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ANTIOXIDANT, HEPATOPROTECTIVE, ANTI-INFLAMMATORY EFFECTS OF AGARICUS CAMPESTRIS EXTRACT AGAINST DICLOFENAC-INDUCED HEPATIC DAMAGE IN WISTAR RATS

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Abstract:

Background: Agaricus campestris (AC), commonly known as the field mushroom, has long been used in traditional medicine for the treatment of hepatic disorders, diabetes mellitus, hypercholesterolemia, and gastrointestinal diseases. However, its antioxidant and hepatoprotective properties have not been scientifically validated.
Objective: To evaluate the anti-inflammatory, hepatoprotective, and antioxidant activities of the methanolic extract of Agaricus campestris (AEAC) in a rat model of diclofenac sodium (DFS)-induced liver injury.
Methods: Hepatoprotective activity was assessed by estimating serum biochemical markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin. Antioxidant activity was evaluated by free radical scavenging activity, lipid peroxidation (LPO), and reduced glutathione (GSH) levels. Histopathological examination of liver tissues and hyaluronidase inhibition assay were also performed.
Results: Administration of DFS caused a marked increase in serum AST, ALT, ALP, and total bilirubin levels. Treatment with AEAC significantly reduced these biomarkers, indicating restoration of normal liver function. AEAC exhibited significant free radical scavenging activity, decreased LPO, and increased liver GSH levels, demonstrating improved oxidative balance. Histopathological examination confirmed near-normal hepatic architecture in AEAC-treated groups. AEAC also significantly inhibited hyaluronidase ($p < 0.005$), indicating anti-inflammatory activity. These biological effects were attributed to the presence of flavonoids, polyphenols, and tocopherols.
Conclusion: Agaricus campestris possesses significant hepatoprotective, antioxidant, and anti-inflammatory activities against DFS-induced liver injury and may serve as a promising natural therapeutic agent for the management of hepatic disorders.

Keywords: Agaricus campestris; hepatoprotection; antioxidant; anti-inflammatory; diclofenac.

INTRODUCTION

The ‘Agaricaceae’ family includes the cultivated mushroom ‘Agaricus campestris; (AC), which grows naturally in pastures and grasslands in the summer and fall. Its pleasant flavor and typical glossy white cap, which is usually 3 to 5 cm in diameter, make it easy to identify, this species has been prized as a traditional remedy and a wholesome food for centuries (Davis et al., 2012). In addition to its nutritional value, A. campestris has been castoff extensively in old-style medicine to treat a numeral of illnesses, such as burns, hypercholesterolemia, stomach ulcers, and liver problems. Aqueous extracts of the mushroom have also been shown in recent studies to increase insulin secretion, indicating a possible therapeutic role for the mushroom in metabolic regulation (Gray & Flatt, 1998; Brondz et al., 2014).

‘Diclofenac sodium’ (DFS) is a frequently utilized NSAID(nonsteroidal anti-inflammatory drug) that knowledgesimportant hepatic metabolism via hydroxylation and glucuronidation pathways. However; excessive or prolonged use of DFS has been linked to hepatotoxic possessions, which can range from mild increases in serum transaminase levels to severe hepatocellular necrosis (Bethesda et al., 2017). The underlying mechanisms of DFS-induced liver injury involve the formation of reactive intermediates, depletion of hepatic glutathione reserves, and induction of oxidative stress.

The contemporarytraining aimed to assess the hepatoprotective, antioxidant, &anti-inflammatory activities of a AEAC in view of the ethnomedicinal qualities ascribed to the plant and the rising incidence of drug-caused liver damage. In models of DFS-induced liver damage, biochemical markers including total bilirubin, lipid peroxidation (LPO), glutathione (GSH), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and others remained gauged. Anti-inflammatory mechanisms and protective activity of AEAC were further investigated using hyaluronidase inhibition tests and histopathological examination (Chatterjee et al., 2009; Sanapala & Kumar et al., 2013; Sunil et al., 2010;Sanapala & Kumar et al., 2020; Sunil et al., 2015;Sharma & Gautam et al., 2015).

MATERIALS AND METHODS

Chemicals

DFS, 100 mg/kg was provided by Dr. Reddy's Laboratories, HYD, India. Silymarin was procured by Micro Labs, Tamil Nadu. All biochemical test kits were bought from Span Diagnostics. All additionalsubstances were analytical grade.

