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Original Article

Antioxidant-Rich *Vitex agnus-castus* Fruit Extract Attenuates Toluene-Induced Hepatic Apoptosis and Autophagy Dysregulation

Kosar Khosravi[1], Mohammad Karimian[2*], Fereshteh Mir Mohammadrezaei[1*], Ehsan Nazifi[3]

[1]Department of Biology, Faculty of Science, University of Mazandaran, Babolsar, Mazandaran, Iran
[2]Department of Cellular and Molecular Biology, Faculty of Science, University of Mazandaran, Babolsar, Mazandaran, Iran
[3]Department of Plant Sciences, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

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Abstract

Introduction: Toluene can trigger oxidative stress, cellular injury, and dysregulation of apoptosis and autophagy pathways in the liver. *Vitex agnus-castus* (VAC) fruit extract, known for its rich antioxidant, flavonoid, and phenolic contents, may counteract these effects through its free radical-scavenging and cytoprotective actions. This study aimed to evaluate the protective role of VAC fruit extract on key apoptotic and autophagy-related marker genes in toluene-exposed mice, supported by bioinformatics analysis.

Methods: Flavonoid and phenolic contents of VAC fruit extract were measured colorimetrically, and antioxidant activity was assessed using the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Adult male mice received toluene (100 mg/kg), VAC fruit extract (100 or 200 mg/kg), or their combination. The evaluation included gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR), measurement of serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and molecular docking analysis along with gene network construction using bioinformatics tools.

Results: The flavonoid, total phenolic content, and antioxidant potential of the VAC fruit extract were 60.24, 126.35, and 455.23 mg equivalents per gram of extract, respectively, indicating its high antioxidant capacity. Toluene upregulated Bax ($P<0.01$), Lc3 ($P<0.05$), and Beclin1 ($P<0.05$) gene expression, which was significantly downregulated by VAC (100 or 200 mg/kg; $P<0.05$ to $P<0.01$), while Bcl2 gene expression, as well as AST and ALT levels, remained unchanged. Molecular docking confirmed the strong binding of VAC compounds to the target proteins, which may contribute to their functional alterations.

Conclusion: The strong antioxidant and bioactive features of VAC fruit extract contributed to the modulation of oxidative stress-induced apoptotic and autophagy gene expression in the liver of toluene-exposed mice, highlighting its potential as a hepatoprotective and regulatory compound against toluene-induced toxicity.

Introduction

Volatile organic compounds are widespread environmental contaminants present in industrial wastewater, vehicle emissions, and household products. [1] Among these, toluene is a frequently used volatile aromatic hydrocarbon solvent found in paints, adhesives, printing inks, and fuels. [2] Because of its high volatility and extensive usage, toluene can easily contaminate air and water, leading to pervasive human exposure via inhalation or ingestion. [3] Although the central nervous system (CNS) is a well-established target of toluene toxicity, the liver also carries a substantial metabolic load and can be susceptible to injury under prolonged or high-level exposure conditions.[4,5] Toluene rapidly accumulates in lipid-rich tissues like the liver.[6] In hepatocytes, cytochrome P450 enzymes metabolize toluene to benzyl alcohol, benzaldehyde, and ultimately hippurate for excretion. [7,8] These metabolic processes can produce reactive oxygen species (ROS) as byproducts. [9] When ROS generation exceeds the capacity of cellular antioxidants, oxidative stress occurs, characterized by lipid peroxidation, damage to proteins and DNA, and disruption of mitochondrial membrane structure. [10,11] Mitochondrial membranes are highly vulnerable to oxidative damage, and intense oxidative stress may cause the disruption of membrane potential, triggering the release of molecules that promote apoptosis. [12,13] Oxidative stress activates cell-death pathways in hepatocytes, mediated by proteins like Bax (pro-apoptotic) and Bcl2 (anti-apoptotic). [14,15] Under

Corresponding Authors: Mohammad Karimian, Email: mdkarimian@gmail.com; Fereshteh Mir Mohammadrezaei, Email: fereshteh.mmrezaei@gmail.com

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stress conditions, Bax translocates to the mitochondria to promote cytochrome c release, while Bcl2 on the mitochondrial membrane prevents this event; thus, an elevated Bax/Bcl2 ratio indicates apoptotic activation. [16,17] Cells may simultaneously induce autophagy to eliminate damaged organelles or misfolded proteins and maintain cellular balance. [18] The onset of this process begins with the triggering of Beclin1, which forms a core complex with class III PI3K (Vps34) to nucleate the autophagosomal membrane. A critical step in autophagosome formation involves the lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3), converting LC3-I to LC3-II, which associates stably with the autophagosomal membrane and serves as a widely used autophagy marker. [19,20] Notably, the anti-apoptotic protein Bcl2 can bind directly to the BH3 domain of Beclin1, thereby suppressing its pro-autophagic activity. This interaction highlights the molecular crosstalk between the autophagic and apoptotic pathways, wherein Bcl2 not only prevents cell death but also inhibits autophagy under certain circumstances. [21] The delicate balance between these pathways is essential for determining cell fate, especially under stress conditions such as nutrient deprivation or oxidative insult. [22]

Vitex agnus-castus (VAC), commonly called chasteberry, is a herbal plant traditionally used to treat a variety of health conditions. [23] Research indicates that the fruits and leaves of VAC contain abundant bioactive substances, with a notable concentration of flavonoids and phenolic compounds. Major flavonoids identified in VAC include orientin, luteolin, casticin, and vitexin, which are abundant in the fruit. [24-28] These polyphenols have potent antioxidant capabilities; they scavenge free radicals and enhance endogenous antioxidant defenses. [29] In animal models, VAC extracts have demonstrated hepatoprotective activities by upregulating antioxidant enzyme activities and reducing mitochondrial ROS levels. [30] In addition to its antioxidant potential, VAC exhibits anti-apoptotic and cell-protective effects. [31] VAC exhibits notable apoptosis-modulating properties, mainly through its active constituents such as vitexins and casticin. [32,33] Based on reported evidence, vitexin, one of the active compounds in *Vitex agnus-castus*, plays an inhibitory role in the autophagy pathway by suppressing the autophagic process and reducing the expression levels of key proteins related to autophagy, including Beclin1 and LC3-I/II. [34] In addition, other bioactive compounds in this plant have also shown the potential to regulate autophagy mechanisms, which could be important in various disorders, including inflammatory and fibrotic conditions. [35] Together, these properties make VAC a promising hepatoprotective substance against toxin-induced injury. Therefore, it can be inferred that a parallel assessment of apoptosis- and autophagy-related genes in the livers of mice exposed to toluene can provide a comprehensive understanding of the protective role of VAC at the molecular level and enable a more precise identification of the mechanisms of action of its compounds. Accordingly, this study aimed to examine the effect of VAC fruit extract on the expression of *Bax*, *Bcl2*, *Beclin1*, and *Lc3* genes in mice exposed to toluene, using a complementary computational approach.

