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Research Article……!!!

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FT-IR ANALYSIS AND IN VITRO CYTOTOXICITY ASSAY OF METHANOLIC EXTRACT OF ROOTS OF *ALSTONIA SCHOLARIS*

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ABSTRACT

The present study was aimed to investigate the functional groups involved in extract-pathogen interaction and the cytotoxic effect of the methanolic extract of roots of *Alstonia scholaris* on cell lines. In order to draw the comparison and shift in the spectrum, the FT-IR of dried powdered methanolic root extract was done followed by the *E.coli* cell pellet before and after being treated with methanolic root extract. Among the various functional groups involved, Alkanes, Alkenes, Nitro compounds, Alkynes, Phenols, Carboxylic acids , Amines played a major role in interaction of extract with *E.coli* cells. MTT assay performed to study the cytotoxic effect of methanolic extract on mouse fibroblast cell line showed that the cell viability changed as a function of concentration of the methanolic extract and time - upto 72 hrs. The cell viability decreased with the increase in the concentration of the methanolic extract of the roots.

INTRODUCTION:

The rich Indian biodiversity of flora has been the source of herbs against various ailments like diabetes and heart diseases. Strong traditional knowledge of ayurveda has been used for centuries. Apocynaceae is a family of medicinal plants which includes genera like *Rauvolfia canescens*(Rauwolscine), *Alstonia boonei*(Alstonine), *Rauwolfia serpentine*(Reserpine) and *Vinca rosea*(Vinblastine) which have been used to isolate the drugs used in treatment of various diseases like schizophrenia and cancer[1]. Because of the rich knowledge of medicinal plants, traditional medicine has been integrated in the health care systems. In India and China use of traditional herbal preparations accounts for almost 40-50% of the total medicinal consumption which is increasing day by day due to the growing concern over the side effects of the modern chemical based formulations.

Many workers proposed that different functional groups which are involved in biosorption of heavy metals are the dominant metal binding sites which are present on the cell wall. The functional groups include phosphate, carboxyl, amine and amide etc [2,3,4,5]. Cell wall composition differs among different groups of microorganisms, viz. algae, bacteria, cyanobacteria and fungi which can bring about significant change in the pattern of the type and amount of metal ions that binds to their cell wall. The principle compositions of cell walls of bacteria consist of peptidoglycans which are made up of linear chains of the disaccharide N-acetylglucosamine-β 1, 4-N-acetylmuramic acid with peptide chains. In some strains of *E.coli*, peptidoglycan was found to be a potent binder of the metals tested and carboxylate groups were the principal components which were involved in metal binding[6,7] . Owing to the work that has been done on various plant parts of *Alstonia scholaris*[8,9], it is now easier to understand the mechanism of action of plant extracts on various organism and which functional groups are involved in the interaction of microbe and the extract. The functional groups and their corresponding properties owe it to several compounds like alkaloids, steroids etc [10]. Previously no significant work was done on roots of *Alstonia scholaris* to study which functional groups are involved to impart its corresponding properties or to evaluate its pharmaceutical importance. There is no single bioassay method to determine all the medicinal biological properties. Therefore in this paper, we have also reported the cytotoxic properties of the methanolic extracts of roots of *Alstonia scholaris by* MTT assay[11].

MATERIAL AND METHODS:

Collection and processing of plant materials:

The fresh plant parts of *Alstonia scholaris* were separated , dried and pulverized to obtain fine powder which were then subjected to solvent extraction. The phytochemical investigation and antimicrobial screening of the various plant extracts were done. The solvent extract with best antimicrobial activity was chosen for the spectral analysis and further study on the cytotoxicity on cell lines.

Instrumental Analysis

Fourier Transform Infrared Spectroscopy (FT-IR)

E.coli cells were grown in nutrient broth (2 g/L) both in the absence and presence of methanolic root extracts of *Alstonia scholaris*. The cells were harvested at the end of 14 hrs. The pellet was dried in hot air oven at 45° C for 24 hrs. Dried powder of methanolic root extract was also considered for instrumental analysis. Samples were analysed in Central Instrumentation Facility, Pondicherry University. For the FT-IR study, approximately 20 mg of finely ground bacterial cell pellet and dried powder of methanolic root extract was encapsulated in 1000 mg of KBr pellet (Sigma, USA) in order to prepare translucent sample disks. The FT-IR spectra of the *E.coli* cell pellet (before and after interaction with methanolic root extract) along with dried powdered methanolic root extract were recorded.

