In vitro Antibacterial and Cytotoxic activities of Polyherbal extracts on 3T3 Murine Fibroblast cell lines

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Abstract

Medicinal plants by its virtue of possessing phytoconstituents exhibit promising therapeutic potential in infection control and management of burn wounds. In the present study, Alcoholic consortia extract of Achyranthes aspera linn (Apamarga) shoot, Withania somnifera (Ashwagandha) root, Ocimum sanctum (Tulsi) leaves was evaluated for its antibacterial activity on burn wound sepsis causing antibiotic sensitive and resistant strains. The cytotoxic & proliferative studies were carried out on 3T3 murine fibroblast cell line. The consortium of aqueous extract was assessed for antibacterial activity and Minimum Inhibitory Concentration (MIC) was determined at the concentration range of 0.5% (5 mg/ml) to 2% (20 mg/ml). 1.5 % (15 mg/ml) of extracts showed effective in inhibition of pathogens. Cytotoxicity, percentage viability and proliferative effects were studied using 3T3 murine fibroblast cell lines under the influence of consortium at the concentration range of 25 µg/mL to 500 µg/mL. Percentage viability analysis was conducted after 24 hours of exposure to the prepared consortial extract using the MTT [3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl -2H - tetrazolium bromide)] salt based colorimetric assay. The cytotoxicity studies of alcoholic consortium have no effect on percentage viability of fibroblast cells beyond 50% and even at higher concentration of 500 µg/mL. Migration of fibroblast cells is related to the proliferative effects in response to treatment with Polyherbal extracts. Thus, to conclude, that consortium of aqueous extract of Apamarga, Ashwagandha and Tulsi exhibited promising antibacterial activity and is found to be non-toxic as per the results from in vitro studies.

Keywords: *Medicinal Plants, Achyranthes aspera linn (Apamarga), Withania somnifera (Ashwagandha), Ocimum sanctum (Tulsi), Antibacterial potential, Cytotoxicity, % viability, Proliferation, Non-toxic.*

1: Introduction

Herbal remedies practiced in traditional folk medicine are still largely unexplored source for the development of potentially new drugs which might help to overcome the growing problem of bacterial resistance. Even though synthetic drugs have proven to be effective in treatment and management of burns, but the occurrence of resistance to antibiotics has led to paradigm shift from modern medicine to traditional therapies [1, 2].
For thousands of years, Medicinal plants are used worldwide in treatment of numerous skin ailments [3] due to their effectiveness, negligible side effects, wide range of action and relatively low cost. The plant-based products are abundant source of phytoconstituents having wide range of applications in pharmaceutical industry for the development of newer agents for treatment of skin ailments [4, 5].

As per the ethno-botanical literature survey, a great number of medicinal plants are still being used to treat microbial infections particularly in the rural areas where the traditional folk medicine remains a major source to cure various skin ailments [6]. In order to add knowledge about the possible therapeutic agents in burns, cell and tissue culture studies for better understanding mechanism of action and to evaluate efficacy of therapeutic agents are done which adds up to the scientific validation of these agents. However, in vivo experiments study of biological phenomena and clinical trials are required for verification of security aspects [7, 8].

The objective of the study was to prepare herbal consortia using *Achyranthes aspera linn* (Apamarga), *Withania somnifera* (Ashwagandha*)*, *Ocimum sanctum* (Tulsi) and screen its antibacterial efficacy against sepsis causing burn wound pathogens. In this study, we have also investigated *in vitro* toxicity of Polyherbal formulations at different doses using 3T3 cell lines. The 3T3 fibroblast cell line was chosen as the fibroblasts play a major role in the wound healing process and are one of the major cell types in dermis. The toxicity studies of these Polyherbal extracts are prerequisite to ensure safety of these extracts for further development of topical agents that can be formulated for burn wound management.

2: Materials and Methods

2.1: Collection of Herbals

Individual fresh plant parts were collected and authenticated from a botanist. The plants parts used for the study were as follows: Ocimum *sanctum* (Tulsi) leaves, *Withania somnifera* (Ashwagandha*)* roots, *Achyranthes aspera linn* (Apamarga) Shoots. Plant parts were cleaned thoroughly with water to remove dirt and were placed in the oven for drying. Dried plant parts were ground to a fine powder using a blender and were stored in containers at room temperature till extract preparation.

2.2: Preparation of Extract of herbal consortium

2.2.1: Aqueous extract of Polyherbal - Apamarga Ashwagandha Tulsi

The aqueous extract was prepared by soaking 30 grams of finely ground powder (10 grams of each powder) of Apamarga, Ashwagandha and Tulsi with distilled water. The mixture was boiled in a water bath till the quantity was reduced to ¼th of original volume. It was then filtered through muslin cloth and further heated till honey-like consistency was obtained. Then the extract was kept at 37℃ till the water evaporated completely and it was then stored in the refrigerator [9].

