

BIOCHEMICAL CHARACTERIZATION OF TRIPHALA EXTRACTS FOR DEVELOPING POTENTIAL HERBAL DRUG FORMULATION FOR OCULAR DISEASES

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ABSTRACT

In recent years, multiple drug resistance has been developed due to indiscriminate use of existing drugs in the treatment of infectious diseases. The major thrust is to establish alternative antimicrobial agent in order to treat microbial infections with less or no toxicity and less or negligible side effects. The herbal medicines have shown potential to overcome the limitation associated with conventional drugs. One such herbal drug is triphala (formula consists of three myrobalans) that possesses potential pharmaceutical activities and used in several ayurvedic formulations. The present work focused on study of triphala in ophthalmic disorder management; to reduce growth of microorganisms, minimizing the risk of infection, while optimizing the conditions to encourage healing. In our study we found, ethyl acetate and acetone extracts of triphala have better bactericidal and antioxidant property than methanolic extract. Furthermore, cytotoxic effect of triphala was checked on monolayer corneal cells (SIRC). The cells were found to be metabolically active in presence of triphala extract with negligible cytotoxicity. The cell morphology and cell viability was evaluated by staining the cell nuclei using DAPI and Hoechst stain respectively. These results suggested the safety and efficacy of these extract in formulation of the new drug. These findings may provide scientific rationale for the use of triphala as a new drug compound for preparation of eye drops, also as potential antioxidant and antimicrobial agent.

Keywords: Triphala; Myrobalans; Rabbit corneal cells (SIRC); Antimicrobial; Cytotoxicity.

INTRODUCTION

The antibiotics and steroid-based eye drops for the management of ophthalmic disorder has been found clinically unsafe in recent times and a current demand is to investigate alternative drug. A serious adverse drug reactions (ADRs) occurring due to use of herbal drugs is very rare event. A common argument that favours the use of herbal medicines is that they have a longstanding history of traditional use (1). Even after being used from ancient times, plant-derived medicines can results in some adverse reaction so needs have been raised for safety evaluation. Microorganisms develop resistance to present day antibiotics that has created immense clinical problem in the treatment of infectious diseases (2). Based on the experience of many generations of physicians and traditional systems of medicine from different ethnic societies, medicinal plants have been used to cure many ailments. The use of medicinal plants in modern medicine suffers only from the fact that, scientific evidence in terms of modern medicine is lacking in most cases. However, today it is necessary to provide scientific proof as to whether or not it is justified to use a plant or its active principles. It is still a problem to use these medicinal plants for ophthalmic problems (3).

Triphala ['three' (tri) 'fruits' (phala)] is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants, *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica*, also known as the 'three myrobalans'. This formulation, rich in antioxidants, is a frequently used ayurvedic medicine to treat many diseases such as anaemia, jaundice, constipation, asthma, fever and chronic ulcers. It is an important medicine of the 'rasayana' group and is believed to promote health, immunity and longevity (4). Methanolic extract of triphala (70%) has shown high antioxidant activity in the *in-vitro* studies. Some reports have shown the radio- protective activity of triphala in mice exposed to gamma radiation (5). The chloroform, water and acetone extracts of triphala have shown distinct anti-mutagenic activity against *Salmonella typhimurium* (6) and is found to act as purgative (7). *Terminalia bellirica* is reported to provide protection from myocardial necrosis (8). Fruits of *T. bellirica* were found to be useful in many ocular diseases. Fully ripe or dried fruit, mixed with honey is used as an external application in ocular diseases (5). *Emblica officinalis* is found to possess significant anti-inflammatory (9), cytoprotective (10), antimutagenic (6), hypolipidaemic (11) and

gastroprotective (12) activity. The exudates obtained from the incisions of *E. officinalis* fruits are used as an external application for ocular inflammation (7). An aqueous extract of *E. officinalis* was found to be a potent inhibitor of lipid peroxide formation and as a scavenger of hydroxyl and superoxide radicals *in vitro* (9). It also possesses significant antimicrobial activity against both gram-positive and gram-negative organisms (6). Similarly, *Terminalia chebula* is potent antibacterial (13), anticaries (14), anticancer (15), antimutagenic (16) agent and also inhibits occurrence of local anaphylaxis (17).