Cytotoxicity Studies On Mouse Fibroblast Cells (MTT Assay)

Cell Line

We used L929, an adherent type mouse fibroblast cell line, approved by ISO 10993 for cytotoxicity assessment of biomedical devices and materials which are supposed to come in contact with human subject directly or through indirect means. L929 cells used in all the experiments of the present investigations were originally procured form NCCS, Pune and since then has been maintained at *In Vitro* Toxicology Laboratory, IITR, Lucknow.

Initiation of Culture from Frozen Cells

Frozen vials of L929 cells were taken out from liquid nitrogen and immediately transferred to a pre warmed (37°C) water bath for thawing. The cells were then transferred to sterile centrifuge tube containing complete medium (MEM (Minimum Essential Medium) + 10% FBS (Foetal Bovine Serum) + Antibiotic-Antimycotic solution (1.5 ml/100 ml of medium)) and centrifuged at 600 rpm for 10 minutes at

room temperature. The supernatant was discarded & the loosely bound pellet was suspended in 5 ml of complete medium & appropriate numbers of cells were seeded in T-75 tissue culture flask and incubated in a CO_2 incubator (5% CO_2 - 95% air at high humidity) at 37°C. Initially the medium was changed post 24 hr. and subsequently at an interval of 2-3 days till sufficient growth was observed.

Cell Viability Test (Dye Exclusion Test)

Well-mixed cell suspension (0.8 ml) was added to a test tube already containing 0.2 ml of trypan blue (0.4%) and the contents mixed by gentle shaking. Without any time gap in between, 20 µl of dye-cell mixture was placed on the edge of both the chambers of the haemocytometer prefixed with the cover slip and allowed the cell suspension to fill the chambers by capillary action. The counting of unstained (viable cells) and stained cells (nonviable cells) in the four large corner squares in both counting chambers was made using a 10X microscope objective. The percent cell viability was calculated by deducting the number of all stained cells from total number of cells counted (stained cells and unstained cells) in the haemocytometer. Only the batches with greater than 95% cell viability were used in further experimentation.

Plating of L929 Cells

Cells in the flasks showing high confluence were harvested by addition of trypsin and centrifugation at 600 rpm for 5 minutes. The pellet was re-suspended in fresh culture medium and clumps were broken if any by a tip of 20 µl by up and down several times. Cells were then counted with the help of coulter electronic cell and particle counter and plated in the frequency of 10,000 cells per well in 100µl of culture medium.

Percent Cell Viability by MTT Assay

Cells (10,000 in number) were seeded in 96-well tissue culture plates and incubated in the CO_2 incubator for 24 hr. at 37°C prior to experiment for the proper attachment of the cells. Then the medium was aspirated & cells were exposed to different concentrations (0.005 to 1.0 mg/ml) of extracts prepared as discussed earlier for different periods of time i.e. 24, 48, 72 and 96 hr. Tetrazolium (10µl/well containing 100µl of cell suspension; 5mg/ml of stock in PBS) salt was added 4 hr. prior to completion of each incubation period. After incubation, the reaction mixture was carefully taken out to which 200 µl of DMSO was added to by pipetting up & down several times to facilitate rapid homogenization. After 10 minutes, the colour was read at 530 nm, using Multiwell microplate reader. The untreated sets were also

run parallel under identical conditions & served as basal control, whereas cells treated with manganese (10^{-4} M) served as positive control.

RESULT AND DISCUSSION

FT-IR Studies

Spectral analysis like FT-IR has been extensively used by earlier researchers [12, 13, 14] to yield valuable information regarding the chemical groups possessed by the biosorbents for identification of functional groups present on the biosorbent.

