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Evaluating the antiviral efficacy of *Azadirachta indica* and *Moringa oleifera* against hepatitis B and C: Implications for alternative therapeutic approaches

Amna Arshad[1#], Raheela Adeeb[1#], Nyla Jabeen[1]*, Sadaf Anwaar[1], Syed Zaheer Hussain[2], Tauseef Anwar[3*], Huma Qureshi[4], Jameel M. Al-Khayri[5]*, Othman Al-Dossary[5], Bader Alsubaie[5], Mohammed I. Aldaej[5], Wael Fathi Shehata[5], Mustafa I. Almaghasla[6] and Muneera Q. Al-Mssallem[7]

[1]Applied Biotechnology and Genetic Engineering Lab, Department of Biological Sciences, International Islamic University, Islamabad, Pakistan.
[2]Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.
[3]Department of Botany, The Islamia University of Bahawalpur, Bahawalpur, Pakistan.
[4]Department of Botany, University of Chakwal, Chakwal, Pakistan.
[5]Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia.
[6]Plant Pests and Diseases Unit, College of Agriculture and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia.
[7]Department of Food Science and Nutrition, College of Agriculture and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia.

Abstract: **Background**: Chronic hepatitis B (HBV) and C (HCV) remain major global health burdens due to high morbidity, treatment costs, and the emergence of antiviral resistance. Plant-derived compounds offer a potential alternative or complementary therapeutic approach. This study evaluated the antiviral effects of *Azadirachta indica* (neem) and *Moringa oleifera* (drumstick tree) leaf extracts on peripheral blood mononuclear cells (PBMCs) obtained from HBV- and HCV-infected patients. **Objectives**: To determine and compare the phytochemical profiles of *A. indica* and *M. oleifera* leaf extracts and assess their antiviral activity through induction of apoptosis and necrosis in virus-infected PBMCs. **Methods**: Leaf extracts were subjected to phytochemical screening. PBMCs isolated from HBV- and HCV-infected patients were treated with each extract and analyzed by flow cytometry to quantify live, apoptotic, and necrotic cell populations. Statistical analysis was performed using one-way ANOVA with significance set at $P < 0.05$. **Results**: Phytochemical analysis revealed that *A. indica* contained flavonoids, alkaloids, tannins, saponins, glycosides, and steroids, whereas *M. oleifera* contained flavonoids, alkaloids, and tannins but lacked glycosides and saponins. In HBV-infected PBMCs, *A. indica* significantly reduced live cell percentages from 24.3% to 11.35% and increased necrotic cells from 18.98% to 55.43%. In HCV samples, live cells decreased from 40.27% to 37.78%, while necrosis increased from 21.35% to 30.1%. *M. oleifera* demonstrated comparatively moderate effects consistent with its simpler phytochemical profile. **Conclusion**: *A. indica* exhibited strong antiviral potential, markedly enhancing necrotic responses in HBV- and HCV-infected PBMCs, while *M. oleifera* showed moderate activity. These results highlight the therapeutic promise of phytochemical-rich extracts, particularly *A. indica*. Further investigations—including in-vivo validation, dosage formulation, cost-effectiveness assessments, and evaluation of synergistic effects with existing antiviral therapies—are warranted to advance their development as complementary treatments for chronic viral hepatitis.

Keywords: Antiviral activity; Hepatitis treatment; Medicinal plants; Phytochemicals; Viral resistance.

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INTRODUCTION

Hepatitis is a viral infection that primarily affects the liver, presenting a clinical spectrum ranging from mild, self-limiting illness to chronic, life-threatening conditions. The etiological agents of hepatitis vary in genetic composition; hepatitis B virus (HBV) is a DNA virus, whereas hepatitis A, C, D and E viruses are RNA-based (Grant and Purres, 2025, Albadr *et al.*, 2025). Chronic HBV and hepatitis C virus (HCV) infections are among the foremost global health challenges, affecting approximately 328 million individuals worldwide, with an annual incidence of 1.5 million new infections (Stroffolini and Stroffolini, 2024). In Pakistan, the situation is particularly alarming, with the World Health Organization (WHO) estimating that nearly 12 million people are currently infected with HBV or HCV (Saleem *et al.*, 2022). These infections are strongly associated with progressive liver disorders, including fibrosis, cirrhosis, hepatocellular carcinoma and chronic liver failure (Ning *et al*., 2023; Wang *et al*., 2023).

**Corresponding author: e-mail:* tauseef.anwar@iub.edu.pk, nyla.jabeen@iiu.edu.pk, jkhayri@kfu.edu.sa
#Authors have equally contributed.

The transmission pathways of these viruses differ. HBV is commonly spread through mother-to-child transmission, sexual contact, blood transfusion and unsafe injections, while HCV transmission is primarily attributed to unsafe medical practices and transfusion of unscreened blood (Wiktor, 2017). Although antiviral drugs are available, their use is often constrained by high cost, limited accessibility, side effects and the emergence of resistant viral strains. Additionally, the lack of a vaccine for HCV further emphasizes the need for innovative, affordable and safer alternatives to conventional therapies (Zhang *et al*., 2022; Chen *et al*., 2019).

Natural products derived from medicinal plants have historically played an important role in the treatment of infectious diseases and recent evidence suggests that they may serve as effective antiviral agents. Plant-derived compounds such as flavonoids, alkaloids, terpenoids, saponins and tannins have shown potential in modulating key molecular pathways involved in viral entry, replication and protein synthesis (Atampugbire *et al.*, 2024). Among these plants, *Moringa oleifera*, a member of the Moringaceae family, has attracted significant attention due to its broad spectrum of bioactivity (Yang *et al.*, 2025). Its phytochemical constituents-including flavonoids, isothiocyanates, alkaloids and anthocyanins-have demonstrated antiviral efficacy against viruses such as HIV, SARS-CoV-2, influenza and HBV (Ghada *et al.*, 2025; Pareek *et al.*, 2023). Emerging *in-vitro* studies indicate that *M. oleifera* extracts may downregulate HBV DNA levels and inhibit viral replication (Jose-Abrego *et al.*, 2023). Similarly, *Azadirachta indica* (commonly known as neem), a widely used plant in traditional medicine, contains potent bioactives such as nimbin, quercetin, saponins and triterpenoids. Neem extracts have demonstrated inhibitory effects against various viruses, including coxsackie B, HIV, polio, HCV and HBV, largely through the disruption of viral enzymes and replication cycles (Alzohairy, 2016; Bhamare *et al.*, 2020). Despite these promising findings, comparative studies evaluating the relative antiviral efficacy and potency of *A. indica* and *M. oleifera* against HBV and HCV remain limited. Moreover, few studies have investigated their selectivity indices or cytotoxic thresholds on normal cells, which are essential to establish their therapeutic relevance.