Methods

Ethical consideration

All animal experiments in this study were performed in strict compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and in accordance with the Animals (Scientific Procedures) Act 1986 and its associated regulations, the European Union (EU) Directive 2010/63 on the protection of animals used for scientific purposes, and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The experimental protocols were thoroughly reviewed and authorized by the Ethics Committee of the University of Mazandaran, thereby ensuring full adherence to relevant ethical standards (Ethical code: IR.UMZ.REC.1402.024).

Chemicals

All the necessary materials for the extraction procedure, along with toluene, were procured from Sigma-Aldrich (St. Louis, MO, USA). Molecular biology reagents and consumables (including kits and enzymes) were obtained from SinaClon (Tehran, Iran).

Preparation of Vitex agnus-castus fruit extract

The plant sample of *Vitex agnus-castus* was collected from Maragheh, Iran, during the summer of 2022 and was deposited at the Herbarium of the University of Mazandaran (HUMZ). The voucher number of the sample is HUMZ-8007. To prepare the extract, 300 g of dry *Vitex agnus-castus* fruit powder was mixed with 1000 mL of 80% methanol. This mixture was kept for 24 hours at room temperature, in the absence of light, on a shaker at 120 rpm. Then, the solution was passed through filter paper and concentrated using a rotary evaporator under vacuum at 40°C. Finally, the concentrated extract was completely dried in an oven at 37°C and stored at 4°C until further use.

Determination of total flavonoid and phenolic contents and antioxidant activity

Flavonoid content was determined using the aluminum chloride colorimetric assay. For this purpose, 200 µL of the extract (at a concentration of 1 mg/mL) was mixed with 40 µL of 10% aluminum chloride, 40 µL of 1 M potassium acetate, 600 µL of 80% methanol, and 1120 µL of distilled water. After 30 minutes of incubation, the absorbance of the samples was measured at 415 nm using a spectrophotometer. Finally, the flavonoid content was calculated based on the rutin standard calibration curve and expressed as mg per gram of extract. [36]

Total phenolic content was measured using the Folin-Ciocalteu colorimetric method. First, 200 µL of the extract (at a concentration of 1 mg/mL) was mixed with 1000 µL of 10% Folin reagent, and after five minutes, 800 µL of 7.5% sodium carbonate was added. After incubation for 60 minutes in darkness, the absorbance of the samples was read at 765 nm using a spectrophotometer. Finally, the total

phenolic content was determined in milligrams per gram of extract using a gallic acid standard curve. [37]

The antioxidant capacity of the plant samples was assessed through the ABTS cation radical scavenging assay. To generate the $ABTS^+$ radical cation, 88 µL of 40 mM potassium persulfate was added to 5 mL of 7 mM ABTS solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The mixture was then incubated in the dark at room temperature for 16 hours to allow complete generation of the free radical. The resulting solution was diluted with distilled water until its absorbance reached 0.70 ± 0.05 at 734 nm. For the assay, 200 µL of the plant extract (at a concentration of 1 mg/mL) was mixed with 1800 µL of the prepared ABTS solution. After 10 minutes, the absorbance was measured at 734 nm using a spectrophotometer. The percentage of inhibition was calculated using the following equation, and antioxidant activity was expressed in mg per gram of extract based on the ascorbic acid standard curve. [38,39]

$$\text{Inhibition } (\%) = (A_0 - A_s) / A_0 \times 100$$

A_0: Absorbance of the control, A_s: Absorbance of the sample

Experimental design and grouping

A total of 48 male laboratory mice (weighing between 20 and 25 g) were sourced from the Pasteur Institute in Amol, Iran. The animals were housed in the animal facility of the Biology Department of the University of Mazandaran at a temperature of 24°C, a 12-h light/dark cycle, and optimal humidity for one week to acclimate to the conditions. The mice were randomly assigned to 6 groups (n = 8 for each group). 1- The sham group received saline as the extract solvent alongside corn oil as the toluene solvent. 2- The VAC100 group received 100 mg/kg of VAC fruit methanolic extract. 3- The VAC200 group received 200 mg/kg of the same extract. 4- The TL group was given toluene dissolved in corn oil at 100 mg/kg. 5- The TL+VAC100 group first received toluene (100 mg/kg), followed by VAC extract at 100 mg/kg two hours later. 6- The TL+VAC200 group similarly received toluene (100 mg/kg) and then VAC extract at 200 mg/kg after a two-hour interval. All treatments were administered via intraperitoneal injection once daily for 21 consecutive days. [40,41] The dose of toluene for the study of hepatic toxicity in male mice was selected based on acute toxicity data. The intraperitoneal LD_{50} of toluene in mice has been reported to be approximately 1.15 g/kg over 24 hours. [42] Considering the prolonged duration of the study (21 days) and the risk of cumulative toxicity, a sublethal dose of 100 mg/kg (less than 1/10 of the acute LD_{50}) was used to prevent animal mortality while still allowing detectable hepatic effects.

Sample collection

At the end of the treatment period, all animals were anesthetized with ketamine (100 mg/kg) combined with xylazine (10 mg/kg). After achieving deep anesthesia, the mice were euthanized by decapitation. Their liver tissues were carefully excised and stored at -80°C to analyze gene expression changes. Also, blood samples were taken via cardiac puncture from all mice using sterile syringes, and their serum was stored in a -80°C freezer to measure liver enzymes.

