



Antiviral Activities and Cytotoxicity Assay of Seed Extracts of *Piper longum* and *Piper nigrum* on Human Cell Lines

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ABSTRACT

Traditional plant based compounds are seeking more attention in pharmaceutical companies to treat incurable diseases as they have no side effect, ease of recovery in large amount and high magnitude of activity. The present study deals with anti-viral assay of methanolic and chloroform extract from seeds of *Piper longum* and *Piper nigrum* which are commonly used in food. From the seeds of *Piper longum* and *Piper nigrum* chloroform and methanolic extracts were collected by reflux method and extracts were evaluated against Vesicular stomatitis virus and human para influenza virus on HeLa cell lines. Cytotoxicity assay was carried out by MTT assay and LDH measurement. Anti-viral activity of *Piper nigrum* in chloroform extract showed higher activity than *Piper nigrum* in methanolic extract against Vesicular stomatitis Indiana virus and Human para influenza virus on HeLa cell line. *Piper longum* in methanolic extract showed higher anti-viral activity than in chloroform extract against both viruses. Cytotoxicity assay of *Piper longum* treatment showed significant dose-dependent inhibition of growth of HeLa cells at IC₅₀ values of 46.24, 33.43 and 38.49 µg/ml at 24, 48 and 72 hours of incubation respectively. Similarly, extract of *Piper nigrum* treatment also showed inhibition of growth of HeLa cells at IC₅₀ values of 551.58, 24.18 and 17.47 µg/ml at 24, 48 and 72 hours of incubation respectively. LDH measurement showed 100% of inhibition at 260 mg/ml for both extracts of *Piper nigrum*. *Piper longum* in methanolic extract showed 100% of inhibition at 240 mg/ml and no activity were observed in the case of chloroform extract. These results suggest that both *Piper longum* and *Piper nigrum* have significant anti-viral and anti-cancer activity in HeLa cells. HPTLC analysis of both the pepper in chloroform extract at 254nm and 366 nm was done which produces different bands. At 254 nm resolutions of bands were poor and at 366 nm well resolved bands with different intensity of color of bands confirmed the presence of piperidine.

Keywords: *Piper longum*, *Piper nigrum*, HeLa cells, MTT assay, LDH activity, HPTLC.

INTRODUCTION

Spices are the plant substance which is derived from exotic or indigenous origin. It is also used as a taste enhancer in food which provides strong taste and is also aromatic.¹ Some spices have antimicrobial property against the food spoilage causing pathogenic bacteria or some have antioxidant property which will prevent the rancidity and thereby increase the shelf life of foods. Spices provide protection against some diseases and promote healing as they are rich in phytonutrients and other active ingredients.² One of the commonly used spices are fruits of black pepper (*Piper nigrum*) which has been used to treat asthma, colon toxins, obesity, sinus, congestion, chronic indigestion, and fever.³ *Piper longum* Linn. which comes under the family Piperaceae sometimes called Long Pepper. It is a flowering vine cultivated for its fruit which is used as a spice and for seasoning after drying. It has a close relation to black pepper plant, generally hotter in taste.⁴ They produce salivation and numbness of the mouth and having a pungent pepper taste. Both have many medicinal uses, especially the fruits and roots are used to treat digestive tract and respiratory tract related diseases⁵

Now-a-days incidences of viral infections are increasing. The major routes of entry are the respiratory and oral routes and it cause severe infections. These viruses are

impossible to treat by drugs and to prevent by vaccines. It is estimated that 3.9 million deaths worldwide is due to acute respiratory infections (ARI- including both upper and lower - pneumonia, bronchiolitis and bronchitis) every year. It was reported that India, Indonesia, Nepal and Bangladesh together account 40% of global ARI mortality. According to the hospital records 13% of inpatient deaths was reported in paediatric wards due to ARI.⁶ Reports showed 19% of children die with pneumonia in hospitals.⁷ Enteric viruses is next to respiratory infection which results in mortality in children. Some of the enteric viruses cause mild or asymptomatic infections, but certain wide range cause serious and life threatening illnesses in children. Enterovirus and infectious hepatitis may results in foetal death or serious illness.⁸ To evaluate the traditionally proposed ailment, extract from fruit of pepper as anti-respiratory and anti-enteritis viral activity was analysed, Vesicular stomatitis Indiana virus (an enteric virus) and Human para influenza virus (a respiratory infection causing virus) were cultured in cell lines and antiviral activity against pepper extracts were evaluated. Chloroform and methanolic extract contains major phytochemicals at high concentrations so these extracts⁹ were chosen for cytotoxicity assay on HeLa cell lines.



