An Investigation into the Antifungal Property of Fabaceae using Bioinformatics Tools

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Abstract

Chemodiversity in plants provides sources of great value which might be helpful for finding new leads in drug discovery programs. Fabaceae as the third largest family of flowering plants was chosen to investigate its possible antifungal activity. In order to increase the effectiveness of the result, molecular similarity methods and chemical data were used. Twelve plants were selected from Fabaceae and collected from the North and South of Iran. Percolation method with 80% ethanol was used for extraction of collected plants. Antifungal activities of these extracts were determined using broth microdilution method against *Candida albicans* (*C. albicans*) ATCC 10231, *Aspergillus fumigatus* (*A. fumigatus*) AF 293 and *Asperigillus niger* (*A. niger*) ATCC 16404. Extracts with promising activity were screened for toxicity with larvae of *Artemia salina* (brine shrimp). *Dalbergia sissoo, Lathyrus pratensis, Oreophysa microphyalla, Astragalus stepporum, Ebenus stellata, Sophora alopecuroides, Ammodendron persicum* and *Taverniera cuneifolia* showed activity against at least one of the microorganisms used in this study. According to the results of our experiment, the extracts of these plants can be used for further investigation in therapeutic research.

Introduction

There has been an increasing incidence of fungal infections in recent years, largely due to an increase of AIDS-related opportunistic fungal pathogens and the emergence of resistance strains [1,2]. Despite several available antimycotic drugs, the treatment of immunocompromised patients is still limited due to a number of factors. These include low drug potency, poor solubility of drugs, emergence of resistant strains and drug toxicity.

This situation, coupled with the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections is a serious medical problem. It sounds essential to find new sources of antifungal agents.

Plants are invaluable sources of pharmaceutical products that have drawn the attention of many scientists [3]. Fabaceae is the third largest family of angiosperm plant with approximately 730 genera* and over 19400 species worldwide, which includes the plants commonly known as legumes [4]. The family

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* a genus (plural: genera) is one of the lowest levels in taxonomic ranking and is used in the classification of living and fossil organisms

includes horticultural varieties and many species harvested as crops and for oils, fiber, fuel, timber, medicines, and chemicals. Several types of alkaloids, non-protein amino acids, amines, flavonoids, isoflavonoids, coumarins, phenylpropanoids, anthraquinones, di-, sesqui- and triterpenes, cyanogenic glycosides, protease inhibitors and lectins have been described in this family [5]. Additionally, in recent years, reports exist on antifungal activity of peptides and proteins isolated from medicinal plants that have mainly concerned species of Fabaceae family [6].

Legumes are able to fix atmospheric nitrogen via symbiotic Rhizobia in root nodules. Thus nitrogen is easily available for secondary metabolism and it is probably not surprising that nitrogen-containing secondary metabolites (alkaloids, non-protein amino acids, cyanogens, protease inhibitors, lectins) are a common theme in legumes.

The therapeutic effect of medicinal plant is related to the presence of alkaloids, terpenoides, glycosides, phenolic compounds, polysaccharides [7,8]. Fabaceae, containing several compounds of these types, can serve as a possible source for new therapeutic agents. Wide variety of species and their compounds and high cost of experimental techniques forced scientists using strategies to reduce the number of plants.

Important criteria which were previously used include *Chemotaxonomy:* When an interesting lead is found, and either a new (richer) source of the compound or related structures are sought, chemotaxonomy can point to related plant species to screen [9], *Traditional use*: Many products have traditional uses that are now being investigated to create an evidence base that will facilitate their inclusion in general medical practice [10] and many plant-derived medicines used in traditional medicinal systems have been recorded in pharmacopeias as agents used to treat infections [11], *Plant ecological observations:* Ecological theories of plant defense can increase the probability of discovering compounds with activity in bioassays against

human disease targets [12] and *Phylogenies:* Have great explanatory power and also enable a predictive perspective not offered by previous classifications of plants.

Phylogenetic selection of target species is a new approach to drug discovery in which one strategy could be to select close relative of the most active species for further investigation [13]. In order to introduce new methods with higher efficiency, we used chemical data with bioinformatics tools.

