

**ASSESSMENT OF ANTIFUNGAL ACTIVITY OF ALCOHOLIC
EXTRACT OF *CALVATIA CRANIIFORMIS* MUSHROOM ON
MALASSEZIA PACHYDERMATIS IN GERMAN SHEPHERD DOG
(*IN VITRO*)**

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ABSTRACT

This study was conducted to assess the antifungal activity of alcoholic extract of *Calvatia carniiformis* mushroom (*invitro*) on *Malassezia pachydermatis* fungus which isolated from German Shepherd dog came to outpatient clinic of Veterinary College of Diyala University Iraq. The characteristic features of the colony of *Malassezia pachydermatis* were round, convex and cream to yellowish in color develop on Sabouraud's dextrose agar. Microscopical examination of direct smear from the lesion show large number of organisms (yeast resembling a footprint or a peanut), combination clinically with erythema, irritation and pruritus. As the determination of growth were more at 750 mg in comparison with 250 mg and 100mg, and the widest were at 1000 mg against *Malassezia pachydermatis*. The

present study show significant difference at $P < 0.05$ between susceptibility of *M. pachydermatis* to different concentration of alcoholic extract of *Calvatia carniiformis* mushroom when compared with common antifungal drug as fluconazole.

KEYWORD: Antifungal activity, *Calvatia carniiformis*, *Malassezia pachydermatis*.

INTRODUCTION

Pityrosporum (Malassezia) pachydermatis, which is lipophilic but not lipid-dependent, is a yeast found on the skin and ears of dogs. Though a normal inhabitant of these regions, an abnormal overgrowth of the yeast can cause dermatitis, or inflammation of the skin. The exact reasons behind this disease are not yet known, but it has been linked to allergy, seborrhea, and possibly congenital (born with) and hormonal factors. *Malassezia* dermatitis can affect any breed of dog, but the following breeds are predisposed to this disease: poodles, basset hounds, cocker spaniels, and dachshunds. The disease is characterized by irritation of skin, loss of hair (alopecia), greasiness, scaly skin, redness of affected areas, malodorous discharge from lesions, patches of skin becoming darker (hyperpigmentation) and epidermal thickening (seen in chronic cases) (Guého *et al.*, 1996).

High humidity and temperature may increase the frequency of the cases. Other factors that may be a predisposing factor to this hypersensitivity disease include concurrent infections and food and flea allergies. Genetic factors are also suspected for young onset in predisposed dog breeds. You will need to give a thorough history of your dog's health, including the onset and nature of the symptoms, to your veterinarian. He or she will then perform a complete physical examination as well as a biochemistry profile, urinalysis, and complete blood count the results of which are typically normal unless the dog has a concurrent disease (Srivastava, 2008).

More specific testing includes a culture of the causative organism as well as taking a small skin tissue sample for a skin cytology test. In this test your veterinarian will touch a sterilized cotton swab to the affected area and stain it with Diff-Quik stain on a glass slide. After staining, the glass slide is observed under a microscope to demonstrate the yeast in the sample. This will help him or her identify the causative organism (Guillot and Bond, 1999). Identification of *Malassezia* on skin has been aided by the application of molecular or DNA-based techniques. These investigations show that the *Malassezia* species causing most skin disease in humans, including the most common cause of dandruff and seborrheic dermatitis, is *M. globosa* (though *M. restricta* is also involved) (DeAngelis *et al.*, 2007). The skin rash of tinea versicolor (pityriasis versicolor) is also due to infection by this fungus (Inamadar and Palit, 2003).

The aims of the present study are to evaluate activity of *Calvatia craniiformis* mushroom in treatment of Malasseziosis in dogs (*In vitro*).

The figure (1a) reveals the *Calvatia craniiformis* mushroom has been classified nationally and, the figure (1b) reveals the discovered mushroom in Jadidat Al-shat village in Hibhib city and Bani saad city- Diyala province for first time in Iraq according to the diagnosis of laboratory of fungus researches and plant disease in the college of Agriculture- Baghdad university.

