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RESEARCH ARTICLE

HERBAL BIOSYNTHESIS OF ZINC NANOPARTICLES AND EVALUATION OF THEIR ANTIFUNGAL AND ANTIBACTERIAL EFFECT FOR BUFFALOES SKIN AFFECTIONS

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ABSTRACT

Herbal biosynthesis of zinc oxide nanoparticles (ZnONPs) using extract of *Corriandrum sativum* plant leaf and evaluation of their antimicrobial potential against some fungal and bacterial causes of skin affection in buffaloes were investigated. Out of 100 buffaloe cases that showed skin lesions and 30 apparently healthy cases, the fungi of *Trichophyton verucosum*, *T. mentagrophytes* and *C.albicans* were recovered from (50%, 31% and 15%) of hair and from (65% ; 37% and 13%) of skin scales samples of affected animals, respectively. The apparently healthy animals had comparatively lower rate of infection that ranged from 0.3-1%. On the other hand, bacterial species of *Dermatophilus congolensis* and *S. aureus* were recovered from 15% and 13% of hair samples and from 17% and 14% of skin scales samples of the affected animals, respectively. Whereas, in case of apparently healthy animals, all cases were not affected by *Dermatophilus congolensis* and only 1.8% of hair and 6 % of skin scraping samples were affected by *S. aureus*, respectively. The ZnONPs was synthesized using extract of *Corriandrum sativum* plant, identified and characterized by visual inspection; in a UV-visible spectrophotometer and Scanning by transmission electron microscope (TEM) and scanning electron microscope (SEM) for detection of their particle size and the purity of the prepared powder. The particle size of prepared ZnO nanoparticles was 50 nm. The antimicrobial potential of prepared ZnONPs was evaluated by broth microdilution methods against recovered microbial species from skin affection of buffaloes. As the concentrations of ZnONPs increased, the optical density and turbidity of treated spore suspension decreased and reached 100% transmittance and clear medium at the MIC of ZnONPs. The MIC that inhibited 50% of the growth of *T.verucosum* and *T.mentagrophytes* was 11 -12 mg/ml and it was 17 and 14 mg/ml for MIC 100%, respectively. The MIC that inhibited 50% of the growth of *D.congolensis*, *S. aureus* species was 12.5-25 ug/ml and it was 150 ug/ml for MIC 100%, respectively. The treated fungal and bacterial cells were subjected to SEM, the damage and rupture of their cell wall was detected or membrane damage and some pits that have been caused leakage in inter cellular components and finally cell death. Further studies are needed to investigate the efficacy of pharmaceutical preparation of ZnONPs as ointments, skin lotions and synergistic therapy with other traditional antibiotics in the treatment of animal skin diseases.

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1. INTRODUCTION

The bacterial and fungal skin affections of animals cause many economic loses among affected animals and may cause zoonotic infection to the contact human. Dermatophytosis or ringworm infection and bacterial skin affection are considered as the most common cutaneous conditions all over the world, these infections are related to the stratum corneum of the

epidermis and keratinized tissues such as skin, hair and nails of humans and animals (Popoola et al., 2006 ; Ameen, 2010 and Hassan et al., 2015). The diseases are caused by keratinophilic filamentous fungi, called dermatophytes, belonging to the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* (Deacon, 1988). In cattle, dermatophytosis is most often caused by *T. verrucosum* called ringworm which denotes the clinical entity characterized by annular skin lesions (Weiss et al., 1979 and Stenwig, 1985). It is transmitted easily from one animal to another and its spores may survive in the environment for 2 to 3 years and calves or purchased animals introduced into the herd are likely to contract infection. According to Hay (2003)

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and Havlickova *et al.* (2008), a number of factors including geographic location, prevailing climate as (temperature, humidity, wind, water activity etc.), overcrowding, health care, immigration, environmental hygiene culture and socioeconomic dispositions have great implication for the proliferation of dermatophytosis. The inflammation of the skin is associated with unthriftiness and general discomfort in affected animals. Dermatophytosis also has important economic significance, as apparently healed skin lesions re-appear after the tanning process (Haab, 1991).

On the other hand, the bacterial skin affections of buffaloes particularly with the *dermatophilus* species, can cause skin disease of animal (Radositis *et al.*, 1994) and man (Harman *et al.*, 2001). This disease in cattle caused by agram-positive actinomycetes, *Dermatophilus congolensis* is characterized by acute or chronic, local or progressive and sometimes fatal exudative dermatitis; it starts as an erythema, progressing through serous exudation and drying to form characteristic matting of the hair (Harman *et al.*, 2001; Ambroso *et al.*, 1999; Abdullahi, 2001 and Loria *et al.*, 2005)

There is definitely a need for effective prophylaxis against the disease as hygienic and other preventive measures often fail. Consequently, a national vaccination program was advocated by the Swedish hide industry and restrictions on sale of breeding animals and on the access of livestock to common pastures are imposed on affected herds. Treatment of clinical ringworm in cattle is expensive and time-consuming (Shams-Ghahfarokhi, 2009).

The emerging infectious diseases and the development of drug resistance in the pathogenic bacteria and fungi at an alarming rate is a matter of serious concern. Despite the increased knowledge of microbial pathogenesis and application of modern therapeutics, the morbidity and mortality associated with the microbial infections still remains high (Kolar *et al.*, 2001). Therefore, there is an urgent demand to discover novel strategies and identify new antimicrobial agents from natural and inorganic substances to develop the next generation of drugs or agents to control microbial infections. Prior to the extensive use of chemotherapeutics in modern health care system, inorganic antimicrobials such as silver, zinc and copper were used since ancient times to treat microbial infections (Moghimi, 2005). In the recent times, the advances in the field of nanosciences and nanotechnology has brought to form the nanosized inorganic and organic particles which are used for many applications as amendments in industrial, medicine and therapeutics, synthetic textiles and food packaging products (Gajjar *et al.*, 2009).