In the present study, the triphala was extracted by using various solvents of increasing polarity like ethyl acetate, acetone, methanol and water. These extracts were characterized to demonstrate antimicrobial property against microbes causing eye infections such as *Streptococcus pyogenes*, *S. pneumoniae*, *S. mutans*, *Staphylococcus aureus*. The active antioxidants property and scavenging free radical activity was evaluated using FRAP assay. Further *in-vitro* cytotoxic test were performed on SIRC (Rabbit Corneal Epithelial Cells) for its application developing drug formulation for eye infections.

MATERIALS AND METHODS

Materials

Triphala powder was purchased from local market of Mumbai, India. Pathogens of MTCC grade were obtained from IMTECH (Chandigarh, India). Gallic acid and Glutaraldehyde was purchased from s.d. fine-chemicals limited (Mumbai, India). Gentamicin and 2,4,6-tripyridyl-s-triazine (TPTZ) were procured from Hi-Media (Mumbai, India), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 98%) reagent, 4'-6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and nystatin was purchased from Sigma (St. Louis, USA). Fetal bovine serum (FBS) and streptomycin-penicillin antibiotic solutions were bought from HyClone (Utah, USA). All other chemicals used were of analytical grade. SIRC (rabbit corneal epithelial cells) cell line was obtained from NCL, Pune, INDIA.

Methods

Preparation of triphala extracts

25 g of triphala powder was taken in a thimble. Extracts were prepared in the series of 500ml of different solvents based on

increasing polarity (Ethyl acetate, Acetone, Methanol, and Water) using Soxhlet extraction method. The soxhletting cycle was completed after the solvents turned colorless in the tube indicating the complete extraction of the phytosignatures. The solvents were evaporated to dryness using rotary evaporator and the extract obtained was lyophilized at -50°C . The lyophilized solvent-free extracted powder was stored in airtight bottles at 4°C till further experimentation. (18).

High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out using C18 PCX 500 Dionex analytical column (WATER 2414 refractive index detector and 515 HPLC pump) with 0.1 M KCl, 0.05 M HCl and 10% acetonitrile as the mobile phase. The samples (solvent-free) were prepared in DMSO (1mg/ml). 20 μl of the samples was injected and the programme was processed for 15min (15min runtime). Gallic acid was used as a standard marker compound. The detection was carried out at 260nm using UV detector (19).

Determination of antimicrobial activity of triphala extracts

Antibacterial activities of all the solvent free triphala extracts of ethyl acetate, acetone, methanol and aqueous were determined by agar well diffusion method. The extracts were dissolved in DMSO (dimethylsulphoxide). The microbial lyophilized cultures were revived at 37°C for 18h in a broth medium and the culture was adjusted to 5×10^5 cfu/ml in accordance with the McFarland Turbidity standards (20). The 20 μl of the culture was spread on Muller Hinton agar plates and wells of 9mm diameter were punched into the agar plates. 100 μl of the solvent-free extracts of concentration 0.5mg/100 μl and 1mg/100 μl were used for determination of ZOI (Zone of Inhibition). After holding the plates at room temperature for 2 hours to allow diffusion of the extract into the media, the plates were incubated at 37°C for 24h. DMSO and Gentamicin was used as a negative control and as reference antibiotic (positive control) respectively. The test was performed in triplicates and the final results were presented as the mean zone of inhibition.

Inhibition Zone was observed after 24h and Zone of inhibition was recorded for all the extracts and gentamicin. Inhibition Zone Diameter (IZD) was measured to the nearest millimeter (mm) by reducing the IZD value with Diameter of the well bored.

Total Zone Of Inhibition = Inhibition Zone Diameter- Diameter of the well.

Determination of Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MICs) of the extracts of triphala was determined against the tested bacteria by macro broth dilution assay method (21). Two-fold serial dilutions of all the extracts (based on their IZD results: 0.5mg/100 μl) were prepared in 24well plates with MHA as diluents. Each dilution was seeded with 20 μl of test microorganisms to the standard concentration (5×10^5 cfu/ml). Two-fold serial dilution of Gentamicin was used as experimental positive control. The plates were incubated at 37°C for 24h. The least concentration of the extract or standard drug showing no visible growth was taken as the MIC. After 24h of incubation period, mean MIC values were calculated. The test was performed in triplicates for each microorganism used and the final results were expressed as the arithmetic average of triplicate experiments. 20 μl test media from each MIC broth tube was spread over the MHA plates. Plates were incubated at 37°C for 24h. The test MIC concentration showing no bacterial growth on agar plates was considered as Minimum Bactericidal Concentration (MBC) of the extract.

