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Allophylus cobbe Attenuates Oxidative Stress and Liver Damage in Chemically-Induced Hepatotoxicity in Wistar Rats

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ABSTRACT

Liver disorders, including cirrhosis and hepatitis, are major health concerns worldwide. Nowadays, many herbal remedies are reported to be promising against liver disorders. There is an urgent need to find new herbal remedies that are highly effective for liver problems. Therefore, the present research was aimed at characterizing and screening the hepatoprotective activity of *Allophylus cobbe* leaf extract in both acute and chronic hepatic disorders in Wistar rats. Ethanol leaf extract of *Allophylus cobbe* was prepared. The extract was subjected to phytochemical screening, acute oral toxicity, and hepatoprotective testing against chemically-induced hepatotoxicity and evaluated against a standard liver protective agent (silymarin). The concentration of different serum and tissue biochemical parameters was estimated. Using column chromatography, the active phytoconstituent from the plant extract was isolated and confirmed by UV, FTIR, NMR, and mass spectrometry. The qualitative phytochemical analysis showed the presence of various phytochemicals. Oral administration of the extract at a dose of 2000 mg/kg was shown to be safe, with no signs of behavioral, neurological toxicity, or mortality. Significant restoration of serum and tissue biochemical parameters supported the dose-dependent hepatoprotective capacity. The groups that were given the leaf extract had regular hepatic architecture, suggesting that it had a hepatoprotective function. The presence of quercetin flavonoid was revealed by the analytical techniques, and the hepatoprotective activity was attributed to the chemical compound, quercetin. The findings of this study confirmed that the ethanol leaf extract of *Allophylus cobbe* possesses hepatoprotective activity and may be used for the treatment of hepatotoxicity.

Keywords: *Allophylus cobbe*, Antioxidant, Hepatoprotective, Hepatotoxicity, Histopathology.

Introduction

One of the important organs of humans and other living organisms is the liver, which plays a very important function in carbohydrate, lipid, protein, bilirubin, porphyrin, amino acid, hormone, vitamin, and bile acid metabolism.[1] Certain biochemical functions and metabolic activity may help to preserve the liver's functional integrity. Oxidative stress, tissue damage, inflammation, and fibrosis are also prevalent causes of liver disorders, which are major public health problems.[2] Cirrhosis is a chronic liver disorder resulting from fibrosis or architectural changes in the liver, leading to the development of regenerative nodules.[3] The diseased condition can have a wide range of clinical symptoms and complications. Over the past few years, liver disorders have grown to become one of the main causes of death and sickness around the world.[4]

Liver damage is mainly caused by the chronic use of alcohol, chemicals such as carbon tetrachloride (CCl_4), paracetamol, rifampicin, ethanol, nitrosamines, thioacetamide (TAA), and infections.[5] CCl_4 administration can stimulate acute or chronic liver injury in rats and is the most commonly used experimental model for liver damage. It is metabolized by cytochrome P450 to produce trichloromethyl radicals and reactive oxygen species (ROS) which damage the liver.[6] Thioacetamide (C_2H_5NS; TAA), an organosulfur and white crystalline compound commonly used in laboratories, leather processing, textile, and paper industries, have also been implicated in causing liver damage. TAA is a model hepatotoxicant, administered to stimulate acute and chronic liver damage due to its effects on protein synthesis, RNA, DNA, and Gamma-glutamyl transpeptidase activity.[7] Alcohol is a psychoactive substance, and its excess is associated with different health-related issues globally. It is metabolized mainly by the liver, and its chronic consumption is associated with hepatotoxicity by inducing oxidative stress.[8] Thus, substances like TAA, CCl_4, and ethanol are used to induce liver damage in rats to screen the hepatoprotective potential of different drugs.

Allophylus cobbe is a small tree and an ethnomedicinally essential plant species from the *Sapindaceae* family that has been used to treat several ailments in the past. There are approximately 255 species of *Allophylus* (family *Sapindaceae*) found worldwide, with nine species found in India. *Allophylus cobbe* shows a long line of ethnobotanical and ethnopharmacological properties. It can be used in the treatment of ulcers, burns, dyspepsia, anorexia, diarrhea, stomach ache, fever, bruising, and inflammation.[9] It has anti-inflammatory, digestive, carminative, and constipating effects. The leaf extract is used to treat stomach aches, and the leaf pest is used to treat scabies. For diarrhoea, the root powder is blended with honey.[9]*Allophylus cobbe* includes several bioactive compounds, including tannins, terpenoids, tannins, steroids, cardiac glycosides, and flavonoids.[9,10] Extensive research work has been done to prove the antioxidant, anti-inflammatory, anticancer, analgesic, antimicrobial, insecticidal, and anthelmintic activities of the *Allophylus cobbe*.[10-13]

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Plant extracts are widely used as herbal medicines to treat a host of chronic illnesses, including liver disorders. Plant products have long been utilized to combat liver cancer, and they are still employed in some form or another all around the world.[14] When no authorized hepatoprotective treatment is available in western medicine, a wide range of natural herbal compositions are used to treat liver diseases.[15] Despite substantial scientific advances in hepatic studies in recent years, liver disease is on the rise. In various parts of the world, a number of effective local herbal therapies for the treatment of liver disorders are available, the majority of which have yet to be clinically confirmed. If done right, it could lead to the production of low-cost medications.[16]

According to an extensive literature study, no phytochemical screening, isolation of bioactive components, or hepatoprotective potential of *Allophylus cobbe* has been proven. Furthermore, the acute and chronic hepatoprotective capacity of this plant has not been tested in numerous animal models. In a study by Chavan *et al.*, the hepatoprotective potentials of *Allophylus cobbe* leaves were screened in rats with promising findings against paracetamol-induced hepatotoxicity.[17] The present study was aimed at screening hepatoprotective potentials of *Allophylus cobbe* leaf extract in both acute and chronic conditions using some hepatotoxicants such as CCl_4, ethanol, and TAA in rats.

