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Hepatoprotective Activity of Syzygium Cumini Aqueous Leaf Extract Against DEN Using RT-PCR-Gene Expression and Molecular Docking Studies Using Zebrafish (Danio Rerio)

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Abstract

Hepatic issues caused by oxidants and hazardous materials are one of the largest health concerns in the world, thereby highlighting demand for safer and effective natural treatment medicines. A zebrafish (Danio Rerio) was used as an experimental model for the current work to evaluate the molecular hepatoprotective activity of the aqueous extract of the leaves of Syzygium cumini on Diethyl nitrosamine (DEN) induced liver injury. DEN is an effective carcinogenic and hepatotoxic agent that has a tendency to induce inflammation, oxidative stress and renal cell damage. Aqueous leaf extract of Syzygium cumini was administered to adult zebrafish that had been exposed to DEN to create hepatotoxic conditions to evaluate its molecular protection effect. To assess the regulation of oxidative stress and inflammation-related genes in liver tissues of the control and the treatment groups, Reverse Transcription Polymerase Chain Reaction (RT-PCR) gene expression analysis was performed. The results between the leaf extract-treated groups and the DEN-induced groups indicated a significant change in the expression of stress-related genes, indicating the restoration of hepatic cellular integrity and

antioxidant defense system. The results imply that Syzygium cumini's bioactive phytoconstituents may lessen liver damage caused by oxidative stress. To further elucidate the molecular mechanism of hepatoprotection, molecular docking studies were performed with certain phytocompounds present in the aqueous extract of leaves of D. donax against proteins associated with liver. This docking study result of good binding affinities and stable ligand-protein interactions validated the therapeutic potential of Syzygium cumini bioactive chemicals in the modification of hepatic damage pathways. Based on the findings of RT-PCR and molecular docking investigations, Syzygium cumini leaf extract shows promising hepatoprotective effect against DEN-induced liver damage in zebrafish. The study confirms that Syzygium cumini is a potential natural source for the development of therapeutic agent against liver diseases and supports the plant's traditional medicinal applications.

Keywords: Syzygium cumini, Zebrafish, RT-PCR, Molecular docking

I. INTRODUCTION

Liver diseases rank as one of the most important causes of morbidity and mortality around the world, as a result of exposure to toxic drugs, harmful environmental influences, alcohol, narcotics and oxidative stress. The liver's unique nature for metabolism, detoxification, excretion and maintenance of physiological homeostasis makes it highly vulnerable to xenobiotic-induced damage. Liver injury due to chemical carcinogens often leads to oxidative stress, inflammation, lipid peroxidation, hepatocyte injury and cirrhosis, fibrosis and HCC. Diethylnitrosamine (DEN), one of many hepatotoxic substances, is well known for being a powerful hepatocarcinogenic and hepatotoxic chemical that is frequently used to cause experimental liver injury in animal models. Reactive oxygen species (ROS), oxidative stress and cellular necrosis are the main mechanisms by which DEN-induced hepatotoxicity is mediated. These mechanisms disrupt the natural liver antioxidant defense mechanisms. Liver diseases can be treated with a number of synthetic medicines but prolonged consumption of such medicines may have undesirable effects and may not be as therapeutically effective. Medicinal herbs have been more attractive for potential natural hepatoprotective drugs due to their safety, availability, and pharmacological properties.

Java plum or jamun, Syzygium cumini, belongs to the Myrtaceae family and can be found in tropical or subtropical regions. In ancient medicine systems such as Ayurveda, Siddha and Unani, the plant has found extensive use in the treatment of various diseases such as diabetes, inflammation, gastrointestinal problems and liver ailments. Leaves, fruits, seeds and bark of various plants contain a rich source of

biologically active chemical constituents such as flavonoids, alkaloids, tannins, glycosides, terpenoids and phenolic chemicals.

