

Exploring Southern Italian Traditional Remedies: In Vitro Antifungal Activity of Plant Extracts Against *Candida albicans*. A Preliminary Study

Giovanni Russo¹, Marco Ferraro¹, and Elena Bianchi^{2*}

¹ Department of Biomedical Sciences, University of Calabria, Cosenza, Italy

² Department of Agricultural Sciences, University of Calabria, Cosenza, Italy

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.

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

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 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 (OD₆₀₀) 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 OD₆₀₀ 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 OD₆₀₀ 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 (OD₆₀₀) 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**.

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]

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

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	<i>Punica granatum</i>	October	Fruit – peels	PW
			Fruit – seeds	PR
Rue	<i>Ruta graveolens</i>	Early July	Leaves	R
Walnut	<i>Juglans regia</i>	Early June	Leaves	W
Tomato	<i>Solanum lycopersicum</i>	Early July	Leaves	T
Garlic	<i>Allium sativum</i>	End of June	Bulb	G

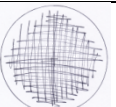
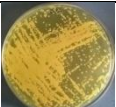
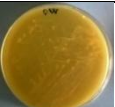
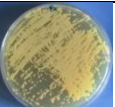




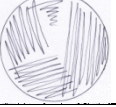






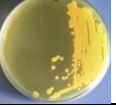
Table 2. Plant extracts description: color and pH

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 x 10 ⁷
PW	0.718	7 x 10 ⁶
PR	1.785	1.7 x 10 ⁷
R	1.845	1.8 x 10 ⁷
W	1.877	1.8 x 10 ⁷
T	1.727	1.7 x 10 ⁷
G	1.765	1.7 x 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.

Reference

1. Nur Y. Epidemiology and risk factors for invasive candidiasis. *Ther Clin Risk Manag.* 2014; 10: 95-105.
2. Colombo AL, Júnior JNA and Guinea J. Emerging multidrug-resistant *Candida* species. *Curr Opin Infect Dis.* 2017; 30(6): 528-538.
3. Nett JE and Andes D. Fungal Biofilms: In vivo models for discovery of anti-biofilm drugs. *Microbiol Spectr.* 2015; 3(3): E30.
4. Kojic EM and Darouiche RO. *Candida* Infections of Medical Devices. *Clin Microbiol Rev.* 2004; 17(2): 255-267.
5. Pfaller MA, Andes DR, Diekema DJ, Horn DL, Reboli AC, Rotstein C, Franks B and Azie NE. Epidemiology and outcomes of invasive candidiasis due to non-*albicans* species of *Candida* in 2,496 patients: data from the Prospective Antifungal Therapy (PATH) registry 2004-2008. *PLoS One.* 2014; 9(7): e101510.
6. Pilmis B, Puel A, Lortholary O and Lanternier F. New clinical phenotypes of fungal infections in special hosts. *Clin. Microbiol. Infect.* 2016; 22,681-687.
7. Garber G. An overview of fungal infections. *Drugs.* 2001;61 Suppl 1:1-12.
8. Manzoor S, Ugena L, Tornero-López J, Martín H, Molina M, Camacho JJ and Cáceres JO. Laser induced breakdown spectroscopy for the discrimination of *Candida* strains. *Talanta.* 2016; 155: 101-6.
9. Pfaller MA and Diekema DJ. Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem. *Clin Microbiol Rev.* 2007; 20(1): 133-163.
10. Lohse MB, Gulati M, Johnson AD and Nobile CJ. Development and regulation of single- and multi-species *Candida albicans* biofilms. *Nat Rev Microbiol.* 2018; 16(1):19-31.
11. Quindós G. Epidemiology of candidaemia and invasive candidiasis. A changing face. *Rev Iberoam Micol.* 2014; 31(1):42-8.
12. Gong X, Luan T, Wu X, Li G, Qiu H, Kang Y, Qin B, Fang Q, Cui W, Qin Y, Li J and Zang B. Invasive candidiasis in intensive care units in China: Risk factors and prognoses of *Candida albicans* and non-*albicans* *Candida* infections. *Am J Infect Control.* 2016; 44(5):e59-63.
13. Tanwar J, Das S, Fatima Z and Hameed S. Multidrug resistance: an emerging crisis. *Interdiscip. Perspect. Infect. Dis.* 2014; 541340.
14. Xiao W (ed.). *Yeast Protocols. Methods in Molecular Biology*, Humana Press. 2014; vol. 1163.
15. Brogan DM and Mossialos E. A critical analysis of the review on antimicrobial resistance report and the infectious disease financing facility. *Global Health.* 2016; 12:8.
16. Alexandre EMC, Silva S, Santos SAO, Silvestre AJD, Duarte MF, Saraiva JA and Pintado M. Antimicrobial activity of pomegranate peel extracts performed by high pressure and enzymatic assisted extraction. *Food Res Int.* 2019; 115:167-176.
17. Sukumar MR and König B. Pomegranate extract specifically inhibits *Clostridium difficile* growth and toxin production without disturbing the beneficial bacteria in vitro. *Infect Drug Resist.* 2018; 11:2357-2362.