Materials and Methods

Source of plant material

Fresh leaves of the *Allophylus cobbe* were collected from surrounding areas of Katyayani, Kolhapur District, Maharashtra, India in the month of June 2018. The authentication and identification of the collected plant samples were done at the Botanical Survey of India, Pune, with a voucher specimen number: BSI/WRC/IDEN.CER. /2018/H3-69.

Sources of chemical reagents

Thioacetamide, ethanol, and carbon tetrachloride were obtained from the Research Lab in Islampur, Sangli. Silymarin was purchased from Spectrum Chemical Manufacturing Corp and Ranbaxy Laboratories Ltd., Baddi, H.P. India. A serum glutamic-pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), acid phosphatase (ACP), and creatinine were estimated by using a diagnostic kit obtained from Pathozyme Diagnostic Ltd., Kagal, Kolhapur, and Medsource Ozone Biomedicals Pvt. Ltd. All the reagents were of analytical grade.

Source of experimental animal

Wistar rats (male or female) weighing 150-180 g were purchased from Crystal Biological Solutions, Uruli Devachi, Pune, Maharashtra, India. All the animals were handled according to the Animal Ethical Committee guidelines of Rajarambapu College of Pharmacy, Kasegaon (1290/PO/Re/S/09/CPCSEA, Protocol No. RCP/P-18/18-19). The animals were kept comfortably in standard polypropylene cages (3 animals in one cage). The standard hygienic environment was maintained at 25-28^{o}C with a 12-h light/dark cycle and provided with a standard pellet diet procured from Pranav Agro, Sangli. Water and food were given *ad libitum* till the end of the investigation.

Preparation of ethanol plant extract

The collected fresh leaves were shade-dried at room temperature until they were completely dried and pulverized with an electrical grinding machine to form a fine powder. This powder was sieved through a 100 mesh to get a uniform particle size and stored in sealed polythene bags. About 100 g of powder was extracted with 500 mL of 95% ethanol using Soxhlet extraction for up to 6 hours. The extract was filtered through Whatman filter paper and further subjected to a rotary evaporator and vacuum dryer to get a concentrated extract which was used for preliminary phytochemical evaluation and animal studies.

Phytochemical analysis of Allophylus cobbe leaf extract

By qualitative phytochemical analysis following standard procedures, flavonoids, phenolic compounds, carbohydrates, tannins, proteins, free amino acids, saponins, steroids, triterpenoids, and glycosides were traced in the ethanol extract by qualitative phytochemical analysis.[18,19]

Acute toxicity evaluation of Allophylus cobbe leaf extract

The toxicological profile of *Allophylus cobbe* leaf extract was analyzed according to the regulations of the Organization for Economic Cooperation and Development (OECD), and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Female non-pregnant Wistar albino rats were kept on fasting before the administration of the test substance by oral feeding tube. After the treatment, the animals were monitored at least daily for the first 30 minutes, then frequently for the first 24 hours. Changes in skin, fur, eyes, mucous membrane, respiration, circulation, and behavioral patterns were observed for a period of 14 days.[20]

In vivo hepatoprotective activity testing in acute and chronic hepatotoxic models

Acute and chronic hepatotoxic models were developed by the administration of CCl_4, TAA, and ethanol to the albino Wistar rats.

Thioacetamide-induced acute hepatotoxic model

All the animals (n=30) were placed into five different groups; each comprising six animals. Group I was fed with saline solution (2 mL/kg of b.w.) and was considered as the control. TAA was administered subcutaneously (s.c.) to Group II animals at a rate of 50 mg/kg for 21 days. Group III animals were given Silymarin (100 mg/kg per oral [p.o.]), a standard drug, for 21 days, and TAA at a dose of 50 mg/kg b.w. subcutaneous administration; 1 hour later, the pre-determined treatment was administered every 72 hours. Group IV and V were given ethanol plant extract at doses of 200 and 400 mg per kg total body weight, respectively, orally for 21 days, while concurrently given TAA at a dose of 50 mg/kg b.w. subcutaneously every 72 h.[21]

Carbon tetrachloride-induced acute hepatotoxic model

The Wistar rats (n = 30) of either sex weighing 175-200 g were divided into five groups, each containing six animals. The Group I animals were treated as negative control and received only vehicle. Groups II to V received 0.125 mL of CCl_4 in the solution of liquid paraffin (1:1) per hundred grams of total body weight intraperitoneally (i.p.). Group II was treated as a positive control and was given CCl_4 treatment. The animals from Group III were administered Silymarin (100 mg/kg) as a standard medicine for up to a week. Groups IV and V were administered with ethanol plant extract at doses of 200 and 400 mg per kg total body weight, respectively, up to a period of one week.[22]

Ethanol-induced chronic hepatotoxic model

All the experimental animals (n=30) were randomly grouped into five, each containing six test animals. The animals in Group I were administered with distilled water once a day for 28 days and were considered the control group. Group II was considered the toxicant group and was administered with 10 ml/kg of ethanol (5 g/kg, 20% w/v p.o.) once a day for 21 days. The animals in Group III were kept on Silymarin, a standard drug at a dose of 100 mg per kg per oral daily for 28 days and 10 ml/kg ethanol (5 g/kg, 20% w/v per oral) each day up to 21 days (8-28 days). Groups IV and V were administered with ethanol extract at doses of 200 and 400 mg per kg total body weight orally each day up to 28 days and 10 ml/kg ethanol (5 g/kg, 20% w/v per oral) daily for 21 days from day 8 to day 28.[23]

Blood sample collection and preparation

Blood samples were collected from the test animals 48 hours after CCl_4, TAA, and ethanol administration by heart puncture or plexus puncture of the retro-orbital. The serum from the blood sample was obtained using a centrifuge (4000 rpm) at 40°C for 15 min and then examined for various biochemical parameters. All the experimental animals were then euthanized with a dose of mild ether anesthesia. The livers of the animals were obtained and small fragments were stored in a solution of 10% formalin for histological testing.[24]

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Estimation of serum biochemical parameters

Various biochemical parameters (liver enzyme markers) were estimated by reported standard methods.[25,26] Serum glutamic-oxaloacetic transaminase (SGOT) and Serum glutamic pyruvic transaminase (SGPT) were evaluated by Reitman and Frankel's method.[27] Alkaline phosphatase **(**ALP) and Acid phosphatase (ACP) were quantified by a modified Kind and King's method.[28] Creatinine was estimated by a modified Jeff's method.[29] Serum enzyme activities were measured using the diagnostic strips via colorimetric analysis at a different wavelength.

