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Research Article

EVALUATION OF BACTERICIDAL AND ANTICANCER PROPERTIES OF FRUITS OF *PIPER LONGUM.*

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ABSTRACT

The resistance of bacteria against the traditional antibiotics needs urgent attention and thus necessitates for the development of the new drug molecule against bacterial infections. The whole study focused on investigating the therapeutic and medicinal properties of the fruits of *Piper longum.* The antioxidant properties of the plant extracts was determined using FRAP method. The anticancer activity of the different extracts of fruits of *Piper longum* on human lung epithelial adenocarcinoma cell line (HCC-827) has been assessed in vitro using(4,3 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT assay). The potency of plant extracts to inhibit the cancerous growth was recorded in terms of decrease in viable cell count as compared to the control value. The inhibition of the growth of human lung epithelial adenocarcinoma cell line (HCC-827) has been found to be dose dependent. Phalloidin staining of the control and treated cells were performed to check the changes in the structure of cytoskeleton which appeared to be distorted and disorganized in control as compared to treated cancerous cells. In our study we have focused on working on the causes and search for herbal medicine which has less or no side-effects, is cost effective and reliable source of treatment.

Keywords: *Piper longum,* Invitro, Antioxidant, Human lung epithelial adenocarcinoma cell line (HCC-827), MTT, Phalloidin**,** FRAP.

INTRODUCTION

Prevalence of multi drug resistance phenomenon among human pathogens against commonly used antibiotics has led to the search for new plant-based therapeutics as a cure. Plants have been explored for the presence of active ingredients and the strong inhibitory activity which promoted its use in various herbal preparations. According to a report by World Health Organization (WHO), interest has been increased towards the use of herbal medicines in both developing and industrialized countries. A large percentage of people residing in developing countries lack opportunities to use essential medicines and the availability of safe and cost-effective plant based medicines could provide more ways to improve health care in underdeveloped nations. WHO launched its first traditional medicine strategy in 2002 (1). Plants have been the source of therapeutic agents for thousands of years and many potential modern drugs have been isolated from this natural source, which is based on their traditional medicinal values (2). These plants effectively cure various diseases, including the infection caused by microbes in various forms, powder, crude extracts, decoction or infusion. The importance of traditional therapeutics has gained importance in today's world and is noticeable. (3). Exploration of the biological properties of traditionally used medicinal plants to protect against the diseases caused by microbes is still required. (4) In order to analyze and promote the usage of plant based medicine as a new therapeutic agent, it is necessary to have knowledge of the bioprotective properties of medicinal plants, with a folklore reputation in a more effective way (5, 6, 7). The extensive use of antibiotics has lead to the inefficiency of current antimicrobials to control some bacterial diseases due to the prevalence of resistance against antibiotics (8). Natural constituents can be derived from any plant parts, which are naturally toxic to bacteria (9, 10). The pharmacological actions of plants vary in every plant species or group has been proved because of the presence of variation in secondary products. (11) Variety of phytosignatures like alkaloids, saponins flavonoids, cyanogenic glycosides, phenolic compounds, tannins and lignins are synthesized by plants and stored in their cells (12). *Piper,* belonging to the family Piperaceae is among the important medicinal plants used for its medicinal values (13, 14). Habitat of *Piper longum* L. (Piperaceae), common name "long pepper", is tropical and subtropical regions of the world, including the Indian subcontinent, Sri Lanka, Middle Eastern countries and the America. The plant has been used in traditional medicine as well as in the Ayurvedic system against various disorders and infections. Unripe, dried fruits are used as an alternative to tonic. Various plant preparations like decoction of young fruits and roots are used for treating chronic bronchitis, cough, and cold also used as antidote in snake biting and scorpion sting (15, 16, and 17). A use of combination of fruits of *Piper longum*, seeds of *Emblica ribes* and borax powder has already been cited in Ayurveda as contraceptive. (18).