Several functional groups were found to be present in methanolic root extracts of *Alstonia scholaris* (Fig 1,Table 1). A peak of 3412.2 cm^{-1} indicated the presence of alcohols and phenols. Peaks of 2929.7 , 1609.4 cm^{-1}, 926.4 cm^{-1} indicated the presence of alkanes (C-H stretch) , carboxylic acids (O-H stretch) and amines (N-H bend) and alkenes (O-H bend,=C-H bend). Presence of aromatic and nitro compounds was indicated by the peaks 1442.8 and 1518.9 cm^{-1}. Alkyl halides (C-Cl stretch) were found to be present by the presence of peaks 554.5,632.1,767.0, 828.3 cm^{-1}. Peaks of 1110.3 ,1059.8 cm^{-1} and 1259.9 cm^{-1} indicated the presence of Aliphatic amines (C-N stretch),Alcohol, Carboxylic acids,Esters,Ethers (C-O stretch) and aromatic amines (C-N stretch). Peaks 828.3 and 767.0 cm^{-1} indicated alkenes, aromatic compounds and amines.

In the present study, the main effective functional groups on *E.coli* before being treated with methanolic root extracts of *Alstonia scholaris* have been identified by FTIR (Fig 2,Table 2). The band indicted at 3309.2 cm^{-1} were due to bounded hydroxyl (-OH), (-NH) groups, amides ,alkynes and phenols of the biomass. The peaks observed at 2958.0 and 2928.2 cm^{-1} could be assigned to the presence of carboxylic acids (O-H stretch) and alkanes (–C-H stretch) group of the biomass. The peaks at 1653.6 and 963.9 cm^{-1} indicated the presence of alkenes due –C=C– stretch and =C–H bend .Peak positions at 1232.5 and 1077.2 cm^{-1} indicated the presence of alcohols, carboxylic acids, esters, ethers, alkyl halides, aliphatic amines due to C–O stretch ,C–H wag (–CH_2X), C–N stretch. Presence of nitro compounds, aromatic compounds and alkanes were indicated by N–O asymmetric stretch and C–C stretch (in–ring),C–H bend due to the peaks 1540.4 and 1454.3 cm^{-1}. In case of *E.coli* cells treated with methanolic extracts of roots of *Alstonia scholaris* (Fig 3,Table 3), a major difference that was noted was the presence of the peak of 616 cm^{-1} which indicated the role of alkyl halides and alkynes in the interaction between the cells and extract. This was due to C–Cl stretch, C–H: C–H bend, C–Br stretch. A peak shift from 963.9 to 956.3 cm^{-1}((=C–H bend)) and 1653.6 to 1650.4 cm^{-1} (–C=C– stretch) indicated the involvement of alkenes. A shift in peak from 3300.9 to 3302.4 cm^{-1} and also from 2928.2 to 2926. cm^{-1} indicated the role of alcohols, phenols (O–

H stretch) , amines (N-H stretch),alkynes (C-H stretch),carboxylic acids (O-H stretch) and alkanes (C-H stretch) in the interaction between cells and extract. A peak shift from 1540.4 to 1538.6 cm^{-1} confirmed the role of nitro compounds in this case (N–O asymmetric stretch). A major shift from 1454.3 to 1403.9 cm^{-1} indicated the role of aromatic compounds and alkanes in the present interaction. A slight shift of peak from 1232.5 to 1232.2 cm^{-1} and 1077 to 1111.7 cm^{-1} indicated the role of Alcohols, carboxylic acids, esters, ethers, alkyl halides, aliphatic amines due to C–O stretch,C–H wag (–CH_2X), C–N stretch.

Table 1: FT-IR analysis of methanol extract of root of *Alstonia scholaris*.

Peaks(cm^{-1})	**Functional Groups**	**Remarks**
3412.2	OH-stretch, H-bonded	Alcohols, Phenols
2929.7	C-H stretch, OH-stretch	Alkanes, Carboxylic acids
1609.4	N-H bend	1˚ amines
1518.9	N-O asymmetric stretch	Nitro compounds
1442.8	C-C stretch(in – ring)	Aromatics
1259.9	C-N stretch, C-O stretch, C-H wag(-CH_2X)	Aromatic amines, Alcohol, Carboxylic acids, Esters, Ethers, Alkyl halides
1110.3	C-N stretch, C-O stretch	Aliphatic amines, Alcohol, Carboxylic acids, Esters, Ethers
1059.8	C-N stretch, C-O stretch	Aliphatic amines, Alcohol, Carboxylic acids, Esters, Ethers
926.4	O-H bend,=C-H bend	Carboxylic acids, Alkenes
828.3	C-Cl stretch, N-H wag, C-H “oop”,=C-H bend	Alkyl halides,1˚ and 2˚ amines, Aromatics, Alkenes
767.0	C-Cl stretch, N-H wag, C-H “oop”,=C-H bend	Alkyl halides,1˚ and 2˚ amines, Aromatics, Alkenes
632.1	C-Cl stretch,C-H bend,C-Br stretch	Alkyl halides, alkynes
554.5	C-Cl stretch, C-Br stretch	Alkyl halides

