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Demonstration of the antifungal activity of the *aqueous macerated extract* of pomegranate (*Punica granatum Linn.*) bark against *Rhodotorula sp.*

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ABSTRACT

Phyto-therapy is considered as a complementary approach, to prevent and treat diseases, for this reason, we have tried in this study, to evaluate the antifungal activity of aqueous macerated extract of Punica granatum bark (MAEG),against Rhodotorula sp.; provided by the Medical Analysis Laboratory of the Sidi-Bel-Abbés University Hospital Center(Algeria);this yeast is considered as one of the emerging pathogenic yeasts, particularly in immune-compromised patients, responsible for meningitis, Moreover; according to our results, we noticed that the inhibition of Rhodotorula sp., was marked by the MAEG at 50 mg/ ml, where the inhibition zones diameters was 29.5±0.5mm. In addition, Rhodotorula sp. presented a Minimum Inhibitory Concentration (MIC) range of (0.39≥MIC≥0.195)mg/ ml for this extract with a Minimum Fungicidal Concentration MFC≥0.39mg/ml.

Keywords: antifungal, *aqueous extract macerated* bark (MAEG), *Punica granatum, Rhodotorula sp.,* MIC.

INTRODUCTION

Plant diversity serves humanity as a renewable natural resource, for a variety of biologically active chemicals products. These phyto-chemicals products have a variety of antifungal and other properties (Parajuli *et al*., 1998), these active constituents of aromatic and medicinal plants are less phytotoxic and readily biodegradable (Fawcett and Spencer, 1970). A portion of the world's population uses traditional medicine to heal. This widespread use is explained by, its accessibility and availability of this medicine in developing countries on the one hand, and the harmful effects of synthetic drugs on the other. Hence the current craze for the study, valorization and exploitation of the wealth of medicinal plants. For this reason, it seemed interesting to study a medicinal plant already used in traditional medicine. During this work within the framework of the valorization of by-products of vegetable origin, we chose the pomegranate (*Punica granatum*), which is a popular fruit of tropical and subtropical regions, belonging to the family *Punicaceae* (Ghosh *et al.,*2015); whose bark was the subject of valorization and exploitation of its antifungal properties against *Rhodotorula sp.*; this yeast has emerged as an opportunistic agent over the last twenty years, particularly in immune-compromised patients (Hazen,1995), It has also been reported, that this yeast causes eye infections and endophthalmitis, especially in immune-compromised patients (Gyaurgieva *et al*., 1996).

MATERIALS AND METHODS

Plant material

The plant material was made from pomegranate bark *(P. granatum Linn)*. To recover the peels, the pomegranate fruit was harvested during the month of November 2008, in the locality of Sidi-Ali-Benyoub, 25 km south of the town of Sidi-Bel-Abbés ; the peels were authenticated, the Reference specimens of the plant, were sent for identification and confirmation to the Plant Taxonomy Laboratory by Professor Benhassaini.H, at the Environment Department of the Faculty of Natural Sciences and Life, Djillali Liabés University of Sidi-Bel-Abbés, where the bark samples were deposited and coded under N°. PGE 2008 (Kanoun *et al*., 2014 and 2016).

Preparation of powder

The fruit was washed with tap water; the peels were dried in the shade, away from humidity and light, and at room temperature for one (01) month, on a wooden plate. After drying, they were crushed in a traditional mortar and then pulverized by manual grinding, until a very fine powder was obtained. This powder was stored in a hermetically sealed container, in the refrigerator at +4°C, and will be used later for the preparation of the studied *extract* (Kanoun *et al.,* 2014 and 2016).

Aqueous cold maceration of *Punica granatum* peel MAEG

Two hundred (200) g of peel powder was added to 1000 ml of distilled water; the mixture was stirred for 24 hours at laboratory temperature, using a magnetic stirrer at a speed of 500 rpm. This mixture underwent a double filtration on hydrophilic cotton and then a filtration on Whatman paper (No.3). After 24 hours of stirring, the macerate was filtered on Whatman paper (No.3) under reduced pressure, the separated pomace was subjected to a second extraction, by mixing 800 ml of the latter with 100 ml of distilled water, under the same operating conditions as at the start, the recovered supernatants were concentrated by the lyophilizer; the lyophilized *extract* was labelled MAEG (Kanoun *et al.,* 2014 and 2016).