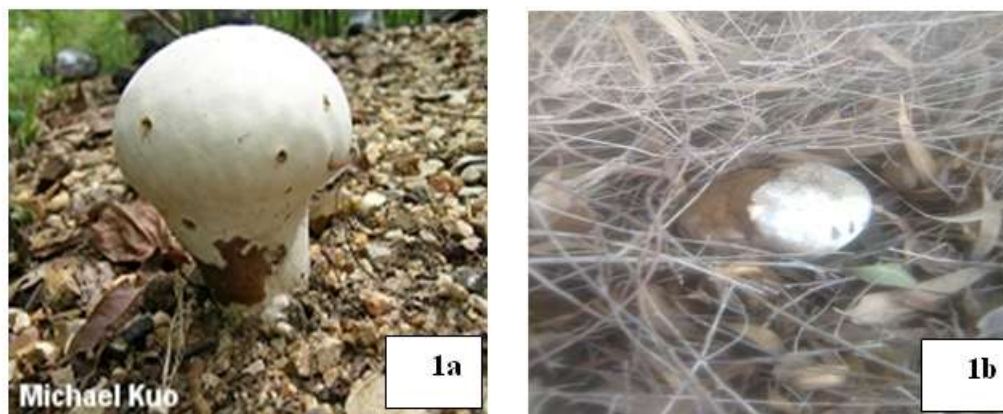


Figure- 1a: represent the mushroom in the world(Smith,1951).

1b: represent the mushroom in the Iraq(Jameel *et al.*,2014).

Perhaps the most frequently collected puffball in Kansas, *Calvatia craniiformis* is also quite common in southern and eastern North America. Arora (1986) reports that to his knowledge it does not occur on the west coast, but it was reported at the 1986 North American Mycological Association foray in Idaho. It is usually found in dry, scrubby woods and along forest edges in late summer and fall, and occasionally in the spring, but it is not uncommon even in open grassy areas.

Fruiting Body: 8-20 cm broad; 6-20 cm tall; skull-shaped; white to tan; smooth becoming wrinkled; skin cracking and flaking with age. *Calvatia craniiformis* owes its distinctive appearance to its stemlike, sterile base and its smooth to wrinkled surface. The skin will eventually slough away, exposing a powdery yellow-brown spore mass. After the spores blow away the cup-shaped sterile base remains, often over winter. The flesh is white and firm when young, becoming yellow-brown and powdery in maturity(Smith,1951).Of late, mushrooms have emerged as wonderful source of nutraceuticals, anti-oxidants, anti-cancer, prebiotic, immunomodulating, anti-inflammatory, cardiovascular effect, anti-microbial, and anti-diabetic (Barros *et al.*, 2007; Kirmizigul *et al.*, 2008; Wang *et al.*, 2004; Kim *et al.*, 2007; Synytsya *et al.*, 2009). The ongoing research projects are aimed to promote mushrooms as new generation “biotherapeutics”.

MATERIAL AND METHODS

Samples collection

Sample (Ear swab) is taken from dog ear suffering from skin lesion characterized by erythema, crusting, edema, irritation, pruritus and wax products by pressing a slide against the skin lesion and by using cotton swabs deeply or performing dry skin scrapings with a blade. Preparation of direct smear with methyl alcohol for 2-3 minute fixation, then staining with Geimza stain for 30 minute. The slide can be examined under oil emersion 100x. A few yeast organisms (1-2 yeast organisms per microscopic field) found on the skin or ear are usually considered normal. However, a larger number of organisms in combination with erythema, irritation and pruritus is considered abnormal.

Preparation of extract

a-Fruiting body of *Calvatia craniiformis* mushroom is dried and crushed in sterile Petri dish to obtain a yellow – brown powder.

b-By using a balance weigh 10 gm from the powder.

c-The weigh is dissolved in 10 ml ethanol and mixed perfectly. The concentration become 1 gm / 1 ml and considered as stock solution.

d-By disposable syringe pull (0.1 , 0.25 , 0.50, 0.75,1) ml from stock solution and diluted by (9.9 , 9.75 , 9.50,9.25,9) ml distill water to obtain (0.1 , 0.25 , 0.5, 0.75, 1) % concentrations respectively then, mixed with prepared media for cultivation .

RESULTS

The figure(2a,b) were revealed the dog and the lesion in the ear respectively.



Fig.(2 a,b) German shephard dog infected by *Malassezia pachydermatis* show erythem, alopecia and accumulation of waxy material.

The figure(3a,b) were represents the longitudinal section of the mushroom in the world and in Iraq respectively.

