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CELLULAR TOXICITY INDUCED BY ALCOHOL IN VITRO: COMPARATIVE STUDY OF ANTIOXIDANT, HEPATOPROTECTIVE, ANTI-HEMOLYTIC AND DNA PROTECTIVE ACTIVITY OF NATURAL VS SYNTHETIC VITAMIN C

Abstract

Alcohol-induced oxidative stress is a major contributor to alcoholic liver disease (ALD). In this study short term alcohol induced cyto-toxicity model was used to simulate some attributes of ALD *in vitro*, producing hepatocellular injury, red cell (RBC) haemolysis and DNA damage. The antioxidant, hepatoprotective, anti-hemolytic and DNA protective efficacy of fresh *Phyllanthus emblica* (amla) juice, *Citrus limon* (lemon) juice, and synthetic vitamin C was compared. Antioxidant activity was assessed through ABTS and DPPH radical scavenging assays, hepatoprotective effects using goat hepatocytes exposed to ethanol (MTT assay and AST assay), membrane stabilization through RBC hemolysis assay and DNA protective potential through comet assay. Results demonstrated that both amla and lemon juice exhibited strong antioxidant activity, with efficacy broadly comparable to synthetic vitamin C. Amla juice extract showed slightly greater hepatoprotective, anti-hemolytic, and DNA protective activity, while lemon exhibited higher catalase-stabilizing effects. These findings suggest that fresh dietary intake of amla and lemon may serve as accessible, cost-effective alternatives to synthetic supplements for mitigating alcohol-induced oxidative damage. Further *in vivo* and clinical studies are recommended to validate their preventive and therapeutic potential in ALD management.

Key words: Alcohol-induced oxidative stress, Hepatoprotective activity, Comet Assay, RBC Hemolysis, *Phyllanthus emblica*

Introduction

The liver is considered to be one of the most vital organs that functions as the centre of metabolism of nutrients such as carbohydrates; proteins and lipids; and excretion of waste metabolites (1). Chronic alcohol abuse is a significant cause of cirrhosis and liver failure in adults worldwide, especially in India where it is prevalent between 9% to 32% (2). Alcoholic liver disease (ALD) is a pathological process characterized by progressive liver damage that leads to steatosis, steatohepatitis, fibrosis and, finally, cirrhosis (3). Eventually, cirrhosis may progress to hepatic decomposition and hepatocellular cancer. Oxidative stress plays an important role in this process.

Many medicinal plants contain bioactives which can be used for therapeutic purposes. However, the development of bioactives from medicinal plants is a long and costly process in particular due to regulatory requirements for quality, safety and efficacy. Therefore, non-clinical toxicity testing is important and a mandatory part towards drug development and introduction of alternative methods for toxicity testing is necessary.Additionally, over the past decade, herbal medicines have attracted much attention as potential therapeutic agents in the prevention and treatment of (ALD), due to their multiple targets and less toxic side effects (4) Several herbs such as *Cnidiummonnieri* (L.) Cusson (Apiaceae), *Curcuma longa* L. (Zingiberaceae) and *Pueraria lobata* (*Willd.*) Ohwi (Leguminosae), etc., have been shown to be quite effective and are being widely used. (5**)** Standardized extracts of these herbs are used for therapeutic purposes. But unlike standardized commercial extracts, amla and lemon juice were tested in their raw, non-extracted, edible form to mimic real-world dietary consumption. This approach was adopted to reflect actual consumer usage rather than purified extracts, avoid exposure to additives and preservatives typically present in market-available preparations, and to evaluate their direct antioxidant and hepatoprotective potential in a simple, reproducible model.

By experimenting on *Phyllanthus emblica* (amla) and *Citrus limon* (lemon), widely available plants in India, this study aims to evaluate and compare the efficacy of these botanicals in mitigating alcohol-

induced oxidative liver damage. The findings may contribute to the growing body of evidence supporting the integration of dietary interventions into preventive strategies against ALD.

