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Basic nutritional investigation

In vitro bactericidal activity of promising nutraceuticals for targeting multidrug resistant *Pseudomonas aeruginosa*

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ABSTRACT

Objective: We evaluated the bactericidal activity of nutraceuticals against multidrug resistant *Pseudomonas aeruginosa.* The nutritionally valued herbs were screened on the basis of a matrix modeling approach and molecular docking based validation analysis.

Methods: The database of 38 herbs developed earlier using fuzzy logic based scoring analysis was subjected to molecular docking based validation. The molecular docking (Hex 6.12) analyses of predominant phytoligands (~10 per herb) against exoenzyme S of *P. aeruginosa* filtered potent herbs were selected. The preauthenticated bacterial inoculum (10^8 CFU/mL) was added to the sterile nutrient broth impregnated with standardized aqueous-alcoholic herbal extracts (1–1600 μg/mL). After overnight incubation at 37°C, antibacterial activity was evaluated in terms of minimum inhibitory and minimum bactericidal concentrations.

Results: Five herbs were selected on the basis of fuzzy set scoring, an herbal informatics model, and validation analysis based on energy of docking (i.e., E_{value} of 380) phytoligands with maximum scoring obtained by *Glycyrrhiza glabra.* Among the 5 nutraceuticals, *G. glabra* showed maximum bactericidal activity significantly ($P < 0.05$) higher than Amikacin, a standard antibiotic, which was in consonance with in silico bioprospection. *Zingiber officinale*, despite a low E_{value}, showed considerably higher inhibition attributed to its higher flavonoid content as compared to other herbs.

Conclusion: G. glabra (licorice), a flavoring agent; *Z. officinale* (ginger), a condiment; and *Mentha piperita* (mint), a fragrance component, showed significant therapeutic potential against multidrug resistant strains of *P. aeruginosa.*

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Introduction

Pseudomonas aeruginosa is a ubiquitous gram-negative opportunistic pathogen, ranked second for nosocomial infection (18%), third for urinary tract infection (16.3%), and eighth for bloodstream infection (3.4%) among various infections [1,2]. Hospitalized patients, especially those in the intensive care unit, were reported to be a carrier of these infections. Its propensity to grow on wet and dry surfaces makes it epidemiologically prevalent: 20% to 70% of the global death rate is associated with nosocomial pneumonia [3–6]. In India, the situation is alarming because 55% of cases caused by *P. aeruginosa*–induced

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pneumonia could be fatal if not managed properly [7,8]. Furthermore, the vulnerability profile of patients is also a deciding factor as nosocomial pneumonia is a major cause of death in case of cystic fibrosis patients, for example.

P. aeruginosa, with its arsenal of virulent factors, infects the host by either bypassing the first line of defense or initiates cutaneous or mucosal infection, which is attributed to their various extracellular enzymes. Type III secretion system is an important virulent factor. It works as an effector protein, injecting apparatus into the host cell. These effector proteins include exoenzyme S (ExoS), exoenzyme T, exoenzyme U, and exoenzyme Y, which cause phagocytosis inhibition by affecting the intracellular vesicular trafficking, apoptosis, actin cytoskeleton reorganization, and acute toxicity in mammalian cells by disrupting the cell membrane of infected host cell. Among them, ExoS plays a pivotal role in accelerating the cell mortality by inactivating Rho, Rac1, and Cdc42, and therefore is responsible for regulating the shape and movement of the cell by utilizing their GTPase activating protein process. Ras and Rab proteins are also affected by their ADP-ribosylation activity, resulting in inactivation of a signal transduction pathway in infected host cells that has a direct effect on the intracellular vesicular transport system [9–11]. Aminoglycosides, fluoroquinolones, and cephalosporin are current treatments; however, due to the ongoing challenge of these infections developing resistance to these drugs, finding new potent medications is necessary. ExoS provides a pharmacologically relevant target to validate novel leads against *P. aeruginosa*, playing a central role in this virulence cascade.

The holistic interventions offered by 5 nutraceuticals selected earlier using an herbal informatics approach were validated by molecular docking analyses against preselected virulence factor ExoS, an effector virulent protein of *P. aeruginosa* attributed to its multidrug resistance [1]. The nutraceuticals include *Glycyrhhiza glabra* (licorice, a flavoring agent); *Zingiber officinale* (ginger, a condiment); *Mentha piperita* (mint, fragrance component); *Terminalia chebula* (haritaki, a pickled or candied form); and *Aloe vera* (ghritakumari, a natural fiber-enriched herb) were used for evaluation of bactericidal activity at in vitro level. This study will provide the lead herbal candidates with probable therapeutic efficacy to counter such reemerging multidrug resistant threats.

Material and methods

Chemicals

All chemicals and reagents used for the study were of high purity. Ferric chloride, folin ciocalteau, sodium carbonate, sodium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, potassium ferricyanide, aluminium chloride, trichloroacetic acid, ascorbic acid, sodium hydroxide, Mueller Hinton agar, MacConkey agar, and quercetin were procured from Sigma Chemicals (St. Louis, MO, USA). Metal-free micro centrifuge tubes, pipette tips, and sterilized petriplates were obtained from Tarsons (Kolkata, India). The rest of the chemicals utilized in this study were of analytical reagent grade and were procured from reputed suppliers within India.

