

VOLUME 10 ISSUE 1 2024

ISSN 2454 – 3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

***Forum for Biological and
Environmental Sciences***

Published by Saran Publications, India



Pharmacognostic, Phytochemical and Hepatoprotective Activity of Berries of *Vitex agnus-castus*

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Received: 22nd December, 2023; Accepted: 28th January, 2024; Published online: 7th February, 2024

<https://doi.org/10.33745/ijzi.2024.v10i01.018>

Abstract: The aim of this study was to assess the hepatoprotective properties of several *Vitex agnus-castus* berry extracts *in vitro* utilizing a normal Chang liver cell line and the MTT test. Pharmacognostical investigations identified a number of distinctive traits found in the plant. The ethyl acetate extract yielded a larger percentage than the other extracts when the berries of *Vitex agnus-castus* were extracted using solvents with increasing polarity. The antioxidant activity was determined using the reducing power ability assay and the H₂O₂ scavenging method. The ethyl acetate extract showed the highest antioxidant activity compared to the other two extracts; in all processes, its IC₅₀ value was almost equivalent to that of the reference drug ascorbic acid. The highest level of protection against paracetamol-induced hepatotoxicity was provided by the ethyl acetate extract, according to *in vitro* hepatoprotective experiments conducted on Chang liver cell line. Our research confirmed the hypothesis that *Vitex agnus-castus* may be a beneficial hepatoprotective herb. It is recommended to conduct more research on identifying the phytoconstituents causing the action.

Keywords: Hepatoprotective activity, *Vitex agnus-castus*, Extraction, *In vitro*

Citation: Sundhararajan R., Raman S.G., Bharath V. and Dhasarathan M.: Pharmacognostic, phytochemical and hepatoprotective activity of berries of *Vitex agnus-castus*. Intern. J. Zool. Invest. 10(1): 148-156, 2024.

<https://doi.org/10.33745/ijzi.2024.v10i01.018>



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Introduction

There has been a connection between plants, sickness, and life ever since the dawn of humanity. There is no evidence that early men employed artificial medications to treat illnesses when they first began researching illnesses and cures (Chhabra and Kulkarni, 2020). When they began employing plants, the knowledge of how to make herbal treatments was passed down through the

centuries as folk medicine. Herbal medicine has thus existed for as long as there have been humans (Ibrahim *et al.*, 2017).

Traditional medicine encompasses a wide spectrum of age-old, all-natural health care methods, such as Ayurveda, Siddha, and Unani, as well as tribal practices. Over 7500 plants are said to be utilized, primarily in India's rural and tribal

regions (Toplan *et al.*, 2020). Home remedies based on Ayurveda, Siddha, and Unani medicine, together with traditional and ethical herbal medicine formulations marketed "over the counter," bring in over \$1 billion a year, with the export of herbal crude extracts bringing in around \$80 million (Rani and Sharma, 2013).

The World Health Organization defines traditional medicine as treatment techniques that have been practiced for hundreds of years before the development and spread of modern medicine and are still in use today (Tewari *et al.*, 2022). This includes the use of herbal drugs. Medicinal herbs, minerals, organic materials, and other materials which are used in traditional medicines. Herbal pharmaceuticals are limited to traditional medicines that predominantly employ preparations made from medicinal plants for therapeutic purposes (Chan *et al.*, 2018). In the developed world, herbal medications are highly sought because of their effectiveness, safety, and low incidence of adverse effects. These medications promote economic growth and are produced using bio- and environmentally-friendly techniques and renewable raw materials (Sahib *et al.*, 2019). Because the chemical components they contain are engaged in the physiological processes of living plants, it is believed that they are more suited to human bodies. Ancient literature also mentions the use of herbal medicines for a variety of age-related ailments, such as memory loss, liver issues, osteoporosis, diabetic wounds, and immune system issues (Kamal *et al.*, 2022).

The liver performs several of the body's primary tasks, including xenobiotic excretion and metabolism. As such, the liver is susceptible to several insults related to metabolism, circulation, and neoplastic processes (Meena *et al.*, 2011). The four primary liver ailments are viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease, and hepatocellular cancer. Additionally, liver damage can result from some of the most prevalent illnesses in humans, including extra hepatic infections, disseminated malignancy, and cardiac breakdown (Al Saka *et al.*, 2017).