RNA extraction and synthesis of cDNA and primer design

Total RNA was isolated using a commercial kit (Parstoos, Iran). The integrity and concentration of RNA were assessed by spectrophotometry (the ratio of absorbance at 260 nm to 280 nm) and agarose gel electrophoresis. The cDNA was synthesized from the extracted RNA using a dedicated kit (Parstoos, Iran) following the manufacturer's instructions. The materials in the kit were incubated with 30 ng of total RNA for 10 minutes at 25°C, then for 60 minutes at 47°C, and finally for 5 minutes at 85°C. Specific primers for *Bax*, *Bcl2*, *Lc3*, and *Beclin1* and the housekeeping control gene (*Gapdh*) were designed with Primer 3 software and validated by the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. The sequence and characteristics of the primers are presented in Table 1.

Quantitative Real-Time Polymerase Chain Reaction

To evaluate gene expression levels, a quantitative real-time polymerase chain reaction (qRT-PCR) process was performed using a Bio-Rad CFX96 system (Bio-Rad, CA, USA) and a SYBR Green Real Master Mix kit (Parstoos, Iran). The thermal cycling protocol consisted of an initial

Table 1. Sequence and characteristics of the specific primers

Gene name	Accession no.	Primer sequence (5′ to 3′)	Annealing temperature (˚C)	PCR product size (bp)
Bax	NM_007527	F: AGATCATGAAGACAGGGGCC R: GTCCACGTCAGCAATCATCC	59	200
Bcl2	NM_009741	F: AACTCTTCAGGGATGGGGTG R: ATCAAACAGAGGTCGCATGC		227
Lc3	NM_026160	F: GTGGTTCTGACAGCTGTTGG R: ACGAGTGAGTGAGTGACCAG		181
Beclin1	NM_001359820	F: TGAAATCAATGCTGCCTGGG R: TGCACACAGTCCAGAAAAGC		228
Gapdh	NM_001411844	F: CGACTTCAACAGCAACTCCC R: GAGTTGGGATAGGGCCTCTC		217

denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds, concluding with a final extension phase at 72°C for 5 minutes. To confirm specific amplification, the melting curve was generated and analyzed by a Roche LightCycler 96 real-time PCR system (Roche, Germany). The Ct values of the target genes (*Bax*, *Bcl2*, *Lc3*, and *Beclin1*) were compared with the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) reference gene, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Measurement of serum AST and ALT activity

Blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum. The activity levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes in the serum were quantified by UV-Vis spectrophotometry using Bionik assay kits (Tehran, Iran). Finally, the results were reported in units per liter (U/L) to allow for accurate and standardized comparisons.

In silico analysis

Molecular docking of the main components of VAC fruit extract, namely vitexin and casticin, with the Beclin-1, Lc3, and Bax proteins was carried out. The three-dimensional structures of mouse Beclin-1, Lc3, and Bax were constructed using SWISS-MODEL (v2023.10, https://swissmodel.expasy.org/) based on amino acid sequences retrieved from UniProt (accession numbers O88597, Q91VR7, and Q07813, respectively). Homology modeling was performed using template Q91XJ1.1.A (98.66% sequence similarity) for Beclin-1, 8t35.1.A (100% sequence similarity) for Lc3 and 4s0o.1.A. (91.67% identity) for Bax. The reliability of the models was assessed with GMQE scores of 0.77 for Beclin-1, 0.87 for Lc3, and 0.78 for Bax, demonstrating their quality. The resulting Beclin-1, Lc3, and Bax structures were examined in Molegro (v6.0) to identify key regions, including binding sites. Crystal water molecules, ligands, and redundant ions were removed using UCSF Chimera (v1.15rc) and PyMOL (v3.1.1), followed by the incorporation of polar hydrogens and assignment of partial charges using the Assisted Model Building with Energy Refinement (AMBER) ff14SB force field. Energy minimization was conducted with 1,000 steepest descent steps to optimize the structure.

Vitexin and casticin, the two main bioactive constituents of VAC, were sourced from the PubChem database with PubChem CIDs 5280441 and 5315263, respectively, in SDF format. Their structures were then energy-minimized using the UFF force field within Avogadro 1.2.0 software. Subsequently, the files were converted from SDF to PDB using Open Babel 2.3.90. [43]

Molecular docking was performed using AutoDock Vina (v1.1.2) integrated within Chimera. The grid box dimensions and center coordinates were carefully defined around the active site of each protein (Table 2). Docking parameters, including the number of runs and exhaustiveness, were optimized to ensure comprehensive sampling. Each ligand was docked individually with Beclin-1, Lc3, and Bax. The optimal binding pose was selected based on the lowest binding affinity (kcal/mol), and the ligand orientation within the active site was evaluated. Interactions, including hydrogen bonds, hydrophobic contacts, and van der Waals forces, were analyzed using PDBsum (https://www.ebi.ac.uk/pdbsum/), Protein-Ligand Interaction Profiler (PLIP, v2.2.2), Discovery Studio Visualizer (v21.1.0.20298), and PyMOL. Two- and three-dimensional visualizations of the complexes were generated, emphasizing key residues involved in hydrogen bonding and hydrophobic interactions.

Table 2. Coordinates of the Grid Box around the protein active site

Protein/Ligand	Coordinates/Size	X	Y	Z
Beclin1/Vtx	Center	84.2653	10.027	-115.988
	Size	25.8818	24.0108	30.4779
Beclin1/Cas	Center	-22.8258	-1.97491	24.0358
	Size	18.0234	28.7788	25.0276
Lc3/Vtx	Center	-5.01599	5.67863	-0.22322
	Size	19.4769	21.603	21.5498
Lc3/Cas	Center	7.01888	4.24174	-11.1437
	Size	23.6026	16.5429	20.458
Bax/Vtx	Center	21.2603	-11.7905	26.7018
	Size	19.1728	16.5098	14.973
Bax/Cas	Center	28.9995	6.45275	3.84945
	Size	15.9192	24.4186	17.0654

Vtx: vitexin, Cas: casticin

To further explore functional interactions, four target genes, Bax and Bcl2 (apoptosis pathway), and Beclin1 and Lc3 (autophagy pathway), were analyzed using GeneMANIA. This platform integrates multiple types of evidence, including co-expression, biological pathways, protein-protein interactions, and shared protein domains, to construct a network of functionally related genes. The analysis revealed complementary genes and overlapping pathways between apoptosis- and autophagy-related genes, providing deeper insights into their potential molecular cross-talk.