The plant phytochemical with antioxidant properties is accountable for the preparation of metal nanoparticles. Recently, nanoparticles synthesis was achieved with bacteria, fungi and actinomycetes (Holmes *et al.*, 1995 and Mukherjee *et al.*, 2001) and use of plant extract such as neem, *camellia sinensis* and *Corriandrums* and several others which are compatible with the green chemistry principles (Badri *et al.*, 2008; Begum *et al.*, 2009 and Gnanasangeetha and Sarala, 2013). The biosynthetic approach using plant extracts is a compensation of adverse effects of chemical methods such as

easily available to be applied, safe to handle and possess a broad viability of metabolites.

Therefore, the present study was undertaken to study the prevalence of some fungal and bacterial causes of skin affection of buffaloes in some Egyptian farms. The herbal biosynthesis of ZnO nanoparticles and investigation of their antimicrobial potential were also evaluated.

2. MATERIAL AND METHODS

2.1. Animals and samples

One hundred Egyptian buffaloes clinically showing skin lesions and 30 apparently healthy animals were selected from different farms at Giza and Cairo governorates for mycological and bacteriological examination. Hair and skin scraping scales samples were collected from all animals. The affected skin areas of the animal body were cleaned with 70% alcohol and skin scraping were taken from the edge of the lesion by scalpel blade and hair as mentioned by Refai *et al.* (2014 a). The scales and hairs were collected in clean labeled envelopes or in clean sterile labeled Petri dishes.

2.2. Antifungal and antibacterial agents

Fluconazol (20 ug) and Entrofloxacin (20%) were purchased from Sigma Chemical Company (USA) and used as a comparable control.

2.3. Mycological and bacteriological examination

2.3.1. Direct microscopical examination

The hair and skin scraping scale specimens from all animals were examined microscopically as described by Refai *et al.* (2014 a) using KOH wet preparation method.

2.3.2. Isolation of dermatophytes and *C.albicans* from infected or apparently healthy animals

The samples from clinically affected or apparently healthy animals were cultured as described by Larone (1995) and Refai *et al.* (2014 a &b) in Petri dishes or test tubes containing Sabouraud Dextrose Agar supplemented with chloramphenicol and cycloheximide. A light inoculum of each hairs and skin scrapings scale specimen was scattered over the surface of the medium and gently pressed down into the agar. The inoculated plates were incubated at 37°C for 24 hours up to 2-3 weeks. After the establishment of dermatophyte or yeast of candida growth, a subculture was made on SDA with cycloheximide for further identification and preservation of the obtained colonies.

2.3.3. Identification of the dermatophyte and *C. albicans* isolates

The isolated colonies were identified by macroscopical morphology and microscopical examination using Lactophenol cotton blue (LPCB) wet mount (Refai *et al.*, 2014a and b), to demonstrate the presence of hyphae, macroconidia,

chlamydospores, pseudohyphae, germ tube and other fungal structure.

2.3.4. Isolation and identification of *S. aureus* and *dermatophilus* species

A. Direct Microscopical Examination (Quinn *et al.*, 1994):

Small pieces were taken from the underside of the scab and softened in few drops of distilled water on a clean microscope slide, a smear was made and stained by Loeffler's methylene blue and Gimsa or Gram's stains.

B. Culturing Method for isolation and identification of *S. aureus* :

A part of skin scales and hair samples was inoculated into the test tubes containing nutrient broth and were incubated at 37 °C for 24 hrs. The subcultures were also made on nutrient agar, 5% sheep blood agar and Baird Parker and incubated at 37 °C for overnight. Based on morphological and staining characteristics, hemolytic activities on blood agar, biochemical characteristic and antibiotic sensitivity test, the bacteria were isolated and identified as described by (Quinn *et al.*, 1994; Anon, 2007)

C. Culturing Method for isolation and identification of *dermatophilus* species (Haalstra, 1965):

A small amount of scab material was ground up, placed in a screw capped bottle, moistened with one ml sterilized distilled water and allowed to stand open for 3 and half hours on the bench. Then the opened bottle transferred to candle jar with a candle was burned within the jar to obtain 10- 20% CO₂ tension (so the motile zoospores were chemo-tactically attracted to the CO₂ enhanced atmosphere and move to the surface of distilled water). After 15 minutes, the bottle was carefully removed and a drop was taken from the water surface with a bacteriological loop and cultivated on brain heart infusion agar plates which were incubated at 37°C in 20% CO₂ tension for 24 to 48 hours. The suspected colonies were identified as recommended by (Silva *et al.*, 2003; Yardley, 2004; Anon, 2007).

2.4. Herbal biosynthesis and characterization of Zinc oxide nanoparticles

A. Preparation of the leaf extract (Harborne, 1973; Trease and Evans, 1989):

Fresh leaves were collected from *Corriandrum sativum* plants and washed several times with water to remove the dust particles and then sun dried to remove the residual moisture. The used extract for the reduction of zinc ions (Zn²⁺) to zinc nanoparticles (ZnONPs) was prepared by placing 50 grams of washed dried fine cut leaves in 250 mL glass beaker along with 100 ml of sterile distilled water. The mixture was then boiled for 60 minutes until the color of the aqueous solution changes from watery to light yellow. The extract was cooled to room temperature and filtered using filter paper. The extract was stored in a refrigerator in order to be used for further experiments.

B. Preparation of zinc nanoparticles (Gnanasangeetha and Sarala, 2013):

50 ml of *Corriandrum sativum* leaves extract were taken and boiled to 60-80°C using a stirrer-heater. 5 grams of Zinc acetate dehydrate (99% purity) were added under constant stirring for 10 minutes. Then, 2.0 M NaOH was added

to make pH 12 resulted in a formation of pale white aqueous solution. This was then placed in a magnetic stirrer for 2hrs. The pale white precipitate was then taken out and washed several times with distilled water followed by ethanol to get rid of the impurities. Then a pale white powder of ZnONPs nanoparticles was obtained after drying at 60°C in vacuum oven over night. Complete conversion of Zn (OH)₂ into ZnO NPs took place during drying. The obtained powder in the filter paper was dried in hot oven at 50-60 °C.