These phytochemicals have been shown to possess a wide range of beneficial health-promoting properties, including antioxidant, anti-inflammatory, antibacterial, antidiabetic, and hepatoprotective effects. However, in particular, Syzygium cumini leaves are rich in polyphenols and flavonoids which can trap free radicals and protect the tissues from oxidative stress-induced damage.The therapeutic value of Syzygium cumini in controlling oxidative stress and enhancing antioxidant defense mechanisms has been documented in earlier research; however, comprehensive molecular studies about its hepatoprotective mechanism are still few.

Zebrafish has many characteristics that make it a valuable vertebrate model organism for toxicologic and medicinal studies, including its genetic similarity to humans, rapid development, translucent early development, and low maintenance requirements. The zebrafish liver is a good model for researching hepatotoxicity and hepatoprotective treatments because it has anatomical, physiological and functional characteristics with the liver of mammals. Furthermore, due to their cost-effectiveness and repeatability, zebrafish models are being used more and more in molecular biology, pharmacological screening and drug development research.

Recent advances in molecular and computational biology have led to a better understanding of disease mechanisms and drug interactions. RT-PCR is a sensitive molecular approach to measure gene expression of markers of oxidative stress, inflammation and apoptosis associated with liver damage. Moreover, the use of Molecular Docking as a computational tool to predict the interaction of phytocompounds and target proteins of disease pathways has become significant. A deeper comprehension of the molecular mechanisms underpinning hepatoprotective action is made possible by combining RT-PCR and molecular docking techniques.

Hence, in the present study, zebrafish were used as an experimental model and gene expression analysis and molecular docking studies were performed, to evaluate the hepatoprotective effect of Syzygium cumini aqueous leaf extract against DEN-induced liver injury at the molecular level. The findings of the current study can be helpful in suggesting the potential application of Syzygium cumini as a natural therapeutic agent in the management of hepatic diseases, and can also provide scientific validation of the traditional medicinal applications of this plant.

Materials and Methods:

Collection and Preparation of Plant Material (syzygium cumini):

Fresh leaves from Syzygium cumini were harvested, washed with distilled water to wash off the dust particles and shade dried for few days at room temperature. Powdered dried leaves were kept in air tight containers for further analysis using mechanical grinding. The powdered leaf material was extracted with water by using the standard extraction procedures. The extracted was filtered and concentrated for experimental use.

Experimental Animal:

Approximately 0.3-0.5 g adult zebrafish (Danio rerio) were purchased and reared in standard laboratory conditions. The fish were acclimatised for two weeks in the aerated aquaria with the controlled temperature with 12/12 h L/D cycle. Commercial feeds were fed twice a day to the fish. The water quality for the fish was not compromised during the experimental period.

Induction of Hepatotoxicity:

To induce hepatotoxicity, Diethylnitrosamine (DEN), a potent hepatotoxic and carcinogenic compound was used. Experimental zebrafish were separated into various groups; control, DEN induced and treated. The aqueous extract of the leaves of Syzygium cumini was given to the treatment groups at various concentrations to the experimental period and DEN was administered to induce liver damage. The fish were killed at the end of the treatment period and the liver tissues were taken for molecular analysis.

Gene Expression Analysis by RT-PCR

Under RNase-free condition, the liver tissues of experimental zebrafish were used for total RNA extraction. Tri Reagent (acidic phenol–guanidinium thiocyanate) was used to homogenise liver tissues, with a DEPC-treated homogeniser. After thoroughly lysing the homogenate with repetitive pipetting, 200 µL chloroform was added, mixed vigorously and centrifuged for 15 min at 4°C at 12,000 × g, which resulted in the phase separation. Aqueous phase with RNA was carefully transferred to sterile DEPC treated microcentrifuge tubes.

The RNA was precipitated with 250 µL of ice-cold isopropanol, vortexed and left at room temperature for 15 minutes. RNA pellets were obtained by centrifuging the samples at 12,000 × g for 15 minutes at 4°C. After two washing with 75% ice-cold ethanol, the pellets were centrifuged for 10 min at 14,000 × g. The pure RNA pellets were dissolved in DEPC-treated water and kept at -80°C for further examination after air-dried and heated for 20 min at 55°C. The concentration and purity of RNA were determined by using a biophotometer.

