BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e18825

Article

Efficacy of an herbal compound in decreasing steatosis and transaminase activities in non-alcoholic fatty liver disease: A randomized clinical trial

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Hepatoprotective effects of many herbal agents have been reported in animal studies and clinical trials. In this study, five hepatoprotective plants with potent antioxidant, anti-inflammatory, and hypolipidemic effects were chosen to prepare a polyherbal compound for managing NAFLD. Sixty patients with NAFLD were randomly divided into treatment and control groups (2:1 ratio). Both group were advised to take healthy diet and exercise. The treatment group also received herbal capsules containing 400 mg of the mixture of *Anethum graveolens*, *Citrus aurantium*, *Cynara scolymus, Portulaca oleracea*, and *Silybum marianum* (2 capsules, thrice daily, for two months). The liver ultrasound and biochemical markers including the serum lipids, liver enzymes, and glucose were evaluated before starting the study and at the end of the treatment. Thirty patients in the treatment group and sixteen patients in the control group completed the study. The herbal compound significantly decreased the serum level of alanine transaminase (ALT), aspartate transaminase (AST), and total cholesterol. Treatment with the herbal compound significantly improved the grade of the fatty liver, but no significant change was found in the control group. In conclusion, the formulated herbal compound appeared to be effective in biochemical improvement and decreasing the grade of the fatty liver in the patients with NAFLD.

KEYWORDS: Anethum graveolens. Citrus. Cynara scolymus. Fatty liver. Portulaca oleracea. Silybum marianum.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common hepatic disorder in industrialized countries, with the prevalence of 6-35% worldwide (Bellentani, 2017). This disease has emerged as a major health problem because of its high prevalence, complex pathogenesis, and lack of approved therapies (Bellentani, 2017; Neuschwander-Tetri, 2017; Rotman, Sanyal, 2017). If NAFLD is not managed at early stages, it progresses to nonalcoholic steatohepatitis, which is a strong predictor of cirrhosis and hepatocellular carcinoma (Neuschwander-Tetri, 2017; Reeves, Zaki, Day, 2016).

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To date, no ideal therapeutic drug has yet been proven for NAFLD. The main approach is lifestyle modification with a focus on healthy diet and physical activity for weight loss (Stavropoulos *et al*., 2018). Since losing weight to the optimal level is hard for many patients, the search for new complementary and alternative medications is of great interest. Because oxidative stress, inflammation, lipotoxicity, and insulin resistance are involved in the pathogenesis of NAFLD (Buzzetti, Pinzani, Tsochatzis, 2016), natural agents that target one or some of these pathologic events may have therapeutic potentials.

Beneficial effects of several medicinal plants and natural compounds have been shown on NAFLD (Baghernya *et al*., 2017). Based on the literature data and on our earlier experience in formulating polyherbal compounds (Ghorbani, 2014; Shafiee-Nick *et al*., 2012; Zarvandi *et al*., 2017), five hepatoprotective plants (*Anethum graveolens, Citrus aurantium, Cynara scolymus, Portulaca oleracea, Silybum marianum*) with potent antioxidant (Bahramikia, Yazdanparast, 2009; Ben Hsouna *et al*., 2018; Colak *et al*., 2016; Speroni *et al*., 2003), anti-inflammatory (Aghazadeh *et al*., 2011; Kazemi, 2015; Samarghandian, Borji, Farkhondeh, 2017; Shen *et al*., 2017), and hypolipidemic (Bundy *et al*., 2008; El-Sayed, 2011; Mirhosseini, Baradaran, Rafieian-Kopaei, 2014; Moradi *et al*., 2012; Nazni *et al*., 2006) effects were selected for formulating a compound for managing NAFLD. The plants *A. graveolens* (dill), *C. scolymus* (artichoke), and *P. oleracea* (purslane) have been shown to reduce serum lipids in hyperlipidemic patients and improve insulin sensitivity in type 2 diabetic patients (Bundy *et al*., 2008; El-Sayed, 2011; Mirhosseini, Baradaran, Rafieian-Kopaei, 2014; Mobasseri *et al*., 2014; Moradi *et al*., 2012; Nazni *et al*., 2006). They have potent antioxidant and anti-inflammatory effects and showed hepatoprotective activities in different animal models of the liver damage (Bahramikia, Yazdanparast, 2009; Chen *et al*., 2012; Colak *et al*., 2016; Kazemi, 2015; Samarghandian, Borji, Farkhondeh, 2017; Speroni *et al*., 2003). *C. aurantium* is a rich source of flavonoids with antioxidant and anti-inflammatory effects (Ben Hsouna *et al*., 2018; Shen *et al*., 2017). This plant is able to induce lipolysis, inhibit adipogenesis, and protect liver against chemical-induced toxicity (Ben Hsouna *et al*., 2018; Kim *et al*., 2012). Similarly, several studies have reported that *S. marianum* (milk thistle) has a hepatoprotective effect and is a viable medicinal plant for treating the patients with NAFLD (Abenavoli *et al*., 2011).

