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Study on Antimicrobial Efficacy of an Indigenously Prepared Herbal Ophthalmic Solution against Selected Eye Pathogens Associated With Eye Diseases

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Abstract: Aqueous plant extracts in ophthalmic preparations is not uncommon. Folklore (Indigenous) herbal preparations in this regard claim to cure eye diseases need to be explored. Antimicrobial potency of such herbal formulation is studied with comparison of its efficacy against selected pathogens associated with eye diseases helps for its standardization and validation as well. Hence Herbal formulations were taken and their antimicrobial efficacy was compared with standard treatises against wide range of eye pathogens. Both agar well and disc diffusion methods were followed for this purpose. Effect of extract on viability of microbes was also studied taking absorbance data at 600nm spectral wavelength. The multidrug resistance of the strains was also tested prior to their use in antimicrobial sensitivity test. The antibacterial efficacy of standard eye drop showed highest zone of inhibition against *E. coli* and lowest against *P. vulgaris. S*imilarly the indigenously prepared eye drop was found to be with fewer efficacies. However the 50% diluted ophthalmic preparation showed higher degree of inhibition was revealed. The study explored that the diluted and standardized indigenously prepared ophthalmic solution can be used as a more potential drug at least against microbe associated and induced eye diseases.
Keywords: Ophthalmic preparation, antibacterial efficacy, multidrug resistance, zone of Inhibition.

INTRODUCTION

The use of medicinal plants and their preparations in health care is as old as human civilization. Out of the total 4, 22, 000 flowering plants reported from the world, more than 50,000 are used for medicinal purposes [1].

In India, more than 43% of the total flowering plants are reported to be of medicinal importance [2]. Indian people had an incredible knowledge of phyto-medicine which is evident both in the living folklore cultures and from the codified ancient system of medicines namely Ayurveda, Siddha, Unani etc. Indians have depth of knowledge on medicinal plants and their applications as well. In the recent years' there has been a great demand for the plant derived traditional formulations in the developed countries. These formulations are increasingly being required as medicinal products, neutraceuticals, and cosmetics. Herbs and prepared herbal compounds are available in different forms. Each of which has its own particular characteristics. They may be available as individual herbs and as complex herbal formulations, including raw herbs, tinctures, extracts, capsules, tablets and ointments etc. Different aqueous plant extracts are used in eye drops or in ophthalmic preparations for treatment of eye diseases. The phytochemicals and other therapeutic agents present in them act as antimicrobials for fighting against microbial pathogens and rectification of functional attributes of organs and systems of the body.

Plants contain numerous biologically active compounds, many of which have been shown to have antimicrobial properties. Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years and there is increasing interest in plants as sources of agents to fight microbial diseases. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents.

The disorders of eye are broadly categorized into 4 types based on pathological events like Inflammation, Infection, Free radical damage and Decreased local immunological potential. These four types cover more than 84 kinds of disorders. The disorders virtually start with each alphabet such as amblyopia, blepharitis to vitreorethnopathy and xerophthalmia etc.

The medications should be having antimicrobial, anti-inflammatory along with antioxidant property so as to enhance the local immunity to cure any diseases borne thereof.

Use of herbal drugs in curing eye diseases is not new. It has been a practice from ancient times of Vedas to the modern times of pharmacopeias [3]. Rig-Veda, Bhrigutantra, Charak Samhita and Sushrut Samhita provide evidences for the therapeutic efficacy of the herbs in curing ophthalmic disorders. Hence seeing the importance of herbs W.H.O. has been active in framing guidelines and standards of botanical medicine. Herbs cure mostly eye disorders of infective, allergic and inflammatory etiology. Some herbs are also effective against degenerative disorders such as ARMD, Diabetic cataract etc.

The prepared ophthalmic drop contains many ingredients of plant extracts. Here is a list of plants and their properties based on which these plants have been selected as the constituents in the eye drop. One parameter of infective eye was considered and the response of the eye drop was studied. Comparative potency of one marketed and the self indigenously prepared eye drop for microbiological analysis was undertaken.

