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Original Article

UNVEILING THE ANTICANCER EFFECT OF SYRINGIC ACID AND ITS DERIVATIVES IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

Objective: Traditionally, syringic acid has been used as a medicine for a wide range of ailments. The current study aims to look at its potential therapeutic benefits against experimentally generated hepatocellular carcinoma in wistar rats, and in order to better understand how syringic acid interacts with apoptosis proteins in hepatocellular carcinoma, the molecular docking has been performed by using argus lab 4.0.1 software.

Methods: The hepatocellular carcinoma (HCC) targets such as P53, BAX, Bcl-2, capase 3 and 9, Cytochrome-C, TNFα, NFκB, and TRAF1 were docked with syringic acid. The syringic acid derivatives such as acetosyringone, syringaldehyde, syringol, sinapinic acid, sinapyl alcohol, sinapaldehyde, sinapine, and canolol were docked with caspase3. Thirty male wistar rats were randomly assigned to five groups. The control group was given normal saline. Group 2 obtained a single oral dose of diethylnitrosamine (DEN) (200 mg/kg) body weight. Groups 3, 4, and 5 received diethylnitrosamine (DEN), and furthermore daily administration of syringic acid orally at 25 mg/kg for 14 w. Serum samples were used for the determination of liver marker levels. Liver tissue samples were used for histopathological determination, apoptotic and anti-apoptotic protein expression.

Results: The syringic acid and its derivatives exhibited excellent energy values and satisfied the drug-likeness property of Lipinski's rule of five. Syringic acid significantly reduced the serum liver marker levels, and in contrast, it increased the expression of apoptotic proteins in the diethylnitrosamine (DEN)-induced treated group.

Conclusion: It has been demonstrated that syringic acid had a protective effect against diethylnitrosamine (DEN)-induced hepatocellular carcinoma in Wistar rats, and a docking study exhibited that it has good anticancer activity.

Keywords: Molecular docking, Argus lab, *In vivo* study, Apoptotic proteins, Hepatocellular carcinoma (HCC), Syringic acid

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common liver malignancy caused not only by the persistent hepatitis C (HCV) and by hepatitis B (HBV) viral infection but also by other factors such as heavy alcohol intake, aflatoxin-contaminated foods, smoking, obesity and type 2 diabetes. The World Health Organization reports that the prevalence of hepatocellular carcinoma (HCC) has increased considerably over the past 20 y, making it the fifth most prevalent malignancy and a major contributor to cancer-related mortality in the US [1], and it is anticipated that by 2025, more than one million people will be impacted by liver cancer each year [2]. Median survival of only 2-3 mo has been recorded in patients with advanced hepatocellular carcinoma (HCC), even with the finest supportive care. Radiotherapy, stem cell therapy, hormone therapy, and chemotherapy like Atezolizumab, Bevacizumab, Nivolumab, Sorafenib, and Lenvatinib are the common treatments for hepatocellular carcinoma (HCC); however, the window for therapy is limited, because they cause adverse effects, which affect the life quality of patients [3]. Consequently, it is necessary to provide an affordable yet delicate and equally effective medication to assure a major influence on the prevalence of hepatocellular carcinoma (HCC) internationally. Medicinal plants and their phytochemicals have long been used as low-cost alternative medications with little or no adverse effects. In 2015, Aranya Manosroi *et al.* studied that the plants like *Stemona collinsae, Gloriosa superb, Caesalpinia sappan*, and *Ventilago denticulate* has anti-liver cancer activity [12]. Recently it was found that the phytochemical phyllanthin has anticancer activity, and it was proved by studying its effect in both *in vivo* and *in vitro* (Hep G2 cells) [13]. Similarly, medicinal plants such as *Wedelia calendulacea, Andrographic paniculata, Cassia roxburghii, Ficus carica, Azadirachta indica, Curcuma Longa, Eclipta alba, Camellia sinesis, Cassia roxburghii,* has the hepatoprotective activity [14].

Plant-derived secondary metabolites such as flavonoids, tannins, carotenoids, limonoids, phytosterols, and phytoestrogen has anticancer activity. In earlier studies, it was found that the phytochemicals naringenin and quercetin have the cytotoxic effect against the Hep G2 liver cancer cell line [15]. Similarly, Seungmo Park *et al.* (2017) reported that resveratrol has a potent hepatoprotective effect by lowering the amount of cyclin D1 mRNA, and it can prevent tumor cells from entering the G1 phase in Huh7-HBx cell line [16]. Another study showed that curcumin increases the p53 protein expression, which declines the proliferation of hepatitis B viral (HBV) infections [17]. It is imperative that these collaborative initiatives start to identify new substances that will improve the therapeutic index management and support justified usage considering the current focus on alternative therapies.

Syringic acid, a phenolic compound present in many fruits and vegetables, has been utilized extensively in medicine and has demonstrated a wide range of potential applications in the treatment of cancer. Many earlier studies showed that syringic acid has anti-inflammatory, anti-diabetic, anti-oxidant, and anti-microbial activity. Syringic acid and its derivatives exert its anticancer impact through a number of mechanisms, which includes the downregulation of proliferating cell nuclear antigen (PCNA), CyclinD1, and mutant P53, the cell cycle proteins cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase 6 (CDK6), and cyclins B, C, and H, and the upregulation of apoptotic genes Bax, Caspase 2, 3, and 9. Additionally, syringic acid also has anti-mitogenic and chemo sensitizing effects by interfering with cell migration, NFκB-DNA binding, apoptosis regulation, and cell cycle arrest [4]. Remarkably, no research has been conducted to examine the impact of syringic acid on hepatic cancer.

Here in this study, we examined the anticancer effect of syringic acid using a molecular docking study and a diethyl nitrosamine (DEN)-induced hepatocellular carcinoma model in wistar rats. Docking facilitates the prediction of the intermolecular binding created when a protein binds to its ligand. Therapeutic targets in HCC are increasingly based on signaling pathways. In order to better

understand how syringic acid interacts with various apoptotic proteins in hepatocellular carcinoma, and the binding of a major apoptotic protein caspase3 with syringic acid and its derivatives, molecular docking studies of this interaction are performed and evaluated the Lipinski's Rule of Five's drug-likeness for syringic acid. The results exhibited syringic acid derivatives acetosyringone and syringol has a binding energy of-4.9Kcal/mol with caspase3. The sinapinic acid, sinapyl alcohol, sinapine has a binding energy of-6.0,-6.0, and-6.1 Kcal/mol with caspase3. The syringaldehyde, sinapaldehyde, and canolol has binding energy of-5.4,-5.6, and-5.5 Kcal/mol with caspase3, and syringic acid has an excellent binding energy with P53, cytochrome C, and caspase3 with-4.16,-4.17, and-4.2 Kcal/mol respectively. In an animal experiment, the biochemical parameters such as aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and mitochondrial enzyme assays such as succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) exhibited the dramatic increase in the blood serum of den-induced hepatocellular carcinoma group of Wistar rats and exhibited the declined levels in the den-induced syringic acid group. The immunohistological study showed the presence of apoptotic proteins (cyt-C, caspase3, Bax), and the absence of anti-apoptotic protein (Bcl2) in the den-induced syringic acid-treated group as compared with the control. Thus, in this study, it has been proved that syringic acid has anticancer activity against den-induced hepatocellular carcinoma.