Total RNA (0.5–1.0 μg) was used for cDNA synthesis by mixing with random hexamer primer, and subsequent incubation at 72°C for 10 min after which it was immediately put on ice. dNTP mix, M-MLV reverse transcriptase buffer, M-MLV reverse transcriptase enzyme and nuclease-free water (water) were added to the reaction mixture to a final reaction volume of 50 μL. Reverse transcriptions were performed following standard procedures.

Polymerase chain reaction (PCR) was used to do gene expression analysis with SYBR Green chemistry. The housekeeping gene used for normalisation of gene expression was Beta-actin. The relative expression levels of target genes were analysed and compared between control and experimental groups to assess the molecular hepatoprotective effect of Syzygium cumini leaf extract against DEN-induced hepatic damage.

Molecular Docking Analysis

The binding affinity and the molecular interaction of these bioactive phytocompounds with the specific proteins involved in hepatotoxicity and apoptosis were evaluated through molecular docking studies. The three-dimensional structures of the target proteins such as STAT2, CDKN1A, BAX and BCL2 were obtained from SWISS-PROT database. The selected phytocompounds identified from the leaf extract of Syzygium cumini were used as ligands for docking analysis.

Protein and ligand structures were prepared and optimised prior to docking studies. The molecular docking simulation was performed with AutoDock Vina software to forecast the binding affinity and the interaction pattern of the ligand–protein. Binding stability and strength of the ligands to the target proteins was determined from docking scores obtained from the analysis.

Discovery Studio Visualizer software was used to visualize the ligand–receptor interaction profile, such as hydrogen bonding and hydrophobic interactions. Docking analysis gave insights into the possible molecular mechanism of hepatoprotective effect of Syzygium cumini leaf phytocompounds against DEN induced liver injury.

Results:

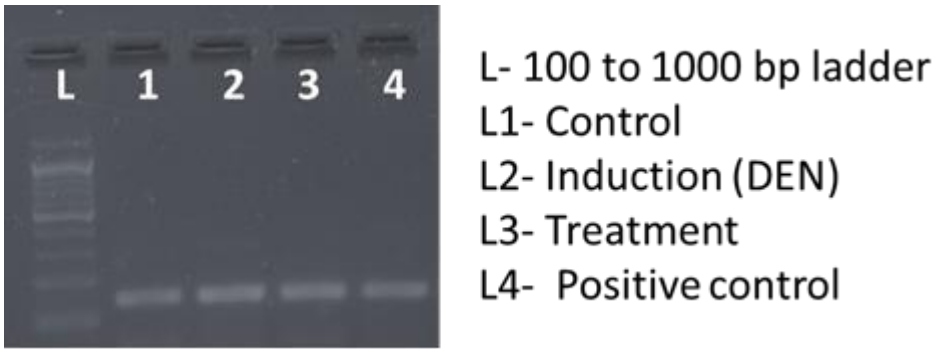
Gene Expression Analysis (RT-PCR):

The liver tissues of control and experimental zebrafish groups were used for the evaluation of the expression of the KEAP1 gene expression by RT-PCR and Agarose gel electrophoresis. The Control (C), Induction (I), Treatment (T) and Positive Control (PC) groups had distinct bands, with L being the molecular ladder. In physiological conditions, the band intensity of the Control group was moderate with normal expression of the KEAP1 gene. The Induction group, on the other hand, showed a higher band intensity that suggested that Nrf2 was down regulated

after DEN exposure leading to decreased antioxidant defence mechanisms. The expression of KEAP1 was comparatively lower in the treatment group when compared with the induction group indicating that Jamun leaf extract may regulate the activity of KEAP1 and restore the Nrf2-mediated antioxidant signalling. In the Positive Control group, there was also regulated KEAP1 expression like the treatment group.

The results presented here show that exposure to DEN caused a significant oxidative stress in the liver tissues of the zebrafish and resulted in upregulation of KEAP1 expression and changes in the antioxidant status. The decrease in expression of KEAP1 in the Jamun leaf extract–treated group indicates that Jamun leaf extract may exert a protective role in the cells by modulating the KEAP1–Nrf2 pathway, which in turn helps to improve antioxidant defence and maintain cellular redox balance.

