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BIOCHEMICAL AND HISTOMORPHOMETRICAL ANALYSIS OF HEPATOPROTECTIVE AND HYPOLIPIDEMIC POTENTIAL OF COMBINED HERBAL EXTRACT IN DIABETIC RATS

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Abstract

Identical anti-diabetic effect-producing herbs (*Syzygium cumini* and *Ficus racemosa*) were administered in different doses for improved hypolipidemic and hepatoprotective effects. Experimental rats collected from icddr'b were divided into six treatment groups (control, diabetic control, standard drug, and combined herbal extracts of low, moderate, and high dose) and arranged in a Completely Randomized Design. The doses were- low (SC @ 100 mg/kg b.wt & FR @ 100 mg/kg b.wt); moderate (SC @ 300 mg/kg b.wt & FR @ 200 mg/kg b.wt) and high (SC @ 500 mg/kg b.wt & FR @ 250 mg/kg b.wt). After 30 days of experiment, blood was collected for biochemical assay and the liver was harvested for histopathologic and histomorphometric analysis. Elevated biochemical indices were significantly improved after low and moderate dose. Like biochemical parameters, histopathological abnormalities of diabetic liver were also restored within the normal range after herbal extract administration in a dose-dependent manner. Histomorphometric findings further corroborate biochemical and histological results with an increased number of healthy hepatocytes. However, high dose herbal extract did not improve these parameters satisfactorily. Collectively, these results indicated that among the three doses, moderate dose of combined extract had the best hypolipidemic and hepatoprotective effect.

Keywords: *Syzygium cumini, Ficus racemosa,* hepatoprotection, hypolipidemia, histomorphometry, biochemical analysis.

Introduction

Lipolysis, the lipid breakdown process, occurs only during limited insulin action (Duncan *et al*., 2007). Insulin deficiency also inhibits the lipoprotein lipase enzyme responsible for triglyceride hydrolysis (Ahmed and Urooj, 2009). Moreover, the absence of insulin hampers apolipoproteins synthesis in hepatocytes (Krishnaswami, 1996). Thus, insufficient insulin release in diabetes mellitus causes promotion in serum lipid profile indices, namely total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL) and the demotion of high-density lipoprotein (HDL)- which are identical characteristics of hyperlipidemia (Firdous *et al*., 2021).

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In terms of lipid metabolism, the liver plays a unique role. It synthesizes lipoproteins which allow lipid transportation through the blood (Nguyen *et al*., 2008). Cholesterol and phospholipids are also synthesized in the liver. It converts excess calories into fatty acids and triglycerides to store them in adipose tissue (Richard, 2022). Thus, irreversible damage to liver cells through oxidative stress and inflammation in diabetes can hamper lipid metabolism, which increases the concentrations of LDL, VLDL and triglycerides in the blood, contributing to dyslipidemia (Jamaludin *et al*., 2016). Moreover, damaged hepatocytes release liver enzymes- AST (aspartate aminotransferase) and ALT (alanine aminotransferase) into the blood, hence elevating their concentration (Ramesh *et al*., 2010).

There is a causal relationship between hyperlipidemia and atherosclerosis, the prime cause of cardiovascular diseases (CVDs) (Frostegård, 2013; Rathi *et al*., 2019). World Health Organization (WHO) reported 17.9 million deaths from CVDs in 2019; more than 75% occurred in low- and middle-income countries (WHO, 2021). Therefore, the elimination of CVDs risk factors such as hyperlipidemia with locally available medicine could save many valuable lives.

Synthetic drugs counter hyperlipidemia by reducing lipid production or gut absorption. However, their use increases gallstone formation, myopathy or rhabdomyolysis (Firdous *et al*., 2021). On the contrary, herbal drugs have antidiabetic and hepatoprotective phytochemicals such as flavonoids, terpenoids and α-amyrin acetate, which could stimulate insulin release and prevent hepatic cell injury (Parameswari *et al*., 2012). Therefore, herbal drugs, which are trusted by many as safe and sound, could be another possible solution to hyperlipidemia and liver damage.