The Table (I) given below provides much information which has led us to investigate and compare the properties of indigenously prepared and a standard marketed eye drop particularly for their antibacterial property against standard strains of bacteria associated with eye pathogens. Let us have an idea about some herbal medicament to combat eye infection available in the market with special emphasis on the eye drop "BriteSite". The ingredient rationale of BriteSite is based on scientific research of the therapeutic action of medicinal plant extracts as claimed in the website [4].

Table-I: List of active ingredients extracted from herbs and their effect on eye (composition of aqueous extracts in v/v)

Sl. No.	Name of the plant	Local name	Active ingredient	Plant properties	Effect on eye	% used
1	Rosa damascena	Satapatri	Citranool	AI, AM, and AO	Soothing and antisolar effect	15%
2	Honey	Madhu	Quercetin	AI, AM, AO and IM	Anti-Cataract, trace nutritient provider	15%
3	Curcuma longa	Harida	Curcumin	AI, AM, AO and IM	Anti Cataractogenic	12%
4	Glycyrrhiza glabra	Yasti madhu	Glycyrrhizin and Glabridin	AI, AM, AO and IM	Anti-inflammatory	10%
5	Coriander sativum	dhanyak	coriandrol	AM, AO and IM	Strong antioxidant	10%
6	Terminalia chebula	Bibhitak	Gallic acid	AM and AO	antioxidant	5%
7	Piper longum	Pippali	Piperine	AI, AM, AO and IM	antiinflammatory	5%
8	Phyllanthus niruri	Bhuin amla	Phyllanthin	AI, IM, and AO	immunomodulatory	5%
9	Albizzia lebbeck	shirish	albezziahexicide	AI, AM, and IM	astringent	5%
10	Ocimum sanctum	Tulsi	Euginol	AI, AM, AO and IM	Anti-Cataract	5%
11	Butea frondosa	palash	Butin , butrin	AI and AM	Anti-bacterial and anti-fungal	5%
12	Berberis aristata	rasanjan	berberine	AI, AM, and IM	Anti C.trachomatis	5%
13	Vitex negundo	Nirgundi	- -	AI, AM, and AO	Anti-oxidant	3%

AI- anti-inflammatory, AM- anti microbial, AO- anti oxidant, IM- immunomodulatory

The indigenously prepared eye drop also contains almost same ingredients as information collected and written to be there in the list of composition but the processing methodologies and trade related secretes varies similarly as varies among the marketed products.

Different antibiotic and synthetic preparations are also used in treatment of eye infection but as herbal preparations are given importance, only comparative study for its antimicrobial efficacy against selected pathogens associated with eye diseases was undertaken between two herbal indigenously prepared and marketed branded eye

drops. The indigenously prepared eye drop was named as "Vision Care" and the marketed eye drop available entitled as "Brite Site" by Centaur Pharmaceuticals.

The herbal aqueous formulation was prepared after thorough search of the therapeutic potentials of different constituting plants and other materials used in the eye drop preparation.

MATERIALS AND METHODS

Microorganisms used for the study

For antibacterial activity study of the aqueous extracts of the eye drops (both indigenously prepared and BriteSite) bacteria that causes infectious diseases both in humans and animals were used. They were both gram positive and gram negative. Five gram negative bacteria such as *E.coli, Proteus vulgaris, S.pneumoniae, Pseudomonas aerogenosa, salmonella typhimurium* and two gram positive bacteria such as *Bacillus subtilis & Staphylococcus auerus* were used.

All the microorganisms were collected from PGI, Chandigarh and the few numbers from M.K.C.G Medical College, Berhampur, Odisha (India).

