



"Phyto-Chemical and Antiviral Study on *Glycyrrhiza Glabra*, *Phyllanthus Amarus* and *Solanum Nigrum* Against Hepatitis C Virus"

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ABSTRACT

Hepatitis C virus (HCV) is a serious public health concern that affects millions of people worldwide, causing liver cirrhosis and Hepatocellular carcinoma (HCC). While there are no effective anti-HCV vaccines, the availability of potent anti-HCV drugs has streamlined the management of viral hepatitis and its complications. There are currently many DAA's available to help keep HCV infections under control. DAA's, on the other hand, are known to cause untoward side effects in addition to being expensive. Furthermore, DAA's role in treating drug-resistant HCV is known to be difficult. These facts necessitate the development of newer anti-HCV drugs, which are currently being pursued. Looking for alternative drugs from natural resources is one approach to discovering novel anti-HCV drugs. In this study, we evaluated the anti-HCV and phytochemical properties of extracts derived from *G. glabra*, *S. nigrum* and *P. amarus*. The anti-HCV activity of plant extracts was tested on the FL-J6-JFH replicon system and found a strong anti-HCV activity elicited by *S. nigrum* aqueous extract (IC₅₀-95.8µg/ml) followed by *G. glabra* methanolic

extracts (IC₅₀-97µg/ml). All plants had comparable levels of flavonoids, tannins, phenols, carbohydrates, proteins, and lipids, according to phytochemical analysis. Our study disclosed a novel plant i.e., *S. nigrum* as an effective drug against HCV.

Key Words: Anti-HCV, Plant extracts, Toxicity, RT-PCR, Phytochemical, Anti-oxidant.

INTRODUCTION

Hepatitis C Virus (HCV) is a major cause for liver diseases all over the world. Patients with chronic HCV will develop liver fibrosis, cirrhosis, HCC. HCV is an enveloped, positive sensed single stranded RNA genome comprising 9.6kb of uncapped RNA. HCV is primarily transmitted through blood, sharing contaminated devices for injection drug use, mother to infant transmission and sexual transmission. In Europe and the United States, the greater risk for HCV infection, which accounts for 50-60% of acute infections (Stanaway, J. D *et al*, 2016). Even though there are various drugs for treating HCV there is also increasing evidence that viral cure does not eliminate the risk for developing progressive liver disease once fibrosis is established. In the late 2000s, FDA has approved antivirals such as a combination of Ribavirin and pegylated Interferon- α (IFN- α) which were effective initially, owing to drastic side effects that have been exhibited is an escalating global health issue. These new drugs include direct-acting antiviral (DAA) agents that specifically block a viral enzyme or function protein, and host-targeted agents (HTA) that blocks a viral component that are essential to the viral life cycle. In fact, DAAs might not be effective once chronic HCV infection reaches liver fibrosis, cirrhosis and HCC stages. These drugs do have various demerits, which serves as a main purpose to find an alternative natural source of drug treatment to inhibit the virus (El-Serag *et al*, 2016).

DAAs targets against various steps in the HCV life cycle. The NS3/4A protease was major target on the intracellular life cycle by inhibiting maturation of the viral polyprotein. Replication has been identified as a major target for antiviral drugs that can be inhibited by NS5B inhibitors. The long-time use of these drugs has adverse side effects and hence to find a new alternative drug is essential (Yousaf, 2018). Herbal medicines have been applied for centuries against different ailments including viral diseases and establishment of focal point to identify, isolate and purify new entities to treat diseases like HCV. According to an estimate, 25% of the medicines used has compounds of plant origin derivative. Medicinal plant compounds derived from plant extracts, are of lifelong interest to the pharmaceutical industry. Plants contain a variety of chemically active compounds such as flavonoids, alkaloids, saponins and proteins. Which interferes by inhibiting the replication cycle of various DNA/RNA viruses. For this reason, plant material from different traditional medicinal plants can be used as an alternative (Tariq Javed *et al*, 2011). Hence, the main aim of the study was finding an effective drug from natural source which could be less expensive and easily available against HCV.

MATERIAL AND METHODS

Collection and extraction of medicinal plants

The Roots of *Glycyrrhiza glabra* (*G. glabra*), leaves and fruits of *Solanum nigrum* (*S. nigrum*) and leaves of *Phyllanthus amarus* (*P. amarus*) were collected from a commercial source in Chennai, authenticated at the Department of Botany, University of Madras, Guindy. The roots were washed with sterile distilled water, air dried, powdered and stored at 4°C. The aqueous and

Methanolic extracts of plant extracts were prepared using Soxhlet extraction method. The dried materials were scrapped off and stored at 4°C until next use.