Collection and Extraction of the Fungal Material

Fresh specimens of AC were collected from Telangana, India. The material was acknowledged by Rana Kausar, Department of Botany, Osmania University, HYD, and voucher samplestayedstored in the herbarium. Obtained mushrooms were washed, shade-dried, powdered, and stored for extraction.

The powdered sample (100 g) was treated with Soxhlet extraction employing 90% methanol at 50–60°C for 24 hours, thrice in repetition. The extracts were combined & concerted under abridged pressure at 50–60°C employing a 'rotary-evaporator' to yield a crude methanolic extract, labelled as AEAC. The produce of the extract stayed determined, and the sample was treated with preliminary phytochemical screening for bioactive compounds.

BIOACTIVE EVALUATION

Fatty Acid Composition

AC's fatty acid profile was analysed employing a standard methylation and gas chromatographic protocol. First, 45 g of sodium hydroxide pellets were dissolved in 300mL of 50% methanol &mixed by vortexing for 1minute to fully dissolve. The mixture was thereafter heated to 100°C for 5 minutes, vortexed again for 1 minute, and later kept in a water bath at the same temperature for 25 minutes to allow saponification.

After dissolving the 1 mL of powdered fungal samples in the prepared solution, 6 N hydrochloric acid (HCl) was added to methanol to perform methylation. To finish the methylation process; the mixture was vortexed for one minute and then heated to 80 °C in a water bath.The top organic layer, which made up about two-thirds of the volume after extraction, was carefully separated and put into gas chromatographyvials for examination.As a measure of the fungal extract's nutritional value and antioxidant capacity, this index sheds light on the level of unsaturation in the fatty acid profile (Ghanem et al., 2016)

Finding δ-Tocopherol

A consistent analytical method was used to quantity the amount of δ-tocopherol in AC. Butylated-hydroxytoluene; (BHT) in hexane (10mg/mL;100µL) and an internal standard solution in hexane (1.6 g/mL;250 µL) were further to each sample to stop oxidative degradation and make sure the measurements were accurate.

To get a full mix, each 500 mg sample was mixed with 4 mL of methanol and vortexed for one minute. Then, 4 mL of hexane remainedextra, and the mixture was vortexed for one more minute to help the phases separate. Centrifugation at 4000 × g for five minutes separated the top hexane layer, which contained the tocopherol fraction.

We used chromatographic methods to carefully analyze the collected hexane phase. We compared the retention times to those of real δ-tocopherol standards to identify and measure the amount. The grades were shown as micrograms of δ-tocopherol each gram of AEAC (µg/g), which proved that 'AC' had measurable amounts of this powerful antioxidant.

Evaluation of Additional Bioactive Substances

To ascertain the presence of different bioactive ingredients, 5 g of powdered 'AC'sample was extracted by hundred mL methanol at 25 °C for 24hours using a 150-rpm rotary shaker.To fully recover the soluble components, the resulting mixture remainedclean using Whatman-filter paper and the residue was extracted double in succession using additional 100mL volumes of methanol. The extracts were combined, dried at 42°C, redissolved in 50 mg/mL alcohol, and kept at 4°C until further analysis. Carotenoid estimation was done by mixing 100mg of the dry extract with 10mL acetone:hexane solution (4:6 v/v) &shaking for one minute. The subsequent

filtrate was analyzed spectrophotometrically and absorbance was measured at A_{453}, A_{505}, and A_{663} to estimate carotene content.

The TPC (total phenolic content) was resolutevia the 'Folinciocalteu;colorimetric method. Briefly; 1mL of the extract was mixed with 1mL of 'Folinciocalteu' reagent, shadowed by the adding of 10mL of distilled H_2O and 1mL of saturated sodium carbonate (Na_2CO_3) solution after three minutes. Following; a 90-minute dark incubation period, the optical density of the reaction mixture was read at A_{725}. A calibration plotwas set using standard solutions of gallic acid ranging from 0.01 to 0.4mM, showingoutstanding linearity ($R^2 = 0.9999$). The 'TPC' was uttered as milligrams of gallic acid equivalents (GAE) per gram of AEAC

TFC (total flavonoid content) was resolute using $AlCl_3$ colorimetric assay. This was accomplished by mixing 0.3 mL of a 5% $NaNO_2$ solution with 1.5mL of aqueous extract and 5 mL of deionized water. 1.5 mL of 2% $AlCl_3$ solution was added after 5 minutes of room temperature incubation, and 2 mL of 1 M NaOH was added after 6 minutes. After five minutes of vigorous shaking at 200 rpm on an orbital shaker, the absorbance at A_{510} was measured in comparison to the reagent blank. The calibration curve was constructed using quercetin standards.

