RESEARCH PAPER

Swertiamarin Ameliorates HFD+DMN-Induced Hepatic Steatosis and Fibrosis Markers via Downregulation of ASK1/MAPK Signaling Pathway

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Abstract
Background: Nonalcoholic fatty liver disease (NAFLD) and liver fibrosis are major health concerns globally, especially in patients with type 2 diabetes mellitus (T2DM). Dysregulated insulin signaling and chronic inflammation are central to disease progression.
Objective: This study evaluates the hepatoprotective and anti-fibrotic efficacy of Swertiamarin, a secoiridoid glycoside from *Enicostemma littorale*, in a rat model of diet- and toxin-induced liver injury.
Methods: Hepatic injury and fibrosis were induced in Wistar rats using a high-fat diet (HFD) in combination with dimethylnitrosamine (DMN). The protective effects of Swertiamarin (100 mg/kg) were compared with the ASK1 inhibitor Selonsertib (50 mg/kg). Key biochemical, histological, and molecular endpoints were evaluated.
Results: Swertiamarin (100 mg/kg) significantly attenuated hepatic steatosis and fibrosis, reduced insulin resistance, and lowered pro-inflammatory cytokine levels. Mechanistically, Swertiamarin downregulated the ASK1/MAPK signaling pathway and suppressed the expression of α-SMA and collagen I.
Conclusion: Swertiamarin demonstrates promising anti-fibrotic and anti-inflammatory properties via ASK1/MAPK inhibition, offering a natural alternative to current pharmacological approaches for NAFLD-associated fibrosis.

Keywords: NAFLD, liver fibrosis, Type 2 diabetes mellitus (T2DM), ASK1, Swertiamarin
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1. Introduction

Liver fibrosis is a prevalent form of chronic liver disease mostly caused by nonalcoholic steatohepatitis (NASH). NAFLD can lead to hepatic inflammation and advance to NASH, fibrosis, cirrhosis, or hepatocellular carcinoma (HCC) [1]. Approximately 55% of individuals worldwide diagnosed with T2DM are also affected by NAFLD [2]. In addition, almost 37% of individuals with T2DM already exhibit advanced NASH, characterized by the presence of steatosis with hepatocyte ballooning degeneration and possibly fibrosis [3]. There is a strong interconnection between the disorders, and when diagnoses are made in both directions, as expected, there are similar associations. Over 50% of individuals diagnosed with NASH also receive a diagnosis of T2DM. In addition, individuals who do not have diabetes may have either prediabetes or insulin resistance syndrome [4, 5]. Furthermore, it has been documented that a significant proportion of individuals with T2DM also suffer from NAFLD or NASH, which may potentially exacerbate their risk of developing cardiovascular and metabolic disorders [6].

The development of NAFLD and liver fibrosis is intricate, and the molecular mechanisms behind this process are starting to be understood. Over the past decade, many clinical studies have examined the safety and efficacy of NAFLD/NASH medications. These medications include diet tablets, antioxidants, insulin sensitizers, lipid-lowering agents, anti-inflammatory cytokines, cytoprotectants, and intestinal probiotics [6]. Nevertheless, the US Food and Drug Administration (FDA) has not yet granted approval for pharmacotherapy in the treatment of NAFLD. Insulin resistance is a significant factor that causes the liver to accumulate excessive fat and is crucial in the development and advancement of steatohepatitis and fibrosis [7]. The presence of elevated oxidative stress and impaired antioxidant defense mechanisms has been widely investigated throughout the various phases of NAFLD. These factors are thought to play a role in liver damage and the progression to NASH [8].

This study used a well-established experimental approach to induce NAFLD/NASH in Wistar rats. The approach entailed delivering an HFD to induce steatosis. We also used dimethylnitrosamine (DMN), a chemical known to cause liver cancer and increase hepatic fibrosis [9, 10], in conjunction with the HFD. ASK1 has recently been recognised as a key element in the development of a number of diseases, including liver disorders. This has sparked greater interest in targeting ASK1 for therapeutic purposes. The extended MAPK kinase (MAP3 K) group activates downstream MAPKs such JNKs and p38 MAPKs via ASK1. It controls stress responses like apoptosis, cell death, differentiation, and inflammatory cytokines [11]. ASK1 activates JNK to worsen acetaminophen-induced liver damage. In the NASH model, ASK1 causes liver fibrosis and cell death. Furthermore, overexpressing ASK1 promotes the

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activation and proliferation of hematopoietic stem cells (HSCs) by increasing p38 and JNK phosphorylation [12]. Given ASK1's proven function in the progression of liver fibrosis, addressing it through pharmaceutical development is critical for effectively treating advanced liver diseases [13]. Selonsertib (Selon), a new small molecule inhibitor of ASK1, has antifibrotic and anti-inflammatory properties. It is currently undergoing clinical trials for the treatment of NAFLD/NASH and liver fibrosis [11].