MATERIALS AND METHOD

Collection and drying of pepper samples

The dried fruits of *Piper nigrum* and *P. longum* were collected aseptically, sun dried for 3 days and powdered. The powdered samples were hermetically sealed in separate polythene bags until the time of the extraction.

Extraction of *Piper nigrum* and *Piper longum* using methanol and chloroform¹⁰

The dried powder from *Piper nigrum* and *P. longum* were extracted using organic solvents such as methanol and chloroform. One gram of powdered *Piper nigrum* and *P. longum* were extracted successively with 20 ml of methanol and chloroform using reflux method separately. The extracts were then filtered through Whatman filter paper and stored in a sterile container.

Antiviral assays¹¹

The cells were collected when they reach about 70-80% confluency. The viability were checked and centrifuged. About 50,000 cells/ well were seeded in a 96 well plate and incubated for 24, 48 and 72 hrs at 37°C, 5% CO₂ incubator. The samples to be tested were added from 0 - 320 µg/ml concentration of *Piper longum* and *Piper nigrum* in DMEM without FBS & are incubated for 24 hr. Viral cytopathogenicity was recorded as soon as it reached completion in the untreated virus-infected cell cultures. Antiviral activity was expressed as minimal inhibitory concentration (MIC₅₀) required reducing virus induced cytopathogenicity by 50%. Control prepared by applying 100 mcg of Acyclovir and virus infected cell lines.

Invitro cytotoxicity analysis

Invitro cytotoxicity study was conducted using HeLa cell lines by MTT Assay and LDH Assay

MTT assay¹²

The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) or MTT, is a yellowish solution converted to an insoluble purple formazan by mitochondrial dehydrogenase enzymes of viable cells. The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

The cells were collected by centrifugation when they reach about 70-80% confluency. About 50,000 cells /well were seeded in a 96 well plate and incubated for 24, 48 and 72 hrs at 37°C, 5% CO₂ incubator. The samples to be tested were added from 0 - 320 µg/ml concentration of *Piper longum* and *Piper nigrum* in DMEM without FBS & are incubated for 24 hr. After incubation with test samples, 100 µl/well (50 µg /well) of the MTT (5 mg/10 ml of MTT in 1X PBS) was added to the respective wells and

incubated for 3 to 4 hours. After incubation with MTT reagent, discard the MTT reagent by pipetting without disturbing cells and then 100 µl of DMSO was added to rapidly solubilize the formazan. The absorbance was measured at 590 nm. The % of inhibition was calculated using the formula; % of inhibition = 100 - (OD of sample/OD of control) × 100.

LDH Measurement¹³

The cells were treated with different concentrations of pepper extracts and supernatants were collected from each treatment. About 50 µl of the reconstituted 2X LDH assay buffer was added to the supernatants. The reagent was mixed gently by shaking for 30 seconds. The assay plate was protected from light and the plate was incubated at room temperature (22 – 25°C) for 10 –30 minutes, then 50 µL of Stop solution was added to the well. The reagent was mixed by shaking for 30 seconds. The absorbance was measured between 490 – 520 nm. The percentage of cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = 100 \times \frac{(\text{corrected reading from test well} - \text{corrected reading from untreated well})}{(\text{corrected maximum LDH release control} - \text{corrected reading from untreated well})}$$

HPTLC¹⁴

The stationary phase was aluminum packed pre-coated Merck silica gel plate 60 F 254 plate (10×10 cm). Toluene : Ethyl acetate: formic acid (7: 3: 0.3) act as the solvent system. Samples were applied on the plate using Camag automatic TLC sampler 4 attached to camag HPTLC system. 10 µl of test solutions was applied on a precoated silica gel 60 F254 TLC plate (E. Merck) of uniform thickness of 0.2 mm plate in the form of bands with width 8 mm using Hamilton syringe (100µl). Developed the plate in the solvent system in a twin trough chamber to a distance of 9 cm. The plates were observed under UV light at 254 nm, 366 nm. Densitometric scanning of the plates was done by using camag TLC scanner at 254 nm, 366 nm.

RESULTS

Antiviral assays

The *Piper nigrum* in chloroform extract shows higher activity than compared to *Piper nigrum* in methanolic extract in the case of Vesicular stomatitis Indiana virus and Human para influenza viruses. *P. longum* in methanolic extract have higher activity than in chloroform extract in the case of both viruses in HeLa cell lines which are shown in table 1.