Preliminary phytochemical examination assured the availability of variegated secondary metabolites in A. campestris methanolic extract such as fatty acids, amino acids, tocopherols, phenolic compounds, flavonoids, steroids, tannins, and terpenoids (Ghanem et al., 2016; Eesha et al., 2011;Ling et al., 2003). These bioactive components are presumable accountable for the antioxidant and hepatoprotective activities reported in follow-up analysis**.**

AID Analysis

AEAC's AI capacity was assessed through the inhibition of hyaluronidase assay by the procedure reported by (Ling et al., 2003). The absorbance was measured at A_{600} in the assay, and the absorbance values for the enzyme's presence and absence were compared to determine the degree of enzyme inhibition.By contrasting; the absorbance of the reaction mixture with that in the absence of the enzyme, the percentage inhibition of hyaluronidase activity was determined. The AEAC was tested at concentrations ranging from 10µg/mL to 100µg/mL in order to determine its dose-dependent inhibitory potential. The standard reference medication for comparison was DFS. A quantitative charge of AEAC ability to inhibit hyaluronidase, an enzyme connected to an 'AID' and increased tissue permeability, demonstrated the extract's possible anti-inflammatory efficacy.

Design of Experiments and Animal Model

The study employed healthy adult Wistar albino rats, evaluatingamid 200 & 250 g, of both sexes. The animals stayed kept in a typical laboratory location with a 12-hour light/dark cycle and unrestrictedadmittance to food and water. The 'IAEC'(Institutional Animal Ethics Committee) approved all experimental trials, which were carried out in submission with ethical standards for the use & care of laboratory animals. The procedures were registered under the number 926/PO/Re/S/06/CPCSEA.

6-groups (n = 6) were created from the animals: Group-I:Vehicle-only normal control; Group-II:100 mg/kg oral DFS; Group-III:100 mg/kg oral DFS + Silymarin; Group-IV:100 mg/kg oral DFS + AEAC; Group-V:250 mg/kg oral DFS + AEAC; Group-VI:500 mg/kg oral DFS + AEAC.

Treatment was given for 28 days. Day 29 blood & liver tasters were collected for Histological & biochemical analysis.

Biochemical Estimation

Liver damage was determined through the estimation of serum ALT, AST, ALP, and bilirubin levels. LPO, estimated as malondialdehyde content, & reduced GSH were determined in liver homogenates (Baali et al., 2016; Heidarian & Rafieian-Kopaei, 2013; Gevrenova et al., 2015; Hafez, 2014; Cristani et al., 2016).

Histopathological analysis of formalin-fixed liver tissue stained with hematoxylin and eosin was done after following standard protocols (Standish et al., 2006; Sunil et al., 2017).

Statistical analysis

The mean ± SEM is used to represent the values (n = 6). Statistical significance was assessed using Origin Pro 7.6's Dunnett's multiple assessment after a one-way ANOVA. It was deemed significant when $p < 0.05$.

RESULT

Extraction Yield and Phytochemical Composition

Methanolic extract (AEAC) of approximately 15.5% (w/w) of dry fungal mass was obtained. Preliminary phytochemical screening revealed the occurrence of amino acids, fatty acids, tocopherols, terpenoids, phenolic compounds, and flavonoids (Table-1).

Table 1: % chemical composition and bioactive constituents of AC

% Chemical composition	
Component	**%**
Protein	19.36 ± 0.5
Crude Fat	0.13 ± 0.0
Fiber	1.16 ± 0.0
Ash	0.85 ± 0.0
Carbohydrate	44.08 ± 2.3
Bioactive constituents of AC	
	Concentration
β-Carotene (µg/100g)	0.60 ± 0.0
Phenolic Compounds (mg/100g)	44.16 ± 1.6
Flavonoids (mg/g)	2.24 ± 0.0
Ascorbic Acid (ASC) (mg/100g)	0.55 ± 0.0
δ-Tocopherol (µg/g)	2.95 ± 0.0

FRSA (Free radical scavenging action)

DPPH-scavenging activity

The concentration-dependent antioxidant activity of AEAC was demonstrated via DPPH radical assay. The IC_{50} assess of AEAC and ASC were 195.34µg/mL (Y= 0.217x+1.028) &181.77µg/mL (Y=0.2332x+7.61), correspondingly, which depicted potent free-radical scavenging activityThe findings for each test sample's DPPH scavenging activity are shown in Figure 1.