Determination of antioxidant activity of triphala extracts

The ferric-reducing antioxidant power (FRAP) assay measures the antioxidant potentials of "antioxidants" to reduce the $\text{Fe}^{3+}/2,4,6\text{-tripyridyl-s-triazine}$ (TPTZ) complex present in stoichiometric excess to the blue colored $\text{Fe}^{2+}\text{-TPTZ}$ form. (22). The stock solution of various extracts of concentration 0.3mgml $^{-1}$ was prepared. 10 μl -100 μl (6×10^{-4} g/l - 60×10^{-4} g/l) of extract

was mixed with 1.5 ml of FRAP reagent and the volume was adjusted to 5ml with distilled water. The test tubes containing the test solutions were incubated at 37°C for 15min. The absorbance was recorded at 593nm.

Effect of triphala extracts on corneal cell (SIRC) proliferation

Rabbit Corneal Epithelial (SIRC) cells were cultured and maintained in 90% DMEM media substituted with 10% Foetal Bovine serum and 1% antibiotic for 48h. 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. Corneal (3×10^4) cells were seeded on each of 24-well plate and allowed to proliferate in 1ml DMEM for 24h.

After 24h (23), various extracts of triphala (extracted in different solvents based on increasing polarity: ethyl acetate-S1, acetone-S2, methanol-S3, and aqueous-S4) were added to each well. Following five different concentrations, 10 μgml^{-1} , 20 μgml^{-1} , 30 μgml^{-1} , 40 μgml^{-1} , and 50 μgml^{-1} were prepared from each extract and added to each well containing grown SIRC cells (24; 4; 25). After incubation with triphala extracts for 24h, MTT assay was performed to check the cytotoxic effect of various extracts on these cells.

Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT-0.5mgml $^{-1}$) was prepared in DMEM media. Media with extracts were removed after 24h of cell growth. The corneal cell monolayer was washed with PBS twice in order to remove traces of media or extracts. MTT (0.5mg/ml) reagent was then added in each well and incubated for 4h in CO_2 incubator. After 4h, MTT reagent was removed and 1ml of DMSO (cell culture grade) was added in each well to dissolve purple colour formazon crystals. The absorbance was observed at 490nm in a spectrophotometer (26). Each experiment was performed in triplicates.

Image analysis of cell proliferation

Corneal cells seeded with triphala extract were fixed with 2.5% glutaraldehyde for 2h followed by 20 min incubation in nuclear stain; DAPI (200ngml $^{-1}$) working solution was prepared in PBS. After the incubation, samples were gently washed twice with PBS (0.1M, pH 7.4). The morphology of the cell nuclei was observed under fluorescence microscope (Nikon, TE-2000 U) at excitation wavelength of 350nm. Nuclei are considered to have the normal phenotype when glowing bright and homogeneously. Similarly cells were stained using DNA staining Hoechst dye and visualised under fluorescence microscopy (27).

RESULTS

Phytochemical Analyses of extracts of triphala

In extraction procedure, the phytosignature components were not compromised which was evaluated through HPLC analysis. Gallic acid was identified in extracts as one of the components as it is a marker component of triphala. The use of HPLC with UV detector at a wavelength of 260nm to identify compounds of the extracts (solvent-free) in reference (S1-S4) revealed many peaks of different phytosignatures in the chromatogram of the extracts (Fig 1). Emerging results clearly led us to believe the prominence of gallic acid as the principal component of the ethyl acetate and acetone extracts of triphala in addition to small presence of other phytosignatures which could not be identified in different extracts. The presence of the higher concentration of other phytosignature other than gallic acid could not be ruled out as observed in HPLC spectrum of methanolic and aqueous extracts of triphala extracts where gallic acid appeared to be either in small quantity or negligible amount. A comparison between the spectra of the sample peaks with those obtained from gallic acid standards confirmed that the retention time of gallic acid at 6.573 min.