Estimation of tissue biochemical parameters

One part of the liver tissues from the sacrificed experimental animals was washed and homogenized in ice-cold 50 mmol/L tris buffer (pH 7.4) in a ratio of 1:10 (w/v). The contents were centrifuged at 10,000 × g for 20 min at 4^{o}C and the supernatant obtained was analyzed for various tissue parameters. Then, lipid peroxidation (LPO) was estimated by malondialdehyde (MDA) using Okhawa's procedure.[30] Glutathione (GSH) was analyzed by its reaction with 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB) by Ellman's method.[31] Catalase (CAT) was evaluated by Aebi's method and superoxide dismutase (SOD) was determined by Marklund's procedure.[32,33] The total level of protein was measured by Lowry's method.[34]

Histopathological analysis

The liver samples from each group were separated and inspected visually for any pathological changes. Then, they were patched in a solution of 10% formalin, dehydrated in a graduated ethanol solution (50-100%), cleaned in a solution of xylene, and fixed in paraffin. About 5 µm thick sections were cut and stained using a dye known as hematoxylin and eosin, and histopathological changes were analyzed microscopically.[35]

Isolation and identification of phytoconstituents from Allophylus cobbe

Dried leaf powder of *Allophylus cobbe* (200 g) was allowed to macerate in the presence of acetone: water (7:3) for 3 days, followed by filtration and removal of the solvent by evaporation to get a concentrated filtrate. This was further extracted three times with dichloromethane. The acetone: water fraction was once again extracted thrice with ethyl acetate. Then, the acetone: water fraction was evaluated for flavonoid content. The obtained extract of *Allophylus cobbe* containing flavonoids was then subjected to column chromatography using silica gel as the stationary phase and benzene: pyridine: formic acid (72:18:10) mobile phase. The eluted fractions were further analyzed by UV, IR, NMR, and mass spectrometry for the presence of flavonoids.

Characterization of active components

The fractions obtained through column chromatography were analyzed spectrophotometrically using a UV spectrophotometer (Jasco). The yellow band obtained from column chromatography was evaporated and dried in a desiccator to obtain a dry powder. This product was mixed with KBr and pressed to get the pellets. The samples were then analyzed using Thermo Nicolet, Avatar 370 at SAIF, Cochin. The isolated and dehydrated sample was subjected to NMR analysis using DMSO as the solvent at 400 MHz. The NMR study was performed at SAIF in Cochin on Bruker type AV 400 MHz equipment. Also, the compounds obtained from the column chromatographic analysis were subjected to mass analysis by dissolving them in methanol. Mass spectrometric analysis was performed using API-2000 Applied Biosystem mass spectrometer (Canada).

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using the GraphPad Prism software package (version 9). The ANOVA was used to compare the studied parameters between the groups, and p values of <0.05 were considered significant.

Results and Discussion

Physicochemical and phytochemical analysis of Allophylus cobbe extract

The physicochemical analysis of *Allophylus cobbe* extract was performed to determine the quality of the plant. The percentage of extraction yield was found to be 22%. The loss on drying (0.04 g), total ash content (0.18 g), extractive value (4.6% [water-soluble]), extractive value (8.25% [alcohol-soluble]) for 4 g, acid insoluble ash content (0 g), and water-insoluble ash content (0.1 g) were observed. The qualitative phytochemical analysis revealed the traces of alkaloids, sterols, triterpenoids, glycosides, flavonoids, and tannins in varying amounts. Carbohydrates, proteins, and saponins were found to be absent in the ethanol extract of the plant.

Acute toxicity analysis of Allophylus cobbe leaf extract

The safe dosage regimen was determined by conducting acute pharmacological toxicity studies. The limit test dose of 2000 mg/kg of total b.w. was carried out using an ethanol extract of leaves as specified by the OECD 423 guidelines. Oral administration at this dose level did not show any sign of behavioral toxicity, neurological toxicity, or mortality. Also, all the animals were alive, active, and healthy during the observation period, hence, ⅕th and ⅒th part of the dosage regimen which was considered as the maximum tolerant level was selected for the efficacy study **(**Table 1). From the results obtained, 200 and 400 mg/kg of total b.w. were accepted as safe for use in *in vivo* studies.

Serum biochemical parameters of albino rats in a chemically-induced hepatotoxicity study

The various serum biochemical parameters, including SGOT, SGPT, ACP, ALP, and creatinine in the CCl_4-, TAA-, and ethanol-induced acute and chronic hepatotoxicity, are presented in Tables 2, 3, and 4, respectively. CCl4, TAA, and ethanol are routinely prescribed hepatotoxic agents that have long been used as a model for testing the hepatoprotective activities of plant extracts and drugs.[36] The hepatotoxicity was marked by elevated levels of serum hepatic markers when observed in the rats treated with CCl_4. There are two steps by which CCl_4 causes hepatic damage. In the first step, cytochrome P450 2E1 transforms CCl_4 to the poisonous trichloromethyl radical ($CCl_3^{\bullet}$) which undergoes metabolism to become trichloromethyl peroxy radical ($CCl_3OO^{\bullet}$). This induces lipid peroxidation and cellular membrane peroxidation, eventually contributing to hepatocyte necrosis.[37] In the second stage, the free radical stimulates kupffer cells, resulting in the production of pro-inflammatory mediators such as TNF (which induces cytotoxicity and cell death) and nitric oxide (NO), which limits mitochondrial respiration and DNA synthesis).[38] The groups were provided with pre-treatment of ethanol extract (200 and 400 mg/kg b.w.) to influence the biochemical parameters in a dose-dependent manner (Table 2). When compared to lower doses, these extracts were found to have a lot of activity. Biological and chemical parameters of serum, such as SGOT, SGPT, ACP, ALP, and creatinine levels, were significantly reduced ($P < 0.01$) in the group of animals given the ethanol extract of *Allophylus cobbe* (EEAC) compared to the standard group given Silymarin. Table 3 shows the findings of serum biochemical parameters of the animals pretreated with the ethanol solution to increase hepatotoxicity utilizing TAA. There was a rise in the concentrations of SGOT, SGPT, ACP, ALP, and creatinine ($P < 0.01$) for the groups administered with TAA, compared to the normal control.

