



MOLECULAR TYPING OF *MALASSEZIA* SPECIES BY RFLP-PCR AND EVALUATE ANTIFUNGAL ACTIVITIES OF SOME PLANT EXTRACTS

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Abstract

Malassezia is opportunistic Basidiomycete yeasts, attacked human and animal, Fungal Identification based on molecular evidence has obtained a great attention in recent years. The aim of this study was to perform molecular identification of *Malassezia* yeast and evaluated antifungal activity of three medicinal plant extracts: *Peganum harmala* (Hermel), *Syzygium aromaticum* (Clove) and *Juniperus communis* (Juniper). The molecular identification was achieved by amplification of the partial sequence of 28S rDNA with flanking region of the Mal1F/ primer pair. PCR of 24 isolates of *Malassezia* was 605bp. RFLP of PCR products were performed using *Hin6I* (*Cfo1*) restriction enzyme. Five species of *Malassezia* was recognized out of 24 yeasts isolates. The RFLP-PCR is the best molecular marker for identification mixed cultures of *Malassezia* species. The Clove extract plant considered a more antifungal activity inhibitor of *Malassezia* in glass compare with other extracts.

Key words: *Malassezia*, RFLP-PCR, Antifungal activity, plant extract

Introduction

Malassezia is a normal flora Basidiomycete yeast and an important causes a type of skin infection called pityriasis versicolor disease. The infection appears usually as a pale patches dark or light on the skin color (hypo or hyper pigmentation). The recorded *Malassezia* species ranged between 7-14 species, and those considered to be the threat yeasts associated with animal infection (Kindo *et al.*, 2004). The pathogenicity of *Malassezia* yeasts in human and animal has obtained a great attention in recent years from dermatologists, other clinicians, veterinarians and mycologists (Ashbee, 2007; Gaitanis *et al.*, 2012). There were some difficulties in detecting of *Malassezia* Yeasts Crespo *et al.* (2000).

Many restriction enzymes use in RFLP-PCR typing, and this method is rapid, easy, and reliable to overcome problems with Morphological and Microscopic identification methods. PCR-RFLP technique was used in to determine the occurrence rate of *Malassezia* yeasts in human. This method has been successfully used in a previous study (Mirhendi *et al.*, 2005), appears to be reliable for the identification of nearly all the known *Malassezia* species, requires only PCR and enzymatic digestion by any enzyme, and is technically less demanding than most other molecular biological methods (Mirhendi *et al.*, 2005; Gaitanis *et al.*, 2006).

Herbal has medically important since long time, due to their important role in the pain treatment and antifungal activity against several diseases, and it is still used by people until this time in about 75% from drug. The importance of plant extract includes compounds had antifungals as natural products against

microorganisms like Alkaloids, Phenols, Saponines, Tannins, Resins and others (Alves *et al.*, 2013).

Material and Methods

1. Sampling

A total of 145 specimens (tape strips and swab techniques) were collected from the skin of different age and gender of patients with pityriasis versicolor. Questioner chart was included in sampling collection; patient history, Job, age and gender, symptoms, duration of the disease, occupation, living style. All suspected samples were cultured on SDA and modified Dexon agar supplemented with chloromphenicol 250ml/L and cyclohexamide 500ml/L. and incubated for 7 days at 32 ±2°C, followed by isolated colonies in pure culture on the same previous media.

2. DNA Extraction

Twenty four isolates of *Malassezia* subjected to DNA extraction. In brief; A loop full of *Malassezia* colonies was suspended in the lysis buffer supplemented by Favrogen Yeast Extraction Kit (Korea) and following up the extraction steps based on the instruction of this Kit. A dry DNA pellet dissolved in diluted rinse and preserved in - 20 °C until use (Imran and Al-Shukry 2014; Imran, 2015).

3. PCR assay

The following primer pairs (Mal1F :5-TAACAAGGATTCCCCTAGTA-3, Mal1R: 5-ATTACGCCAGCATCCTAAG-3), targeting partial sequence of 28S rDNA was used for PCR amplification and amplified using thermal cycler PCR System

(Labnet, USA). The PCR mixture was amplified. Initial denaturation temperature 95°C for 5 min, 30 cycles, 95°C for 30 Sec, annealing temperature of 55°C for 40Sec., extension temperature of 72°C for 1 min, final extension temperature 72°C for 5min, cool step by 4°C according to Imran *et al.* (2016).