There is a long history of using herbs outside of traditional treatment. It is becoming more widely accepted as the usefulness of herbal medicine in treating and preventing illnesses has been highlighted by advancements in clinical research, analysis, and quality control. For the treatment of liver problems, several botanicals and traditional mixtures are available (Tiwari *et al.*, 2022). No particular synthetic medication is utilized as hepatoprotective. Many plants include biomolecules, which are biodegradable and capable of dissolving into their component parts. Therefore, natural remedies are more favoured than synthetic ones while treating liver disease. The plant *Vitex agnus castus* (VAC), which belongs to the Verbenaceae family, has long been used as a supplementary medicine in Europe (Ogaly *et al.*, 2021). Antioxidants are known to have hepatoprotective effects, and VAC has also been demonstrated to have antioxidant activity. It is well known that plants with flavonoids have increased hepatoprotective action. Although no research has been published in the scientific literature, this herb has been historically used to heal enlarged livers. Therefore, the goal of this investigation was to assess the hepatoprotective potential of *Vitex agnus-castus* berries (Das *et al.*, 2022).

Materials and Methods

Phytochemical Investigation:

Using the appropriate chemical tests, phytochemical assessment was performed to identify the type of phytoconstituents that are present in the plant. It can be accomplished by the use of certain reagents for qualitative analysis, which was then confirmed using various chromatographic methods such as TLC and HPTLC. Consequently, a thorough examination was done to qualitatively and quantitatively characterize the phytoconstituents (Yeola *et al.*, 2023).

Extracts Preparation:

The first stage in the phytochemical research is extraction. It releases the primary and secondary

metabolites into the extraction solvent based on its polarity (Tiwari *et al.*, 2023). The dried, coarsely ground berries of *Vitex agnus-castus* were extracted with solvents of increasing polarity, such as hexane, ethanol, and ethyl acetate, for 16 to 18 h at 55 to 75°C using a Soxhlet apparatus. A rotating vacuum evaporator was used to redistilled and concentrate each extract, after which the % yield was computed. Testing was done on the extracts for both qualitative and quantitative analysis (Tiwari *et al.*, 2023).

Pharmacological studies:

Antioxidant activity:

Hydrogen peroxide scavenging method:

The ability of *Vitex agnus-castus* to scavenge hydrogen peroxide was evaluated using this method. A solution of hydrogen peroxide was made using 2 mmol/l of phosphate buffer (pH 7.4). A 0.6 ml hydrogen peroxide solution was combined with 10–100 µg/ml *Vitex agnus-castus*. A blank solution containing phosphate buffer but no hydrogen peroxide was compared to the absorbance of hydrogen peroxide at 230 nm after 10 min (Rashmeei *et al.*, 2022).

Ferric Reducing Antioxidant Power Assay:

The diminishing power was ascertained using the previously mentioned method. Plant extracts were mixed with 1 ml of 1% potassium ferricyanide, 1 ml of 200 mmol/l sodium phosphate buffer (pH 6.6), and various concentrations of 1 ml each. The mixes were incubated at 50°C for 20 min. 1 ml of 10% w/v trichloroacetic acid was added, and the mixture was centrifuged at 2000 rpm for 10 min. The top layer solution was mixed with 2.5 ml of deionized water and 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm (Tiwari *et al.*, 2022).

MTT assay-based in vitro hepatoprotective efficacy employing a normal Chang liver cell line:

The cells were cultured in Minimal Essential Media supplemented with 10% FBS, penicillin 100 U/ml, and streptomycin 100 µg/ml at 37°C in a humidified atmosphere with 50 µg/ml CO₂. The

culture media was changed twice a week (Sonawane *et al.*, 2023).

Preparation of Toxicants solutions:

Using minimal necessary medium, 100 mg of paracetamol were diluted to 100 ml after being dissolved in 10 ml of DMSO. Solutions containing 1000, 500, 250, and 125 µg/ml were created by diluting them with distilled water (Thakur and Ashawat, 2023).

Standard drug:

Using minimal necessary medium, 100 mg of silymarin was diluted to 100 ml after dissolving in 10 ml of DMSO. Solutions containing 1000, 500, 250 and 125 µg/ml were prepared by diluting them with distilled water (Tiwari *et al.*, 2022).

Sample solutions preparation:

100 mg of several test sample extracts, including hexane, ethanol, and ethyl acetate, were dissolved in 10 ml of DMSO and then diluted to 100 ml using the minimal necessary medium. Solutions containing 1000, 500, 250, and 125 µg/ml were created by diluting them with distilled water.