The increase in the level of hepatic biological markers for serum in the animals with TAA showed hepatotoxicity.

Table 1: Acute toxicity study of *Allophylus cobbe leaf extract*

Plant	Extract	Dose (mg/kg)	Observation	Safe dose (mg/kg)
Allophylus cobbe (leaves)	Ethanol	2000	O	> 2000

O: No mortality

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Table 2: Effects of ethanol extract of *Allophylus cobbe* leaves on blood biochemical parameters in a CCl_4-induced hepatotoxicity model

Treatment	SGOT Activity in IU/L	SGPT Activity in IU/L	ACP KA units	ALP KA units	Creatinine mg/dl
Normal control	133.33 ± 19.23	56.67 ± 18.56	1.72 ± 0.32	4.50 ± 0.52	0.40 ± 0.00
CCl_4 (0.125ml, i.p.)	437.33 ± 29.69####	329.67 ± 29.16###	8.67 ± 0.94####	12.93 ± 0.39####	2.67 ± 0.71##
Standard (Silymarin 100 mg/kg)	181.33 ± 14.11****	70.00 ± 11.55***	2.83 ± 0.08****	5.49 ± 0.19****	0.80 ± 0.23**
Allophylus cobbe 200 mg/kg	298.67 ± 29.69*	270.00 ± 61.10ns	4.83 ± 1.01**	8.43 ± 0.52**	1.60 ± 0.40ns
Allophylus cobbe 400 mg/kg	208.00 ± 16.00***	143.33 ± 29.63**	4.08 ± 0.22***	7.25 ± 0.34***	1.07 ± 0.13*

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the CCl_4, treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

Table 3: Effects of ethanol extract of *Allophylus cobbe* leaves on blood biochemical parameters in a thioacetamide-induced hepatotoxicity model.

Treatment	SGOT Activity in IU/L	SGPT Activity in IU/L	ACP KA units	ALP KA units	Creatinine mg/dl
Normal control	74.67 ± 29.69	96.67 ± 8.12	3.58 ± 0.79	6.47 ± 0.34	0.67 ± 0.13
Thioacetamide 50 mg/kg, s.c.	362.67 ± 23.25####	356.67 ± 29.06####	9.58 ± 0.60###	15.09 ± 0.52####	4.80 ± 1.54###
Standard (Silymarin 100 mg/kg)	165.33 ± 37.33**	190.00 ± 11.55***	4.42 ± 0.36***	7.64 ± 0.34****	1.47 ± 0.35**
Allophylus cobbe 200 mg/kg	250.67 ± 41.66ns	243.33 ± 24.04**	6.17 ± 0.60*	12.35 ± 0.52**	3.73 ± 0.61ns
Allophylus cobbe 400 mg/kg	186.67 ± 19.23**	210.00 ± 17.32**	5.25 ± 0.66**	8.23 ± 0.34***	2.40 ± 0.13*

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the thioacetamide treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

Table 4: Effects of ethanol extract of *Allophylus cobbe* leaves on blood biochemical parameters in an ethanol-induced hepatotoxicity model.

Treatment	SGOT Activity in IU/L	SGPT Activity in IU/L	ACP KA units	ALP KA units	Creatinine mg/dl
Normal control	48.00 ± 9.24	113.33 ± 18.56	1.83 ± 0.30	3.13 ± 0.19	1.73 ± 0.35
Ethanol (5 g/kg, 20% w/v p.o.)	298.70 ± 50.88###	280.00 ± 17.32###	7.17 ± 0.58####	14.50 ± 0.52####	5.87 ± 0.58####
Standard (Silymarin 100 mg/kg)	85.33 ± 29.69**	136.67 ± 8.82**	2.25 ± 0.38***	5.68 ± 1.09****	2.80 ± 0.61**
Allophylus cobbe 200 mg/kg	197.33 ± 29.69ns	176.67 ± 21.86*	4.92 ± 0.30ns	9.02 ± 1.97**	4.00 ± 0.23*
Allophylus cobbe 400 mg/kg	133.33 ± 34.97*	160.00 ± 34.64**	3.08 ± 0.74**	7.84 ± 0.52***	3.07 ± 0.48**

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the ethanol treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

TAA is an essential hepatotoxin that belongs to the group of organosulfuric compounds and is used in animal hepatotoxicity models. Based on adequate data in laboratory animals, TAA is considered a potential human hepato-carcinogen. More toxic metabolites, such as thioacetamide-S-oxide (TASO), thioacetamide-S, S-dioxide (TASO2), and their derivative radicals, are formed by the bio-oxygenation of TAA's S-atom by mammalian P450E1 (Phase-I cytochrome) and other monooxygenases.[39] TAA also causes a rise in intracellular Ca^{2+} concentration in hepatocytes via TNF (tumour necrosis factor) and IFN (interferon). The combined effects of oxidative stress on hepatocytes contribute to the establishment of macrophage populations (Kupffer cells).[40] These conditions induce liver damage and fibrosis by altering the normal histology of hepatocytes. If left unchecked, liver fibrosis can lead to portosystemic shunts, hepatitis, and hepatocellular carcinoma. Pretreatment with ethanol extracts at doses of 200 and 400 mg per kg total body weight primarily regulated the change in biochemical parameters in a dose-dependent manner. All the extracts at a dose level of 400 mg/kg showed remarkable activity when compared to lower doses. There was a significant decrease ($P < 0.01$) in the serum biochemical parameters like SGOT, SGPT, ACP, ALP, and creatinine in the animals pretreated with the EEAC compared to the standard group, treated with Silymarin.