Phytochemical analysis and Anti-Oxidant property

The Phytochemical analysis of extracts were performed to examine the presence of alkaloids, steroids, saponins, flavonoids, Phenols, glycosides, proteins and for reducing sugars using standard protocols (Gudimella *et al.*, 2022). The evaluation of ABTS radical scavenging assay was carried out by modifying the method of (Lee *et al.*, 2015). The DPPH radical scavenging activity was determined by the method of (Lee *et al.*, 2015).

Cell line

Huh 7.5 cells (provided by Dr. Saumitra Das, IISC, Bangalore) were grown at 37°C in Dulbecco's Modified Essentials Medium (Gibco- No.11995040) with 10% Fetal bovine serum (Gibco No. 2384431), 100 IU/ml of Penicillin and Streptomycin (Gibco No. 15140122), Non-essential amino acid (Gibco No. 2219146) and 100µg/ml Plasmocin (Invitrogen No. MPP39-02A).

Plasmid construction

The plasmid (FL-J6-JFH) was procured from Dr. Charles M Rice, Rockefeller University after signing MTA. Transformation was done using Dh5α *E. coli* cells and extracted by using Favorprep™ plasmid extraction mini kit according to manufacturer's instructions. To linearize the cells, FL-J6-JFH plasmid was digested with the Xba1 (10U/µL) Restriction enzyme (Invitrogen). The In-vitro RNA transcription was performed with a MEGAscript T7 kit (Promega No-P1320) according to the manufacturer's instruction.

Transfection of RNA into cells

The Huh 7.5 cells were seeded at a density of 2×10^4 cells/well in a 24 well plate, then cultured in DMEM medium overnight in 5% CO₂ at 37°C. RNA transfection was carried out according to manufacturer's instructions using Invitrogen™ Lipofectamine™ 2000 Transfection reagent. After 72 hours, the culture supernatant was collected, filtered through 0.45-µm membrane, and stored in small aliquots at -80°C (Wakita, 2005, Ganta *et al.*, 2017).

Anti-HCV evaluation of plant extracts on Huh 7.5 cells

The antiviral effects of plant extracts (*G. glabra*, *S. nigrum* and *P. amarus*) was evaluated at a varying concentration (1mg/ml to 1.95µg/ml). Sofosbuvir (Positive control) and Distilled water (Negative control) were tested for their ability to inhibit HCV by measuring HCV RNA levels. The plant extracts were pre-incubated at 37°C with FL-J6-JFH viral particles for 1 hour. The virus compound complex was then added to Huh 7.5 cells and incubated for 4 hours at 37°C followed by subsequent washed twice with PBS and complete medium were added. The antiviral activity was assessed 48 hours post-infection using real-time PCR (Ganta *et al.*, 2017). Total RNA was isolated from the Huh 7.5 cells using Nucleospin® RNA Virus kit (Macherey-Nagel- Catalogue No-740955.250) RNA extraction kit, cDNA converted using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Catalogue No-K1622) and Real-Time PCR were done to quantify the HCV using the artus® HCV RG RT-PCR Kit (Qiagen®, 96 Catalogue No-4518265) according to manufacturer's protocol.

Cytotoxic study

Huh7.5 cells were incubated at 37°C on 96 well plates at a density of 2×10^6 cells per well, with 5% CO₂ in a humidified atmosphere with 10% Dulbecco's modified essentials medium. After 24 hours, extracts ranging from 1000µg to 1.95µg/ml were added to a monolayer (70-80% confluency). The plate was incubated for further 5 days. 200µl MTT (Sigma- Aldrich, catalogue No.m2003) solutions (5mg/ml in phosphate buffer) was added to each well and incubated at 37°C for 4 hours. The MTT solution was decanted and formazan was extracted from cells using 100µl of DMSO in each well. The color was measured using a 96-well ELISA plate reader at 590nm. As a toxicity control, 1% Triton X-100 (Qualigens, Catalogue No.10655) was used and Distilled water was added as a Negative control. MTT assays were done in triplicates (Mosmann, 1983).

