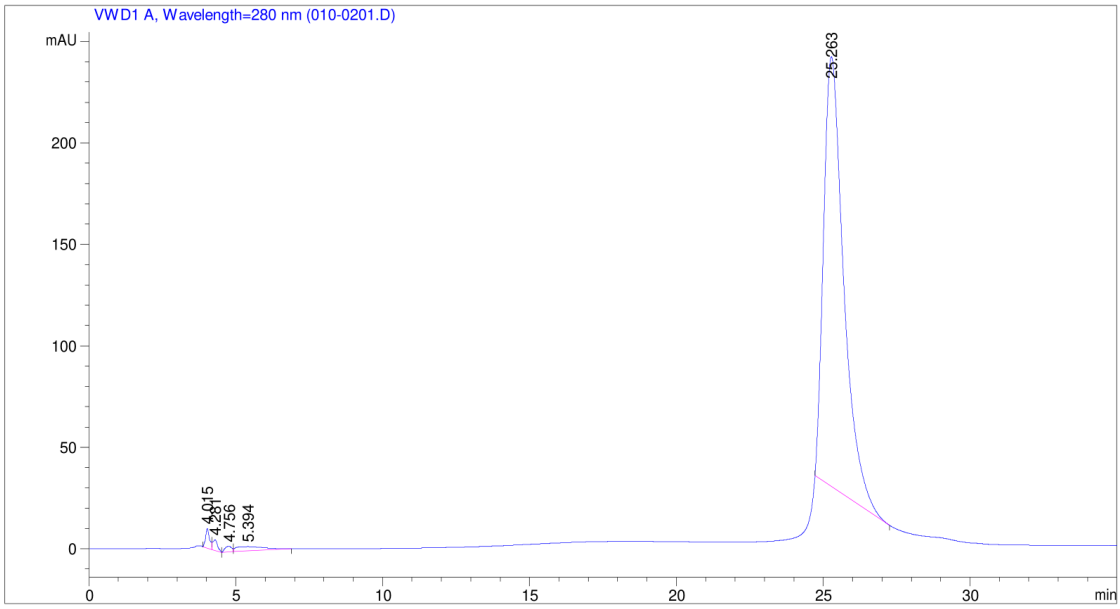

Figure 10: HPLC chromatogram of chlorogenic acid (tR: 25.263).

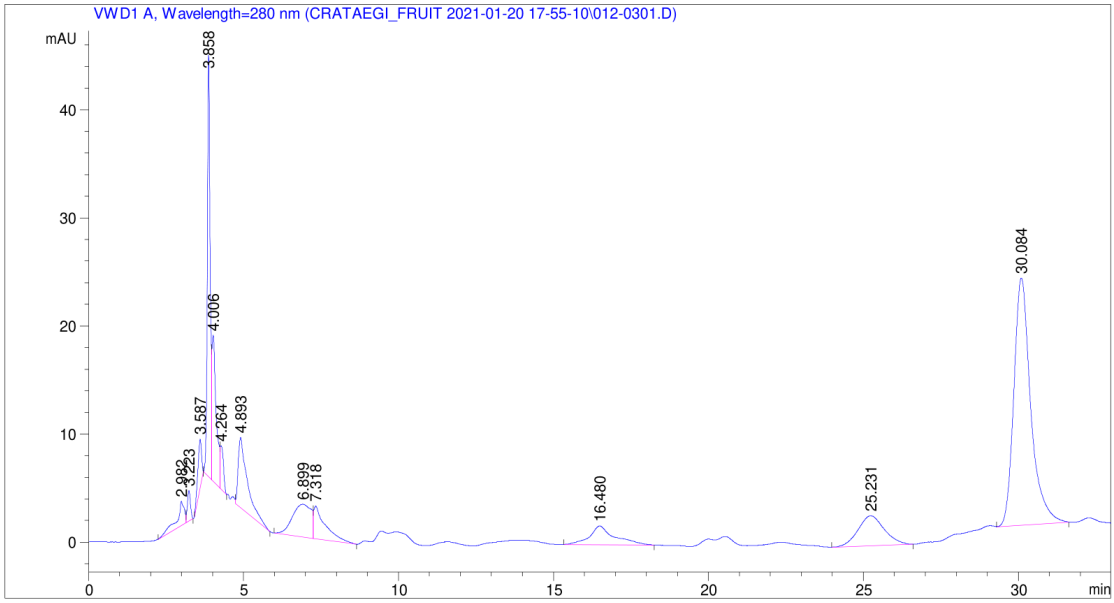

Figure 11: HPLC chromatogram of *T. officinale* extract.