In the present investigation, p.o. administration of ethanol at a concentration of 5 g/kg, 20% w/v p.o. once a day for 28 days showed changes in serum hepatic biochemical parameters, mainly SGOT, SGPT, ACP, ALP, and creatinine (Table 4). Chronic ethanol ingestion induces cellular and tissue damage, as well as cellular enzyme leakage and other biochemical parameters.[41] Alcohol causes oxidative damage to the liver by creating a lot of reactive oxygen species (ROS) and adducts. Also, alcohol dehydrogenase (ADH) produces acetaldehyde, which is then oxidized by aldehyde dehydrogenase (ALDH) to acetate. Acetaldehyde can result in the development of hybrid adducts by reacting with reactive residues (such as the malondialdehyde [MDL] adduct) that work on proteinaceous, tiny particle-like cysteines, triggering peroxidation of lipid, as well as oxidation of nucleic acid. During the metabolism of alcohol, further oxidations are followed by an unnecessary reduction of nicotinamide adenine dinucleotide

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(NAD), resulting in a changed proportion of NADH to NAD. Their shuttering into mitochondria is caused by a rise in NADH levels. The development of reactive oxygen species (ROS) in mitochondria results in cell damage and necrosis.[42] Each extract with a dose range of 400 mg/kg showed remarkable action compared to lower doses. There was a significant decrease ($P < 0.01$) in the biological and chemical parameters of serum, including SGPT, ACP, ALP, SGOT, and creatinine in the animals pretreated with a lesser dose of the ethanol extracts compared to that of the standard group, administered with Silymarin. All these findings support the previously proposed hypothesis that healing in the parenchyma in the hepatic region with a re-appearance of hepatocytes assists in normalization of transaminase and other biochemical markers. The findings of the normalization of multiple biochemical markers are outstanding evidence of *Allophylus cobbe's* hepatoprotective potential in both acute and chronic hepatotoxicity.

Tissue biochemical parameters of albino rats in a chemically-induced hepatotoxicity study

The tissue biochemical parameter changes in CCl_4-, TAA-, and ethanol-induced acute and chronic hepatotoxicities are presented in Tables 5, 6, and 7, respectively.

Table 5: Effects of ethanol extract of *Allophylus cobbe* leaves on tissue biochemical parameters in a CCl_4-induced hepatotoxicity model

Treatment	SOD U/min/ g protein	CAT U/mg of protein	GSH nmol/ min / mg protein	LPO moles MDA/ mg proteins/mL	Total proteins mcg/mL
Normal control	269.23 ± 7.69	4.63 ± 0.07	0.42 ± 0.04	66.77 ± 6.70	175.25 ± 4.67
CCl_4 (0.125ml, i.p.)	92.30 ± 13.32####	1.50 ± 0.09####	0.10 ± 0.01####	252.57 ± 8.87####	48.96 ± 5.20####
Standard (Silymarin 100 mg/kg)	223.08 ± 7.69***	2.37 ± 0.16****	0.29 ± 0.03***	131.61 ± 15.84**	99.69 ± 9.73***
Allophylus cobbe 200 mg/kg	176.92 ± 15.39*	1.68 ± 0.03ns	0.23 ± 0.02*	196.45 ± 5.39ns	67.04 ± 1.89ns
Allophylus cobbe 400 mg/kg	199.99 ± 20.35**	1.91 ± 0.09*	0.25 ± 0.01*	149.03 ± 37.19**	76.48 ± 5.06*

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the CCl_4, treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

Table 6: Effects of ethanol extract of *Allophylus cobbe* leaves on tissue biochemical parameters in a thioacetamide-induced hepatotoxicity model

Treatment	SOD U/min/g protein	CAT U/mg of protein	GSH nmol/min/mg protein	LPO moles MDA/ mg proteins/mL	Total proteins mcg/mL
Normal control	287.50 ± 6.25	4.34 ± 0.03	0.42 ± 0.02	46.45 ± 7.31	161.76 ± 15.02
Thioacetamide 50 mg/kg, s.c.	87.50 ± 6.25####	1.502 ± 0.09####	0.07 ± 0.01####	174.19 ± 19.76###	27.10 ± 2.14####
Standard (Silymarin 100 mg/kg)	243.75 ± 10.83***	2.33 ± 0.07****	0.25 ± 0.01***	81.29 ± 15.99**	81.34 ± 6.74**
Allophylus cobbe 200 mg/kg	187.50 ± 21.65*	1.74 ± 0.07ns	0.17 ± 0.01*	141.28 ± 12.36ns	67.04 ± 3.78*
Allophylus cobbe 400 mg/kg	218.75 ± 12.50**	2.13 ± 0.09***	0.18 ± 0.02*	99.67 ± 8.96*	71.89 ± 4.34**

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the thioacetamide treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

Table 7: Effects of ethanol extract of *Allophylus cobbe* leaves on tissue biochemical parameters in an ethanol-induced hepatotoxicity model