Calculation of yield

The yield in g of *extract* relative to the mass of the plant material to be treated, was calculated according to the following formula: R=m 100/m°, R: yield of the crude *extract* in percentage (%), m: the mass of the crude *extract* obtained after extraction (g), m°: the mass of the plant material (g) (Caree, 1953), the *extract* obtained was preserved and stored in tubes covered with aluminum foil, in the refrigerator at + 4 ° C and protected from light, until it use for bioassays (Berahou *et al.,* 2007).

Preparation of the tested *extract*

The lyophilized *extract* was dissolved in *aqueous* DMSO at 10%, knowing that this concentration is not toxic to germs (Pujol *et al.,* 1990), a final concentration of 50 mg /ml, was filtered through a sterile *Millipore* Membrane *Filter* of *0.45 µm* pore size. The concentration range of the plant *extract* was prepared in test tubes by using, a geometric progression of 2 dilution method ,with concentrations ranging from 50 mg/ml to 0.195 mg/ml (Siri *et al.,* 2008; Kanoun *et al.,* 2014 and 2016).

Preparation of the inoculum

The suspension of *Rhodotorula. sp* vegetative cells was prepared in 10 ml of Tryptone-salt solution (De Billerbeck *et al.,* 2002), then vortexed for 3-5 min (Kannan *et al*., 2010). The 24-hour culture, which was seeded on Sabouraud agar and incubated at 30°C, was removed in the needle of Platinum loop. The fungal suspension was well homogenized, its opacity should be equivalent to 0.5 Mc Farland (10^6 cells/ml) at 530 nm optical transmission (Amoo *et al.,* 2009; Kanoun *et al.,* 2016).

Susceptibility tests

Agar diffusion method

Method of diffusion in a solid medium: (disc method test)

The used method principle (Kirby-Bauer Method, 1996) is to measure the diameter of the microbial growth inhibition zone, around a source of antimicrobial deposited on the surface of the agar. As soon as the discs are applied, the antifungal agents diffuse uniformly. After incubation, the discs are surrounded by circular inhibition zones, corresponding to an absence of culture. A concentration gradient is obtained by diffusion of the antimicrobial product from the source zone. Measurement of the inhibition zone took place after 24 h of incubation at 37°C (Kanoun *et al.,* 2014 and 2016).

Inoculation of microbial strains

The inoculation was carried out by flooding with 5 ml of the tested strains solutions on Petri dishes, containing 10 ml of Muller Hinton agar, cast at a thickness of 4 mm, it was carried out within 15 minutes following the inoculums preparation (Berahou *et al.,* 2007; Kannan *et al.,* 2010; Kanoun *et al.,* 2014 and 2016).

Disks deposits and incubation

Using sterile forceps, sterile discs of Whatman paper No.1 (5 mm of diameter) (Voravuthikunchai

et al., 2005), were impregnated with 10 µl (500 µg/disc) of *extract* (Senhaji *et al.,* 2005) at different concentrations from the stock solution, prepared in DMSO (10%) (Thitilertdecha *et al.,* 2008), these discs were then deposited on the agar surface, and then pressed to ensure their application. Discs impregnated with DMSO (10 µl) served as a negative control, were also deposited on the surface of the inoculated agar, each test was repeated 3 times (Berahou *et al.,* 2007), this yeast was incubated in oven at 30°C (De Billerbeck *et al.,* 2002), for 48 h (Senhaji *et al.,* 2005; Hammer *et al.,* 1996; Kanoun *et al.,* 2014 and 2016).