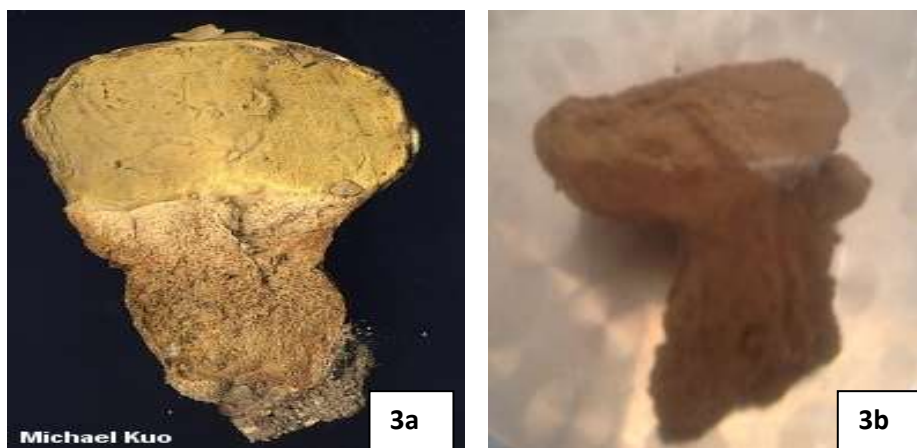
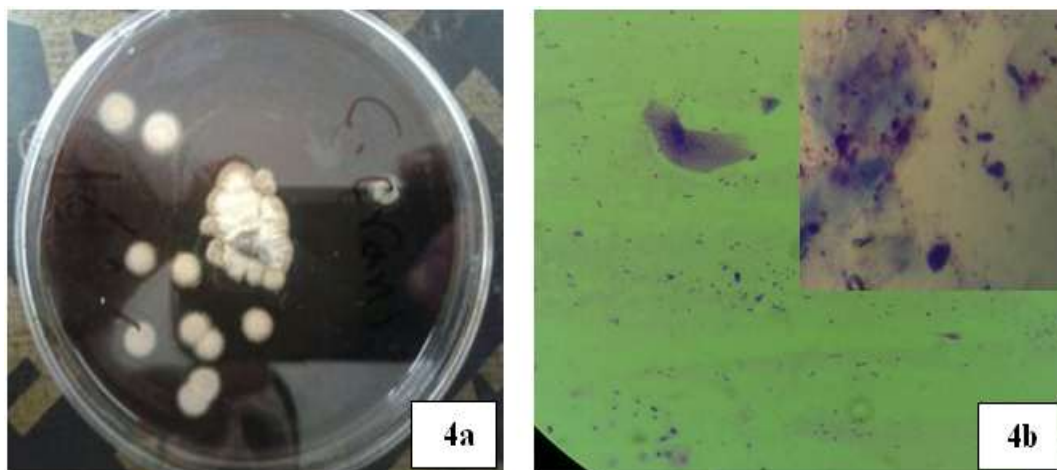


Figure- 3a: represent the longitudinal section of the mushroom in the world.

3b: represent the longitudinal section of the mushroom in the Iraq (Jameel *et al.*,2014).

Characteristic features of *Malassezia pachydermatis* colony was round, convex and creamy to yellow color develop on Sabouraud's dextrose agar as in figure (1a). Microscopical examination of direct smear show large number of organisms (yeast resembling a footprint or a peanut). When examined under the microscope at 100x (under oil) reveal the conidia in purple color look like peanut or small footprint as in figure (1b).



**Fig.(4a,b) a :Characteristic of *Malassezia pachydermatis* colony on Sabouraud's dextrose agar .
b :*Malassezia pachydermatis* fungus isolated from infected ear. Viewed under the microscope at 100x, the conidia are purple in color that look like peanuts or footprints .**

The results in table (1) were represent the sensitivity of the causative fungus to the common antifungal drugs which are used in treatment of the *Malassezia pachydermatis* infection and also reveals the effect of the five concentrations which were prepared

from the *C. craniiformis* mushroom powder in addition to the diameters of fungus growth calculated by millimeter displayed in the figures (3 and 4) after the addition of fluconazole and our extract in different concentrations respectively.

Table(1): Represent the effect of common antifungal drug and mushroom extracts on *Malassezia pachydermatis* fungus

Concentrations of <i>Calvatia craniiformis</i> extract					
Test	100 mg M(ml)± SE	250 mg M(ml)± SE	500 mg M(ml)± SE	750 mg M(ml)± SE	1000 mg M(ml)± SE
Extract of <i>Calvatia craniiformis</i>	24± 0.00 - a*-	20.5 ± 0.33 -b*-	15.5 ± 0.33 *-c-	12.75± 1.0 *,**d	10.5± 0.33 *,**e
Antifungal Fluconazole	150 mg M± SE				
	28 ± 0.33				

Values : M± S.E.M= Mean± Standard error of the mean . a, b, c, d, e, f, g, h; significantly different level of P < 0.05

- a*- mean the significance was between concentration 100mg and 250 mg
 -b*- mean the significance was between concentration 250mg and 500 mg
 -c*- mean the significance was between concentration 500 mg and 750 mg
 -d*- mean the significance was between concentration 750 mg and 1000 mg
 d**-* mean the significance was between concentration 100 mg and 750 mg
 -e* mean the significance was between concentration 100mg and 1000 mg
 -e- mean the significance was between concentration 100mg and 750 mg