Treatment	SOD U/min/ g protein	CAT U/mg of protein	GSH nmol/ min /mg protein	LPO moles MDA/ mg proteins/mL	Total proteins mcg/mL
Normal control	278.57 ± 12.37	4.25 ± 0.08	0.31 ± 0.03	59.99 ± 5.89	150.96 ± 9.35
Ethanol (5 g/kg, 20% w/v p.o.)	85.71 ± 12.37####	1.42 ± 0.23####	0.08 ± 0.01####	155.80 ± 23.78##	46.53 ± 1.69####
Standard (Silymarin 100 mg/kg)	192.85 ± 12.37***	2.44 ± 0.07***	0.19 ± 0.02**	66.77 ± 8.38**	86.19 ± 4.67***
Allophylus cobbe 200 mg/kg	142.85 ± 14.28*	1.73 ± 0.13ns	0.14 ± 0.01ns	85.15 ± 15.21*	59.48 ± 2.14ns
Allophylus cobbe 400 mg/kg	164.28 ± 14.29**	2.15 ± 0.08**	0.15 ± 0.01*	78.38 ± 17.74*	69.47 ± 2.82*

All the values are expressed in mean ± S.E.M. (n=06). *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$ was considered statistically significant value when compared with the ethanol treatment groups and #: $p < 0.05$, ##: $p < 0.05$ and ###: $p < 0.001$ was considered statistically significant value when compared with the normal control group. ns: Non significant value where $p > 0.05$.

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In contrast to normal controls, the results of tissue biological and chemical criteria during the pre-treatment of solution of ethanol extract to induce hepatotoxicity using CCl_4 showed significant reductions ($P < 0.01$) in total protein, SOD, as well as the level of GSH, followed by an increase in the levels of CAT and LPO as observed in animals treated with carbon tetrachloride. A remarkable change in the tissue biochemical markers in the animals treated with CCl_4 (hepatotoxicant) is considered an indication of hepatic toxicity. As observed in the tissue biochemical estimations, there was a significant increase ($P <0.01$) in total proteins, SOD, and GSH levels in the animals administered with the ethanol extracts compared to the toxic control group. As indicated in Table 5, the result was comparable to that of a normal animal given Silymarin. Simultaneously, CAT and LPO concentrations were significantly reduced ($P< 0.01$) in the EEAC extract groups.

Table 6 highlights the results of tissue biochemical estimates of animals pretreated with ethanol extracts due to TAA-induced liver damage. In comparison to the normal control, the animals treated with TAA showed significant reductions (P 0.01) in total protein, SOD, and GSH concentrations, followed by a significant increase in CAT and LPO levels. A noticeable change in the biochemical indicators in the animals administered with TAA was an indication of hepatic toxicity. Pretreatment of ethanol extracts normalized the altered tissue biochemical parameters. A significant increase ($P < 0.01$) in the levels of total proteins, SOD, and GSH was observed compared to the toxic control group. Meanwhile, the results were similar to those of the standard silymarin-treated animal group, in which tissue biochemical estimates revealed that CAT and LPO levels were significantly (P 0.01) reduced. Furthermore, it also reduced the total proteins, SOD, and GSH and increased CAT and LPO levels in the animals treated with ethanol. This result indicates an altered permeability of the plasma membrane. These observations might be the result of lipid peroxidation by free radical oxygen. Pretreatment of ethanol extracts normalized the altered tissue biochemical parameters in the ethanol-induced hepatotoxic animal model. Total proteins, SOD, and GSH levels increased significantly (P 0.01) when compared to the control group, with results comparable to those observed in the standard silymarin administered group, but CAT and LPO levels declined significantly (P 0.01) in tissue biochemical estimation (Table 7). Reactive oxygen species (ROS), scavengers of free radicals, and terminators of sequence reactions are all responsible for protecting the liver from harmful and toxic substances.[43] However, it has been discovered that the administration of CCl_4, TAA, and ethanol lowers the activity of SOD, GSH, and CAT in the liver, increasing the concentrations of ROS, free radicals, and hepatic cell damage.[44] In contrast, feeding animals with ethanol plant extract in acute and chronic hepatotoxic rat models, increases the influence of CAT, SOD, and GSH, while decreasing LPO levels. The antioxidant properties of flavonoids and phenolic compounds found in ethanol extracts may help to explain why the extract is hepatoprotective.

In previous research, similar observations were made in paracetamol-induced hepatotoxic rat models.[17] As a result of ethanol extract treatment, serum and tissue biochemical parameters are considerably stabilized. The 400 mg per kg extract groups had substantial protection against the development of chronic ethanol-, CCl_4- and TAA-induced toxicity in the experimental animals, which was similar to the effect observed in the silymarin group. This finding was linked to the widely held assumption that as the hepatic parenchyma heals and hepatocytes regenerate, serum transaminase levels and other biochemical signs will return to normal.[45]

Histopathological observation of rat livers in a chemically-induced hepatotoxicity study

The hepatic protective potential exhibited by EEAC was further determined through histopathological analysis.

Histopathological observations (Figure 1A) indicated that in the normal control group of animals, normal liver cells with lob structure, normal liver cell structure, and no alterations in disease were observed. Meanwhile, treatment of animals with CCl_4 shows degeneration of hepatic cells with moderate to severe hepatic cell necrosis as a result of CCl_4 toxicity (Figure 1B).

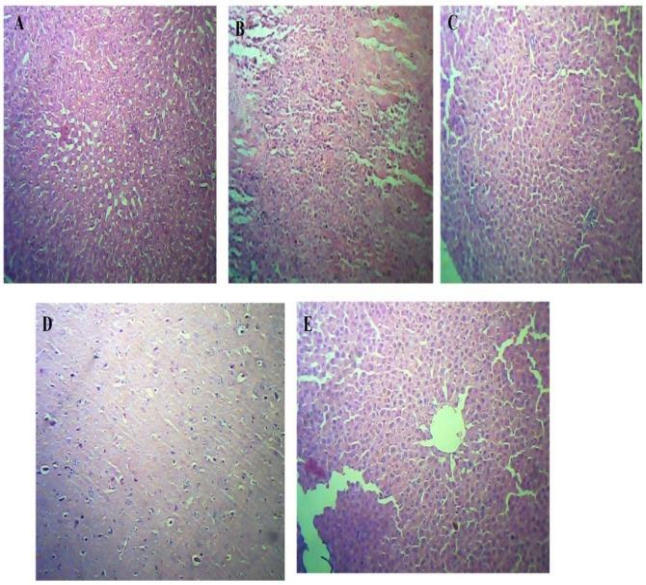

Figure 1: Histopathology of rat liver in a CCl_4-induced hepatotoxicity model.
A: Normal control group, showing well defined liver architecture with normal hepatic cells without any damage; B: CCl_4-treated group, showing the degenerative hepatocytes with necrosis causing severe degree of liver damage; C: Standard silymarin (100 mg/kg)-treatment group, showing the mild degree of liver damage and restoring hepatocytes; D and E: Groups treated with *Allophylus cobbe* (200 mg/kg and 400 mg/kg, , respectively) showing the regeneration of liver cells with mild degree of liver damage.