MATERIALS AND METHODS

Molecular docking analysis

Preparation of proteins

The target proteins p53 (1AIE), Bax (4QVF), apoptosis regulator Bcl-2 (2O2F), capase3 (3DEH) and caspase9 (1NW9), Cytochrome-C (3ZCF), TNFα (1TNF), NFκB (1SVC) and TRAF1 (1EXT) were selected and their structure was retrieved from the protein data bank for docking. The protein data bank (PDB) structures are coordinated in a file format. The water molecules were removed from the target protein structures. The resulting protein data bank (PDB) structures were 3D optimized before docking.

Preparation of ligand

The syringic acid and its derivative's 3D structure was annotated from the PubChem database, analyzed in structured data file (SDF) format and then further converted to protein data bank (PDB) format using IQmol software.

Molecular docking of syringic acid and syringic acid derivatives with target proteins

The argus lab 4.0.1 were used for the docking study [5]. The target protein binding sites were grouped, and the grid size was calculated. The grid was created with a resolution of 0.4 units. For docking, the target protein ligand-binding site was positioned in the center of the grid. Prior to docking, the target protein and ligand were both refined for the correct geometry. The target proteins and syringic acid was simulated using their optimal structures. Energy values were computed using the intermolecular flexible docking simulations to produce the docked conformations. Docking analysis results were saved as protein data bank (PDB) files. The optimal ligand pose's binding energy was expressed in terms of kcal/mol.

Drug likeness

Lipinski's Rule of Five was used to analyze the molecular characteristics and drug-likeness of syringic acid. The Mol Inspiration software was used to determine whether the syringic acid molecule satisfies all the five Lipinski rules. Lipinski's rule is a general guideline to assess whether the drug similarity stipulates an orally bioavailable drug has no more than one violation of the following factors, as shown in table 1**.**

Table 1: Lipinski rule of five

01.	A maximum of 10 hydrogen bond acceptor molecules.
02	molecules under 500 Daltons in weight
03.	Partition less than a log P coefficient of 5.
04.	Not more than 5 hydrogen bond donor

In vivo study

Chemicals

The syringic acid and diethyl nitrosamine was purchased from Sigma Aldrich., USA.

Animal

Male wistar albino rats were obtained from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), weighing 100-150 g were housed in a controlled laboratory environment (25±2 °C, 12 h light/dark cycle) with adequate food and water for a week prior to the experiment. The Institutional Animal Ethics Committee (IAEC) (XXV/VELS/PCOL/07/2000/CPCSEA/IAEC/09.10.2021) ethically approved the study.

Experimental design

The rats were housed in cages and divided into five groups, with six rats per group (n=30). Group I: Animals in the control group were treated with normal saline orally. Group II: served as drug (syringic acid) control. Animals from group III to group V were treated with a single dose of DEN (200 mg/kg), then the animal's in-group IV was treated with syringic acid (25 mg/kg), and group V was treated with 6 mercapto purine [20]. After the experimental period at the end of week 14, animals were injected with xylazine (10 mg/kg) [21], and blood was drawn for biochemical analysis. The organ weight of the liver of each group was recorded. Then a few of the tissue pieces were exercised for histological and molecular studies.

Biochemical parameter

The serum was used to assess the biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alpha-fetoprotein (AFP), and gamma-glutamyl transferase (GGT) as per the guidelines in the enzyme-linked immunosorbent assay (ELISA) kit, and ultraviolet-visible (UV-Vis) Spectrophotometer was used to measure the wavelength at 450 nm.

Mitochondrial segregation

The excised liver was washed with cold saline, and the liver tissue was subsequently homogenized (10% w/v) in a homogenizing solution made up of 0.44 M sucrose, 10 mmol Tris-HCl, 10 mmol ethylenediamine tetraacetic acid (EDTA), and 0.1% bovine serum albumin (BSA) at a pH of 7.4. This tissue homogenate was centrifuged at 4 °C for 15 min. After centrifugation, the supernatant was spun again at 12,000 rpm for 20 min (4 °C). After washing the pellet with homogenizing buffer and centrifuging it for 15 min at 16,000 rpm 4 °C, the mitochondrial fractions were recovered in suspension buffer (10 mmol Tris-HCl and 0.44 M Sucrose), pH 7.4, and used for further study [18].

Succinate dehydrogenase

To determine the activity of the mitochondrial enzyme, succinate dehydrogenase (SDH), the mixture of succinic acid (0.6M), bovine serum albumin (BSA) (1%), 0.03M potassium ferricyanide, and 0.2M phosphate buffer was added to a mitochondrial fraction. The optical density was measured at 420 nm using an ultraviolet-visible (UV-Vis) spectrophotometer [19].

Malate dehydrogenase

The malate dehydrogenase (MDH) activity was measured by adding 6 mmol oxaloacetic acid, 0.25M sucrose buffer, and 3.75 mmol nicotinamide adenine dinucleotide hydrogen (NADH) to the mitochondrial fraction. The optical density was measured at 340 nm using an ultraviolet-visible (UV-Vis) spectrophotometer.

Histopathology

Hematoxylin and eosin (H and E) staining was used to analyze the histological alteration in liver tissues. The liver tissue from each group was preserved in 10% formaldehyde diluted in phosphate buffer, pH 7.2, for 24h. Following fixation, the sample was treated

with increasing alcohol concentrations before embedding in paraffin wax. A section of the implanted samples of 5 µm thickness was stained with Hematoxylin and eosin (H and E). The stained samples were analyzed using a light microscope [19].

Immunohistochemistry

The sections were deparaffinized in xylene and dehydrated using ethanol at various concentrations. The sections were warmed in a 0.01 mol/l citrate buffer in an autoclave for 20 min after the inhibition of endogenous peroxidase activity with 3% hydrogen peroxide for 15 min. After allowing the slide to cool, non-specific binding was stopped by blocking it with regular horse serum for 20 min at room temperature. The primary antibody against Bax, Bcl2, Cytochrome C, and Caspase 3 was added to the sections and incubated for 30 min. Following this, the sections were stained with secondary antibody using an HRP technique. The binding sites interpreted by using avidin/biotin complex (ABC) chromogens. Between each procedure, phosphate buffer saline was utilized for washing, and at last, Mayer's hematoxylin had used to counterstain each section [22].

Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) and significant results were determined by post-hoc Tukey test ($P<0.05$). The data were shown as mean±SD.

RESULTS

Molecular docking analysis

Syringic acid

We explored the interaction between syringic acid and various apoptotic proteins. Fig. 1 shows the structure of syringic acid.