MATERIALS AND METHODS

Plant collection

The dried mature fruits of *Piper longum* were obtained from local market of Dehradun, India.

Chemicals and Reagents

2, 4, 6-tripyidyl-s-triazine (TPTZ), Mueller Hinton Broth were purchased from Hi-Media (Mumbai, India), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, 98%) reagent, Fetal bovine serum (FBS) and streptomycin-penicillin antibiotic solutions were purchased from Sigma Aldrich, South Korea. The chemicals and reagents used for the study are of pure grade. The human lung epithelial adenocarcinoma cell lines (HCC- 827) were procured from Korean cell bank, South Korea.

Preparation of plant extracts

25g of powder was taken as a thimble charge and organic (ethyl acetate, methanol) and water extraction was done in succession using soxhlet extraction method **(19).** All the extracts were made solvent free and concentrated using rotary evaporator and preserved at 4°C in airtight bottle until further use.

Antimicrobial Activity

Agar well diffusion method

Antibacterial activities of all the extracts (ethyl acetate, methanol, aqueous) of plant were determined by agar well diffusion method. The extracts were dissolved in DMSO (dimethylsulphoxide). The microbial lyophilized cultures were revived at 37^0C for 18h in a broth medium and the culture was adjusted to 5 x 10^5 cfu/ml in accordance with the McFarland Turbidity standards **(20).** The 20µl of the culture was spread on Muller Hinton agar plates and wells of 9mm diameter were punched into the agar plates. 100µl of the extracts concentration (0.5mg/100µl and 1mg/100µl) were used for determination of ZOI (Zone of Inhibition). The plates were incubated at 37^0C for 18h – 24h. Commercial antibiotic (Gentamicin) and

DMSO was used as positive and negative control respectively. The test was performed in triplicates and the final results were presented as the mean zone of inhibition.

Broth Dilution MIC tests (NCCLS, 1993)

The Minimal Inhibitory Concentration (MIC) of the plant extracts was determined by macro broth dilution assay. On the basis of the results obtained from Agar well diffusion method (ZOI) two-fold serial dilutions of all the extracts were prepared in well plates with Muller-Hinton Broth (Hi-media, Mumbai, India) as diluents. 20µl of test microorganisms of the standard concentration (5 x 10^5 cfu/ml) was inoculated in the each dilution. Two-fold serial dilution of DMSO and Gentamicin was used as experimental negative and positive control respectively. The plates were incubated at 37ºC for 24hours. The lowest concentration at which the extract or standard drug showed no visible growth (turbidity) was taken as the MIC. 20µl of the MIC test broth tube solutions were spread over MHA plates and incubated for 18-24h at 37ºC. The plates showing no single bacterial growth, the dilution was considered as MBC (Minimum Bactericidal Count) concentration of the extract that is bactericidal in nature. The test was performed in triplicates and its mean MIC and MBC values were calculated.

Antioxidant power (Ferric Reducing Ability of Plant)

The FRAP assay was performed in accordance to the standard method (**22**). The stock solution of various extracts of 2.5mg/ml concentration was prepared in DMSO. 10µl-100µl of extract was mixed with 1.5ml of FRAP reagent and the volume was adjusted to 5ml with distilled water. The tubes were incubated at 37ºC for 15minutes and absorbance was noted at 593nm.

Anticancer Activity of *P.longum*

The human lung epithelial adenocarcinoma-HCC-827 cells were cultured and maintained in 90% DMEM (Dulbecco's Modified Eagle's Medium) substituted with 10% Foetal Bovine serum and 1% antibiotic for 24h. The media was then removed and the cell layer was washed with phosphate buffer saline PBS (0.1M pH7.0) to remove the traces of media. Later, 500 µl of trypsin-EDTA was added to the culture flask to remove the adherent cell layer from the flask. After 5min, 2ml of the media was added and single cells were collected. The cells were counted on the haemocytometer to get the exact viability and cell count for the experiments. 1 X 10^5 cells of the human lung epithelial adenocarcinoma-HCC-827 were used for the anticancer study of extracts of *P.longum*.

