

# The Antiviral Efficacy of *Withania somnifera* (Ashwagandha) against Hepatitis C Virus Activity: *In Vitro* and *in Silico* Study

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## Abstract

**Objective:** Evaluation antiviral effects of *Withania somnifera* (Ashwagandha) leaf extract against HCV. **Methods:** cell proliferation was assessed using MTT assay after isolation of lymphocyte cells and treated with Ashwagandha water extract (ASH-WX) (6.25 mg/ml - 100 mg/ml). Assessment of quantitative Real-time PCR, Colony forming assay, TNF- $\alpha$  and molecular docking studies after infection of normal lymphocyte cells with 1 ml ( $1.5 \times 10^6$  HCV) serum then incubated with ASH-WX at concentration 25 mg/ml & 50 mg/ml. **Results:** MTT assay revealed a significant increase ( $p < 0.001$ ) in normal lymphocyte proliferation at all concentration's particularity at 25 mg/ml with SI (6.06) and at 50 mg/ml with (5.8). While TNF- $\alpha$  significantly decreased following ASH-WX treatment compared with control untreated infected cells ( $p < 0.05$ ). PCR results showed a marked viral load reduction after treatment by ASH-WX at concentration 25 mg/ml to  $6.241 \times 10^3$  IU/mL. Colony formation assay test revealed colony formation reduction compared to positive untreated control. Molecular docking analysis revealed good prediction of binding between Ashwagandha and NS5B and PKN2 compared to Sovaldi. **Conclusion:** ASH-WX may be a powerful antiviral against HCV infection.

## Keywords

Antiviral, Ashwagandha, Hepatitis C Virus, Docking, Lymphocyte

## 1. Introduction

Hepatitis C is an infectious disease caused by hepatitis C virus (HCV) that essentially influences the liver [1] [2]. The global prevalence of HCV infected adults is estimated at 2.5% (177.5 million) ranging from 2.9% in Africa to 1.3% in Americas, with a global viraemic positive cases of 67% (118.9 million), varying from 64.4% in Asia to 74.8% in Australia [3] [4]. Most of the cases are caused by HCV genotypes 1 (70%) and 4 and less frequently by genotypes 2 and 3 [5]. HCV is epidemic in Egypt that has the highest prevalence in the world (15%) [6]. Genotype type 4 is the most prevalent in Egypt of about 73% followed by genotype 1 (26%), whereas 15.7% of HCV infection in Egypt were mixed genotypes [7] [8]. There is no protective vaccine available for HCV treatment but there are several recent drugs that could be used as a treatment for HCV including pegylated interferon (PEG IFN), boceprevir, ribavirin, Sofosbuvir (Sovaldi) and telaprevir [9]. Every drug has its mechanism against HCV, for example, Pegylated IFN is used due to its increased stability in vivo that activates cellular antiviral responses. Approximately 50% of responders relapse will appear upon withdrawal of treatment. Ribavirin had a broad-spectrum activity against several RNA and DNA viruses, however the treatment of chronic HCV using ribavirin alone had no significant effect on HCV RNA levels, so it has been used in combination with IFN-alfa [10] [11] [12]. Sofosbuvir (Sovaldi) can mimic the physiological nucleotide and competitively blocks the NS5B polymerase which is one of the non-structural proteins essential for viral RNA replication and inhibits the HCV-RNA synthesis by RNA chain termination. Due to the high-cost and severe side effects of current HCV treatments such as fatigue, hematologic toxicity, ophthalmologic disorders, cardiac diseases, myocardial infarction and the probability of virus recurrence [12], scientists are in great need to find new agents that are less expensive and less non-toxic and highly effective in combating HCV. Natural products have been used as traditional medicines in many parts of the world like Egypt, China, Greece, and India since ancient times [13]. Ayurvedic medicine eliminates many symptoms of different human diseases, including infectious diseases, and has been used for thousands of years [14]. One of the important natural products is *Withania somnifera*. *Withania somnifera* belongs to family Solanaceae and is commonly known as Ashwagandha or Indian ginseng and considered as a valuable medicinal herb in the Ayurvedic and indigenous medical systems [15]. Ashwagandha and its pharmaceutical derivatives; Withaferin A (WA) have vital role as antiviral agents against different types of viruses like; Infectious Bursal Disease Virus (IBDV) [16], HIV-1 [17], HPV [18], HSV [16] and the only one study investigated the effect of WA against HCV where, Sen *et al* showed that WA inhibits phosphorylation of PKC substrate peptide HCV [19] and suppresses HCV replication. Therefore, the present study was conducted to check antioxidant and antiviral activity of Ashwagandha against HCV replication.