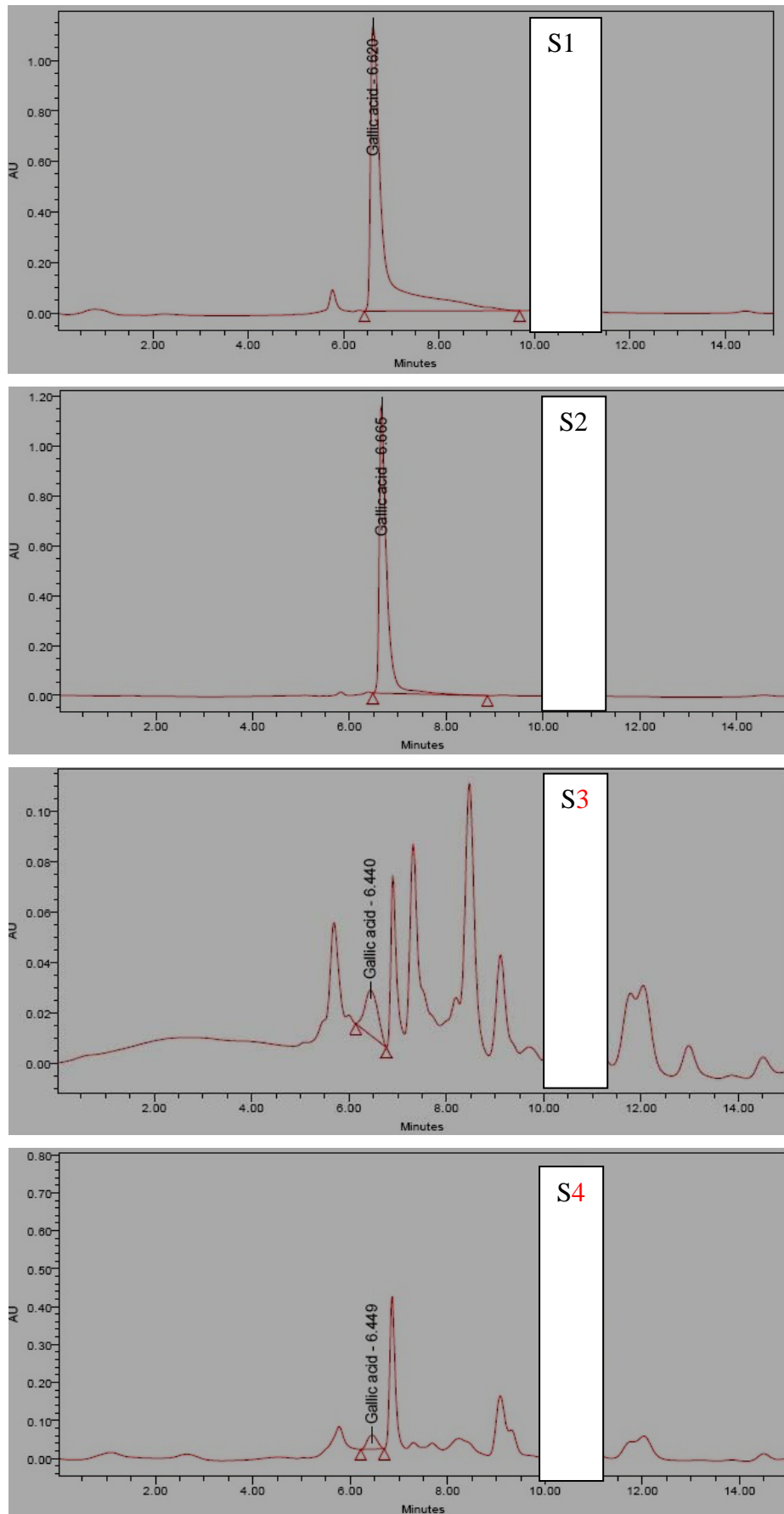


Fig. 1: HPLC analysis of triphala extracts S1 (ethyl acetate), S2 (acetone), S3 (methanol) and S4 (aqueous) detecting gallic acid as their principle component.

Determination of antimicrobial activity and MIC of triphala extracts:

Triphala is a blend of equal proportion of seed powder of three myrobalans. It was tested for its potency as an antimicrobial. The solvent-free extracts of triphala were subjected to antimicrobial study (agar well diffusion method) and the results obtained were measured to nearest millimetre (mm) and recorded in terms of its IZD values. S1 extract of triphala was found to be most effective against the chosen microbes followed by acetone extract. The S1 extract of triphala was found to be most effective against *S.aureus* showing a zone of inhibition (ZOI) of 24.00±0.82mm at a concentration of 1mg/100µl, whereas against *S. pyogenes* and *S. pneumoniae* ZOI was determined to be 22.33±0.47mm and 23.67±0.94mm respectively. S2 extract inhibited *S.aureus* as effectively as S1 extract having the same ZOI value 24.33±0.82mm at