Table 2: FT-IR analysis of *Escherichia coli* cell biomass before being treated with methanol extract of root of *Alstonia scholaris*.

Peaks(cm^{-1})	Functional Groups	Remarks
3300.9	O–H stretch, H–bonded, N–H stretch, C–H stretch	Alcohols, phenols, 1°, 2° amines, amides, alkynes (terminal)
2958.0	O–H stretch, C–H stretch	Carboxylic acids, alkanes
2928.2	O–H stretch, C–H stretch	Carboxylic acids, alkanes
1653.6	–C=C– stretch	Alkenes
1540.4	N–O asymmetric stretch	Nitro compounds
1454.3	C–C stretch (in–ring),C–H bend	Aromatics, alkanes
1398.3	C–C stretch (in–ring)	Aromatics
1232.5	C–O stretch,C–H wag (–CH_2X), C–N stretch	Alcohols, carboxylic acids, esters, ethers, alkyl halides, aliphatic amines
1077.2	C–O stretch,C–N stretch	Alcohols, carboxylic acids, esters, ethers, aliphatic amines
963.9	=C–H bend	Alkenes

Table 3 : FT-IR analysis of *Escherichia coli* cell biomass after being treated with methanol extract of root of *Alstonia scholaris*.

Peaks(cm^{-1})	Functional Groups	Remarks
3302.4	O–H stretch, H–bonded, N–H stretch, C–H stretch	Alcohols, phenols, 1°, 2° amines, amides, alkynes (terminal)
2958.0	O–H stretch, C–H stretch	Carboxylic acids, alkanes
2926.1	O–H stretch, C–H stretch	Carboxylic acids, alkanes
2855.8	O–H stretch, C–H stretch	Carboxylic acids, alkanes
1650.4	–C=C– stretch	Alkenes
1538.6	N–O asymmetric stretch	Nitro compounds
1403.9	C–C stretch (in–ring)	Aromatics
1232.2	C–O stretch,C–H wag (–CH_2X), C–N stretch	Alcohols, carboxylic acids, esters, ethers, alkyl halides, aliphatic amines
1111.7	C–O stretch, C–N stretch	Alcohols, carboxylic acids, esters, ethers, aliphatic amines
956.3	=C–H bend	Alkenes
616.6	C–Cl stretch, C–H: C–H bend, C–Br stretch	Alkyl halides, alkynes ,alkyl halides