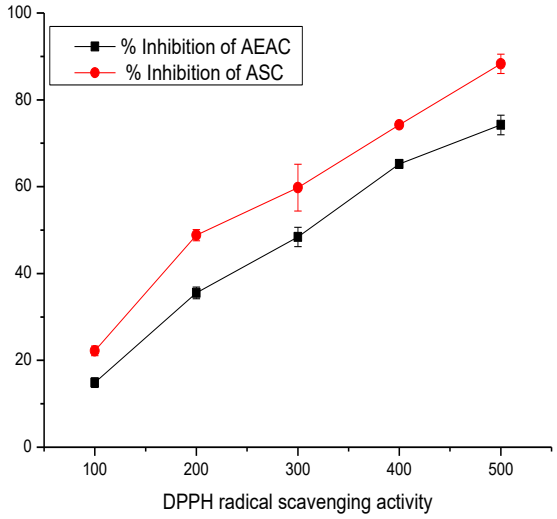

Figure 1: DPPH of AEAC&ASC

Activity of FRAP-scavenging

The FRAP of fungal extracts in comparison to ASC, a common antioxidant data set, is displayed in Figure 2. The FRAP test revealed IC_{50} values of 181.27µg/mL (Y= 0.2346x+7.47) for ASC& 239.67µg/mL (Y= 0.1861x+5.39) for AEAC.

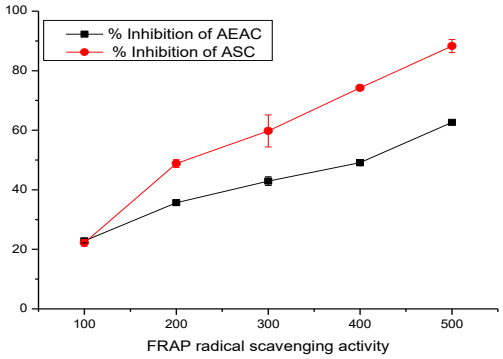

Figure 2: FRAP of AEAC and ASC

Rat liver homogenate's in-vitro LPO inhibitory activity:
Alcohol-treated animal groups exhibited noticeably higher MDA levels and lower GSH activities. Using silymarin (100 mg/kg b.wt.) and AEAC (100, 250&500 mg/kg b.wt.) as pretreatment against DFS-toxicity successfully maintained and restored MDA levels close to normal. The findings are shown in Table 3.

Table 3: In-vitroLPOinhibition and GSH activity

Groups	LPO	GSH
Group-I	1.93 ±0.99	6.71 ±1.22
Group-II	5.18 ±0.98[a]	3.16 ±1.32[a]
Group-III	3.15 ±2.27	6.98 ±2.12
Group-IV	3.11 ±1.07**	4.98 ±1.10**
Group-V	3.21 ±1.11**	5.40 ±0.98**
Group-VI	3.55 ±1.03***	5.84 ±0.89***

LPO=(nM MDA/mg); GSH =µg/mg

AID Analysis
The AID capacity of the AEAC was evaluated by the hyaluronidase inhibition assay, and the data is presented in Figure 3. The extract exhibited an appreciable ($p < 0.005$) inhibitory activity against hyaluronidase, reflecting significant AID potential. Nonetheless, the inhibitory activity of AEAC was lower in comparison with that of the standard reference drug, DFS.

Hyaluronidase is an enzyme that is involved in the breakdown of hyaluronic acid, and it therefore enhances tissue permeability and allows for spread of inflammation. Inflammation; frequently results in improved hyaluronidase activity, whereas inflammation that has stopped is characterized by decreased hyaluronidase activity. 'AC' may have 'AID' properties by stabilizing connective tissue matrices and reducing enzyme-mediated inflammatory responses, as evidenced by the observed inhibition of hyaluronidase activity by 'AEAC'. The application of 'AEAC', a naturally occurring anti-inflammatory substance made from a 'AC', demonstrates its pharmacological significance and therapeutic potential.