This study addresses these gaps by conducting a direct, comparative evaluation of *A. indica* and *M. oleifera* leaf extracts for their antiviral efficacy against HBV and HCV. The research focuses on quantifying potency using key virological assays, assessing cytotoxicity to evaluate selectivity and identifying phytochemical components responsible for activity. This study contributes to the growing body of evidence supporting the development of plant-based antiviral strategies and sets the stage for future preclinical and clinical investigations into herbal therapeutics for hepatitis B and C.

MATERIALS AND METHODS

Plant material collection and extraction

Mature leaves of *Azadirachta indica* and *Moringa oleifera* were collected from Hyderabad (Sindh) and Vehari (Punjab), respectively. The plant materials used in this study *Azadirachta indica* (Voucher No.: AI-2025-IUB) and *Moringa oleifera* (Voucher No.: MO-2025-IUB) were taxonomically authenticated by Dr. Tauseef Anwar and the voucher specimens were deposited in the Herbarium of the Department of Botany, The Islamia University of Bahawalpur, Bahawalpur. The leaves were thoroughly washed under running tap water to eliminate dust and surface contaminants, followed by air-drying in a shaded, dust-free environment to preserve thermolabile bioactive constituents. Dried leaves were ground into fine powder using a sterile laboratory grinder. For extraction, 45 g of powdered leaves were soaked in 300 mL of analytical grade methanol and incubated in a shaking incubator at 37°C for 24 hours. The extracts were filtered through Whatman No. 1 filter paper and sterile muslin cloth. Filtrates were passed through bacterial filters (0.22 μm) under aseptic conditions and then concentrated using a rotary evaporator (Heidolph) at 31°C under reduced pressure. The concentrated methanol extracts were stored at 4°C for further phytochemical and antiviral evaluation.

Phytochemical analysis

Qualitative phytochemical analysis was carried out using standard colorimetric procedures as described by Harborne (1973) and Sofowora (1993). Flavonoids were confirmed using the sodium hydroxide test, where a yellow coloration disappearing upon acidification indicated their presence. Alkaloids were detected using Mayer's reagent, forming a cream-colored precipitate. Tannins were identified by the ferric chloride test, producing a dark blue-green coloration. Saponins were determined via the foam test, were persistent froth formation upon vigorous shaking signified presence. Steroids were detected using the Salkowski reaction, showing a red upper layer and yellow-green lower phase. Glycosides were confirmed by Fehling's test, with a brick-red precipitate indicating a positive result.

Blood sample collection and lymphocyte isolation

Blood samples were collected from HBV- and HCV-positive patients, along with healthy controls, from Holy Family Hospital, Rawalpindi, after obtaining informed consent and ethical clearance. The collection procedure followed standard venipuncture protocols described by Clinical and Laboratory Standards Institute (CLSI, 2020). Peripheral blood mononuclear cells (PBMCs), including lymphocytes, were isolated using density gradient centrifugation. Equal volumes of blood and phosphate-buffered saline (PBS, pH 7.4) were layered over lymphocyte separation medium (Ficoll-Hypaque; density = 1.077 g/mL, verified using pycnometric method) in sterile Falcon tubes. Samples were centrifuged at 1000 rpm for 30

minutes at 20°C. The mononuclear cell layer was harvested from the plasma-medium interface, washed twice with PBS (10 mL) at 700 rpm and 4°C and finally suspended in 0.5 mL PBS. While HBV and HCV primarily replicate in hepatocytes, PBMCs have been shown to harbor viral genomes or experience immune-mediated cytopathic effects in chronically infected individuals (Yuki & Chen, 2003). Therefore, lymphocyte apoptosis can reflect systemic cytotoxicity and indirect antiviral responses.

Cell viability assay using trypan blue exclusion

Cell viability was assessed via the Trypan blue exclusion method (Strober, 2001). An equal volume of cell suspension and 0.4% Trypan blue solution was mixed and loaded onto a hemocytometer. Viable (unstained) and non-viable (blue-stained) cells were counted under a light microscope and cell density was calculated using:

$$Cell\ Concentartion = \frac{No.\ of\ live\ cells\ counted}{No.\ quadrant\ counted} \times (diluation\ factor) \times 10^4 \quad (1)$$

A total of 1×10^6 cells/mL were seeded and incubated for 24 hours at 37°C in a 5% CO_2 incubator. Cells were exposed to varying concentrations of plant extracts based on previously determined IC_{50} values (serial dilution method), with concentrations ranging from 10 to 100 µg/mL. The initial IC_{50} values were determined using MTT assays (not shown here) and subsequent extract concentrations for apoptosis assays were derived accordingly to ensure dose-dependent responses.

Flow cytometry analysis

Post-treatment, cells were stained using Annexin V-FITC and propidium iodide (PI) following manufacturer instructions (eBioscience) and analyzed by flow cytometry as described by Vermes *et al.* (1995). Cells were washed in 1% FBS and Annexin V binding buffer, incubated in the dark for 20 minutes with 5 µL each of Annexin V and PI and washed again in PBS. Stained cells were analyzed on a BD FACSCalibur™ flow cytometer. Gating strategies excluded debris and populations were classified as live (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), or necrotic (Annexin V-/PI+). While apoptosis in PBMCs does not directly confirm antiviral action against HBV/HCV, apoptosis induced selectively in infected lymphocytes may reflect cytotoxic selectivity or indirect modulation of viral persistence. However, this remains an indirect proxy and future studies should include viral load quantification and assays in hepatocyte lines for confirmation.