Statistical analysis

All assays were done in triplicate to check the reproducibility of the results. All statistical comparisons were made by means of Student's t-test and $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

HCV infection continues to be a global health risk. Despite the fact, that various drugs are available on the market at various times, there is overwhelming evidence that viral cure does not eliminate the risk of developing progressive liver disease once fibrosis is established. In fact, DAA's may be ineffective once chronic HCV infection has progressed to the stages of liver fibrosis, cirrhosis, and HCC. These drugs have a number of drawbacks, which is why the main goal is to find an alternative natural source of drug treatment to inhibit the virus.

Herbal medicine has been used for centuries to treat a variety of ailments, including viral diseases, and has become a focal point for identifying, isolating, and purifying new entities to treat diseases such as HCV. According to estimates, 25% of commonly used medicines contain compounds isolated from plants. The pharmaceutical industry has long been interested in medicinal plant compounds derived from plant extracts. The alteration of a therapeutic drug by a medicinal herb is known as herb-drug interaction. Drug-herb interactions can be pharmacokinetic or pharmacodynamic. The effect of a medicinal herb on the absorption, distribution, metabolism, or excretion of a therapeutic drug is referred to as a pharmacokinetic interaction, whereas pharmacodynamic interactions are often attributed to the pharmacological activity of herbs, which results in potentiated or decreased action of drugs.

Phytochemical analysis

The preliminary phytochemical analysis of aqueous and methanolic plant extracts of *G. glabra*, *S. nigrum* and *P. amarus* revealed the presence of phytoconstituents such as phenols, flavonoids, tannins, proteins, carbohydrates, lipids (Table 1). The phytocompounds were present in all samples and total phenolic content of *P. amarus* aqueous and methanolic extracts is higher (62 ± 1.2 mg/10g and 34.2 ± 1.4 mg/10g) than that of *G. glabra* and *S. nigrum*. Flavonoid content in *S. nigrum* aqueous extract is higher (27.5 ± 0.8 mg/10g) than the aqueous extracts of *G. glabra* and *P. amarus*. The methanolic extract had highest flavonoid content (39.1 ± 2.1 mg/10g) in *P. amarus* other than *G. glabra* and *S. nigrum*. The tannin content of *P. amarus* methanolic and aqueous extracts is higher (16 ± 0.8 mg/10g and 10.9 ± 0.7 mg/10g) than that of *G. glabra* and *S. nigrum*. The protein concentration of *P. amarus* aqueous and methanolic extracts is higher than

other extracts (23.2 ± 1.9 mg/10g and 50 ± 2.4 mg/10g). The carbohydrate content of *G. glabra* aqueous and methanolic extracts is higher (118.4 ± 4.9 mg/10g and 70 ± 2.9 mg/10g) than that of *P. amarus* and *S. nigrum*. The lipid content of *P. amarus* aqueous and methanolic extracts is highest (17.2 ± 1.4 mg/10g and 14.0 ± 1.2 mg/10g). This was consistent with Zain *et al.*, 2018, who found that phyto-compounds from methanolic extracts of *P. amarus* had higher concentrations than aqueous extracts. According to Veerapagu *et al.*, (2018), the methanolic extracts of *S. nigrum* had the highest concentration of Phyto-compounds. Adianti *et al.*, 2014 described *G. glabra* extracts with the highest concentrations of phytochemicals. Our results correlated with the results, and all plant extracts had the highest concentrations in methanolic extracts.

Table 1. The phytochemical constituents of the *G. Glabra*, *S. Nigrum* and *P. Amarus*

S. No.	Chemical	<i>G. glabra</i>		<i>S. nigrum</i>		<i>P. amarus</i>	
		Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol
1.	Total Phenol	23.1 ± 1.9	39.6 ± 1.4	25.6 ± 0.8	47.5 ± 1.3	34.2 ± 1.4	62 ± 1.2
2.	Total Flavonoid	19.26 ± 0.8	27.3 ± 1.3	27.5 ± 0.8	39.8 ± 1.2	21.1 ± 1.3	37.1 ± 2.1
3.	Total Tannin	8.6 ± 0.6	14.4 ± 0.4	7.6 ± 0.2	21 ± 0.4	10.9 ± 0.7	16 ± 0.8
4.	Total Protein	33.1 ± 1.9	9.6 ± 0.6	55.6 ± 2.8	17.5 ± 1.3	50 ± 2.4	23.2 ± 1.9
5.	Total Carbohydrate	118.4 ± 4.9	70 ± 2.9	92.5 ± 4.2	53.8 ± 1.9	88.3 ± 3.5	46.9 ± 2.9
6.	Total Lipid	15.1 ± 1.9	11.6 ± 1.4	12.6 ± 0.8	7.5 ± 1.3	17.2 ± 1.4	14 ± 1.2