The most efficacious pharmacotherapies for NAFLD/NASH and liver fibrosis not only prioritise enhancing lipid metabolism, but also emphasise the augmentation of hepatic anti-oxidative and anti-inflammatory actions. Currently, the majority of serious illnesses such as cancer, AIDS, kidney damage, cardiovascular diseases, and others can be effectively treated with medicinal herbs [14]. *Enicostemma littorale* is a species that is particularly noteworthy for its significant contribution to human healthcare. The plant exhibits antioxidant, hypoglycemic, hepatomodulatory, and hepatoprotective characteristics, and aids in the reduction of obesity [15, 16]. The plant contains several antioxidative phytochemicals, such as alkaloids, glycosides, saponins, sterols, catechins, xanthones, phenolic acid, flavonoids, and triterpinoids [17]. Swertiamarin, a secoiridoid glycoside, is a key component found in the crude extract of this medicinal plant. Swertiamarin has diverse pharmacological properties, with a particular focus on its antioxidant, anti-inflammatory, anti-diabetic, and antihyperlipidaemic effects, as well as its ability to protect against hepatic injury [18]. Although the potential of Swertiamarin to improve liver fibrosis induced by DMN has been discussed, no conclusive data has been provided. Thus, this work aimed to investigate the potential of Swertiamarin in reducing liver fibrosis generated by DMN. The focus was on examining how Swertiamarin inhibits the ASK1/MAPK receptor-mediated apoptotic pathways and understanding the underlying processes in vivo.

2. Materials and methods

2.1. Chemicals

Swertiamarin (Purity ≥ 98%, Pub Chem CID: 442435) was extracted and preserved from complete dried *Enicostemma littorale* plant. Selonsertib (Selon) was purchased from Sigma Aldrich, USA. Triglycerides quantification colorimetric assay kit (MAK266-1KT, Sigma-Aldrich, USA) and Shanghai Yuanye Bio-Technology Co., Ltd. (R21819-100T and R21814-100T) were also used.

2.2. Isolation of Swertiamarin from *Enicostemma littorale*

The entire botanical specimen of *Enicostemma littorale* was gathered from the state of Tamil Nadu in India. The plant was identified by examining its morphological and microscopical features, as specified in authoritative literature and floras published by the NISCAIR department in New Delhi, India. The process of extracting and analysing Swertiamarin was carried out using a method that has been previously described in a published study [19, 20]. Swertiamarin was isolated using column chromatography. After being separated, the resultant solid was passed through a filter and then washed with ethyl acetate. Afterwards, Swertiamarin was separated and purified using a filter column with ethyl acetate as the mobile phase. The purity of isolated Swertiamarin was verified through the use of Mass and NMR spectroscopy methods.

2.3. Animal study

2.3.1. Animals

The experiment was conducted with the previous approval of the Institutional Animal Ethical Committee (IAEC) of Delhi Pharmaceutical Sciences and Research University (DPSRU), situated in New Delhi, India (DPSRU/IAEC/2019/III-R02). A total of thirty-two male Sprague-Dawley rats, aged six weeks and weighing between 150 and 200 grams, were acquired from the animal house facility at the university. The rats were housed under standard laboratory conditions.

2.3.2. Experimental Protocol

The investigation lasted 12 weeks. With 0.5% methylcellulose (MC), Selonsertib (Selon) and Swertiamarin were taken orally. In 2010, Jaishree et al. [21] found that 100 and 200 mg/kg Swertiamarin protected rats against galactosamine-induced liver damage. This information and our first findings led us to choose 100 mg/kg of Swertiamarin for the experiment.

8 rats were given equal quantities of saline and 0.5% MC in the normal control group [14]. Twenty-four rats were fed a HFD and DMN for 12 weeks to induce hepatosteatosis and fibrosis. DMN was injected intraperitoneally six times, once every two weeks. The first and second DMN injections were 15 mg/kg, the third and fourth 10 mg/kg, and the fifth and sixth 5 mg/kg [9]. After eight weeks, HFD and DMN-induced rats (n = 24) were randomly divided into three l groups. This comprised the HFD+ DMN, Selonsertib 50 mg/kg [11], and Swertiamarin 100 mg/kg groups. In the ninth to twelfth week, animals in the Selonsertib and Swertiamarin groups received oral medication once daily [14].

At the conclusion of week 12, the animals in each group were euthanized after being fed the experimental diets. The liver and adipose tissue around the epididymis were promptly removed, weighed, and sliced into sections measuring 3 to 4 mm in thickness, Stored in 10% formalin. Excess liver tissue was frozen and kept at a temperature of -80 °C until it was ready to be processed for biochemical estimation [9].

2.3.3. Biochemical analysis

Blood was obtained by puncturing the abdominal aorta during the sacrifice. Triglycerides (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were quantified using established protocols on an automated analyzer with a commercially available laboratory kit. The Ultra-Sensitive Rat Insulin ELISA Kit (Morinaga) was utilised to analyse insulin. The HOMA-IR scores were computed to test insulin resistance using the formula [9, 22].

2.3.4. Cytokine assay

Recently obtained sera were rapidly extracted from blood samples taken from sacrificed rats. The levels of IFN-γ and TNF-α cytokines were evaluated using the Sandwich ELISA method. The required capture and detection antibodies (R&D system, Minneapolis, MN, USA) were utilised alongside a visualisation system consisting of streptavidin-conjugated HRP and TMB microwell peroxidase substrate (Vector Laboratories, CA, USA)[11].