ALD is the most prevalent chronic liver disease in the global population (6). The liver is responsible for most detoxification processes in the body, making any damage to the liver have significant effects on the entire body. Alcohol is absorbed from the gastrointestinal tract and circulated throughout the body. Less than 10% is removed from the lungs, kidney and sweat. Alcohol dehydrogenase (ADH) in the liver metabolizes a large proportion of the alcohol to acetaldehyde which is further oxidized, with the help of the enzyme mitochondrial aldehyde dehydrogenase ($ALDH_2$) to acetate (7). Most of the acetate, along with some of the acetaldehyde is then removed from the liver and enters the bloodstream where it is peripherally metabolized further. Chronic alcohol consumption results in higher than regular levels of acetaldehyde in the blood. This is highly toxic due to mechanisms such as oxidative stress, glutathione depletion and lipid peroxidation. There is a decrease in fatty acid oxidation and an increase in the synthesis of triglycerides. The disease progresses from alcoholic fatty liver (AFL) to alcoholic steatohepatitis (ASH). AFL develops in most individuals that consume >40 g alcohol per day (8). Chronic ASH may lead to cirrhosis, or irreversible scarring of tissue, in some cases, liver failure, which may be life threatening. With the established prevalence of ALD and increased research into Drug Induced Liver Injury, more people are seeking 'alternative' or 'complementary' treatment (9). Herbal or botanical treatments, such as tea have been widely used for this purpose, particularly in India. Thus, this project was designed to test the effectiveness of the extracts of common plants available in India- Amla and Lemon.

***Phyllanthus emblica L.* (Amla)**

Phyllanthus emblica, commonly known as amla or Indian gooseberry, is a deciduous tree whose fruit is a staple in traditional Indian diets and medicinal systems. Amla is especially prized for its exceptionally high vitamin C content and an array of potent bioactive phytochemicals, including alkaloids, ellagitannins, gallic acid, emblicanin A and B, as well as flavonoids such as rutin and quercetinc(10)These compounds are at the heart of amla's well-documented antioxidant, anti-inflammatory, and hepatoprotective effects. Multiple studies confirm that amla extracts can attenuate alcohol and toxin-induced liver injury by scavenging free radicals, reducing oxidative stress, and restoring hepatic antioxidant enzymes (11, 12, 13). The fruit's efficacy in lowering serum ALT and AST levels and preventing hepatic fat accumulation underscores its protective effect on liver health. Traditionally valued for its broad health benefits, the scientific exploration of *Phyllanthus emblica* provides robust support for its inclusion in hepatoprotective interventions, especially in populations with elevated risk factors for liver disease.

***Citrus limon (L.) Osbeck* (Lemon)**

Citrus limon, or lemon, is a widely cultivated citrus species whose fruit is a familiar component in daily Indian cuisine and remedies. Rich in vitamin C, lemons also offer an impressive spectrum of bioactive substances, including flavonoids (notably hesperidin), limonin, and essential oils. These physiologically active constituents endow lemon with strong antioxidant, anti-inflammatory, and antimicrobial abilities. Of particular interest is its hepatoprotective potential—lemon extracts and juice have been shown to protect against alcohol-induced hepatic injury by reducing liver enzyme (ALT, AST) elevations, decreasing lipid peroxidation, and enhancing endogenous antioxidant defences such as glutathione. The widespread culinary use of lemon is enhanced by these additional therapeutic benefits, with research increasingly supporting its role in liver health management. Continued scientific validation of *Citrus limon* in hepatoprotective studies may further encourage its integration as a natural agent for preventing and ameliorating liver damage, especially in environments where conventional remedies may present risks or side effects.

Usage of *in vitro* assay procedure to test short term cyto-toxicity of different drugs and cyto-protection by different agents has now been widely accepted. Although there are some reservations among scientists whether *in vivo* mechanisms can be simulated *in vitro*, it is now established that precious information can be obtained from appropriately designed *in vitro* experimental procedures and diverse agents can be screened in one go. Thus, these studies are cost effective and time saving yet generate information regarding toxicity, cell membrane damage, status of oxidative stress, loss of enzymes, damage to nucleic acids, etc.

With this background our aim was to evaluate and compare the *in vitro* antioxidant activity of *Phyllanthus emblica* (amla juice), *Citrus limon* (lemon juice) and synthetic Vitamin C and to study cytoprotective activity of these test samples against short term cyto-toxicity induced by alcohol in *ex vivo* model with freshly isolated hepatocytes from goat liver. We also aim to delineate RBC membrane stabilising activity and DNA protecting activity of amla juice, lemon juice and synthetic vitamin C.Most of the studies have been done using purified extracts, but this study has been done with raw, unpurified extracts, thereby mimicking daily dietary consumption and these can form an important part of the diet.