The reversal of these changes was observed in the animals administered with an extract of ethanol, with a lower rate of degeneration, as well as liver necrosis (Figures 1D and E) similar to that of silymarin-treated animals (Figure 1C). The histological observations were strongly supported by biochemical analysis of serum and tissue outputs, and the hepatic protective effects of ethanol extracts in TAA-mediated hepatic toxicity were also confirmed by comparing the histopathological analysis of treated liver tissues with normal, toxic, and standard drug tissues. The histopathological analysis of the normal group showed normal liver cellular structure without any damage to hepatocytes (Figure 2A), while liver tissues treated with TAA exhibited noticeable fatty changes, necrosis, as well as cellular boundary loss (Figure 2B). These defaults in histopathological observations in the structure of the liver were visibly normalized in the ethanol extracts at 200 and 400 mg per kg administered animals (Figures 2D and E), equal to the concentration of silymarin administered groups (Figure 2C), demonstrating its prominent hepatic protective action.

There was a micro-vascular change in fat deposits in the groups with ethanol administration (Figure 3B). The animals treated with EEAC and silymarin (Figure 3D, E, and C) had fewer micro-vascular alterations in lipids with normal structure, indicating that the extracts provide better hepatic protection than other extracts. Similarly, the histological architecture of the normal and ethanol-treated groups revealed normal liver cellular structure without any damage to hepatocytes (Figure 3A). Meanwhile, liver tissues from animals treated with ethanol showed more micro-vascular changes in fatty deposits, necrosis, and loss of cellular boundaries (Figure 3B), while their architectures in standard drugs were normal (Figure 3C). The EEAC -treated animal tissues showed potent hematopoietic activity (Figures 3D and E). Organ destruction and elevated serum enzyme levels can cause mixed hepatic cellular injury. In the current research, elevated serum levels of various markers, including SGOT, SGPT, ACP, ALP, and creatinine, revealed hepatotoxicity as a mixed hepatic cell type injury. However, EEAC decreased serum enzyme levels, suggesting that they were hepatocyte-defensive. Also, they preserved natural liver physiology, inducing plasma membrane stabilization and

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regeneration of weakened liver cells by restoring hepatic parenchymal cells and normalizing the permeability of the altered cells. An ethanol extract of *Allophylus cobbe* demonstrated hepatoprotective efficacy, which was confirmed by histopathological observations.

Identification and characterization of active phytoconstituents of Allophylus cobbe extract

The spectral analysis of the isolated compound revealed the following characteristics: UV λmax: 371; IR: 3405.42 (O-H stretching), 2840.45 (CH_2 stretching), 2794.01 (C-H stretching), 1665.03 (C=O stretching), 1611.10 (C=C stretching); ^{1}H-NMR (DMSO; 500 MHz; δ ppm); 3.301, 3.317 (d, 2H, O-H), 4.631 (d, 2H, O-H), 4.906 (s, 1H, O-H), 6.177, 6.182 (d, 1H, Ar-H), 6.284, 6.389 (d, 1H, Ar-H), 6.872, 6.894 (d, 1H, Ar-H), 7.623, 7.644 (m, 1H, Ar-H), 7.729, 7.735, (d, 1H, Ar-H), MS (*m/z*) 302.23. Using ethanol, the UV spectra of the extracted compound from *Allophylus cobbe* leaves were obtained. The UV spectrum of the isolated fraction is presented in supplementary data file figure which produced a peak of isolated compound at 371 nm. The standard showed a characteristic peak at 369 nm. As compared with the standard, it was observed that the compound belongs to the group of flavonoids and was quercetin. Furthermore, the structure of the isolated compound was identified by the Fourier Transformed-Infra Red (FT-IR) spectrophotometer. The values of the infra-red spectra were measured in cm^{-1} and the results are shown in supplementary data file figure. The characteristic peaks are associated with quercetin as previously reported by Catauro *et al.*[46] These observations in the IR study confirmed the presence of quercetin. FT-NMR (BRUKER AMX 400MHZ) study was used to deduce the structural data from the isolated compound. The proton NMR spectrum of the isolated compound is given in supplementary data file figure. The proton NMR spectra showed peaks for aromatic and non-aromatic protons which resembles the structure of quercetin (Figure 4).

The mass spectrum of the isolated fraction is given in supplementary data file figure which produced a molecular ion peak at *m/z* 303.04 (M+1) ($C_{15}H_{11}O_7$). The production of ion peaks was observed at m/z 285.03 ($C_{15}H_9O_6$), 257.04 ($C_{14}H_9O_5$), 247.06 ($C_{13}H_{11}O_5$), 229.04 ($C_{13}H_9O_4$), 201.05 ($C_{12}H_9O_3$), 165.01 ($C_7H_5O_4$), 153.01 ($C_7H_5O_4$), 137.02 ($C_7H_5O_3$), 121.02 ($C_7H_5O_3$), 111.00 ($C_7H_3O_3$), and 95.04 (C_6H_7O). From the above fragmentation pattern, the structure of the isolated compound confirmed its identity as quercetin ($C_{15}H_{10}O_7$).