In this present study, the chlorogenic acid content of the *T. officinale* methanol extract was confirmed by the HPLC method, where the amount of chlorogenic acid was determined as 0.3% (w/w). HPLC chromatograms of all standards and extracts were illustrated in Figures 1-13. The importance and quality difference of plant materials from known sources and Pharmacopeia standards were also comparatively studied.

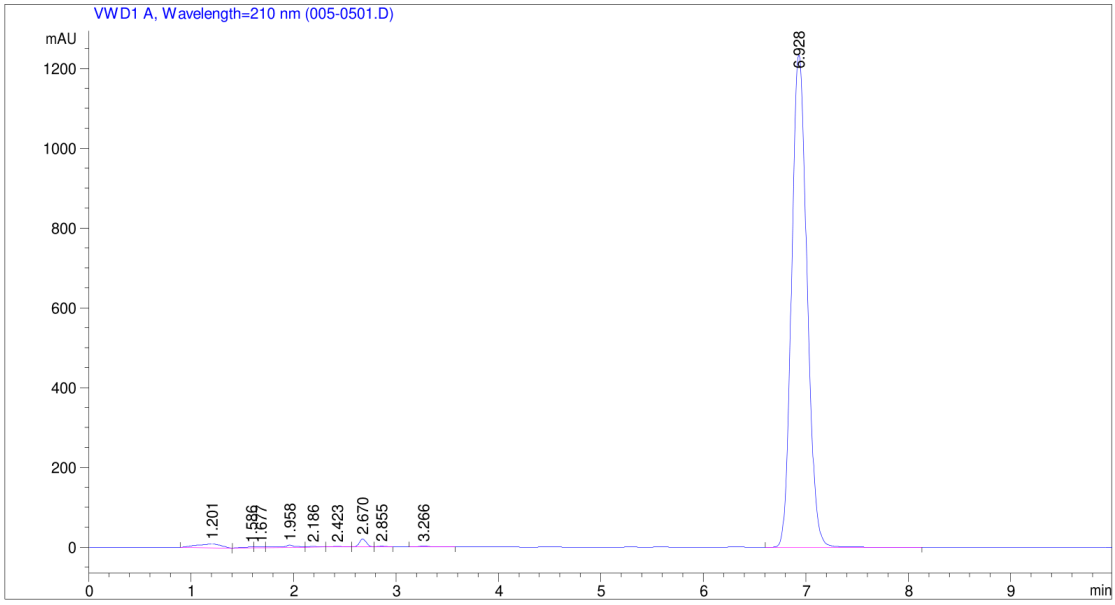

Figure 12: HPLC chromatogram of parthenolide (tR: 6.948).

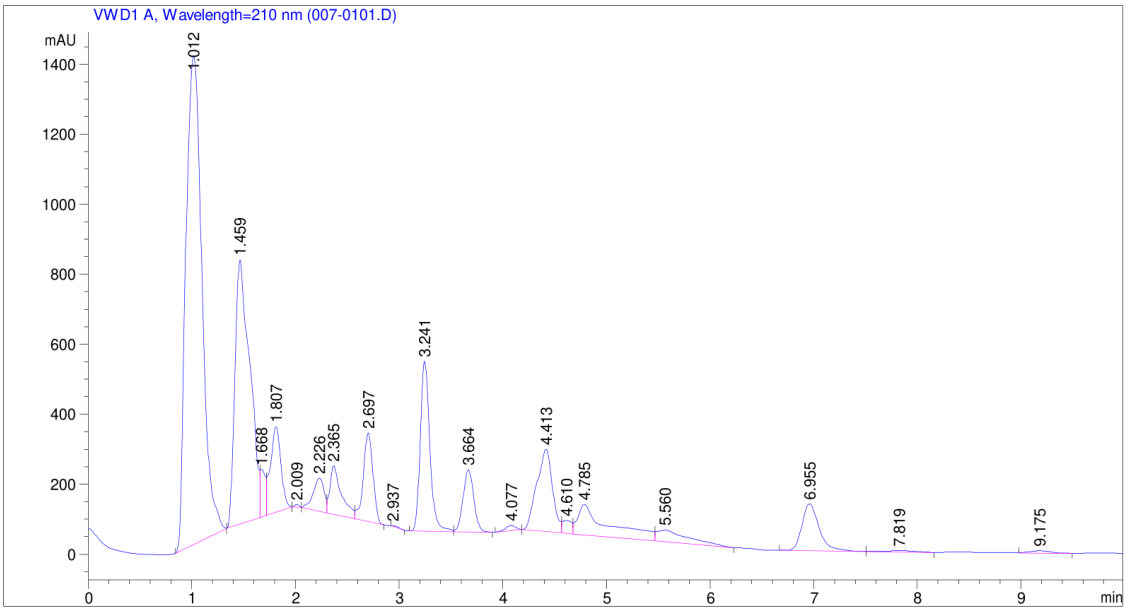

Figure 13: HPLC chromatogram of *T. parthenium* extract.