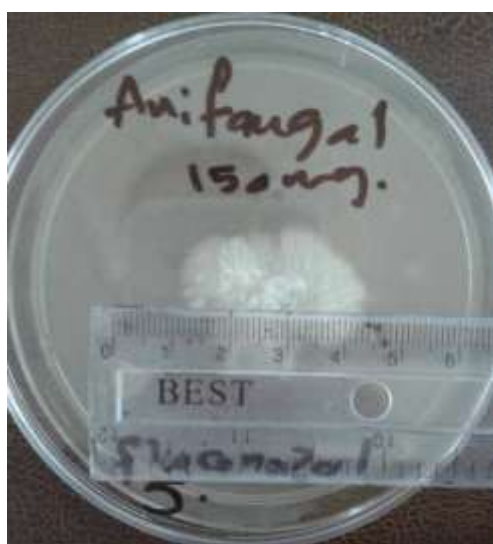


Fig.(3) Effect of antifungal (Fluconazole 150 mg) against growth of *M. pachydermatis*



Fig.(4): Effect of different concentration of Alcohol extract of *C. craniiformis* on growth of *M. pachydermatis* on Sabouraud dextrose agar

DISCUSSION

Malassezia pachydermatis is considered the most common causative agent of external otitis in dogs, having an increased predilection for certain breeds, kind of ear and hair and

according to case history and clinical signs of patient with *M. pachydermatis* the result agreement with (Crespo *et al.*,2002 and Cafarchia *et al.*,2005). Depending on characteristic morphology growth of *M. pachydermatis* on Sabouraud's dextrose agar these finding agreement with other author (Kaneko *et al.*,2006). On the other hand microscopical observation show large number of *Malassezia pachydermatis* organisms viewed under the microscope at 100x, will noticed purple shapes that look like peanuts or small footprints this closed agreement with(Hernández, 2005);(Gupta *et al.*, 2004); (Cabañes *et al.*,2005) and (Indianapolis *et al.*,2011).

The result of antifungal study of extract of *Calvatia craniiformis* performed against *Malassezia pachydermatis* show significant difference at ($p < 0.05$) of different concentrations of extract as in table (1) reveals that 750mg & 1000mg determinate the growth of *M. pachydermatis* fungus more than 500mg & 250 mg when compared with common antifungal drug fluconazol shown In table(1).

The medical analysis of the powder of mushroom proved the presence of three components; the first is calvatic acid which has chemical formation P-carboxyphenyl-azoxycarbonitrile (Okuda and Fujiwara, 1982). This calvatic acid reveals strong action against the yeast and fungi like *Saccharomyces cerevisiae* and some *Candida* species and *Trichophyton asteroides* (Hamao *et al.*, 1976).

The second components from chemical analysis and spectroscopic means of the mushroom powder is hydroxyphenylazoforamid derivatives which has three chemical compounds, 4-hydroxyphenyl-1azoforamid, 4-hydroxyphenyl-ONN-azoforamid and 2-methylsulfonyl -4-hydroxy-6-methylthiophenyl-1-azoforamid, it is known craniformin (phenolic tautomer of rubroflavin), and also three components are known steroids, ergosta-4,6,8 (14), 22-tetraene- 3-one, ergosta-7,22-diene-3-01 and ergosterol peroxide (Takaishi *et al.*, 1997).

The hydroxyphenylazoforamid derivatives or craniformin have phenolics in its formation which are endowed with interesting biological activities as a broad spectrum bactericidal and fungicidal effect represented by *Candida albicans*, *Aspergillus niger* (Bouaziz *et al.*,2008). Also the craniformin has azol compound which inhibit the synthesis of ergosterol by blocking the action of 14-alpha-demethylase and stop proliferation of the fungus (Fugmann *et al.*,2001) . The action of azol compounds reveals inhibition fungal mRNA transcription (Lewis, 2006). Foiani *et al.*, (1994) proved that the B subunit of the DNA polymerase alpha-

primase complex in *Saccharomyces cerevisiae* has essential function at initial stage of DNA replication and this should be inhibited by ergosterol peroxidase which results in inhibition of the proliferation of the yeasts and fungus.

Also the chemical analysis of mushroom powder which is done in White Fields Company for Chemical and Engineering Studies and Consultations in Baghdad – Iraq proved the presence of different materials as gallic acid and others. Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid and, found both free and as part of tannins . Gallic acid seems to have anti-fungal and anti-viral properties (Jeremy and Nuansri, 2007).

CONCLUSION

We concluded that extract of *Calvatia craniiformis* is used as antifungal against some fungus and yeast specially *Malassezia pachydermatis* locally.

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