2.3.5. Western blotting

Following DPBS washing, the liver tissue of each rat was lysed using RIPA buffer. Then, the solution was supplemented with 2 mM Xpert protease and phosphatase inhibitor, and centrifuged for 30 minutes at 15,000 rpm. Using SDS-PAGE, we separated 30 µg of protein lysate, and then we used a wet transfer kit to transfer it to PVDF membrane. Primary antibodies diluted in PBS-T with 5% BSA were then incubated overnight at 4°C after the membrane was blocked with 5% skim milk in PBS and 0.5% Tween 20 (PBS-T). Santa Cruz Biotechnology, Genetex, Sigma-Aldrich, and Cell Signalling Technology were the suppliers of anti-JNK (9252S) and anti-ASK1 (107921). Additionally, we estimated the expression of other markers using standards of anti-SMA (F3777), anti-β-Actin (A5441), and anti-collagen I (ab34710). The blots were visualised using Cell Signalling Technology secondary antibodies and an improved chemiluminescence system from Bio-Rad Laboratories [11].

2.3.6. Histopathological analysis

The liver sections were prepared for paraffin embedding after being stored in a 10% formaldehyde solution and rehydrated to water through a graded series of alcohol in the initial. The sections with a thickness of around 4 µm are then stained with hematoxylin which binds to the acidic components of the cell such as nuclei and imparts a blue-purple color. After rinsing the sections are treated with eosin which stains the cytoplasmic components and the extracellular matrix in pink shades. The stained sections are then dehydrated through graded alcohols, cleared in xylene and mounted with coverslip. Under the microscope fat vacuoles appear as clear spaces within hepatocytes due to the dissolution of fat during tissue processing providing a clear indication of steatosis. Image analysis was used to determine the severity of fibrosis [11].

2.4. Statistical Analysis

Differences in quantitative data, expressed as mean ± standard deviation (SD), between groups were compared by one-way ANOVA followed by Dunnett's comparison tests using Graph Pad Prism 8. P value < 0.05 was considered significant.

3. Results

Swertiamarin was extracted from *Enicostemma littorale* employing a well-documented isolation procedure. The resultant compound underwent characterization through Mass Spectrometry and Nuclear Magnetic Resonance (NMR) techniques, and its purity was duly validated, as illustrated in Supplementary Fig. 1 and 2. (SI Fig. 1 & 2).

3.1. Effect of Swertiamarin on metabolic alterations in HFD+DMN-induced steatohepatitis rats

DMN injections and HFD consumption were examined at 12 weeks. The HFD+DMN-treated group had larger body and liver weights than the control group. Rats receiving Swertiamarin (100 mg/kg) showed significantly lower body weight and liver weight ($P \leq 0.05$) compared to the HFD+DMN group (Fig. 1).

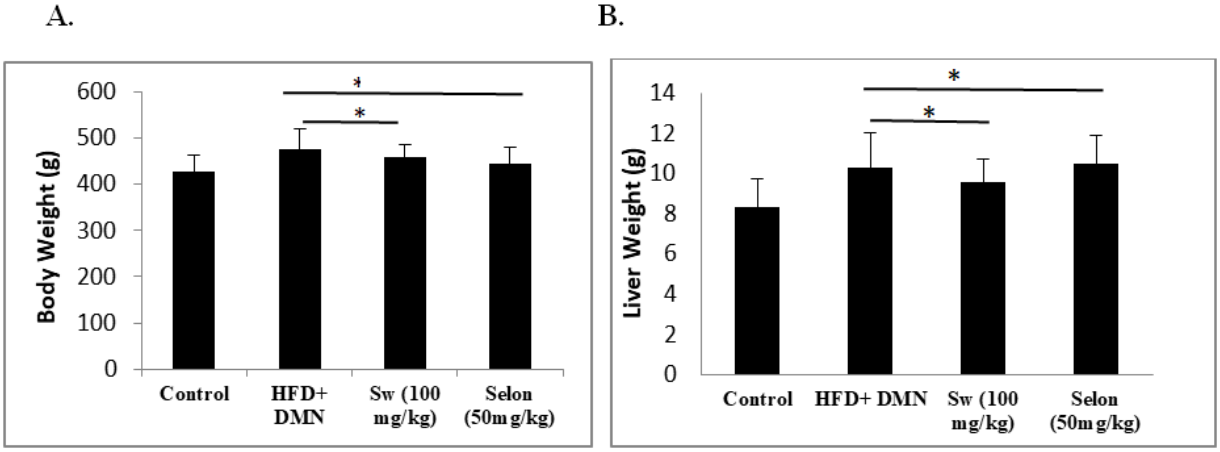

Fig. 1. Effects of Swertiamarin on HFD+DMN -induced body and liver weight changes
A). The body weight gain (g) was measured daily. B). Relative liver weight (g/100 g of body weight) was examined after sacrifice. All data are presented as the mean ± SD. (n=8). *p<0.05 versus HFD+DMN group.

After the experiment, the HFD+DMN group had significantly higher serum TG, AST, and ALT levels than the normal control group (Table 1). We measured serum ALT and AST levels to indicate liver damage. Transaminases in the serum rise when the liver is injured due to hepatocyte cell membrane permeability. Compared to rats in the HFD+DMN and standard medication Selon (50 mg/kg) treatment groups, Swertiamarin (100 mg/kg) significantly decreased these parameters (Table 1). These findings indicate that Swertiamarin decreases HFD+DMN-induced liver hepatotoxicity in treatment group.