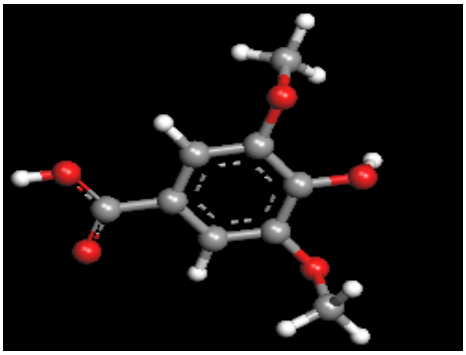

Fig. 1: Structure of syringic acid

Syringic acid docked with apoptotic proteins involved in apoptotic pathway

P53

The interaction between P53 and syringic acid is shown here (fig. 2) with two hydrogen bonds at chain A. The lengths of the hydrogen bonds are 2.95, and 2.00 Å. The best ligand pose energy exhibited-4.16 kcal/mol indicating strong binding.

Bax

The syringic acid interaction with BAX is shown in fig. 3. Three hydrogen bonds interactions occurred in chain A of the molecule with an energy expenditure of–5.21 kcal/mol. The hydrogen bond length measured as 2.87 Å, 2.99 Å, and 2.29Å showed very close proximity with excellent bond strength.

Apoptosis regulator Bcl2

The binding of syringic acid with Bcl2 shown in fig. 4. The interaction occurs with three hydrogen bonds at different sides of chain A with ligand pose energy-6.05 Kcal/mol. The hydrogen bonds are at 2.02, 2.94, and 2.84 Å showing good bond strength.

Caspase 3

The interaction of syringic acid with Caspase 3 is shown in fig. 5. Syringic acid showed the strongest interaction with four hydrogen bonds. The bond length measurements of these hydrogen bonds are 2.72, 2.87, 2.03, and 2.27Å. The B chain of the molecule interacted with syringic acid. The energy spent is-4.2 Kcal/mol.

Caspase 9

Syringic acid binds with caspase 9 very firmly at the binding site with the formation of two hydrogen bonds at the same side of the structure at the B chain (fig. 6). The hydrogen bonds length measured are 2.99 Å and 2.93 Å. The energy expenditure was found to be-4.49Kcal/mol.

Cytochrome C

The interaction between cytochrome C and syringic acid is shown here (fig. 7) with two hydrogen bonds at chain B. The length of the hydrogen bond is 2.54, and 2.95 Å. The best ligand poses energy exhibited-4.17 kcal/mol. It shows strong binding.

Tnf α

TNF alpha and syringic acid were interconnected by three hydrogen bonds as shown in (fig. 8). TNF alpha and syringic acid interact quite significantly. TNF alpha exhibits the strongest interaction with three hydrogen bonds. These hydrogen bonds have the following measurements: 2.75, 2.94, and 2.09 Å. The molecule's A chain has been selected for interaction. The amount of energy used is-5.08 Kcal/mol.

Nfκb

Here, in fig. 9, two hydrogen bonds with the corresponding lengths of 2.52 and 2.12 Å serve to describe the interaction between NFκB and syringic acid. Higher interaction results from shorter hydrogen bonds. Consequently, the interaction is powerful and the best ligand poses energy was found to be-4.79Kcal/mol.

Traf1

The molecule syringic acid binds to the tumor necrosis factor receptor at the TRAF 1 binding site as illustrated in (fig. 10) with three hydrogen bonds on distinct sides; the bonding occurs at chain A of the molecule with an energy expenditure of-5.18 Kcal/mol. The hydrogen bond strengths of syringic acid molecules are 2.20, 2.62, and 2.21 Å, indicating very strong bonds.

Drug likeness

Table 2 illustrates the data on the molecular characteristics and drug-likeness of syringic acid.

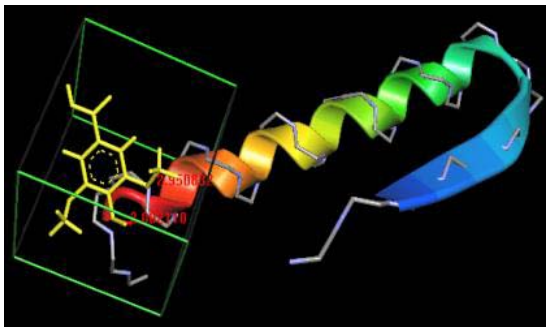

Fig. 2: P53 docked with syringic acid

Table 2: The drug-like properties of syringic acid

Molecular Weight	198.17
Log P	1.20
No. of the hydrogen donor	02
No. of hydrogen acceptor	05

Derivatives of syringic acid docked with caspase 3

Syringic acid derivatives such as *acetosyringone, syringaldehyde, syringol, sinapinic acid, sinapyl alcohol, sinapaldehyde, sinapine, and canolol* were docked with the terminating apoptotic protein caspase3. The results of molecular docking are shown in table 3. Fig. 11 shows the molecular docking of syringic acid and its derivatives with caspase 3.