(*This table explains about the phytochemical constituents of aqueous and methanolic extracts of *S. nigrum*, aqueous and methanolic extracts of *G. glabra*, aqueous and methanolic extracts of *P. amarus*)

Anti-Oxidant property

ABTS radical scavenging activity

The Methanolic and aqueous extracts of *P. amarus* had the highest ATBS radical scavenging activity, with an IC_{50} value of 65.2 ± 1.3 at $20 \mu\text{g/ml}$ (Fig. 1) which was higher than other extracts. The methanolic extracts of *P. amarus* and *S. nigrum* shows higher scavenging activity than *G. glabra*, with an IC_{50} value of 70.2 ± 2.6 at $40 \mu\text{g/ml}$. Methanolic extracts of *S. nigrum* (80.4 ± 4.7) and *P. amarus* (79.5 ± 3.4) had the highest scavenging activity in $60 \mu\text{g/ml}$ while methanolic extract of *G. glabra* and aqueous extract of *P. amarus* had the moderate activity at $60 \mu\text{g/ml}$. Methanolic extract of *S. nigrum* (84.6 ± 1.4) had the highest activity than methanolic and aqueous extract of *P. amarus* at $80 \mu\text{g/ml}$. Methanolic extracts of *S. nigrum* (87.8 ± 1.8) and *G. glabra* (86.6 ± 4.5) had the highest scavenging activity and methanolic and aqueous extract of *P. amarus* shows lowest ABTS scavenging activity than other extracts at $100 \mu\text{g/ml}$.

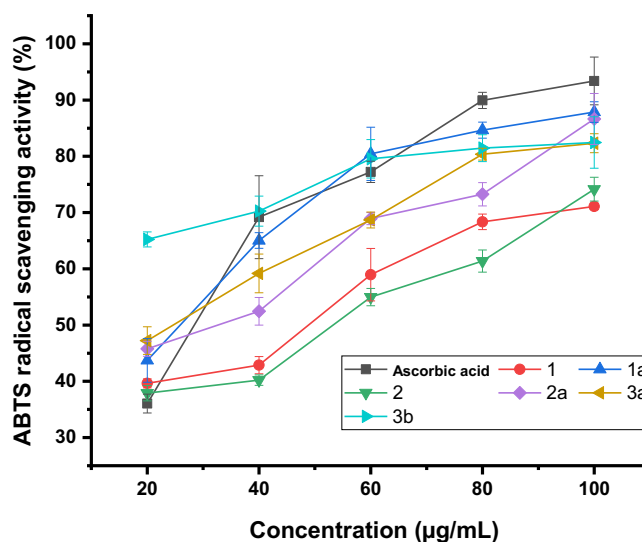


Fig 1. Abts radical scavenging activity

(*Figure 1 explains about the ABTS Scavenging activity of aqueous and methanolic extracts of *S. nigrum* (1, 1a), aqueous and methanolic extracts of *G. glabra* (2, 2a), aqueous and methanolic extracts of *P. amarus* (3a, 3b) and control (Ascorbic acid)

DPPH radical scavenging activity

The methanolic extract of *S. nigrum* had the highest DPPH scavenging activity in all the doses and aqueous extract of *P. amarus* shows second highest activity compared to other extracts (Fig. 2). The variations in anti-oxidant properties of plant species could be attributed to differences in collection season or age of the plants collected in the current study. Veerapagu *et al.*, 2018 reported a similar result in which methanolic fruit extracts of *S. nigrum* L. showed a significant reduction of DPPH radical at a concentration of 200µg/ml and an IC₅₀ value of 70.73µg/ml.