Among the herbs, *Syzygium cumini* seeds and *Ficus racemosa* fruits have many common antidiabetic phytochemicals. They are also renowned for their hepatoprotective and hypolipidemic role (Zulfiker *et al*., 2011; Behera *et al*., 2014). The previous researchers use either *S. cumini* seeds or *F. racemosa* fruits in individual dose. From the literature review it was found that individual dose of *S. cumini* seeds @ 100 mg/kg b.wt, 300 mg/kg b.wt and 500 mg/kg b.wt was used by Sharma *et al*., 2003; Singh and Gupta, 2007 and Nahar *et al*., 2010 respectively. Moreover, *F. racemosa* fruits individually @ 100 mg/kg b.wt, 200 mg/kg b.wt and 250 mg/kg b.wt was used by Zulfiker *et al*., 2011 and Irfan *et al*., 2011. However, the combined application of *S. cumini* seeds and *F. racemosa* fruits could produce improved therapeutic efficacy than the single herb treatment. This may provide synergistic or potentiative pharmacological properties within themselves because of presence of vast range of phytochemicals.

In these backgrounds, the combination of *Syzygium cumini* seeds and *Ficus racemosa* fruits in a dose-dependent manner was investigated to determine its hypolipidemic and hepatoprotective effects by qualitative and quantitative approach in comparison to a standard drug-atorvastatin.

Materials and Methods

Animals and experimental procedures

The experiment was accomplished following Completely Randomized Design (CRD) under

controlled condition at the Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) anatomy laboratory, Gazipur, Bangladesh. Thirty-six icddr'b-bred Long Evans rats (age: 6-7 weeks) were segregated into six treatment groups and each treatment had six replications. After a week acclimation period, all animals except the control were given a single intraperitoneal injection of freshly made alloxan monohydrate (Sigma-Aldrich, Germany) solution to induce diabetes (@ 120 mg/kg b.wt). A drop of blood from the tail vein was placed in a glucometer (Accu-chek® active, Roche Diabetes Care, Germany) on the fifth day morning to check the fasting blood glucose level. Animals were considered diabetic when blood glucose values reached 14 mmol/L or above (Sharma *et al*., 2010). The rats received oral treatment once daily for 30 days as follows:

Group-A: Control (non-diabetic) (NC);

Group-B: Diabetic Control (DC);

Group-C: Standard drug (AT), rats received Atorvastatin @ 10mg/kg body weight (Tablet Anzitor® 10 from Square pharmaceuticals limited);

Group-D: Low dose combined herbal extract (LOW), rats were administered 100 mg/kg body weight *S. cumini* seeds and 100 mg/kg body weight *F. racemosa* fruits;

Group-E: Moderate dose combined herbal extract (MID); rats were administered 300 mg/kg body weight *S. cumini* seeds and 200 mg/kg body weight *F. racemosa* fruits;

Group-F: High dose combined herbal extract (High); rats were administered 500 mg/kg body weight *S. cumini* seeds and 250 mg/kg body weight *F. racemosa* fruits

All standard (international, national, and institutional) animal care and handling criteria were followed in conducting the present experiment.

Extract preparation

Syzygium cumini seeds were gained from locally purchased fruits in May, 2021. *Ficus racemosa* fruits were collected from the BSMRAU campus. After confirming the ethnopharmacological information, *S. cumini* seeds and *F. racemosa* fruits were incised, shade dried and pulverized. The dry powder was dissolved in ethyl acetate (1:3 w/v) for five days, shaking and stirring occasionally. The solvent was then filtered with Whatman filter paper (GE Healthcare UK Limited, UK). Then the filtrate was concentrated under a vacuum rotary evaporator (EV400, Lab Tech, Inc., USA) and finally dried in an automatic lyophilizer (VirTis BenchTop Pro, SP Scientific, USA) to yield the extract (Sharmin *et al*., 2018). The different experimented dose of *S. cumini* seeds and *F. racemosa* fruit extracts (calculated as per the body weight of each animal) were dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) before being used on the animal.