Statistical analysis

All experiments were conducted in triplicate. Comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine significant differences. *P*-values less than 0.05 were considered statistically significant. All data were analyzed with the Statistical Package for the Social Sciences (SPSS) software version 22, ensuring accurate and reproducible results.

Results

Flavonoid and phenolic content and antioxidant activity of the extract

In this study, the flavonoid content of the extract was determined using the aluminum chloride colorimetric

method, which relies on the formation of a stable flavonoid-aluminum complex. The quantification was performed by comparing the absorbance readings to a calibration curve prepared with rutin, a well-known flavonoid standard widely used due to its strong complexation and reliable spectrophotometric response. Based on this rutin standard curve, the flavonoid content of the extract was calculated and found to be 60.24 ± 2.68 mg of rutin equivalents per gram of extract.

The results of this study showed that the total phenolic content of the extract was 126.35 ± 2.63 mg gallic acid equivalent per gram of extract. In addition, the antioxidant activity of the extract was determined to be 455.23 ± 21.72 mg ascorbic acid equivalent per gram of extract. These values indicate a considerable phenolic content and antioxidant activity in the examined sample.

Effect of Vitex agnus-castus fruit extract on the expression of Bax, Bcl2, Lc3, and Beclin1 genes

Analysis of gene expression data revealed that toluene significantly increased *Bax* gene expression compared with the sham group ($P < 0.01$). Nevertheless, the combined treatment of toluene with VAC fruit extract at concentrations of 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) markedly decreased the expression of this gene compared with the toluene group. Administration of VAC fruit extract alone at both doses of 100 and 200 mg/kg did not produce any significant effect on *Bax* gene expression compared with the sham group (Figure 1A). In contrast, analysis of the *Bcl2* gene expression showed that toluene caused a reduction in the expression of this gene compared with the sham group, but this decrease was not statistically significant. Ultimately, VAC fruit extract and toluene did not cause any significant change in *Bcl2* gene expression among the groups (Figure 1B).

Regarding autophagy-related genes, the expression profiles of *Lc3* and *Beclin1* are presented in Figures 1C and 1D, respectively. Evidently, toluene significantly increased the expression of both genes compared with the sham group ($P < 0.05$). However, co-treatment with toluene and VAC fruit extract at a dose of 100 mg/kg was able to reduce the expression of both *Lc3* and *Beclin1* genes compared with the toluene group ($P < 0.05$). Also, co-administration of toluene and VAC fruit extract at a dose of 200 mg/kg only led to a significant downregulation of the *Beclin1* gene compared with the toluene group ($P < 0.05$). It is worth noting that administration of VAC fruit extract alone did not significantly influence *Lc3* and *Beclin1* gene expression compared with the sham group.

Effect of the VAC fruit extract on the level of ALT and AST enzymes

Serum AST and ALT levels in the group receiving toluene at a concentration of 100 mg/kg for 21 days did not exhibit a significant difference compared to the sham group. Similarly, the VAC 100 and VAC 200 groups and the TL+VAC 100 and TL+VAC 200 groups showed no significant changes in AST and ALT levels compared to the toluene group. Moreover, the VAC 100 and VAC 200 groups, as well as the TL+VAC 100 and TL+VAC 200

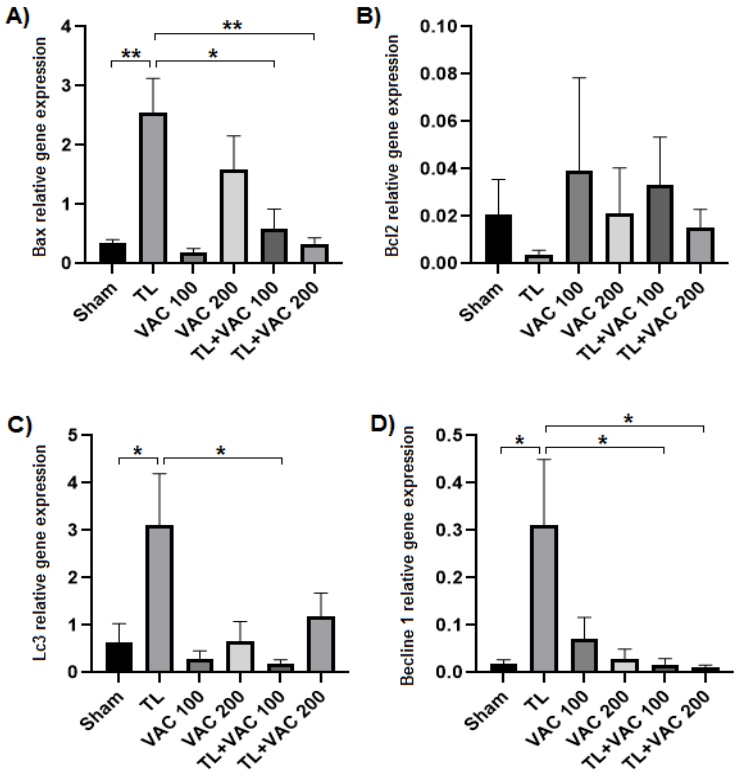


Figure 1. Results of *Bax*, *Bcl2*, *Lc3*, and *Beclin1* gene expression in liver tissue following treatment of mice with toluene and VAC fruit extract. Alterations in *Bax* (A), *Bcl2* (B), *Lc3* (C), and *Beclin1* gene expression after treatments. The symbols * and ** denote significant differences less than 0.05 and 0.01, respectively (n = 8). TL: toluene, VAC: *Vitex agnus-castus*.

groups, did not demonstrate any significant differences in AST and ALT enzyme levels when compared to the sham group (Figure 2).