Procedure

Cytotoxicity evaluation by tetrazolium (MTT) assay:

Using trypsin-ethylene Diamine tetra acetic acid (EDTA), single cell suspensions of Chang liver monolayer cells were obtained. After that, a hemocytometer was used to count the live cells, and they were diluted with medium and 5% FBS to get a final density of 1×10⁵ cells/ml. 1×10⁵ cells were plated in 5 ml of media per well in 96-well plates. The cell reaches confluence 48 h after it is cultured. After that, cells were cultivated with different dosages of silymarin, hexane, ethanol, and ethyl acetate for 24 to 48 h at 37°C. A phosphate-buffered saline solution (1 ml) containing 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) was added to each well (5 mg/ml) (pH 7.4) following the removal of the sample solution. Added of 0.04 M HCl/isopropanol which followed a 4 h incubation period. Using a UV spectrophotometer,

Table 1: Yield percentage of *Vitex agnus-castus* berries extracted successively using solvents

S. No.	Extracts	Extraction Method	Physical Nature of extract	Colour of extract	Percentage yield (w/w)
1.	Non polar (Hexane)	Continuous hot percolation method	Semisolid	Pale green	9.2
2.	Semi polar (Ethyl acetate)		Sticky	Greenish black	18.4
3.	Polar (Ethanol)		Semisolid	Brownish black	12.3

the absorbance at 570 nm was used to measure the viability of the cells, and wells devoid of material were considered blank. Following measurements, a visual representation of the concentration needed to provide a 50% inhibition of viability (IC_{50}) was produced. Every concentration was maintained in triplicate (Somtimuang *et al.*, 2018; Khan *et al.*, 2021).

In vitro hepatoprotective activity using various extracts against paracetamol induced hepatotoxicity (MTT assay):

Trypsin-ethylene diamine tetra-acetic acid (EDTA) was used to separate the Chang liver monolayer cells into single cell suspensions. Viable cells were then counted using a hemocytometer and diluted with medium and 5% FBS to achieve a final density of 1×10^5 cells/ml. In 96-well plates, cells (1×10^5 /well) were plated in 5 ml of medium per well. After being incubated for 48 h, the cell reaches confluence. Subsequently, 125 μ g/ml of the hepatotoxicant paracetamol was given to the cells, along with varying quantities of other extracts (hexane, ethanol, and ethyl acetate) and the standard medication silymarin. The cells were cultured at 37°C for 24 to 48 h. A volume of 1 ml per well (5 mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-terazolium bromide cells (MTT) phosphate-buffered saline solution was added following the removal of the sample solution and washing with phosphate-buffered saline (pH 7.4). After incubation for 4 h, 0.04 M HCl/isopropanol was added. The UV Spectrophotometer was used to detect the absorbance at 570 nm. The sample-free wells served as blanks. Every concentration was

maintained in triplicate (Surana and Mahajan, 2022).

Statistical analysis:

Mean $\pm$ S.E.M. was used to express the data. The t test was used to compare the groups statistically after one way analysis of variance (ANOVA) was performed. P-values less than 0.05 were deemed noteworthy (Shukla *et al.*, 2020).

Results and Discussion

Phytochemical Investigation:

The yield percentage of Vitex agnus-castus berries after serial solvent extraction:

The berries of *Vitex agnus-castus* were extracted using solvents with higher polarity, and the % yield was calculated and recorded (Table 1). In comparison to the other extracts, the semi-extract (ethyl acetate) had a higher percentage yield.

Pharmacological Studies:

Anti-oxidant Activity:

Hydrogen peroxide scavenging method:

Table 2 illustrates the % inhibition of the standard and the different extracts.

The standard (ascorbic acid) IC_{50} values were determined to be 34.35 μ g/ml, ethyl acetate extract to be 35.11 μ g/ml, ethanol extract to be 37.12 μ g/ml, and hexane extract to be 41.23 μ g/ml (Fig. 1). Comparing the three extracts, it is evident from the data that the ethyl acetate extract has the strongest antioxidant activity. The ethyl acetate extract's IC_{50} value is the same as that of ascorbic acid, a common medication.