C. Characterization of zinc nanoparticles (Awodugba and Ilyas, 2013):

The prepared ZnO nanoparticles were characterized for their optical and structural properties by using a UV-Vis spectrophotometer (Lambda-25; PerkinElmer; Waltham, Massachusetts) and the particle sizes and morphology were observed and measured under transmission electron micrograph (TEM) HITACHI H-800 (Hitachi) and scanning electron microscope (SEM) (Joe, JSM-5600LV, Japan).

2.5. Measurement of MIC of prepared ZnO nanoparticles against isolated dermatophytes and bacteria from skin affection of buffaloes (CLSI 2008)

The minimum inhibitory concentration (MIC) of ZnO-NPs for the tested isolated was determined by a broth micro-dilution method based on the National Committee for Clinical Laboratory Standards (NCCLS) for bacteria (Balachandran *et al.* 2015), for filamentous fungi (CLSI 2008) and for yeasts (NCCLS, M27-A2 2002). In sterile 12- x 75-mm plastic test tubes, 900 ul of Roswell Park Memorial Institute broth medium (RPMI 1640) or Sabouraud dextrose broth medium (SD broth) (for fungi) or nutrient broth (for bacteria) was inoculated separately, then, 100 ul of spore suspension to adjust the inoculum of dermatophytes spp and *S. aureus* to (2.5 x 10³ cells/ml) and dermatophytes to (5 x 10⁴ spores/ml). 100 ul of zinc oxide nanoparticles concentrations (250, 200, 150, 100, 50, 25, 12.5 and 6.25 ug/ml) for bacteria and (10, 11, 12, 13, 14, 15, 16, 17 & 18 mg/ml) for fungi, were added. The antifungal agent Fluconazole 20 ug and antibacterial agent Entrofluxacin 20% were included in the assays as positive controls. For fungi, the tubes were incubated for 48 hr-14 days at 30-37° C and for bacteria the tubes were incubated for 24-48 h at 37° C. The experiment was repeated twice. The MIC for fungi and bacteria was defined as the lowest zinc oxide nanoparticles concentration that showing no visible fungal or bacterial growth after incubation time. After end of incubation period, 5 ul of tested broth were inoculated on the sterile nutrient agar plates for bacteria and SDA plate for fungi and incubated at 37° C for 24 hr- 2 weeks. The MIC was determined as the lowest concentration of ZnONPs inhibiting the visual growth of the test cultures on the agar plate. The turbidity of the growth in tubes was observed every 24 hrs. The growth was assayed by measurement of optical density and transmittance percentage of each tubes content at 405 nm using spectrophotometer.

2.6. Scanning Electron Microscopy (SEM) (Gong *et al.*, 2007)

The morphological changes of *T. mentagrophytes*, *T. verrucosum*, *Dermatophilus congoleensis* species and

S. aureus which were treated by zinc oxide -NPs were observed with a scanning electron microscope (SEM). All the content of tubes were centrifugated and the sediments of each tube were dehydrated separately through a graded series of ethanol (30, 50, 60, 70, 80, 90, 95, and 100%), each level was applied twice for 15 min each time, then the ethanol :isoamyl acetate (3:1, 1:1, 1:3) and 100% isoamyl acetate applied twice for 30 min). The solutions in wells were dried with a critical-point drier using liquid CO₂ and coated with gold-coater for 5 min. The coated samples were observed under SEM, JSM-5600LV with accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

Recently, the prevalence of the skin affection has significantly reduced in many developed nations of the world compared to the developing countries due to improved social, economic, health care and hygiene practice factors evident in the former (Loria *et al.*, 2005; Havlickova *et al.*, 2008 and Ilkit, 2010). Numerous medical and mycological literatures reported the incidence of dermatophytic infections around the globe with high prevalence rate recorded in many developing countries within the tropical and subtropical regions of the world. The disease can be epizootic and can result in considerable economic losses as a result of lost production, public health concern, premature culling and treatment costs and down grading of hides and skins (Wabacha *et al.*, 1998; Nweze, 2010 and Hassan *et al.*, 2015). The clinical disease has been recognized in several African countries (Efuntoye and Fashanu, 2002) and the distribution of dermatophytosis consequently reflected the distribution of the causative agents in these countries (Rahbar *et al.*, 2010) with countries variations in dominant etiologic species. Dermatophytic infections may be symptomatic or asymptomatic pose public health problems in many parts of the world causing cutaneous disfigurement (Havlickova *et al.*, 2008). On the other hand, the fungal dermatophytosis infection in buffaloes (ringworm) and bacterial dermatophilosis are important skin infections which have received major consideration not only for economical losses in the animal breeding industry but also in regards to their zoonotic transmission to humans which represent the widest spread and most prevalent diseases of man and animal (Loria *et al.*, 2005; Hassan *et al.*, 2008 and 2015). The occurrence of these serious skin affections have public health problem. Despite aggressive treatment with new or more established licensed antimicrobial agents, these infections are important causes of morbidity and mortality in animals, Espinel-Ingroff (2009). The prevalence of dermatophytes infection in buffaloes was investigated by (Hassan *et al.*, 2008) who recovered *T.verrucosum* and *T.mentagrophytes* from cases of suspected ringworm in cattle at incidence rates of 70% and 30% in skin scraping samples, respectively. Whereas, Shams-Gahfarokhi (2009) reported that the most frequent dermatophyte isolated from cases of cattle ringworm was *Trichophyton verrucosum* (99% of total isolates) which was obtained from all cultures of positive cases except five cases (1.0%) infected with *Trichophyton mentagrophytes*.

Recently, Hassan *et al.* (2015), investigated the microbial causes of 120 cases of cattle suffering from obvious skin lesions. The recovered fungal species from samples included

Trichophyton verrucosum and *T. mentagrophytes* and bacterial species of *Dermatophilus congolensis*. In this work, 100 cases of Egyptian buffaloes clinically showed skin lesions and 30 apparently healthy animals were selected from different farms in Giza and Cairo governorates for mycological and bacteriological examination. Hair and skin scraping scale samples were collected from all animals (Table 1). The fungi of *Trichophyton verrucosum*, *T. mentagrophytes* and *C.albicans* were recovered from (50%, 31% and 15%) of hair and from (65%; 37% and 13%) of skin scales samples of affected animals, respectively. While, the apparently healthy animal had comparatively lower rate of infection, ranged from 0.3-1%.