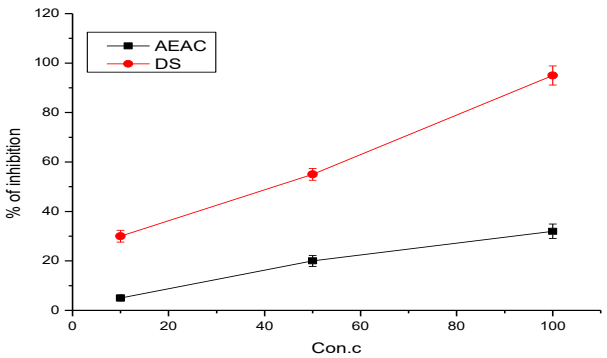

Figure 3: AID of AEAC

Hepatoprotective Activity

DFS-Induced Hepatotoxicity

Significant hepatic damage brought on by the administration of 'DFS' was established by raised levels of serum biomarkers including 'AST', 'ALT', 'ALP', and 'total bilirubin' in the toxic control group. These parameters were successfully normalized by treatment with silymarin (100 mg/kg b.w., p.o.), a well-known reference hepatoprotective agent, confirming its proven protective efficacy. Similar to silymarin, the AEAC also demonstrated a dose-dependent hepatoprotective effect upon administration, suggesting a possible function for it in reducing liver damage brought on by DFS.

Pre-administration of 'AEAC' restored the balance of hepatic enzymes affected by oxidative stress caused by 'DFS' and preserved the membranous integrity of hepatocellular membranes.GSH levels are lowered by 'DFS' metabolism, which has been shown to cause membrane instability and cell necrosis. By maintaining GSH levels and bringing significant liver biomarkers back to baseline, 'AEAC' therapy has been shown to reverse this effect.

The formula for calculating the percentage of inhibition of hepatic marker elevation was as follows:

$$\% \text{ of inhibition} = \frac{[(\text{value of toxic control}) - (\text{value of test sample})]}{[(\text{value of toxic control}) - (\text{value of control})].} X100$$

As shown in Table-4, the results clearly demonstrate that 'AEAC' exhibits exceptional hepatoprotection against 'DFS'-induced liver toxicity, suggesting that its protective effect is due to its antioxidant constituents, particularly polyphenols and flavonoids.

Table 4:Elevated levels of serum biomarkers

Groups	SB (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
Group-I	0.56 ± 0.02	53.01 ± 2.88	35.00 ± 1.02	108.03 ± 5.52
Group-II	2.05 ± 0.17[a]	180.34 ± 3.02[a]	205.67 ± 1.52[a]	210.18 ± 5.38[a]
Group-III	0.93 ± 0.07***	135.50 ± 3.68***	164.05 ± 1.73***	185.5±3.83***
Group-IV	1.65 ± 0.036***	162.15 ± 4.06***	197.66 ± 5.17***	218.14±5.73*
Group-V	1.25 ± 0.97	142.18 ± 5.22	181.32 ± 2.72	178.20 ± 2.64
Group-VI	0.96 ± 0.16	131.12 ± 3.58	168.57 ± 4.23	189.32 ± 4.56

*P<0.05, **P<0.01, *** P<0.001 in evaluation to the equivalent DFS-treated control groups; [a]P<0.001 vs. vehicle control.

Histopathological Examination

Microscopic examination of the liver tissues offered more proof of 'AEAC' hepatoprotective effectiveness against 'DFS'-induced liver damage. Histopathological analysis of liver sections in the 'DFS'-treated control group confirmed widespread hepatocellular injury by revealing severe hepatic necrosis, noticeable fatty degeneration, and disarray of the normal hepatic architecture. Conversely, animals pre-treated with AEAC, especially with amounts of 250 mg/kg & 500 mg/kg, showed well-preserved hepatic architecture with almost normal hepatocyte morphology, minimal fatty changes, and intact central vein structures. These results were very like to those in the Silymarin-treated group, indicating equal hepatoprotective potential. The slices stained with hematoxylin and eosin and detected microscopically at 100× magnification to assess morphological changes.