Table 2: H₂O₂ scavenging method

S. No.	Concentration (µg/ml)	% Inhibition			
		Standard(Ascorbic Acid)	Semi polar Extract (Ethyl Acetate)	Polar Extract (Ethanol)	Non polar Extract (Hexane)
1.	10 µg/ml	14.22	12.70	13.19	10.11
2.	20 µg/ml	25.99	26.11	25.11	23.32
3.	30 µg/ml	49.84	48.12	47.27	44.22
4.	40 µg/ml	58.98	59.11	58.23	49.14
5.	50 µg/ml	72.25	70.15	63.25	58.13

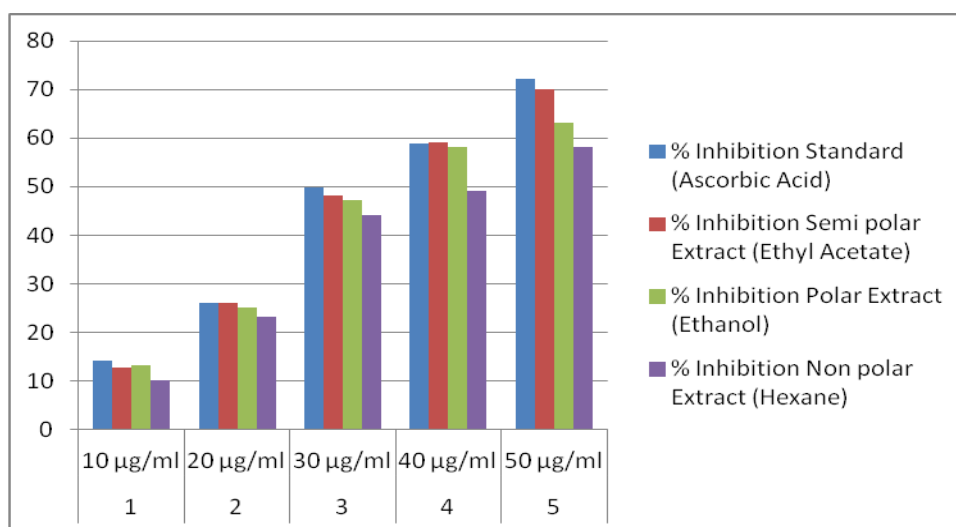
Fig. 1: H₂O₂ scavenging assay.

Table 3: Reducing power ability assay

S. No.	Concentration µg/ml	% Inhibition			
		Standard(Ascorbic Acid)	Semi polar Extract (Ethyl Acetate)	Polar Extract (Ethanol)	Non polar Extract (Hexane)
1.	10 µg/ml	14.17	13.95	13.12	12.45
2.	20 µg/ml	27.56	25.86	25.67	22.89
3.	30 µg/ml	49.53	48.75	47.34	42.73
4.	40 µg/ml	60.27	59.12	53.62	49.12
5.	50 µg/ml	72.31	70.45	68.45	57.91

Reducing power ability assay:

Table 3 presents the % inhibition of the standard and the different extracts.

Standard (ascorbic acid) - 34.35 µg/ml, Ethyl acetate extract - 37.25 µg/ml, Ethanol extract-

39.13 µg/ml, and Hexane extract - 42.36 µg/ml were found to have IC₅₀ values (Fig. 2). Comparing the three extracts, it is evident from the data that the ethyl acetate extract has the strongest antioxidant activity. The ethyl acetate extract's IC₅₀ value is the same as that of ascorbic

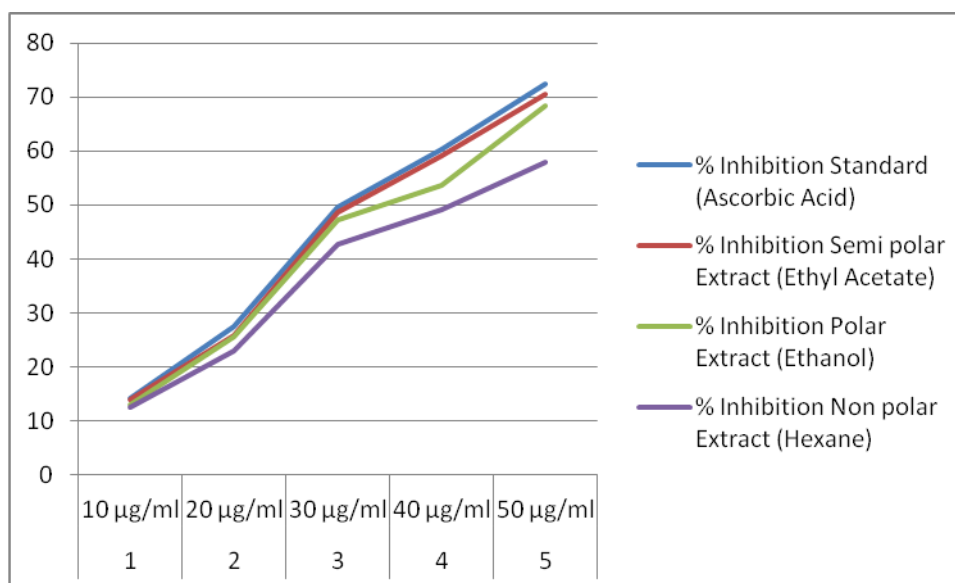


Fig. 2: Reducing power ability assay.

