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THOMSON REUTERS
International Journal of Medical Research and Pharmaceutical Sciences
Volume 6 (Issue 1): January 2019 ISSN: 2394-9414
DOI- 10.5281/zenodo.2551767 Impact Factor- 4.174

REVISITING A SOUTHERN ITALIAN TRADITIONAL PRACTICE: IN VITRO ANTIFUNGAL ACTIVITY ASSAY BY SOME PLANT EXTRACTS AGAINST CANDIDA ALBICANS. PRELIMINARY DATA REPORT

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Keywords: *Candida albicans, antifungal activity, plant extracts, aqueous extracts*

Abstract

Candida species represent the most common cause of fungal infections worldwide. In this study, we have evaluated the antifungal activity of plant aqueous extracts against a standard strain of *Candida albicans*, to assess a widespread practice in their allegedly healing use in southern Italian tradition. The tested plants were: pomegranate (*Punica granatum*), rue (*Ruta graveolens*), walnut (*Juglans regia*), tomato (*Solanum lycopersicum*), garlic (*Allium sativum*). Results showed a potential antifungal and anti biofilm-forming activity in the pomegranate (fruit peels extract). This study is a preliminary antifungal activity data report and further evaluations are needed to obtain more definite information and to eventually allow the development of plants-derived new potential antifungal drugs.

Introduction

In last few years, *Candida* species infections incidence has significantly increased, with higher mortality rates and hospital acquired infections [1]. These species can cause both mild cutaneous or mucosal infections and severe systemic infections. Even though they are generally responsible for mucosal infections, including thrush and vaginitis in immunocompetent subjects, in immunocompromised patients they can lead to invasive candidiasis, with multi-drug resistant emerging species [2]. Moreover, systemic *Candida* species infections are favored by a growing use of invasive medical devices (such as intravascular and urinary catheters or implanted prostheses), an increased use of broad-spectrum antimicrobial and immunosuppressive therapies [3-5]. Predisposing or debilitating diseases (as diabetes), or compromised immune system related diseases may also increase their pathogenic potential among individuals [6-7]. Although *Candida* species counts more than 200 strains, about 10% are human pathogens [8]. Among these species, *Candida albicans* results the most frequent isolated fungus from hospitalized patients [9-10]. However, recently *Candida non-albicans* increased as a cause of fungal infections' proportion [5,9,11-12]. Despite advances in antifungal therapy and patient's clinical management, *Candida* species infections have a major impact on mortality and morbidity and on length and cost of hospitalization [13]. These issues led to research new antifungal agents. Plants can constitute a good alternative, being a safe and effective sources of antifungals. This strategy aims to solve synthetic drugs development issues, as well as their potential side effects, rapid increase in fungal infections and emergence of multidrug-resistant fungal pathogens. In the present study, different plants aqueous extracts were tested against a standard *C. albicans* strain. These plants, known as antimicrobial, were chosen by southern Italian traditions to evaluate their antifungal potential.

Materials and methods

Plant material and collection

Plant materials were collected from fully developed plants growing in Molise (Italy), from biological cultivations, during 2016, as shown in ***Table 1***. Samples collected came from previously identified species and signaled plants. Chosen plants were: pomegranate (*Punica granatum*), rue (*Ruta graveolens*), walnut (*Juglans regia*), tomato (*Solanum lycopersicum*), garlic (*Allium sativum*). Collected samples were cleaned from impurities (dust, dry or

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rotten parts) and washed three times in distilled water. The parts to use were separated, washed in distilled water once again and dried. To preserve the potential plants properties, the samples were weighed, divided in plastic bags, stored and frozen at -20 °C.

Fungal strain and medium

The *Candida albicans* strain used in this study was provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Strain DSM code number was 11225 (ATCC 90028 or CBS 8837 for other collection). Sabouraud broth and Petri Sabouraud plates were the selected mediums. Microorganism was incubated 24 hours (overnight) at 37 °C. Standard medium preparation followed the data sheet (Biolife Italiana srl), some variations were applied in order to test the plant extracts.