Results reading

The biological activity is manifested by the appearance of a clear zone, or a halo of microbial growth inhibition, around the discs containing the *extract* to be tested, the larger the zone surrounding the disc, the more sensitive the germ is. The reading results was taken by measuring the observed inhibition diameter, expressed in mm (Govinden-Soulange *et al.,* 2004, Al-Zorky, 2009; Kanoun *et al.,* 2014 and 2016).

Determination of antifungal parameters by the macrodilution technique

Preparation of the concentration range of the herbal substance

The concentration range of the plant *extract* was prepared in test tubes, by the dilution method (NCCLS, 1993) in a geometric progression of 2, with concentrations ranging from 50 mg / ml to 0.195 mg /ml (Benjilali *et al.,* 1986). Based on the results of the susceptibility tests, and in order to specify the MIC values, a series of *extract* dilution was carried out in sterile distilled water to *Rhodotorula sp*. (Kanoun *et al.,* 2014 and 2016).

Inoculation of microbial strains

In a series of test tubes numbered Tt1, Tt2, Tt3 ... etc, we introduced 1ml of liquid Sabouraud, inoculated by *Rhodotorula sp*. Then we added in these same tubes, 1ml of MAEG concentration, according to the concentration range prepared. For the last tube, instead of the *extract*, it received 1 ml of sterile distilled water, which served as a growth control. The first tubes were called experimental tubes, or tests and the last tube was noted growth control tube, or TC (Moroh *et al.,* 2008), these inoculated tubes were incubated at 30°C for 48 h (Johann *et al.,* 2008; Motiejûnaitë and Peèiulytë, 2004), this after having noted the values of their initial turbidity read by UV-visible spectrophotometer (Kanoun *et al.,* 2014 and 2016).

Reading and enumeration of germs

The enumeration of the germs after incubation was carried out directly on liquid medium (Sabouraud broth), by measuring turbidity, with a densimeter designed to measure the density of the inoculums, produced in a micro-cell with 1 cm of optical path, the enumeration was carried out by making the difference between the value of optical density (D.O), measured before and after incubation for each tube (Moroh *et al.,* 2008; Kanoun *et al.,* 2014 and 2016).

Determination of Minimum Inhibitory Concentration (MIC)

The evaluation of the Minimum Inhibitory Concentration consists of determining the lowest concentration, of an antimicrobial agent, required to completely inhibit fungal growth (Senhaji *et al.,* 2005, Vasconcelos *et al.,* 2006) ; the MIC was determined by using a dilution range of the agent, added to a series of tubes of liquid culture medium Sabouraud (Andrews, 2001; Sarker *et al.,* 2007), its determination was based on the measurement of turbidity induced by the growth of *Rhodotorula sp.* (Moroh *et al.,* 2008), inhibition resulted in the absence of visible culture in the tubes (Sanou, 1997). Consequently, it is the first tube where the value di was equal to df (di = df), with (di: the D.O value of the test tube before incubation and df: the D.O value of the test tube after incubation).The minimum inhibitory concentrations were expressed in mg /ml (Johann *et al.,* 2008); so, the MIC was indicated in the dilution tube from which no fungal growth was observed. That is, no turbidity was observed in the medium (Moroh *et al.,* 2008; Kanoun *et al.,* 2014 and 2016).

Determination of the Minimum Fungicidal Concentration (MFC)

The Minimum Fungicidal Concentration MFC is the concentration of the antimicrobial that leaves at most 0.01% of surviving yeasts. For its determination, the control tube was diluted to 10^{-4}.