Cell Viability Assay

The viability of the cells was assessed by MTT (3,4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) assay (23). 1 X 10^5 cells (The human lung epithelial adenocarcinoma-HCC-827) were incubated in DMEM containing extracts of various concentrations (10µg/ml, 50µg/ml, and 100µg/ml) at 37ºC. The metabolic activity of each concentration was assessed using MTT assay at 570nm after 24h and 48h.

Image analysis

Phalloidin staining of the control and treated cells was performed to check the changes in the structure of cytoskeleton of the cancerous cells. Cells were fixed in 4% paraformaldheyde for 10 min, and were then permeabilized with 0.1% Triton-X 100 for 5min and after each step thorough rinsing with 0.01M PBS at room temperature (in LAF) was performed. The working solution of FITC labeled Phalloidin stain was made up in 1:200 dilutions with 1% BSA and cells were incubated for 15 min before imaging. The morphology of the cell cytoskeleton was observed under microscope (Nikon, TE-2000 U) (24).

RESULTS AND DISCUSSION

Antibacterial activity

Extracts of the fruits of *P. longum* were subjected for antibacterial study. *Streptococcus mutans* was found to be most resistant microbe as extracts (S1, S2 and S3) of *P.longum* could not inhibit the bacterial growth. S2 extract was found to possess strong inhibitory activity against *Staphylococcus aureus,* but moderately inhibiting the growth of *S.pyogenes* and *Streptococcus pneumoniae* and least effective against *K. pneumoniae*. *S.aureus* was potentially inhibited by S1 extract of the plant having the maximum ZOI followed by *S.pyogenes*, *S.pneumoniae* and *K.pneumoniae.* S3 extract effectively inhibited *S.aureus* in comparison with *S.pyogenes*, *S.pneumoniae* and *K.pneumoniae.* MIC is the lowest concentration of the test sample or drug at which it shows the highest inhibitory activity against microorganisms. The extracts that showed high efficacy against microorganisms were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2) (25; 26).

The extracts in reference showed varied MIC values (table 6, 7, 8) whereas MBC is the bactericidal concentration of the extracts or drug, which kills the microbial population up to 99.9%. From the MBC data obtained it is observed that MBC values ranged from 0.0156-0.0625mg/ml (table 6, 7, 8). The MIC index (MIC/MBC) was performed to determine whether an extract is bactericidal (MIC/MBC <4) or bacteriostatic (MIC/MBC >4) in nature. MIC index values of greater than 4 and less than 32 are considered as bacteriostatic.

The S1, S2 and S3 extracts obtained from the fruits of *P.longum* were found to be strongly active against the selected human microbes while inactive against *Streptococcus mutans* (tables 3, 4, 5). Bactericidal effects of the phytoconstituents may be of four types: 1. They inhibit cell wall synthesis 2. They stop microbial protein and nucleic acid synthesis 3. They disrupt microbial membrane structure and function 4. They block metabolic pathways through inhibition of key enzymes (27; 28). The ZOI and MIC values of different extracts of *P.longum* have exhibited strong inhibitory activity against the selected microbes, which cause various types of respiratory disorders in body like pneumoniae, bronchitis, cough, sore throat and in chronic form leading to lung cancer. Our data obtained on antimicrobial activity against various microbes can be compared with the other study like the isolates of fruits of *Piper longum* showing potent antimicrobial activity against Gram-positive bacteria and Gram-negative bacteria (29). It can be concluded that plant extracts with low MIC and MBC values may serve as sources for compounds with therapeutic potency. The fruits of *P.longum* are known to possess volatile oil, starch, protein and alkaloids, saponins, carbohydrates and amygdalin etc (30).