## 2. Material and Methods

### 2.1. Ashwagandha Water Extracts Preparation

Egyptian Ashwagandha leaves were collected from Rafah, El-Arish, Egypt in September 2015, as fresh wet leaves, which were then sun-dried, grounded and filtered by sieving to get a fine dry powder [20] [21] and ASH-WX was prepared as previously described [21] [22].

### 2.2. Normal Lymphocyte Cells Isolation by Ficol Separation Media and Treated with ASH-WX

Peripheral blood mononuclear cells (PBMC) were isolated from a healthy donor by Cell separation media (Ficoll-Paque™, BiochromAG company, Berlin, Germany) [23] had no history or molecular evidence of HCV exposure, as confirmed by HCV RNA analysis of sera by **Quantitative Real time RT-PCR**. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) [24] supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 0.1 mM nonessential amino acids, all from Invitrogen Life Technologies (Burlington, Ontario, Canada), the cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 h. The cells (1 × 10<sup>6</sup> cells/ml) were then treated with a range of concentrations of ASH-WX (6.25 mg/ml - 100 mg/ml), 24 h for every concentration.

### 2.3. Proliferation Analysis by MTT Assay

Lymphocyte proliferation assay was used as an indicator of cellular immune function. Briefly, after isolation of normal lymphocytes, 100 µl of lymphocytes (2 × 10<sup>4</sup> cells/ml) were placed into each well of a sterile 96-well flat-bottom plate for 24 h then treated with gradual concentrations of Ashwagandha water extract (6.25 mg/ml - 100 mg/ml) in triplicate for each sample. Also, an untreated negative control 100 µl of lymphocytes were kept in triplicate. The plates were incubated at 37°C in a 5% CO<sub>2</sub> for 24 h. Then, 10 µl MTT solutions (5 mg/1ml of 1.0 M PBS, pH 7.4) were added and the cells incubated for 4 h at 37°C in a 5% CO<sub>2</sub> incubator. The medium was discarded, and each well was supplemented with 100 µl of dimethyl sulphoxide, mixed thoroughly using a pipette, and incubated in a dark room for 2 h at 37°C in a 5% CO<sub>2</sub> incubator. The absorbance of each well was read at 570 nm with a plate reader (Sunrise™; Tecan Group, Männedorf, Switzerland). GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used to calculate the percentage viability and the half-maximal inhibitory concentration (IC<sub>50</sub>) of ASH-WX.

Percentage of proliferation was calculated separately for concentration by using the lymphocyte stimulation index (SI) and compared with untreated control and by ANOVA tests [25].

The stimulation Index was calculated according to the formula:

$$SI = E570(\text{stimulated cells})/E570(\text{unstimulated cells})$$

In addition, lymphocyte cells are counted by hemocytometer (60 cell/10 $\mu$ l) at every concentration of ASH-WX (6.25 mg/ml - 100 mg/ml) for 24 h & 48 h.

#### **2.4. Assessment of Anti-Oxidants' Activities Using Colorimetric Analysis**

Antioxidants are synthesized or natural compounds that may avoid or delay some kinds of cell damage [26]. Total antioxidant, Glutathione S transferase, and Glutathione reductase were measured in normal lymphocytes after isolation and treated with ASH-WX at different concentrations (25 mg/ml & 50 mg/ml) and incubation for 48 h at 37°C in a 5% CO<sub>2</sub> incubator using colorimetric assay kits (Biodiagnostic, Giza, Egypt) following the manufacturer's instructions.