In a previous study it was reported that the Indian medicinal plant extract, *Swertia chirata* showed antiviral properties against Herpes simplex virus type-1.¹⁵ Antiviral activity has been reported earlier on plant products against DNA viruses, including herpes viruses.¹⁶ Indian medicinal plant i.e., *Swertia chirata* showed antiviral



properties against HSV-1. Basic experiments conducted such as, plaque reduction assay and time kinetics of HSV-1 antigen expression showed that Swertia plant product has a potential to have antiviral activity as compared to acyclovir drug treated virus control. It was also reported that the aqueous and methanolic extracts from leaves, stems and roots of *V. indica* (Asteraceae) were tested

against HSV – 1 replication. Among these extracts, aqueous extract of roots was found to have more potent inhibitory effect. The less concentration (20µg/mL) of methanolic extracts was cytotoxic to vero. The aqueous extract of root at a concentration of 100 µg/ml exhibited strong activity against HSV-1.¹⁷

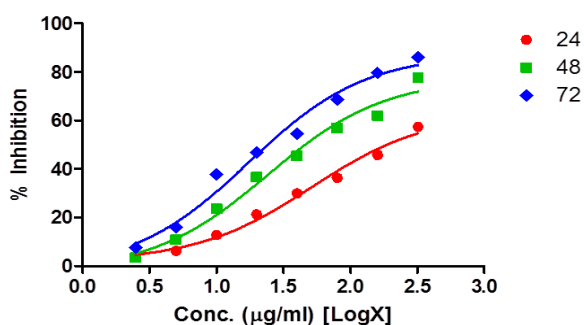
Table 1: Antiviral assay of methanolic and chloroform extract of *P. nigrum* and *P. longum*

Conc of sample (mcg)	<i>P nigrum</i> -methanol		<i>P nigrum</i> -chloroform		<i>P longum</i> -methanol		<i>P longum</i> chloroform	
	Vesicular Stomatitis Indian virus	Human para influenza viruses	Vesicular Stomatitis Indian virus	Human para influenza viruses	Vesicular Stomatitis Indian virus	Human para influenza viruses	Vesicular Stomatitis Indian virus	Human para influenza viruses
200	0 mm	0 mm	2 mm	0 mm	4 mm	2 mm	0 mm	0 mm
600	2 mm	0 mm	4 mm	2 mm	6 mm	4 mm	0 mm	0 mm
1000	4 mm	4 mm	6 mm	4mm	8 mm	4 mm	4 mm	2 mm
2000	8 mm	10 mm	8 mm	10 mm	8 mm	8 mm	6 mm	8 mm
MIC	600 mcg	1000 mcg	200 mcg	600 mcg	200 mcg	200 mcg	1000 mcg	1000mcg
Acyclovir 100 mcg	5 mm	10 mm	5 mm	10mm	5 mm	10 mm	5 mm	10 mm

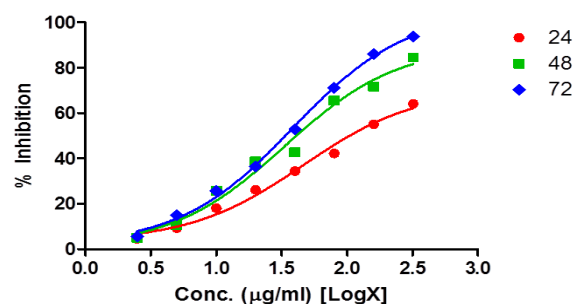
In vitro cytotoxicity analysis

MTT assay

The test compounds namely *Piper longum* treatment showed significant dose-dependent inhibition of growth of HeLa cells at IC₅₀ values of 46.24, 33.43 and 38.49 µg/ml at 24, 48 and 72 hours of incubation respectively. Similarly, the test compounds namely *Piper nigrum* treatment also showed significant dose-dependent inhibition of growth of HeLa cells at IC₅₀ values of 551.58, 24.18 and 17.47 µg/ml at 24, 48 and 72 hours of incubation respectively. These results suggest that both *P. longum* and *P. nigrum* have cytotoxicity on HeLa cells. Cytotoxic effect of *Piper longum* and *Piper nigrum* extract on HeLa cells are shown in graph 1 and 2 at 24, 48 and 72 hrs of incubation.



Graph 1: Cytotoxic effect of *Piper nigrum* extract on HeLa cells



Graph 2: Cytotoxic effect of *Piper longum* extract on HeLa cells

LDH measurement

The LDH was measured using the *Piper nigrum* and *P. longum* in methanolic and chloroform extract. % inhibition was calculated. The values are given in table 2. In this *Piper nigrum* in both the extract shows % inhibition at 260 mg/ml and in the case of *P. longum* in methanolic extract shows inhibition at 240 mg/ml. No activity was observed in *P. longum* in chloroform extract.