Softwares

The software includes Molecular Modeling Database, PubChem, OpenBabel graphical user interface (GUI), online simplified molecular-input line-entry system (SMILES) translator and structure generator (http://cactus.nci.nih.gov/translate/), and Hex 6.12.

In silico studies

Receptor

The three-dimensional crystal structure of receptor "ExoS" was taken from the Molecular Modeling Database (http://www.ncbi.nlm.nih.gov). Identification of active site/pocket of the receptor was determined by the DoG Site Scorer (a web-based server).

Ligands

Phytoconstituents from 38 herbals (selected in previous study [1]) were taken as ligands. The structural data format of ligands (phytoconstituents and tobramycin) was obtained from PubChem and was converted into SMILES formula by using OpenBabel (http://openbabel.org/docs/dev/GUI/GUI.html), respectively [12]. The Protein Data Bank (PDB) file format of all ligands was attained by online SMILES translator and structure generator.

Molecular docking

Molecular simulation was performed by using Hex 6.12 with PDB file pattern filter for both receptor and ligand. The parameters involved were shape complementarity, 3D-Fast Fourier transform mode, 0.6 grid dimension, and 180° range angle of receptor and ligand to calculate the linear relationship based binding energy of receptor-ligand complex.

Collection, authentication, extraction and chemical fingerprinting of herbal extracts

Collection and authentication of plant material

The shade-dried plant materials (1.0 kg) were procured from a reputed supplier of medicinal herbs in New Delhi, India. Fresh and dried stems of *G. glabra* Linn. (mulethi) was collected from Punjab, Northern India; rhizome of *Z. officinale* (ginger) and leaves of *M. piperata* (mint) were collected from the low altitude cultivation sites of Himachal Pradesh; dried fruits of *T. chebula* (harad) were collected from wild forests of Kanha National Park of Madhya Pradesh, Central India, and dried leaves of *A. vera* (gheekumari) were collected from agricultural farms in Gujarat plains as per the supplier's information manual. The plant material was identified and further authenticated on the basis of its botanical characteristics by ethnobotanist Dr. Rajesh Arora from the Defence Research and Development Organisation, Delhi. Care was taken to free the plant material of foreign matter like soil, dust, insects, and other extrinsic contaminants. Accordingly, voucher specimens of the plant material were stored at the Institute of Nuclear Medicine and Allied Sciences, Delhi.

Preparation of natural plant products

The dried plant material (~200 g) was pulverized in a pestle mortar until a coarse powder was obtained, which was then stored at room temperature until extraction. The powdered plant material was transferred to a Soxhlet apparatus and extracted with aquomethanolic solvent system (30:70). The extract was prepared using hot continuous percolation method for three consecutive cycles, then the respective filtrates were combined. The pooled filtrate was filtered through Whatman Paper No.1 and concentrated by the solvent evaporation in a rotary evaporator (EYELA SB-1200, Hyderabad, India) at ~65 ± 2°C, 160 to 180 rpm and reduced pressure of 40 torr. The residual solvent was removed using vacuum oven and the yield was estimated. The extracts of *G. glabra*, *Z. officinale*, *M. piperata*, *T. chebula*, and *A. vera* were designated as ACRC-14, ACRC-22, ACRC-29, ACRC-23, and ACRC-01 respectively. In the ensuing manuscript, extracts will be addressed with codes only.

Qualitative chemical Fingerprinting

The qualitative estimates of various extracts with respect to alkaloids (Wagner test); flavonoids (sulphuric acid test); saponins (Froth test); tannins (Ferric Chloride test); steroids (Lieberman test); proteins (Ninhydrin test); carbohydrates (Benedict test); glycosides (Keller-Killani test), and terpenoids (Salkowski test) were evaluated [13,14].

Quantitative estimation of total phenolic content

The total phenolic content in herbal extracts was estimated using the method of Singleton and Rossi [15]. To an aliquot of 10 μL of stock solution (1 mg/mL) of the extracts, 50 μL of 10% Folin Ciocalteau reagent, and 75 μL of double distilled water were added. The mixture was kept for 5 min at room temperature, and then 100 μL of 20% sodium carbonate solution was added. The mixture was kept for 30 min and absorbance was recorded at 765 nm using ultraviolet (UV)-visible spectrophotometer (Electronics Corporation of India Ltd, Hyderabad, India). Based on the measured absorbance, the concentration of phenolic contents was calculated in terms of milligrams of tannic acid per gram of dried extract.

Quantitative estimation of flavonoid content

Aluminum chloride colorimetric method was used for the determination of total flavonoid contents in the extracts [16]: 200 μL of double distilled water was added to 50 μL of extracts and mixed with 15 μL each of 5% sodium nitrite and 10%

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