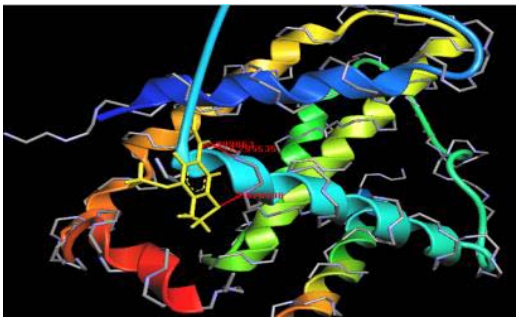

Fig. 3: Bax docked with syringic acid

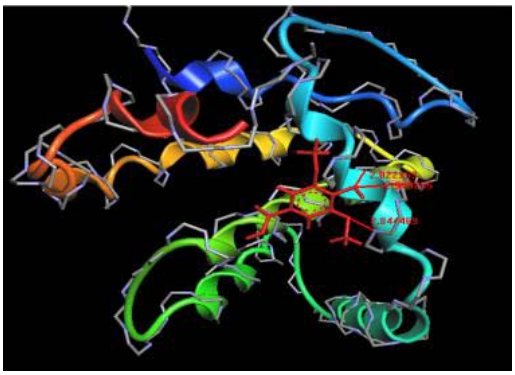

Fig. 4: Bcl2 docked with syringic acid

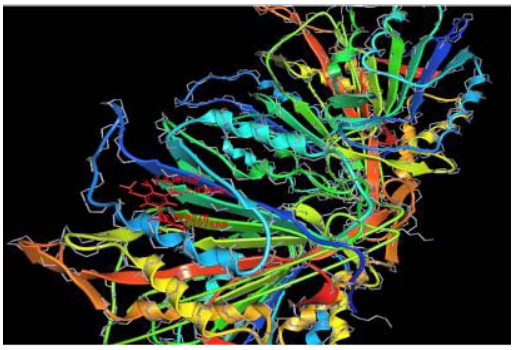

Fig. 5: Caspase 3 docked with syringic acid

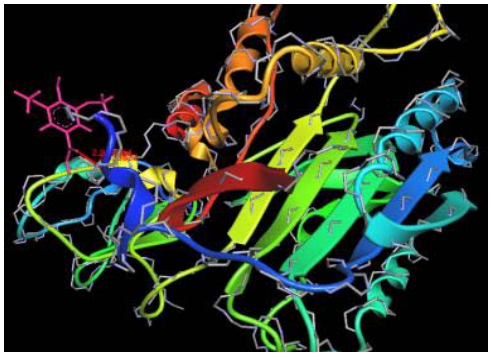

Fig. 6: Caspase9 docked with syringic acid

Fig. 7: Cytochrome C docked with syringic acid

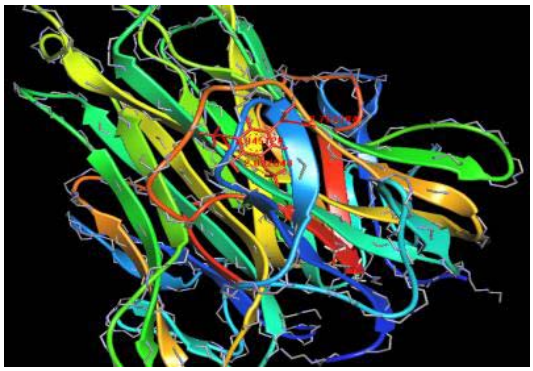

Fig. 8: TNF α docked with syringic acid

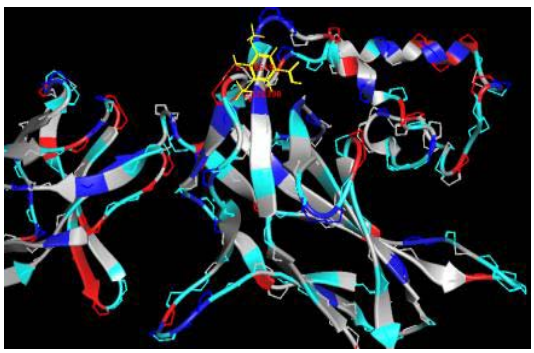

Fig. 9: NFKB docked with syringic acid

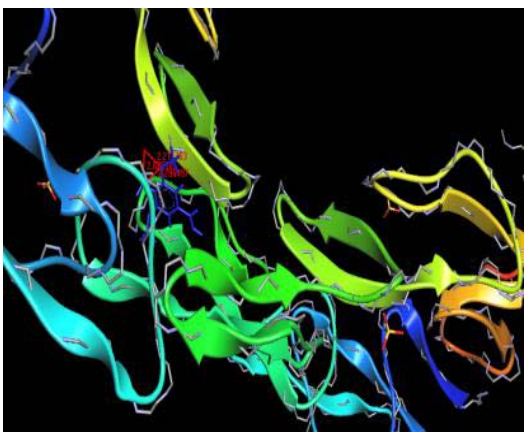

Fig. 10: TRAF1 docked with syringic acid

Table 3: Docking of syringic acid derivatives with caspase 3

S. No.	Syringic acid derivatives	Name of the protein (2J30)	Energy in Kcal/mol	Hydrogen bonds	Length
1.	Acetosyringone	Caspase3	-4.9	1	H1: 2.8 Å
2.	Syringaldehyde	Caspase3	-5.4	3	H1: 2.91 Å H2: 2.37 Å H3: 2.92 Å
3.	Syringol	Caspase3	-4.9	1	H1: 2.34 Å
4.	Sinapinic acid	Caspase3	-6.0	4	H1: 2.37 Å H2: 2.03 Å H3: 2.84 Å H4: 2.67 Å
5.	Sinapyl alcohol	Caspase3	-6.0	4	H1: 2.91 Å H2: 2.75 Å H3: 2. 90Å H4: 2.84 Å
6.	Sinapaldehyde	Caspase3	-5.6	4	H1: 2.75 Å H2: 2.52 Å H3: 2. 82Å H4: 2.99 Å
7.	Sinapine	Caspase3	-6.1	5	H1: 2.87 Å H2: 2.33 Å H3: 2.61 Å H4: 2. 83Å H5: 2.99 Å
8.	Canolol	Caspase3	-5.5	2	H1: 2.83 Å H2: 2.84 Å

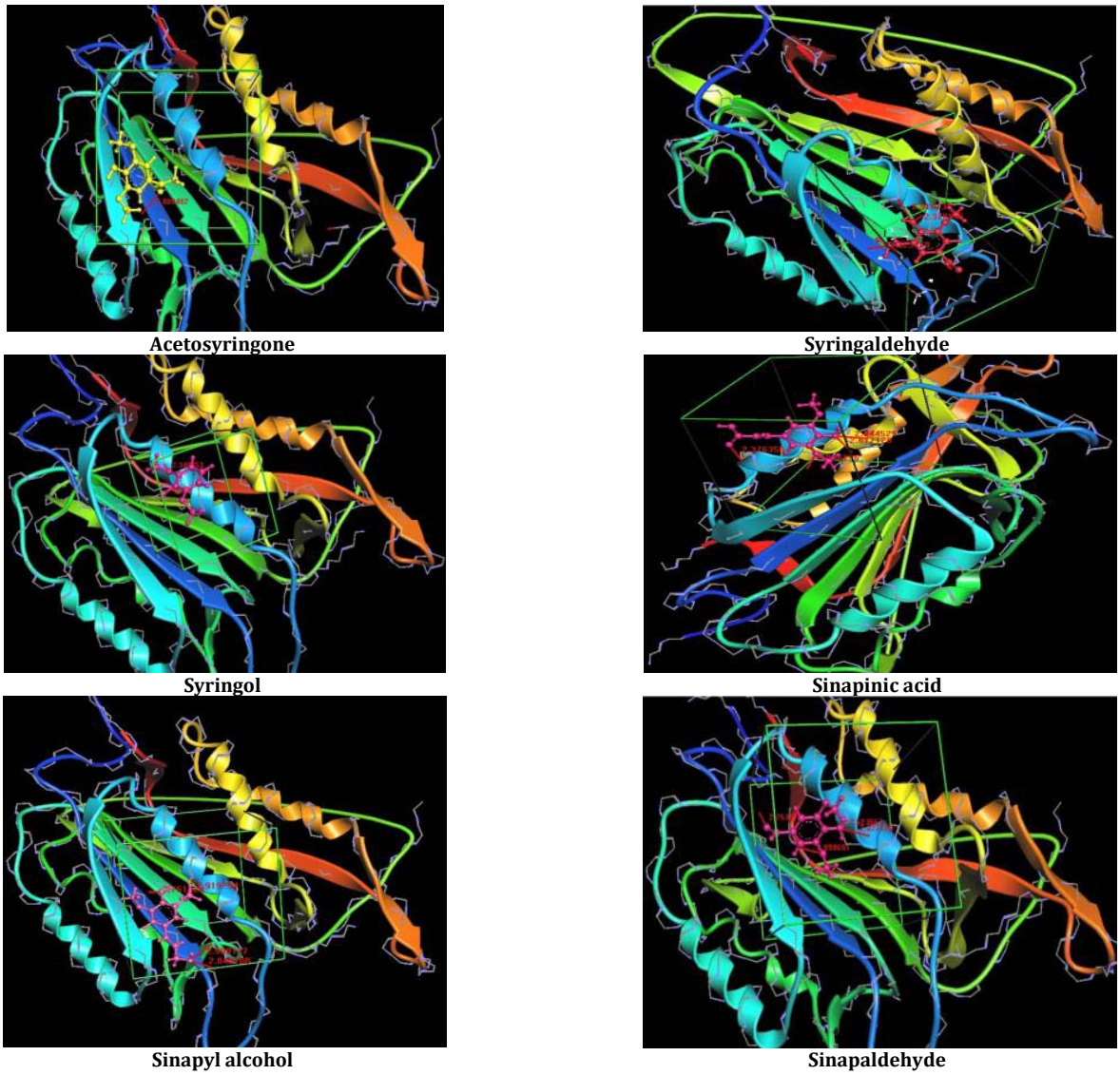

Fig. 11: Docking of syringic acid derivatives with caspase 3

***In vivo* study**

Effect on weight (Body and liver)