Table 1. Effects of Swertiamarin on serum levels of AST & ALT in HFD+DMN-treated rats

Group	TG mmol/L	AST (U/L)	ALT (U/L)
Control	426.5 ± 35.4	71.7 ± 15.1	33.3 ± 4.0
HFD+DMN	465.4 ± 44.1**	102.5 ± 25.5**	64.4 ± 15.0***
Swertiamarin (100mg/kg)	452.6 ± 27.5*	74.9 ± 13.8	35.5 ± 6.2
Selon (50 mg/kg)	458.6 ± 34.9**	78.9 ± 13.7	52.0 ± 9.3***

Control, HFD high-fat diet+DMN, Swertiamarin 100 mg/kg, Selon 50 mg/kg. One-way ANOVA followed by Dunnett's comparison tests*P < 0.05, **P < 0.01, ***P < 0.001 vs control.

We examined how Swertiamarin affected insulin sensitivity in HFD+DMN-induced NASH rats. In rats, HFD+DMN feeding significantly increased blood sugar and insulin levels (Table 1; P < 0.05 and 0.001, respectively), leading to a higher HOMA-IR score.

Table 2. Effects of Swertiamarin on insulin resistance in HFD+DMN-treated rats

Group	Blood Sugar (mg/dl)	Insulin (µU/ml)	HOMA-IR
Control	137.0 ± 16.5	15.6 ± 4.6	5.4 ± 2.1
HFD+DMN	162.3 ± 24.8*	44.3 ± 25.8***	18.7 ± 13.0**
Swertiamarin (100mg/kg)	147.3 ± 15.6*	18.4 ± 3.6**	7.3 ± 2.4**
Selonsertib (50 mg/kg)	152.0 ± 21.4*	22.3 ± 9.6**	8.6 ± 4.3***

Control, HFD high-fat diet+DMN, Swertiamarin 100 mg/kg, Selonsertib 50 mg/kg, HOMA-IR homeostasis model assessment-insulin resistance, One-way ANOVA followed by Dunnett's comparison tests*P < 0.05, **P < 0.01, ***P < 0.001 vs control.

3.2. Swertiamarin inhibits fibrotic response by ASK1/MAPK signaling in HFD+DMN- induced liver fibrosis model

ASK1 activates the MAPK signalling cascade, including p38 and JNK, to cause liver disease. Through collagen and other ECM buildup, p38 and JNK drive hepatic fibrosis. Thus, we tested whether Swertiamarin would decrease fibrosis-related protein expression via MAPK signalling in treatment group. As seen in Fig. 2, Swertiamarin reduced ASK1 phosphorylation and downstream p38/JNK and MAPK signalling compared to control. Swertiamarin was tested for its ability to reduce profibrotic mediators such α-SMA and collagen by suppressing ASK1 phosphorylation in an HFD+DMN-induced liver fibrosis model (Fig. 2). The HFD+DMN group exhibited elevated α-SMA and collagen levels. Swertiamarin treatment groups had less fibrosis-related indicators. HFD+DMN induction increased ASK1, p38, and JNK expression in control group, but Swertiamarin (100 mg/kg) decreased it. Our findings showed that Swertiamarin is effective against HFD+DMN-induced liver fibrosis by indicating a significant reduction in protein expressions of fibrotic markers as comparison to disease control (DC) and standard group (Selon 50 mg/kg).

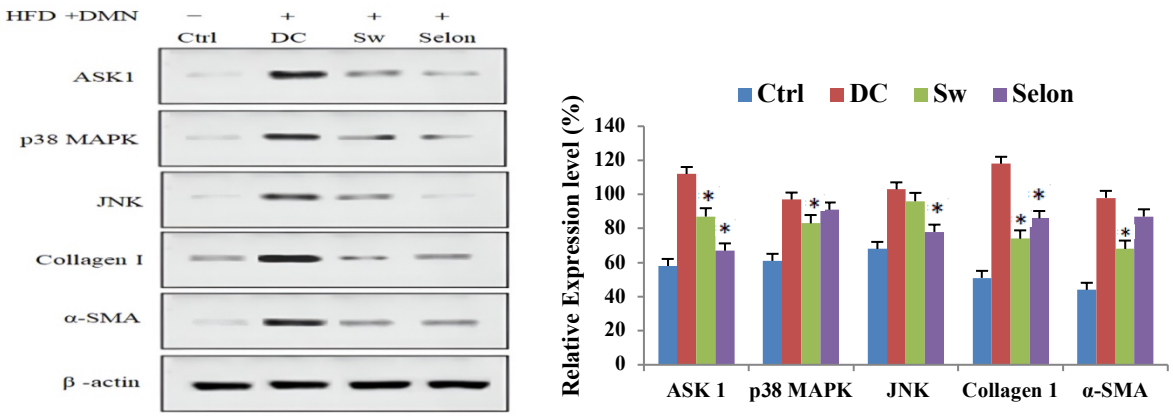

Fig. 2. Effect of Swertiamarin (100 mg/kg) on ASK1/MAPK signaling and expression of other fibrogenic proteins such as; β-actin, α-SMA, collagen 1 and JNK. Data represented as the mean ± SD (n=4). *p<0.05 versus the Disease control (DC).