2.3: Isolation, Identification and Antibiogram profile of isolates

2.3.1: Bacterial strains

The standard reference strains used for the study were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC *700603*. The bacterial strains were revived by sub-culturing in fresh nutrient broth for 24hr before use. The clinical isolates were collected by a sterile cotton swab

from ICU admitted Burn patients. Collection of clinical samples were carefully carried out following safety protocols and samples were transported to laboratory in a transport medium.

2.3.2: Culture medium for isolation and identification

Clinical isolates were cultured on selective and differential media such as MacConkey's agar, Mannitol Salt Agar, CLED Agar and the plates were incubated at 37°C for 24 hours. Isolates obtained were studied for their growth and cultural characteristics. Isolates were further identified by biochemicals such as Sugar fermentation, methyl red and indole production, citrate utilization, catalase and oxidase reaction [10].

2.3.3: Studying antibiogram profile of clinical isolates

Susceptibility to a commercially available antibiotic disc was tested according to Clinical and Laboratory Standard Institute guidelines [11] by the agar disk diffusion test using Mueller–Hinton (MH) agar against the following antibiotics: Imipenem, Meropenem, Doripenem, Ertapenem, Cefepime, Ampicillin, Kanamycin, Nitrofurantoin, Gentamicin, Vancomycin, Oxacillin, Cefoxitin, Methicillin, Penicillin G, Amoxicillin, Piperacillin-Tazobactam, Amikacin, Nalidixic acid and Tetracycline.

2.4: Evaluation of antibacterial efficacy

2.4.1: Agar ditch method

Antibacterial efficacy was evaluated by agar ditch method using standard as well as clinical strains. Different concentrations of combinations of extracts ranging from 10 mg/mL to 50 mg/mL were mixed separately with molten agar butt and was poured in the ditch created on the nutrient agar plate followed by streaking of isolates perpendicular to the ditch [12].

2.4.2: Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration of the test compounds were determined by plate dilution method. The different concentration of extracts 5 mg/ml to 20 mg/ml were mixed with agar and poured in the sterile empty Petri plates. Isolates were spot inoculated onto the plates and were incubated at 37°C for 24 hours. The lowest concentration of the antibacterial agent at which the organism is completely inhibited is called its **Minimum Inhibitory Concentration** [13]**.**

2.5: Assessment of cytotoxicity *in vitro*

2.5.1: *Maintenance of Cell Lines*

Assessment of cytotoxicity of the Polyherbal formulation was studied on mouse 3T3 fibroblast cells using MTT assay. 3T3 cells were revived and 0.05 million cells were seeded in 96 well plate and were incubated overnight at 37°C in a CO_2 incubator with 5% CO_2. Post incubation, the plate was observed under microscope for observing the fully confluent cells. The cells were treated with the different concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL & 750 µg/mL) of aqueous and alcoholic extracts of Polyherbal [14].

2.5.2: MTT Assay

The MTT assay is a colorimetric method that involves measuring the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate

dehydrogenase. The principle is based on the number of cells present and the assumption that those dead cells or their products do not reduce tetrazolium. The MTT enters the mitochondria in the cells and is reduced to purple-colored formazan crystals, which are insoluble. The cells were solubilized with dimethyl sulfoxide (DMSO) and then released; the solubilized formazan reagent was measured spectrophotometrically [14, 15]. Cell viability was evaluated by the MTT assay with 3T3 mouse fibroblast cells. The cells were incubated overnight in the presence of test samples in a CO2 incubator at 37° C. After observing the cells under microscope 10µL of 5mg/mL MTT reagent was added in the wells and incubated for 4 hours. Following 4 hours incubation, the media was discarded, and the formazan crystals were dissolved by adding 100 µL of DMSO and the absorbance was measured at 595 nm