In a previous study, cytotoxicity of ethanolic extract of *P. nigrum* was evaluated using four different cancer cell lines. Murine cell lines chosen were Ehrlich Ascites Carcinoma (EAC) and Melanoma B-16 cells, and the human cell lines were HeLa and Raji cells. It was observed that *P. nigrum* displayed cytotoxicity towards both normal and tumor cell lines. However the toxicity

elicited on tumor cells was significantly higher than that on normal cells, indicating a selective toxic effect of the plant extract on tumor cells. The IC₅₀ values for EAC and Raji cells were comparatively lower than that for Melanoma B-16 and HeLa cells, indicating lesser toxic effect of the extraction on them.¹⁸ previous study showed that piperine exhibited immunomodulatory and antitumor activities. Peperine was able to inhibit the growth of solid tumors induced by DLA cells and as cites tumors induced by EAC cells and suggested that it may be due to the combined action of humoral and cell mediated immune responses. They stated that peperine acts as a non-toxic immunomodulator, which also possess an antitumor property. Peperine also elicited cytotoxicity towards DLA, EAC, L929 and B16 cells.¹⁹

In vitro cytotoxicity was studied previously in black pepper extracts for esophageal squamous cell line TE-13 and breast cancer cell lines like MCF-7, MDA-MB-231 and MDA-MB-468.²⁰ The Cytotoxicity of *P. longum* extracts was also reported in lung epithelial adenocarcinoma cell line HCC-827.²¹ *P. chaba* extracts were also checked for their cytotoxicity in large lung carcinoma cell line COR-L23, cervical cancer cell line, HeLa and liver cancer cell line HepG2.²² In a previous study In vitro cytotoxicity of the oils was also checked on cervical cancer cell line CaSki for three time intervals (24, 48 & 72 hrs) and found that there was a time dependent increase in the cytotoxicity of oils on CaSki. By considering cytotoxicity for three time intervals, black pepper essential oil was more toxic to Caski.²³

In vitro cytotoxic activity of peperine on HeLa cell lines was previously reported. The dried fruit of *P. nigrum* was used for the study. The MTT values obtained demonstrated that piperine has cytotoxic effect. The IC₅₀ value was found to be 61.94±0.054 µg/ml.²⁴ Cytotoxic effect of chloroform extracts of *Waltheria indica* leaves on cervical cancer cells has been reported. The cytotoxicity study clearly showed the dose dependent cytotoxic effect of extract in HeLa cell line with a CTC₅₀ of 103.33±5.77 for MTT. The LDH leakage was observed in a dose dependent manner with an LDH IU/mg of total protein of 36.18. The data in the study clearly demonstrated cytotoxic effects of extracts on human cervical cancer cells.²⁵ It has been reported that from the extract of *P. nigrum* they have isolated an alkaloidal constituent piperidine. In vitro antitumour activity of isolated compound, piperidine was performed by MTT assay. The study confirms the in vitro antiproliferative property of piperidine against HEP2 cancer cell line.²⁶ It was also reported that the extract of *P. nigrum* and *P. bettle* have cytotoxicity activity against HL60 and HELA cell line.²⁷

HPTLC

HPTLC results showed best separation of components by using different layers and solvent system. At 254 nm resolutions of bands were poor and at 366 nm well resolved bands with different intensity of color of bands were recorded. The peak showing the components of both pepper at 254nm and 366 nm is shown in graph 3, 4, 5 and 6.

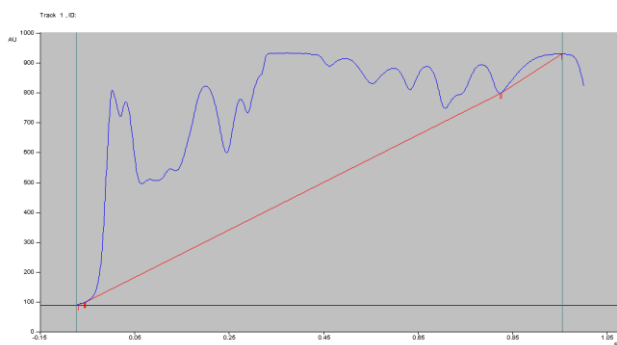
Table 2: LDH assay of *Piper nigrum* and *P longum* in methanol and chloroform extract