Bioinformatics outputs

Molecular docking was employed to assess the binding affinity and key interactions between vitexin and casticin, and the Beclin-1, Lc3, and Bax proteins. Vitexin demonstrated binding affinities of -7.2 kcal/mol for Beclin-1 and Lc3, and -7.1 kcal/mol for Bax, whereas casticin showed binding affinities of -7.1, -6.7, and -7.3 kcal/mol for Beclin-1, Lc3, and Bax, respectively, with docking precision confirmed by root mean square deviation (RMSD) values of 0.0 for all proteins. Two-dimensional interaction analyses revealed that both vitexin and casticin establish hydrogen bonds as well as van der Waals interactions with each of the target proteins. In three-dimensional models, these compounds established hydrophobic and hydrophilic interactions with distinct residues in their active sites. These interactions are depicted in two-dimensional and three-dimensional diagrams (Figures 3 and 4). Detailed binding energies and interacting residues are summarized in Tables 3 and 4.

Molecular interaction network analysis using GeneMANIA indicated that all four genes studied were associated with a large number of genes related to the apoptosis and autophagy pathways (Figure 5). Notably, a significant portion of these interactions (45%) involved physical contacts, indicating the possibility of direct interactions between the protein products of these genes and other components of the aforementioned biological pathways. Additionally, interactions resulting from gene

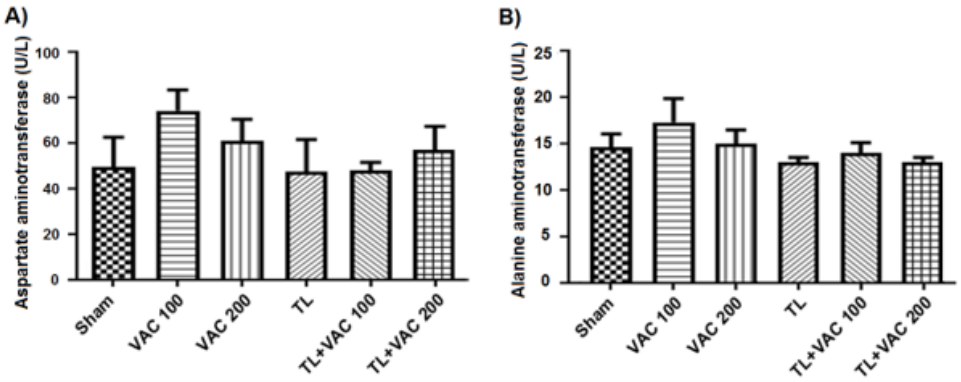


Figure 2. Results of AST and ALT levels in serum after treatment of mice with toluene and VAC fruit extract. Illustration of changes in the AST (A) and ALT (B) levels after treatments (n=8). TL: toluene, VAC: *Vitex agnus-castus*, AST: aspartate aminotransferase, ALT: alanine aminotransferase.

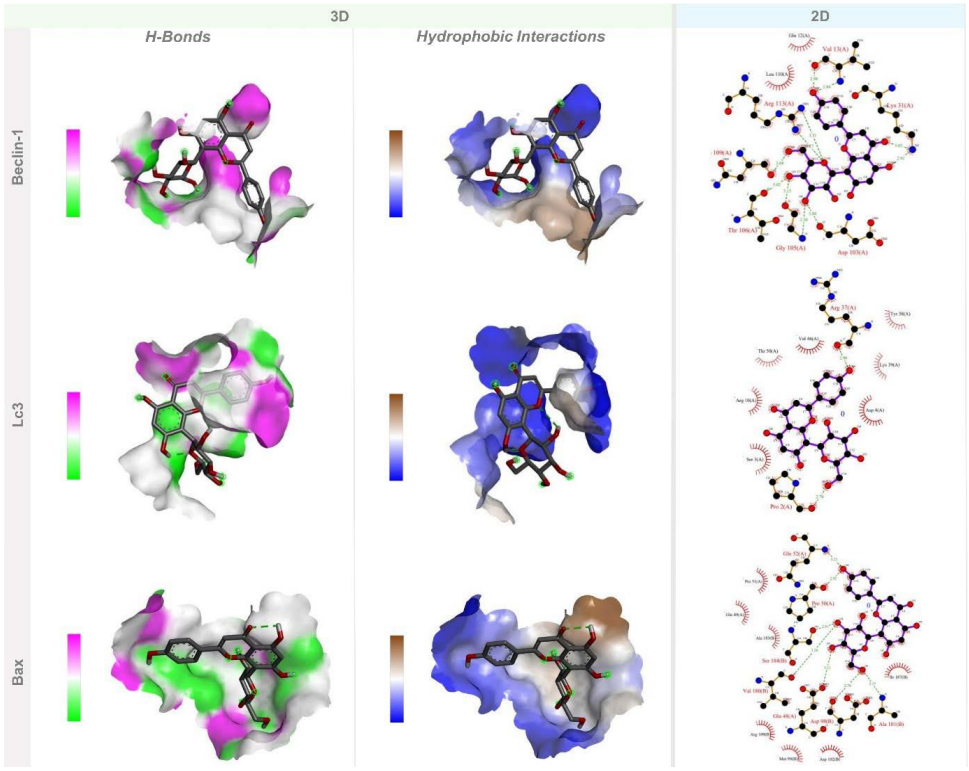


Figure 3. Molecular interactions of vitexin with Beclin-1, Lc3 and Bax proteins, presented in three panels. A 3D representation highlights hydrophobic interactions, with color intensity ranging from blue (low) to brown (high). A detailed 2D interaction map identifies key amino acid residues involved in the binding interface. Scale and interaction types are annotated accordingly.