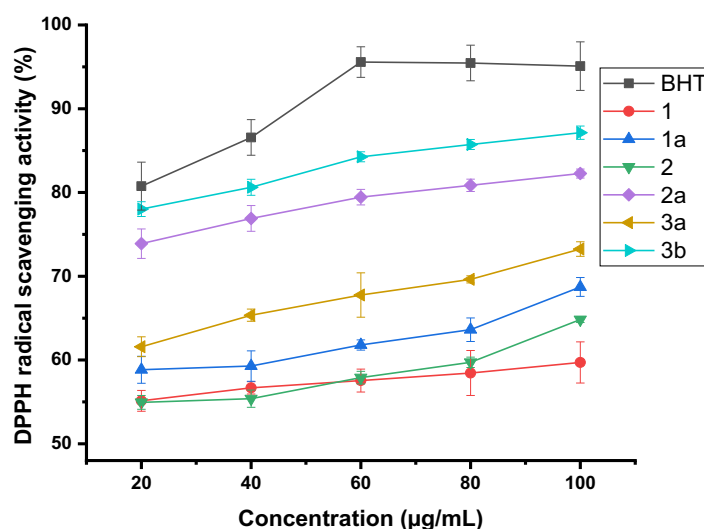


Fig 2. Dpph radical scavenging activity

(*Figure 2 explains about the DPPH Scavenging activity of aqueous and methanolic extracts of *S. nigrum* (1, 1a), aqueous and methanolic extracts of *G. glabra* (2, 2a), aqueous and methanolic extracts of *P. amarus* (3a, 3b) and control (BHT-Butylated hydroxytoluene)

Anti-HCV activity of plant extracts

Real-time PCR was carried out to evaluate infectivity 48 hours after infection, the RNA viral copy number was compared to the virus control. The GAPDH is used as an internal control infection to investigate the effect of treatment. The antiviral activity was observed in aqueous *G. glabra*, which showed potent activity (above 90%) at 125µg/ml compared to aqueous extracts of *S. nigrum*, which showed 500µg/ml, followed by and *P. amarus*, which showed very minimal activity at 1000µg/ml. The Methanolic extracts of *G. glabra* had potent anti-HCV activity (above 90%) at 250µg/ml, *S. nigrum* has 500µg/ml and *P. amarus* has 1000µg/ml. The aqueous and methanolic extracts of *S. nigrum* showed reduction of HCV (above 90%) at a concentration of 500µg/ml. The overall percentage inhibition of *S. nigrum* aqueous and Methanolic extracts was found to be higher with 95.8µg/ml and 94.8µg/ml, followed by *G. glabra* aqueous and Methanolic extracts with 96.5µg/ml and 97µg/ml, and *P. amarus* showing activity against HCV but at a lower level with 91.6µg/ml and 98.6µg/ml when compared to other extracts.

A careful review of the literature revealed that there are no comparable publications to ours that allow us to compare our data side by side. These plants, however, were tested for anti-HCV activity using various solvents. Tariq Javid *et al.*, (2011) demonstrated that chloroform extracts of *S. nigrum* seeds reduced the expression or function of HCV NS3 protease in a dose-dependent manner. Furthermore, the HCV RNA of the 3a genotype was reduced by 37% in this study. They concluded that their extracts were effective in reducing HCV replication. Despite the fact that we did not use chloroform as a solvent, we found a comparable level of HCV inhibition. In a study conducted by Usman A Ashfaq *et al.*, (2011) he found, that glycyrrhizin derived from *G. glabra* reduced HCV by 50% at a concentration of 13µg/ml in a study. However, our study used different plants and found that 250µg/ml of anti-HCV activity was effective. Our study's end point was 90% inhibition, so we achieved HCV inhibition at a higher concentration. In their study, they found that anti-HCV activity by 13µg/ml that too with 50% inhibition. These facts accentuates that our extracts were superior to this work.

There are few reports in the literature that describe the effectiveness of *P. amarus* against HCV. Phyllanthin derived from *P. amarus* inhibited HCV replication and HCV NS3 protease activity at sub-micromolar concentrations and inhibited NS5B-RdRp activity at higher concentrations, according to Uma Reddy *et al.*, (2020). Despite the fact that we did not use any of the isolated compounds like phyllanthin, we consistently obtained a positive result.

The IC₅₀ values of *S. nigrum* in aqueous and methanolic extracts were found to be less than 31.2µg/ml and 20.5µg/ml, respectively, followed by *G. glabra* with IC₅₀ values of 60.1µg/ml and 31.2µg/ml and *P. amarus* with IC₅₀ values of 123µg/ml and 121µg/ml. As a result, *G. glabra* was found to have greater antiviral activity, whereas *S. nigrum* had a higher inhibitory concentration due to lower dosage (Table 2). These findings are fascinating and intriguing. However, no comparable papers exist to compare, making proper validation impossible. After finding the plants' anti-HCV activity, we assessed their toxicity to determine the utility value of these drugs.