#### **2.5. Lymphocyte Cell Infection with HCV Serum**

After isolation of Lymphocyte from normal cells as described above, cells were grown ( $1 \times 10^6$  cell/ml) in a sterile falcon 15 ml with 1 ml ( $1.5 \times 10^6$  HCV) serum for 3 h, then 1 ml of RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. Cells were washed with 1.0 M PBS, pH 7.4 for RNA extraction from HCV and quantitative Real-time PCR to detect the copy number of HCV in infected normal lymphocyte cells before and after treatment with ASH-WX. The following day, the cells were washed with of 1.0 M PBS, pH 7.4, then treated in 24 well plate with drugs (ASH-WX) at concentrations (25 mg/ml & 50 mg/ml) for 24 h at 37°C in a 5% CO<sub>2</sub> incubator, then the cells were centrifuged and washed with 1.0 M PBS pH 7.4 for RNA extraction from HCV and quantitative real-time PCR.

#### **2.6. RNA Extraction**

Total RNA from cultured lymphocyte cells was extracted using QIAamp® RNA Blood Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

#### **2.7. Quantitative Real Time RT-PCR**

The RNA copy number of HCV in the supernatant of infected normal lymphocyte cells was determined by using HCV quantitative Real Time PCR Kit according to the manufacturer's instructions (Agpath-ID One-Step RT-PCR). Samples were run on 7500 fast Real-Time PCR System instrument (Applied Biosystems, USA, Cat# 2750142R) using the 40-cycle RT-PCR protocol. The established standard curve was used to calculate viral load using CobasAmpli-Prep/CobasTaqMan HCV test (CAP/CTM HCV) assay.

#### **2.8. Colony Forming Assay for HCV Replication**

Colony-forming assays could be used as a pre-clinical tool to assess HCV colony formation as a reflection of the antiviral drugs effectiveness as previously described [27]. Briefly, lymphocyte normal cells ( $1 \times 10^6$  cell/ml) were isolated and

infected with 1 ml ( $1.5 \times 10^6$  HCV) serum as described above, then treated in 24 well plate with ASH-WX at concentrations (25 mg/ml and 50 mg/ml), the cells were centrifuged and washed with 1.0 M PBS, pH 7.4. Coomassie blue stain (Coomassie® Brilliant blue G 250, Sigma-Aldrich, St Louis, MO, US) was added as follows: fixing solution: 50% methanol and 10% glacial acetic acid, staining solution: 0.1% Coomassie® Brilliant blue G 250, 50% methanol and 10% glacial acetic acid, storage solution (5% glacial acetic acid) on the cells. The cells were then incubated at 37°C in a 5% CO<sub>2</sub> incubator with fixing solution for 1 h to overnight with gentle agitation, then staining solution was added for 20 minutes with gentle agitation and finally destaining solution was added, the solution was replenished several time until background of the gel was being fully destained and the cells ( $1 \times 10^6$  cell/ ml) were divided in 24 well plates. Finally, the cells in each panel were examined under an inverted microscope (ZeissAxio Vert.A1; Zeiss; Gottingen, Germany) at 40× magnification, morphological changes were observed, and cells were photographed using the digital camera of an inverted microscope (Color Digital Imaging-SPOT Idea 3MP).

### 2.9. Assessment of Protein Concentration of TNF- $\alpha$ , Using ELISA

Tumor necrosis factor (also known as TNF- $\alpha$  or cachectin) is one of the most vital cytokines which regulate the cell signaling. The TNF- $\alpha$  system is enhanced in patients with HCV chronic infection with high levels of circulating TNF- $\alpha$  and a parallel increase in the level of the soluble TNF receptors [28]. TNF- $\alpha$  levels were measured using an ELISA Kit (K0331131P; KomaBiotech, Seoul, South Korea) was measured in lymphocytes normal cells after isolation and infection with 1ml ( $1.5 \times 10^6$  UI HCV). HCV serum as described above and treated with ASH-WX (25 mg/ml & 50 mg/ml) following the manufacturer's instructions.