Materials and Methods

Selection strategies

The strategy for selection of cases include informatics based steps and query; the second strategy is application of chemotaxonomy, which are explained in detail below.

1- Cheminformatics strategy

A) Database formation: Literature search was performed to find plants from Fabaceae with antifungal activity. A database was made from the results of the literature search including genera and their antifungal compounds. Non-protein constituents of this database were drawn and converted to SMILES (Simplified Molecular Input Line Entry System) codes using Chem. Draw Ultra (Version 7.01, 2002 Cambridge Soft). The SMILES codes were used for similarity search.

B) Similarity search: Similarity search was performed on SMILES codes of the structures [14]; a Tanimoto cutoff score of 0.8 was applied. Following servers were used for similarity search: http://pubchem.ncbi.nlm.nih.gov, http://cactus.nci.nih.gov.

In this way, a subset of compounds similar to antifungal constituents of our database was prepared. These compounds were examined for the presence in Fabaceae as the plant source. Then, the species which existed in flora of Iran were selected.

2- Chemotaxonomy

Since secondary metabolites are often similar within members of a clade, their occurrence or absence might be taken as an

indication of common descent and thus relatedness [5].

In our strategy, genera with high antifungal values were chosen from our database. Plant species of tribes from Fabaceae which include these active genera, were selected to achieve either new sources of antifungal compounds or higher concentrations of previously known compounds.

Plant material

Plant materials were collected in March 2007-2008 in the South and August in the North of Iran. The identification of the plants was carried out in our group and for any suspected sample further validation was carried out in Department of Botany of Shahid Beheshti University by Dr Mehrabian.

The voucher specimens were deposited in the herbarium of Pasteur Institute. Aerial parts of plants were air-dried for seven days in shade at room temperature and powdered using an electric grinder. Scientific names of collected plants from Fabaceae family, location and voucher specimen numbers are shown in table 1.

Preparation of extracts

One hundred *gm* of each plant was extracted using percolation method at room temperature with 300 *ml* 80% ethanol. This procedure was repeated three times at room temperature away from sunlight. The extracts

Table 1. Scientific names of collected plants from Fabaceae family, location and voucher specimen numbers

Plant name	Province (collected from)	Voucher specimen
Dalbergia sissoo	Khusestan	86-99
Lathyrus pratensis	Mazandaran	86-72
Hippocrepis unisiliquosa	Khuzestan	85-50
Oreophysa microphylla	Tehran	86-88
Onobrychis altissima	Ardebil	88-15
Argyrolobium roseum	Hormozgan	85-36
Hymenocarpus circinnatus	Lorestan	85-59
Astragalus stepporum	Khuzestan	74-41
Ammodendron persicum	Khorasan	810-3
Sophora alopecuroides	Isfahan	85-6
Taverniera cuneifolia	Kerman	85-28
Ebenus stellata	Fars	74-8

were evaporated under vacuum at 40 ℃ by rotary evaporator (Ika, Germany). The dried residue was subjected to liquid-liquid partition using dichloromethane (DCM, Merck, Germany) and water (3:1) [15]. These two fractions were dried in vacuum for further investigation.

Antifungal activity assay

Candida albicans ATCC 10231, *Aspergillus niger* ATCC 16404, *Aspergillus fumigatus* AF293 were used as test strains. Plant extracts were dissolved in Dimethyl Sulfoxide (DMSO, Merck, Germany) to prepare the concentration of 20 *mg/ml*. Broth microdilution was carried out as a standard method for the determination of antifungal activity of plant extracts [16].

Inoculum preparation of yeast

Candida albicans was subcultured on Sabouraud dextrose agar (SDA, Merck, Germany) for 3 days at 35 ℃. The colonies were taken and suspended in sterile saline.

The resulting suspension was adjusted to produce a turbidity of 75-77% of transmittance at 530 *nm* using spectrophotometer (Bio-Rad, USA). Then the suspension was diluted 1/1000 in Sabouraud maltose broth (SMB, DIFCO, USA) [17].