2.2. *In vitro* fatty liver assay

After the extractions and analytical work, six different extracts were evaluated separately using the cell culture methods and by Nile Red staining in fatty liver models as well as the GSH, MDA, ALT, and AST measurements, respectively. To determine the non-toxic concentrations of all extracts on HepG2 cell, cytotoxicity studies were performed using the MTT method (Figure 14). Results showed that the maximum concentration that can be applied was determined as 0.25 mg/mL. Thus, all extracts of this study were standardized to a concentration of 0.25 mg/mL.

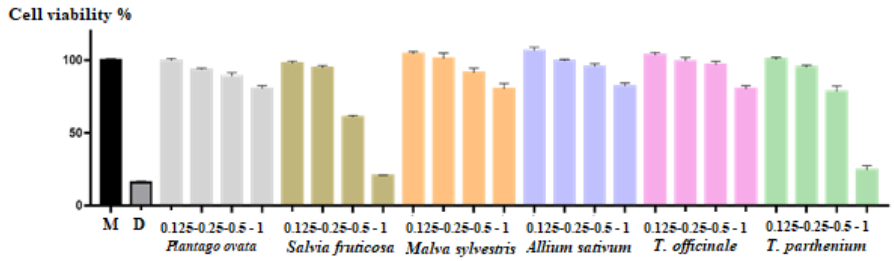

Figure 14: Cytotoxicity results of tested extracts.

In human HepG2 cells, an increase in MDA levels was observed when treated with Oleic acid, Palmitic acid and their combinations compared to the normal control group. The highest efficiency was observed in A. sativum extract by reducing the levels of MDA, which is a lipid peroxidation product, at the tested concentration of 0.25 mg/mL. The results obtained from *P. ovata* and *T. parthenium* extracts did not differ according to the free acid-induced cell environment. Results of lipid peroxidation levels were compared with the positive control resveratrol, which decreased the MDA level (nmol/g protein) by 31.8%, while *A. sativum* extract decreased it by 48.2%, and *M. sylvestris* extract by 36.4%. The *S. fruticosa* extract caused a decrease of 27.3%, a value close to the control resveratrol (Figure 15). The results were found statistically significant.

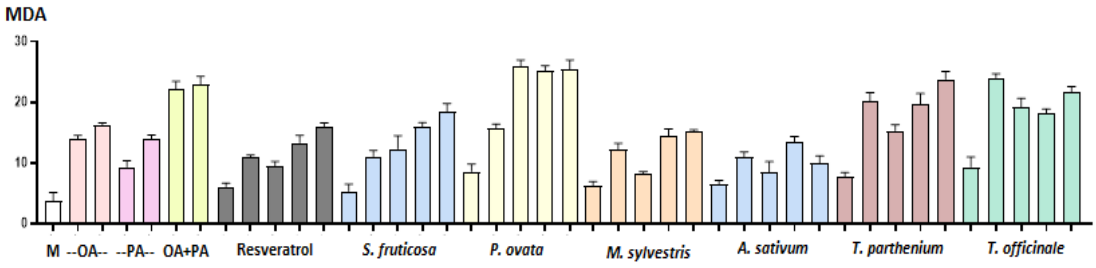

Figure 15: MDA levels of after and before treatment with resveratrol and tested extracts (nmol/protein). OA: Oleic acid; PA: Palmitic acid. $p < 0.05$ was considered statistically significant.