Table 1. Prevalence of dermatophytes in affected and apparently healthy buffaloes

Type of fungi	Apparently healthy (30 cases)				Affected with skin lesion (100 cases)			
	Hair		Skin scales		Hair		Skin scales	
	No.	%	No.	%	No.	%	No.	%
<i>T. verrucosum</i>	2	0.6	3	1	50	50	65	65
<i>T. mentagrophytes</i>	1	0.3	1	0.3	31	31	37	37
<i>C.albicans</i>	3	1	1	0.3	15	15	13	13

Currently, the isolation of *Trichophytone verrucosum*, *T. mentagrophytes* and *C.albicans* from cases of buffaloes ringworm indicated that these fungi are the most common dermatophytes affecting animals in Egypt (Refai *et al.*, 2014 a; Hassan *et al.*, 2015). The primary dermatophyte isolation in this work was achieved by using a commercial Sabouraud-cycloheximide medium and direct microscopy for any given specimen type as recommended by Kane *et al.* (1997). The dermatophytes are able to survive in skin scales of infected animals for up to several months in moist and dark places where they can be easily transmitted to human and other animals (Weitzman and Summerbell, 1995). The zoophilic fungi, *T. verrucosum* and *T. mentagrophytes* isolated in this study may be associated with frequent contacts of the buffaloes with rats and pet animals like dogs and cats as it was reported in cattle cases from traditional type farms, Moretti *et al.* (1998).

Regarding, the bacterial skin affection of buffaloes, several factors are involved in the pathogenesis of dermatophilosis and staphylococcus infections, among them are the mechanical injury to the skin, rainfall, tick infestation, concurrent diseases and or stresses that compromise the hosts immune system (Amabrose, 1996).

Hassan *et al.* (2014) isolated 6 species of bacteria namely, *Staphylococcus* species., *Escherichia coli* species, *Klebsiella* species, *Coryne bacterium* species, *Enterococcus* species and *Streptococcus* species from diseased buffalo. While, Hassan *et al.* (2015) recovered *D. congolensis* from cattle cases suffered from skin affection.

The infection occurs when the integrity of the skin is impaired, as in case of long exposure to rain or traumatic injuries resulting from arthropod bites which serve as mechanical transmitters of microorganisms into epidermal layers, where germination zoospores takes places (Zaria 1993). The high relative humidity has a significant influence on the maturation and motility of the infective zoospores and it has been claimed

to be a major predisposing factor in the spread and epizootiology of dermatophilosis and other bacteria (Zaria, 1993) and latently infected animals may serve as the major reservoir of infection (Stewart, 1972). The organism persists in dry scabs and crusts and can survive in the environment for long periods (up to 42 months) (Oduyeo, 1989; Martinez and Prior, 1991). It may be transmitted by direct contact with a carrier host, by contaminated fomites or by biting and non-biting flies and is also encountered in a wide range of hosts such as buffalo, rabbit and sheep and rarely in man (Pal, 1989 and 1995 and Nagpal *et al.*, 2012).

The current results in Table (2) revealed that the *Dermatophilus congolensis* and *S. aureus* were recovered from 15% and 13% of hair samples and from 17% and 14% of skin scales of the affected animal, respectively. Whereas, in case of apparently healthy animal, all cases were not affected by *Dermatophilus congolensis* and only 1.8% of hair and 6 % of skin scraping samples were affected by *S. aureus*, respectively. Similar results were obtained by (Hassan *et al.*, 2015).

Table 2. Prevalence of some bacterial infection in buffaloes with or without skin affection

Type of Bacteria	Apparently healthy (30 cases)				Affected with skin lesion (100 cases)			
	Hair		Skin scales		Haire		Skin scales	
	No.	%	No.	%	No.	%	No.	No.
<i>Dermatophilus congolensis</i>	0	0	0	0	15	15	17	17
<i>S. aureus</i>	6	1.8	20	6	13	13	14	14

Several studies indicated that there was significant prevalence of *Dermatophilus* infection in cattle and buffaloes that infested with ticks. This may be due to the fact that toxins present in saliva of ticks result in immunosuppression of the animals. In cattle, it causes high economic losses as body weight loss, decreased milk yield and the acceptance of live animals in the market would also be reduced (Hassan *et al.*, 2015).

The alarming rate for antimicrobial resistance is growing poses great challenge, especially by bacteria and fungi which is one of the major concerns to public health and scientific communities worldwide (Dismukes, 2006 and Goossens, 2005). One promising approach is the application of nanotechnology in the battle against microorganisms and is being applied not only to the treatment of infectious diseases but also to diagnostics of infections and to prophylaxis against its effect (Klabunde *et al.*, 1996). In addition, Zinc oxide nanoparticles had been utilized in patients for both diagnosis and therapy, leading to more effective medication with less unfavorable effects (Richards *et al.*, 2000).

In the chemical synthesis of nanoparticles, high temperature and/or high pressure, inert atmosphere protection are required for initiating the reaction or using toxic matters such as H_2S and metallic precursors which lead to non-eco-friendly byproducts Garima *et al.*, 2011; Manish *et al.*, 2012). Hence, the growing need of environmental friendly biosynthesis methods of metal nanoparticles interested the researchers for using green methods for pharmaceutical applications and biological approaches using microorganisms and plant extracts

have been suggested as valuable alternatives to chemical methods (Akl *et al.*, 2012). Several biological systems including bacteria, fungi and yeast have been used in synthesis of nanoparticles (Alagumuthu and Kirubha, 2012) and the use of herbal methods in the synthesis of zinc oxide nanoparticles has increasingly become a topic of interests (Cynthia *et al.*, 2012). In addition, the use of environmentally benign plant leaf extract (Rajasekharreddy *et al.*, 2010) for the synthesis of zinc oxide nanoparticle offers copious profit of eco-friendliness, where toxic chemicals are not used (Vijayaraghavan and Nalini, 2010).