Blood collection and biochemical assay

The rats were starved overnight after the experiment period to collect blood from the retro-orbital sinus under light anesthesia (pentobarbitone @ 35 mg/kg, i.p.). After centrifuging the blood sample at 2500 rpm for 15 minutes, serum was obtained. Commercially available reagents from Human Diagnostic Worldwide, Germany were used to estimate lipid profile indices- TC, TG and

HDL as well as liver function biomarkers-AST and ALT. These biochemical parameters were measured by Hitachi 7180 automatic analyzer (Hitachi, Tokyo, Japan) as per the manufacturer's guidelines (Rathi *et al*., 2019; Firdous *et al*., 2021).

Histopathological study

Following blood collection, all animals were ethically sacrificed by cervical dislocation and their livers were removed for histological examination. The liver samples were then fixed in 10% neutral buffered formalin, dried in a series of alcohols and embedded in paraffin. The paraffin blocks were then sliced into 6 µm thickness using a rotary microtome (Leica, RM2245, Germany) and stained with hematoxylin and eosin (Alam *et al*., 2014). Using a Leica ICC50 E microscope (Leica microsystems, Wetzlar, Germany) coupled with a digital camera, the sections were viewed and images were captured at a magnification of 40X.

Histomorphometric analysis

To obtain quantitative information about the hepatic parenchyma, 6 histological sections from each group were examined with an ocular micrometer and a calibrated graticule. The liver area was calculated in each section at 40X magnification with the help of an area calibrated ocular grid. The number of healthy and necrotic hepatocytes were determined by direct counting method at 40X magnification using the ocular grid. The total number of healthy and necrotic hepatocytes was expressed as N/1000 μm^2 of the hepatic parenchyma (Rajesh *et al*., 2017).

Statistical analysis

All the recorded data were analyzed using Graph pad prism software (version 9.0). One-way analysis of variance (ANOVA) was followed by Tukey's multiple comparison test in the statistical analysis. The results were reported as mean ± standard error of the mean (SEM). Differences between groups were treated significant at $p < 0.05$ level.

Results and Discussion

Hematological findings

Throughout the experiment period, the diabetic rats had significantly higher ($p < 0.05$) glucose levels in comparison to the control (Table 1). However, alloxan-induced hyperglycemia was considerably decreased ($p < 0.05$) by antidiabetic medications and

Table 1. Changes in fasting blood glucose level in different groups

Group	Day 0 (mmol/L)	Day 30 (mmol/L)
NC	5.8 ± 0.3^{a}	6.0 ± 0.3^{a}
DC	20.7 ± 1.4^{b}	22.7 ± 1.5^{b}
AT	21.1 ± 1.9^{b}	10.6 ± 0.4^{c}
LOW	20.3 ± 1.3^{b}	14.0 ± 0.8^{d}
MID	20.6 ± 1.7^{b}	11.1 ± 0.5^{c}
HIGH	20.8 ± 1.1^{b}	13.5 ± 0.7^{d}

Note: Results are Mean ± SEM of 6 rats in each group. The mean with the different upper case letters are significantly different at $p < 0.05$. [One way-ANOVA followed by Tukey's multiple comparison test].

combined herbal extracts. With standard drug, blood sugar levels reduced the most, while the moderate dose combined extract had statistically comparable but marginally less hypoglycemic effects.

Alloxan is a toxic glucose analogue which selectively destroys insulin producing pancreatic *β-cells* and resulting in hyperglycemia or increased blood glucose level (Yamamoto *et al*., 1981). However, the presence of phyto-chemicals such as mycaminose, jambosine, jambolin or antimellin, saponins, flavanoids, phenols could be the reason for significant hypoglycemic activity of *S. cumini* seeds and *F. racemosa* fruits combined extract. The individual and/or synergistic action of these phytochemicals might be responsible for such anti-diabetic activity.