The present clinical trial was designed to evaluate the effects of a combination of these five plants on serum lipids, hepatic enzymes, and severity of steatosis in the patients with NAFLD.

MATERIAL AND METHODS

Preparing the herbal compound

The *A. graveolens* seeds, *C. aurantium* flower, *C. scolymus* leaf, *P. oleracea* seeds, and *S. marianum* seeds were purchased from Herbal Medicine Division of Imam-Reza Pharmacy (Mashhad, Iran) and identified by Mohammad Sadegh Amiri (Department of Biology, Payame Noor University, Tehran, Iran). The plant materials were powdered separately and, then, mixed together equally to prepare 400 mg capsules (containing 80 mg of each plant material).

Standardizing the herbal compound

A sample of the herbal compound (20 g) was suspended in 70% ethanol (200 mL) and shaken for 48 h at 40 °C. The obtained extract was filtered through a metal mesh (106 µm pore size) and centrifuged for 5 min at 500 g. The supernatant solution was dried in an oven at 40 ºC. Then, the content of phenolic agents in the extract was determined by the Folin-Ciocalteu method (Hosseini *et al*., 2017). Briefly, 20 µL of the extract (10 mg/mL) was added to a test tube containing 1580 µL deionized water, 100 µL of Folin-Ciocalteu reagent, and 300 µL of sodium carbonate solution (1 mol/L). The absorbance of the complex solution was measured by spectrometer at 765 nm. The standard curve was created for gallic acid as standard at concentrations of 0, 50, 100, 150, 250, and 500 mg/L. The experiment was performed in triplicate.

Study design

This study was performed as a double-blind randomized controlled trial in accordance with

CONSORT guidelines. Ethics Committee of Mashhad University of Medical Sciences approved the study protocol (ethics committee reference number: IR.MUMS.fm.REC.1394.114). Also, the clinical procedures were performed according to principles of Declaration of Helsinki and its subsequent revisions. Written consent was obtained from all the participants after describing the aim of the study.

Participants

The participants were derived from Clinic of Internal Medicine, Imam-Reza Hospital, Mashhad University of Medical Sciences. Patients were screened for the inclusion and exclusion criteria with a detailed medical history, liver sonography, and serological markers of liver damage. The participants were included in the trial if they had grade ≥ 2 of NAFLD on sonography or had grade 1 of NAFLD + elevated alanine transaminase (ALT) level (> 40 U/L). They were excluded if they had any of the following criteria: alcohol intake within the previous 10 years; cancer; pregnancy; lactating; severe infection; renal insufficiency; and other forms of the liver disease such as cirrhosis, hepatitis B, and hepatitis C.