Inoculum preparation of molds

Each strain of *Aspergillus* was cultured on Potato dextrose agar (PDA, Merck, Germany) medium for 7 days at 35 ℃. Spores were collected and suspended in a sterile of saline containing Tween 20 (Merck, Germany) (0.1% *w/v*). Tween 20 was added to facilitate the dispersion of the lipophilic fungal spores in water. After the heavy particles were allowed to settle the turbidity of the supernatants was measured spectrophotometrically at 530 *nm* and the transmittance was adjusted to 80–82% for *Aspergillus*.

Then, the suspension was diluted 1/100 in SMB [17]. Sabouraud Maltose Broth (SMB) was used for the screening of antifungal activities. Broth medium (100 *μl*) was added to all wells of a 96-well plate and then 40 *μl* of plant extract and 60 *μl* broth were added to

well (A), then a solution (100 μl) serially diluted from well (A) by taking 100 μl into (B) was obtained. This two-fold dilution was continued down the plate and 100 μl from the last well (H) was discarded. Then all the plates were filled with 100 μl of the inocolum. Amphotericin B (Sigma, Germany), and Itra-conazole (Zahravi, Iran) were used as positive controls and 10% DMSO as a negative control, respectively.

The plates were covered and incubated at 35 °C for 24 - 48 hr. The Minimal Inhibitory Concentration (MIC) values were defined as the concentration of the extract with no visible growth after incubation.

Toxicity screening

Extracts with promising activity were screened for toxicity with larvae (nauplii) of *Artemia salina* (brine shrimp) [18]. The eggs were placed in an aerated bottle, using electric pump, containing 33 gm/L NaCl (Merck, Germany) saline and lighting for 28 hr. After 28 hr at room temperature, the nauplii were ready for the experiment. The test was per-formed in triplicate in 96-well plate with extract concentration of 25, 50, 100, and 200 $\mu g/ml$. Ten shrimps were added to each well. The saline and gallic acid were used as negative and positive control solutions. After 24 hr, the survivors were counted under microscope and recorded. The data were analyzed by SPSS version 11.5 for probit analysis to determine LC_{50} values.

Results

Dalbergia sissoo, Sophora alopecuroides, and *Lathyrus pratensis* were selected using cheminformatic strategy and for the rest chemical data were used. Antifungal activity of ethanolic extracts of these plants was determined. The results of this assay are shown in table 2. The antifungal activity of ethanolic extracts was considered weak. The antifungal assay was repeated using DCM and water phases.

The inhibitory fungal growth by these extracts is summarized in tables 3 and 4. At the same time, control agents were used to

Table 2. Antifungal activity of plant ethanolic extracts expressed as Minimum Inhibitory Concentration, MIC ($\mu g/ml$)

Plant /Drug tested	C. albicans		A. fumigatus		A. niger	
	24hr	48hr	24hr	48hr	24hr	48hr
D. sissoo	500	2000	1000	2000	1000	2000
L. pratensis	1000	20 00	1000	2000	1000	2000
H. unisiliquosa	1000	2000	1000	2000	1000	2000
O. microphylla	1000	2000	1000	2000	1000	2000
O. altissima	1000	2000	1000	2000	1000	2000
A. roseum	1000	2000	1000	2000	1000	2000
H. circinnatus	1000	2000	1000	2000	1000	2000
A. stepporum	1000	2000	1000	2000	1000	2000
A. persicum	500	1000	1000	2000	1000	2000
S. alopecuroides	500	2000	1000	2000	1000	2000
T. cuneifolia	1000	2000	1000	2000	1000	2000
E. stellata	1000	2000	1000	2000	1000	2000
Amphotericin B	1	2	>8	>8	>8	>8
Itraconazole	< 0.156	< 0.156	0.156	0.312	0.312	0.625

Table 3. Antifungal activity of DCM phase expressed as Minimum Inhibitory Concentration, MIC ($\mu g/ml$)