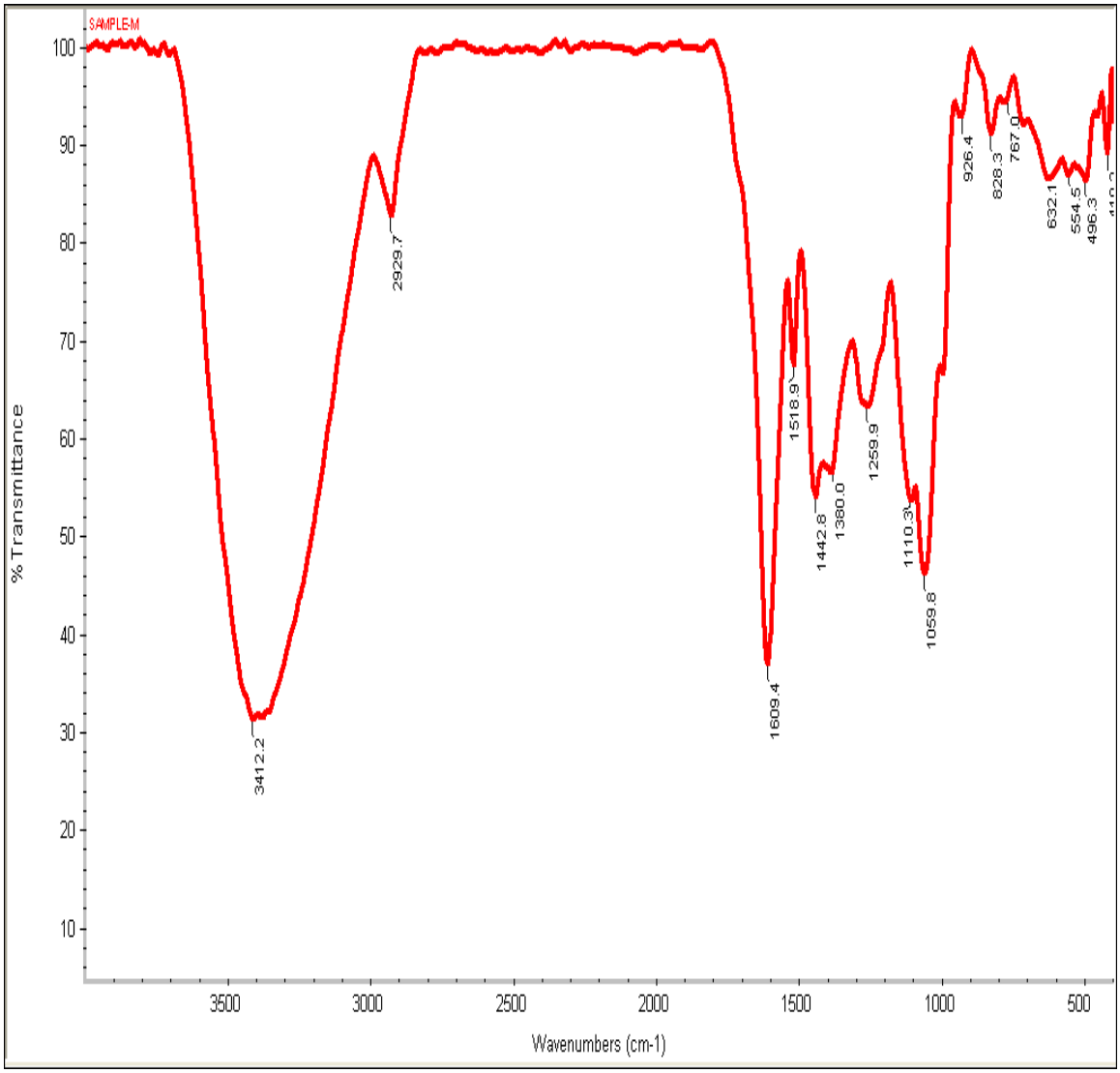

Fig 1: FT-IR spectrum of methanol extract of root of *Alstonia scholaris*.