Statistical Analysis

All experiments were performed in triplicate and results are presented as mean ± standard deviation (SD). One-way ANOVA was performed using IBM SPSS Statistics v26, followed by Tukey's HSD post hoc test to determine significance between treatment groups. A p-value of <0.05 was considered statistically significant.

RESULTS

Extract preparation and phytochemical composition

Methanol extracts of *Azadirachta indica* and *Moringa oleifera* were prepared using the maceration method (Table 1). The leaves were qualitatively assessed for the presence of major phytochemicals. As shown in table 2, *A. indica* tested positive for all six tested classes-alkaloids, saponins, flavonoids, tannins, steroids and glycosides-while *M. oleifera* lacked saponins and glycosides but was otherwise phytochemically rich.

Evaluation of antiviral activity using flow cytometry

Flow cytometric analysis was employed to evaluate the antiviral potential of *A. indica* and *M. oleifera* extracts against HBV and HCV infections. In the initial dot plots, three major populations—lymphocytes, granulocytes and monocytes—were identified based on size and granularity. Lymphocytes were gated for quadrant-based analysis using Annexin V-FITC/PI dual staining, categorizing cells into viable (Q4), early apoptotic (Q3), late apoptotic (Q2) and necrotic (Q1) populations.

Antiviral effects of azadirachta indica extract

Treatment with *A. indica* extract induced marked apoptosis and necrosis in HBV- and HCV-infected cells, as visualized through quadrant statistics (Fig. 1–3) and histogram peak shifts (Fig. 4–5). Compared to untreated normal cells, which showed high viability (94–95% live cells), HBV-infected samples treated with *A. indica* exhibited a drastic reduction in live cells (as low as 11.35%) and a significant increase in late apoptotic (up to 71.68%) and necrotic populations (up to 55.43%).

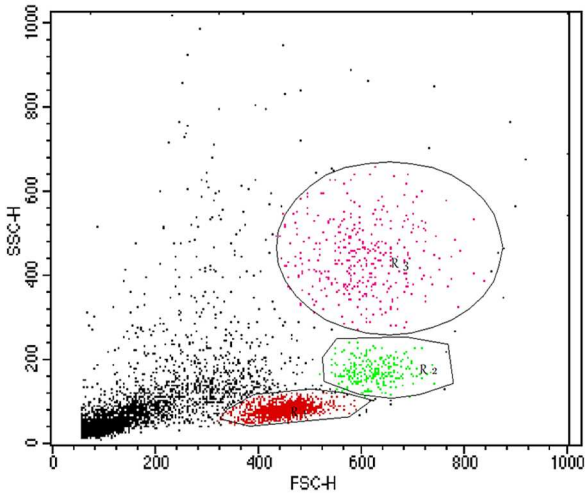

Fig. 1: Gating of lymphocytes (R1) based on SSC-H versus FSC-H in the initial scatter plot.

Similarly, HCV-infected cells showed reduced viability and increased apoptosis and necrosis after *A. indica* treatment. The effect was particularly pronounced in HCV

Table 1: Details of plant collection and extraction parameters

Plant name	Place of collection	Used part Powder (15g)	Used solvent (100ml)	Plant powder color	Procedure
A. indica	Hyderabad	Leaves	Methanol	Dark green	Maceration
M.oleifera	Vehari	Leaves	Methanol	Vibrant green	Maceration

Table 2: Phytochemical composition of methanolic extract of *Azadirachta indica* and *Moringa oleifera*.

Extracts	Alkaloids	Saponins	Flavonoids	Tannins	Steroids	Glycoside
Azadirachta indica	+	+	+	+	+	+
Moringa oleifera	+	-	+	+	+	-

Table 3: Comparative antiviral activity of *Azadirachta indica* and *Moringa oleifera* methanolic leaf extracts against HBV and HCV-Infected cells (Flow Cytometry Analysis)

Extract	Sample	Live cells (%)	Dead cells (%)	Early Apoptotic (%)	Late Apoptotic (%)
	N1	94.71	0.78	2.56	1.87
	N2	93.10	2.13	2.49	2.28
	N3	95.44	2.42	1.10	1.03
	N4	80.89	15.60	0.92	2.60
	N5	83.82	3.43	4.57	8.18
	HBV 1	24.30	18.98	3.79	52.93
A. indica	HBV 2	11.35	13.12	3.85	71.68
	HBV 3	22.82	55.43	1.71	20.04
	HCV 1	40.27	21.35	9.53	28.85
	HCV 2	67.52	16.55	7.42	8.70
	HCV 3	50.87	18.53	6.45	24.15
	HCV 4	76.79	15.15	2.67	5.38
	HCV 5	37.78	30.10	13.48	18.56
	N1	88.22	4.04	2.50	5.23
	N2	93.45	2.94	1.16	2.90
	N3	88.37	8.40	0.54	2.70
	N4	82.12	4.02	5.07	8.79
	N5	95.37	0.83	2.90	0.90
	HBV 1	56.01	18.80	6.56	18.62
M. oleifera	HBV 2	32.92	64.04	0.03	3.01
	HBV 3	43.17	2.22	11.95	42.66
	HCV 1	23.19	9.85	32.08	34.88
	HCV 2	26.07	10.88	31.33	31.72
	HCV 3	27.73	63.27	0.15	8.85
	HCV 4	18.72	0.43	65.80	15.05
	HCV 5	27.40	58.72	0.22	13.66

5, which showed only 37.78% live cells and high proportions of early (13.48%) and late (18.56%) apoptotic cells (Table 3). Notably, statistical analysis using one-way ANOVA confirmed significant differences ($P < 0.05$) in treated versus untreated groups.

Antiviral effects of moringa oleifera extract

Similar to *A. indica*, *M. oleifera* extract also exerted antiviral activity, albeit with somewhat variable potency. In HBV-infected cells, treatment led to decreased viability and increased late apoptosis, particularly in HBV 3, where only 43.17% of cells remained viable and 42.66% entered late apoptosis.