Table 1: Appearance and Performance of MAEG

Extract	Texture, color and odor	Yield ± SD (%)
Peel pomegranate *aqueous extract* (MAEG)	Powder brown	54,36±0,06

SD = Standard Deviation

Table 2: Categorization criteria according to the critical (D and d), I (intermediate), R (resistant), S (Sensitive) values of *Rhodotorula sp.* with MAEG

		Phenotypic characteristics of the strains tested (S, I, R)				
Pathogenic yeast	***Extract***	**50 mg/ml**	**25 mg/ml**	**12,5 mg/ml**	**6 ,25 mg/ml**	**3,125 mg/ml**
Rhodotorula sp.	**MAEG**	S	S	I	I	R

This dilution represented 0.01% of survival. It was sub-cultured by 5 cm streaks on Mueller Hinton agar and incubated at 30°C for 48 h (Espinel-Ingroff, 1998; Portillo *et al.,* 2005). The number of germs obtained on the streak of the 10^{-4} dilution, was compared to that of each experimental tube, also transplanted by streaks of 5 cm, so that the first experimental tube whose number of germs present on its streak was less than or equal to that of the 10^{-4} dilution corresponded to the MFC (Moroh *et al.,* 2008; Kanoun *et al.,* 2014 and 2016).

Statistical study

Statistical analysis of the results was performed using, the SAS Institute's Statview 5.0 statistical program. Means were compared using the ANOVA test. The test was considered significant for values of $p<0.05$. We used the correlation coefficient r and covariance to compare the variables and their associations. The mean inhibition diameter of the 3 assays for each concentration was calculated for the extract and strain tested (Sineenat *et al.,* 2008).

RESULTS AND DISCUSSION

Yield of the *extract* obtained

Based on the results obtained, we noticed that the MAEG *extract* is characterized by its yield (Table 1), and by its color (Fig. 1)**.** Our extraction resulted in a high yield of 54.36 ± 0.66%, which could be explained by the high water miscibility of the substances contained in the plant *extracts*. Our results did not agree with those found by (Voravuthikunchai *et al.,* 2004), who showed in their study, that the bark *aqueous extract* presented a yield of 8% compared to the bark ethanolic *extract* of the same fruit that gave a yield of 13.0%.

Thus, the different results obtained in our study and those of other researchers could be explained by, the difference in solubility of the components of the raw plant *extracts*, which vary according to the solvent used, and the manner in which they are prepared (Ciulei, 1982). However, the extraction yields depend on several factors including, the part of the plant used (leaves, stems, roots, whole plants, etc.), the extraction method and the concentration of active ingredients (Wagner, 1993; Thangara *et al.,* 2000).Water seems to be the best solvent for extracting the majority of chemical constituents from the bark of this fruit, responsible for different biological activities, demonstrating the relevance of the traditional form of use (Sanogo *et al.,* 2006).

This water extraction capacity has been proposed to be attributed to high levels of polypolyphenols, which are mostly soluble in water (Samy and Gopalakrishnakone 2008, Tzulker *et al.,* 2007, Gil *et al.,* 2000).

Study of the antifungal activity of MAEG (diffusion in solid medium)

The antifungal activity of pomegranate bark was evaluated by, measuring the diameters of growth inhibition zones on *Rhodotorula sp*. By testing the MAEG *extract*, we found that there was an apparent inhibitory effect. Moreover, as the concentration of the *extract* increases, the diameter of the inhibition zone increases; this was confirmed by, calculating the covariance which was greater than 0. This means that there is a positive correlation.

We noticed that the inhibition of *Rhodotorula sp.* was marked by, MAEG at 50 mg/ml with 29.5 ± 0.5mm. The values obtained in our study were much higher than those of (Duman *et al.,* 2009),

Fig. 1 : MAEG *extract* obtained after concentration

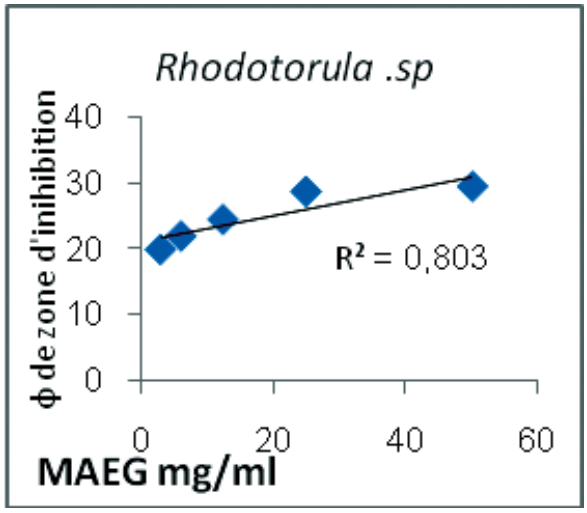

Fig. 2: Linear correlation between the diameters of the inhibition zones (mm) of the growth of *Rhodotorula sp.* and concentration of MAEG ($p \leq 0.05$)

whose averages of the zones of inhibition produced by the Turkish variety (Serife) of the pomegranate, were 17 mm on *Rhodotorula rubra.*