The initial body weight of all groups was nearly identical. At the end of the study, both the normal control group and the syringic acid (25 mg/kg) group of rats had increased body weight. After the experiment, the rats treated with diethyl nitrosamine (DEN) showed a decreased body weight. Furthermore, both the diethyl nitrosamine (DEN)-induced syringic acid (25 mg/kg) and positive control treated groups of rats had improved body weight. Normal control and syringic acid alone groups had comparable liver and relative liver tissue weight. The liver weight of rats in the diethyl nitrosamine (DEN)-induced HCC group was higher than in the control group. The syringic acid and positive control (mercapto purine) treated group of rats had lower liver weight than the diethyl nitrosamine (DEN) control [29].

Table 4: Body weight and liver weight of rats

	Initial weight (g)	Final weight (g)	Liver weight (g)
Control	172.09±1.25	263.67±1.12	9.70±0.55
Syringic acid	172.38±0.89	261.29±2.17	8.66±0.44
DEN	179.41±2.89	214.16±2.08	12.16±1.46
DEN+syringic acid	173.34±0.83	241.84±3.09	11.84±0.58
DEN+mercapto purine	175.78±3.34	231.64±3.37	11.21±0.83

Notes: The mean data in table 4 exhibited a significant difference of *p<0.05 based on the posthoc Tukey test. The initial weight of the rat has a p-value of 0.000042; the final weight showed a p-value of 0.00001, and the liver weight showed a p-value of 0.0013. Therefore the significant difference between and within the groups is acceptable. The data represent mean±SD (n=6), diethyl nitrosamine (DEN).

Effect on biochemical parameters

The alpha-fetoprotein (AFP) is a key parameter widely used to estimate the progression of hepatocellular carcinoma (HCC). Due to spill over into the serum during HCC, the level of hepatic parameters has risen. The aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) levels in the DEN-induced group rats were higher, as indicated in the table 5. Treatment with Syringic acid (25 mg/kg) and 6-mercapto purine (group V) treatment significantly reduced alpha-fetoprotein (AFP), aspartate aminotransferase (AST), and alanine transaminase (ALT) levels.

Table 5: Biochemical parameters

	AST(U/l)	ALT (U/l)	ALP (ng/dl)	GGT(U/l)
Control	54.18±0.84	59.94±4.22	4.03±1.03	8.62±0.47
Syringic acid	57.85±2.49	58.5±2.87	5.60±0.40	10.17±1.67
DEN	99.01±4.42	113.83±8.19	32.84±3.43	44.52±3.31
DEN+Syringic acid	60.2±4.09	73.17±6.48	10.82±2.65	10.86±1.66
DEN+mercapto purine	67.84±5.44	75.83±3.25	12.17±2.21	12.80±1.69

Notes: The mean data in table 5 exhibited a significant difference of *p<0.05 based on the posthoc Tukey test. The aspartate aminotransferase (AST) has a p-value of 0.00001, the alanine transaminase (ALT) showed a p-value of 0.00001, the alkaline phosphatase (ALP) showed a p-value of 0.00013, and the gamma-glutamyl transferase (GGT) has a p-value of 0.0002. Therefore, the significant difference between and within the groups is acceptable. The data represent mean±SD (n=6), diethyl nitrosamine (DEN).

Activity of mitochondrial enzymes

In comparison to the Control group, DEN dramatically reduced the activities of succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) in the mitochondria. In comparison to the diethyl nitrosamine (DEN) group, the DEN-induced syringic acid and positive control treatment groups substantially enhanced the activity of succinate dehydrogenase and malate dehydrogenase. There was no significant difference in the activity of mitochondrial enzymes between the syringic acid-alone treated group and the control group. Fig. 12 and 13 shows the effect of syringic acid on mitochondrial enzyme activity.

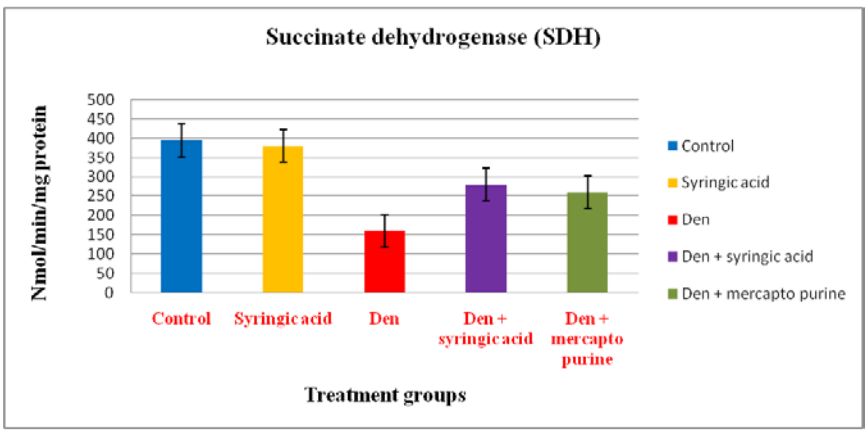

Fig. 12: Succinate dehydrogenase activity, Note: The mean data in fig. 12 exhibited a significant difference of *p<0.05 based on the post-hoc Tukey test. The data represents mean±SD (n=6). Diethyl nitrosamine (DEN)

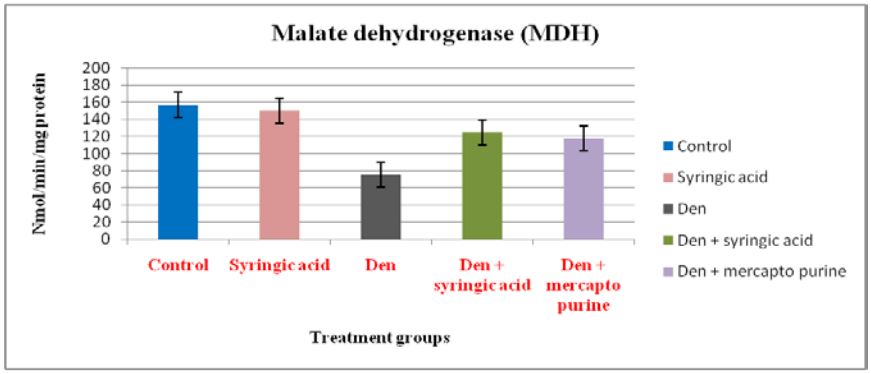

Fig. 13: Malate dehydrogenase activity, Note: The mean data in fig. 13 exhibited a significant difference of *p<0.05 based on the post-hoc Tukey test. The data represent mean±SD (n=6). Diethyl nitrosamine (DEN)

