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In vitro Antibacterial and Antifungal activity of Hydro-alcoholic extract of Polyherbal Formulation

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Abstract

For the past several years, microbial drug resistance has been doubly increasing. Antimicrobials from natural sources are become alternate to reducing microbial infections in human. The aim of present work is to evaluate antimicrobial activity of hydro-alcoholic extract of polyherbal formulation in different bacterial and fungal species. To determine the zone of inhibition different concentrations (31.25 – 500 µg/ml) of extract were tested by disc diffusion method. Minimum Inhibitory Concentration (MIC) was determined by broth microdilution method. The results indicate that the hydro-alcoholic extract showed good antibacterial activity against *Salmonella typhimurium* with zone of inhibition 15.40 mm at the concentration 500 µg/ml and MIC was 7.81µg/ml. It also shows antifungal activity against *Candida albicans* at the concentration of 500 µg/ml with zone of inhibition 12.17 mm and MIC 7.81 µg/ml. In other bacterial and fungal species extract showed antimicrobial activity in dose dependant manner. Gentamicin 10 µg/ml and Clotrimazole 10 µg/ml were used as standards for bacteria and fungus. In this research work it has been concluded that, the hydro-alcoholic extract can be used as antimicrobial agent to reduce the microbial infections. Evaluation is going on to confirm that the phytoconstituents said present in the formulation are responsible for the above activity. Further studies towards quantification of phytoconstituents and explain the mechanism of action through *in silico* methods were in progress.

Key Words: Antimicrobials, Polyherbal formulation, Zone of Inhibition, Minimum Inhibitory concentration (MIC).

INTRODUCTION

There is an increasing awareness and demand for natural product-based therapeutics in both developing and developed countries due to fewer side effects in most cases and easily available at affordable price [1, 2]. Most of the pathogenic organisms respond slowly and getting resistance against available drugs. Individual ingredients are proven to be potent antimicrobial agents. *Picrorhiza kurroa* rhizome was used for skin diseases, urinary tract, gastrointestinal infections, diarrhea, antioxidant, anti-allergic, antihyperglycaemic, hepatoprotective, immunostimulating, anticancer and anti-inflammatory agent [3, 4 & 5] .Anjum Gahlaut and Anil K Chhillar [6] has reported this plant water and methanolic extracts are effective in *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa* with the MIC of 1.25 mg/ml. Hari Venkatesh K R and Chethana G. S [7] also reported that methanolic extract of *Picrorhiza kurroa* is effective in *Escherichia coli and Staphylococcus aureus* with the zone of inhibition of 16 mm and 12 mm. *Phyllanthus niruri* as a potential plant for the treatment of Hepatitis B virus which suppresses growth and replication. Methanolic extract of *Phyllanthus niruri* acts as potent antibacterial agent against in various Gram positive and Gram negative bacteria [8]. Three compounds from *Eclipta alba* such as wedelolactone, luteolin, and apigenin exhibited dose-dependent inhibition of HCV replication *in vitro* and anti-HCV replication activity in the cell culture system and thereby used as antiviral agent [9]. Different fractions of *Eclipta alba* were effective against the bacterial species tested [10].

Aqueous and ethanolic extract of *Azadirachta indica* leaves were found effective against *Candida albicans* and shows sensitivity at the concentration of 15% and 7.5% aqueous extract and the MIC was 7.5%. In the ethanolic extract, *Candida albicans* were found to be susceptible at the concentration of 15%, 7.5%, and 3.75%, besides the MIC was 3.75%. It also possesses an effective antibacterial effect against various bacterial species [11]. Neem leaves are found to be effective against Dengue virus type – 2 which halts the replication of the virus itself in an *in vitro* environment and in the laboratory animals [12]. Aqueous and methanolic extracts of *Swertia chirata* were screened for antibacterial activity with *E.coli* and found effective [13]. Various studies around the globe found *Swertia chirata* ethanolic and methanolic extracts possess antibacterial and antifungal activities [14, 15 & 16]. Methanolic leaf extracts of both *Swertia chirata* and *Swertia cordata* are found to be potent antioxidant, antimicrobial and antidiabetic agent [17]. The present study was undertaken to prove the polyherbal formulation whether it possess antibacterial and antifungal efficacy by *in vitro* methods.