The coefficient of determination R^2 is an indicator for judging the quality of a linear regression; if it is high, the R^2 is > 0.5, if it is low, the R^2 is < 0.5. Thus in our tests we obtained R^2 greater than 0.5, which means the existence of a strong relationship between the diameter of the *Rhodotorula sp*. inhibition zone and the *extract* concentration (Fig. 2).

The results of the aromatogram showed that, the measured diameters around the disks can be evaluated. The sensitivity results of *Rhodotorula sp*. showed that, according to the diameters measured around the disks, the microbial strains are classified in three categories: the sensitive strains (S), the intermediate strains (I), and the resistant strains (R), these characteristics are determined by the diameters obtained and by the formula : $(ZI) \geq D \rightarrow S / (ZI) \leq d \rightarrow R / d \leq (ZI) \leq D \rightarrow I$. (D, d) (ZI, Inhibition Zone), are respectively critical values of upper and lower inhibition diameters in the test, the categorization criteria were according to the critical values (D and d), I (intermediate), R (resistant), S (Sensitive) (EUCAST, 2013) (Table 2).

Determination of the MIC and MFC

A gradual decrease in the intensity of the bacterial disorder induced by fungal growth was observed as the concentration of the plant *extract* increased in the experimental tubes. MIC was determined to be the lowest concentration of the *extract*, where fungal growth is completely inhibited. *Rhodotorula sp***.** presented a MIC range of ($0.39 \geq CMI \geq 0.195$) mg/ml with a MFC ≥ 0.39mg/ml (Fig. 3).

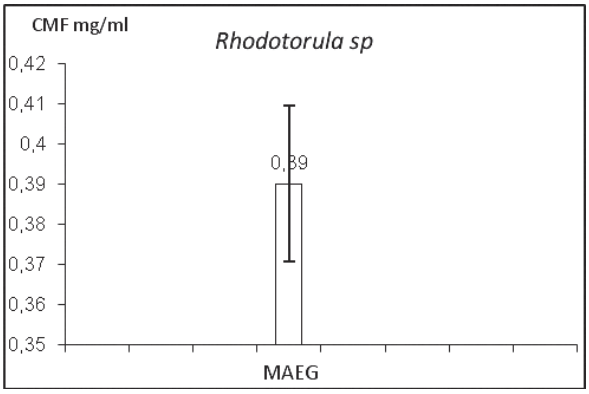

Fig. 3: MFC of MAEG on *Rhodotorula sp.*

CONCLUSION

The results presented here are very encouraging, for the development of a new antifungal treatment in an area, where there is a clear lack of effective antifungal medications. Many other studies must be done, in order to begin to discern the growth inhibition mechanism, by the purified compounds of this plant; in this case the phenolic compounds (punicalagin) alone or in combination, to study the mechanism of synergy between them. This work complements the promising results obtained in previous work on *Punica granatum*. It just needs

to be finalised by fractionation and purification tests, toxicity studies and *in-vivo* tests.

The concentrations at which the *aqueous macerate* of pomegranate bark, remains active and the broad spectrum of actions observed lead us to affirm that, the pomegranate is an interesting plant in the fight against infectious diseases. It would be important to make a more complete *screnning* of the main potentially active chemical groups, to identify and isolate the active ingredient(s), which could after further studies, strengthen the *antibiotherapy* in medical pathology. This study justifies the traditional use of this plant, in the treatment of many diseases.

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