As seen in Figure 15, while the MDA levels of the empty medium (M) were quite low, an increase was observed in the MDA level as the medium was induced with oleic (OA), palmitic acid (PA), and oleic acid+palmitic acid (OA+PA) treated groups, respectively. According to the results from the lipid peroxidation experiments, while *A. sativum* extract decreased MDA levels at the highest rate, *T. parthenium* extract caused an increase in MDA levels. As it is known, the determination of MDA is based on the principle that thiobarbutyric acid (TBA) and MDA react to a colored outcome. Tetramethoxypropane (TMP) was used as a standard, where the results were reported as nmol/g. In previous studies, different extracts of *A. sativum* were also evaluated enzymatically in terms of lipid peroxidation, and the extracts reduced the levels of lipid peroxidation products [25,26]. In a recent work of Bontempo et al. (2021), lipid peroxidation products in HepG2 cells interacted with *A. sativum* extracts, which were effective and inhibitory [27]. In another previous work, the effects of *M. sylvestris* flower extracts on lipid peroxidation levels were tested enzymatically, where better results were obtained when compared with *M. sylvestris* leaf extracts [28]. It was also determined that extracts obtained from both flowers and leaves of *M. sylvestris* reduce the levels of lipid peroxidation products in different organ damages such affecting kidney, brain, heart, etc. [28–32]. The *S. fruticosa* methanol and water extracts were previously experimented by Kyriakou et al. (2021) for lipid peroxidation inhibition using the A375 cell line. It was observed that the extract statistically significantly reduced lipid peroxidation in the cell medium, however, no phytochemical analysis of the extract was performed to justify the chemistry.

The *in vitro* experimental work showed in HepG2 cells a decrease of GSH levels, when treated with Oleic acid (OA), Palmitic acid (PA), and their combinations (OA+PA) compared to the normal control group. Resveratrol increased the GSH level (µmol/g protein) in the cell medium by 27.9%, while the *A. sativum* extract

increased by 59.1%, and M. sylvestris extract by 49.7%, statistically significant, respectively. *P. ovata* extract was also effective at a rate of 29.1%, comparable to the positive control resveratrol. According to the experimental data *M. sylvestris* and *A. sativum* extracts increased the GSH level, which decreases with the influence of oleic acid and palmitic acid. While the *T. officinale* extract did not change the experimental data, *T. parthenium* extract influenced a decrease in GSH levels as shown in Figure 16.

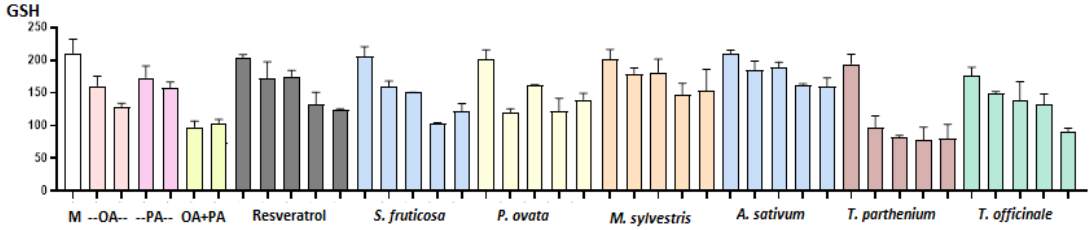

Figure 16: Glutathione levels (µmol/protein) of after and before treatment with resveratrol and tested extracts. OA: Oleic acid; PA: Palmitic acid. $p < 0.05$ was considered statistically significant.

It is well known that GSH is a thiol that plays a critical role in cellular defense against oxidative stress in tissues and cells. DTNB also known as Ellman's reagent reacts with the thiol groups and disulfide bonds. The formed TNB- turns into TNB-2 in neutral or alkaline environment and gives a yellow color [33]. The glutathione, a low molecular weight thiol, was also measured based on this principle.

Steatosis status in cell culture medium was determined by the fluorescence microscopy method. Nile Red, which is a fluorescent and lipophilic dye can specifically be used to stain fat cells [34]. For the detection of oil droplets, samples were stained using the Nile Red dye, and resulting fluorescent images were illustrated in Figure 17. The results suggested that the Nile red is a more specific marker in the fatty liver model in cell culture. Based on the fluorescent microscopy image analyses, a relatively less amount of oil droplets was detected in the cell media treated for the *M. sylvestris* and *S. fruticosa* extracts, respectively.