Quercetin has been identified as a primary phytochemical in *Allophylus cobbe* using analytical elucidation techniques. A flavonoid that is available in numerous quantities, known as quercetin, is found in vegetables, as well as fruits. The drug's therapeutic activity can be used to prevent and treat a variety of diseases, including cardiovascular, cancer, and neurological diseases. Quercetin has been shown to inhibit oxidation, inflammation, and carcinogenic action in a variety of cellular, animal, and human models by modifying the signaling and gene expression pathways involved in these processes.[47] Yuan-Lu *et al*, reviewed the antioxidant activities of quercetin and its complexes for medicinal applications.[48] Marjia *et al*. demonstrated the drug's antioxidant and anti-inflammatory properties, concluding that after quercetin intake, quercetin derivatives in the bloodstream can act as powerful antioxidants and anti-inflammatory agents, contributing to the complete biological potential of foods with higher quercetin content.[49] The antioxidant and liver-protective properties of quercetin 7-rhamnoside (Q7R) were demonstrated *in vitro* and *in vivo* by Li *et al*. In a dose-dependent manner, Q7R inhibited the upward regulating role of serum activity of AST, LDH, TG, and ALT. In an animal model, Q7R lowered MDA generation while raising liver GSH levels and antioxidant enzyme CAT activity, suggesting that it could be a hepatoprotective drug.[50] CCl_4, TAA, and ethanol mediated acute and chronic hepatotoxicity in albino Wistar rats were significantly reduced as a result of the presence of quercetin antioxidant, a hepatoprotective flavonoid constituent in ethanol extracts of *Allophylus cobbe* leaves.

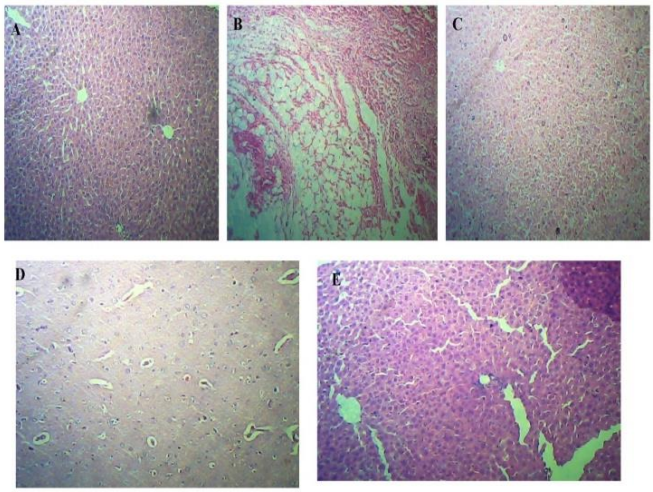

Figure 2: Histopathology of rat liver in a TAA-induced hepatotoxicity model.
A: Normal control group, showing well defined liver architecture with normal hepatic cells without any damage; B: TAA-treated group, showing the degenerative hepatocytes with necrosis causing severe degree of liver damage; C: Standard silymarin (100 mg/kg)-treatment group, showing the mild degree of liver damage and restoring hepatocytes; D and E: Groups treated with *Allophylus cobbe* (200 mg/kg and 400 mg/kg, respectively), showing the regeneration of liver cells with mild degree of liver damage.

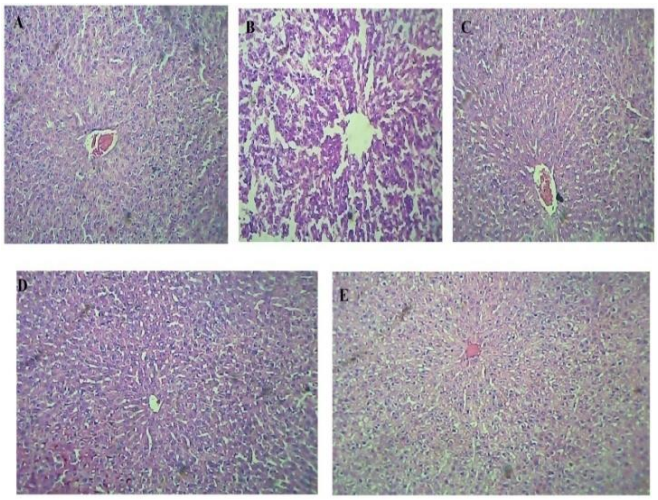

Figure 3: Histopathology of rat liver in an ethanol-induced hepatotoxicity model.
A: Normal control group, showing well defined liver architecture with normal hepatic cells without any damage; B: Ethanol-treated group, showing the degenerative hepatocytes with necrosis causing severe degree of liver damage; C: Standard silymarin (100 mg/kg)-treatment group, showing the mild degree of liver damage and restoring hepatocytes; D and E: Groups treated with *Allophylus cobbe* (200 mg/kg and 400 mg/kg, respectively), showing the regeneration of liver cells with mild degree of liver damage.

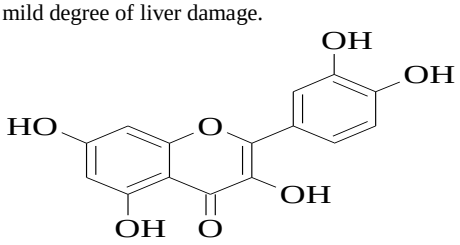

Figure 4: Chemical structure of the isolated compound from *Allophylus cobbe*

Conclusion

In the CCl_4-, TAA-, and ethanol-mediated hepatotoxicity models, ethanol extracts of *Allophylus cobbe* leaves demonstrated outstanding hepatoprotective capacity in acute and chronic conditions. The hepatoprotective potential was substantiated by a significant decrease in SGOT, SGPT, ACP, ALP, creatinine, and LPO levels, as well as improvements in total proteins and defense enzymes including SOD, CAT, and GSH. According to the histopathological observations, the administration of the plant's ethanol extract resulted in the healing of hepatic cellular damage. Chromatographic analysis of the extract led to the isolation of quercetin which may be responsible for the hepatoprotective properties of the plant.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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