Against HCV, *M. oleifera* demonstrated even more pronounced antiviral activity. In HCV 1 and HCV 2, live cells dropped below 27%, while early and late apoptotic cells combined exceeded 60%, indicating robust apoptosis induction. Fig. 6–9 display the corresponding dot plots and histogram shifts confirming these findings.

Interestingly, neither *A. indica* nor *M. oleifera* exhibited cytotoxicity against normal cells, as evidenced by minimal peak shifts and consistently high viability (>88%) in untreated and extract-treated controls. This selective cytotoxicity toward virus-infected cells highlights the therapeutic potential of both extracts.

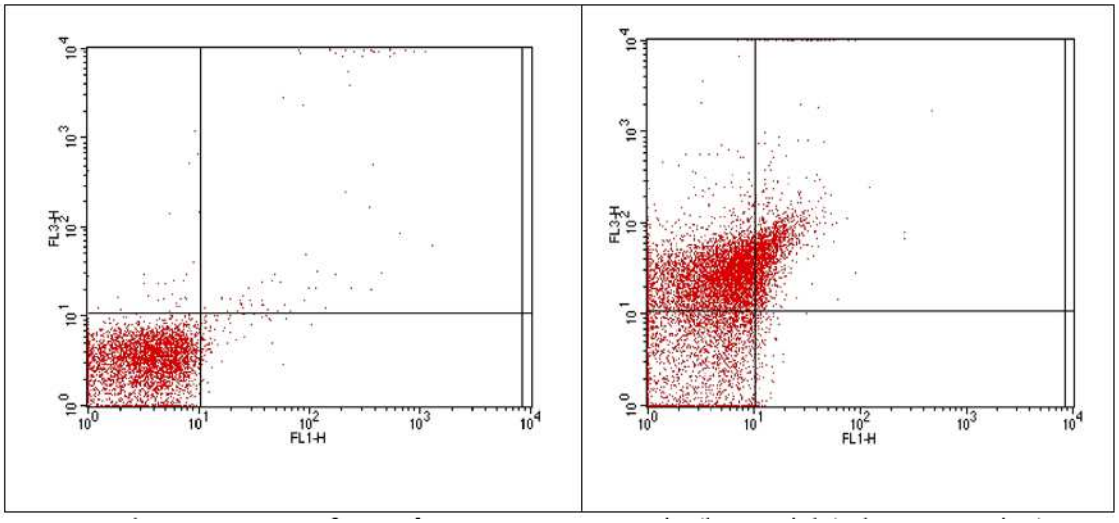

Fig. 2: Flow cytometry of *A. indica* treatment: necrotic (lower right), late apoptotic (upper right), early apoptotic (upper left), viable cells (lower left).

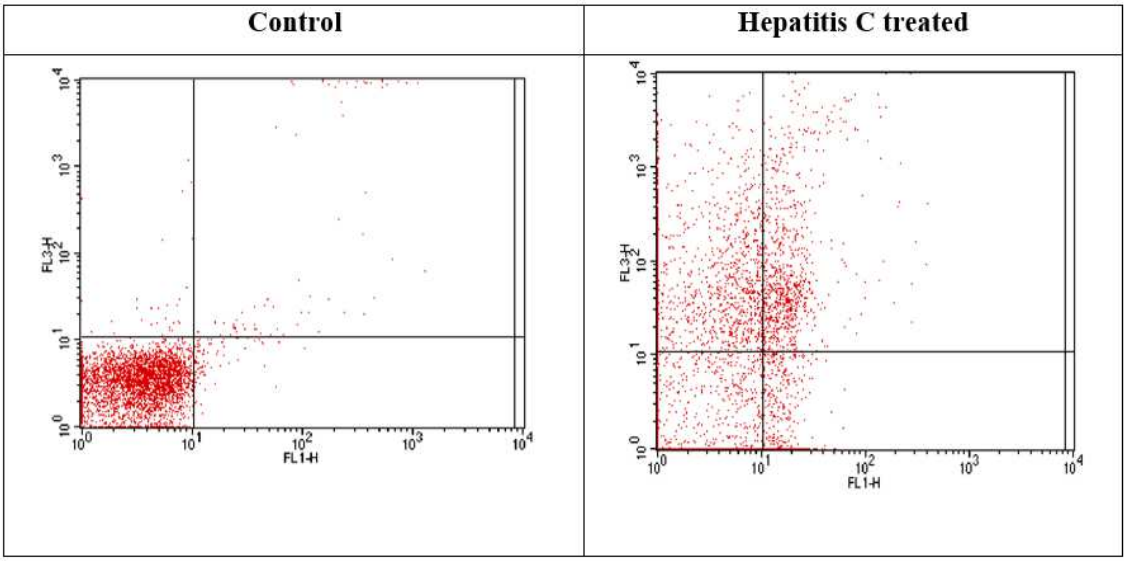

Fig. 3: Flow cytometry with *A. indica* extract for 24 hours, displaying necrotic, apoptotic, and viable cell states.

DISCUSSION

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major global health burdens, with an estimated 296 million and 58 million affected individuals worldwide, respectively (Zhou *et al.*, 2024). Although antiviral drugs such as nucleos(t)ide analogs and direct-acting antivirals have improved clinical outcomes, limitations including incomplete viral clearance, drug resistance, high cost and adverse effects underscore the urgent need for alternative or adjunct therapeutic strategies. In this context, plant-derived phytochemicals offer promising antiviral alternatives due to their structural diversity, multi-target action and low cytotoxicity. The plant materials used in this study—*Azadirachta indica* (Voucher No.: AI-2025-IUB) and *Moringa oleifera* (Voucher No.: MO-2025-IUB)—were taxonomically authenticated by Dr. Tauseef Anwar and the voucher specimens were deposited in the Herbarium of Department of Botany, The Islamia University of Bahawalpur, Bahawalpur.

This study evaluated the antiviral efficacy of *A. indica* and *M. oleifera* methanol leaf extracts against HBV and HCV using flow cytometry-based apoptosis assays. Phytochemical screening confirmed the presence of multiple classes of secondary metabolites in *A. indica*, including alkaloids, flavonoids, tannins, saponins, glycosides and steroids. In contrast, *M. oleifera* lacked saponins and glycosides but was rich in alkaloids, flavonoids, tannins and steroids. These results areconsistent with prior reports (Divya *et al.*, 2024; Islas *et al.*, 2020), reinforcing the reproducibility and reliability of the extraction and analytical methods.