The histopathological findings of all experimental groups are given below (Figures 4–9)

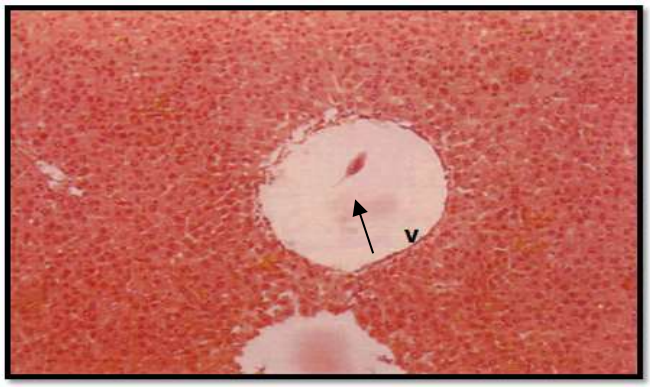

Figure 4: Group I

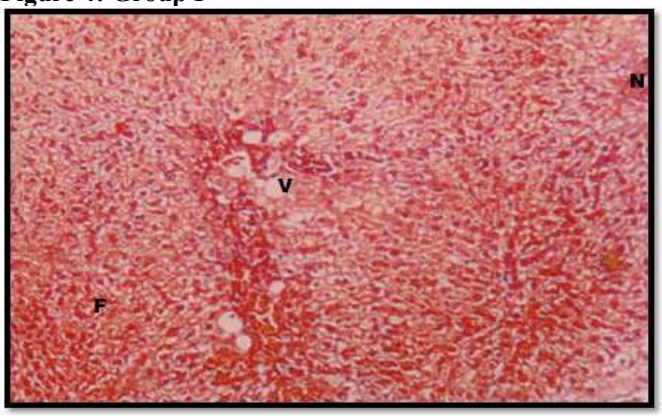

Figure 5: Group II

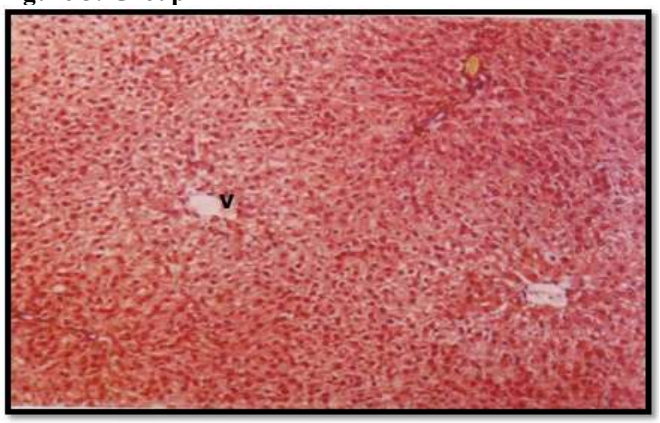

Figure 6: Group III

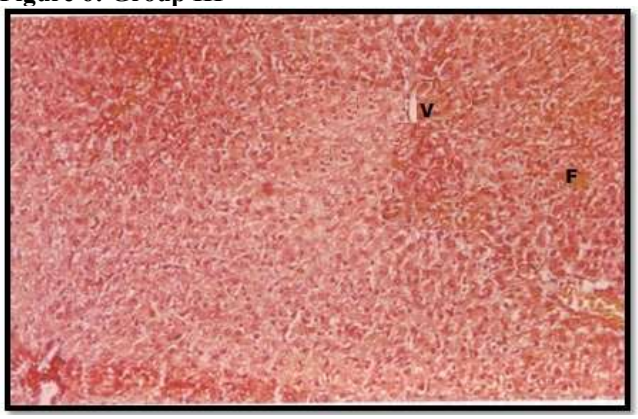

Figure 7: Group IV

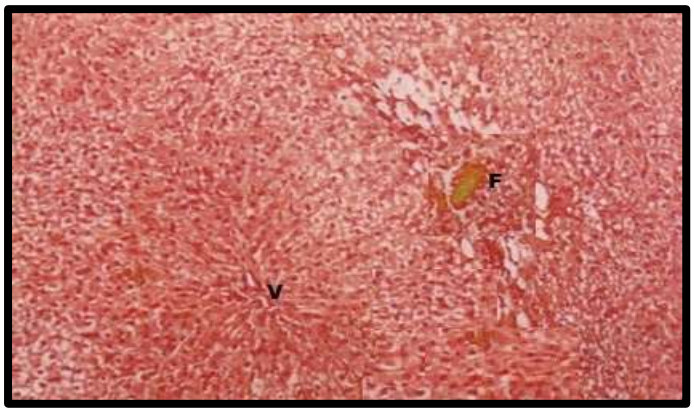

Figure 8: Group V

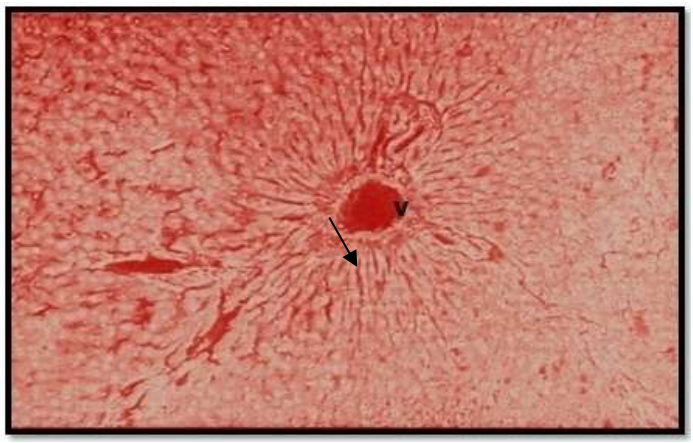

Figure 9: Group VI

Figure 4: Liver tissue from the control group (Group I) with normal hepatic architecture and a well-defined portal triad, which consists of the portal vein (V), portal artery (arrow), and bile duct (arrowhead).
Figure 5: DFS-treated group (Group II) section with widespread hepatocellular necrosis (N), fatty vacuolation (F), central vein congestion (V), reflecting extensive hepatic damage.
Figure 6: Liver tissue of the Silymarin-treated group (Group III) with normal hepatocytes , distinct lobular structure with fewer degenerative changes.
Figure 7: Section from AEAC-treated rats (100 mg/kg; Group IV) with regular procedure of hepatocytes around portal vein (V), no necrosis,& mild growth of fatty material.
Figure 8: Liver section of rats treated with AEAC (250 mg/kg; Group V) showing mild fatty infiltration, absence of necrotic areas, and almost normal hepatic parenchyma.
Figure 9: Hepatocytes were uniformly distributed, the central and portal veins (V) were intact, and the bile ducts (arrowheads) were normal in liver sections from the AEAC high-dose group (500 mg/kg; Group VI). These results show significant protection against hepatotoxicity caused by 'DFS'.

The biochemical results are strongly supported by the microscopic observations, which show that 'AEAC' effectively protects hepatic tissue by maintaining structural integrity and preventing degenerative changes linked to oxidative and inflammatory damage caused by 'DFS'.