Materials and methods

2,2';-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Fetal bovine serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were obtained from Sigma, USA. Reagents for Hanks' Balanced Salt Solution (HBSS), Ethydium Bromide (EtBr), Low melting point agarose (LMPA), Normal Melting Point Agarose (NMPA) and all the other reagents were of analytical grade and purchased from Merck, India. Aspartate aminotransferase (AST) kit was procured from a local supplier made by Autospan, India. Amla and lemon were procured from the local market. Those were crushed, pressed and filtered to obtain fresh juice. Synthetic Vitamin C was procured from Merck Millipore, India.

Screening of *in vitro* antioxidant activity

ABTS and DPPH assay

In 2,2';-azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS) assay, the antioxidant reduces bluish green coloured ABTS radical to colourless ABTS which can be measured spectrophotometrically (14). For ABTS, test samples, amla juice, lemon juice and vitamin C (at a concentration of 10-40µl) were added to 1 ml ABTS solution, mixed thoroughly and kept for 4 minutes. After that absorbance was recorded at 734 nm using UV-VIS spectrophotometer (UV 1800, Shimadzu, Japan).

DPPH assay is based on scavenging of the purple chromogen radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) by the antioxidants, which produces a light yellow to colourless solution that is measured at 515 nm (15). Different test samples at a concentration of 10-40**µ**l were added to 1 ml DPPH solution, mixed properly and kept for 15 minutes followed by measuring the absorbance at 515 nm. The anti-oxidant potential of test samples has been calculated from the regression curve deducing the IC_{50} (inhibitory concentration) for each sample for both ABTS and DPPH. A lower IC_{50} meant better radical scavenging activity or antioxidant activity.

Hepatoprotective activity

The *in vitro* hepatoprotective activity of the test samples was tested on fresh hepatocytes isolated from goat liver obtained from local abattoir. Goat liver was obtained from a local abattoir and brought on ice within 30 minutes. Approximately 2 g of liver was taken and minced into minute pieces and were strained using a cell strainer of 40 µl pore size (Genetix cell strainer, S.Korea). Hepatocytes were then washed in HBSS. After removal of debris by centrifugation, cells were kept in 10% FBS supplemented 1% RPMI and preincubated with individual test samples (amla juice, lemon juice and vitamin C) for 30 minutes followed by exposure to 80 % ethanol for 2 hours at 37°C. After incubation, samples were centrifuged at 1000 rpm for 5 minutes, supernatant were kept and the cells were resuspended in MTT solution (0.5mg/ml) and incubated for another 1 hour at 37°C, followed by centrifugation and dissolution of formazan crystal in dimetyhl sulfoxide (DMSO) to obtain dark violet colour, which were measured spectrophotometrically at 570 nm (16). Results were expressed as the percentage of cell viability relative to the control cells (assuming 100%) in our study.

The concentration of liver enzyme aspartate aminotransferase (AST) in the cell supernatant indicates degree of cell membrane damage/protection and enzyme loss and status of hepatic health. In this study, AST was estimated from the cell supernatant using aspartate aminotransferase (AST) assay kit.

Protection against RBC haemolysis

To evaluate the potential of amla juice, lemon juice and synthetic vitamin C against alcohol induced haemolysis, *in vitro*Malagoli's (17) method was followed. Healthy male mice were obtained from animal house of Union Drug Company Limited, Kolkata (721/PO/RC/S/02/CPCSEA dated 25.01.2023). Blood was collected on EDTA by puncturing the orbital plexus of healthy mice and centrifuged at 1000 rpm for 5 minutes. Then 10% erythrocyte suspension was prepared in phosphate buffered saline (PBS, pH 7.4) for haemolytic studies. Test samples were added in a varied concentration (from 0.10 µl/ml to 2µl/ml for amla & lemon juice while 5 to 20 µg/ml for Vitamin C) to 100 µl of 10% RBC suspension and preincubated for 30 minutes at 37 °C followed by exposure to 80% ethanol for 1 hour.. Then cells were centrifuged and the supernatant was used to measure the absorbance for the liberated hemoglobin or protection against it spectrophotometrically at 540 nm. Two controls were prepared without extracts; negative control with PBS, while 80% ethanol was taken as the positive control. The protection against haemolysis was calculated by obtaining IC_{50} from the regression equation. Catalase activity was assayed using RBC haemolysate following the method of Aebi et al (18).