Figure -01
Gene Expression Analysis (RT-PCR) in the liver tissue of the control and experimental groups



Molecular Docking Analysis:

Selected phytocompounds of Jamun leaf extract were docked with KEAP1 (PDB ID: 6TYM) to examine the interaction of these phytocompounds with the Kelch domain with which they are likely to interact in the human body. The docking results showed that the phytocompounds have strong binding interactions with the active binding cavity of KEAP1, indicating their potential in modulating oxidative stress response pathways in hepatotoxicity caused by DEN.

The binding affinity score of phytol with the active site of KEAP1 was calculated and found to be −6.1 kcal/mol, which was considered to be stable. The compound was docked in the C1 binding cavity with a volume of 1259 $Å^3$ and was mainly hydrophobic to the residues such as Val420, Ala466 and Ile560. Furthermore,

a classical hydrogen bond between hydroxyl group and Gly367 was noted, which helped to stabilise the ligand–protein complex. The three-dimensional docking visualisation showed that Phytol was deeply inserted into the electrostatic surface pocket of KEAP1 and interacted with several hydrophobic and van der Waals interactions, which is in accordance with the lipophilic nature of the terpenoid compound. The 2D interaction map also showed the existence of alkyl interactions between Val420, Val465 and Ala366, confirming proper docking of the ligand in the binding groove. The results indicate that Phytol could disrupt the binding between KEAP1 and Nrf2, and enhance the activation of antioxidant defence mechanisms.Likewise, α-Cadinol exhibited a positive binding affinity score of −6.4 kcal/mol with the Kelch domain of KEAP1 suggesting energetically favourable and stable interaction with the binding pocket of the target. The compound was located in the active pocket which is surrounded by amino acid residues Ala510, Val512, Gly464, Ile559, Ala556 and Val606. The hydroxyl group of α-Cadinol also formed conventional hydrogen bonds with Ala510 and Gly464, and a number of nonpolar and van der Waals contacts with the ligand with other residues, such as Val512, Ile559 and Val606, contributed to further ligand–protein complex stabilization. The surface electrostatic analysis showed that α-Cadinol was primarily hydrophobic in the binding region of KEAP1, which indicated that it had terpenoid properties and a preference for nonpolar amino acid residues. The observed interactions suggest that α-Cadinol could interfere with the inhibition of Nrf2 activation by KEAP1, thus strengthening the anti-oxidative signalling pathways and preventing damage to hepatocyte tissues caused by oxidative stress.

The KEAP1 active site also showed a strong interaction with Ledol with a binding affinity score of −6.9 kcal/mol, indicating comparatively higher ligand stability amongst the selected phytocompounds. The docking analysis showed several hydrophobic contacts with important residues such as Val512, Val606 and Ile559 that helped stabilize the ligand in the active site pocket through van der Waals interactions. In addition, the specificity and stability of the interaction between the ligand and the receptor were increased by the formation of hydrogen bonds with Ala510. The binding conformation of Ledol in the binding groove of KEAP1 is favourable as it may be able to disrupt the binding of KEAP1–Nrf2 and promote activation of Nrf2-mediated antioxidant responses. This modulation of the KEAP1–Nrf2 pathway could help to increase the cell's ability to withstand oxidative stress and xenobiotic-induced liver damage.

The overall result of molecular docking showed that all the selected phytocompounds from Jamun leaf extract had good affinity towards the target protein KEAP1 and could be used as natural modulators of the antioxidant pathway KEAP1–Nrf2. The hepatoprotective activity of phytocompounds is attributed to

these interactions, which seem to enhance antioxidant defence mechanisms and alleviate oxidative stress resulting from liver injury caused by DEN. The moderate docking scores observed are comparable with the other standard inhibitors, but the consistency of hydrophobic and hydrogen bonding interactions strongly suggests their therapeutic relevance. These computational results should be confirmed by further in vitro/in vivo studies to validate their findings and to establish the molecular mechanisms of their hepatoprotective activity.