Histopathology analysis

The influences of syringic acid on the livers of diethyl nitrosamine (DEN)-induced experimental rats are illustrated in fig. 14. Significant enlargement and localized nodular hyperplasia were seen in the liver tissues of a diethyl nitrosamine (DEN)-induced rat when compared with the control. Remarkably, both the syringic acid (group III) and the positive control groups had less cellular expansion, nodules, and hyperplasia. Similarly, when syringic acid was administered alone in-group IV, there was no significant difference in the shape of liver cells compared to the animal's in-group I. Histological findings suggest that syringic acid has therapeutic benefits on the liver in rats with diethyl nitrosamine (DEN)-induced liver cancer.

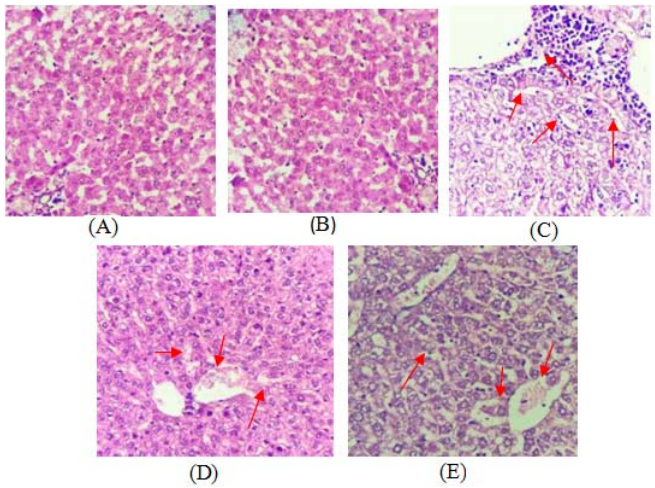

Fig. 14: Hematoxylin and eosin (H and E) staining control group (A) and syringic acid group (B) show no hepatic tissue injury. Hematoxylin and eosin (H and E) staining diethyl nitrosamine (DEN) group (C) show enlarged nodular hyperplasia. Hematoxylin and eosin (H and E) staining of syringic acid and positive control treated groups (D and E groups) show less cellular enlargement and nodular hyperplasia (magnification 400X)

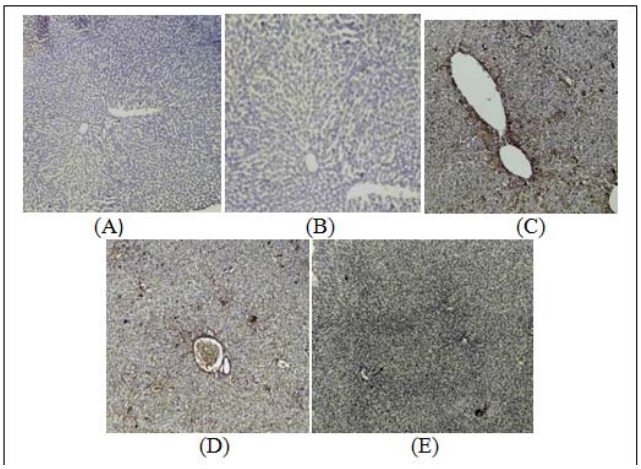

Fig. 15: BCL2 group: control (A) shows the absence of Bcl2, Syringic acid (B) shows the absence of Bcl2, DEN-induced HCC (c) shows the Presence of Bcl2, DEN-induced and Syringic acid treated (D), and Positive control-treated (E) shows the absence of Bcl2. DEN: diethyl nitrosamine, HCC: hepatocellular carcinoma

Immunohistochemical analysis

Syringic acid's potential for inducing apoptosis in HCC was evaluated by analyzing the expression of antiapoptotic protein Bcl2 activity as well as activated caspase-3, BAX, and cytochrome C activity as apoptotic markers. When compared to the control group, the diethyl nitrosamine (DEN)-treated group exhibited the lowest apoptotic protein (Bax, cytochrome C, caspase3) activity and the greatest Bcl2 activity, leading to cell proliferation and the development of hepatocellular carcinoma. Notably, the expression of Bax, cytochrome C, and caspase 3 increased dramatically in the syringic acid and positive control treatments. However, as compared to the control group, the syringic acid alone treated group revealed substantial downregulation of Bcl2 expression and elevation of apoptotic proteins. Thus, these analyses demonstrate the effect of syringic acid against HCC by promoting apoptotic proteins in cancer cells. The immunohistochemical images of Bcl2, Bax, cytochrome-C, and caspase 3 was shown in fig. 15,16,17,18 respectively.

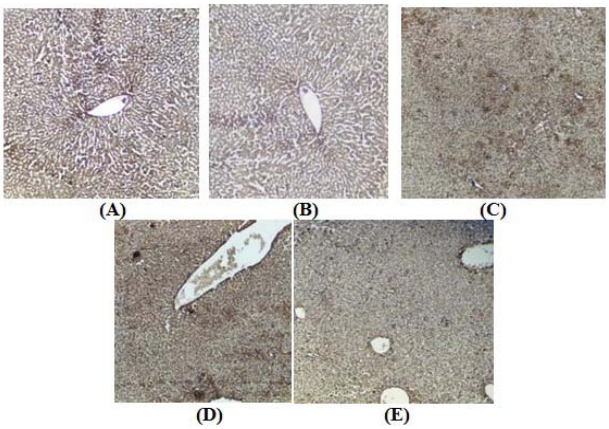

Fig. 16: Bax group: (A) control shows the presence of Bax, (B) Syringic acid shows the presence of Bax, (c) DEN-induced HCC shows the absence of Bax, (D) DEN-induced and Syringic acid treated, (E) Positive control treated shows the cytoplasmic and membranous positivity of Bax. DEN: diethyl nitrosamine, HCC: hepatocellular carcinoma