1mg/100µl. *Pyogenes* was resistant for S3 and S4 extract of triphala. *S.mutans* was found to be least susceptible by all the extracts but resistant for S3 extract giving the minimum zone of 12.00mm at a higher concentration of 1mg/100µl. Growth of *S.pneumoniae* was effectively affected with all the extracts of triphala. ZOI obtained for each bacterium by gentamicin and different extracts of triphala are tabulated in Table 1 to 5. All microbes were found highly susceptible to reference antibiotic. Gentamicin being a pure antibiotic inhibited all bacteria in reference giving highest ZOI of inhibition than the extracts of triphala (Table 1). The minimal inhibitory concentration required for inhibiting the growth of the pathogens and the concentration of extract obtained to be bactericidal was found to vary with different organisms (Table 6). The experiments were performed in triplicates with calculated standard deviation that was found to be insignificant in all the cases in MIC studies.

Table 1: Represents the antimicrobial activity (IZD value) of gentamicin (positive control) was tested against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4) at 0.5mg/100µl and 1mg/100µl concentration

Microorganisms	Zone of Inhibition (mm)	
	Concentration of Gentamicin (mg/100µl)	
	0.5	1
M1	27.00±0.00	29.00±0.47
M2	31.33±0.82	32.00±0.82
M3	27.00±0.47	28.00±0.94
M4	31.00±0.00	32.00±0.00

Table 2: Represents the antimicrobial activity (ZOI value) of ethyl acetate extract of Triphala against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4) at 0.5mg/100µl and 1mg/100µl concentration.

Microorganisms	Zone of Inhibition (mm)	
	Concentration of the S1 extract (mg/100µl)	
	0.5	1
M1	19.00±0.47	20.67±0.47
M2	22.33±0.94	23.67±0.94
M3	21.67±0.82	22.33±0.47
M4	23.67±0.20	24.00±0.82

Table 3: Represents the antimicrobial activity (ZOI value) of acetone extract of Triphala against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4) at 0.5mg/100µl and 1mg/100µl concentration

Microorganisms	Zone of Inhibition (mm)	
	Concentration of the S2 extract (mg/100µl)	
	0.5	1
<i>Streptococcus mutans</i>	15.00±0.00	17.67±0.47
<i>Streptococcus pneumoniae</i>	25.00±0.82	26.67±0.94
<i>Streptococcus pyogenes</i>	15.00±0.47	17.67±0.47
<i>Staphylococcus aureus</i>	23.67±0.82	24.33±0.82

Table 4: Represents the antimicrobial activity (ZOI value) of methanol extract of Triphala against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4) at 0.5mg/100µl and 1mg/100µl concentration

Micro-organisms	Zone of Inhibition (mm)	
	Concentration of S3 extract (mg/100µl)	
	0.5	1
<i>Streptococcus mutans</i>	Nil	12.00±0.00
<i>Streptococcus pneumoniae</i>	20.67±0.82	21.33±0.82
<i>Streptococcus pyogenes</i>	Nil	Nil
<i>Staphylococcus aureus</i>	17.67±0.47	18.00±0.00

Table 5: Represents the antimicrobial activity (ZOI value) of aqueous extract of Triphala against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4) at 0.5mg/100µl and 1mg/100µl concentration

Micro-organisms	Zone of Inhibition (mm)	
	Concentration of S4 extract (mg/100µl)	
	0.5	1
<i>Streptococcus mutans</i>	12.00±0.00	13.00±0.00
<i>Streptococcus pneumoniae</i>	15.00±0.47	17.33±0.47
<i>Streptococcus pyogenes</i>	Nil	Nil
<i>Staphylococcus aureus</i>	17.33±0.82	18.00±0.00

Table 6: Represents the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of various extracts of Triphala against *Streptococcus mutans* (M1), *S.pneumoniae* (M2), *S.pyogenes* (M3) and *Staphylococcus aureus* (M4)

Microbes	MIC, MBC and MIC Index Of Various Extracts Of Triphala in mg/ml											
	S1			S2			S3			S4		
	MIC	MBC	MIC Index	MIC	MBC	MIC Index	MIC	MBC	MIC Index	MIC	MBC	MIC Index
M1	0.25	0.5	2	0.063	0.125	2	0.125	0.25	2	0.063	0.125	2
M2	0.063	0.125	2	0.063	0.125	2	0.063	0.125	2	0.063	0.125	2
M3	0.063	0.125	2	0.125	0.25	2	Nil	Nil	Nil	Nil	Nil	Nil
M4	0.125	0.25	2	0.125	0.25	2	0.063	0.125	2	0.125	0.25	2

Determination of antioxidant power using FRAP

The ability of triphala to scavenge the free ions were tested using FRAP assay. The results obtained are tabulated in table 7. From the results obtained it was confirmed that the antioxidant power of S2 extract of triphala was better than other extracts in reference and

the property was concentration dependent. The trend that followed among various extracts to convert and neutralise Fe³⁺ ions to Fe²⁺ ions was S2 > S1 > S3 and S4. With increase in concentration, the S3 and S4 were almost at par with each other with negligible variations in the antioxidant power (Table 7).