Antibacterial Study

Preparation of antibiotic disc

Sterile empty antibiotic disc were prepared by using Whatman filter paper number-4. The aqueous extract of the eye drops were directly taken in the discs amounts to 100 µl extract of the plant was added to the disc individually and aseptically. Each disc contains of extract respectively. Then the discs were allowed to drying at room temperature. After drying they were used for screening the antibacterial activity.

Preparation of culture media

Muller Hinton agar medium was used to study the antibacterial activity of the aqueous extract of the formulations in the form of eye drops.

Inoculum Preparation

Pure culture aliquots of bacterial pathogens were taken from nutrient agar slant and inoculated into respective culture broths prescribed by for each strain by MTCC and incubated at 37°c for 24 hours. The turbidity was adjusted to that of standard level by adding sterile respective broth.

Assay of Antibacterial Activity

Antibacterial assay was carried out by disc diffusion and agar well diffusion method [5]. 0.1 ml of 24 hour old culture of bacterial pathogen was placed on Muller Hinton agar medium and spread throughout the plate by spread plate technique. The sterile disc containing respective aqueous extract of the eye drops were placed on the surface of the medium at equidistance. The plates were kept at room temperature for 45 minutes which helps to diffuse the extract on the medium. Aqueous extract was tested in triplicate and calculation of mean value was taken.

For well method, a single bacterial colony was suspended in 1ml of sterilized saline, added into 20 ml of media at 45^0C, mixed thoroughly and poured into plates. After solidification, wells were dug in it and different doses of aqueous extract from the eye drops were loaded into wells. The plates were left at room temperature for 1 hour for drug diffusion into media and then incubated overnight at 37^0C.

Methodology to study the Effect of extract (eye drops) on viability of microbes

- Make ready 9 sterile test tubes each containing 5 ml sterile broth. Label them according to the conc. and type organism to be inoculated.
- Add the test drug to respective tubes at 0 hour and incubate them at 37 ^{0}c after taking O.D. at 600 nm and plating of a single or replica conc. carrying test tubes in previously prepared NAM plates as a reading conc. at 0 hour.
- Incubate the treated tubes for 4 hours and take out the tubes for further plating and take O.D. with respective blanks.
- Continue the process at 4 hrs. Interval i.e. 4 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs, 24 hrs, 28 hrs, 32 hrs, 44 hrs and 48 hrs.
- The NAM plates prepared by spread plate method are incubated at 37 ^{0} C in BOD for counting of colonies after 36-48 hrs.
- Of vigorous growth is seen in broth Dilution of the broth alignment is to be done and then spreading is to be done.

(N.B.: there is no problem in dilution as the dilution factor noted will be multiplied while counting colonies and CFU/ml determination)

RESULTS & DISCUSSION

Antibiotic Sensitivity Test

Different procured standard strains of bacteria were tested for their response to varied standard antibiotics by Antibiotic Sensitivity Test before they were to use for antibacterial activity tests against standard and indigenously developed eye drops. Day to day increased resistance of bacteria to antibiotics led scientists to discover new herbal remedies. This test aims in finding the multidrug resistance strains or sensitivity of bacteria that certainly will speak the efficacy of the herbal drug under study.

The strains taken as the experimental microbe for antibiotic sensitivity test are mostly multidrug resistance varieties which is evident from the experimental result table - 1. The inhibitory zone found out in case of each and every organism is shown in the Table-1. The bacterial strains *E.coli, S. pneumonia, C. albicans, P. aroginousa, P. vulgaris* are resistant to the antibiotics Streptomycin except *S. typhimurium* which is not associated with any of the eye diseases. The

mostly eye disease associated bacteria are resistant to one or the other type of antibiotics. *C. albicans* and *P. aroginousa* are resistance to *chloramphenicol* while *P. vulgaris* is resistant to Tetracycline. *S. pneumonia* is resistant to ciprofloxacin while *C. albicans* is resistant to Gentamycin. According to the approved standards of NCCLS, USA the sensitivity of the bacteria as R-Resistance, I-Intermediate, S-Susceptible was predicted [6 &7].