Interventions

Sixty patients with NAFLD were randomly divided into two groups (2:1 ratio): treatment (n = 40) and control (n = 20). Since diet and regular exercise are the basic approaches for managing the patients with NAFLD, both groups were advised to take healthy diet (vegetables, fruits, low fat, low carbohydrates) and exercise. The treatment group also received the herbal compound for two months (2 capsules, thrice daily).

Outcomes

The primary outcome measure included change in grade of NAFLD in sonography. Secondary outcomes were the levels of ALT, aspartate transaminase (AST), serum lipids, and fasting blood glucose (FBG).

Statistical analysis

Data were analyzed using IBM SPSS statistics 20 software. Comparisons of parametric values within groups and between groups were performed using paired-sample and independent-sample t-tests, respectively. Non-parametric values were compared by Wilcoxon signed-ranks test (within groups) and Mann-Whitney U-test (between groups). Categorical variables were compared by the Fisher's exact test. The results were shown as mean ± standard deviation (SD) and p-value of less than 0.05 was considered statistically significant.

RESULTS

Participant flow

Of the 60 randomized participants, 46 completed the study (16 control and 30 treatment) and were included in the analysis (Figure 1). Four patients in each control and treatment groups were excluded from the study because of failure to perform final paraclinical tests on time. Three patients in the treatment group were excluded from the study because of their unwillingness to continue the trial. Also, one participant in the treatment group was excluded because of taking another herbal drug during the study.

Baseline demographics and clinical variables are shown in Table I. No significant differences were found in these variables between the control and treatment groups.