Table 1: The yield, yield% and physical properties of *Piper longum*

S. No	Solvent Used	Yield (g/500ml)	Yield %	Color	State
1.	Ethyl Acetate (S1)	0.74	2.96	Black	Viscous
2.	Methanol (S2)	10.17	40.68	Black	Viscous
3.	Water (S3)	3.79	15.16	Brown	Solid

Table 2: The ZOI in mm of Positive Control (Gentamicin) against various bacteria

S. No	Micro-Organisms	Zone of Inhibition (mm) Gentamicin	
		0.5mg	1.0mg
1.	*Klebsiella pneumoniae*	28	29
2.	*Staphylococcus aureus*	31	32
3.	*Streptococcus mutans*	27	28
4.	*Streptococcus pyogenes*	31	32
5.	*Streptococcus pneumoniae*	29	30

Table 3: The ZOI in mm of S1 extract of *P.longum* against various bacteria

S. No	Micro-Organisms	Zone of Inhibition (mm)	
		0.5mg	1.0mg
1.	*Klebsiella pneumoniae*	13	17
2.	*Staphylococcus aureus*	20	22
3.	*Streptococcus mutans*	Nil	Nil
4.	*Streptococcus pyogenes*	16	19
5.	*Streptococcus pneumoniae*	15	18

Table 4: The ZOI in mm of S2 extract of *P.longum* against various bacteria

S. No	Micro-Organisms	Zone of Inhibition (mm)	
		0.5mg	1.0mg
1.	*Klebsiella pneumoniae*	14	19
2.	*Staphylococcus aureus*	25	27
3.	*Streptococcus mutans*	Nil	Nil
4.	*Streptococcus pyogenes*	20	22
5.	*Streptococcus pneumoniae*	17	21

Table 5: The ZOI in mm of S3 extract *P.longum* against various bacteria

S. No	Micro-Organisms	Zone of Inhibition (mm)	
		0.5mg	1.0mg
1.	*Klebsiella pneumoniae*	8	15
2.	*Staphylococcus aureus*	25	27
3.	*Streptococcus mutans*	Nil	Nil
4.	*Streptococcus pyogenes*	16	21
5.	*Streptococcus pneumoniae*	12	14

MIC and MBC: Broth dilution assay

Table 6: The MIC, MBC and MIC Index values of S1 extract against different pathogens

Organism	Incubation period	MIC (mg/ml)				
		Range	MIC (control)	MIC (extract)	MBC(extract)	MIC Index
Stapylococcus aureus	37^{0}C	0.5- 0.0156	0.0156	0.0625	0.125	2
Streptococcus pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.0625	0.125	2
Streptococcus pyogenes	37^{0}C	0.5- 0.0156	0.0156	0.0156	0.0312	2
Klebsiella pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.0156	0.0312	2

Table 7: The MIC, MBC and MIC Index values of S2 extract against different pathogens

Organism	Incubation period	MIC (mg/ml)				
		Range	MIC (control)	MIC (extract)	MBC(extract)	MIC Index
Stapylococcus aureus	37^{0}C	0.5- 0.0156	0.0156	0.0156	0.0132	2
Streptococcus pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.0132	0.0625	2
Streptococcus pyogenes	37^{0}C	0.5- 0.0156	0.0156	0.0156	0.0312	2
Klebsiella pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.0156	0.0312	2

Table 8: The MIC, MBC and MIC Index values of S3 extract against different pathogens

Organism	Incubation period	MIC (mg/ml)				
		Range	MIC (control)	MIC (extract)	MBC(extract)	MIC Index
Stapylococcus aureus	37^{0}C	0.5- 0.0156	0.0156	0.0625	0.125	2
Streptococcus pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.0625	0.125	2
Streptococcus pyogenes	37^{0}C	0.5- 0.0156	0.0156	0.0312	0.0625	2
Klebsiella pneumoniae	37^{0}C	0.5- 0.0156	0.0156	0.125	0.25	2