Sample Conc (mg/ml)	OD Values			
	<i>Piper nigrum</i>		<i>Piper longum</i>	
	Methanol	Chloroform	Methanol	Chloroform
Control	3.20	3.20	3.20	3.20
100	2.80	2.60	2.20	2.92
200	2.45	2.10	1.80	2.84
300	1.20	1.20	1.20	2.65
400	0.84	0.80	1.00	2.20
500	0.62	0.60	0.60	1.80

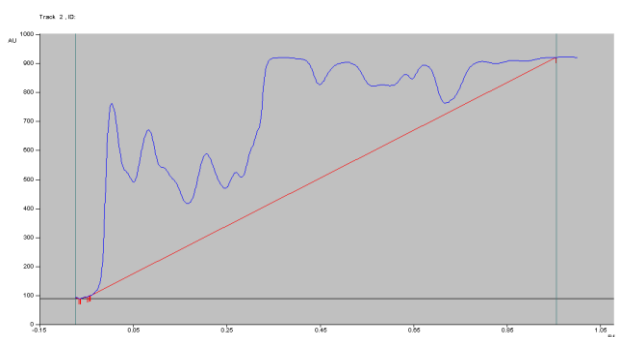
Previous studies using standard peperine in HPTLC^{28, 29} were standardised and confirmed that Rf value of standard piperine is 0.54 at 254 nm and 0.51 at 366 nm. The present HPTLC study results given above were compared with these results and confirmed the presence of piperine at 254 nm and 366 nm. Compared to 254 nm more precise grey to blue color bands were observed at 366 nm. By comparison, intensity of peak revealed that concentration of peperine in extracts may range from 0.104-0.34 % dry weight. So the extraction and purification methods were validated. Apart from peperine, presence of other related alkaloids, tannins

were confirmed by the presence of red, yellow and green color bands. Results also supported the highest percentage of extraction of peperine by methanol or chloroform. It was reported that dried fruit of *P. nigrum* (Black pepper) contains piperine, piperidine, piperettine and piperanine.³⁰ From the above results the other alkaloids may piperidine, piperettine and piperanine. Previously it was reported that *P. longum* showed the presence of Piperlongumine, piperlonguminine, piperine and piplartine.³¹

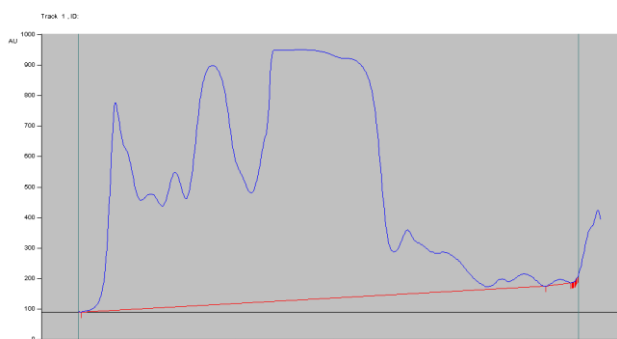




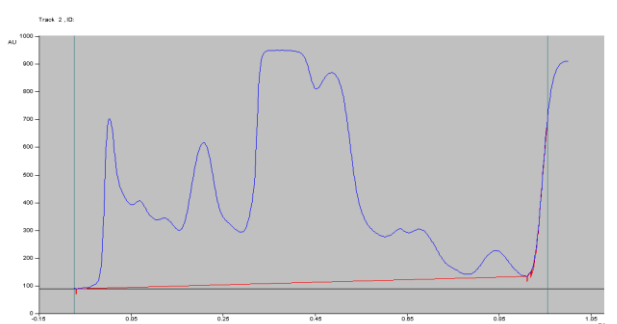
Graph 3: HPTLC of *Piper nigrum* at 254nm



Graph 4: HPTLC of *Piper longum* at 254nm



Graph 5: HPTLC of *Piper nigrum* at 366 nm



Graph 6: HPTLC of *Piper longum* at 366nm

CONCLUSION

Here we conclude that the chloroform extract of *P nigrum* have highest activity when compared to that of the other extracts of both *Piper nigrum* and *P.longum*. The reason for this may be due to the presence of higher content of alkaloids.

The MTT assay which we carried out was also supporting the above result by showing a lower lethal dosage for 50%

killing. Finally we conclude that the alkaloids present in the chloroform extract are responsible for the above specified activities. So the identification and structural elucidation of alkaloids are remaining in this work. The identification of new cytotoxic alkaloids will be a boon for the entire pharma industry.

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