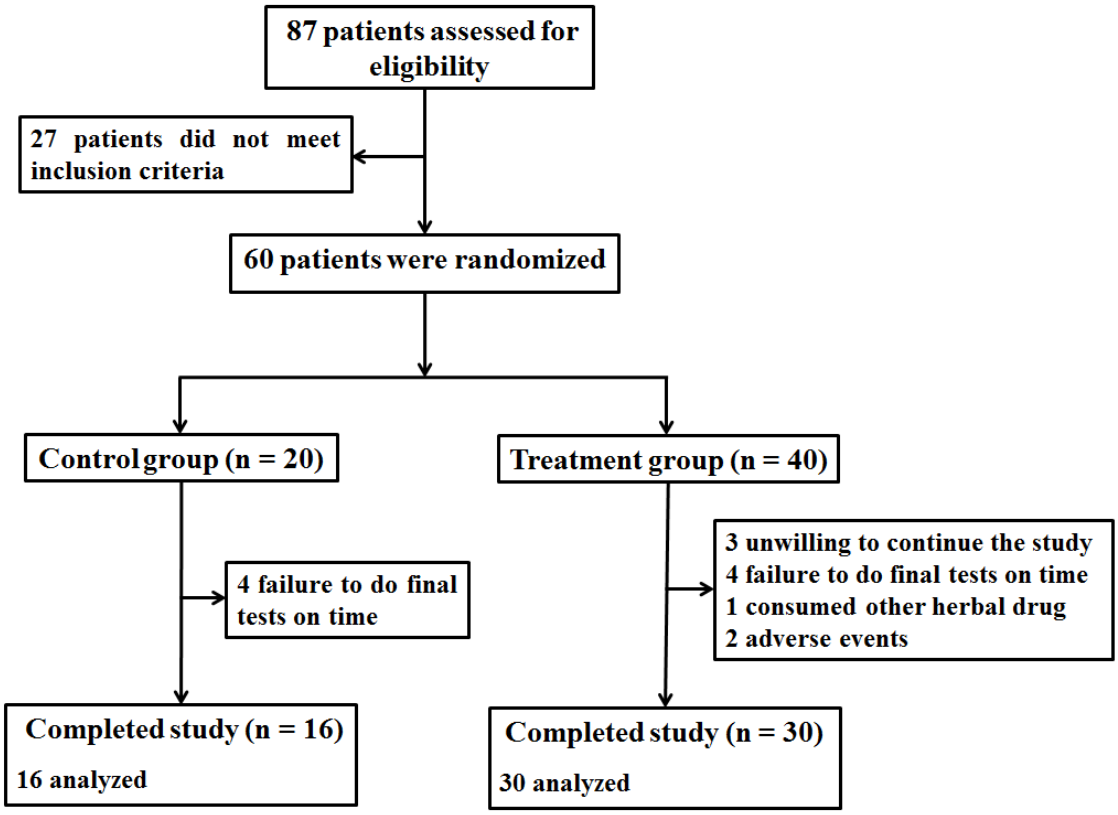

FIGURE 1 - Flowchart of the patients′ distribution, treatment, follow up, and analysis.

TABLE I - Patient demographics and clinical characteristics

Variable*	Control group (n = 16)	Treatment group (n = 30)
Age (Mean ± SD)	44 ± 8	49 ± 11
Sex (%)		
Female	38	50
Male	62	50
Race (%)		
White	100	100
Black	0	0
Others	0	0
Comorbidities (%)		
Diabetes mellitus	18.7	20
Hyperlipidemia	68.7	66.6
Thyroid disease	6.2	3.3
End-stage cardiovascular disease	0	0
Renal insufficiency	0	0
Malignancy	0	0

*We lost data of the body weight of most of the participants and, therefore, failed to report the body mass index.

Liver sonographic findings

Before starting the study, there was no significant difference in the severity of NAFLD between the two groups (Table II). Treatment with the herbal compound significantly improved the grade of the fatty liver ($p < 0.001$), but no significant change was found in the control group. At the end of the study, the severity of NAFLD in the treatment group was lower than that in the control group ($p < 0.01$).

TABLE II - The severity of steatosis in the patients with nonalcoholic fatty liver disease (NAFLD) before and after treatment with the herbal compound. *Within-group comparison (before vs after); #Between groups comparison

	NAFLD severity	Control group	Treatment group	p-value#
Before study	Grade 0	0%	0%	0.740
	Grade 1	31.2%	17.9%	
	Grade 2	62.5%	67.8%	
	Grade 3	6.3%	14.3%	
After study	Grade 0	0%	21.4%	<0.01
	Grade 1	26.7%	60.8	
	Grade 2	60%	14.3%	
	Grade 3	13.3%	3.5%	
p-value*		0.334	< 0.001	

Serum biochemical findings

Administration of the herbal compound was associated with significant reductions in the ALT and AST activities ($p < 0.05$), but no significant changes were found in the control group (Table III). In addition, a significant decrease in total cholesterol level was observed in the treatment group ($p < 0.05$), but not in the control patients. Although the level of FBG was decreased in the treatment group, the effect was not statistically significant. Also, no significant changes were observed in the levels of low-density lipoprotein, high-density lipoprotein, and triglyceride in either control or treatment groups.

TABLE III - The blood biochemical parameters of the patients before and after the study. Values are expressed as mean ± SD. ALT: Alanine transaminase; AST: Aspartate transaminase; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; FBG: Fasting blood glucose; *$p < 0.05$ compared to before study in the corresponding group; #$p < 0.05$ compared to control group after study

Parameters	Group	Before study	After study
ALT (U/L)	Control	61 ± 49	76 ± 59
	Treatment	42 ± 23	33 ± 17*#
AST (U/L)	Control	40 ± 25	47 ± 31
	Treatment	33 ± 14	28 ± 13*#

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TABLE III - The blood biochemical parameters of the patients before and after the study. Values are expressed as mean ± SD. ALT: Alanine transaminase; AST: Aspartate transaminase; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; FBG: Fasting blood glucose; *p < 0.05 compared to before study in the corresponding group; #p < 0.05 compared to control group after study