Plant /Drug tested	C. albicans		A. fumigatus		A. niger	
	24hr	48hr	24hr	48hr	24hr	48hr
D. sissoo	15.62	62.5	31.25	62.5	62.5	125
L. pratensis	1000	2000	1000	2000	1000	2000
H. unisiliquosa	1000	2000	1000	2000	1000	2000
O. microphylla	125	500	1000	2000	1000	2000
O. altissima	1000	2000	1000	2000	1000	2000
A. roseum	1000	1000	1000	2000	1000	2000
H. circinnatus	1000	2000	1000	2000	1000	2000
A. stepporum	500	1000	1000	2000	1000	2000
A. persicum	62.5	250	1000	2000	1000	2000
S. alopecuroides	125	250	1000	2000	1000	2000
T. cuneifolia	125	250	1000	2000	1000	2000
E. stellata	125	125	1000	2000	1000	2000
Amphotericin B	0.25	0.5	1.56	3.12	<0.5	1
Itraconazole	< 0.12	0.25	0.156	0.312	0.312	0.625

compare the effect of extracts. There was no inhibition by DMSO solvent for microorgan-isms tested at the highest concentration tested (10% *V/V*).

DCM phase of *Dalbergia sissoo* showed strong antifungal activity against three micro-organisms. Water phase of *Lathyrus prat-ensis, Ebenus stellata, Taverniera cuneifolia*

Table 4. Antifungal activity of water phase expressed as Minimum Inhibitory Concentration MIC ($\mu g/ml$)

Plant/ Drug tested	C. albicans		A. fumigates		A. niger	
	24hr	48hr	24hr	48hr	24hr	48hr
D. sissoo	250	500	1000	2000	500	1000
L. pratensis	15.62	15.62	1000	2000	1000	2000
H. unisiliquosa	1000	2000	1000	2000	1000	2000
O. microphylla	1000	2000	1000	2000	1000	2000
O. altissima	1000	2000	1000	2000	1000	2000
A. roseum	1000	2000	1000	2000	1000	2000
H. circinnatus	1000	2000	1000	2000	1000	2000
A. stepporum	1000	2000	1000	2000	1000	2000
A. persicum	1000	2000	1000	2000	1000	2000
S. alopecuroides	500	1000	1000	2000	1000	2000
T. cuneifolia	15.62	15.62	1000	2000	1000	2000
E. stellata	62.5	125	1000	2000	1000	2000
Amphotericin B	0.25	0.5	1.56	3.12	<0.5	1
Itraconazole	< 0.12	0.25	0.156	0.312	0.312	0.625

Table 5. The result of toxicity assay on *Artemia salina* expressed as LD_{50} ($\mu g/ml$)

Plant	LD_{50} ($\mu g/ml$)
D. sissoo	389.28
L. pratensis	484.03
O. microphyalla	190.22
A. stepporum	258.83
E. stellata	263.3
S. alopecuroides	1679.93
A. persicum	538.86
T. cuneifolia	892.76
Gallic acid	20.00

and DCM phase of *Oreophysa microphyalla, Astragalus stepporum, Sophora alopecuroides, Ammodendron persicum* had strong or intermediate activity against *C. albicans*. Results of toxicity tests which were performed for active phases are shown in table 5.

Discussion

An emergence of multiple drug resistance in human pathogenic fungi and small number of antifungal classes available stimulated research directed towards the discovery of novel antifungal agents from different sources such as medicinal plants [15]. Plants are not only important in traditional medicine, 25%

of modern medicines originate from plants first used traditionally [13]. Owing to wide variety of plants, over 250,000 species of angiosperms alone [19], it is necessary to develop strategies of selecting plants.

In this paper, we used bioinformatics handling of plant chemical data to come up with a plant selection strategy. In such cheminformatics strategy, antifungal compounds of plant sources with strong antifungal activity were used as queries to find similar structures.

SMILES strings of the structures were applied to search databases of chemical structures. A SMILES string is human understandable, very compact, and if canonicalized, represents a unique string that can be used as a universal identifier for a specific chemical structure. In addition, a chemically correct and comprehensible depiction can be made from any SMILES string symbolizing either a molecule or reaction [20].

Instead of synthesizing and testing similar compounds to find out what effect they have, plant species of Fabaceae which contain similar molecules of the query were selected as new leads for our experiment. *Dalbergia sissoo, Lathyrus pratensis* and *Sophora alopecuroides* which were selected by this strategy, showed moderate to strong antifungal bioactivity. This strategy led us to a more reliable way for selection of new target species. It also reduces high cost of experiments and number of plants to be chosen.