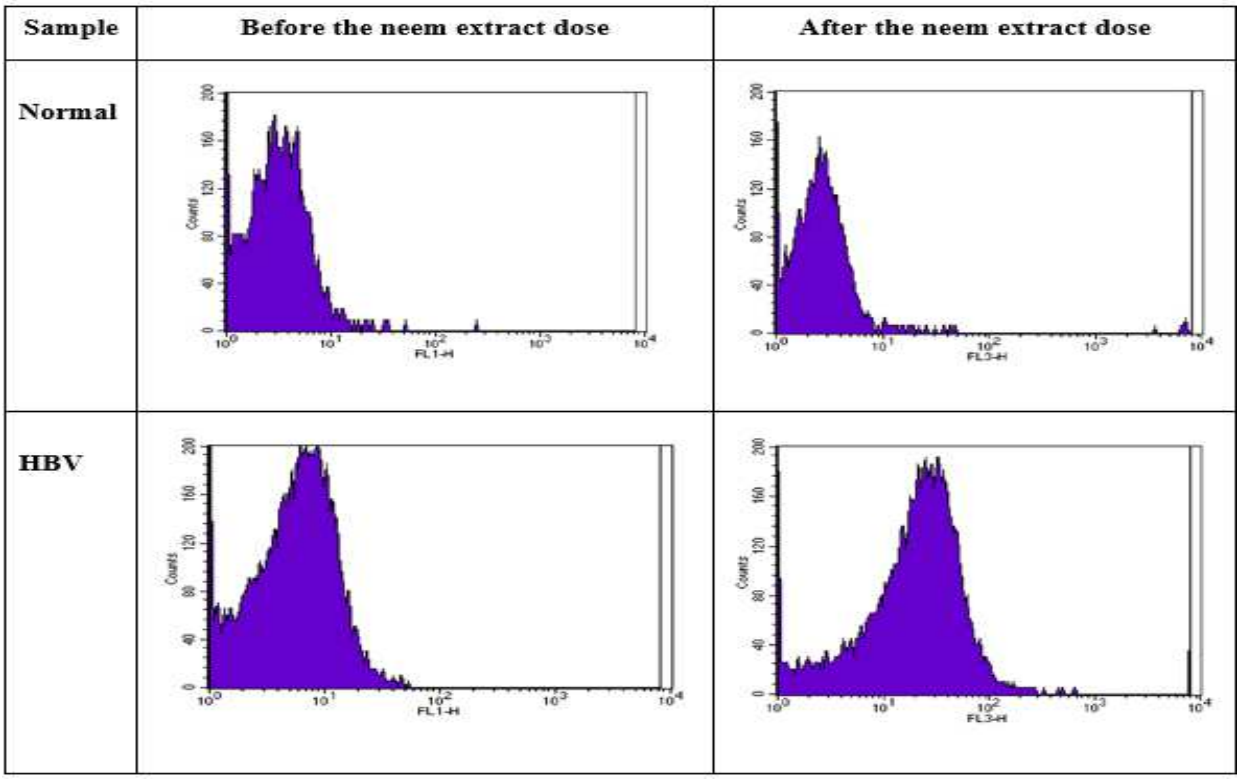

Fig. 4: Peak shift analysis showing *Azadirachta indica* extract induces apoptosis in HBV cells.

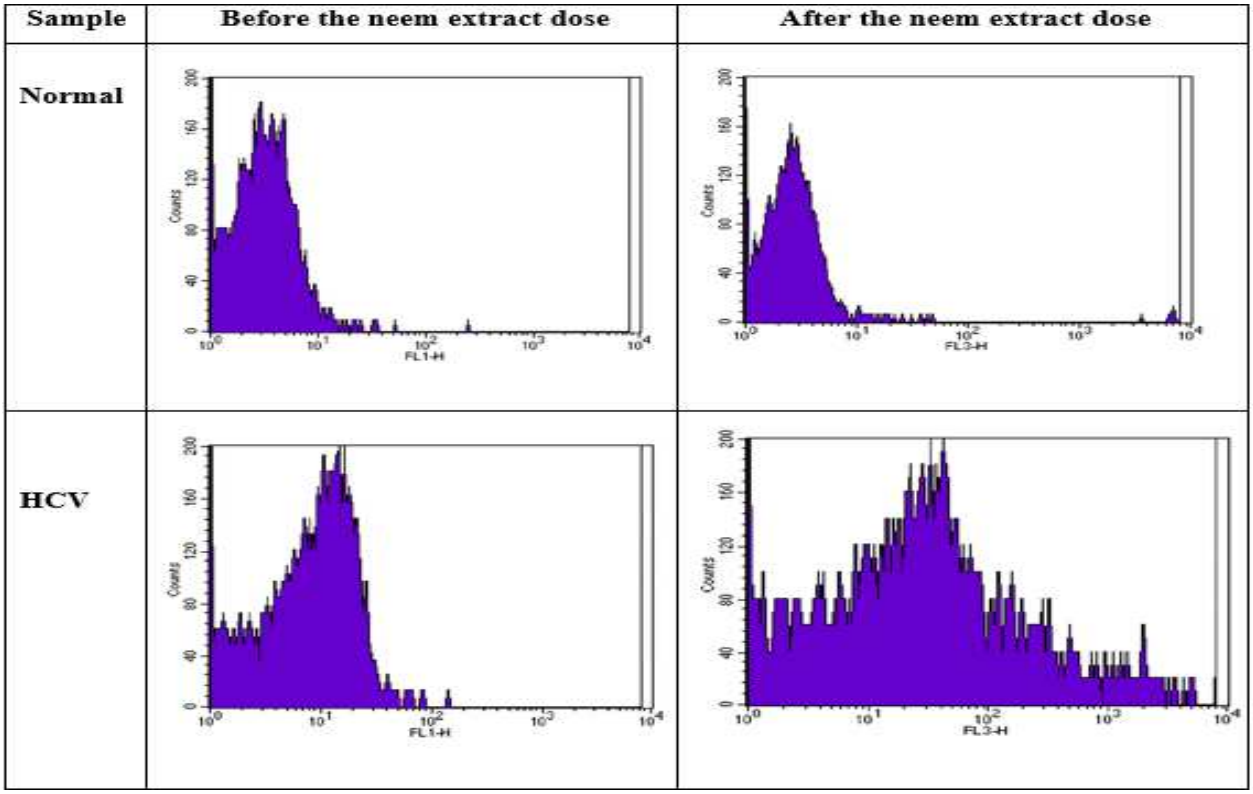

Fig. 5: Peak shift analysis showing *Azadirachta indica* extract induces apoptosis in HCV cells while sparing normal cells.