DNA protective activity using Comet Assay

The geno-protective potential of the test samples was assessed by single-cell gel electrophoresis comet assay following the method of Singh et al. 1988 (19). One (1) ml blood was collected from orbital plexus puncture of healthy mice. 100µl of heparinised whole blood was incubated with 20µl 80 % alcohol and 50 µl test samples at 37°C for 2h in a CO_2 incubator. The negative and positive controls were included. 25 µl cell suspensions were embedded in 75 µl of 0.5% low melting point agarose (LMPA) and then spread on a slide pre-coated with a film of 1% normal melting point agarose (NMPA). Two slides were prepared for each sample in which agarose cell suspensions were allowed to solidify at 4°C. After solidification, slides

were immersed to cold lysis buffer, (2.5 M NaCl, 100 mM EDTA, 10 mM Tris buffer, 10% DMSO, Triton X-100 0.8%, pH 10) for 1 h. The slides were removed from the lysis buffer and placed on a horizontal gel electrophoresis chamber, filled with alkaline electrophoresis buffer (1mM EDTA, 0.3N NaOH, pH 13.0) for 20 min for unwinding of DNA. Then, electrophoresis was performed for 30 min at 25V/300mA and electrophoresis slides were neutralized (three times) and stained with ethidium bromide solution (20mg/ml). The stained nuclei were visualized under fluorescent microscopy and photographed. Olive Tail Moment (OTM) of individual stained nuclei was calculated using comet assay software (CaspLab). A lower percentage tail DNA indicated a lower level of DNA damage and higher level of genoprotective activity of test samples.

Results

Table 1. *in vitro* antioxidant activity of different test sample using ABTS and DPPH radical scavenging assay

Groups	ABTS (IC_{50})	DPPH (IC_{50})
Amla juice	3.37 µl/ml	9.09 µl/ml
Lemon juice	2.82 µl/ml	9.79 µl/ml
Vitamin C	1.61 µg/ml	3.8 µg/ml

Experiments were done in triplicate sets, values are mean of three data

Hepato-toxicity (MTT assay)	
Groups	**% viability**
Amla juice	77.6
Lemon juice	70.4
Vitamin C	79.4

Alcohol	42.7
Control	100

Table 2. Assay of hepato-protective activity of different test samples against alcohol induced liver damage, by MTT assay

Experiments were done in triplicate sets, values are mean of three data

Table 3: Effect of alcohol on AST release and protection with test samples

AST	
Groups	**AST (IU/L)**
Amla juice	116.4 ± 36.6*
Lemon juice	121.8 ± 39.4*
Vitamin C	108.1 ± 38.8*
Alcohol	292.0 ± 11.9
Control	111.7 ± 47.9

Data are in Mean ± SD,n=3, *p<0.005-0.001, Statistical analysis have been done using Students Paired 't' test, and comparison was done between alcohol and treatment groups: amla, lemon, vitamin C individually

Table 4. Assay of protection of different test samples against alcohol induced haemolysis

Protection against Haemolysis	
Groups	**IC_{50}**
Amla juice	0.283µl/ml
Lemon juice	0.369 µl/ml
Vitamin C	0.120 µg/ml

All experiments were done in triplicates, values are mean of three data

Table 5. Effect of different test samples on Catalase (CAT) activity of RBC haemolysate

Catalase activity	
Groups	**Catalase (mU)**
Amla juice	11.81 ± 4.96*
Lemon juice	8.83 ± 4.09**
Vitamin C	7.89 ± 3.15
Alcohol	29.82 ± 3.45
Control	8.74 ± 1.73

Data are in Mean ± SD, *p<0.005-0.001, **p<0.0005, n=3, Statistical analysis have been done using Students Paired 't' test, and comparison was done between alcohol and treatment groups: amla, lemon, vitamin C individually

Table 6. Effect of different test samples on Olive Tail Moment (OTM) in DNA Comet Assay

Comet Assay	
Groups	**OTM**
Amla juice	6.46 ± 2.65*
Lemon juice	6.93 ± 1.79*
Vitamin C	7.17 ± 2.57*
Alcohol	22.48 ± 2.60
Control	2.77 ± 1.35

Data are in Mean ± SD, *p<0.0005, n=3,Statistical analysis have been done using Students Paired 't' test, and comparison was done between alcohol and treatment groups: amla, lemon, vitamin C individually