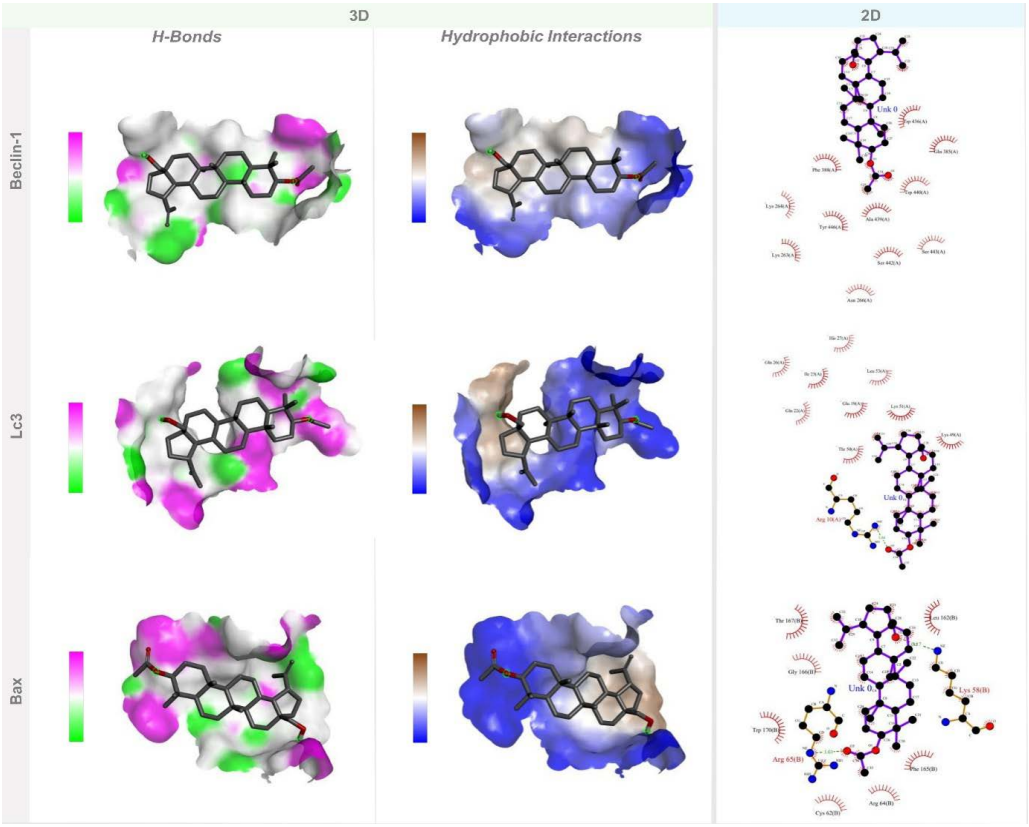


Figure 4. Molecular docking results of casticin with Beclin-1, Lc3, and Bax proteins, displayed across three panels. The 3D visualization reveals the distribution of hydrophobic interactions, where the gradient from blue to brown indicates increasing intensity. Additionally, a detailed 2D interaction diagram highlights the key residues at the binding sites, with annotated labels for interaction types and distance scales.

Table 3. Binding energy and key interactions of vitexin with Beclin1, Lc3 and Bax proteins

Vitexin interactions											
Receptor	Binding affinity (kcal/mol)	H-Bond Count	RMSD l.b.	RMSD u.b.	Hydrophobic interactions			Hydrogen bonds			
					Residue	AA	Distance	Residue	AA	Distance H-A	Distance D-A
Beclin1	-7.2	9	0.0	0.0	110A	LEU	3.73	13A	VAL	2.47	2.84
								31A	LYS	1.95	2.91
								103A	ASP	2.99	3.88
								103A	ASP	2.43	3.08
								105A	GLY	2.48	3.30
					110A	LEU	3.70	105A	GLY	2.28	3.15
								110A	LEU	2.97	3.39
								113A	ARG	2.73	3.31
								113A	ARG	2.68	3.27
Lc3	-7.2	2	0.0	0.0	4A	ASP	3.91	2A	PRO	2.34	2.70
					37A	ARG	3.69				
					46A	VAL	3.74	37A	ARG	2.12	2.98
Bax	-7.1	10	0.0	0.0	-	-	-	48A	GLU	2.83	3.21
								50A	PRO	2.41	2.92
								52A	GLN	2.36	3.23
								98B	ASP	2.70	3.33
								99B	MET	2.83	3.48
								101B	ALA	2.38	3.17
								102B	ASP	2.72	3.66
								102B	ASP	2.86	3.59
								109B	ARG	2.61	3.05
								184B	SER	3.64	4.04

RMSD l.b. and RMSD u.b. = 0.0 indicate high docking precision; AA: Amino acid residue number; H-A: Hydrogen-acceptor distance; D-A: Donor-acceptor distance.

Table 4. Binding energy and key interactions of casticin with Beclin1, Lc3 and Bax proteins

Casticin interactions											
Receptor	Binding affinity (kcal/mol)	H-Bond Count	RMSD l.b.	RMSD u.b.	Hydrophobic interactions			Hydrogen bonds			
					Residue	AA	Distance	Residue	AA	Distance H-A	Distance D-A
Beclin1	-7.1	1	0.0	0.0	266A	ASN	3.49	266A	ASN	2.50	3.38
					388A	PHE	3.96				
					388A	PHE	3.53				
Lc3	-6.7	0	0.0	0.0	19A	GLU	3.50	-	-	-	-
					23A	ILE	3.65				
					23A	ILE	3.70				
					51A	LYS	3.29				
					51A	LYS	3.74				
					53A	LEU	3.56				
Bax	-7.3	2	0.0	0.0	58B	LYS	3.67	58B	LYS	2.26	3.17
					162B	LEU	3.83				
					162B	LEU	3.55	58B	LYS	2.64	3.17
					167B	THR	3.66				
					170B	TRP	3.51				
					170B	TRP	3.89				

RMSD l.b. and RMSD u.b. = 0.0 indicate high docking precision; AA: Amino acid residue number; H-A: Hydrogen-acceptor distance; D-A: Donor-acceptor distance.

co-expression also accounted for a substantial fraction (17.96%) of network connections, which could indicate the simultaneous regulation of these genes under specific cellular conditions or in response to similar biological stimuli.

Discussion

Toluene is a commonly used volatile organic solvent that, when metabolized in the liver, produces reactive oxygen species and triggers cell death pathways, raising concerns about its potential hepatotoxic effects in both occupational and environmental settings. [44] In the present study, we examined the effects of *Vitex agnus-castus* fruit extract on the expression of key genes involved in apoptosis and autophagy within the liver of mice exposed to toluene. Additionally, we assessed serum ALT and AST levels following treatment. Our results showed that toluene increased the expression of the Bax gene, while the extract reduced its expression. At the dose of 200 mg/kg of *Vitex agnus-castus* extract alone, a slight increase in Bax gene expression was observed; however, this change was not statistically significant. Therefore, this increase cannot be considered a definitive biological effect attributable to the extract, and accordingly, only statistically significant changes were taken into account in the interpretation of the results. On the other hand, the variability observed in Bcl2 gene expression is likely attributable to the condition-dependent nature of this gene and the heterogeneous cellular responses to stress. Therefore, although some changes were observed, these responses were not uniform and should be interpreted with caution. However, toluene caused an upregulation of the *Lc3* and *Beclin1* gene expression, and the VAC fruit extract also influenced the expression of these genes. In contrast, analysis of serum liver enzymes showed no significant differences among the experimental groups treated with toluene and/or VAC extract.