In the present study, the green synthesis of ZnONPs using aqueous leaf extracts of *Corriandrum sativum* plant was undertaken. The prepared ZnONPs were identified and characterized by visual inspection; in a UV-visible spectrophotometer and Scanning by transmission electron microscope (TEM) and scanning electron microscope (SEM) for detection of their particle size and the purity of the prepared powder. The particle size of prepared ZnO nanoparticles was 50 nm (Fig.1). It was reported that the characterized absorption peak of ZnONPs is detected at wave length of 350 nm due to electron transition from valence band to conduction band (Fig 2). The results revealed that ZnONPs with spherical and granular morphology had uniform distribution. Because of their unique properties and large number of applications, zinc oxide nanostructures are one of the main subjects of the nowadays research. ZnO nanoparticles are durable, free from affecting the soil fertility in comparison to traditional antifungal agents (Lopes *et al.*, 2002 and Hassan *et al.*, 2012 and 2013 a & b).

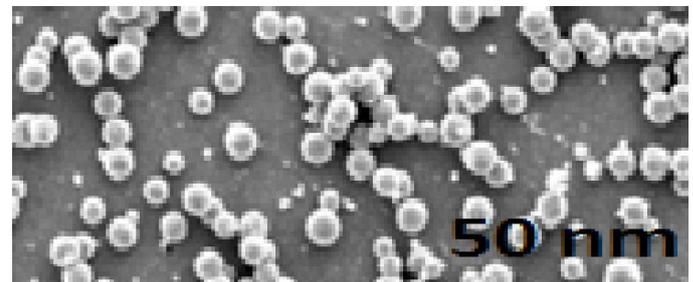


Fig.1. The TEM image of the size and distribution of the particles of zinc oxide (50 nm) (x 20 000)

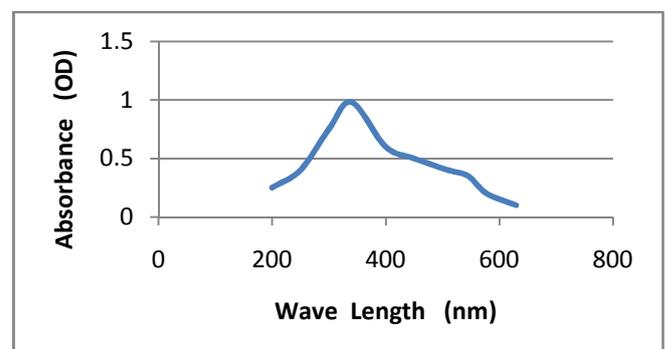


Fig. 2. The UV-VIS absorbance spectra of ZNO-NPS (the optimal W.L was 350 nm)

Several studies evaluated the antimicrobial activity of metal oxide NPs particularly ZnO powder against fungi in culture

media. Metal oxides nanoparticles are characterized by very high surface area to volume particularly ZnONPs which is of the least hazards to the environment (Sawai and Yoshikawa, 2004; Hassan *et al.*, 2013 b and Nabawy, 2015). Zinc oxide is one of five zinc compounds that are currently listed as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Zinc salt has been used for the treatment of zinc deficiency (Lopes *et al.*, 2002).

Recently, the antifungal activities of ZnONPs was evaluated, the yeast of *C. albicans* was more sensitive for relatively lower concentrations (100 µg/ml). While, *A. niger* and *A. ochraceus* required higher concentration of ZnONPs to inhibit their growth. The diameters of inhibition zones of ZnONPs (MIC) against *A. flavus* and *A. ochraceus* were 7 and 15 mm at the concentration of 300 µg/ml using well diffusion test, whereas, *A. niger* required relatively lower concentration (200 µg/ml) of nanoparticles to inhibit their growth (Hassan *et al.*, 2014). Other study by Hosseini *et al.* (2011) reported that the MIC of ZnONPs against *Aspergillus spp.* and *C. albicans* was 1.013-296 µg/ml and for SDS and of Fluconazole were 0.001-0.56 and 0.062-128 µg/ml, respectively. They added that lower concentrations of ZnONPs were most effective as antifungal and antibacterial agent. Furthermore, different studies conducted in different laboratories showed that the antimicrobial activity is influenced by not only nanoparticles concentration but also by the size of the ZnO particles (Violeta *et al.*, 2011 and Shawky *et al.*, 2014).

On the other hand, Hassan *et al.* (2013 b) evaluated the inhibition of aflatoxigenic mold growth and aflatoxins production on yeast extract sucrose medium by zinc oxide NPs and they detected that a lower concentration of (8 µg/ml) was required to obtain effective inhibition. While, Hassan *et al.* (2013 c) investigated the inhibitory effect of prepared iron oxide nanoparticles against *C. neoformance*. They detected that the MIC of Fe₂O₃-NPs by the use of disc diffusion test was efficient than well diffusion test, where the MIC of Fe₂O₃-NPs for *C. neoformance* was 50 ppm but in case of well diffusion test the MIC was 100 ppm or more. However, EL-Diasty *et al.* (2013) evaluated the antifungal activity of zinc oxide nanoparticles against species of *Trichophyton mentagrophyte*, *Microsporum canis*, *Candida albicans* and *Aspergillus fumigatus* that were isolated from diseased cases. They detected that the largest inhibition of the germination of all the tested fungi was observed at largest ZnO nanoparticles concentration (40 mg/ml).

Nabawy *et al.* (2014) evaluated the antifungal potential of ZnO nanoparticles in comparison with commercial antifungal feed additives. The authors revealed that the mean of growth inhibition zone diameters for non-aflatoxigenic strains ranged from (11.0±0.5 mm) to (21.3±0.96 mm) when the concentration of ZnO nanoparticles elevated from 25-250 µg/ml using well diffusion test. Whereas, at the same concentrations of ZnO nanoparticles, the mean growth inhibition zone diameters of aflatoxigenic strains were relatively smaller (ranged from 8.2±0.4 to 19.3±0.94 mm).