### 2.10. Molecular Modelling (Docking Study)

The purpose of this study is to analyze the inhibitory action of Ashwagandha & Sovaldi on the Hepatitis C virus NS5B RNA-dependent RNA and Human protein kinase N2 (PKN2, PRKCL2) using Molecular docking. Docking experiment was carried using with Schrodinger 16.4 software Glide's Extra Precision (XP) [29]. The following ligands including (Ashwagandha and Sovaldi) were downloaded from PubChem Bioassay. Ligand preparation was carried out using Maestro 9.2 and LigPrep 2.4 software. Crystallographic structure of Human protein kinase N2 (PKN2, PRKCL2) and the Hepatitis C virus NS5B RNA-dependent RNA were retrieved from the Protein Data Bank with PDB codes: (PKN2-4CRS.pdb) and (NS5B-4KHM.pdb). The size of grid box for each protein was set to 20 Å by default. The binding site residues of each protein are summarized in **Table 1**.

#### Statistical analysis:

All statistical analyses were performed using the SPSS® statistical package, version 22.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data are presented as mean  $\pm$  standard deviation (SD) or median (range). Comparison of numerical

**Table 1.** Binding site residues of Sovaldi (1) and withaferin (2) with Human Protein Kinase N2, and NS5B.

PDB (Protein)	Binding site residues
<b>4CRS (Human Protein Kinase N2)</b>	Ala 923, Arg 758, Arg 917, Asp 920, Glu 756, Ile 919, Leu 918, Lys 930, Phe 761, Pro 757, Pro 931, Pro 932, Sep 755, Ser 881, Val 760 and Val 929
<b>4KHM (NS5B)</b>	Arg 394, Asn 142, Asn 406, Asn 411, Glu 143, Glu 398, Gly 410, Lys 141, Met 414, Pro 404, Ser 39, Ser 407, Thr 40 Thr 41, Thr 403, Trp 397 and Val 144, Val 405

variables between 2 study groups was done using Mann-Whitney *U*-test for independent samples while analysis of variance (ANOVA) with Bonferroni correction was done for more than 2 groups' comparison Two-tailed *P*-values < 0.001 were considered statistically significant.

### 3. Results

#### 3.1. Lymphocyte Proliferation Using MTT Assay

Proliferation of lymphocyte cells increased following treated with gradual concentrations (6.25 mg/ml - 100 mg/ml) of ASH-WX, particularly at concentration 25 mg/ml (**Table 2**). The mean of cell proliferation concentration was  $0.75 \pm 0.01$  with SI of 6.06 and at concentration 50 mg/ml of ASH-WX, the mean of cell proliferation concentration was  $0.72 \pm 0.05$  with SI of 5.8. While the untreated cell proliferation concentration was  $0.13 \pm 0.04$  with the stimulation index of 1. Also, increase the number of cells after incubation with ASH-WX at gradual concentration (6.25 mg/ml - 100 mg/ml) for 24 h and 48 h compared to untreated cells was observed, particularly at concentration 25 mg/ml and 50 mg/ml of ASH-WX (10 g/100ml distilled water).

#### 3.2. Assessment of Anti-Oxidant Activities

Normal lymphocyte cells treated with ASH-WX at different concentrations (25 mg/ml & 50 mg/ml) for 48 h revealed a significant increase in anti-oxidant activities compared with the untreated control cells ( $P < 0.001$ ). The highest activity of total antioxidant ( $331.8 \pm 9.6$ ) was observed when lymphocyte normal cells were treated with 25 mg/ml of ASH-WX, while the highest activity of Glutathione reductase ( $946.3 \pm 26.1$ ) was observed when lymphocyte normal cells were treated with 50 mg/ml of ASH-WX. Glutathione-S-transfers revealed the highest activity ( $1534.6 \pm 9.7$ ) when treated with 25 mg/ml of ASH-WX (**Table 2, Table 3**).

#### 3.3. Real-Time PCR (Normal Infected Lymphocyte with HCV)

Real time PCR results revealed a reduction of the viral load from a very high viral titer which recorded  $1.5 \times 10^6$  IU/mL to  $3.71 \times 10^5$  IU/mL and after treatment by ASH-WX at concentration 25 mg/ml, viral load was reduced to  $6.241 \times 10^3$  IU/mL and at concentration 50 mg/ml, viral load was reduced to  $2.6878 \times 10^4$  IU/mL (**Table 4**).