4. PCR-RFLP test

The PCR-RFLP test was previously described by Imran (2015). In brief; incubation of 8 µl aliquots of PCR products were incubated for the 28S rRNA genes (amplified by primer pairs(Mal1F/ Mal1R) with 10 µl of cocktail restriction enzymes *CfoI* (Promega, USA) in one reaction, at 37°C for 3 hours. After that, 8 µl of RFLP-PCR products were run on 1.5% agarose gel at 70 V for 60 min. The gel was pre-stained using 0.05% ethidium bromide and was visualized under ultraviolet light and photographed by the imaging range of the ultraviolet 21 imaging device (Korea).

5. Antifungal activity of plant extracts against growth of *Malassezia* spp.

Three medicinal plant extracts: *Peganum harmala* (Hermel), *Syzygium aromaticum* (Clove) and *Juniperus communis* (Juniper), were evaluated their antifungal activities against *Malassezia* spp., according to Imran *et al.* (2016). This method involved pouring 20 ml of modified Dixon agar medium in a petri dish (9 CM). Pick up 20µl from *Malassezia* yeast suspension (1×10^5 cells /ml) and mixed with 5ml of normal saline and it was spreading by the spreader on the surface of medium and left the plates to dry. 50µl of aqueous plant extracts (Clove, Juniper and Hermel) for each, 6 mm bore diameter were made by cork borer, and fill each one by different concentration of plant extracts and then incubated the plates at (28-30 °C) for (24-48 hour). Five *Malassezia* spp., were subjected to the antifungal activity of the plant extract under interest, then measured inhibition zone by metric ruler, (Imran and Al-Karrem 2016).

6. Statistical calculation

This study follows up the mean values for all measurement and CRD analysis based on Al-Rawi and Khalfalla (2000).

Results and Discussion

1. Prevalance of *Malassezia* isolates

Our results conducted that 86.2% (125/145), shown positive infection with *Malassezia* yeast, seven species was identified based on molecular assays. This results consistent with Gupta *et al.* (2001). The results shown that the colony characters and Microscopic *Malassezia* yeasts unreliable to depend them in identifying, These interpretations agreed with Crespo

et al. (2000) when he pointed to some difficulties in detecting of *Malassezia* Yeasts. Many cultures of *Malassezia* plates, shown mixed culture for different *Malassezia* species Figure (1). *Malassezia* isolates and species occurring in many site of patients, sometimes more than species recovering from sampling sites. Our results agree with Gupta, *et al.* (2000).

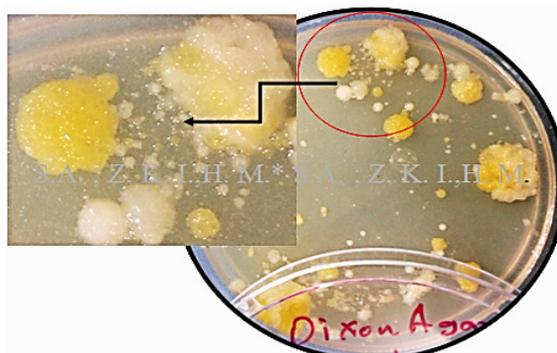


Fig. 1 : *Malassezia* colonies in mixed culture grown on Dixon Agar, incubation period 5-7 days at 32°C ± 2°C. (Lettering represent the author names).

2. Moleculare genotyping

(A) PCR results

The results of PCR typing of the LSU rDNA region amplified by primer pairs (Mal1F/ Mal1R), shown that all *Malassezia* spp., shown monomorphic bands, approximate 605bp of PCR products. Figure (2), these results consistent with Mirhendi *et al.* (2005).