Biochemical study

In the diabetic control group, serum lipid profile indices- total cholesterol (TC), triglyceride (TG) and liver function biomarkers- aspartate aminotransferase (AST), alanine aminotransferase (ALT) were significantly increased, accompanied by a decrease in high-density lipoproteins (HDL) level as compared to the control group (Table 2). In comparison to diabetic rats, low and moderate dose combined extract treatment significantly improved TC, TG, HDL, AST and ALT levels, whereas the biochemical values were below the normal range with high dose combined extract treatment. Interestingly, moderate dose combined extract outscored the effect of low dose and showed statistically equivalent effects to the standard drug.

In diabetic rats, hyperglycemia-induced oxidative stress increase lipid oxidation and liver cell damage, which is manifested by the elevated TG, TC, AST, ALT and diminished HDL levels (Firdous *et al*., 2021). On the other hand, the antidiabetic and antioxidant phytochemicals present in the *S. cumini* seeds and *F. racemosa* fruits may stimulate insulin secretion, lower lipid oxidation and regenerate hepatocytes in the low and moderate dose combined extract-treated animals, resulting in improved biochemical parameters (Dheer and Bhatnagar, 2010). When worked with individually administered extract, such antidiabetic and hypolipidemic effects from

Table 2. Changes in biochemical parameters in different study groups

Group	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	AST (u/dl)	ALT (u/dl)
NC	101 ± 4.1^{a}	90 ± 3.2^{a}	38 ± 2.8^{a}	40 ± 3.2^{a}	53 ± 2.6^{a}
DC	193 ± 6.7^{b}	181 ± 6.1^{b}	20 ± 1.9^{b}	75 ± 3.7^{b}	97 ± 2.9^{b}
AT	123 ± 4.9^{c}	106 ± 4.3^{c}	35 ± 2.7^{ac}	43 ± 1.9^{ac}	64 ± 2.0^{c}
LOW	145 ± 5.3^{d}	125 ± 4.7^{d}	27 ± 2.4^{d}	50 ± 2.4^{d}	72 ± 2.7^{d}
MID	131 ± 4.8^{c}	114 ± 4.6^{c}	34 ± 2.5^{c}	46 ± 1.6^{c}	62 ± 1.9^{c}
HIGH	70 ± 3.3^{e}	60 ± 2.1^{e}	22 ± 2.2^{b}	23 ± 2.2^{e}	33 ± 1.4^{e}

Note: Results are Mean ± SEM of 6 rats in each group. The mean with the different upper case letters are significantly different at $p < 0.05$. [One way-ANOVA followed by Tukey's multiple comparison test].

S. cumini seeds and *F. racemosa* fruits were previously reported (Ahmed and Urooj, 2009; Yadav *et al*., 2015). However, the negative biochemical value in high dose herbal extract treated rats may indicate harmful consequences of high doses (Singh and Gupta, 2007).

Histopathological changes in the liver

In the control liver, anastomosing cords of hepatocytes were found to extend from the clear central veins (Fig. 1). However, the diabetic liver was shown to have significant structural changes, including necrosis of hepatocytes,