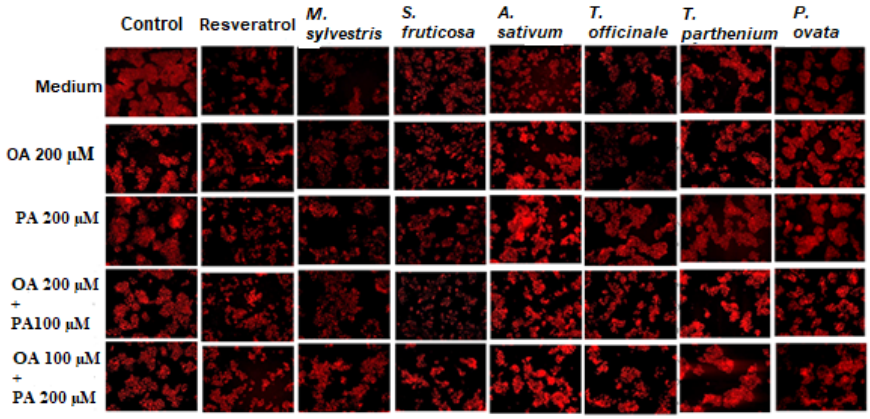

Figure 17: The appearance of oil droplets and steatosis result of Nile red staining after extracts and resveratrol application. OA: Oleic acid; PA: Palmitic acid. $p < 0.05$ was considered statistically significant.

In HepG2 cells, an increase in AST and ALT enzyme levels was observed when treated with oleic acid, palmitic acid, and their combinations compared to the blank medium. According to the results from this experiment, *A. sativum* and *M. sylvestris* extracts decreased the AST level by 65% and 46.5%, respectively as illustrated in Figure 18. The results were found statistically significant. For the ALT experiment, the results from *S. fruticosa* and *M. sylvestris* extracts were more effective, with 53.5% and 38.5%, respectively (Figure 19).

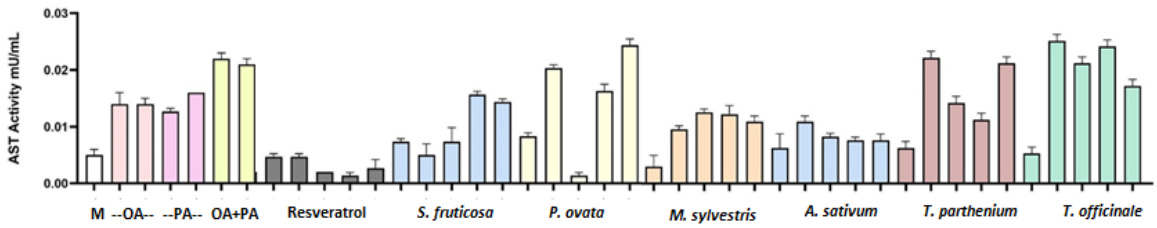

Figure 18: AST levels (mU/mL) of after and before treatment with resveratrol and tested extracts. OA: Oleic acid; PA: Palmitic acid. $p < 0.05$ was considered statistically significant.

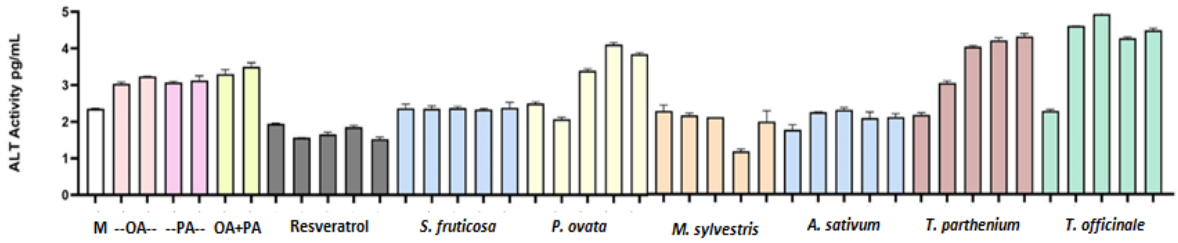

Figure 19: ALT levels (pg/mL) of after and before treatment with resveratrol and tested extracts. OA: Oleic acid; PA: Palmitic acid. $p < 0.05$ was considered statistically significant.

It was observed that *M. sylvestris* extract showed remarkable results for both enzymes. Furthermore, it was determined that the results of *T. officinale* and *T. parthenium* extracts for both experiments increased ALT and AST levels contrary to expectations. Resveratrol, on the other hand, decreased ALT levels by 30.9% and AST levels by 93%, respectively. Comparative *in vitro* test results are given in Table 1.

Table 1. Comparative *in vitro* test results in percentages (%).