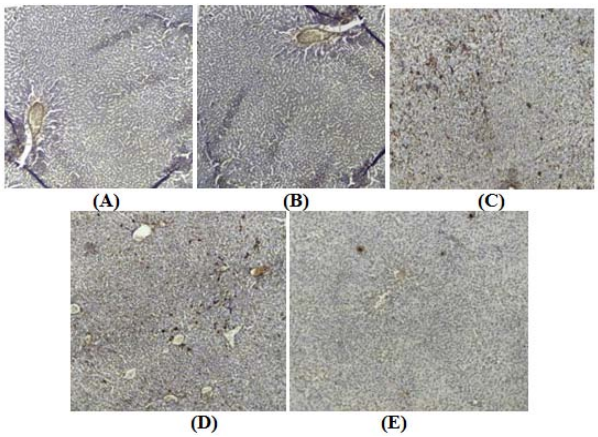

Fig. 17: Cytochrome C group: (A) control group shows the presence of cyt-C, (B) Syringic acid shows the presence of cyt-C, (c) DEN-induced HCC shows the absence of cyt-C,(D) DEN-induced and Syringic acid treated, (E) Positive control treated shows the patchy cytoplasmic positivity of cyt-C. DEN: diethyl nitrosamine, HCC: hepatocellular carcinoma, cyt-c: cytochrome C

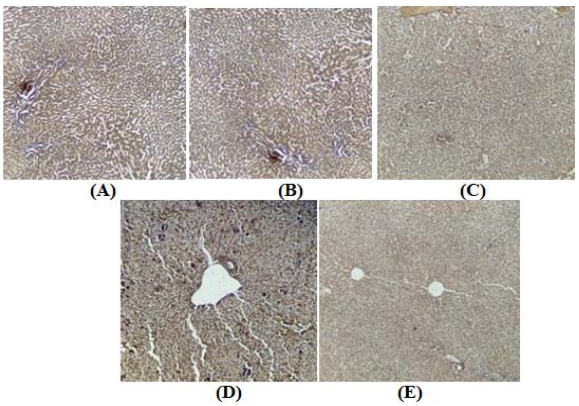

Fig. 18: Caspase 3 group: (A) control shows the presence of caspase 3, (B) Syringic acid shows the presence of caspase 3, (c) DEN-induced HCC shows the absence of caspase 3,(D) DEN-induced and syringic acid treated, (E) Positive control treated shows the cytoplasmic positivity of caspase 3. DEN: diethyl nitrosamine, HCC: hepatocellular carcinoma

Immunohistochemical analysis

Syringic acid's potential for inducing apoptosis in HCC was evaluated by analyzing the expression of antiapoptotic protein Bcl2 activity as well as activated caspase-3, BAX, and cytochrome C activity as apoptotic markers. When compared to the control group, the diethyl nitrosamine (DEN)-treated group exhibited the lowest apoptotic protein (Bax, cytochrome C, caspase3) activity and the greatest Bcl2 activity, leading to cell proliferation and the development of hepatocellular carcinoma. Notably, the expression of Bax, cytochrome C, and caspase 3 increased dramatically in the syringic acid and positive control treatments. However, as compared to the control group, the syringic acid alone treated group revealed substantial downregulation of Bcl2 expression and elevation of apoptotic proteins. Thus, these analyses demonstrate the effect of syringic acid against HCC by promoting apoptotic proteins in cancer cells. The immunohistochemical images of Bcl2, Bax, cytochrome-C, and caspase3 was shown in fig. 15,16,17,18 respectively.

DISSCUSSION

Molecular docking analysis

By utilizing computational tools, molecular docking can determine the ligand-receptor complex's structure. In this study, we predicted the possible interaction and targets of syringic acid against liver apoptotic proteins from the Protein Data Bank using a molecular docking approach. All the apoptotic proteins 1AIE, 4QVF, 2O2F, 3DEH, 1NW9, 3ZCF, 1TNF, 1SVC, and 1EXT were docked with the syringic acid ligand. Table 6 includes all the data on hydrogen bond counts, binding energies (kcal/mol), and bond lengths.

Table 6: Interaction of different molecules of apoptotic proteins with syringic acid

S. No.	PDB id	Name of the proteins	Chain	Energy in Kcal/mol	Hydrogen bonds	Length
1.	1AIE	P53	A	-4.16	2	H1: 2.95 Å H2: 2.00 Å
2.	4QVF	BAX	A	-5.21	3	H1: 2.87 Å H2: 2.99 Å H3: 2.29 Å
3.	2O2F	Bcl2	A	-6.05	3	H1: 2.02 Å H2: 2.94 Å H3: 2.84 Å
4.	3DEH	Caspase 3	B	-4.2	4	H1: 2.72 Å H2: 2.87 Å H3: 2.03 Å H4: 2.27 Å
5.	1NW9	Caspase 9	B	-4.49	2	H1: 2.99 Å H2: 2.93 Å
6.	3ZCF	Cytochrome C	B	-4.17	2	H1: 2.54 Å H2: 2.95 Å
7.	1TNF	TNFα	A	-5.08	3	H1: 2.75 Å H2: 2.94 Å H3: 2.09 Å
8.	1SVC	NFKB	B	-4.79	2	H1: 2.52 Å H2: 2.12 Å
9.	1EXT	TRAF1	A	-5.18	3	H1: 2.20 Å H2: 2.62 Å H3: 2.21 Å

P53, Bax, Bcl2, Cytochrome c, Caspase 3 and 9

A frequently impacted mechanism in the development of hepatocellular carcinoma (HCC) is the P53 cell-cycle pathway. The most frequent mutation in P53 is found in 12–48% of hepatocellular carcinoma (HCC) patients, and inactivation of this tumor suppression cascade promotes uncontrolled cell-cycle activity [6]. The P53 protein inhibits cell development and activates an apoptotic pathway that leads to programmed cell death. P53, directly and indirectly, promotes the activity of BCL-2 family proteins in apoptosis. BCL-2 forms heterodimers with the BAX protein and the up-regulation or downregulation of the BAX/BCL-2 ratio indicates whether apoptosis is triggered or suppressed. The altered BAX/BCL-2 ratio, results in the release of cytochrome C from mitochondria into the cytosol. Cytochrome C in turn activates the caspase enzyme activity, which is closely related to apoptosis signaling via intrinsic and extrinsic mechanisms. In the intrinsic mechanism, caspase-3 has activated by caspase-9 (Intrinsic pathway). Subsequently, in extrinsic mechanism, caspase 8 triggers caspase 3, which is the final step leading to cell death (Apoptosis) [7].

Tnfα, tnfr1 and nfκb

Activated monocyte macrophages, endothelial cells, and lymphocytes are the primary producers of TNFα, which binds to TNF-binding receptors (TNFR1 and TNFR2) to exert their biological action. TNF interacts with its receptor on the cell membrane and initiates the intracellular downstream signaling pathways, such as the proapoptotic pathways [8]. TNFα and the signal-induced degradation of IkB proteins trigger the activation of the NFκB by the enzyme IκB kinase. The NFκB complex penetrates the nucleus with the degradation of IκB, where it activates the expression of particular genes. Genes that activate by NFκB promote cell survival and cell proliferation. NFκB exerts an auto-regulatory impact by activating the expression of its own repressor, IκBα. Members of the NFκB family mostly mediate the interaction of genes involved in different biological phenomena, such as carcinogenesis and inflammatory response [9]. The interaction between the NFκB subunit and TNFα may improve the liver's condition [10].