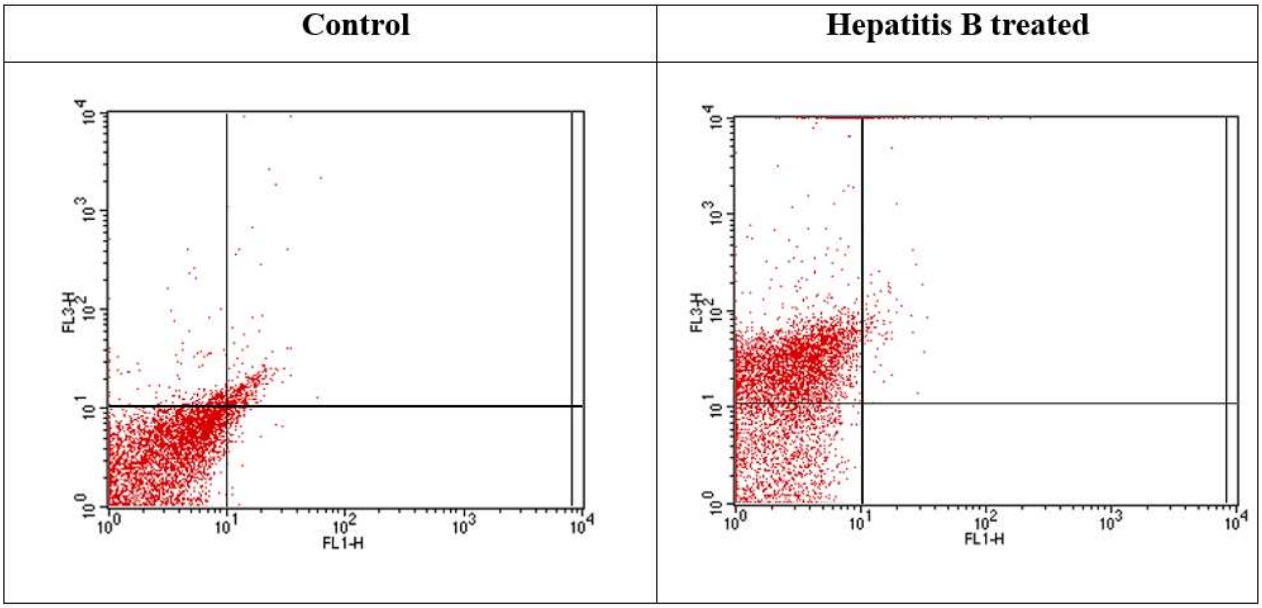

Fig. 6: Dot plot comparison between healthy and HBV-infected individuals.

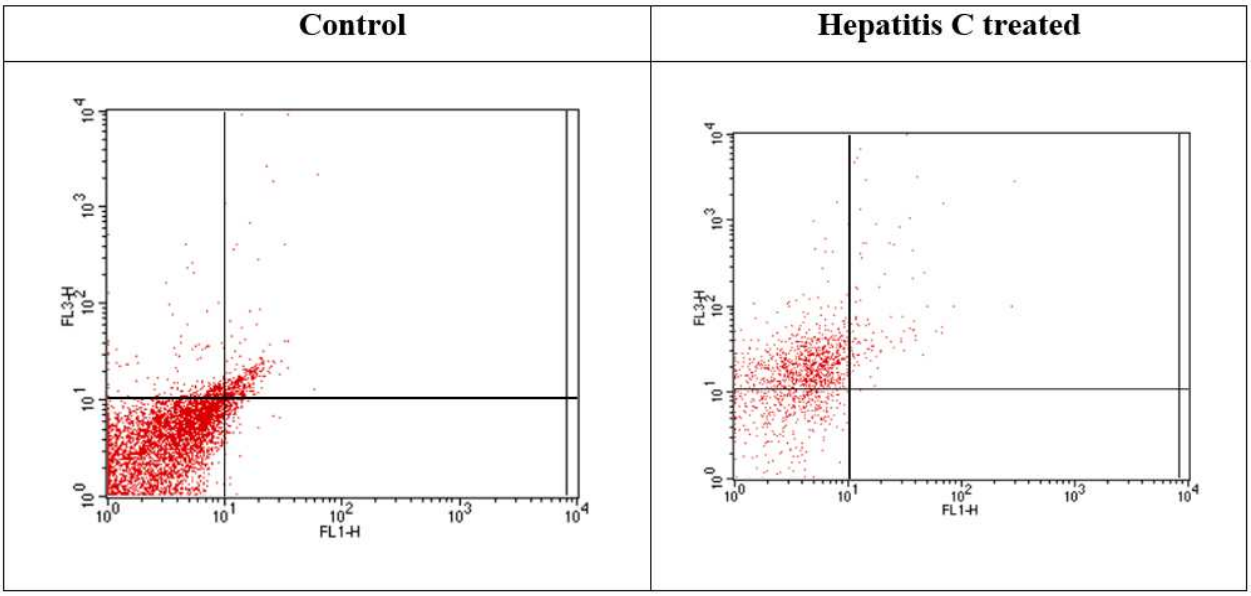

Fig. 7: Dot plot comparison between healthy and HCV-infected individuals.