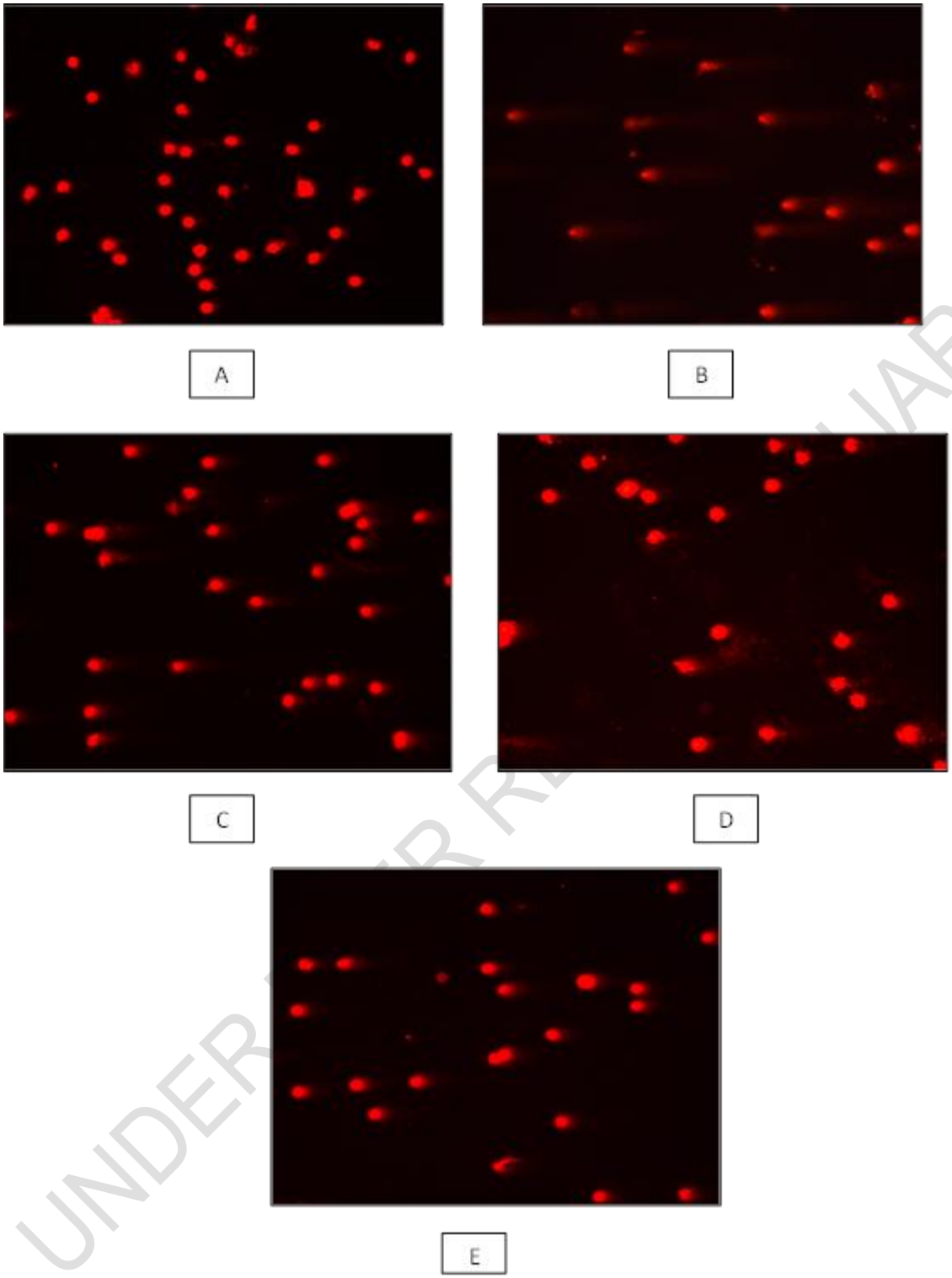

Figure 1. Representative DNA Comet image of different treatment groups.
A = Control group, B = Only Alcohol, C = Alcohol + Lemon juice treated group, D = Alcohol + Amla juice treated group, E = Alcohol + synthetic Vit. C treated group

Discussion

The findings demonstrate that both juices exhibit bioactivity, with efficacy broadly comparable to synthetic vitamin C, suggesting their potential as cost-effective dietary requirements on alcoholic liver disease (ALD).

Antioxidant Assays

The ABTS and DPPH radical scavenging assays revealed strong antioxidant activities of both amla and lemon, though vitamin C showed highest potency (it had the lowest IC_{50} of 1.61 and 3.8 in ABTS and DPPH value). Lemon juice demonstrated slightly better scavenging activity in ABTS assay (2.82 in ABTS and 9.79 in DPPH), while amla performed better in DPPH assay (3.37 in ABTS and 9.09 in DPPH). These results (table 1) are consistent with the phytochemical compositions of both fruits. Amla is rich in antioxidant compounds such as gallic acid, ascorbic acid and phenolic compounds. Lemon also contains natural compounds such as phenolic compounds (mainly flavonoids). Therefore, these substances which demonstrate strong radical scavenging activities are critical in mitigating liver damage.