Parameters	Group	Before study	After study
Total cholesterol (mg/dL)	Control Treatment	205 ± 35 204 ± 50	208 ± 38 185 ± 41*
LDL (mg/dL)	Control Treatment	128 ± 25 112 ± 39	117 ± 38 104 ± 30
HDL (mg/dL)	Control Treatment	43 ± 9 46 ± 9	45 ± 15 43 ± 7
Triglyceride (mg/dL)	Control Treatment	188 ± 100 201 ± 123	200 ± 108 170 ± 76
FBG (mg/dL)	Control Treatment	122 ±39 134 ± 70	123 ± 49 116 ± 53

Side effects

No serious adverse events were observed during the study, except for one patient who reported allergic symptoms and one patient who suffered from dizziness following the consumption of the herbal compound.

Content of phenolic agents in the herbal compound

The solid residue of the hydroalcoholic extract of the herbal compound was 16%. The level of phenolic agents in the extract was 105 ± 12 mg gallic acid equivalent per gram of the extract.

DISCUSSION

NAFLD is a major health problem because no ideal therapeutic drug has yet been proven for it. If patients with NAFLD are not managed at early stages, they can progress to steatohepatitis, cirrhosis, and even hepatocellular carcinoma (Neuschwander-Tetri, 2017; Reeves, Zaki, Day, 2016). This disease has complex pathogenesis involving oxidative stress, inflammation, lipotoxicity, and insulin resistance (Buzzetti, Pinzani, Tsochatzis, 2016). Therefore, no single agent may be perfect to treat the fatty liver in all the patients. A combination of herbal agents can be more effective when each one acts through different mechanisms and shows a synergistic effect. Beneficial effects of several herbal agents and natural compounds have been shown on NAFLD (Baghernya *et al.*, 2017). In our current study, five plants with hypolipidemic and hepatoprotective effects were chosen for preparing a polyherbal compound to manage NAFLD. The results showed that the mixture of *A. graveolens*, *C. aurantium*, *C. scolymus, P. oleracea*, and *S. marianum* significantly improved the grade of fatty liver and decreased the serum levels of the liver enzymes and total cholesterol.

Consistent with our results, earlier clinical studies have shown that *A. graveolens*, *C. scolymus*, and *P. oleracea* have a hypolipidemic property and also can improve insulin sensitivity (Bundy *et al.*, 2008; El-Sayed, 2011; Gheflati, Adelnia, Nadjarzadeh, 2019; Mirhosseini, Baradaran, Rafieian-Kopaei, 2014; Mobasseri *et al.*, 2014; Moradi *et al.*, 2012; Nazni *et al.*, 2006). Several studies have reported that *S. marianum* has a hepatoprotective effect and is a viable medicinal plant for treating patients with NAFLD (Abenavoli *et al.*, 2011). In addition, all the five plants have antioxidant and anti-inflammatory effects and showed hepatoprotective activities in different animal models of the liver damage (Bahramikia, Yazdanparast,

2009; Ben Hsouna *et al*., 2018; Chen *et al*., 2012; Colak *et al*., 2016; Kazemi, 2015; Samarghandian, Borji, Farkhondeh, 2017; Speroni *et al*., 2003; Zhu *et al*., 2018).