**Table 2.** Relation between Concentration of ASH-WX & No. of cells after incubation 24 h & 48 h, mean of cell proliferation and stimulation index (SI) with significant p value (<0.001).

Statistical significance	Stimulation index (SI)	No. of cells after 48 h	No. of cells after 24 h	Mean of cell proliferation	Conc. of ASH-WX (mg/ml)
<i>p</i> < 0.001	1	60	60	0.13 ± 0.04	Control (0)
	1.016	66	63	0.33 ± 0.01	100
	5.8	77	69	0.72 ± 0.05	50
	6.06	80	70	0.75 ± 0.01	25
	2.24	68	65	0.50 ± 0.06	12.5
	1.05	62	60	0.15 ± 0.04	6.25

**Table 3.** Concentration of Total anti-oxidant, Glutathione-S-transferase and Glutathione reductase activities in lymphocyte normal cells treated with ASH-WX at different concentrations at 37°C in a 5% CO<sub>2</sub> incubator for 48 h.

Antioxidant	Control untreated cells	Cells treated with ASH-WX		Statistical significance
		25 mg/ml	50 mg/ml	
Total anti-oxidant (µM/ml)	132.3 ± 6.4	331.8 ± 9.6	233.5 ± 11.5	<i>P</i> < 0.001
Glutathione-S-transferase (µM/ml)	866.8 ± 0.93	1534.6 ± 9.7	1457.9 ± 4.96	<i>P</i> < 0.001
Glutathione reductase (mg/dl)	5.4 ± 10.9	8.16 ± 11.7	946.3 ± 26.1	<i>P</i> < 0.001

**Table 4.** Results of Real time PCR of infected lymphocyte cells.

Drugs	CT	Titer (IU/mL)
Control (HCV serum)	29.08	1,500,000 = 1.5 × 10 <sup>6</sup>
Untreated infected normal lymphocyte cells	32.7	371,000 = 3.71 × 10 <sup>5</sup>
ASH-WX (25 mg/ml)	36.89	6241 = 6.241 × 10 <sup>3</sup>
ASH-WX (50 mg/ml)	34.81	26,878 = 2.6878 × 10 <sup>4</sup>

### 3.4. Colony Formation Assay

Colony formation assay results revealed that ASH-WX enhanced reduction of HCV colony formation compared to untreated infected lymphocyte cells (positive control), where ASH-WX at 25 mg/ml had more significant effect on the reduction of colony formation than 50 mg/ml (Figure 1).

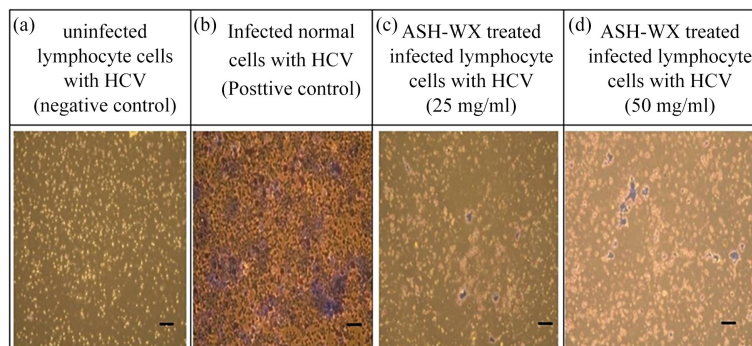
### 3.5. Human Tumor Necrosis Factor Alpha (TNF-α) Activity in Infected Lymphocytes with Hepatitis C Virus

ELISA analysis showed a significant decrease in the TNF-α concentration (*P* < 0.05) of infected lymphocytes cells with Hepatitis C virus treated with ASH-WX at concentrations (25 mg/ml and 50 mg/ml) for 48 h at 37°C in a 5% CO<sub>2</sub> incubator compared with the control untreated cells (Figure 2).