Hepato-protective activity by MTT assay

The degree of cyto-toxicity and protection by different test samples was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, as conversion of MTT into a blue coloured formazan by the mitochondrial enzyme succinate dehydrogenase is potentially useful for assaying cell survival and proliferation. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present.)

In this study, pretreatment with amla and lemon juice increased hepatoprotective activity following alcohol exposure (70-78%) compared to the only alcohol-group (42.7%). The protection achieved by the juices was nearly equivalent to vitamin C (79.4%), highlighting that these fruits can stabilise mitochondrial function and can continue with cell metabolism under oxidative stress. However, the viability for amla juice (77.6%) was greater than lemon juice (70.4%), indicating that a higher number of liver cells are healthier and more viable when treated with amla juice than lemon (Table 2). Furthermore, significantly ($p < 0.005 – 0.001$) reduced AST release from hepatocytes (supported these protective effects (Table 3), indicating decreased membrane disruption and enzyme leakage.

Anti-Hemolytic Effects

Hemolysis is the process by which red blood cells (RBCs) ruptured and leak out hemoglobin into the blood plasma or surrounding fluid. Red blood cells are susceptible to oxidative stress because of their role as oxygen carriers. Free radicals are generated because of the iron metal ion in hemoglobin (20) High concentration of polyunsaturated lipids in the cell membrane facilitates free radicals attack and can result in membrane lipid peroxidation leading to hemolysis (21). In this study, alcohol induced hemolysis, but the protective substance amla and lemon significantly reduced RBC lysis. Amla showed greater potency ($1C_{50}$ 0.283 µl/ml) than lemon (0.369 µl/ml), reflecting greater membrane-stabilizing activity.

Catalase activity (CAT) is an indicator of oxidative stress, as it decomposes hydrogen peroxide into water and oxygen. In this study, alcohol increased catalase activity of RBC (29.82mU) reflecting increased levels of oxidative stress. However, this increased activity was lowered when treated with amla, lemon and vitamin C, showing their efficacy as a protective substance against ALD. Vitamin C (7.89mU) and lemon juice (8.83mU) brought catalase values almost to baseline, while for amla juice (11.81mU) it was still high. This suggests that all three substances exhibited protection against ALD, however vitamin C and lemon showed stronger catalase-stabilizing effects, with the significance being (*p<0.005-0.001, **p<0.0005).

Genoprotective Activity

The comet assay also demonstrated the DNA protective-potential of both fruits (Table 5 and Figure 1). Alcohol showed highest OTM (22.28), meaning that the damaged DNA fragments migrated further from the cell nucleus during electrophoresis. Amla's effect (6.46) was slightly better than lemon (6.93), suggesting that its antioxidant compounds (tannins and flavonoids) might have provided stronger nuclear protection. DNA damage can be done in many ways. For example, Acetaldehyde, another toxic metabolite of alcohol, can also cause DNA damage by forming covalent bonds with DNA, thereby disrupting its structure. Furthermore, DNA damage is a key event in the progression from chronic alcohol liver damage to cirrhosis, these natural substances prove to be effective agents in the prevention of severe damage.

Conclusion

The study demonstrates that fresh juice extract of *Phyllanthus emblica* L. (amla) and *Citrus limon* (L.) Osbeck (lemon) possesses significant antioxidant, hepatoprotective, anti-hemolytic and genoprotective activities against alcohol-induced oxidative stress *in vitro*. Both juices effectively improved the cell viability, reduced AST leakage and stabilized red blood cell membranes from rupturing. It also demonstrated protected DNA integrity. Among the juice extracts, amla exhibited slightly stronger cytoprotective, anti-hemolytic, and genoprotective effects, while lemon showed comparable radical scavenging and catalase-stabilizing activity.

By using fresh juices rather than purified extracts, this study reflects real-world dietary consumption patterns and daily diets of individuals especially in India. It highlights the extensive potential of these commonly available fruits as accessible, cost-effective and natural hepatoprotective agents rather than consuming synthetic medicines. Although further *in vivo* and clinical investigations are necessary to strengthen the findings, the study suggests that regular consumption of amla and lemon could serve as a simple preventive strategy against alcohol induced liver injury and oxidative stress rather than medicines which have other side effects.

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