Table. 2 This shows the Percentage of Inhibition and IC₅₀ Values of Plant extracts

S.NO	PLANT	SOLVENT	CONC. OF EXTRACTS (µg/ml)	PERCENT INHIBITION (%)	IC ₅₀ VALUE (µg/ml)
1	<i>G. glabra</i>	Aqueous	125	96.5	60.1
2	<i>S. nigrum</i>	Methanol	250	97	31.2
		Aqueous	500	95.8	31.2
3	<i>P. amarus</i>	Methanol	500	94.8	20.5
		Aqueous	1000	93.9	123
4	Drug positive control (Sofobusvir-1mg/ml)		1000	91.6	121
5	Drug negative control (Distilled water)		-	98.6	-
			-	<1.0%	-

(*This table explains about percentage of inhibition of and IC₅₀ values of extracts)

Cytotoxicity assay

G. glabra, *S. nigrum*, and *P. amarus* were tested for cytotoxicity at concentrations ranging from 1000µg/ml to 1.95µg/ml and found to be non-toxic to the cells. *G. glabra*, *P. amarus*, and *S. nigrum* cytotoxicity up to 500µg/ml has no effect on the viability of Huh 7.5 cells. However, none of the concentrations demonstrated 100% toxicity, indicating that it is safe for humans (Fig. 3). Because non-toxic plant extracts can be used for biological activities, it was critical to determine non-toxic doses of all plant extracts. There were no comparable to compare the degree of toxicity produced. Taken together *S. nigrum* extracts were found to be most effective in controlling HCV. This was followed by *G. glabra*, and the least was noticed with *P. amarus*.

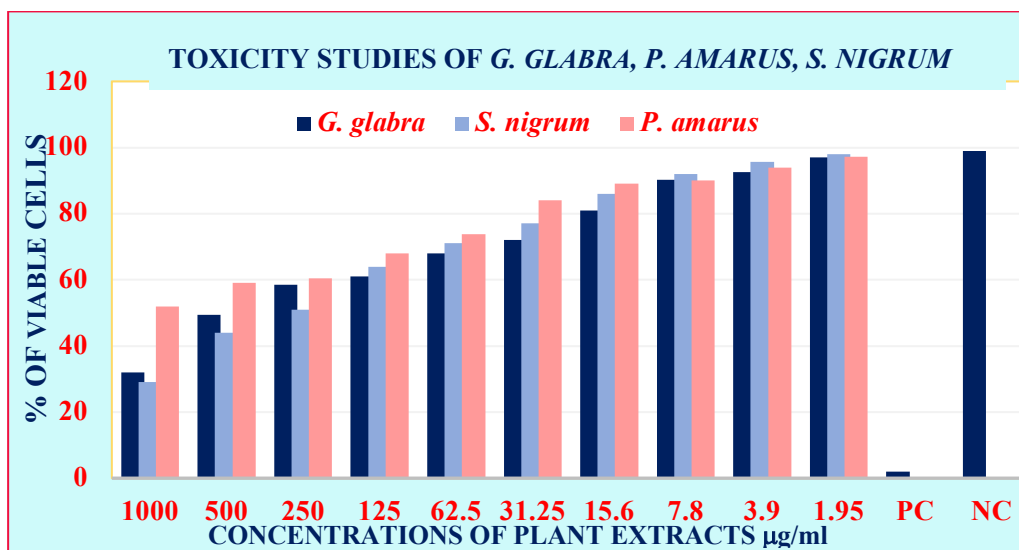


Fig. 3 mtt assay of g. Glabra, p. Amarus, s. Nigrum
(Figure 3 explains about the MTT cell proliferation assay of *G. glabra*, *P. amarus*, *S. nigrum* had no toxic effects up to 500µg/ml concentration).

CONCLUSION

Based on these findings, it is reasonable to conclude that *S. nigrum* outperformed all other plants, with *G. glabra* coming in second. *P. amarus* had the least amount of activity when compared with these plants. Because of its antiviral properties and lack of toxicity, *S. nigrum* could be considered an alternative novel drug for the treatment of HCV. However, adequate pre-clinical data must be available to support the hypothesis proposed in our study.

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