MATERIALS AND METHODS

Polyherbal Formulation

The polyherbal formulation contains *Phyllanthus niruri* (Leaves), *Azadirachta indica* (Leaves), *Picrorhiza kurroa* (Rhizomes), *Eclipta alba* (Whole plant) and *Swertia chirata* (Stem and leaves).

Preparation of Hydro-alcoholic extract and polyherbal formulation

The individual plant ingredients were standardized according to Ayurvedic, Siddha pharmacopoeias and hydro-alcoholic extract were prepared separately by taking

100 g of each herbal ingredient, macerated with 1 L of hydro-alcohol (7:3) for 48 h and shaken vigorously in routine interwell. After the sample was transferred to the round bottom flask connected to the cooling condenser and heated at 65 °C for 2 h. After cooling, the samples were double filtered with a muslin cloth and finally filter through Whatman 1 filter paper. The resulting solution was dried in a vacuum dryer at the temperature less than 50 °C. The greenish black color extract obtained was transferred to airtight glass container and stored in a refrigerator. The extracts were combined in different ratio to form the hydro-alcoholic extract of polyherbal formulation (HAE-LVR05).

Preparation of sample for the experiment

The sample was weighed (1 mg/ml) and dissolved in 1% sterile DMSO to prepare appropriate dilution to get required concentrations (3.90 to 500 µg/ml). The standards such as Gentamicin (10 µg/ml) and Clotrimazole (10µg/ml) in 1% DMSO used to compare the test solution. They were kept under refrigeration and used for the experiments.

Culture medium used

Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) for bacteria, Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) for fungus. The culture media were procured from HiMedia labs, Mumbai.

Microorganisms used for the experiment

For evaluating antibacterial activity the following microorganisms were used. *Escherichia coli (MTCC No. 1687), Salmonella typhimurium (MTCC No. 3231), Staphylococcus aureus, (MTCC No. 737), Pseudomonas aeruginosa (MTCC No. 1688), Streptococcus pyogenes (MTCC No. 1923), Candia albicans (MTCC No. 1637), Trichophyton rubrum (MTCC No. 3272), Microsporum gypseum (MTCC No.2819), and Epidermophyton floccosum (MTCC No. 613).*

Determination of zone of inhibition by disc diffusion method

Preparation of 24 h pure and young culture

Each microorganism was taken with the help of sterile loop and suspended in 5 ml of sterile saline. The organisms were streaked on to the respective culture slants and incubated at 37 °C (bacteria) and 27 °C (fungus) for 24 h. After the growth was observed, microbial slants were kept in 2 – 4 °C until use.

Preparation of dried filter paper discs

Whatman 1 filter paper was used to prepare discs approximately 6 mm in diameter, which are sterilized and placed in a hot air oven. After drying the discs were loaded with different concentrations of prepared sample solutions and again kept under refrigeration (2 – 4 ^{o}C) for 5min.

Antimicrobial screening

The antimicrobial screening was done by disc diffusion method [18 & 19]. Petri plates were prepared by pouring 20 ml of MHA medium for bacteria and SDA for fungus. The test organisms were inoculated on a solidified agar plate with the help of a sterile cotton swab and spread evenly to the entire surface of the culture media and allowed to dry for 5 min. Using sterile forceps, previously prepared paper discs were dispensed onto the surface of the inoculated plates and ensures close contact with culture medium surface. After completion of the process, the plates were inverted and placed in an incubator set to the respective temperature 37 °C (bacteria) for 24 h and 27 ^{o}C (fungus) for 48 h. Each sample was tested in duplicate. The zones of inhibition of extract in the tested microorganisms were measured by using a dial caliper.