Antioxidant power

Radical scavenging activity was estimated by FRAP assay and the results were compared with that of Gallic acid. Gallic acid (Merck) being in the pure form had better antioxidant power than the extracts, which has chemicals either in combined or conjugated form affecting the antioxidant power of the plants. The Aqueous extract of the fruits of *Piper longum* exhibited strong free radical scavenging properties followed by methanol and ethyl acetate extract. Antioxidant power was found to be concentration dependent. The results are represented as graph plotting the concentration of the extracts in X-axis and antioxidant power in µM/l in Y-axis. Fig 1. Medicinal plants have good antioxidant and immunomodulatory properties that lead to its anticancer properties **(31; 32).** Antioxidants neutralize free radicals as the natural by-product of normal cell processes. S1 (ethyl acetate), S2 (methanol), S3 (water) extract had been found possessing the ferric reducing ability expressed as FRAP value or antioxidant power.

The antioxidant properties of these extracts were concentration dependent. In all cases the appreciation of FRAP value was noticed with the increase of extract concentration. The data clearly showed that the active components present in S3 extract (23.58µM/l) had the highest free radical scavenging ability as compared to S2 (8.00 to12.00 µM/l) and S1 (4.00 to 9.41 µM/l) extracts. Aqueous extracts of the fruits of *Piper longum* can be further explored to understand the various phytosignatures present in it which can be used as the cure for the ailments caused by the overproduction of free radicals in the body like Arthritis, Asthma, Heart diseases, respiratory disorders, Parkinson, Alzheimer's, various types of cancers etc.

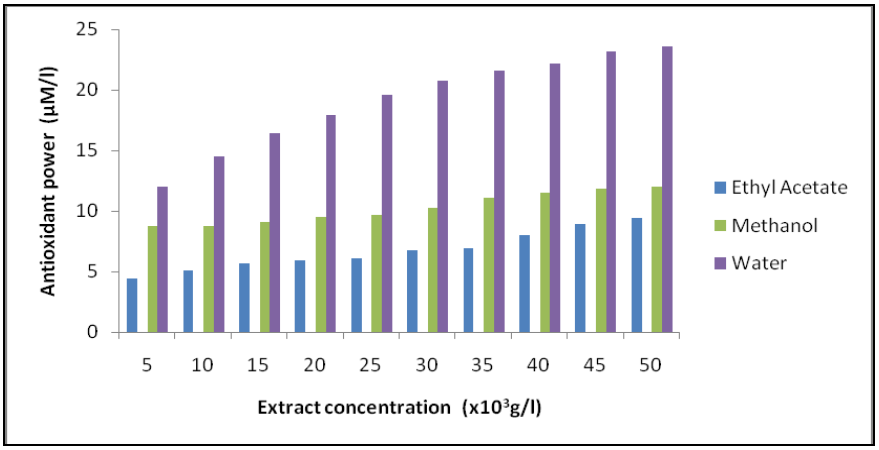

Fig. 1: Demonstrates the antioxidant power (µM/l) of various (S1, S2 and S3) extracts of *P.longum* at increasing concentration (g/l)

Anticancer Study of *P.longum*

Cell Viability assay

Herbal products are used as constituents in the formulations of more than 50% of modern drugs, which possess pronounced antitumour activities without harmful effects **(33).** Plant based therapeutics are preferred due to the fact that steroid based drugs are loaded with adverse effects. The antimicrobial activity was conducted against a wide range of human pathogens responsible for causing various types of respiratory afflictions that may lead to the occurrence of physiological stress in the cells. Damage caused to the DNA and other molecules by the uncontrolled production of free radicals leads to the diseases like cancer. The potential antimicrobial activity and antioxidant properties of *P.longum* have led us to explore its anticancer properties against human lung epithelial adenocarcinoma cell lines (HCC-827).