Flow cytometric quadrant analysis revealed that both extracts significantly induced apoptosis and necrosis in infected cells compared to untreated controls. *A. indica* demonstrated pronounced activity against HBV-infected cells, with a substantial increase in both early and late apoptotic populations and a sharp decline in cell viability. This selective induction of programmed cell death, without affecting normal uninfected cells, highlights the therapeutic specificity of neem extract. The antiviral mechanism may be attributed to its phytoconstituents, particularly flavonoids, saponins and glycosides, which are known to interfere with viral entry, replication and assembly (Ninfali *et al.*, 2020; Ponticelli *et al.*, 2023).

Saponins in *A. indica*, absent in *M. oleifera*, may exert membrane-disruptive properties that facilitate viral envelope breakdown or enhance cellular uptake of other antiviral components. Glycosides and alkaloids can inhibit viral DNA polymerases and proteases, impeding replication. Moreover, neem's immunomodulatory properties-mediated through cytokine regulation and enhanced phagocytic activity-may amplify host defenses against HBV (Hooda *et al.*, 2024). Our findings are consistent with Khurshid *et al.* (2022), who reported significant suppression of HCV RNA levels in hepatocytes treated with neem extract and support the hypothesis that neem exerts antiviral activity through a multifactorial mechanism.

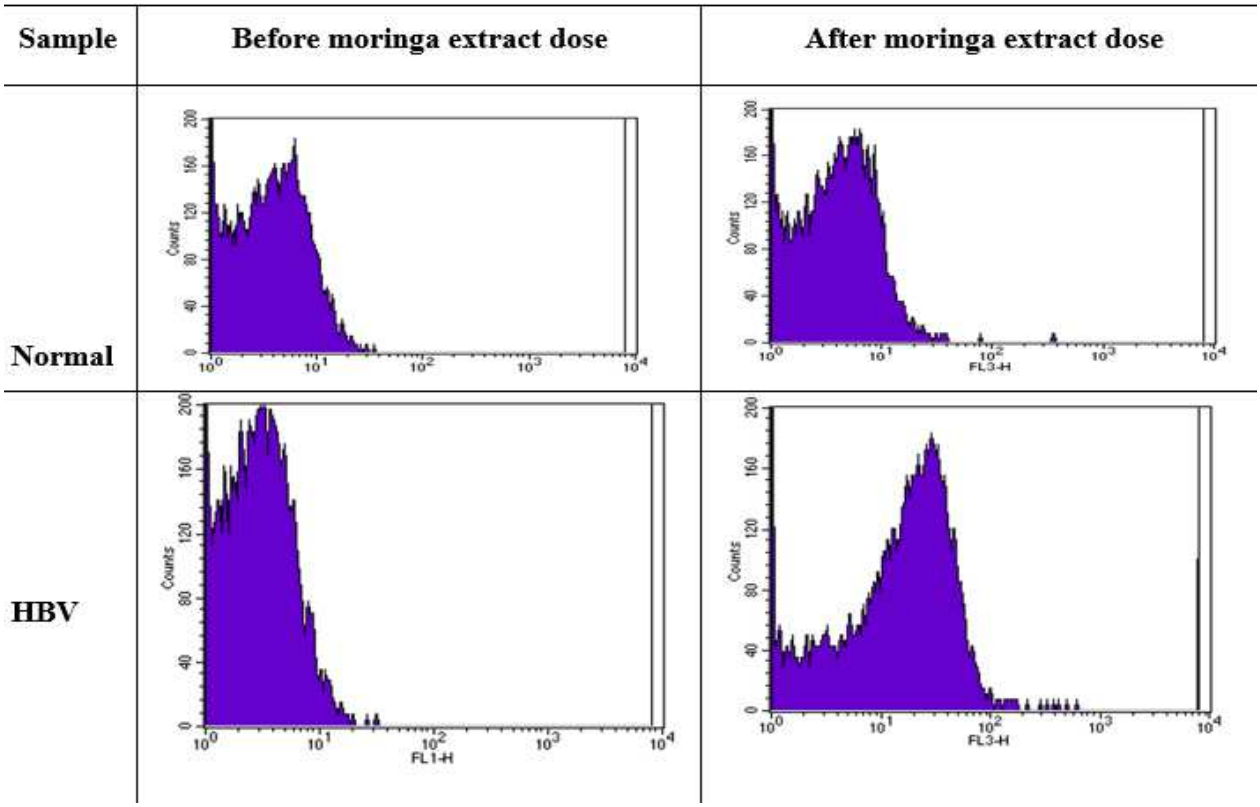

Fig. 8: *M. oleifera* extract promotes apoptosis in HBV cells, with no effect on normal cells.