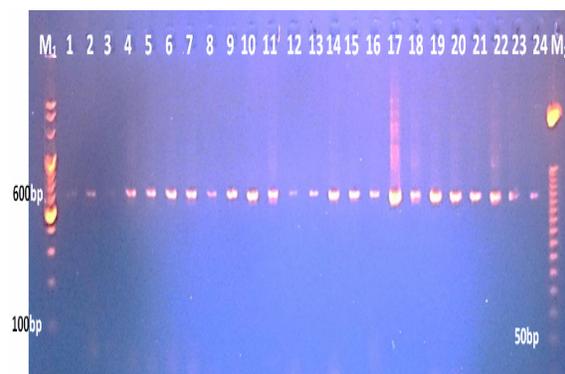


Fig. 2 : Electrophoresis gel profile of PCR-amplification products. All samples yielded a single band of approximately 605 bp., :1-24 isolates of *Malassezia* spp. Molecular marker: M1= (100bp) and M2= marker (50bp).

(B) Fingerprinting RFLP-PCR patterns.

The results of the digestion of the PCR product 605bp) of *Malassezia* by using *Hin6I*(*CfoI*), the samples yielded different bands fragments. Based on this

fingerprinting mode, helping in typing five *Malassezia* spp., especially those grown in mixed culture of *Malassezia* isolates Figure (3).



Fig. 3: Electrophoresis of PCR-RFLP products after restriction digestion of the products with *Hin6I*. 1: 8-10,6-7 : *M. furfur* and *M. sympodialis*; 4: *M. restricta*, 5: Mixture of *M. globosa* and *M. sympodialis*; 11,14,18-20: *M. furfur*; 1: *M. pachydermatis*; 2-5,13,15: *M. restricta*. M1=Molecular marker(100bp each step),M2=(50bp each step).

This band pattern appears to be novel molecular evidence to solving the coexisting of *Malassezia* species through the world, after digestion of the PCR products, different band patterns were observed on the electrophoresed gel. Figure (3), represents a photograph of agarose gel electrophoresis of RFLP products.

The results obtained from RFLP-PCR of amplicon of the Mal primer pair shown that, occurrence of *Malassezia* species through of skin of patient sample under interest, the common members of the *Malassezia* spp. were: *M. furfur*, *M. pachydermatis*, *M. globosa* and *M. restricta* shown percentage reaching about 70% infected human, few of *Malassezia* spp., percentage reaching less than 40% of the samples, our results agree with Sugita *et al.* (2001).

One of the main reasons of the difficulty facing our study in the characterization of these *Malassezia* spp., is the lack of suitable conventional methods for identification of *Malassezia* spp. Our study conducted that, there is no clear cut border line found between species based on colony and yeast cells characters, could be positioned between *Malassezia* spp., A (PCR-RFLP) method was used to detect the distribution of *Malassezia* spp., in patients with pityriasis versicolor successfully and showed that PCR-RFLP was a suitable technique for diagnosing and distinguishing *Malassezia* species. This result was agree with many reports; Mirhendi *et al.* (2005). Gaitanis *et al.*, 2006; Ko *et al.* (2011).

The results of this study were consistent with Gaitanis *et al.* (2002), when he uses PCR-RFLP to detect

and identify *Malassezia* species from skin scales for human patients and reported that this technique is sensitive and enables rapid detection and identification of *Malassezia* species. In addition, in our study, we were able to detect a synchronous occurrence of more than one of *Malassezia* spp., in the same a sample of human.

3. Antifungal activity of some plant water extracts against *Malassezia* spp.

(A) Effects of concentration percentage of plant extract

The results shown that the highest concentration of plant water extract factor under interest had higher antifungal activity, the plant extracts shown inhibition growth of *Malassezia* yeasts. Figure (4).

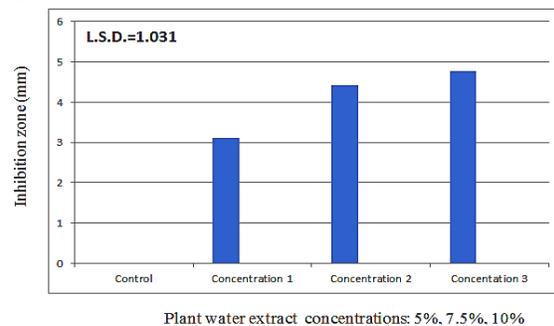


Fig. 4 : The antifungal activity of concentration percentage factor of plant extracts on *Malassezia* spp., growth; concentration= 1 5%, concentration 2 7.5%. (L.S.D. values at 0.05 significant levels=1.031).