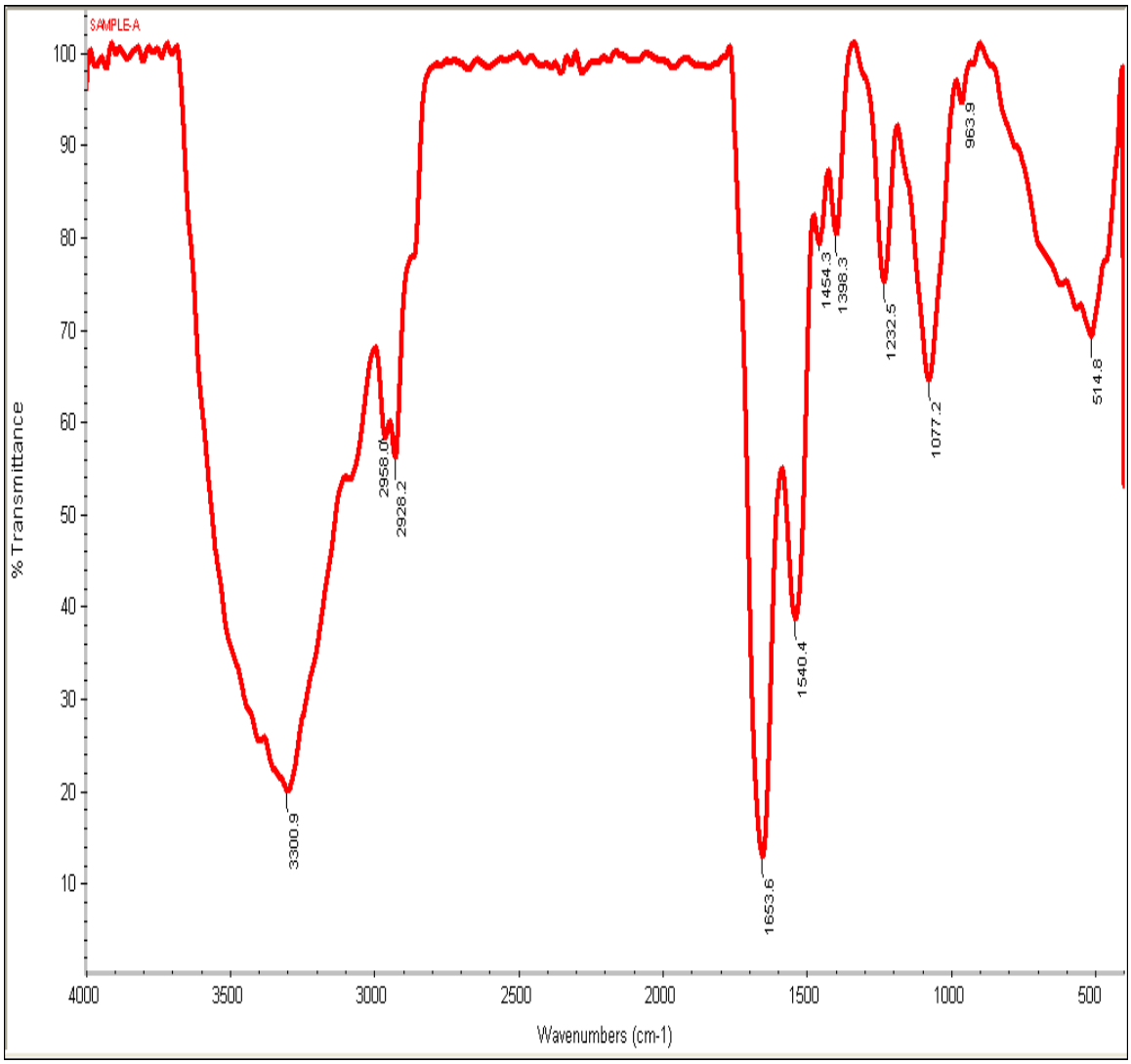

Fig 2: FT-IR spectrum of *Escherichia coli* cell biomass before being treated with methanol extract of root of *Alstonia scholaris*.