There are no previous reports dealing with antifungal activity of plants selected in this study. However, literature is available that discussed the antimicrobial effect of other species of the genera. A herbal preparation containing *Dalbergia sissoo* and *Datura stramoium* with cow urine (DSDS), was evaluated for its antibacterial potential against pathogenic strains of gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and gram-negative (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria and the result shows that the cow urine extract of DSDS may be used as a potent antiseptic preparation for

prevention and treatment of chronic bacterial infections [21]. 5,7-Dihydroxy-3 ethylchromone and its 7-O-methyl ether were reported as phytoalexins of *Lathyrus odoratus* [22] possessing fungitoxicity against *Helminthosporium carbonum*.

Compounds sophoraflavanone G and kurarinone from the root of *Sophora flavescens*, were found to have antifungal activity against *Streptomyces bikiniensis* [23]. Finding leads, drug-like compounds that are worthy of further synthetic or biological studies, is a primary goal in a drug discovery project.

Computational search methods have been very useful in this endeavor. Similarity methods are especially useful because little information is needed to formulate a reasonable query. Many implementations of similarity methods are computationally inexpensive, so searching large databases can be routinely performed. The assumption that molecules that are globally similar in structure should exhibit similar biological activity is generally valid [24].

Chemotaxonomy could be another strategy to select species that are likely to contain antifungal compounds. Five out of nine selected species using chemotaxonomy, *Astragalus stepporum*, *Oreophysa microphyalla*, *Ebenus stellata*, *Taverniera cuneifolia* and *Ammodendron persicum*, had antifungal activity. *Astragalus brachystachys* [25] and *Astragalus verrucosus* [26] are examples of this genus with antimicrobial activity. One study on antimicrobial property of *Ebenus haussknechtii* by the agar disc diffusion showed that the most biologically active fraction was butanol against bacteria, but it was inactive against *Candida albicans* [27]. *Taverniera glabra* is also known to have acceptable antimicrobial and antifungal activity with mild toxicity [28].

It should be emphasized that in our study, antifungal activity of ethanolic extract was not significant, but fractionation using an organic solvent modified the activity of extracts. Ranganathan and Balajee [29] also reported that antibacterial activity was increased on fractionation (petrol, dichloromethane, ethyl acetate), the dichloromethane fraction of the flower extract being the most effective.

This phenomenon may be caused due to the following reasons:

1- Antagonism among the extract ingredients: In antimicrobial combinations, antagonism is defined as ≥2 log10 *CFU/ml* decrease in killing with the combination, as compared with the most active single drug alone. Adwan and Mhanna [30] presented that separation and purification of the crude extracts might show an increase in bioactivity than the crude extracts. This may be due to numerous compounds within the crude extracts which may have interfered with the actions of one another. Once they were separated by various purification methods however, the inhibiting effect of one on the other had reduced significantly.

2- Presence of nutrients in the ethanolic extract: for example sugars, help fungi's growth and reduces the influence of antifungal compounds.

3- Low concentration of antifungal compounds in the ethanolic extracts which are not enough for their antifungal property, but fractionation accumulates antifungal compounds in the active phase and improves the MIC values. Further studies are needed to understand the exact mechanism involved and the relationship between fractionation and its effect on biological activity of compounds.

4- A selective accumulation of compounds with a desired activity could take place during the fractionation to present the higher biological property in the respected fraction. This could happen due to compatible range of hydrophobicity together with other physico-chemical properties of a metabolic family of compounds with the solvent of extraction.

5- In addition to the above factors, there is co-solvation of extracted compounds with other compounds. This means, in case a compound has a preference to be extracted with extraction solvent, it can produce a preferential environment for other compounds it interacts with to be extracted along it; similar to what is seen for detergents that can help the com-

pounds normally do not solve in water, enter into aqueous phase.

Results of toxicity assay showed that all plants showing promising activity were not considered toxic compared with gallic acid. According to these results, the extracts of these plants can be used for further investigation in therapeutic research.

In future studies, we plan to increase the purity of the compounds obtained in this work and further examine our work with more test examples.

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