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Evaluation of phytochemical constituents and antimicrobial activity of a novel polyherbal formulation

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Abstract
The present study was performed for evaluation of antimicrobial activity of a novel polyherbal formulation(PHF) containing plants *viz*. Piper longum, Smilax china, Swertia chirata and Withania somnifera against bacteria (gram positive bacteria: S.aureus, B.subtilis and gram negative bacteria: E.coli and P.aeroginosa) and fungi(A.niger and C.albicans) responsible for various infections. The PHF was extracted using petroleum ether and ethanol to get three different concentrations of 25 µg/ml, 50 µg/ml and 100 µg/ml of each. Evaluation of phytochemical constituents revealed the presence of secondary metabolites like sugars, alkaloids, steroids, glycosides, tannins, saponins and flavonoids. Both the extracts were evaluated for antibacterial and antifungal activity by well diffusion assays. Ciprofloxacin and Miconazole were used as positive controls for antibacterial and antifungal activity respectively. All the extracts showed moderately significant antimicrobial activity. The 100 µg/ml ethanolic extract of the PHF was most effective against Candida albicans showing 30 mm zone of inhibition. The antimicrobial potency of the PHF may be due to the presence of tannins and polyphenols like flavonoids. The PHF showed synergistic broad spectrum antimicrobial activity providing scope for dosage formulation and further investigation for other pharmacological activities.

Keywords: polyherbal formulation, antimicrobial activity, extraction, phytochemical constituents, synergism

Introduction
Despite the advances in science for the treatment of diseases, man has been using plants as medicine. Plant based therapy has been used since ancient times and has attracted the attention of modern researchers to find the cure of various diseases. Traditional medicinal plants have received attention as the phytochemical constituents present maybe leads in the new drug discovery [1]. Herbal medication also called as botanical treatment or phyto medicine refers to the use of seeds, berries, roots, leaves, stem or flowers of a plant for medicinal purpose. Proper identification and appropriate quality with lack of adulteration, sophistication or substitution, is extremely important in the field of herbal medicine [2].Researchers have been exploring plants as a potential source for the development of new leads as new therapeutic agents which are safe, efficient and less toxic. Ayurveda is one of the traditional medicinal systems with a history since ancient times. Ayurveda is known as Mother of all healing [3]. Allopathic medicines use mainly synthetic chemicals designed for specific target receptors which primarily give symptomatic relief whereas ayurveda uses natural means such as diet, herbs, minerals, spices, exercise, meditation, yoga, mental hygiene, sounds, smells and mechanical procedures to eliminate the root cause of the disease. It restores the balance and creates a healthy life style to prevent the reoccurrence of the imbalance. Ayurveda aims to integrate and balance the body, mind and spirit preventing illness, promoting wellness, longevity, vitality and happiness[4]. In Ayurveda, drug formulation can be as a single drug or use of more than one drug known as polyherbal formulation (PHF) [5, 6]. PHF exploits the strategy of combining several medicinal herbs to obtain extra therapeutic effectiveness known as polypharmacy or polyherbalism [7]. PHFs are known to express high effectiveness in a number of diseases. The therapeutic effect of herbal medicines is due to the presence of a variety of phytoconstituents which is furthermore potentiated when compatible herbs are formulated together in PHFs. In a study in UK, the main reason for the usage of herbalism employing single herbs or PHFs is the effectiveness and favourable outcomes of the treatment [8]. Mostly PHFs have wide therapeutic range and are effective at low dose and safe at high dose. Antimicrobial agents are very important in reducing the global burden of infectious diseases [9]. Emergence of multidrug resistant strains in pathogenic bacteria have become a threat as there are fewer effective antimicrobial agents available for infection caused by pathogenic bacteria [10, 11]. A large number of medicinal plants are valuable resources of natural antimicrobial compounds which act as alternatively effective in treatment of resistant strains [12].The literature revealed that Piper longum [13], Smilax china [14], Swertia chirata [15] and Withania somnifera [16] possess antibacterial and antifungal activity. The aim of the present work is to develop a novel polyherbal formulation which has potential antimicrobial activity using above four plants for a synergistic antimicrobial activity.

Material and Methods
Collection of plant material
The raw plant material for the PHF namelyPiper longum, Smilax china, Swertiachirata and with aniasomnifera were bought from local market in Hyderabad and authenticated

by Dr.Abdul Samad, A.S Clinic, Hyderabad, Telanagana, India. Nutrient agar and Sabouraud medium for antibacterial and antifungal activity were purchased from Standard chemicals, Hyderabad, Telangana.