DISCUSSION

The results of the current study clearly demonstrate that 'AC' has potent antioxidant and hepatoprotective activities. The rich phytochemical profile, particularly the occurrence of flavonoids, phenolic acids and tocopherols, greatly contributes to its antioxidant and membrane stabilizing activities. Given that GSH depletion and oxidative stress are known mechanisms by which DFS induces liver toxicity, treatment with the AEAC effectively counteracted these manifestations and restored the liver antioxidant action. The experiential lessening in serum transaminases and bilirubin levels reflects stabilization of hepatocyte membranes and indications of better hepatic repair after damage induced by DFS. The results of histopathological investigation also established these protective effects with less necrosis and evidence of regeneration of hepatocytes. In addition, the ability of AEAC to inhibit the activity of hyaluronidase indicates the possibility of lessening the inflammatory response usually.

CONCLUSION

The AEAC exhibited potent hepatoprotective, antioxidant and AID activities against DFS–caused hepatic damage in Wistar rats. AEAC effectively inhibited lipid peroxidation, restored normal hepatic biochemical parameters to near normal levels and preserved normal liver histoarchitecture. These results promising the ancient claims on the therapeutic value of AC and suggest their potential as a valuable source for the future development of hepatoprotective drugs. More wide pharmacological and toxicological investigations will be needed in the future to clarify the probable mechanisms of its action and to examine the clinical usefulness of this compound in the treatment of liver illnesses.

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