Table 7: Represents the antioxidant power of various extracts of Triphala

Extract Concentration (g/l)	Antioxidant Power (µM/l)			
	Ethyl Acetate	Acetone	Methanol	Water
6x10 ⁻⁴	18.92	23.67	7.6	5.0
12x10 ⁻⁴	20.42	23.83	14.5	13.75
18x10 ⁻⁴	25.33	27.58	20.25	21.25
24x10 ⁻⁴	33.92	35.75	27.00	28.08
30x10 ⁻⁴	41.25	40.75	30.83	33.83
36x10 ⁻⁴	45.08	40.92	37.75	38.08
42x10 ⁻⁴	50.17	45.67	43.58	44.33
48x10 ⁻⁴	54.50	50.50	45.75	47.75
54x10 ⁻⁴	56.75	55.83	50.67	50.75
60x10 ⁻⁴	58.25	57.42	53.08	52.83

In order to determine the concentration required for evaluating the cytotoxic effect of triphala extracts, the MIC values obtained along with antioxidant data formed the basis of our *in-vitro* cytotoxic tests.

Effect of triphala extracts on corneal cell (SIRC) proliferation**Cytotoxic study of Triphala and Image analysis**

The effect of solvent free extracts of triphala on the metabolic activity of corneal cells (SIRC) evaluated using MTT assay demonstrates that the metabolic activity of cells were not significantly compromised thereby extracts are not cytotoxic in nature. The growth of the cells at the varied concentration range (10µg/ml to 50µg/ml) was found to enhance (S2) or be equivalent (S3) or less than that of the control (corneal cells without triphala extracts) (S1 and S4). S2 and S3 extracts shows slightly higher metabolic rate from 20µg/ml - 50µg/ml whereas, the metabolic rate of the SIRC cells were slightly affected in presence of S1 and S4 extract of triphala with increase in concentration of the extracts as compared to the untreated corneal cells as shown in table 8. The treatment of SIRC under the applied conditions at different doses (10µg/ml-50µg/ml of media) registered a fall in the cell count after 24h of

incubation un-proportionate to the dose applied exhibiting slightly cytotoxic nature of S1 and S4 extracts coinciding with the decrease of cell count with the incremental increase (10µg/ml of doses of these extracts). The S2 extract has indicated its cytoprotective nature as with the increase of dose, the cell count increased at the rate of 3%, (10µg/ml), 23% (20µg/ml), 40% (30µg/ml), 63% (40µg/ml) and 83.7% (50µg/ml). The S3 extract also exhibited cytoprotectivity where the cell count showed an appreciation of 20% increase at 10µg/ml but with increase in concentration the percentage increase was 16.67% (20µg/ml), 13.33% (30µg/ml), 10% (40µg/ml) and 6.67% (50µg/ml) but more than the initially seeded cell count. The microscopic images showed the normal cell morphology (Fig 2). The DNA staining performed by DAPI and Hoescht staining procedure showed normal intact nuclei morphology indicating no change in its physical structure resulting in the normal growth of cells thereby confirming the biocompatible nature of triphala (Fig 3).