3.3. Effect of Swertiamarin on cytokine parameters in HFD+DMN-induced steatohepatitis rats

IFN-γ and TNF-α are crucial pro-inflammatory cytokines that play a significant role in the advancement of liver damage. The levels of IFN-γ (Fig. 3A) and TNF-α (Fig. 3B) in the HFD+DMN group showed a substantial rise ($P \leq 0.05$) at the conclusion of the trial. On the other hand, the administration of Swertiamarin (100 mg/kg) resulted in a significant decrease in the levels of IFN-γ and TNF-α compared to the group treated with HFD+DMN. After a duration of 12 weeks, the group of rats treated with HFD+DMN exhibited significant liver injuries in comparison to the normal control group. In certain sections of the liver, the hepatocytes displayed distinct and widespread ballooning degeneration, along with a considerable number of cells with a condensed appearance of the nucleus. These observations were made using a light microscope (Fig. 4).

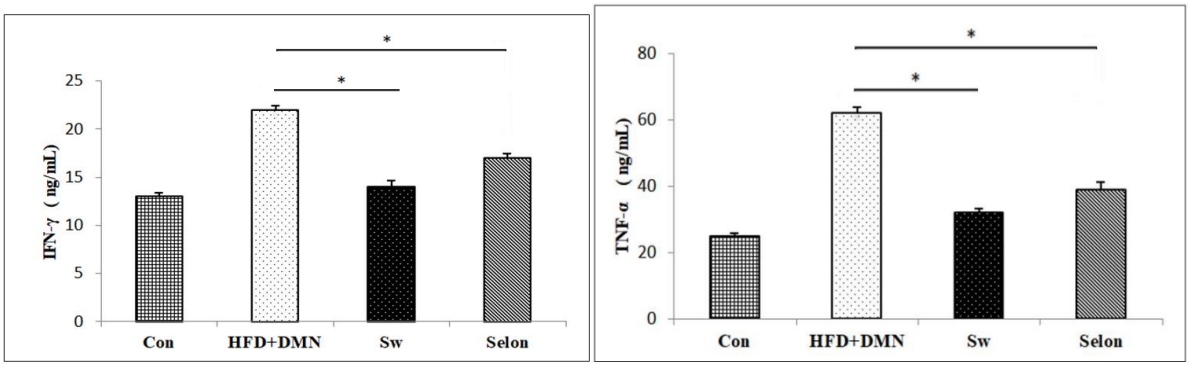

Fig. 3. Effect of Swertiamarin (100mg/kg) on IFN-γ and TNF-α level. Con, HFD+DMN, Swertiamarin (Sw) 100mg/kg, Selonsertib (Selon) 50 mg/kg, (n = 8). Data were analyzed by using One-way ANOVA followed by Dunnett's comparison test. The results are expressed as the mean ± standard deviation (SD), with statistical significance indicated by * ($P < 0.05$), with respect to control group.

3.4. Swertiamarin improves HFD+DMN-induced liver fibrosis in rats

Table 1 & 2 shows how Swertiamarin exhibits promise as a major therapeutic agent for enhancing NAFLD and liver fibrosis, providing a more efficacious alternative to current conventional treatments. Animals body and liver weight increased significantly with HFD-DMN treatment compared to control group. The liver fibrosis caused by HFD+DMN in rats was also examined using H&E staining. The control group had normal liver architecture, but the HFD+DMN group had significant hemorrhagic necrosis, tissue rupture, and inflammatory cell infiltration. These changes were greatly reduced in the Swertiamarin group. Histopathological results showed that Swertiamarin has significantly reduced liver fibrosis, and the results were found to be comparable to the standard drug Selonsertib (50 mg/kg).