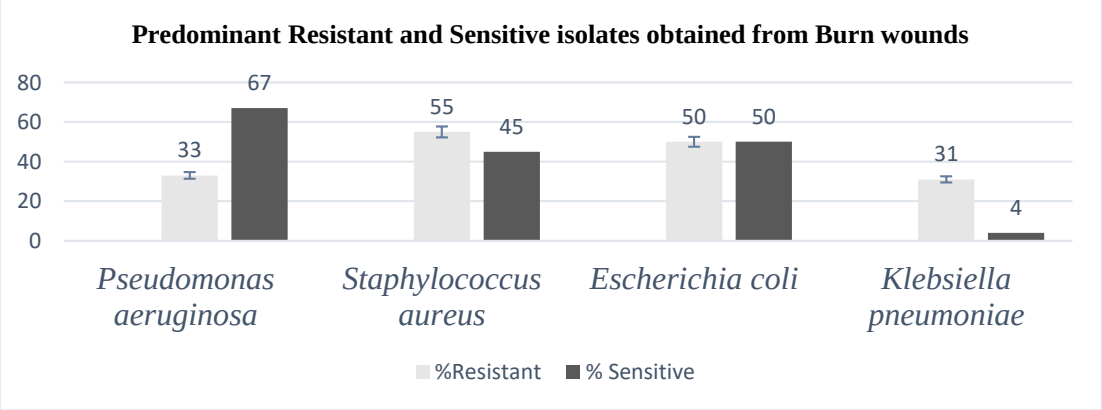
3: Results and Discussion

3.1: Collection of herbals and extract preparation

Individual plant material was collected from the botanical garden followed by washing and grinding to fine powder for further extract preparation. Extraction was carried out by mixing individual herbal powder in 1:1 ratio using water as solvent. Aqueous Extraction yielded 12.00 ± 0.25 grams of Polyherbal extract per 100 g of powder. The extraction yield depends on the type of solvent and the method used for extraction [16]. Hot aqueous extract of herbal consortium was prepared by soaking and boiling herbal powders (mixed in 1:1 ratio) in distilled water till the volume was evaporated to 1/4th of the original volume.

3.2.1: Isolation and identification of isolates

Infection in the burns is a major challenge, thus, timely collection of samples is the crucial step for monitoring and controlling burn wound sepsis [17]. Samples were collected at a weekly interval and were subjected to isolation and identification. The media selected for the study were MacConkey's agar, Salt Mannitol agar, Sabouraud's agar, Leeds Acinetobacter agar, Superimposed Blood agar and Nutrient agar. A total of 136 isolates were obtained which were identified by biochemical testing. Identification of isolates revealed the isolates belonging to genus *Pseudomonas*, *Staphylococcus*, *Klebsiella* and *Escherichia*. *Pseudomonas* spp (36%) was the commonest pathogen isolated from wound of burnt patients followed by Staphylococcus *aureus* (35%), *Escherichia coli* (15%) *Klebsiella pneumoniae* (14 %).



Graph 1: Representation of data of predominant isolates commonly found in burn wound patients

3.2.2: Studying antibiogram profile of clinical isolates

Antimicrobial sensitivity data for the most frequently isolated organisms helped in screening of resistant strains. Out of 136 isolates 63 (46%) were resistant while 73 (54%) isolates were sensitive to antibiotics. Graph 1 represents data of resistant and sensitive isolates. It is observed that the genus *Escherichia* and *Klebsiella* are predominantly resistant as compared to Genus *Pseudomonas* and *Staphylococcus*. Development of antibiotic resistant isolates is a major issue in burn wound healing. It is necessary to study the pattern of resistant development in isolates collected from burn wounds of patients for a continuous period during their hospitalization. High prevalence rates of *Staphylococcus* and *Pseudomonas* is observed due to the ability of these organisms to grow well in a moist environment which is often associated with acquiring resistance to the routinely used antibiotics [18, 19]. Thus, a paradigm shift from medicine to traditional practices is considered in recent times to eliminate the probable chances of emergence of resistance.

3.3: Evaluation of antibacterial efficacy

The antibacterial efficacy of extract consortium was elucidated on Standard (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC *700603*) as well as Clinical isolates obtained from burn wound patients. Herbal consortium showed inhibitory effect on reference strains and isolates indicating positive and effective antibacterial activity.

The consortium was further subjected to MIC determination by plate dilution method and the results are presented in table 1.

Table 1: Determination of Minimum Inhibitory Concentration (MIC) against standard strains and clinical isolates

Concentration (mg/ml) / Strains	**5**	**10**	**15**	**20**	**30**	**40**	**50**
Staphylococcus aureus 25923	+	+	-	-	-	-	-
Escherichia coli ATCC 25922	+	+	-	-	-	-	-
Pseudomonas aeruginosa 27853	+	+	-	-	-	-	-
Klebsiella pneumoniae 700603	+	+	-	-	-	-	-
Resistant isolates	+	+	+	-	-	-	-
Sensitive isolates	+	+	-	-	-	-	-