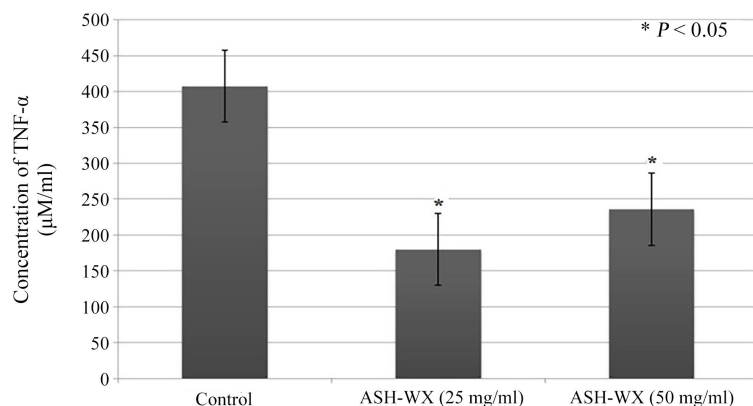


### 3.6. Molecular Docking Study of Antiviral Activity

The binding mode of Sovaldi (1) and Ashwagandha (2) in the active sites of Human Protein Kinase N2, and NS5B, molecular docking was carried using the Glide software. Human Protein Kinase N2 can bind with Sovaldi (1) through two hydrogen bonds with Arg 917, Leu 918 (Figure 3; Table 5). Ashwagandha (2) can form one hydrogen bond with Sep 755. The docking scores of Human protein kinase N2 (PKN2) with compounds Sovaldi (1) and Ashwagandha (2) were  $-2.002$  and  $-3.474$  kcal/mol, respectively. The binding mode of Sovaldi (1) with NS5B showed it can form four hydrogen bonds with Asn 142, Glu 398, Trp 397, Ser 39. It has a calculated docking score of  $-4.688$  kcal/mol. The docking results also showed that Ashwagandha (2) having the highest docking score of  $-5.599$  kcal/mol and maximum inhibitory activity with NS5B (Figure 4, Table 5). It forms hydrogen bonds with Arg 394 and Asn 411.



**Figure 1.** Effect of ASH-WX at concentration (25 mg/ml and 50 mg/ml) on colony formation of lymphocyte cells were infected with 1 ml ( $1.5 \times 10^6$  HCV) serum. (a): uninfected lymphocyte cells with HCV (negative control), (b): infected normal cells with HCV (positive control), (c): ASH-WX treated infected lymphocyte cells with HCV (25 mg/ml), (d): ASH-WX treated infected lymphocyte cells with HCV (50 mg/ml). Scale bar 7  $\mu$ m at magnification 40 $\times$ .



**Figure 2.** The concentration of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) in infected Normal lymphocyte cells infected with 1 ml ( $1.5 \times 10^6$  UI HCV) serum and treated with (25 mg/ml and 50 mg/ml) of ASH-WX at 37 $^{\circ}$ C in a 5% CO $_2$  incubator for 48 h. Data presented as mean  $\pm$  SD. \* $P < 0.05$  compared with the control untreated cells; Mann-Whitney  $U$ -test.





**Table 5.** In silico docking study of Sovaldi (1) and Ashwagandha (2) with Human Protein Kinase N2, and NS5B.

Protein	Ligand	Docking score kcal/mol	Hydrogen bond interactions
<b>Human Protein Kinase N2</b>	Sovaldi	-2.002	Arg 917, Leu 918
	Ashwagandha	-3.474	Sep 755
<b>NS5B</b>	Sovaldi	-4.688	Asn 142, Glu 398, Trp 397, Ser 39
	Ashwagandha	-5.599	Arg 394 and Asn 411

#### 4. Discussion

The present study investigated the anti-viral effects of Egyptian Ashwagandha leaves, a well-known herbal medicine that is full of anti-oxidants, against hepatitis C virus. The phytochemical analysis of Egyptian Ashwagandha leaves suggests that it belongs to chemotype III, which is different to the Indian Ashwagandha regarding the antioxidant activity [21] [30] [31]. To the best of our knowledge, this is the first investigation of this chemotype against HCV.