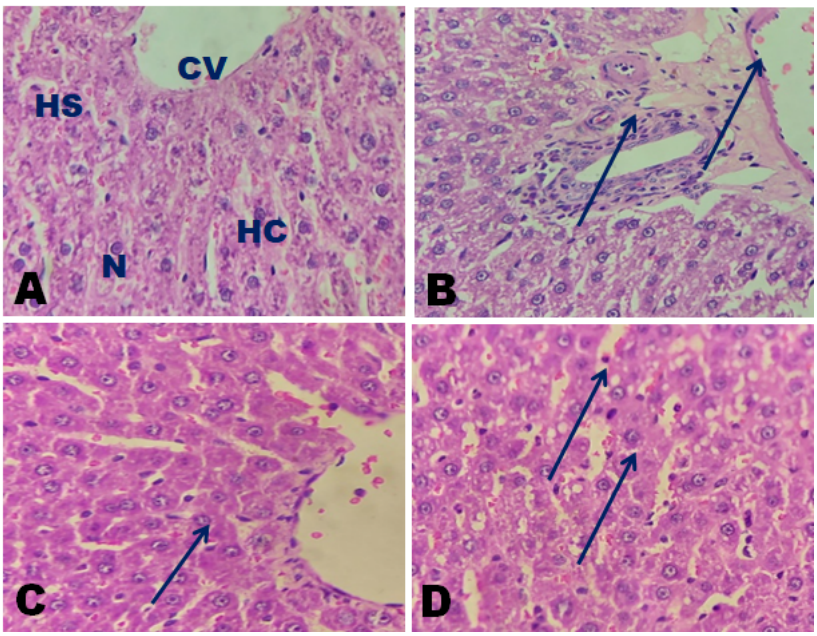

Fig. 4. Histopathological analysis of Liver tissues of Control, Disease control (HFD+DMN), Swertiamarin and Selon treated rats; A) Control, Group1 treated with vehicle showing normal architecture of hepatic lobule. The central vein (CV) lies at the centre of the lobule surrounded by the hepatocytes (HC) with strongly eosinophilic granulated cytoplasm, and distinct nuclei (N). between the strands of hepatocytes the hepatic sinusoids are shown (HS), B) Group 2, Disease Control (HFD+DMN), rats treated with DMN showing interspersed many dilated vessels, mid and peripheral cloudy degeneration, and granular precipitation in the cytoplasm. C) Group 3, a section of liver of DMN intoxicated rat and co-administrated with Swertiamarin (100 mg/kg) shows lobule architecture that appear like normal. Notice the few vacuoles (arrowhead), D) Group 4, a section of liver of HFD+DMN intoxicated rat and co-administrated with Selonsertib (50 mg/kg) shows few focal necrosis, congested and dilated blood sinusoids (arrowhead)(H & E stain X 300)

4. Discussion

Liver fibrosis is a frequent outcome of NAFLD/NASH, resulting in cirrhosis and hepatocellular cancer. The activation of hepatic stellate cells (HSCs) is crucial in this process, since it is marked by their fibrogenic potential and high rate of proliferation [11, 23] . Research has demonstrated that the ASK1 pathway has a role in facilitating and advancing the development of chronic liver fibrosis caused by NASH. Previous studies have revealed the significance of signalling in the progression of hepatic fibrogenesis by activating hepatic stellate cells (HSCs) and promoting the release of pro-fibrogenic mediators. Consequently, researchers have started studying the suppression of ASK1 signalling in HSCs as a promising approach to cure liver fibrosis [12]. Our work focused on assessing the anti-fibrosis properties of Swertiamarin, a secoiridoid glycoside, and investigating the mechanisms by which it acts in liver fibrosis [18]. Furthermore, the obtained data showed that Swertiamarin suppressed the ASK1/MAPK signalling pathway and fibrogenesis via reducing extracellular matrix (ECM)-related molecules in liver fibrosis models produced by a HFD and DMN. Our research indicates that Swertiamarin is a powerful substance that can prevent the formation of fibrous tissue and inhibit the activation of HSCs by blocking the ASK/MAPK signalling pathway in liver fibrosis. The study investigated the effects of Swertiamarin on metabolic changes in rats with HFD+DMN -induced steatohepatitis. The HFD+DMN group showed a remarkable increase in body and liver weight of compared the control group. The Swertiamarin-treated group (100 mg/kg) resulted in a notable reduction in body and liver weight relative to the HFD+DMN group (Fig. 1). The analysis of serum biochemical parameters revealed significant differences among the groups. The HFD+DMN group showed elevated serum triglycerides (TG) levels, AST, and ALT compared to the control (Table 1). Swertiamarin treatment significantly reduced these parameters, indicating its protective effect against liver injury. Further, insulin sensitivity was examined by measurement of blood sugar and insulin levels and the homeostasis model assessment-insulin resistance score. The HFD+DMN group exhibited increased blood sugar and insulin levels, resulting in a higher HOMA-IR score, indicating insulin resistance. Swertiamarin treatment markedly decreased blood sugar, insulin levels, and the HOMA-IR score compared with that in the HFD+DMN group, showing its effect on improving insulin sensitivity. The standard medication, Selonsertib, also showed improvements in these parameters, but Swertiamarin exhibited a more pronounced effect (Table 2).

Multiple studies have documented that activated HSCs enhance the production of ECM-related proteins [3, 12]. Consequently, the ECM plays a role in controlling cell proliferation and apoptosis, ultimately leading to the progression of liver fibrosis. α-SMA, fibronectin, and collagen type I are recognised as pro-fibrotic markers that are crucial in the production of collagen and the deposition of ECM [11]. Furthermore, recent research has indicated that mice with a genetic modification that eliminates the ASK1 gene are shielded against abnormal changes in organ structure and the formation of fibrous tissue by suppressing chemicals associated with the ECM. Hence, we investigated the potential of Swertiamarin to suppress the expression of pro-fibrotic factors, specifically α-SMA and collagen I (Fig. 2). Swertiamarin decreased the expression of these mediators in an animal model of liver fibrosis. Consistent with prior research, the inhibition of ASK1 resulted in decreased expression of fibrotic-related genes, including α-SMA, in pulmonary fibrosis [11]. The pro-inflammatory cytokines IFN-γ and TNF-α crucially drive the process of progression of liver damage. There was a significant rise in these cytokine levels in the HFD+DMN group, indicating intense inflammation. Swertiamarin administration markedly and significantly lowered the levels of these cytokines, again indicative of its anti-inflammatory action (Fig. 3). This suppression of the cytokine levels was proportionately associated with decreased liver injuries, as seen through histopathologic examination, and Swertiamarin-treated rats displayed profound protection from hepatic impairments against HFD+DMN-induced liver damage.