Antibacterial Activity of Aqueous Extracts of Plants Formulated in Brite site

Both disc diffusion and agar well diffusion method was followed to determine the antibacterial efficacy of the herbal standard formulation BriteSite. Disc diffusion test was carried out by Kirby Bauer method. The growth inhibitory zone found in case of each bacterium is presented in the Table: 2. The zone of inhibition in the plates is seems to remarkable from the point that zones are with full clarity and at par with some of the antibiotics. The concentration of the key active principle/ compound present in each aqueous extract is not known to us and still it has given expected zone of inhibition. Against *P. vulgaris, P. arogenousa, C. albicans, E.coli and S. typhimurium* the Brite Site eye drop works well as evident from the Table: 2 the result is found from disc diffusion method. Further this is also again verified in agar well diffusion method. In this method, it gave good inhibitory zones presented in the Table: 3 and 3.1. as in the disc the amount of drug taking and diffusion in the media from the paper disc is less, it is expected the lesser zone of inhibition as compared to well method. The plates of well method shows good zone of inhibition against all most all organisms with the highest against *E.coli* and the lowest against *P. vulgaris.*

Antibacterial activity of aqueous extracts of plants formulated in indigenously developed eye drop "Vision Care"

The indigenously prepared eye drop Vision Care is a locally developed herbal ophthalmic solution containing a series of aqueous extracts of plants having the property related to eye diseases. It was not scientifically developed and hence an attempt was made to examine its antibacterial efficacy against bacteria associated with eye disease. Both disc diffusion and agar well diffusion method was carried out to determine the antibacterial efficacy of the herbal formulation.

In disc diffusion method, when the vision care eye drop was used directly as the drug concentration without any dilution, a very less zone of inhibition was found and for that reason we take a trial and prepared three dilutions and tested for the antibacterial efficacy of different concentrations of the eye drop. To our goodness we got good zone of inhibition even better zone of inhibition than the standard marketed herbal eye drop. The Table: 2, 3, & 3.1 depicts the comparative reading of Brite site, concentrated vision care and the different dilutions of vision care. In disc diffusion method by Kirby Bauer method, the zone of inhibition was very less as compared to the standard marketed drug (Brite site eye Drop). However, when the vision Care eye drop was diluted to 70%. 50% and 30% it produced good zone of inhibition. Out of the three concentrations 50% diluted vision care shows the maximum zone of inhibition and hence is the most effective concentration required to inhibit the eye pathogens. Further the 50% diluted vision care showed inhibitorier diameter zone than it was found out with Brite site. So the vision care eye drop may be having more potency than Brite site to work against microbes associated with eye diseases as evident from the Table-3.1.

Determination of optimum/most effective concentration of composed aqueous extract of the indigenously developed eye drop

There is no need to dilute and prepare the optimum dose of the standard eye drop Brite site. The best efficacy dilution has been marketed which would have been determined by the concerned manufacturer. But the indigenously prepared Vision care required determining its standard effective concentration of the mother stock prepared initially. Whatsoever is the reason, during our test it was found out that 50% dilution became the best effective/ most effective dilution or concentration. From the table-3 and table-3.1 it could be determined which one was the most effective concentration or dilution. However it may also further be made accurate by undertaking tests of gradients ranging in between 50% to 70%.

Effect of extracts on viability of microbes at different time intervals & on different concentrations

The number of viable bacteria in the ophthalmic solution treated culture was found to decrease at each time interval tested in comparison to control culture and the indigenously prepared eye drop 'Vision Care' treated was able to decrease the CFU/ml more effectively than the marketed standard herbal eye drop "Brite site". However this is more conformity with the bacteria that are associated with the eye disease. It was also found that up to 48h the bacteria were still in their stationary phase and the cell number was maintained evident from the graphical representations and in the tables concerned with viability of the bacteria experiments.