	A. sativum	*M. sylvestris*	*S. fruticosa*	*P. ovata*	*T. officinale*	*T. parthenium*
MDA	48,2	36,4	27,3	9,1	11,4	0,5
GSH	59,1	49,7	12,3	29,1	9,2	**-22**
ALT	50,2	38,5	37,5	53,5	29,4	23,4
AST	65	46,5	0,1	27,9	0,3	20,9
Nile Red Oil Droplets	**More**	Less	Less	**More**	Less	**More**

To the best of our knowledge, this is the first *in vitro* experimental study with *S. fruticosa* extract in the NAFLD model. However, limited research on NAFLD was previously reported on *S. hispanica* seeds, known as chia, which is included in special diets. According to a clinical study, non-alcoholic fatty liver disease was prevented in patient groups using *S. hispanica* seed preparations [35]. In another study, where *S. grossheimii* alcohol extracts of the aerial parts were experimented against alcohol-induced fatty liver disease in rats, comparable results to the positive control were observed [36]. *S. plebeia* leaf ethanol extracts were tested in animals using a non-alcoholic fatty liver model, where a decrease in blood and liver enzymes were observed [37].

To the best of our knowledge, this is the first detailed experimental study against fatty liver disease with *M. sylvestris* preparations. In this present study, we also confirmed the efficacy of *A. sativum* preparations against NAFLD, in line with the findings obtained in previous studies. The effect of *A. sativum* preparations against non-alcoholic liver disease were reported both *in vivo* and clinically, however, without the phytochemical profile [38–40]. Also, this is the first experimental fatty liver disease study on *T. parthenium*, and *T. officinale* preparations used against liver diseases ethnobotanically. According to the results of this study, *T. parthenium* and *T. officinale* preparations were ineffective in cell culture experiments related to

NAFLD. In addition, a clinical study with *P. ovata* revealed that *P. ovata* was found ineffective in NAFLD patients [41,42]. Also, in this study, *P. ovata* extract was ineffective in most of the evaluated parameters.

3. CONCLUSION

Overall, in this present study, the *in vitro* effects of six different herbal drugs and their respective preparations rooting to ethnobotanical data were shown as effective against liver disease indicators targeting NAFLD. After toxicity evaluation using cell culture experiments, the extracts were evaluated on different indicative parameters such as MDA, GSH, ALT, AST, Nile Red.

In conclusion, based on traditional medicine knowledge related to liver diseases, the bioactive extracts were spotted experimentally from *A. sativum, M. sylvestris,* and *S. fruticosa* extracts indicating promising *in vitro* efficacy, however with the necessity with further detailed *in vivo* and clinical studies for potential drug development. In addition, the findings obtained from this study can be evaluated as striking in many respects. Because *T. officinale*, which is frequently consumed for liver diseases ethnobotanically, adversely affected liver parameters *in vitro* conditions and gave quite the opposite results. In further studies, the development of individual phytotherapeutic formulations from these three extracts and the testing of the effectiveness of these formulations *in vivo* were determined as new topics for our advanced projects.

4. MATERIALS AND METHODS

4.1. Plant Material and Chemicals

Commercial *S. fruticosa* leaves, *M. sylvestris* flowers, *T. officinale* aerial parts, *P. ovata* seeds, *T. parthenium* aerial parts, and *A. sativum* bulbs were acquired at European Pharmacopoeia Quality from Anoxymer International GmbH, Germany. Voucher samples were deposited at the Istanbul Medipol University (IMEF) Herbarium (Herbarium No: IMEF 1234-1239).

Analytical standards such as rosmarinic acid, parthenolide, alliin, β-sitosterol, chlorogenic acid, and malvin were obtained from Sigma Aldrich (Germany), whereas salvigenin was acquired from Fluka (Germany). The consumables such as toluene, ethyl acetate, formic acid, acetonitrile (HPLC grade), Tween 80, and propylene glycol were purchased from Merck (Germany). Analytical grade and high purity standards and chemicals were used in the experiments if not otherwise stated.

4.2. Extraction

Dried plant material (1000 g) was ground in the range of 200-300 mesh, which was extracted by maceration using different solvents (1 L) as stated below, for 1 hour at room temperature. The solvents were selected according to the European Pharmacopoeia for each plant material separately. Ethanol was used for *S. fruticosa*, 70% ethanol was used for *M. sylvestris*, and methanol was used for the other herbal materials. The extracts were filtered (Whatman No:1) in three equal portions and concentrated using a rotary evaporator for further biological evaluations. The stock test solutions for HPLC analyses were prepared using HPLC grade methanol at a concentration of 10 mg/mL ("European Pharmacopoeia Online").