Our data showed that Swertiamarin suppressed the ASK1/ MAPK signalling pathway by reducing the levels of p-p38 and p-JNK in a liver fibrosis model generated by HFD+DMN. Considering the fact that phosphorylated p38 and JNK have been shown to enhance collagen expression [23], it appears that Swertiamarin effectively reduced liver fibrosis by suppressing ECM formation through the suppression of the ASK1/MAPK signalling pathway. Our findings suggest that Swertiamarin's ability to reduce fibrosis is achieved through the inhibition of ASK1/MAPK signalling. Moreover, Swertiamarin is anticipated to provide anti-fibrotic and anti-inflammatory benefits in several fibrotic disorders. Histopathological examination of liver tissues provided further insights into the protective effects of Swertiamarin. The control group

showed normal liver architecture, while the HFD+DMN group exhibited severe liver damage characterized by hemorrhagic necrosis, tissue rupture, and inflammatory cell infiltration. Swertiamarin treatment significantly mitigated these pathological changes, resulting in a liver architecture comparable to the control group. The histopathological findings corroborate the biochemical and molecular data, reinforcing the potential of Swertiamarin as a therapeutic agent for liver fibrosis and steatohepatitis (Fig.4).

To summarize, the present investigation showed that Swertiamarin (100 mg/kg) effectively reduced the progression of liver fibrosis compared to the standard treatment Selonsertib (50 mg/kg) by inhibiting the ASK1/MAPK signalling pathway in living organisms. Furthermore, this restraint is crucial for triggering HSC death, resulting in the removal of collagen-producing cells in liver fibrosis. Hence, we propose that administering Swertiamarin could be an effective therapeutic strategy for treating chronic hepatic steatosis and fibrosis.

5. Conclusions

In conclusion, the present study demonstrates that Swertiamarin (100 mg/kg) provides protective effects against HFD+DMN-induced hepatic steatosis, inflammation, and fibrosis in rats. Swertiamarin treatment reduced the alterations in body and liver weight, serum triglycerides, AST, and ALT levels and improved glucose homeostasis and insulin resistance, as reflected by reductions in blood glucose, insulin, and HOMA-IR. Swertiamarin also reduced the levels of the pro-inflammatory cytokines IFN-γ and TNF-α and alleviated the histopathological changes associated with HFD+DMN-induced hepatic injury. At the molecular level, Swertiamarin treatment reduced the expression of ASK1/MAPK-related signaling proteins and the fibrosis-associated markers α-SMA and collagen I. These findings suggest that modulation of the ASK1/MAPK signaling pathway may contribute to the anti-fibrotic effects of Swertiamarin. Taken together, the biochemical, molecular, and histopathological findings support the hepatoprotective and anti-fibrotic potential of Swertiamarin in this experimental model. The effects of Swertiamarin were generally comparable to those observed with Selonsertib, although a direct comparison of their efficacy requires appropriate statistical evaluation.

Overall, these findings indicate that Swertiamarin may be a promising natural lead for further investigation in metabolic liver diseases associated with hepatic steatosis and fibrosis. Further studies, including dose-response studies and detailed mechanistic investigations, are needed to confirm its therapeutic potential and to further clarify the role of ASK1/MAPK signaling in its protective effects.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest in this publication.

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† Supplementary data: The supporting information includes spectral data details (NMR and Mass) of Swertiamarin.

Abbreviations:

T2DM	Type 2 diabetes mellitus
ASK-1	Apoptosis signal regulating kinase 1
Swertiamarin	Swertiamarinertiamarin
MC	Methyl cellulose
SEL	Selonsertib
ROS	Reactive oxygen species
α-SMA	Alpha smooth muscle actin
JNK1	c-Jun N-terminal kinase 1
MAPK	Mitogen-activated protein kinase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
HCC	Hepatocellular carcinoma
HFD	High fat diet
DMN	Dimethylnitrosamine
HOMA-IR	Homeostatic model assessment of insulin resistance

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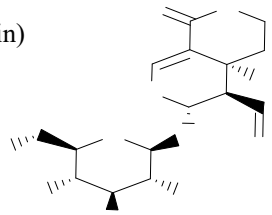
1. Characterization of isolated Swertiamarinertiamarin
Spectral data:
LCMS Data of Swertiamarin (Swertiamarinertiamarin)
1H NMR Data of Swertiamarin (Swertiamarinertiamarin)



Molecular formula: C16H22O10
Molecular weight: 374.34

Spectroscopic methods for mass were used to characterize Swertiamarinertiamarin. During purification, it was discovered that the yield for Swertiamarinertiamarin was 6.5%, respectively. A molecular ion peak at 374 nm in the mass spectra confirmed the isolated Swertiamarinertiamarin's molecular weight (MW. 374.34).