Some previous studies have confirmed the apoptotic effects of toluene. For instance, one investigation reported that acute administration of high doses of toluene (2500 mg/kg) triggered apoptosis in the liver of rats through the mitochondrial pathway. This study also noted elevated serum enzyme levels. [45] Conversely, in our research, serum liver enzyme levels remained unchanged, which may be explained by the difference in dosing. Another study demonstrated that toluene at a dose of 650 mg/kg induced liver apoptosis only after 45 days of exposure to this substance in rats, while shorter exposure periods of 15 and 30 days did not produce significant effects. [46] The discrepancies between their findings and ours could also arise from variations in dose or exposure duration. Additionally, another study reported that 30 days of toluene inhalation elicited apoptotic changes in the rat liver. [47] This study differs from ours in terms of administration route and exposure time. In another study, rats inhaled toluene at 3000 ppm for one hour daily over four weeks, resulting in an increase in Bax gene expression in liver tissue. However, in the aforementioned study, an increase in serum levels of liver enzymes ALT and AST was also reported. [48] In our study, Bax expression was also upregulated; however, unlike that report, serum liver enzymes remained unchanged. This difference could be due to differences in administration routes (intraperitoneal injections versus inhalation or oral administration) and a longer exposure time in the aforementioned study. Notably, most cited studies used a rat model, whereas our research utilized mice. To our knowledge, no direct *in vivo* study has investigated the effects of toluene on autophagy, except for one study

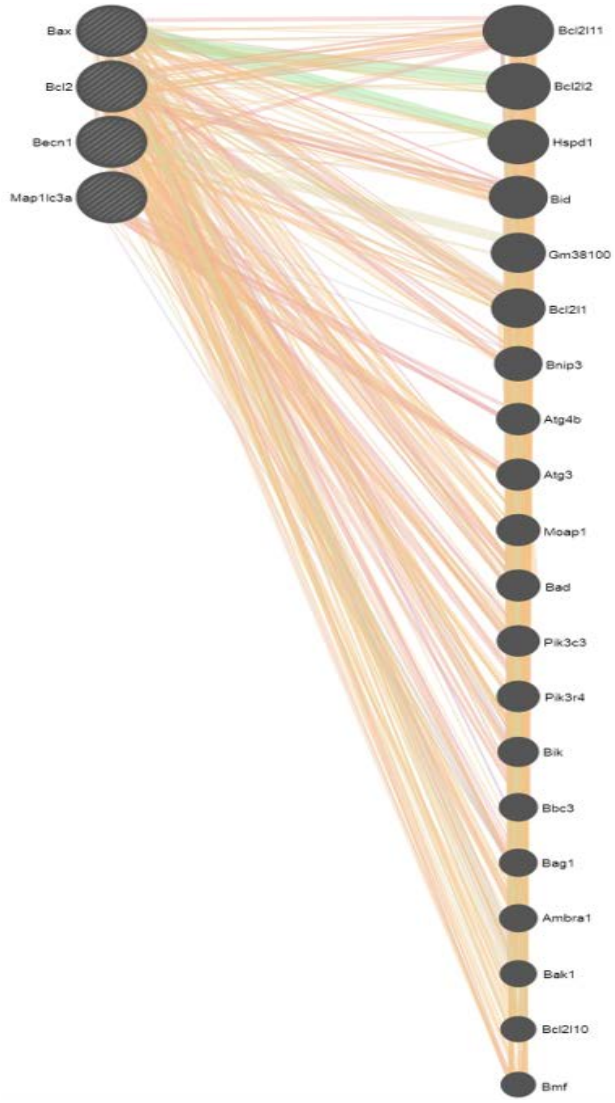


Figure 5. Gene interaction network analysis by GeneMANIA. The data showed that the four studied genes are associated with numerous genes involved in apoptosis and autophagy pathways. Approximately 45% of these interactions were physical, and 17.96% of the network connections resulted from co-expression of the genes.

examining the rat ovary. [49] That study showed that toluene exposure induced both apoptosis and autophagy, consistent with our liver tissue observations.

Moreover, no direct investigation has yet explored the effects of *Vitex agnus-castus* fruit extract on toluene-induced liver toxicity. However, several studies have reported beneficial impacts of this extract on the liver in different contexts. For instance, one study demonstrated that treatment of rats with both the crude extract and the butanolic fraction of VAC resulted in significant improvements, including a reduction in lipid profile markers and a complete reversal of non-alcoholic fatty liver disease. [30] Another study evaluated the efficacy of the ethanolic extract of *Vitex agnus-castus* in ameliorating hematopoietic changes caused by carbon tetrachloride-induced acute liver injury. Their findings showed that the ethanolic extract affected only white blood cell and platelet counts, without altering red blood cell parameters. [50] In contrast, the present study offers a more detailed molecular perspective on the protective effects of VAC fruit extract against toluene-induced liver toxicity.

Based on previous studies, *Vitex agnus-castus* extract has been administered through various routes, including oral and intraperitoneal injection, over a wide range of doses in animal models. [30,50] In one study, the hydroalcoholic extract of this plant was administered intraperitoneally to mice at doses ranging from 65 to 465 mg/kg, resulting in significant biological effects. [40] Additionally, another study reported that intraperitoneal administration of the extract at lower doses (25, 50, and 100 mg/kg) also induced notable behavioral and physiological changes in mice. [51] Collectively, these findings indicate that *Vitex agnus-castus* extract exhibits biological activity over a broad dose range when administered via the intraperitoneal route. Accordingly, the doses of 100 and 200 mg/kg selected in the present study fall within the range of previously reported effective doses and are pharmacologically and experimentally justified.