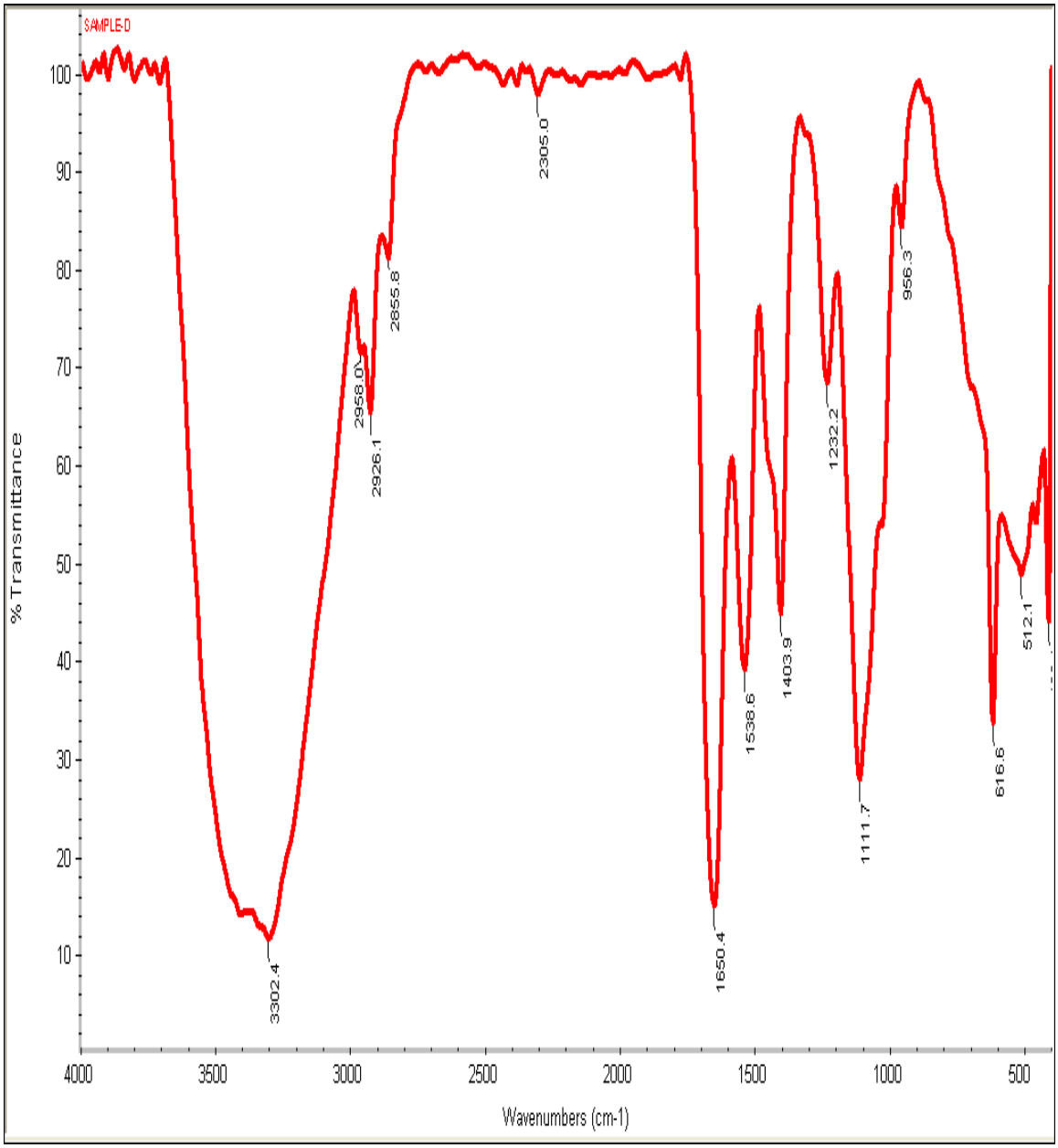

Fig 3: FT-IR spectrum of *Escherichia coli* cell biomass after being treated with methanol extract of root of *Alstonia scholaris*.

Cytotoxicity Studies On Mouse Fibroblast Cell Line (L929)

The data of the present study performed on mouse fibroblast cell line (L929) is presented in Fig 4, Table 4 which indicated that the viability of cell lines decreased with an increase in concentration of methanol extract (0.005 to 1.0mg/ml). As shown in Fig 4, the viability of cells was found to increase from 24 hrs to 72 hrs. A sudden decrease was observed after 72 hrs till 96 hrs. The cytotoxicity results of our study obtained by MTT assay showed less lethal cytotoxic effect on the mouse fibroblast cell line which was comparable to methanolic and ethanolic extract of some Bangladeshi plant and *Zingiber officinalis.*[15,16]