Preparation of Polyherbal Formulation

Each of the plant was cleaned and made free of any earthy matter and powdered separately using a home grinder and mixed in proportions given in the table 1 and stored in an air tight glass container. The powdered PHF was extracted with petroleum ether and ethanol by hot extraction method. Soxhlet apparatus was used to carry out the hot extraction and the temperature was maintained between 60-80^{0}C. Both the extracts were then dried at reduced pressure using rotavapour bath at 40-45^{0}C. The dried extracts were dissolved in petroleum ether (PHFP) and ethanol (PHFE) to get 25 µg/ml (PHFP1), 50 µg/ml (PHFP2), 100 µg/ml (PHFP3) and 25 µg/ml (PHFE1), 50 µg/ml (PHFE2) and 100 µg/ml (PHFE3) which were prepared using distilled water. The above PHF extracts were stored in capped glass vials.

Evaluation of Phytochemical Constituents

Both the extracts were tested for the presence of phytoconstituents like carbohydrates, reducing sugars, alkaloids, sterols, flavonoids, saponins and tannins using standard qualitative tests [17].

Evaluation of Antimicrobial Activity

The antimicrobial activity was performed by agar well diffusion method. The bacterial strains employed were Gram positive bacteria- Staphylococcus aureus and Bacillus subtilis, Gram negative bacteria- Escherichia coli and Pseudomonas aeruginosa. The fungal strains employed were Aspergillus niger and Candida albicans. The microorganisms were obtained from culture collection of Microbiology Department, Anwarul Uloom college of Pharmacy.

Methodology of antibacterial activity [18]

To determine the antibacterial activity pure bacterial culture was sub cultured in nutrient broth at 37ºC for 24 hours. About 100 µl of standardized inoculum of each bacterial strain was spread aseptically on the agar plates.With the help of a borer, cups/bores of about 6 mm diameter were aseptically made into the gelled agar plates. About 0.1 ml of different concentrations of the PHF extract are introduced into the wells/cups. Sterile DMSO was used as negative control. Ciprofloxacin (10µg/ml) was used as positive control. The plates were incubated for 24 hours at 37ºC. The antibacterial activity was calculated by measuring the zone of inhibition. The zone of inhibition (ZOI) was measured to the nearest size in mm [19]. Appearance of ZOI is related to the presence of antimicrobial action in the PHF extracts.

Methodology of Antifungal activity [20]

To determine the antifungal activity of the different concentrations of PHF, pure fungi strains were sub cultured on Sabouraud's agar (SDA) at a temperature of 28ºC for 3-5 days. A sterile borer was used to bore cups (6mm) in the SDA plates. Sterile DMSO was included as negative control while Miconazole (10µg/ml) was used as positive control. About 0.1 ml of the different concentration of PHF extract was transferred aseptically. The plates were incubated at 28ºC for 2-3 days and then the ZOI was recorded and statistically analysed.

Results and Discussion

Results for Phytochemical evaluation of the PHF

The screening of extract revealed the presence of alkaloids, steroids, glycosides, tannins, saponins and flavonoids as shown in table 2.

Results for antimicrobial activity

The petroleum ether and ethanol extract of the polyherbal formulation exhibited moderate antimicrobial activity suppressing the growth of microbes as shown in table 3 and figure 1. The standard drugs exhibited higher antimicrobial activity against all test isolates when compared with both PHFP and PHFE. It was reported previously that ciprofloxacin is effective against E.coli and P.aeroginosa[21]. Evaluation of antimicrobial activity of the PHF extracts was recorded in table 3. Ethanolic extract of the PHF was found to have more potency when compared to the petroleum extract of the PHF. The PHFE3 extract showed moderately significant activity (21-30mm) against all the six strains of microbes followed by PHFP3 (17-26mm). Individual plants have various active phytochemical constituents or secondary metabolites which are responsible for the therapeutic activity exhibited by the plant[22]. The plants with varying potency when combined together as PHF may produce a greater potency as compared to the individual plant or sum of their individual effect known as the synergism[23]. Tannins are known to inhibit many microbial enzymes in raw culture filtrates or in purified forms[24]. The astringent property of the tannins is said to be reported due to its complexation with enzymes or subtrates and metal ions[25]. Polyphenols are said to have antimicrobial activity probably due to enzymes inhibition in the oxidised form or by more nonspecific interactions with the proteins. Flavonoids are hydroxylated polyphenolic compounds known to be produced by plants in response to microbial infections were studied extensively and are reported to show antimicrobial activity against many microorganisms[26]. The PHF showed significant antimicrobial activity due to the presence of secondary metabolities like flavonoids and tannins that may be contributing to the antimicrobial action.