Extract preparation

To obtain the extract we used the water as solvent. The applied ratio was: 5 milliliter of distilled water for each gram of plant material. The preserved portions, ready for use, were combined, still frozen, to distilled water (in above mentioned proportions) in glass containers suitable for autoclaving and then autoclaved (to 121 °C at 100 kPa for 15 minutes). The extracts, brought to room temperature, were transferred into tubes and capped under a sterile hood. Subsequently, they were centrifuged at 3000 rpm for 10 minutes and the supernatant has been collected in sterile and capped test tubes. The tubes containing the plant extracts were subsequently frozen and stored at -20 °C, while a part of each extract was characterized as described in ***Table 2***.

Antifungal evaluation

Antifungal activity of crude extracts (at 20%) was evaluated by two way, on Sabouraud broth and on Petri Sabouraud plates.

Antifungal evaluation on broth

The culture medium with the plant extracts to be tested were prepared according to the following criterion: in glass containers suitable for autoclaving was suspended 1.5 g Souboraud broth preparation dust in 47.5 ml of cold distilled water added with 2.5 ml of plant extract, then autoclaved (to 121 °C at 100 kPa for 15 minutes). The control medium prepared as in the data sheet (1.5 g Souboraud broth preparation dust in 50 ml of cold distilled water). A standardized inoculum was obtained by growing *Candida albicans* 24 hours (overnight) at 37 °C and diluting that starter culture to optical density at 600 nm (OD_{600}) of 0.02. Ended the preliminary phases, evaluation started with the *Candida albicans* inoculum, for the seven condition to test, in 10 ml of media per tube (two control tubes and two tubes for each plant extracts) and incubated 24 hours at 37 °C. Subsequently the OD_{600} was checked for each tube, reporting an average data for each condition, as shown in the ***Table 3***. The colony-forming unit (CFU) per ml has been estimated from OD_{600} values by the ***Formula*** [14].

Antifungal activity evaluation on Petri plate

The Petri plates culture with the plant extracts to be tested were prepared according to the following criterion: in glass containers suitable for autoclaving was suspended 1.5 g Souboraud broth preparation and 0,9 g agar technical dust in 47.5 ml of cold distilled water added with 2.5 ml of plant extract, then autoclaved (to 121 °C at 100 kPa for 15 minutes) and distributed in Petri plates (20 ml per plate) under a sterile hood. The control medium prepared as in the data sheet (1.5 g Souboraud broth preparation dust and 0.9 g agar technical dust in 50 ml of cold distilled water). A standardized inoculum was obtained by growing *Candida albicans* 24 hours (overnight) at 37 °C and diluting that starter culture to optical density at 600 nm (OD_{600}) of 0.02. Ended the preliminary phases, evaluation started with the inoculum for the seven condition to test per Petri plates (divide in two control plates and two plates for each plants extract) and incubated 24 hours at 37 °C. Subsequently the growth has been checked for each Petri plate, reporting a qualitative data for each condition, as shown in the ***Table 4***.

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Results and discussion

The rise in antimicrobial resistance is considered a relevant public health issue, with an estimated 700,000 people dying every year from antibiotic, antiviral, antifungal and antimalarial resistance infections [15]. Therefore, natural products can play an important role as a source in new drugs development. This study wanted to give preliminary data about antifungal activity of some plants extract. Our research started by the southern Italian traditional usage of some plants in poultices and decoctions upon wounds and skin infections. Although the antibiotic properties were known for some among these plants, their potential antifungal activity is little studied [16-26]. Even if more studies worked upon different extractive method, particularly on essential oils [27-29], we evaluated aqueous extraction to reveal a possible scientific explanation of traditional healing use of plants. Indeed, the main extractive method in traditional use was based on the aqueous extraction to prepare poultices and decoctions. Tested plants showed concordance in the results, in the evaluation on broth and on Petri plates (***Table 3*** and ***Table 4***). On broth, among the extracts, pomegranate demonstrated a major antifungal activity, particularly the fruits peels extract. Despite our expectations, the other extract seemed to favor, or not to contrast, the fungal growth.