4.3. HPLC Analysis

HPLC analysis of all extracts was performed with DAD detector connected to Agilent 1100 HPLC system (Santa Clara, CA, USA), where a C18 column (250 x 4.6 mm, 10 µm) was used as stationary phase. The reference standards were defined by matching the retention times with that of the standard analyzed under the same conditions. Column temperatures were set to 25-40°C accordingly to the literature conditions and all injection volumes were 10 µL.

For *S. fruticosa* extract, ultra-pure water containing 10 mM formic acid and acetonitrile (90:10, *v/v*) was used as the mobile phase. A linear gradient flow was used within 40 minutes. The flow rate was set to 0.45 mL/min. The column temperature was kept at 40°C. The peaks were detected at 330 nm [43]. *A. sativum* HPLC analysis; the column temperature was kept at 25°C. The system was operated in isocratic mode (50: 50, *v/v*; methanol:water) at a flow rate of 0.5 mL/min. The peaks were analyzed at 210 nm [44]. *M. sylvestris* analysis; mobile phase A: water: formic acid: acetic acid (100: 0.7: 0.9, *v/v/v*) B: acetonitrile from 10% B between 0-30 minutes, using a linear gradient flow to reach 90% B concentration. Flow returned from 90% B to 10% B (initial conditions) at 30-35 minutes. The column temperature was kept at 40°C. The flow rate was set at 0.5 mL/min. The peaks were analyzed at 530 nm [45]. *T. parthenium* extract was analyzed using the water:acetonitrile (55:45, *v/v*) mobile phase. The flow was isocratic, and the rate was set to 2 mL/min. The column temperature was kept at 30°C. The peaks were analyzed at 210 nm [23]. HPLC analysis of *T. officinale* extract, as mobile phase A: acetic acid:water (2:98, *v/v*), B: acetonitrile:water:acetic acid (44.5:44.5:1, *v/v/v*) gradient flow was used. The

gradient flow chart was adjusted to be 10-18% B for 0-20 min, 18-24% B for 20-30 min, 24-30% B for 30-33 min, and 30-10% B for 33-35 min. The column temperature was kept at 25°C. The flow rate was set to 0.4 mL/min. The peaks were analyzed at 280 nm [46]. *P. ovata* extract, unlike the others, were performed with a UV detector connected to the Shimadzu 2010C HPLC system. The column temperature was kept at 35°C. Isocratic flow was studied with methanol:acetonitrile (90:10, *v/v*) as mobile phase. The flow rate was set to 1 mL/min. The peaks were analyzed at 208 nm [47].

4.4. Cell Culture Studies

The HepG2 cell line was obtained from ATCC (American Type Culture Collection) with the code HB-8065TM. Dulbecco's Modified Eagle's Medium/High glucose (DMEM) was used supplemented with 10% (*v/v*) Fetal Bovine Serum (FBS), 1% penicillin (10.000 units/mL), and streptomycin (10.000 µg/mL) for cell culture studies. The cytotoxicity of the extracts was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method. Cells were plated in 48-well plates with 100.000 cells in each well and incubated for 24 hours at 37°C and with 5% CO_2. Extract was prepared by dissolving in DMSO. By making the necessary dilutions in the cell medium, studied concentrations of the extract (0.125, 0.25, 0.5, and 1 mg/mL with maximum %0.1 DMSO concentration) were prepared and applied on the cells for 24 hours. After incubation, 100 µL MTT solution (0.5 mg/mL) was added to each well and the plates were incubated in incubator at 37°C for 2 hours. After 2 hours, 100 µL isopropanol was added to each well as solvent and absorbance values were read in the Microplate (BioTek, USA) reader at a wavelength of 570 nm. Cell viability percentage (%) was calculated using the following formula:

Cell viability % = (Absorbance $_{\text{test group}}$) / (Absorbance $_{\text{control}}$) × 100%

4.4.1. *Lipid Peroxidation (MDA)*

The HepG2 cells were plated in T-25 flasks (2 x 10^6 cells/flask). Before the MDA level analysis, the cells were washed with PBS and the cells removed with trypsin. The precipitated cell pellet was homogenized in 1 mL of PBS with a tissue homogenizer (Heidolph Silent Crusher, Germany) by gradually increasing the speed from 15.000 rpm to 60.000 rpm for 2 min. Lysed cells were centrifuged at 14.000 rpm at 4°C for 15 minutes and the obtained supernatant was used for MDA determination. 100 µL of supernatant/standard + 200 µL of TBA was mixed and kept in a hot water bath at 90°C for 30 minutes. Distilled H_2O was used as the blank. After 5 min, the pink color was measured in a UV spectrophotometer (Thermo Multiscan Spectrum, Finland) at a wavelength of 532 nm. The experiment was performed as triplicate [48].