The HCC-827 cells were used for anticancer study of fruits of different extracts of *P.longum*. The effect of different concentrations of S1, S2 and S3 extracts on cell viability of cancer cells was assessed by MTT assay. Results clearly showed that different solvent extracts of *P.longum* exhibited inhibitory effect on lung cancer cells. Inhibition of the growth of carcinoma cells was found to be concentration dependent (table 10, 11, 12). The different extracts of fruits of *Piper longum* were tested for its antitumor activity on human lung epithelial adenocarcinoma cell line (HCC-827) using 3-(4, 5-dimethyl thiazø2yl)-2,5-diphenyl tetrazolium bromide (MTT assay), which is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazon product. The potency of each plant extract to inhibit the cancerous growth was recorded in terms of decrease in viable cell count as compared to the control value. The inhibition of the growth of human lung epithelial adenocarcinoma cell line (HCC-827) has been found to be dose dependent.

The S1, S2 and S3 extracts of the plant exhibited moderate inhibitory activity. The human lung cancer cell line ($1x10^5$ cell count) in control exhibited an appreciation of 57% ($1.57x10^5$ cells) and 117% ($2.17x10^5$ cells) after 24h and 48h incubation time respectively. Bright Phase Microscopy shows reduction in number of cancerous growth as shown in Fig 2 (Panel A untreated and Panel B treated cells). The changes in the cytoskeleton shape and reduction in the number of cells is clearly depicted by the Phalloidin staining as shown in fig 3 (Panel A and B). The cytoskeleton of untreated cells (Panel A, Fig 3) looks distorted and number of cancerous cells is more in-comparison to treated cells wherein the cytoskeleton is better organized and even the number of cancer cells are reduced when treated with the extracts (Panel B, Fig 3). The data presented on anticancer studies of fruits of *Piper longum* shows that S2 extract could effectively inhibit the lung cancer cell lines at a concentration of 100µg/ml while in the case of S1 and S3 extract an increase in the dose would show significant inhibition of cancerous cell lines. Thus the anticancer property of *P.longum* can be improved by further increasing the concentration against the human lung epithelial adenocarcinoma cell line (HCC-827).

Table 10: The effect of S1 extract on HCC-827 cells after 24h and 48h

S. No	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$1.05 x10^5$	$2.17x10^5$	$1.00 x10^5$
2.	50	$1.57x10^5$	$1.00 x10^5$	$2.17x10^5$	$0.95 x10^5$
3.	100	$1.57x10^5$	$0.95 x10^5$	$2.17x10^5$	$0.80 x10^5$

Table 11: The effect of S2 extract on HCC-827 cell lines after 24h and 48h

S. No	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$1.14 x10^5$	$2.17x10^5$	$1.10 x10^5$
2.	50	$1.57x10^5$	$0.9 x10^5$	$2.17x10^5$	$0.9 x10^5$
3.	100	$1.57x10^5$	$0.5 x10^5$	$2.17x10^5$	$0.46 x10^5$

Table 12: The effect of S3 extract on HCC-827 cell lines after 24h and 48h

S. No	Concentration (µg/ml)	Control (24h)	Treated (24h)	Control (48h)	Treated (48h)
1.	10	$1.57x10^5$	$1.32 x10^5$	$2.17x10^5$	$1.28x10^5$
2.	50	$1.57x10^5$	$1.15 x10^5$	$2.17x10^5$	$1.10 x10^5$
3.	100	$1.57x10^5$	$0.88 x10^5$	$2.17x10^5$	$0.84 x10^5$