Whereas, nano-silver exhibited potent activity against clinical isolates and ATCC strains of *Trichophyton mentagrophytes* and

Candida species (IC80, 1-7 µg/ml). The activity of nano-Ag was comparable to that of amphotericin B and fluconazole. The antifungal activity of nano-Ag is attributed to its effects on the fungal mycelia (Kim *et al.*, 2009; Hassan *et al.*, 2013 c). There are also reports of the application of nanosilver having antifungal activity in bio-stabilization of footwear materials, wherein, 1% solution inhibited the growth of the majority of yeast-like fungal and mold strains (Falkiewicz and Macura, 2008).

In the present study, the antimicrobial potential of prepared ZnONPs was evaluated by broth micro dilution method as recommended by (CLSI, 2008). The inoculum size, turbidity and transmittance of treated spore suspension of dermatophytes and bacteria was determined spectrophotometrically to provides target percent of transmittance (T%) readings. It is difficult to hold microtiter tests longer than 72 h due to dehydration. Many of the less frequently encountered fungi as dermatophytes may require as long as 120–144 hours before growth is detected in the drug-free growth control well. For this reason, isolates that are known to be slow growers should be tested via the macro broth method (CLSI, 2008). The endpoint determination depend on a reduction in turbidity which typically easy to visualize. Reading the MIC endpoint determined at the lowest concentration that prevents discernable growth, the first clear well (Ghannoum *et al.*, 2004). The dermatophytes are a specialized group of fungi, which infect the keratinized tissues of humans and animals, such as skin, hair, and nails, commonly causing superficial infections. While, there have been several studies involving yeasts which have been used to develop the Clinical and Laboratory Standards Institute (CLSI) reference method and the antifungal susceptibility of dermatophytes has only recently been addressed in the standard for filamentous fungi (CLSI, 2008).

Currently, the tabulated results in Table (3), illustrated that the antifungal potential of ZnONPs against *T. verrucosum* and *T. mentagrophyte* species was concentration dependant, when the concentrations of ZnONPs increased up to 20 mg/ml, the optical density and turbidity of treated spore suspension were decreased till reach 100% transmittance and clear medium. The inhibitory concentration of ZnONPs that inhibited 50% of the growth of *T. verrucosum* and *T. mentagrophytes* was 11, 12 mg/ml and it was 17, 14 mg/ml for IC 100%, respectively. The transmittance percentage and clearance of spore turbidity were confirmed by re-cultivation of inoculums from treated tubes on the specific medium SDA for dermatophyte and NA for bacteria. The accordance of all previous parameters was repeated 2 times to pooled data (Table, 3 and Fig. 3 & 4).

In other study, Hassan *et al.* (2015) evaluated the antimicrobial effect of prepared Fe₂O₃ NPs against isolated *Dermatophytes* and *Dermatophilus* species that recovered from skin affection of cattle and it had an inhibitory effect against the growth of *T. verrucosum* at concentrations of 3 mg/ml and 4 mg/ml, respectively (using well diffusion test). While, in case of *T. mentagrophytes*, iron oxide NPs revealed an inhibitory effect at concentration of 1, 2, 3, 4 and 5 mg/ml by well diffusion test. The treatment by Fe₂O₃ NPs had no effect on the growth of *Dermatophilus sp.* at the concentration ranged from 1- 3 mg/ml

using disc diffusion test. While, the treatment by 4 mg/ml or more resulted in inhibition of bacterial growth.

Table 3. Optical density and degree of turbidity of treated Dermatophytes recovered from skin affection of buffaloes at gradual concentration of ZnONPs

Gradual doses of ZnONPs mg/ml	Dermatophyte Sp					
	<i>T.verrucosum</i>			<i>T.mentagrophytes</i>		
	O.D	T %	D.T.&G.T.	OD	T%	D.T.&G.T.
10	0.60	25.1	4+	0.75	17.7	4+
11	0.56	27.5	3+	0.52	30.2	3+
12	0.30	50.1	2+	0.18	66	2+
13	0.20	63.1	1+	0.05	89.1	1+
14	0.17	67.6	1+	0.04	91.2	1+
15	0.11	77.7	1+	0.00	100	0-
16	0.05	89.1	0-	0.00	100	0-
17	0.00	100	0-	0.00	100	0-
18	0.00	100	0-	0.00	100	0-

Control antifungal: Fluconazol 20 ug (Its OD: 00 and turbidity: 00)- OD : Optical density of treated spores at wave length ; 405 nm.
 - DT : Degree of turbidity of treated suspension.
 - G.T.: Growth after Treatment.
 T%: Transmittance %

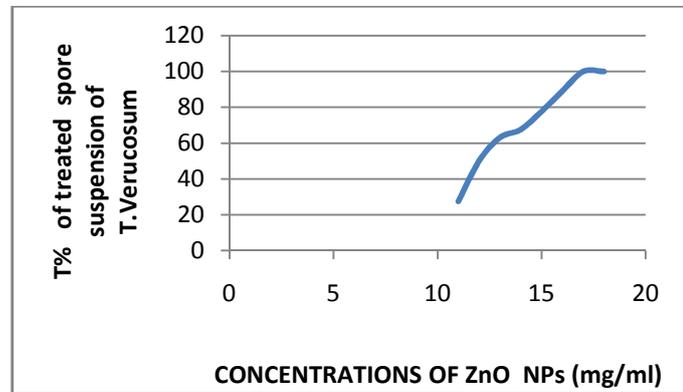


Fig. 3. Transmittance % of treated T.verrucosum at gradual concentration of ZnO NPs