The protein determination method was used described by Lowry et al.[49]. Bovine serum albumin (BSA) was used as a standard. A 0.5 N NaOH solution was prepared. For Na_2CO_3 solution (10%), Na_2CO_3 (Riedel de Haen) was weighed and made up to 5 mL with NaOH solution. For $CuSO_4.5H_2O$ solution (1%), $CuSO_4.5H_2O$ was weighed and made up with distilled H_2O. To prepare the Na-K tartarate (2%) solution, Na-K (Riedel de Haen) tartrate was weighed and made up to 500 µL with distilled H_2O. Copper Reagent was freshly prepared by mixing 1820 µL of Na_2CO_3 solution, 90 µL of $CuSO_4.5H_2O$ solution and 90 µL of Na-K tartrate solution. It was prepared by mixing Folin-Phenol solution (Sigma-Aldrich) with distilled H_2O. A 96-well plate was used, 50 µL of Sample / Standard / Blank (distilled H_2O) + 50 µL of Copper Reagent was added into the wells. The plate was incubated in a shaker for 10 minutes at 25°C. 150 µL Folin-Phenol solution was added to the wells and kept at 50°C for 10 minutes and absorbance was measured at 660 nm.

4.4.2. *Glutathione (GSH) Assay*

Trisma Base-Tris HCl (Sigma) and EDTA (Sigma) were weighed and brought to 50 mL with water. A buffer solution was prepared by adjusting the pH to 8.2 with NaOH. Buffer solution was used as blank. 1.6 mg of DTNB (4.04 µmol) was dissolved in 400 µL methanol. GSH (Sigma) was used as standard. 100 µL supernatant / standard + 50 µL buffer solution + 10 µL DTNB was applied to the 96-well plate and the plate was left at room temperature for 5 minutes. The resulting yellow color was read in a UV spectrophotometer at 412 nm [33]. The experiment was performed in triplicate.

4.4.3. *Oleic-Palmitic Acid Induced Fatty Liver Model*

Preliminary studies were carried out with palmitic acid and oleic acid to determine the values between concentration-steatosis and concentration-viability. Interaction concentrations with fatty acid were calculated as 100, 150, and 200 µM, respectively. Stock solutions of oleic and palmitic acid were prepared in DMSO at a concentration of 20 mmol/L and then diluted with cell culture medium. *S. fruticosa* extract was studied the highest non-toxic concentration at HepG2 cells (0.25 mg/mL). Experimental groups in the cell culture fatty liver model as below:

- HepG2 + Medium control group
- HepG2 cells + 1%DMSO
- HepG2 cells + oleic acid (OA) 100, 150 and 200 µM
- HepG2 cells + palmitic acid (PA) 100, 150 and 200 µM
- HepG2 cells + oleic acid:palmitic acid (2:1, w:w)
- HepG2 cells + oleic acid:palmitic acid (1:2, w:w)
- HepG2 cells + Extract (0.25 mg / mL)
- HepG2 cells + oleic acid (100 µM) + Extract
- HepG2 cells + palmitic acid (100 µM) + Extract
- HepG2 cells + oleic acid:palmitic acid (2:1, w:w) + Extract
- HepG2 cells + oleic acid:palmitic acid (1:2, w:w) + Extract

Nile red staining

Nil Red stock solution was prepared using acetone, and stored at 4°C. The solution for the detection of fat cells (5 mg/mL) was prepared by diluting in PBS. After 24 hours incubation, HepG2 cells were washed 3 times with PBS. Cells were fixed with p-formaldehyde for 15 min followed by addition of the Nil Red dye, further incubated for 5 minutes in the dark. The cells were then washed 3 times with PBS. Steatosis was detected by fluorescence microscopy [34].

4.4.4. *Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Activity Assays*

In the cell culture study, AST and ALT levels were measured with ELISA kits according to the manufacturer's instructions, where 20 µL of cell supernatant was used (Human AST ELISA Kit /ab263881 and Human ALT ELISA Kit/ab234578) [50].

4.5. Statistical Analysis

In vitro groups were analyzed comparatively using GraphPad Prism 6 (Version 6.01; GraphPad software, Inc., San Diego, CA) one-way ANOVA, followed by post-hoc testing by Tukey, where $p < 0.05$ was considered statistically significant.

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