Table-01
Bioactive compounds present in the liver tissue of zebrafish exposed to jamun leaf extract using Molecular docking

S.No.	Compound Name	Canonical SMILES
1	**Phytol**	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C
2	**α-Cadinol (Alpha-cadinol)**	CC1=C[CH]2[CH](CC[C@@]([C@@H]2CC1)(C)O)C(C)C
3	**Ledol**	C[C@@H]1CC[C@H]2[C@@H]1[C@H]3[C@H](C3(C)C)CC[C@@]2(C)O

Figure 02
Molecular interaction of phytol

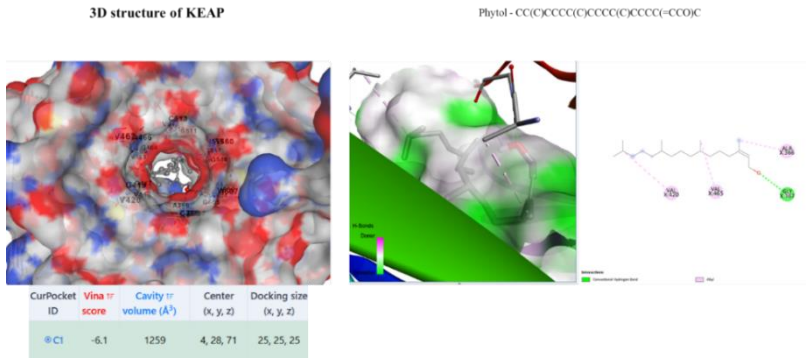


Figure 03

Molecular interaction of α-cadinol

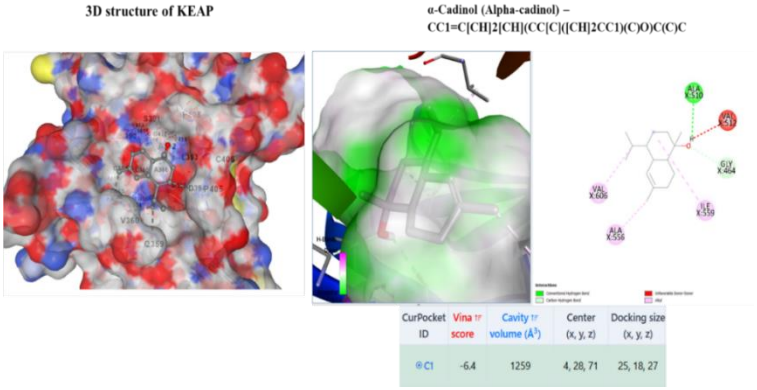
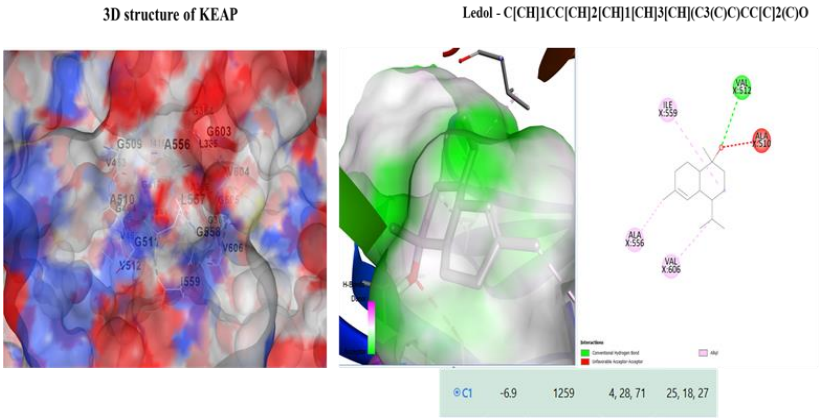


Figure 04

Molecular interaction of Ledol



Discussion:

The present study showed that Syzygium cumini aqueuous leaf extract (SLE) protects the liver against DEN-induced toxicity in zebrafish using molecular and computational approach. In liver tissues, exposure to DEN led to an alteration in antioxidant status and an upregulation in expression of the antioxidant pathway member KEAP1, a sign of oxidative stress. Increased expression of KEAP1 can subsequently cause a downregulation of the antioxidant pathway of Nrf2, which can result in a decrease in cellular antioxidant defence mechanisms and contribute to the progression of hepatic injury. The treatment with the Jamun leaf extract exhibited distinct regulation of the expression of KEAP1, indicating restoration of antioxidant status and protection from liver damage due to oxidative stress.