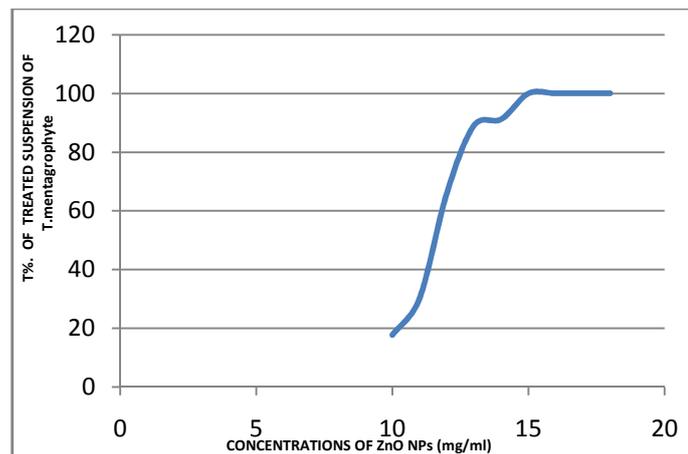


Fig.4. Transmittance % of treated T.mentagrophyte at gradual concentration of ZnO NPs

Several studies showed that nanoparticles as zinc homeostasis is regulated through a number of specific and nonspecific membrane-bound uptake and efflux pumps (Abou-Mohamed *et al.*, 1998) and prevents sulfhydryl groups from oxidation (Lim *et al.*, 2004). The antimicrobial activity of ZnONPs have been studied against the food related bacteria *Bacillus subtilis*,

Escherichia coli and *Pseudomonas fluorescens* (Jiang *et al.*, 2009). Zinc oxide nanoparticles could potentially be used as an effective antibacterial agent to protect agricultural and food safety from food borne pathogens, especially *E. coli* O157:H7 (Zhang *et al.*, 2007). Also, ZnONPs possess antimicrobial activities against *Listeria monocytogenes*, *Salmonella enteritidis* and *E. coli* O157:H7 in culture media (Jin *et al.*, 2009). There are also other studies confirming the strong antimicrobial activity of ZnO nanoparticles where, the nanoparticles could completely lyse the food-borne bacteria *Salmonella typhimurium* and *Staphylococcus aureus* (Liu *et al.*, 2009 and Hassan *et al.*, 2014). In another study, ZnO nanoparticles (12 nm) inhibited the growth of *E. coli* by disintegrating the cell membrane and increasing the membrane permeability (Jiang *et al.*, 2009). The above findings suggest that ZnO nanoparticles can find applications in food systems and can be used to inhibit growth of pathogenic bacteria.

Regarding, the antibacterial potential of prepared ZnONPs in this study, the current data in Table (4) and figures (5, 6), showed the antifungal potential of ZnO-NPs against *D.congolensis* and *S.aureus* species. As the concentrations of ZnONPs increased up to 250 ug/ml, the optical density and turbidity of treated cell suspension were decreased till reach 100% transmittance and clear medium at the IC80-100 of ZnONPs for treated bacteria. The IC that inhibited 50% of the growth of *D.congolensis* and *S.aureus* species was 12.5-25 ug/ml and it was 150 ug/ml for MIC80-100%, respectively. The transmittance % and clearance of cells turbidity were confirmed by re-cultivation of inoculums from treated wells on the nutrient agar medium for *D.congolensis* and *S.aureus* species. The accordance of all previous parameters were repeated 2 times to pooled data (Table, 3 and Fig. 5 & 6).

Table 4. Optical density and degree of turbidity of treated Dermatophilus and Staphylococci bacteria recovered from skin affection of buffaloes at gradual concentration of ZnO NPs

Gradual doses of ZnO NPS ug/ml	Bacterial species					
	<i>D. congolensis</i>			<i>S.aureus</i>		
	O.D	T%	D.T.&G.T.	OD	T%	D.T.&G.T.
0	1.27	5.4	4+	0.80	15.1	4+
50	0.40	39.8	3+	0.75	17.7	3+
100	0.20	63.1	2+	0.17	67.6	2+
200	0.10	79.4	1+	0.11	77.6	2+
300	0.03	93.3	1+	0.08	83.2	1+
500	0.00	100	0-	0.00	100	0-
1000	0.00	100	0-	0.00	100	0-
2000	0.00	100	0-	0.00	100	0-
3000	0.00	100	0-	0.00	100	0-

Control antibacterial as Entrofloxacin 20 ug (Its OD: 00 and turbidity: 00)- OD : Optical density of treated spores at wave length ; 405 nm. - DT : Degree of turbidity of treated suspension.- G.T.: Growth after Treatment.
 T%: Transmittance%

On the other hand, the antimicrobial effect of ZnONPs was reported to occur by 2 ways. The first is the formation of H₂O₂ on the surface of ZnONPs due to the possible formation of hydrogen bond between hydroxyl group of cellulose molecules of fungi with oxygen atom of ZnONPs leading to inhibition of the microbial growth, while the second is the release of Zn²⁺ that causes damages of cell membrane and interacts with intraocular contents (Moraru *et al.*, 2003). Several natural and engineered nanomaterials have demonstrated strong

antimicrobial properties through diverse mechanisms including photocatalytic production of reactive oxygen species that damage cell components and viruses (as ZnO), compromising the cell envelope (e.g. peptides, chitosan carboxyfullerene, carbon nanotubes, ZnO and interruption of energy transduction (Matei *et al.*, 2010; Violeta *et al.*, 2011).