18. Scanone AC, Gsponer NS, Alvarez MG and Durantini EN. Porphyrins containing basic aliphatic amino groups as potential broad-spectrum antimicrobialagents. *Photodiagnosis Photodyn Ther.* 2018; 24:220-227.
19. Sivakamavalli J, Deepa O and Vaseeharan B. Discrete nanoparticles of ruta graveolens induces the bacterial and fungal biofilm inhibition. *Cell Commun Adhes.* 2014; 21(4):229-38.
20. Kavuncuoglu H, Kavuncuoglu E, Karatas SM, Benli B, Sagdic O and Yalcin H. Prediction of the antimicrobial activity of walnut (*Juglans regia* L.) kernel aqueous extracts using artificial neural network and multiple linear regression. *J Microbiol Methods.* 2018;148:78-86.
21. Dolatabadi S, Moghadam HN and Mahdavi-Ourtakand M. Evaluating the anti-biofilm and antibacterial effects of *Juglans regia* L. extracts against clinical isolates of *Pseudomonas aeruginosa*. *Microb Pathog.* 2018; 118:285-289.
22. Irving, GWJ, Fontaine TD, and Doolittle SP. Partial Antibiotic Spectrum Of Tomatin, an Antibiotic Agent from the Tomato Plant. *J Bacteriol.* 1946; 52(5): 601-607.
23. Bellino A, Lofrano G, Carotenuto M, Libralato G and Baldantoni D. Antibiotic effects on seed germination and root development of tomato (*Solanum lycopersicum*L.). *Ecotoxicol Environ Saf.* 2018;148:135-141.
24. Yetgin A, Canlı K and Altuner EM. Comparison of Antimicrobial Activity of *Allium sativum* Cloves from China and Taşköprü, Turkey. *Adv Pharmacol Sci.* 2018; 2018:9302840.
25. Reiter J, Levina N, van der Linden M, Gruhlke M, Martin C and Slusarenko AJ. Diallylthiosulfinate (Allicin), a Volatile Antimicrobial from Garlic (*Allium sativum*), Kills Human Lung Pathogenic Bacteria, Including MDR Strains, as a Vapor. *Molecules.* 2017; 22(10). pii: E1711.
26. Majewski M. *Allium sativum*: facts and myths regarding human health. *Rocz Panstw Zakl Hig.* 2014; 65(1):1-8.
27. Hammer KA, Carson CF and Riley TV. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol.* 1999; 86(6):985-90.
28. Ghafari O, Sharifi A, Ahmadi A and Nayeri Fasaei B. Antibacterial and anti-PmrA activity of plant essential oils against fluoroquinolone-resistant *Streptococcus pneumoniae* clinical isolates. *Lett Appl Microbiol.* 2018; 67(6):564-569.
29. Lemes RS, Alves CCF, Estevam EBB, Santiago MB, Martins CHG, Santos TCLD, Crotti AEM and Miranda MLD. Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria. *An Acad Bras Cienc.* 2018; 90(2):1285-1292.