Table 4: Cytotoxicity studies

S. No.	Concentration (mg/ml)	% cell viability			
		Silymarin	Nonpolar extract (Hexane)	Semipolar Extract (Ethyl acetate)	Polar extract (Ethanol)
1.	Control	100	100	100	100
2.	125	41.66	43.33	45.11	41.60
3.	250	33.21	34.61	34.14	33.21
4.	500	19.32	26.41	23.07	19.25
5.	1000	07.70	16.31	12.91	08.35

acid, a common medication.

In vitro Evaluation:

Assessment of cytotoxicity using the tetrazolium (MTT) test:

The Chang liver cell line was used in cytotoxicity tests for the standard silymarin and several extracts of *Vitex agnus-castus* berries over a range of concentrations (125, 250, 500, and 1000 µg/ml), and the CTC50 value was determined (Table 4).

As the test substances' concentration increased, the viability of the cells dropped. The extracts' CTC50 values of 67.5 µg/ml, which are comparable to those of ordinary silymarin,

demonstrate that they are not hazardous to the normal cell line.

Hepatoprotective action of several extracts in vitro against hepatotoxicity produced by paracetamol:

Initially, 125 µg/ml of paracetamol was added to Chang liver cells, of which 40.89% of the cells survived. The percentage of cell viability was then measured by treating the cell line with standard silymarin and various *Vitex agnus-castus* extracts at concentrations of 100, 50, 25 and 10 µg/ml. The results are listed in Table 5.

Hepatoprotective action of several extracts in toxicity produced by paracetamol:

At the maximum dose of 100 µg/ml, the cell viability in the silymarin-treated group was

Table 5: Hepatoprotective action *in vitro*

S. No.	Treatment	Concentration mg/ml	% cell Viability
1.	Control		100
2.	Standard (Paracetamol)	125	40.89±1.09
3.	Paracetamol + Silymarin	10	56.1±1.07
		25	61.5±1.07
		50	66.1±1.13
		100	70.2±1.11
4.	Paracetamol + Semipolar extract (Ethyl acetate)	10	50.1±1.30
		25	59.5±1.21
		50	67.5±1.51
		100	73.2±1.33
5.	Paracetamol + Polar extract (Ethanol)	10	35.9±1.73
		25	48.1±1.36
		50	54.1±1.14
		100	59.6±1.91
6.	Paracetamol + Nonpolar extract (Hexane)	10	33.1±1.61
		25	42.1±1.19
		50	50.1±1.82
		100	56.3±1.84

Values are expressed as Mean ± S.E.M. (n=3)

70.2%. When compared to the other two extracts, the ethyl acetate extract showed a considerable increase in cell viability of 73.2%, which is quite near to that of the standard. For *in vivo* research, ethyl acetate extract was chosen as a result.

Conclusion

Vitex agnus-castus (Verbanaceae), sometimes referred to as the women's herb, has a long history of use as supplemental medicine in Europe and is also rumoured to have the ability to treat enlarged livers. There are additional reports of antioxidant activity, which is known to enhance hepatoprotective effects. This study revealed the hepatoprotective properties of *Vitex agnus-castus* berries. The ethyl acetate extract yielded a larger percentage than the other extracts when the berries of *Vitex agnus-castus* were extracted using solvents with increasing polarity. The antioxidant activity was determined using the reducing power ability assay and the H₂O₂ scavenging method. The ethyl acetate extract showed the highest

antioxidant activity compared to the other two extracts; in all processes, its IC₅₀ value was almost equivalent to that of the reference drug ascorbic acid.

Toxicity studies on a normal Chang liver cell line revealed that none of the extracts were poisonous. *In vitro* hepatoprotective tests on Chang liver cell line demonstrated that the ethyl acetate extract offered the best level of protection against paracetamol-induced hepatotoxicity. The group that received ethyl acetate plus paracetamol had a cell viability of 70.2%, but the group that received paracetamol only had a cell vitality of 40.89%. Our research confirmed the hypothesis that *Vitex agnus-castus* may be a beneficial hepatoprotective herb. Further investigation is advised to determine which phytoconstituents are responsible for the activity.

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