Determination of Minimum Inhibitory concentration by Broth microdilution method

The MIC endpoint is recorded as the lowest concentration of antimicrobial agent that completely inhibits growth under suitable incubation conditions [20, 21]. The MIC determination was performed by the technique using the calorimetric indicator resazurin [22, 23 & 24] with minor modifications.

Preparation of Bacterial and fungal culture

Antifungal susceptibility testing was performed as per CLSI-M27-A2 recommendations. Inoculum suspension was prepared from fresh cultures in sterile saline matching 0.5 McFarland standards [5 × 10^6 CFU (Colony Forming Units/ml].

Preparation of resazurin solution

The resazurin solution 0.01% (w/v) was prepared by using sterilized distilled water (HPLC grade). A vortex mixer was used to ensure that it was well dissolved and homogenous solution. The resazurin solution was kept in amber color bottle in a refrigerator until the experiment is carried out.

Preparation of microtitre plates

The experiment was carried out under aseptic conditions. Commercially available, presterilized, polystyrene, flat-bottom 96 - well microtitre plates were labeled according to the experiment plan. Different concentration (3.90 to 500µg/ml) of the hydro-alcoholic extract of polyherbal formulation 100 µL was added to the wells. A volume of 100 µL of MHB and 10 µl of resazurin dye was added to the wells and mixed gently. Finally, 10 µl of already prepared broth culture (5 × 10^6 CFU/ml) was added, wrapped the plates with paraffin film loosely to avoid dryness of media and placed in an incubator at 37°C for 24 h. For fungal strains, SDB was used in the place of MHB and incubated separately at 27 ^{o}C for 48 h. The color changes from purple to pink or colorless as the indication of antimicrobial efficacy and it is considered as MIC of respective concentration. The color change was assessed visually. The experiments were carried out in duplicate and average values are represented in table 2.

RESULTS AND DISCUSSION

The results obtained from the study indicate that polyherbal formulation (HAE-LVR05) possess antibacterial and antifungal activity in a dose-dependent manner. Among the bacterial species, the formulation is effective in *S. typhimurium* with the zone of inhibition 15.40 mm in the 500 µg/ml and the MIC is 7.81 µg/ml followed by *P. aeruginosa* with the zone of inhibition 14.63 mm and MIC 15.62 µg/ml (Table 1 & 2, Figure 1).

The mechanism of action of antibacterial agents is inhibition or regulation of enzymes involved in cell wall

biosynthesis, nucleic acid metabolism and protein synthesis known as translation inhibition. Another mechanism is the disruption of membrane structure which leads to alter the cellular functions. Most of the antibiotics are targeted to reduce the multiplication thereby killing the respective organism. Some of the phytoconstituents can bind to the membrane phospholipids of Gram-negative bacteria and disrupt the membrane integrity. The phytoconstituents may reduce peptidoglycans synthesis by inhibiting respective enzymes. The enzymatic targets of popular drugs are transpeptidases, transglycosylases, topoisomerases, RNA polymerase and peptidyltransferases. The phytoconstituents may bind one or more microbial enzymes thereby inhibition or reduction can take place that needs to be proved through research.

Table. 1: Antibacterial and antifungal activity of HAE - LVR05

S.No.	Name of the organism	Conc. of the test sample (µg/ml) and corresponding zone of inhibition (mm)						
A	**Bacteria**	**500**	**250**	**125**	**62.5**	**31.25**	**C**	**Standard**
1	*Escherichia coli*	14.50	12.01	10.24	9.94	7.38	NI*	25.85
2	*Salmonella typhimurium*	15.40	12.40	12.34	10.25	8.87	NI*	29.98
3	*Pseudomonas aeruginosa*	14.63	13.29	12.08	8.66	7.40	NI*	29.75
4	*Staphylococcus aureus*	13.64	10.85	10.14	8.98	6.92	NI*	29.74
5	*Streptococcus pyogenes*	13.45	12.75	10.81	8.97	7.04	NI*	21.58
B	**Fungus**							
1	*Candia albicans*	12.17	11.80	10.60	9.29	7.29	NI*	11.04
2	*Trichophyton rubrum*	12.09	11.18	9.57	7.90	6.24	NI*	11.04
3	*Microsporum gypseum*	10.50	9.42	9.16	8.48	6.50	NI*	11.06
4	*Epidermophyton floccosum*	12.02	10.02	9.85	8.50	6.79	NI*	11.03