Table-1: Antibiotic Sensitivity Test of the strains used in the study

Antibiotics	E.coli	R/I/S	S. pneumoniae	R/I/S	C. albicans	R/I/S	S. typhimurium	R/I/S	P. aeruginosa	R/I/S	P. vulgaris	R/I/S
Streptomycin	8	**R**	10	**R**	7	**R**	15	**S**	9	**R**	11	**R**
Chloramphenicol	13	**I**	16	**I**	12	**R**	13	**I**	11	**R**	13	**I**
Tetracyclin	13	**I**	17	**I**	15	**I**	14	**R**	18	**S**	10	**R**
Ciprofloxacin	18	S	17	**R**	17	I	20	I	18	**S**	21	
Gentamycin	16	**S**	15	**S**	7	**R**	17	**S**	16	**S**	15	**S**

The values represent the diameter of growth inhibition zones in mm. (R-Resistance, I-Intermediate, S- Susceptible)

Table-2: Antibacterial Sensitivity of bacterial strains against eye drops (standard and indigenously prepared) by Disc Diffusion method (Kirby Bauer method) 100 µl per disc

Name of the herbal eye drop	S. pneumoniae	C. albicans	P. vulgaris	S. typhimorium	P. arogenousa	E. coli
Brite site	6 mm	8 mm	7 mm	9 mm	8 mm	10 mm
Vision care (conc.)	4 mm	4 mm	4 mm	4 mm	4 mm	4 mm
Vision care (dil.70%)	6 mm	6 mm	8 mm	8 mm	9 mm	6 mm

The values represent the diameter of growth inhibition zones in mm. Data are represented in the form of mean of three tests. The control disc used for solvent showed no zone of inhibition, so their data was omitted in the table.

Table-3: Antibacterial Sensitivity of bacterial strains against eye drops (standard and indigenously prepared) by Agar well diffusion method. (100 µl per well)

Name of the herbal eye drop	S. pneumoniae	C. albicans	P. vulgaris	S. typhimorium	P. arogenousa	E. coli
Brite site	14 mm	15 mm	11 mm	16 mm	12 mm	17 mm
Vision care	6 mm	7 mm	8 mm	8 mm	7 mm	6 mm
Vision care (dil.70%)	11 mm	11 mm	12 mm	12 mm	14 mm	11 mm

The values represent the diameter of growth inhibition zones in mm. Data are represented in the form of mean of three tests. The control disc used for solvent showed no zone of inhibition, so their data was omitted in the table.

Table-3.1: Antibacterial Sensitivity of bacterial strains against eye drops (standard and indigenously Prepared) by Agar well diffusion method (100 µl per well)

Name of the herbal eye drop	S. pneumoniae	C. albicans	P. vulgaris	S. typhimorium	P. arogenousa	E. coli
Brite site	14 mm	15 mm	12 mm	16 mm	12 mm	17 mm
Vision care (dil.70%)	11 mm	11 mm	12 mm	12 mm	14 mm	12 mm
Vision care (dil.50%)	22 mm	18 mm	17 mm	20 mm	20 mm	21 mm
Vision care (dil.30%)	15 mm	16 mm	12 mm	17 mm	14 mm	17 mm

Table-4: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (S*treptococcus Pneumoniae*) at different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4hr	8hr	16hr	24hr	3hr	48hr
Brite Site	0.05	0.12	0.20	0.30	0.15	0.08	20	35	72	55	20	10
Vision care	0.03	0.06	0.12	0.11	0.08	0.02	28	42	65	35	15	12
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

Table-4.1: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (*P. arogenousa*) at Different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4hr	8hr	16hr	24hr	32hr	48hr
Brite Site	0.06	0.09	0.18	0.31	0.09	0.02	21	32	70	45	21	10
Vision care	0.04	0.08	0.16	0.28	0.07	0.02	15	28	74	46	15	06
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