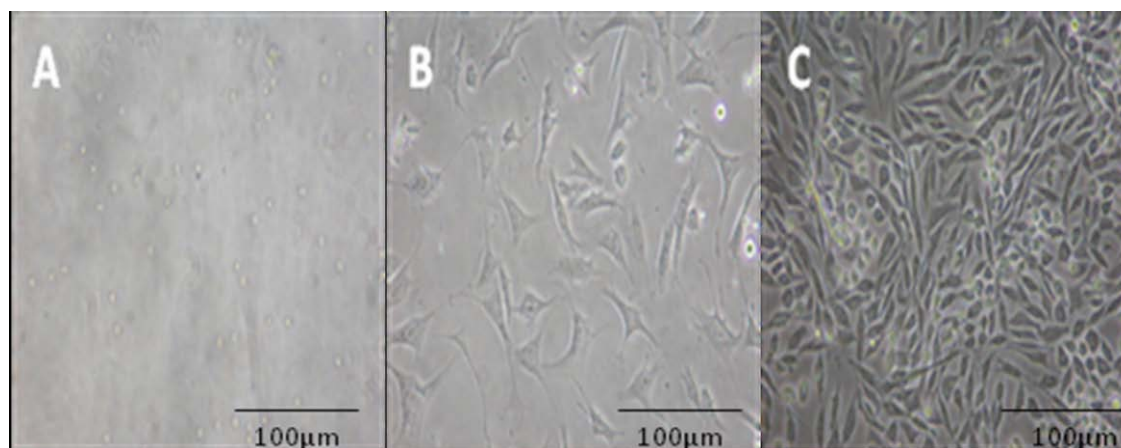
**Fig. 2: SIRC cell proliferation. (A) SIRC cell population after cell seeding, (B) Proliferated SIRC cells without the treatment of triphala extracts (S2) after 48h and (C) SIRC cell proliferation in presence of triphala extract (S2) after 48h.**

Table 8: Represents the MTT assay of various extracts of triphala

S. no	Extracts	Control	Concentration of Extracts in $\mu\text{g/ml}$				
			10	20	30	40	50
1	Ethyl Acetate (S1)	0.279	0.268	0.266	0.219	0.212	0.21
2	Acetone (S2)	0.246	0.254	0.303	0.345	0.401	0.452
3	Methanol (S3)	0.222	0.266	0.258	0.251	0.247	0.24
4	Aqueous (S4)	0.222	0.224	0.222	0.217	0.21	0.205

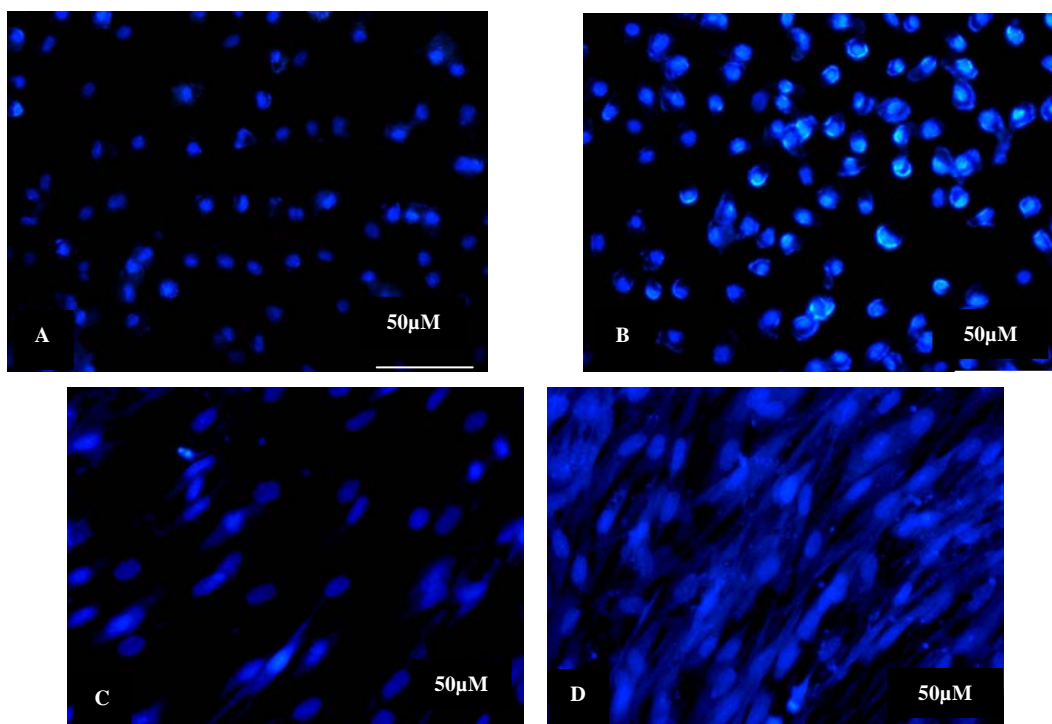


Fig. 3: Shows the distribution of SIRC cells using DAPI (Panel A and B) Panel A shows the control cells without treatment with extracts and Panel B reveals the larger number of SIRC proliferation when treated with S2 extracts of triphala. Panel C and D reveals the viable DNA of untreated and treated cells respectively using Hoechst stain at 48h.