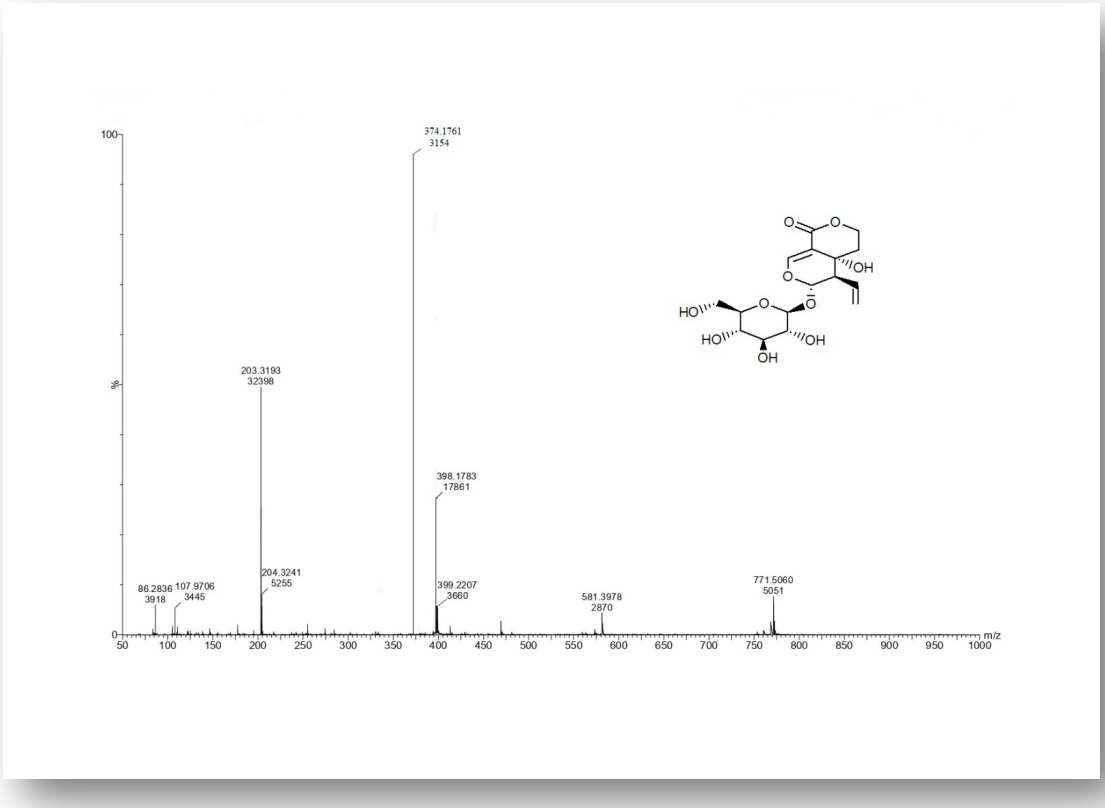


Figure 1: Mass spectra for isolated Swertiamarinertiamarin

Spectroscopic methods for NMR analysis were used to characterize Swertiamarinertiamarin. During purification, it was discovered that the yield for Swertiamarinertiamarin was 6.5%, respectively. Swertiamarinertiamarin (C16H22O10) was effectively isolated from "Enicostemma littorale".
1H NMR (500 MHz, DMSO) chemical shift values (δ): 1.73 (2H, m, CH2); 2.83 (1H, m, CH); 3.81 (1H, m, CH); 4.28 (1H, m, CH); 5.25 (1H, m, CH); 5.35 (2H, d, CH2); 5.63 (1H, s, CH); 7.54 (1H, s, CH); and glycan hydroxyl groups: 2.82 (1H, t, CH); 3.03 (1H, m, CH), 3.15 (2H, m, CH2), 3.43 (1H, m, CH); 3.67 (1H, m, CH); 4.45 (1H, d, CH); ESI-MS m/z found 374.1761, Calcd 374.34.

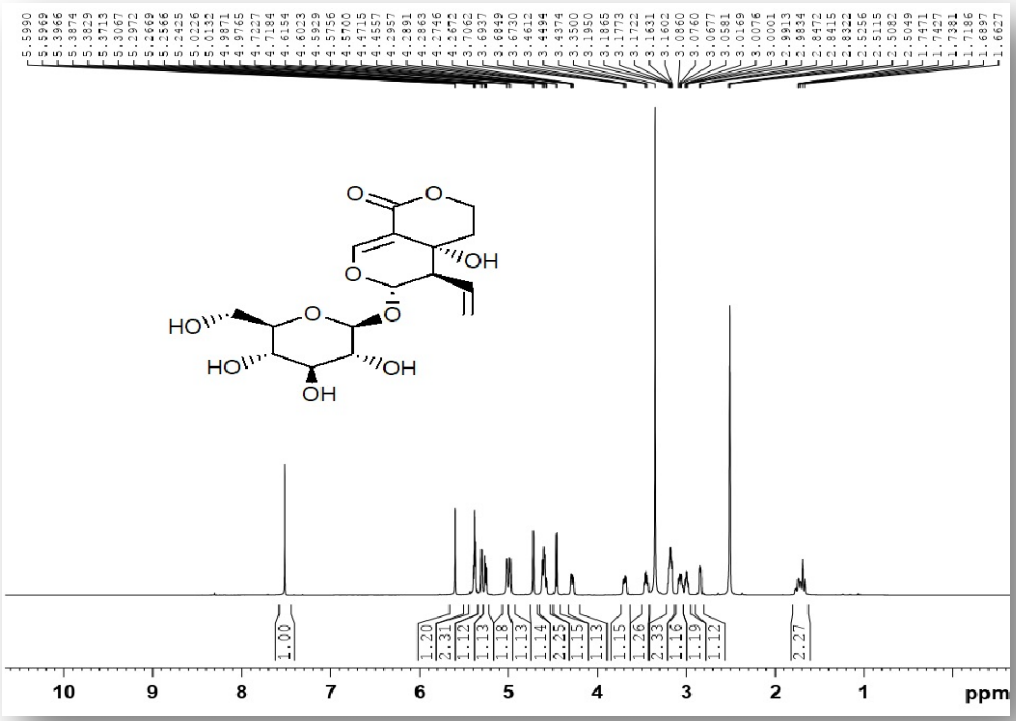


Figure 2: NMR spectra for isolated Swertiamarinertiamarin