Table 1: Phytochemical Screening of Polyherbal Formulation

S. No.	Phytoconstituent	Name of the test	Petroleum Ether extract (PHFP)	Ethanolicextract(PHFE)
1	Carbohydrates	Mohlish test	-	-
2	Reducing Sugars	Benedicts test	-	+
3	Alkaloids	Dragendroffs test	+	+
4	Steroids	Salkowski test	-	+
5	Glycosides	Leibermanburchard test	+	-
6	Tannins	Keller killlani test	-	+
7	Saponins	Soap test	-	+
8	Flavonoids	Sulfuric acid test	+	+

Table 2: Zone of inhibition of different concentration of PHF against microbial cultures.

Concentration of the PHFextract (µg/ml)	Zone of Inhibition(mm) MEAN±SEM					
	Test organism					
	B.subtilis	S.aureus	P.aeroginosa	E.coli	A.niger	C.albicans
Petroluem Ether						
PHFP1	9.83±0.401	11.16±0.600	11±0.575	15.33±0.714	21±0.577	21.83±0.600
PHFP2	11.66±0.421	13.5±0.763	13.16±0.703	18.5±0.428	22.83±0.600	22.83±0.307
PHFP3	17±0.577	15.53±0.763	18.18±0.703	20.66±0.667	25±0.577	26.16±0.307
Ethanol						
PHFE1	10.83±0.307	11.5.±0.763	11.16±0.6009	1.8336±0401.	22±0.577	22.5±0.428
PHFE2	15.16±0.477	13.6±0.843	13.33±0.714	18.66±0.421	23.33±0.494	24.83±0.65
PHFE3	21±0.461	18.66±0.843	18±0.575	24.66 ±0494.	29.5±0.562	30.33±0.557
Standard(Positive Control)						
Ciprofloxacin	31.5±0.428	33.33±0.494	30.33±0.494	35.883±0.792	-	-
Miconazole	-	-	-	-	34.83±0.477	33.16±0.477

*Each value is Mean±SEM of 6 assays

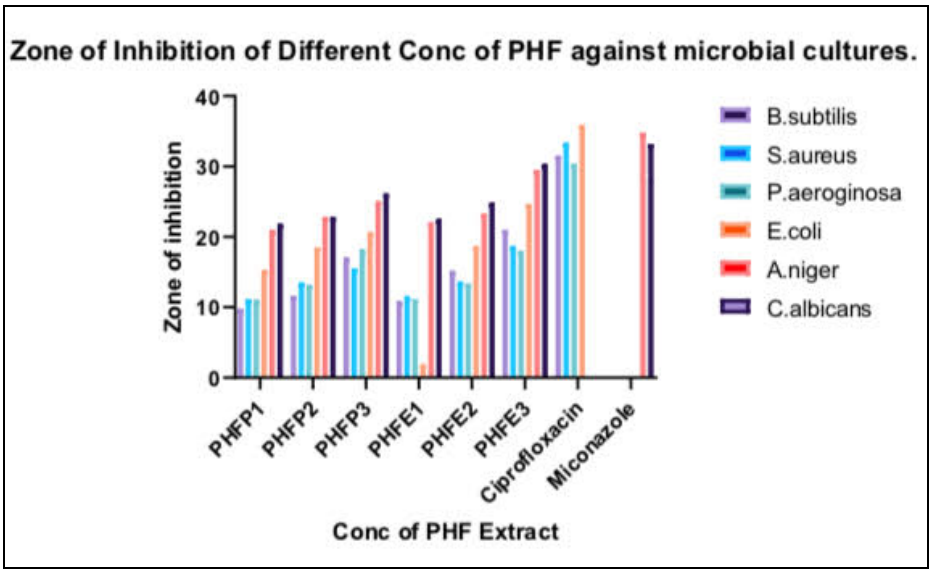

Fig 1: Antimicrobial activity of the Polyherbal Formulation.

Conclusion

The polyherbal formulation developed using a novel combination of plants *viz.* P.longum, S.china, S.chirata and W.somnifera demonstrated significant antimicrobial activity. Development of microbial resistance against antibiotics makes it essential need for effective medicinal plants as a cure from infectious diseases. Moreover PHF used in Ayurveda are plant products and are relatively cheaper, ecofriendly and readily available than the allopathic drugs. The study of herbal and medicinal plants as therapeutic agents is essential to explore the vast array of plant flora and know their efficacy in the treatment of diseases caused by microbes. Our research work can further be modified to get purified secondary metabolites and can be potential source for the discovery of new natural bioactive compounds.

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