In our study, the binding efficiency of syringic acid and its derivatives against liver apoptotic proteins was observed using docking studies. Syringic acid had good binding effectiveness with each of the liver cancer proteins. Likewise, the docking investigation conducted by Holis A. Holik *et al.* in 2022 [30] on the antibiotics ciprofloxacin A and B and chemicals from yodium leaf revealed binding energies of-5.41 and-7.02, respectively. Accordingly, the study demonstrated that the ligand with the lowest binding energy had the greatest affinity. In contrast, in this investigation, syringic acid and its derivatives showed a low affinity for apoptotic proteins. The receptors p53, Caspase 9, cytochrome C, and NFκB bound with strong affinity, have two hydrogen bonds, whereas the receptors Bax, Bcl2, TNFα, and TRAF1 have interacted with three hydrogen bonds and possess the strongest binding. The receptor Caspase3 exhibits strong binding with four hydrogen bonds. Similarly, the syringic acid derivatives with terminal apoptotic protein caspase3 showed excellent docking.

***In vivo* study**

There are many cancer-causing chemicals found in the environment that is utilized as an initial agent for hepatic cancer, including diethyl nitrosamine (DEN), which is often found in smoking products, chemicals, beauty products, roasted foods, and medicinal compounds. A preliminary diagnosis of liver disease has been made by assessing the body and liver mass of animals. Thus, diethyl nitrosamine (DEN)-treated rats (group II) had a noticeable loss of body weight and an increase in liver weight, possibly due to chronic liver damage. Symptoms of liver damage include a reduction in appetite and food consumption. Because of syringic acid treatment, body and liver weight has been restored, indicating enhanced desire and food consumption. Similarly, the study of *Hussain et al.* (2012) [23] has found similar effects with DEN in addition to CCL4 in rats, inducing inflammation and cirrhosis of the liver.

The liver marker enzymes such as aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) in blood serum were assessed in all groups of rats, and compared with control. The diethyl nitrosamine (DEN) induced group (II) showed an elevated level of these liver markers, which indicates liver injury, whereas the diethyl nitrosamine (DEN) treated syringic acid group showed a declined level of these liver markers. In contrast, the study of Veena Sharma *et al.* (2015) [31] reported that N-nitroso pyrrolidine (NPYR) in addition to carbon tetrachloride (CCl4) induced hepatocellular carcinoma (HCC) showed a gradual decrease in liver marker levels, whereas the hepatocellular carcinoma (HCC) group treated with hydrolic extract of *Helicteres isora* exhibited the gradual increase in levels of liver marker enzymes as compared with control. As a result, syringic acid can suppress tumor growth in diethyl nitrosamine (DEN)-induced hepatocellular carcinoma (HCC). Thus, Syringic acid has improved the pathology of liver cell morphology. Many earlier studies showed that the increase in liver enzymes is a result of unstable liver metabolism. Unique markers of HCC like alpha-fetoprotein (AFP), and gamma-glutamyl transferase (GGT) indicators are more accurate in predicting liver malignancy. The increase in these enzymatic activities has been attributed to the release of enzymes from the cytoplasm into the bloodstream and subsequent liver injury [24].

Mitochondria regulate numerous critical cellular processes that include energy generation, signal transduction, redox status modulation, biosynthesis, and apoptosis. Mitochondria regulate the biosynthesis of cholesterol, amino acids, nucleotides, glucose, heme, and FAs [29]. Any changes in these processes can have an impact on biosynthetic pathways and cell signaling pathways, potentially shifting the cells toward carcinogenesis. As a result, safeguarding mitochondria during first carcinogen assaults might be a possible method for preventing carcinogenesis [25]. Thus, in this study, the level of mitochondrial enzymes such as succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) has been evaluated to examine the mitochondrial activity. In this study, it is observed that the diethyl nitrosamine (DEN) induced group showed decreased enzyme activity. The mitochondrial enzyme activity slowly increased upon administration of syringic acid in the diethyl nitrosamine (DEN) treated group (IV) compared to the control. The study of *Sachin Shetty et al.* (2021) [19] also showed that mitochondrial activity was decreased in DEN-induced and mito-TEMPO-treated hepatocarcinogenesis in wistar rats.

In this study, the histopathological evalualtion of the control rat liver showed the normal absence of inflammation and nodules. The diethyl nitrosamine (DEN)-induced hepatocellular carcinoma (HCC) group has shown enlarged nodules and hyperplasia. The syringic acid-treated group exhibited a normal histological condition of the liver as compared with the control. Thus, it has been concluded that syringic acid is effective against hepatocellular carcinoma. Ibrahim A. Komeil *et al.*, [26], reported similar results in the genistein-loaded phytosome group against diethyl nitrosamine (DEN)-induced liver cancer. The histological report of mice liver on diethyl nitrosamine (DEN)-induced hepatocellular carcinoma (HCC) showed hepatic inflammation and nodules and a decrease in nodule formation, inflammation, and hyperplasia in the genistein-loaded phytosome group.

Tumor development, proliferation, and maintenance have been controlled by changes in apoptosis-related proteins. Previous research had revealed that apoptotic dysregulation is a key cause of hepatocellular carcinoma (HCC) development [20]. The mitochondrial-mediated intrinsic and death receptors-mediated extrinsic pathways are the two major apoptotic pathways. The ratio of anti-apoptotic and apoptotic proteins results in cell death. The anti-apoptotic proteins Bcl-2 motivate the proliferation of cancer cell and decrease apoptosis, thus resulting in carcinoma. The apoptotic protein Bax present in the cytosol is activated by stress stimuli and plays a major role in the apoptosis of cancer cells. The Bax protein moves towards mitochondria and induces the release of cytochrome C present in mitochondria. The release of cytochrome C stimulates the expression of caspases. The cytosolic fraction has been found to have caspase-3, 8, and 9. The mitochondrial fraction has been found to have caspase-3 and 9, and the microsome carries caspase-7. The nucleus carries the caspase-2 and 3 [27]. Among all caspases, caspase-3 is involved in the major apoptosis of cancer cells. In the present study, the expression of apoptotic and antiapoptotic proteins was determined by immunohistochemical analysis. The diethyl nitrosamine (DEN)-induced group showed the expression of Bcl-2 and the absence of other apoptotic proteins. The diethyl nitrosamine (DEN)-induced syringic acid-treated group showed the absence of Bcl-2 and the upregulation of other apoptotic proteins Bax, Cytochrome C, and caspase3, as compared to the control. Thus it is found that syringic acid exhibits its anticancer effect by increasing caspase3 expression in diethyl nitrosamine (DEN)-induced hepatocellular carcinoma (HCC) and brings apoptosis of cancer cells.

CONCLUSION

The present study exhibited the docking energy of syringic acid and its derivatives with apoptotic proteins. The ligand molecule, syringic acid, complies with all the properties of Lipinski Rule's of five, which strongly indicates that syringic acid can be used as an oral medication. The study also exhibited the therapeutic output of syringic acid in the liver of diethyl nitrosamine (DEN)–induced hepatocellular carcinoma (HCC) rats through induction of apoptosis. Thus, it has been determined that syringic acid has excellent anticancer activity against hepatocellular carcinoma.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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