DISCUSSION

In the present study we have investigated the antimicrobial, antioxidant activity of triphala that formed the basis for evaluation as potential corneal cell healing and cytoprotective drug for ophthalmic use. Triphala is regarded as the most potential ayurvedic formulation by the practitioner but as far as our knowledge on study of triphala goes, it is exploited for its potential use as an immunomodulatory (23), anti-diabetic (28) anti-mutagenic (6) and anticancer (4) activity. It's potential as an eye formulation is though reported in ayurvedic literatures (29), but still lacks the scientific background to it. In our study we have explored triphala as an effective drug for use in eye diseases caused either by microbes in reference, due to excess of free radical production or due to various physiological activities affecting the normal functioning of the eye. The ZOI and MIC values obtained for various extracts shows triphala to possess phytosignatures which are responsible for scavenging the growth of the microbes in study and the results obtained is at par with the previous study (30). In extraction procedure the phytosignature components were not compromised as shown by HPLC analysis. Gallic acid and tannic acid was identified as the major phenolic acids of *P.emblica*, *T. bellirica*, *T. chebula* and triphala in varying percentages in different solvent (31; 3; 32) and HPLC analysis of extracts of triphala in reference has shown presence of gallic acid to be the major phenolic content. It is possible that the other peaks belong to hydrolysable tannins because these compounds have high polarity due to the presence of hydroxyl group (33), which generally show as initial peaks of chromatograms. Each extract had a variety of bio-signatures with different properties including bactericidal, antioxidant and cytoprotective potentials. We

have selected microbes that were found to cause most common eye infections. These microbes are also responsible for other infections in various body parts. The ZOI and MIC value shows ethyl acetate and acetone extract to possess antimicrobial activity at par with each other.

The ethylacetate (S1), acetone (S2), methanol (S3) and aqueous (S4) extracts of triphala were further tested for its cytotoxicity on Rabbit Corneal Epithelial Cells (SIRC). In study on MTT assay (which is the measure of absorbance of end product i.e., formazon crystals, reflecting the metabolic activity of cells), the S2 extract shows to support higher cell proliferation with enhanced metabolic activity, thereby it has given indication of cytoprotective nature of triphala extract. The cells in the presence of media (defined and described earlier) were incubated for 24h to achieve 70% confluency of cells. The confluent cells were treated with 10 $\mu\text{g/ml}$ -50 $\mu\text{g/ml}$ of media of all extracts and were further incubated for 24h.

The triphala is fully loaded with different variety of phytosignatures drawn from the mixture of seeds of three plants. The above results clearly shows that the each structure of triphala could not be adjudged as cytotoxic or cytoprotective. The enhanced cell proliferation of acetone and methanol extract was clearly visible which may be due to the combined effect of phytosignatures extracted in these extracts, whereas, other extracts could have some cytotoxic phytosignatures in nature. Out of all the extracts tested and tried the acetone extract could suit well for its application in the treatment of ophthalmic disorders. Further, antioxidant study which evaluates the free radical scavenging property of triphala using FRAP assay confirms the antioxidants of extracts. Antioxidants are

important, as they are free radical neutralizers. They are essential as excess of free radical causes diseases like alzheimer's, parkinson's, various kinds of cancers, ophthalmic disorders etc. The result of antioxidant assay confirms the S1 and S2 extract to be more powerful free radical scavenger than S3 and S4 extracts.

Gallic acid either in free form or conjugated form together with other unidentified compounds possessed antibacterial activity (extracts being bactericidal in nature), being a potent antioxidant (scavenging free-radicals) and also cytoprotective in nature.

Antimicrobial, antioxidant activity, viability and better proliferation of cells in the presence triphala extract, provides the concentration that could be used in formulation of new herbal eye drug. The plant based acetone extract may be better suited for the cause in reference as compared to the steroid-penicillin based formulation with side effects, being used presently. The in-vitro study on acetone extract may possibly be extended to in-vivo.

CONCLUSION

The genetically and enzymatically in-built phytosignatures of triphala (a blend of equal parts of 03 myrobalans by weight) have been found as free radical scavengers and bactericides that viably suites to manage and contain the oxidative stress and bacteria that are directing ophthalmic disorder(s) invitro and are safely extendable invivo. It further opens the arena for exploring the active phytosignatures present in herbal plants to be used as a growth rejuvenator or for obtaining the growth regulatory compounds. These plant-based formulations with zero side effects are safer clinically than the steroid and penicillin based eye drop formulation with various side effects.

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