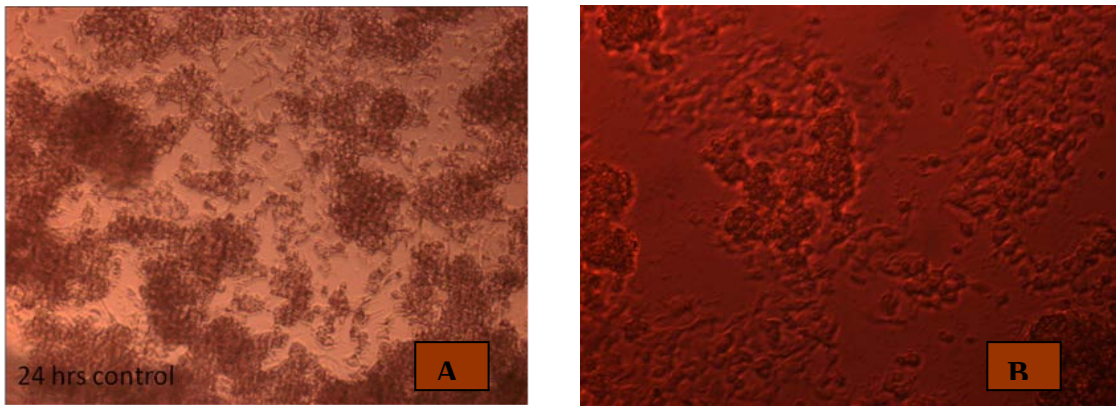

Fig. 2: Bright phase microscopy of Lung cancer cells after 24 h, Panel A shows the untreated cells (Control), Panel B represents treated cells

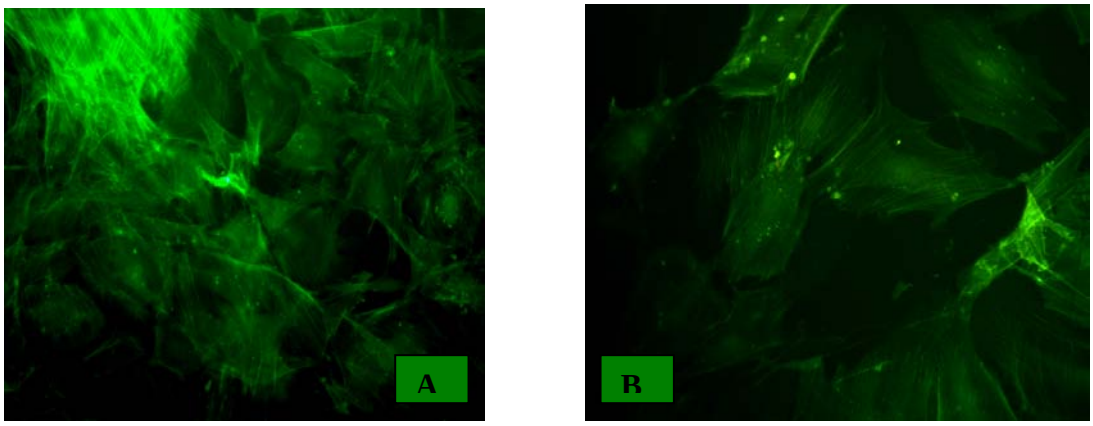

Fig. 3: Phalloidin stained control cells (Panel A) and treated cells (Panel B) shows changes in cytoskeleton morphology and reduction in number of cells after 48h of treatment with extracts

The study clearly demonstrated that the extracts of fruits of *Piper longum* has the anti-oxidative, anti-microbial and anti-apoptotic activity due to the possession of pharmacologically and medicinally significant phytochemicals suggesting its therapeutic usefulness to cure respiratory afflictions caused either by microbial activity or physiological stress caused by the overproduction of free radicals which may lead to the cancer.

CONCLUSION

In our study we have focused on working on the causes and search for herbal medicine, which has less or no side effects, is cost effective and reliable source of treatment. It is of great significance to exploit potent anticancer drugs from medicinal plants. The potentiality in the use of fruits of *Piper longum* holds a great importance in the plant-based formulations as a cure to variety of respiratory disorders and occurrence of cancer due to chronic respiratory infections and overproduction of free radicals. Treatment of respiratory disorders with antibiotics is common but controversial as their use has only moderate benefit weighted against potential side effects, increased resistance, and cost of treatment is a self-limiting condition. Extracts of fruits of *Piper longum* has antimicrobial, antioxidant and anticancer properties that can further be explored to obtain and use its formulations as a cure for various ailments.

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