C: Control (1% DMSO), Standard: Gentamicin (10µg/ml) and Clotrimazole (10µg/ml), NI: No inhibition, Values were expressed as mean

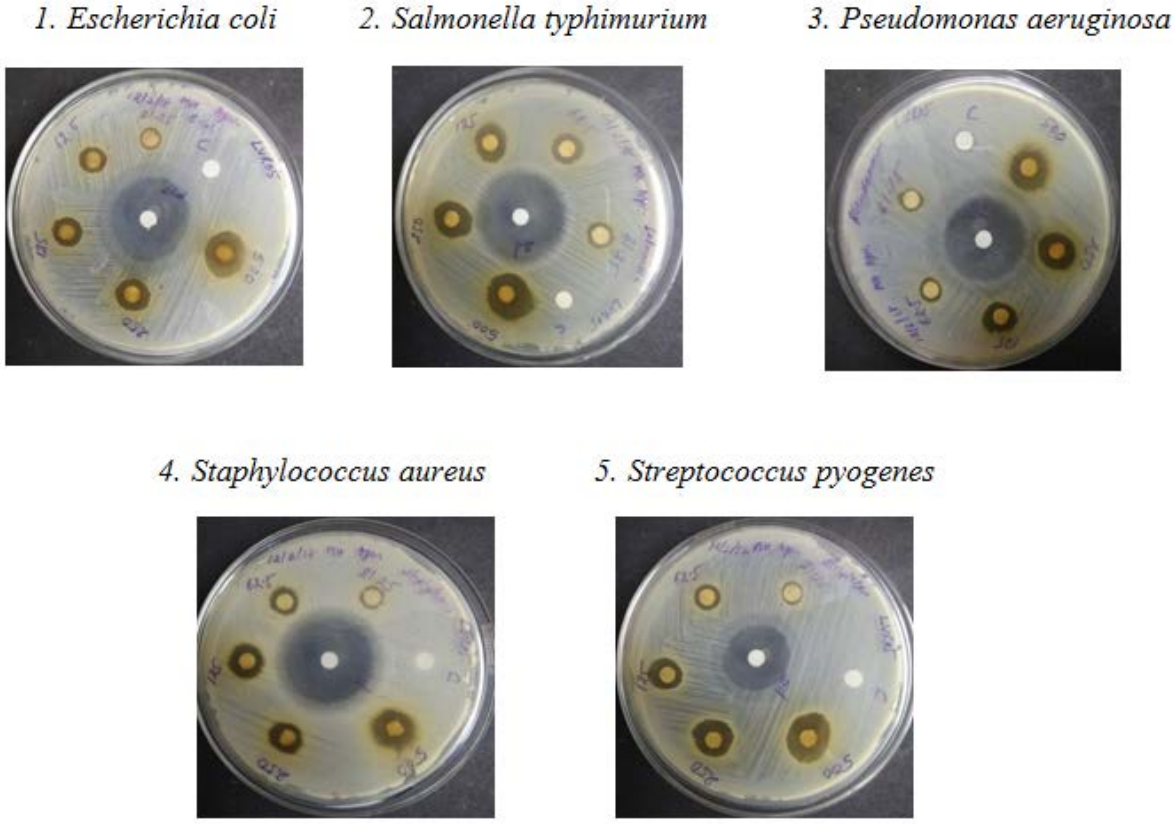

Figure. 1: Effect of HAE - LVR05 on different bacterial species

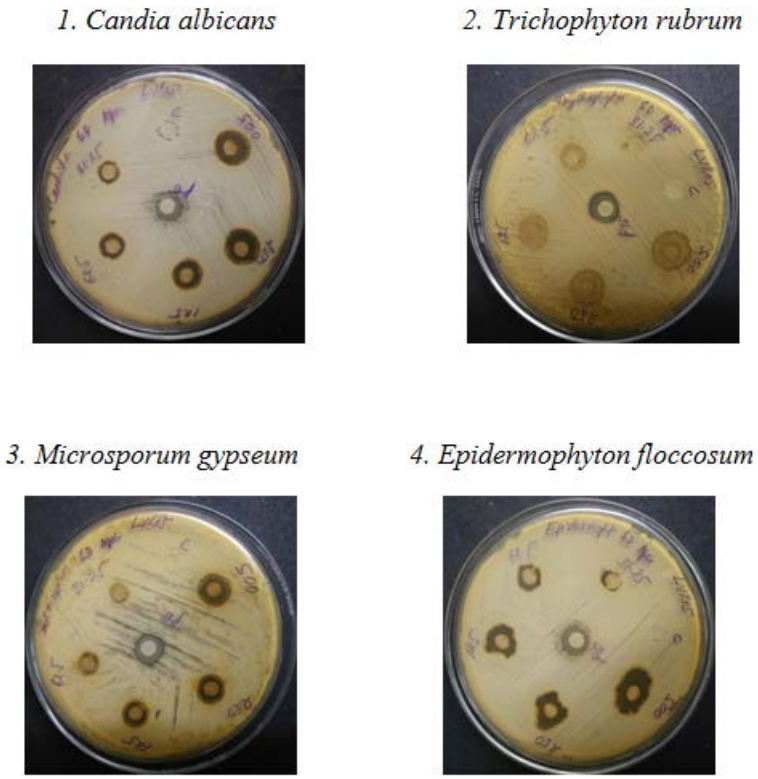

Figure. 2: Effect of HAE - LVR05 on different fungal species

Table. 2: MIC of HAE - LVR05 on different bacterial and fungal species

S. No.	Name of the organism	Test sample MIC (µg/ml)	Standard MIC (µg/ml)
A	**Bacteria**		
1	*Escherichia coli*	15.62	< 3.90
2	*Salmonella typhimurium*	7.81	
3	*Pseudomonas aeruginosa*	15.62	
4	*Staphylococcus aureus*	31.25	
5	*Streptococcus pyogenes*	31.25	
B	**Fungus**		
1	*Candia albicans*	7.81	< 3.90
2	*Trichophyton rubrum*	31.25	
3	*Microsporum gypseum*	15.62	
4	*Epidermophyton floccosum*	31.25	

Standard: Gentamicin and Clotrimazole, Values were expressed as mean

The polyherbal formulation also shows antifungal activity in a dose-dependent manner. Among fungal species *Candia albicans* responded well with a zone of inhibition 12.17 mm at the concentration of 500 µg/ml and MIC was 7.81 µg/ml (Table 1 & 2, figure 2). Mostly UTI, Candidiasis and vaginal yeast infection are caused in humans by *Escherichia coli* and *Candia albicans*. The plant extracts like *Azadirachta indica, Phyllanthus niruri* and *Picrorhiza kurroa* possess antifungal activity [25 & 26]. Most of the antifungal agents are playing a fungicidal role. The antifungal components are bind with sterols (ergosterol) and altering the permeability of fungal cell membrane which leads to membrane instability, less fluid, monovalent ions and small organic molecules are leaked out from the cell and the organism die. Some of the antifungal agents inhibit lanosterol 14 alpha-demethylase (Inhibitors – Imidazole, triazole and thiazoles) which is rate-limiting enzyme for synthesis of ergosterol. β-glucan synthase enzyme (Inhibitors – Echinocandins) [27] involves in glucan synthesis which needs for fungal growth. Squalene epoxidase (Inhibitors–allylamines) [28]. Above said enzymes are the prime targets for phytoconstituents.

CONCLUSION

The results obtained in this work showed hydro-alcoholic extract of polyherbal formulation have significant antibacterial and antifungal activity in dose dependent manner. The antimicrobial activity is due to the presence of phytoconstituents in the formulation. Further studies towards quantification of phytoconstituents and the

mechanism of action through *in silico* methods are in progress.

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