(B) Effects of plant type on *Malassezia* spp., growth

The result showed the water extracts of the clove plant had great effective elements that inhibited the growth of different isolate of *Malassezia* spp., followed by the Juniper plant while Hermel was the less one. These plants had great and several effective compound against this yeast. The efficiency of cloves extract inhibitory is the best result from than other extract, Figure (5).

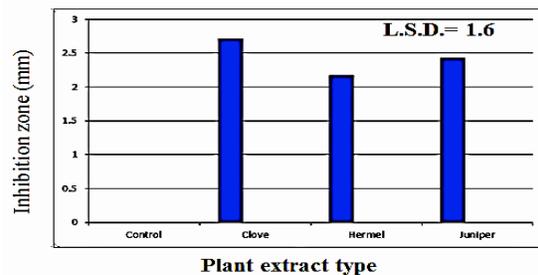


Fig. 5 : The antifungal activity of plant type factor on *Malassezia* spp., growth; plant: Clove, Hermel and Juniper. (L.S.D. values at 0.05 significant levels=1.602).

(C) Response of *Malassezia* spp. To the antifungal activity of plant extracts

The results shown that all *Malassezia* spp., under test not shown any significant difference of response, Figure (6).

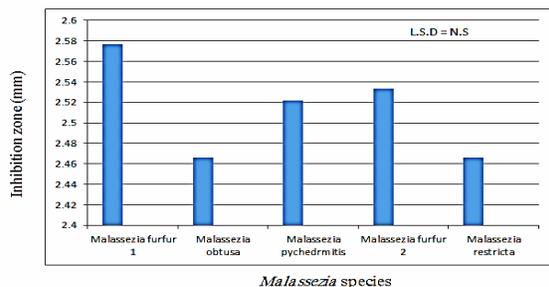


Fig. 6: Response of *Malassezia* spp. Against antifungal activity of plant extract factor (L.S.D. value at 0.05 significant levels=N.S.

These plants have wide and diverse of active elements that inhibited fungi (Briozzo *et al.*, 1989). The clove plant had medical importance because it is having (Eugenol) substance that act as pain killer and antibiotics (Koba *et al.*, 2011). This result is in agreement with several other studies on the active elements in the clove plant against fungi (Aye and Matsumoto (2011). The Hermel plants also had an active component act as an antibiotic, so the percentage of alkaloids in it is seed (4-5%) most of it harmaline, harmalol and hermine ,also it contains resin, banisterin, fatty acid and alkaloid (Nenaah, 2010).