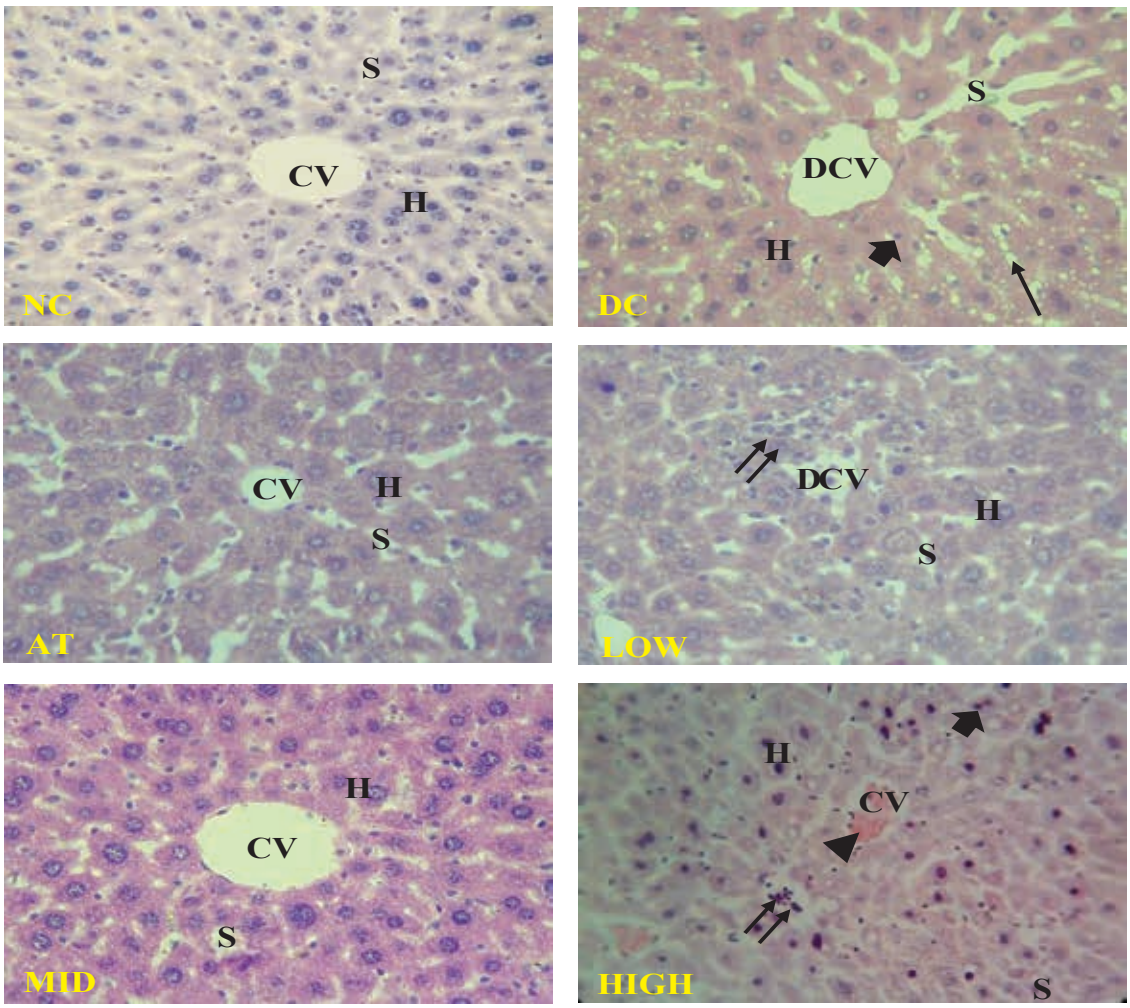


Fig. 1. Histological architecture of the liver of the control (NC) group showing normal hepatocytes (H) with clear central vein (CV); the diabetic control (DC) group showing degenerated central vein (DCV), fatty infiltration (long arrow), necrotic hepatocytes (short arrow) and dilated sinusoids (S); the atorvastatin treated (AT) group presenting almost normal sinusoidal dilatation (S) and clear central vein (CV); the low dose (LOW) herbal extract group showing infiltration of inflammatory cells (double arrow) around the degenerated central vein (DCV); the moderate dose (MID) herbal extract group presenting mild dilated sinusoids (S) and clear central vein (CV); the high dose (HIGH) herbal extract group showing congested central vein (arrow head), necrotic hepatocytes (short arrow) and inflammatory cells infiltration (double arrow).

the degeneration of the central vein, fatty infiltration and dilation of hepatic sinusoids. Restoration of normal hepatic architecture following treatment with the moderate dose combined extract and standard drug-atorvastatin was observed through regenerated hepatocytes, non-dilated hepatic sinusoids and a decreased number of inflammatory cells. In the low and high dose combined extract-treated group, damaged hepatic architecture was partially reversed and few necrotic and inflammatory cells were present.