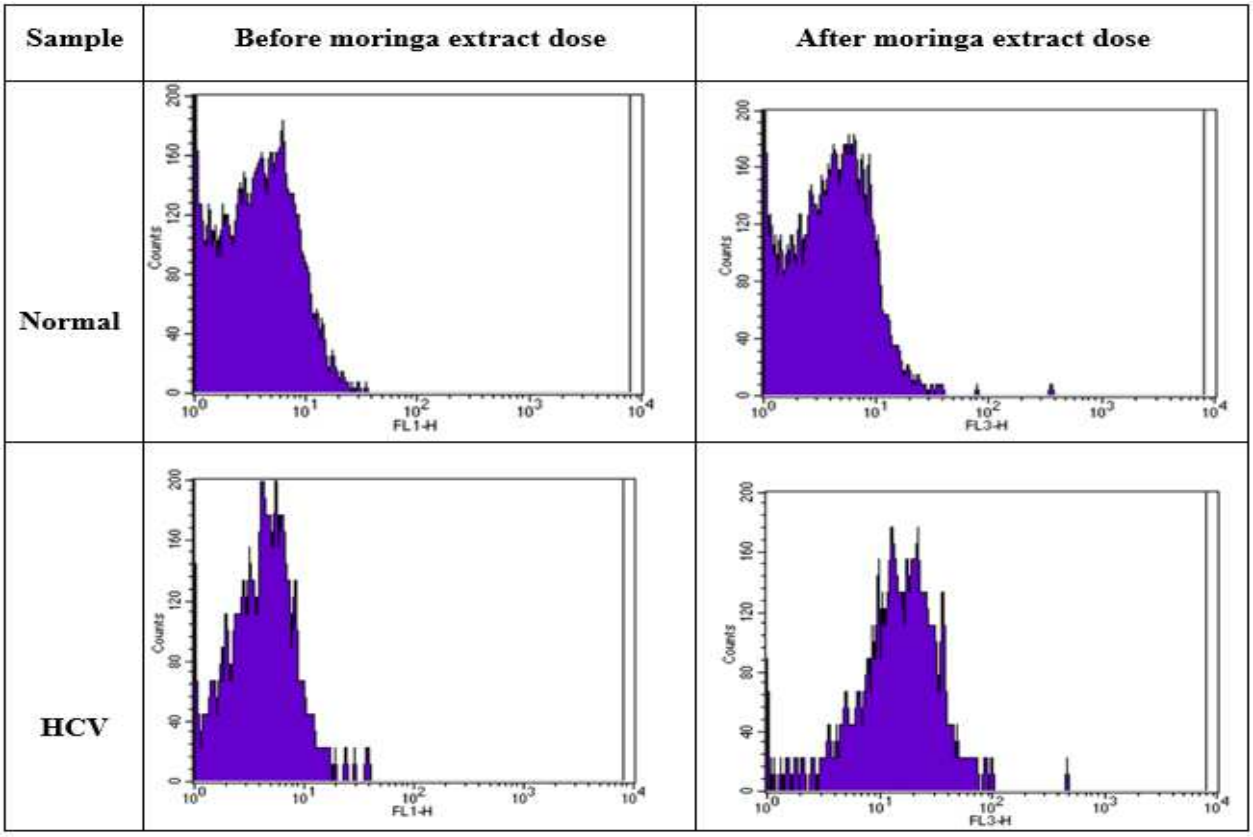

Fig. 9. *M. oleifera* extract promotes apoptosis in HCV cells, sparing normal cells.

M. oleifera extract also exhibited significant antiviral activity, particularly against HCV-infected cells. Despite the absence of saponins and glycosides, it induced marked apoptosis, suggesting that the observed antiviral effects are primarily mediated by flavonoids, alkaloids and tannins. Flavonoids, such as quercetin and kaempferol-previously identified in *M. oleifera*-have been shown to inhibit NS3/NS5 viral proteases and disrupt RNA replication complexes in HCV (Badshah *et al.*, 2021). Tannins may bind viral envelope proteins, blocking cell entry, while alkaloids can interfere with intracellular signaling pathways essential for viral replication.

Interestingly, *M. oleifera* displayed greater efficacy against HCV than HBV, possibly due to virus-specific differences in envelope composition and replication machinery. HCV, an enveloped, positive-sense RNA virus, may be more susceptible to phytochemical-induced membrane disruption or enzyme inhibition than HBV, a DNA virus with a more stable capsid. These differential effects underscore the importance of matching phytochemical profiles to virus-specific targets.

Importantly, both extracts exhibited negligible cytotoxicity toward normal lymphocytes, as evidenced by high cell viability and minimal apoptotic induction in control groups. This selectivity is critical for the development of safe therapeutic agents. Our findings highlight the potential of neem and moringa extracts to serve as virus-specific modulators of apoptosis, rather than broad-spectrum cytotoxins.

The comparative data also suggest that while *A. indica* may be a stronger candidate against HBV, *M. oleifera* holds promise for HCV-targeted applications. This differential activity may guide future research toward phytochemical fractionation, compound isolation and structure-activity relationship (SAR) studies to optimize formulation efficacy.

It is important to acknowledge that measuring apoptosis induction in peripheral blood mononuclear cells serves as an indirect proxy for antiviral activity, rather than a direct measure of viral inhibition. Since HBV and HCV primarily infect hepatocytes, apoptosis observed in lymphocytes may reflect cytotoxic effects or immune-mediated responses rather than direct antiviral action. Future studies employing infected hepatocyte cell lines and viral load quantification assays are necessary to conclusively establish the antiviral efficacy and mechanistic pathways of *A. indica* and *M. oleifera* extracts.

CONCLUSION

This study highlights the significant antiviral potential of *Azadirachta indica* (neem) and *Moringa oleifera* (moringa) against hepatitis B (HBV) and hepatitis C (HCV) viruses, suggesting their use as natural therapeutic alternatives, particularly in drug-resistant infections. Neem showed greater efficacy against HBV, while moringa was more potent against HCV, with both extracts inducing apoptosis selectively in infected cells without affecting normal lymphocytes. The observed antiviral activity is attributed to key phytochemicals such as alkaloids, flavonoids, saponins (in neem) and tannins, which likely inhibit viral replication and modulate apoptotic pathways. To advance these findings toward clinical application, future work should focus on isolating active compounds, optimizing formulations into effective dosage forms such as capsules or topical agents and conducting *in vivo* animal studies to assess pharmacological properties, safety and therapeutic efficacy. These efforts will be instrumental in developing plant-based antiviral agents with the potential to complement or replace conventional treatments for chronic HBV and HCV infections.

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Authors' contribution
AA, RA, NJ, SA, SZH, TA, HQ: methodology, experimentation, supervision, writing, drafting, research design, investigation, data curation; JMAK, OAD, BA, MIA: validation, software; writing, drafting, statistical analysis, validation; WFS, MIA, MQAM: writing, software, resource, research design, validation, data collection, drafting, statistical analysis.

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Data availability statement
The author confirms that all data generated or analyzed during this study are included in this published article.

Ethical approval
All methods were carried out in accordance with relevant guidelines and regulations, which included the collection of blood samples. The experimental protocols were approved by the Research and Ethics Committee of The Islamic International University via Reference No. IIUI-45975, Pakistan. Informed consent was obtained from all subjects and/or their legal guardian (s). All participants willingly engaged and provided informed consent. Participation was entirely voluntary and individuals who chose not to participate were respectfully excluded. The study-maintained principles of transparency, anonymity and confidentiality, with participants briefed on the study's

objectives, significance and commitment to sharing findings without any commercial exploitation.

Conflict of interest

The authors declare no competing interests.

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