Table-4.2: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (*Proteus vulgaris*) at different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4 hr	8 hr	16 hr	24 hr	32 hr	48 hr
Brite Site	0.1	0.18	0.34	0.47	0.19	0.1	50	90	135	108	39	22
Vision care	0.08	0.18	0.31	0.41	0.18	0.13	42	75	118	68	40	28
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

Table-4.3: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (*C. albicans*) at different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4hr	8hr	16hr	24hr	32hr	48hr
Brite Site	0.02	0.05	0.1	0.18	0.12	0.08	30	45	68	50	35	25
Vision care	0.02	0.04	0.09	0.1	0.1	0.06	28	40	70	49	30	18
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

Table-4.4: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (*S. typhimurium*) at different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4hr	8hr	16hr	24hr	32hr	48hr
Brite Site	0.1	0.2	0.41	0.81	0.62	0.60	41	97	199	200	132	110
Vision care	0.12	0.22	0.55	0.97	0.45	0.36	44	102	198	240	143	100
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

Table-4.5: Effects of aqueous extracts (contained in eye drops) on viability of bacteria (*E. coli*) at different time intervals

Aqueous extracts of groups of plants contained in	Absorbance Values						CFU/ml in 10^{-4} dilution					
	4hr	8 hr	16hr	24hr	32hr	48hr	4 hr	8 hr	16 hr	24 hr	32 hr	48 hr
Brite Site	0.1	0.21	0.41	0.3	0.18	0.06	54	120	197	78	40	24
Vision care	0.13	0.17	0.34	0.23	0.11	0.04	62	100	168	105	76	29
Control	0.2	0.4	0.6	0.8	0.6	0.4	81	98	180	122	108	60

PLATE: Showing zone of inhibition of Brite site, Vision care (Concentrated & 70% diluted) by disc diffusion method.

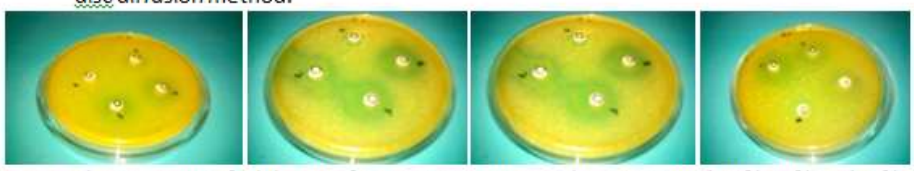

PLATE: Showing zone of inhibition of Brite site, Vision care (Concentrated, 70%, 50% and 30% diluted sample) against (a) by disc diffusion method.

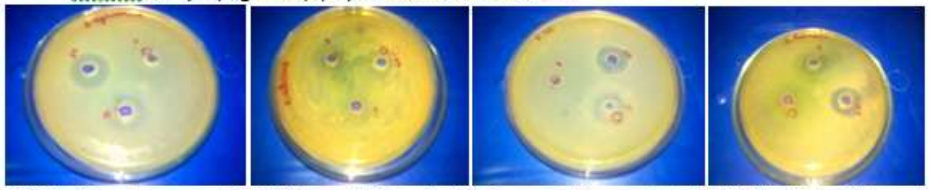

PLATE: Showing zone of inhibition of Brite site, Vision care (Concentrated & 70% diluted) by Agar well diffusion method.

CONCLUSION

This study reports the comparative antibacterial potency of two herbal eye drops. One was a standard marketed eye drop where as another was an indigenously developed local ophthalmic solution. Before its antibacterial property was studied, the bacterial strains against which the drug effect was to study tested for their multidrug resistance. It `was found that most of the organisms under study were multidrug resistance strains. The study reports that both the marketed and indigenously prepared eye drop was having antibacterial property that is evident from the fact and figures obtained from the experiments. The indigenously developed local ophthalmic solution was having more potentiality in encountering the infectious eye disease pathogens. However the eye drop requires to be standardized for its effective potion as revealed in the study. Further better processing technologies and aseptic techniques if employed for its preparation, more efficiency of the eye drop can be expected that can be well adjudicated through field trials/ clinical trials.

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