These observations were confirmed by biochemical antioxidants markers analysed throughout the study. The DEN induced group had decreased levels of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) which showed an oxidative stress condition in the hepatic tissues. Induction group also showed lipid peroxidation and cellular damage. In zebrafish liver tissues, however, antioxidant enzyme activity was enhanced in tissues by administration of Syzygium cumini leaf extract and normal physiological condition was partially restored. The results indicated that the phytoconstituents found in Jamun leaves could contribute to boosting the endogenous antioxidant defence mechanisms and reducing any oxidative injury resulting from DEN exposure.

Some phytochemicals found in the leaf extracts can be crucial to the hepatoprotective activity, such as phytol, α-cadinol, and ledol. These compounds have been previously reported to have antioxidant and anti-inflammatory properties and may be beneficial in mitigating free radical mediated tissue damage. The results of the GC–MS profile of the extract confirmed the presence of biologically active phytocompounds that can interact with proteins involved in the oxidative stress pathways.

The experimental results were further supported by molecular docking analysis, which showed good binding with the target proteins (KEAP1). The binding affinity of the compounds in the analysed set with stability of the hydrophobic and hydrogen bond interactions with the active binding pocket of KEAP1 was comparatively high for compound Ledol. The same interactions were seen with phytol and α-cadinol. Based on these interactions, it is proposed that the phytocompounds could potentially interact with the KEAP1-mediated inhibition of Nrf2 signalling, leading to the activation of the antioxidant response pathways and preventing oxidative stress-induced hepatic tissue damage.

Based on the above observation, the present study revealed that Syzygium cumini leaf extract has a potent antioxidant and hepatoprotective effects against DEN-induced liver injury in zebrafish. The results obtained with combined RT-PCR and molecular docking approaches have been found to be supportive in getting the molecular evidence of the protective effect of phytoconstituents found in Jamun leaves on oxidative stress pathways. However, additional in vivo and clinical studies are needed to establish the exact molecular mechanisms and clinical applicability of these bioactive compounds in liver diseases.

II.CONCLUSION

The present study demonstrated the molecular hepatoprotective potential of Syzygium cumini leaf extract by using molecular docking studies and RT-PCR. Gene expression analysis revealed that induction of DEN significantly up-regulated the expression of KEAP1, which suggests that there is a higher level of oxidative stress and inhibition of the Nrf2 antioxidant signaling pathway. Syzygium cumini leaf extract treatment successfully reduced the expression of KEAP1, suggesting the possibility of modulation of the KEAP1–Nrf2 pathway to restore the antioxidant defence mechanisms. This outcome was further confirmed by the molecular docking experiments, which revealed stable interactions between molecular structures of some of the phytocompounds, (Phytol, α-Cadinol and Ledol) and protein molecules (KEAP1). The docking studies revealed the ability of these phytocompounds to form favorable hydrogen bonding interactions and hydrophobic contacts with the KEAP1 binding pocket, indicating their potential for successful binding. These interactions can help to enhance the antioxidant reaction against oxidative stress-induced liver damage by preventing the reduction of Nrf2 by KEAP1. By controlling the KEAP1–Nrf2 signaling pathway, the combined RT-PCR and molecular docking results indicate that Syzygium cumini leaf phytoconstituents have substantial antioxidant and hepatoprotective potential. The study provides molecular evidence for therapeutic potential of Syzygium cumini as a natural source of bioactive chemicals against liver diseases associated with oxidative stress. Further experimental validation is required to validate specific molecular mechanisms and therapeutic uses of these phytocompounds.

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