The molecular pathways that contribute to the development of hepatic steatosis and its progression to steatohepatitis are multiple and complex. Briefly, accumulation of fatty acids in hepatocytes leads to the formation of lipotoxic species that induces endoplasmic reticulum stress, excessive generation of reactive oxygen species (ROS), and expression of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-18) (Friedman *et al*., 2018). These events alter function/structure of biomolecules and dysregulate signaling pathways, which eventually promotes cell death through apoptosis and/or necrosis processes (Friedman *et al*., 2018; Malaguarnera *et al*., 2009). For instance, inflammatory cytokines can activate c-Jun N-terminal kinases (JNK) and nuclear factor-kappa B (NF-κB) pathways, which in turn promotes the expression of these cytokines and worsens inflammation and insulin resistance (Malaguarnera *et al*., 2009; Zeng *et al*., 2014). Also, Toll-like receptor (TLR) family members such as TLR4 are activated in hepatitis and mediate steatosis, insulin resistance, and fibrosis in the liver (Vespasiani-Gentilucci *et al*., 2015; Zeng *et al*., 2014). Recent studies have suggested that mitochondrial dysfunction plays a significant role in NAFLD (Tarantino, Citro, Capone, 2020). Increase of mitochondrial fatty acid oxidation and stimulation of tricarboxylic acid cycle enhance delivery of reducing equivalents to the electron transport chain. Over-reduction of the respiratory complexes excites the generation of superoxide, which is the precursor of most of the other ROS. Oxidation of biomolecules by ROS may induce mitochondrial damage through disrupting electron transport chain, permeabilizing outer mitochondrial membrane, altering $\Delta\psi_m$, and degrading mitochondrial structural integrity. Under normal conditions, hepatocytes counteract the initiation of a vicious cycle of mitochondrial dysfunction through their antioxidant system. However, in NAFLD, parallel to the enhanced ROS generation, the reduced activity of this system (e.g., catalase, glutathione, and superoxide dismutase) promotes hepatocellular oxidative damage (Caldwell *et al*., 2004; García-Ruiz, Fernández-Checa, 2018).

The exact mechanism of hepatoprotective action of the polyherbal compound is not easy to elucidate, since inhibiting oxidative stress, attenuating inflammation, preventing lipotoxicity, and improving insulin resistance all can contribute. It has been shown that *A. graveolens* extract significantly increases hepatic catalase, glutathione, and superoxide dismutase along with decreased lipid peroxidation in the liver of high-fat diet treated rats (Bahramikia, Yazdanparast, 2009). Silybin, a major active compound of *S. marianum*, was shown to alleviate hepatic steatosis and fibrosis through suppressing the activity of NF-κB and inhibiting apoptosis (Ou *et al*., 2018). In the rat model of CCl4-induced liver injury, *P. Oleracea* and *C. scolymus* could protect hepatocytes against DNA fragmentation and apoptosis (Colak *et al*., 2016; Shi *et al*., 2014). In addition, limonin, a natural agent isolated from the seed of *C. aurantium*, could attenuate hepatic inflammation and oxidative stress via downregulating TLR-4 expression in the rats with D-galactosamine-induced liver injury (Mahmoud *et al*., 2014).

A limitation of our study was the relatively short duration of administering the herbal compound. Therefore, the long-term safety and efficacy of this compound should be further investigated. Another limitation is the lack of placebo, proposing that the beneficial effects may not absolutely result from the tested compound. Considering that numerous studies have shown hypolipidemic and hepatoprotective effects for herbal agents, particularly for the five herbs used in the present work, improving NAFLD can be attributed to the herbal compound. It should be considered that although combination therapy with herbal agents might be more effective when each one acts through different mechanisms, it can also lead to possible drug-drug interaction. A further limitation is that the sample size was not large enough to allow reliable subgroup analysis, for example based on the grade of fatty liver, sex, etc. Yet, a post-hoc power analysis was done on the primary outcome variable to ensure that our study was sufficiently powered. The result showed that sample sizes of 16 and 30 achieved 99% power to detect such a difference was observed in the grade of the fatty liver between our study groups with the significance level of 0.05 and using two-sided Mann-Whitney test.

In conclusion, the results indicated that the formulated herbal compound appeared to be effective in biochemical improvement and decreasing the grade of the fatty liver in the patients with NAFLD. Therefore, this compound may be a candidate for treating or preventing NAFLD progression.

ACKNOWLEDGMENTS

The authors wish to acknowledge Vice-chancellor for Research and Technology of Mashhad University of Medical Sciences for financially supporting this study (grant number: 931243). The authors declare that they have no conflict of interest.

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Received for publication on 08th September 2019
Accepted for publication on 21st September 2020