Mitochondrial apoptosis and autophagy represent two closely linked pathways in the cellular response to oxidative stress. [52] In mice exposed to toluene, Bax expression was markedly increased, indicating enhanced permeability of the outer mitochondrial membrane. Additionally, elevated levels of *Beclin1* and *Lc3* were observed, indicating activation of the autophagy. These molecular alterations support the idea that toluene, as an oxidative stress trigger, may simultaneously trigger both programmed cell death and autophagy pathways. Administration of *Vitex agnus-castus* extract effectively reduced the overexpression of these pro-death transcripts. This protective role is likely due to flavonoid compounds in the extract, which may suppress upstream stress signaling responsible for initiating apoptosis and autophagy. [53]

Toluene, as a member of the aromatic hydrocarbons, has the capacity to induce oxidative stress in various organs, with the liver, being the primary detoxification organ, experiencing greater exposure to this oxidative damage than other tissues. [46,54] The findings of our study demonstrated that the fruit extract of *Vitex agnus-castus* exhibits potent antioxidant activity, which may be attributed to its phenolic and flavonoid content. A previous study showed that *Vitex agnus-castus* extract was effective in preventing non-alcoholic fatty liver disease and oxidative stress, both of which are common causes of impaired liver function during the postmenopausal period. [30] Furthermore, another study reported that *Vitex agnus-castus* extract could mitigate oxidative injury caused by carbon tetrachloride in liver tissue. [55] Therefore, beyond its anti-apoptotic and anti-autophagic effects in the liver, the notable antioxidant capacity of this plant should also be emphasized.

Complementing the biological assays, the molecular docking analysis provides mechanistic insights into how the active compounds of VAC may exert their effects. The two major flavonoids in VAC, vitexin and casticin, were docked to the pro-death proteins Bax, Lc3, and Beclin1. The simulations demonstrated that both ligands exhibited stable binding affinities across all three targets. Notably,

vitexin and casticin formed multiple hydrogen bonds and hydrophobic interactions with critical residues within each protein's structure. For example, vitexin established hydrogen bonds inside the hydrophobic pocket of Bax, firmly anchoring it, while casticin similarly interacted with Beclin1 through a network of polar and nonpolar contacts. These robust binding modes suggest that vitexin and casticin can physically interact with the proteins that regulate apoptosis and autophagy. Functionally, such binding could inhibit or modulate the activity of these proteins. [56-58] In particular, if vitexin binds to Bax's active domain, it may prevent Bax from inserting into the mitochondrial membrane, thereby blocking downstream caspase activation and subsequent cell death. [59] Similarly, interaction with Beclin1 or Lc3 may interfere with autophagosome formation. [60] The docking findings support the *in vivo* results by suggesting a direct molecular mechanism whereby VAC-derived compounds bind to pro-death effectors and reduce their activity.

The protein interaction network analysis revealed that the studied genes are connected to numerous other genes involved in apoptosis and autophagy pathways, especially through physical interactions and co-expression. This highlights the crucial biological role of these genes in regulating cellular pathways. Complementing these findings, gene expression data showed that three of the four studied genes (Bax, Bcl2, and Beclin1) underwent significant changes in expression following exposure to toluene and VAC fruit extract. These alterations in gene expression may reflect an adaptive response to toluene-induced stress, potentially through activation of apoptotic or autophagic mechanisms. On the other hand, it was observed that treatment of mice with VAC fruit extract modulated the expression of these genes, in some instances reducing the toluene-induced increases back to baseline levels. Therefore, the integration of bioinformatics data with gene expression results provides strong evidence supporting the dual role of these genes in regulating both cell survival and death pathways, as well as in modulating the effects of chemical compounds.

Human studies have shown that VAC extract is safe at doses of 8-30 mg per day, with side effects typically being mild and reversible. The most common side effects include nausea, headache, gastrointestinal disturbances, menstrual irregularities, and acne. No drug interactions have been reported, and its use during pregnancy or breastfeeding is not recommended. However, VAC may interact with dopaminergic antagonists. [61,62] In animal studies, including our own, higher doses have been used, and their direct conversion to human doses requires high precision and may need to be evaluated in specific conditions such as hepatic toxicity. While higher doses in animal studies suggest positive effects, further clinical trials are needed to assess their safety and efficacy in humans. Available data suggest that VAC is a safe herbal medicine and can be considered for human treatments, even in cases of liver toxicity, under supervision and with standardized doses.

There are some limitations in this study that should be mentioned. Although a sham group was included, the absence of a non-injected control group limits the ability to fully exclude the potential effects of intraperitoneal injection-induced stress on food intake and body weight. Future studies are recommended to evaluate this factor to better clarify its possible confounding effects. In addition, it should be noted that in humans, exposure to toluene typically occurs via inhalation or oral routes rather than systemic injection. The intraperitoneal injection used in this study allows for precise control of dosage and examination of systemic effects but may not fully mimic real-world exposure scenarios. This limitation suggests the need for complementary studies employing inhalation or oral exposure models to enhance the environmental and occupational relevance of the findings.

Conclusion

Toluene exposure can lead to the upregulation of key genes involved in apoptotic and autophagic pathways within the liver of mice. However, co-administration of *Vitex agnus-castus* fruit extract effectively modulates the expression of most of these genes. Furthermore, molecular docking analyses revealed that the active compounds of VAC, especially vitexin and casticin, form strong hydrogen bonds and hydrophobic interactions with the aforementioned target proteins. Consequently, *Vitex agnus-castus* fruit extract may serve as a promising hepatoprotective agent against the harmful effects of toluene. These findings support further investigation of VAC fruit as a potential dietary supplement or therapeutic option to mitigate toluene-induced liver toxicity.

Authors' Contribution

Conceptualization: Mohammad Karimian, Fereshteh Mir Mohammadrezaei.
Formal Analysis: Kosar Khosravi, Fereshteh Mir Mohammadrezaei, Ehsan Nazifi
Investigation: Kosar Khosravi.
Methodology: Fereshteh Mir Mohammadrezaei, Ehsan Nazifi.
Writing–Original Draft: Kosar Khosravi, Fereshteh Mir Mohammadrezaei, Mohammad Karimian.
Writing–Review & Editing: Mohammad Karimian, Fereshteh Mir Mohammadrezaei.

Competing Interests

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All stages of the experiment were carried out according to the guidelines of the Bioethics Committee of the University of Mazandaran (Bioethics Code: IR.UMZ.REC.1402.024).

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