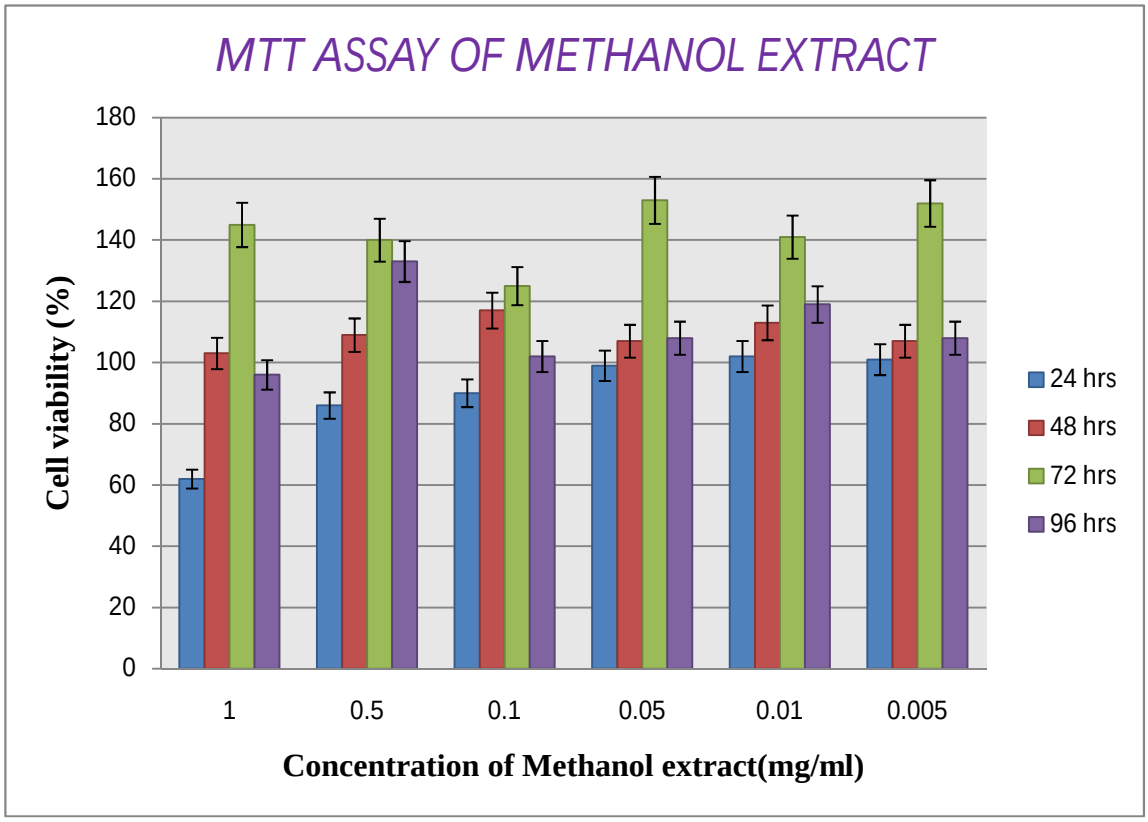

Fig 4: Effect of Methanol extract (0.005 to 1.0mg/ml) on cell viability of mouse fibroblast cells as a function of time (hr).

Table 4: Effect of various concentrations ranging from 0.005 to 1.0 mg/ml of methanol extract on cell viability of mouse fibroblast cells as a function of time (hr).

Treatment period (hr)	Concentration of extract (mg/ml)	Effect of Methanol extract on Cell Viability (%)
24	1.0	62
	0.5	86
	0.1	90
	0.05	99
	0.01	102
	0.005	101
48	1.0	103
	0.5	109
	0.1	117
	0.05	107
	0.01	113
	0.005	107
72	1.0	145
	0.5	140
	0.1	125
	0.05	153
	0.01	141
	0.005	152
96	1.0	96
	0.5	133
	0.1	102
	0.05	108
	0.01	119
	0.005	108

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CONCLUSIONS

Escherichia coli cell pellet was treated with Methanol extract of root of *Alstonia scholaris* .The untreated pellet, treated pellet and dried methanol extract powder were analysed by FT-IR. The results showed that alkanes, alkenes, nitro compounds, alkynes, phenols, carboxylic acids and amines played a major role in interaction of extract with *Escherichia coli* cells. The sample was found to cause lethal cytotoxic effect at a dose of 1mg/ml when the exposure period was 24 hrs. But at longer exposure periods i.e., 48, 72 and 96 hrs the extracts showed no cytotoxic effect.. However, other lower concentrations were found to be non-cytotoxic in L929 mouse fibroblast cells under experimental conditions. The values above 100% might be associated with the xenoestrogenic potential of the extract used in the study. More specific tests are needed to reach any firm conclusion regarding the xenoestrogenic potential of the compounds. Before this plant can be recommended for biopharmaceutical use, the detailed bioassay guided experiments need to be done in order to obtain its potent bioactive principles that can be used for treatment of various disorders and possibly cancer too.

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