From Petri plates, although the results were the same with a more direct and visible reconfirmed antifungal activity, we noted a relevant reduction in biofilm production. Our evaluation on crude extracts is only a preliminary study and a starter point for further research. In prospective, evidenced result could find applications in the disposal and reuse of waste in agri-food industries (pomegranate fruits peels).

Formula. Fungal cells per ml, approximate estimation [14]

$$CFU/ml \cong \frac{OD_{600} x 10^6}{0,1}$$

Table 1. List and description of the plants and their parts used for the study with assigned code

Plant	Scientific plant name	Collection times	Used part	Assigned code
Pomegranate	*Punica granatum*	October	Fruit – peels	PW
			Fruit – seeds	PR
Rue	*Ruta graveolens*	Early July	Leaves	R
Walnut	*Juglans regia*	Early June	Leaves	W
Tomato	*Solanum lycopersicum*	Early July	Leaves	T
Garlic	*Allium sativum*	End of June	Bulb	G

Table 2. Plant extracts description: color and pH

Plants extract - code	Color	pH
PW	ocher yellow	≤ 5
PR	pink	≤ 5
R	straw yellow	5
W	brown	5,5
T	straw yellow	5
G	opalescent yellow	6

Table 3. Optical Density at 600 nm of the tested conditions and CFU/ml (approximate estimation)

Condition tested	OD_{600}	CFU/ml
Control	1.725	1.7×10^7
PW	0.718	7×10^6
PR	1.785	1.7×10^7

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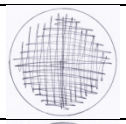
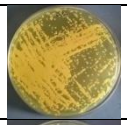
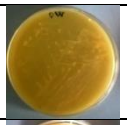
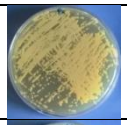
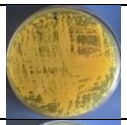
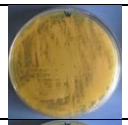
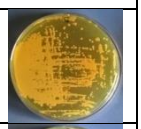
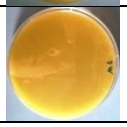
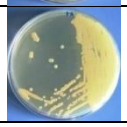
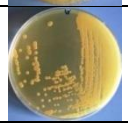
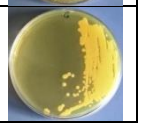
R	1.845	1.8×10^7
W	1.877	1.8×10^7
T	1.727	1.7×10^7
G	1.765	1.7×10^7

Table 4. Petri plates. Inoculum scheme and qualitative features of the tested conditions

Petri plates	Ionoculum scheme	Control	PW	PR	R	W	T	G
Plate 01								
Plate 02								

Conclusion

We can conclude from the current study upon five plants aqueous extracts that pomegranate (peel extract) presents active compounds antifungal against *Candida albicans*. It is necessary a more refined preparation of experimental trials and if these antifungals were effective they could be proposed as experimental therapeutic agents. Indeed, the extracts in our study are crude and the data define a preliminary research line. However, these results can form the basis for further studies on toxicity, active compounds isolation and their evaluation against a wider range of resistant fungal strains with the goal to find new therapeutic principles. Finally, the ultimate result of this study explains the empiric use of plant extracts in the traditional medicine in treating different infections. It also suggests that more attention should be paid to plants which seem to have pharmacological properties that could be considered a natural alternative and an opportunity in new infections treatments' research.

Contributions

Andrea Mariano as corresponding author contributed to literature search, organization and realization of the experimental phase, data collection and interpretation, to prepare tables and writing the article; all other authors contributed equally to the experimental phase and to the article drafting.

Acknowledgements

To my grandfather Andrea, who taught me to respect nature and to learn to observe it to appreciate the wonders and treasures that it gives us.

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Volume 6 (Issue 1): January 2019 ISSN: 2394-9414
DOI- 10.5281/zenodo.2551767 Impact Factor- 4.174

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