Key: +: Growth observed, -: No growth

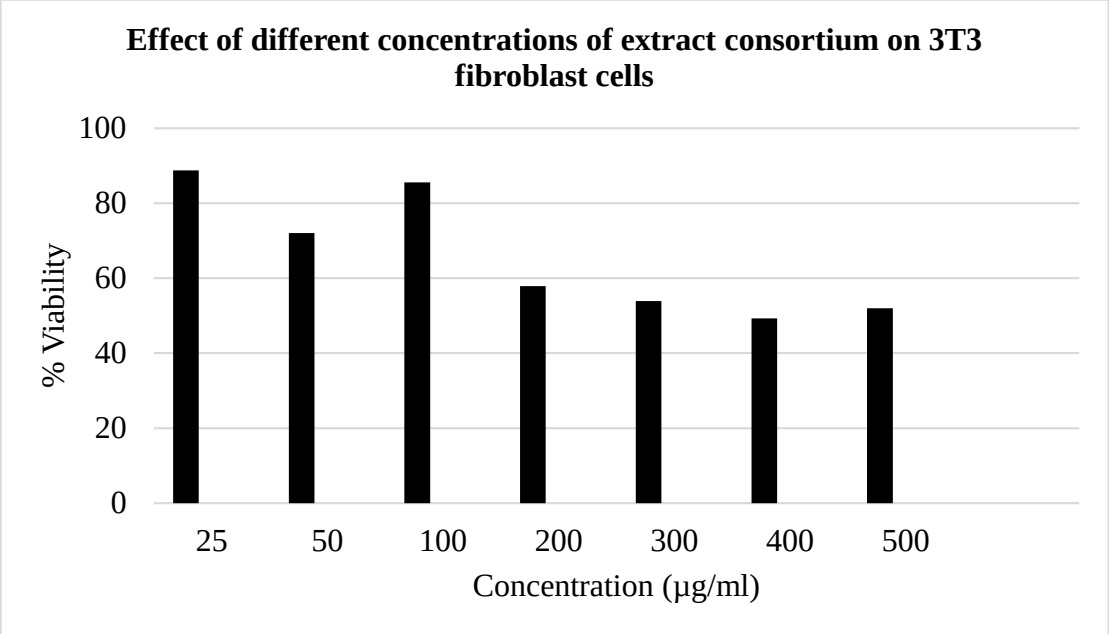
MIC determination was carried out at the concentration range of 5 mg/ml to 50 mg/ml. The range was selected based on preliminary screening of antibacterial efficacy. It was observed that the concentration of 10 mg/ml was effective against reference strains and sensitive isolates while the higher concentration of 15 mg/ml was required to show inhibitory effect against the antibiotic resistant isolates.

3.4: Assessment of cytotoxicity *in vitro*

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay relies on the principle of conversion of MTT into formazan crystals and the determination of mitochondrial activity and cell proliferation by living cells [20]. This *in vitro* method is used to determine cytotoxic effects of test compounds using cells lines. The assay records color change from yellow to purple due to conversion of tetrazolium to formazan, indicating cellular activities that there are such as cells proliferation, mitochondrial and other activities. Percentage cell viability is measured spectrophotometrically, and absorbance is plotted against viability. Stock solution of test extracts was used in the concentration 25 µg/ml to 750 µg/ml as listed below in table no2.

Table 2*:* Concentrations of extracts used for testing *in vitro* cytotoxicity

No.	1	2	3	4	5	6	7	8
Concentration used for the assay (µg/mL)	25	50	75	100	200	300	400	500



Graph 2: Percentage viability of 3T3 cells under the influence of increasing concentration of extract consortium

Graph 2 shows effect of consortium of plant extracts on percentage viability of cells, it is noticeable that the consortium has no cytotoxic effects on the cells. The concentration range of 25 µg/ml to 500 µg/ml was used for percentage viability studies. The results of the MTT test are shown in graph 2, which depicts the effect of extract consortium on 3T3 Fibroblast cells by *in vitro* method used for the cytotoxicity testing.

The results of the MTT assay that represent the cell viability of 3T3 fibroblast cells after 24 hrs of incubation as the concentration goes on increasing, viability of cells tends to decrease. Percentage cell viability in presence of aqueous extracts at the highest concentration i.e., 500 µg/mL was more than 50% while at the lowest concentration i.e., 4µg/mL, the percentage viability was more than 80%. The percent cell viability values of the extract consortium at all concentrations showed low cytotoxicity. Cytotoxicity of the sample is rated based on the percentage viability of cells under the influence of increasing concentrations of extract consortium. The toxicity of the samples is considered based on the following index: The sample is regarded as non-toxic if the viability is > 90% while the value between 60–90% viability indicates a slight toxicity and 30 –59% means a moderate toxicity. The values < 30% indicates that the sample is severely toxic to the cells [21, 22].

4: Conclusion

Extract consortium prepared by mixing equal quantities of Tulsi (leaves), Ashwagandha (root), Apamarga (shoot) exhibited significant antimicrobial activity against resistant and sensitive strains. The results obtained are promising in controlling burn wound sepsis causing pathogens and in combating the problem of resistance. Cell viability over 50% at higher concentration of extract consortium shows the good compatibility of the extract for further ointment formulation. Overall conclusion from the current study is that the consortium of extract can serve as a promising candidate for the treatment of infected burn wounds.

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