References

- Al-Rawi, K.M. and Khalfalla, A.M. (2000). Agricultural experiment analysis and design. Al.Mosul University print 2nd Edt. 488 p.
- Alves, M.J.; Ferreira, I.C.; Froufe, H.J.; Abreu, R.; Martins, A. and Pintado, M. (2013). Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *J Appl Microbiol.* 115: 346–357.
- Aye, S.S. and Matsumoto, M. (2011). Effect of some plant extracts on *Rhizoctonia* spp. and *Sclerotium hydrophilum*. *Journal of Medicinal Plants Research*, 5(16): 3751-3757.
- Ashbee, H.R. (2007). Updates on the genus *Malassezia*. *Med. Mycol.* 45: 287–303.
- Briozzo, J.; Nunez, L.; Chirife, J.; Herszage, L. and D'Aquino, M. (1989). Antimicrobial activity of clove oil dispersed in a concentrated sugar solution. *J. Appl. Bacteriol.*, 66: 69–75.
- Crespo, E.V.; Ojeda, M.A.; Vera, C.A.; Crespo, E.A. and Sanchez, F.F. (2000). *Malassezia globosa* as the causative agent of pityriasis versicolor. *Br J Dermatol* 143: 799–803.
- Gaitanis, G.; Velegriaki, A.; Alexopoulos, E.C.; Chasapi, V.; Tsigonia, A. and Katsambas, A. (2006). "Distribution of *Malassezia* species in pityriasis versicolor and seborrheic dermatitis in Greece. Typing of the major pityriasis versicolor isolate *M. globosa*," *British. Journal of Dermatology*, 154(5): 854–859.
- Gaitanis, G.; Magiatis, P.; Hantschke, M.; Bassukas, I.D. and Velegriaki, A. (2012). The *Malassezia* genus in skin and systemic diseases. *Clin. Microbiol. Rev.* 25: 106–141.
- Gaitanis, G.; Velegriaki, A.; Frangouii, E.; Mitroussia, A.; Tsigonia, A.; Tzimogianni, A.; Katsambas, A. and Legakis, N.J. (2002). Identification of *Malassezia* species from patient skin scales by PCR-RFLP. *Clin. Microbiol. Infect.* 8: 162–173.
- Gupta, A.K.; Kohli, Y.; Summerbell, R.C. and Faergemann, J. (2000). Quantitative culture of *Malassezia* species from different body sites of individuals with or without dermatoses. *Med. Mycol.* 39: 243–251.
- Gupta, A.K.; Kohli, Y.; Faergemann, J. and Summerbell, R.C. (2001). Epidemiology of *Malassezia* yeasts associated with pityriasis versicolor in Ontario, Canada. *Med. Mycol.* 39: 199–206.
- Imran, Z.K. and Al-Shukry, H.N. (2014). Molecular diagnosis of vaginal candidiasis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) in Babylon Province, Iraq. *Afr. J. Microb. Res.* 8(6): 496-502.
- Imran, Z.K. (2015). *Candida albicans* ssp. dubliniensis stat. et comb. nov., a new combination for *Candida dubliniensis* based on genetic criteria. *Afr. J. Microbiol. Res.*, 9(17): 1205-1214.
- Imran, Z.K.; Wtw, S.M.A. and Hussein, O.K. (2016). Molecular typing of dandruff pathogens and EVALUATE THE Antifungal activity of plant extract, *International Journal of Pharm Tech Research*, 9(12): 669-679.
- Imran, Z.K. and Al-Karrem (2016). Evaluation natural cloning of azole-resistant genes CDR1, CDR2, MDR and ERG11 between clinical and soil isolates of *Candida albicans* based on gene expression. *International Journal of Pharm. Tech. Research*, 9(11): 229-236.
- Kindo, A.J.; Sophia, S.K.; Kalyani, J. and Anandan, S. (2004). Identification of *Malassezia* spp. *Indian J Med Microbiol.* 22 (3): 179-181.
- Ko, J.H.; Lee, Y.W.; Choe, Y.B. and Ahn, K.J. (2011). Epidemiologic study of *Malassezia* yeast in patients with *Malassezia* folliculitis by 26S rDNA PCR-RFLP analysis. *Annals.* 23:177–184.

- Koba, K.; Nenonene, A.Y.; Raynauda, C.; Chaumont, J.P. and Sanda, K. (2011). Antibacterial Activities of the Buds Essential Oil of *Syzygium aromaticum* (L.) Merr. & Perry from Togo. *J. Biologically active product from nature*. 1: 42-51.
- Mirhendi, H.; Makimura, K.; Zomorodian, K.; Yamada, T.; Sugita, T. and Yamaguchi, H. (2005). A simple PCR-RFLP method for identification and differentiation of 11 *Malassezia* species. *J. Microbiol. Meth.* 61:281–284.
- Nenaah, G. (2010). Antibacterial and antifungal activities of (beta)-carboline alkaloids of *Peganum harmala* (L) seeds and their combination effects. *Fitoterapia*. 81(7): 779-782.
- Sugita T.; Suto H.; Unno T.; Tsuboi R.; Ogawa H.; Shinoda, T. (2001). Molecular analysis of *Malassezia microflora* on the skin of atopic dermatitis patients and healthy subjects. *J. Clin. Microbiol.* 39: 3486-90.