F. racemosa and *S. cumini* may stimulate hepatic cell regeneration by enhancing protein synthesis (Singh and Gupta, 2007; Irfan *et al*., 2011). Moreover, the free radical scavenging properties of flavonoids, *α*-amyrin acetate and terpenoids may account for their significant hepatoprotective effect (Parameswari *et al*., 2012). Behera *et al*. (2014) mentioned the strong hepatoprotective action of *S. cumini* seeds. Nahar *et al*. (2010) reported restoration of hepatocytic dissociation and sinusoidal dilatation by the treatment with *S. cumini* extract. With *Ficus* plant extract treatment, amelioration of hepatocytes, minimal inflammation and hepatic cords regeneration were observed (Adeyi *et al*., 2012). Irfan *et al*. (2011) also found improved hepatic architecture and normal sinusoids with the administration of unripe *F. glomerata* fruits extract, which justifies the present research findings. However, hepatotoxicity was observed in the form of inflammation and necrosis in the high dose combined extract-treated rats indicating that excessive herbal doses can be hazardous (Singh and Gupta, 2007).

Histomorphometric analysis

The morphometric evaluation showed depletion of healthy hepatocytes numbers in the diabetic control liver (Table 3). However, standard drug and combined extract (moderate and low dose) were sufficient to inhibit this histomorphometric change and bring back the hepatocytes numbers to near normal. Whereas high dose herbal extract showed a slight improvement.

In the diabetic control group, the number of necrotic hepatocytes was detected to increase, which confirmed liver damage by alloxan through oxidative stress and inflammatory response (Jamaludin *et al*., 2016). The hepatoprotective activity of the

Table 3. Changes in histomorphometric parameters in different study groups

Group	Number of healthy hepatocytes (N/1000 μm^2)	Number of necrotic hepatocytes (N/1000 μm^2)
NC	26 ± 2.1^{a}	01 ± 0.4^{a}
DC	10 ± 1.4^{b}	07 ± 1.3^{b}
AT	23 ± 1.9^{c}	03 ± 0.9^{c}
LOW	19 ± 1.3^{d}	03 ± 0.7^{c}
MID	22 ± 1.7^{c}	03 ± 0.4^{c}
HIGH	12 ± 1.1^{b}	06 ± 1.3^{b}

Note: Results are Mean ± SEM of 6 rats in each group. The mean with the different upper case letters are significantly different at $p < 0.05$. [One way-ANOVA followed by Tukey's multiple comparison test].

S. cumini and *F. racemosa* combined extract may be attributed to its antioxidant and anti-inflammatory phytochemicals- saponins, tannins and flavonoids, which scavenge free radicals and prevent oxidative stress (Hasan *et al*., 2016). The present histomorphometric findings were supported by Rajesh *et al*. (2017), who similarly reported a significant increase in the healthy hepatocytes number in the herbal extract-treated group.

Conclusion

A moderate dose of combined extract (*S. cumini* seeds @ 300 mg/kg b.wt and *F. racemosa* fruits @ 200 mg/kg b.wt) was highly effective against hyperlipidemia and liver damage and the effect was comparable to that of the standard drug. Significant improvements in the hepatic architecture, quantity of healthy hepatocytes and biochemical indicators in alloxan-induced diabetic rats justify its hepatoprotective and hypolipidemic role. Thus, this natural and effective combined herbal extract could replace the hazardous synthetic pharmaceutical drugs. Moreover, further studies could establish its long-term efficacy in animals and humans.

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Conflict of Interest

Both the authors declare that they have no conflict of interest.

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