Our results showed increased proliferation of lymphocyte normal cells after treatment with ASH-WX particularly at concentration 25 mg/ml and 50 mg/ml with stimulation index 6.06 and 5.8 respectively. The effect of ASH-WX on proliferation of lymphocyte indicates the possible role of Ashwagandha as immunomodulatory and that agreed with other previous studies which reported that Ashwagandha modulates the immune response, increasing the expression of T-helper 1 (Th1) cytokines, as well as CD4 and CD8 counts, and natural killer (NK) Cell activity [32] [33].

In addition, the current study showed that the effect of ASH-WX on total antioxidant, glutathione-S-transferase and glutathione reductase demonstrated a significant increase ( $P < 0.001$ ) in the activities of these antioxidants when lymphocyte normal cells treated with different concentrations of ASH-WX. The highest activity of total anti-oxidant ( $331.8 \pm 9.6$ ) was observed when lymphocyte normal cells treated with ASH-WX at concentration 25 mg/ml, while the highest activity of glutathione reductase ( $946.03 \pm 26.10$ ) following treatment of lymphocyte with ASH-WX at a concentration of 50 mg/ml. Glutathione-S-transferase showed the highest activity ( $1534.6 \pm 9.7$ ) after treatment of ASH-WX at 25 mg/ml.

These results agreed with previous studies which showed that Ashwagandha has powerful anti-oxidant action as it increased the levels of three natural anti-oxidants; superoxide dismutase, catalase, and glutathione peroxidase in the rat brains [34]. Moreover, agreed with Andallu and Radhika who reported that Ashwagandha is as an important medicinal plant that has good antioxidant potentials throughout its root [35].

The effect of ASH-WX on Hepatitis C virus revealed a reduction of the viral load in infected lymphocyte normal cells before treated with ASH-WX from a very high viral titer which recorded  $1.5 \times 10^6$  IU/mL to  $3.71 \times 10^5$  IU/mL then,

after treated with ASH-WX at concentration 25 mg/ml; viral load reduced to  $6.241 \times 10^3$  IU/ml and at concentration 50 mg/ml; viral load reduced to  $2.6878 \times 10^4$  IU/mL. To confirm the effect of ASH-WX on Hepatitis C virus replication, colony forming assay was performed and the results showed that ASH-WX enhances reduction of colony formation compared to positive control. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pro-inflammatory cytokine produced in response to infectious pathogens. Previous studies demonstrated that the blood level of TNF- $\alpha$  is increased in HCV patients and that correlated with increase of HCV pathogenesis and the severity of liver diseases [36] [37]. Our results of effect of ASH-WX on TNF- $\alpha$  in infected lymphocyte normal cells with Hepatitis C revealed a significant decrease in the TNF- $\alpha$  activity in infected lymphocytes treated with ASH-WX compared to control with significant  $p$  value ( $<0.05$ ) and that agreed with previous studies which demonstrated that ASH-WX has anti-inflammatory effects, specifically reducing gene expression of CCL2 and CCL5 in response to TNF- $\alpha$  stimulation [38]. Another study on effect of ASH-WX on TNF- $\alpha$  showed a significant decrease in the TNF- $\alpha$  concentration ( $P < 0.05$ ) of HepG2 cells treated with ASH-WX at the IC50 concentration (5.0 mg/ml) for 48 h compared with the control untreated cells [21].

Based on the *in-silico* docking study between ASH-WX and Sovaldi, which an example of current drugs in HCV treatment, with Human protein kinase N2 (PKN2, PRKCL2) and NS5B revealed that Ashwagandha, has a better binding affinity and inhibitory activity against PKN2 and NS5B than Sovaldi. Our results on the effect of Ashwagandha on Hepatitis C replication agreed with previous studies that reported that Withaferin A has an effective role in suppression of HCV replication, where it inhibits phosphorylation of PKC substrate peptide HCV [19].

## 5. Conclusion

In conclusion, Ashwagandha (*Withania somnifera*) water extract is a powerful anti-oxidant and has antiviral properties in HCV infected lymphocyte cells. It might have potential as a promising anti-viral agent against HCV and these results should be confirmed in animal studies.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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