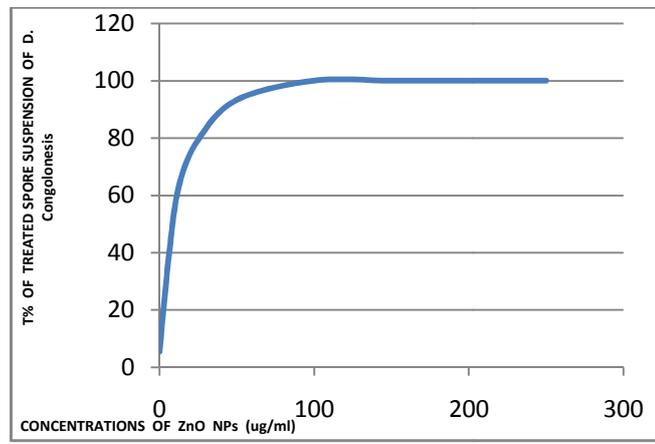


Fig.5. Transmittance % of treated *D. congolensis* at gradual concentration of ZnO NPs

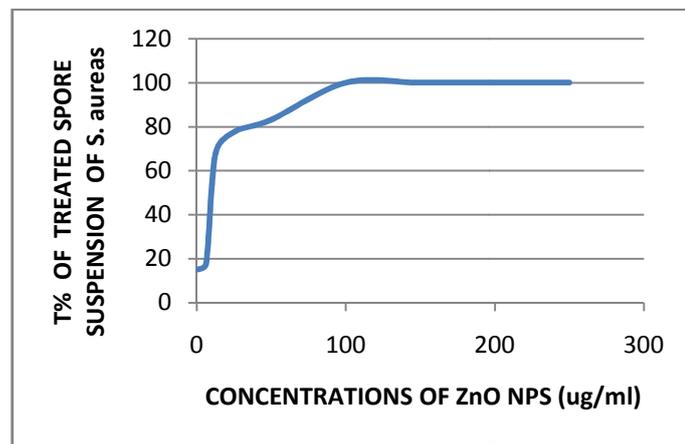


Fig.6. Transmittance % of treated *S. aureus* at gradual concentration of ZnO NPs

There are several mechanisms, which have been proposed to explain the antibacterial activity of ZnONPs. The generation of hydrogen peroxide from the surface of ZnO is considered as an effective mean for the inhibition of bacterial growth (Yamamoto, 2001). It is presumed that with decreasing particle size, the number of ZnO powder particles per unit volume of powder slurry increases resulting in increased surface area and increased generation of hydrogen peroxide. Another possible mechanism for ZnO antibacterial activity is the release of Zn²⁺ ions which cause the damage of cell membrane and interact with intracellular contents (Brayner *et al.*, 2006).

In the present work, it was proved that the antifungal and antibacterial effects of metal nanoparticles was probably due to the damage of the cell wall of the microbial cells leading to leakage of the cell contents and finally cell death. These effects are confirmed when the treated *dermatophytes*, *dermatophilus species* and *S. aureus* were subjected to SEM, the damage and rupture of their cell wall were detected in the area surrounding

growth. The effect of high concentration of ZnO nanoparticles on the treated fungi and bacteria was observed as membrane damage of cells and some pits that have been caused leakage in inter cellular components and finally cell death (Fig.7&8). Similar findings were also reported by (Violeta *et al.*, 2011, Hassan *et al.*, 2014; Hassan *et al.*, 2014, 2015; Shawky *et al.*, 2014).

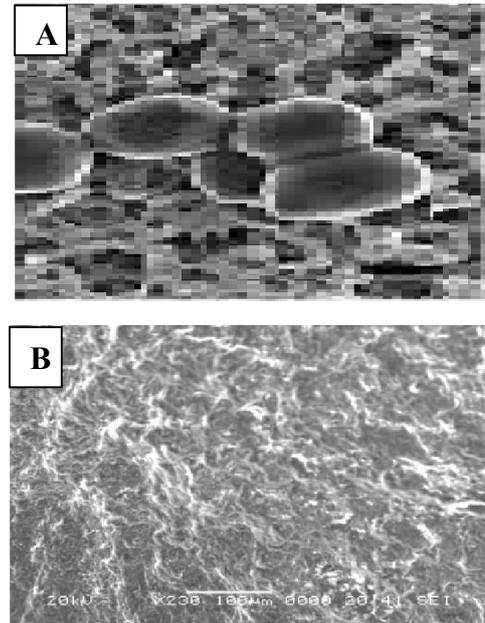


Fig. 7. (A) Scanning Electron Microscopy (SEM) of normal *T. verrucosum* (B): Scanning Electron Microscopy (SEM) of *T. verrucosum* after treatment with ZnO NPS

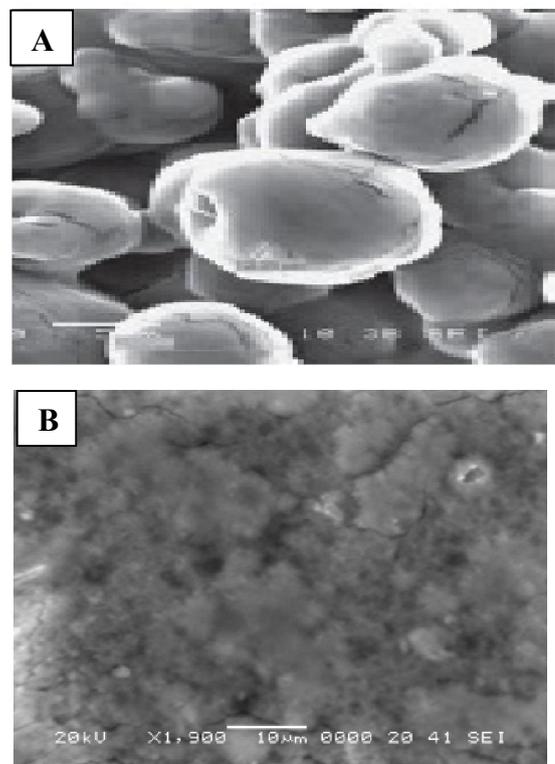


Fig. 8. (A) Scanning Electron Microscopy (SEM) of normal *D. congolensis* bacteria (B): Scanning Electron Microscopy (SEM) of *D. congolensis* after treatment with ZnONPs

Conclusion

From the present study it is concluded that the periodical examination of human and animals for skin affections pathogens should be undertaken. All environmental factors which predispose for these affections must be under hygienic measures and good control. The rapid biosynthesis of zinc oxide nanoparticles using aqueous leaf extract of *Corriandrum sativum* plant provides an environmental friendly, simple and efficient route. The use of plant extracts avoids the usage of harmful and toxic reducing and stabilizing agents. In addition, the antimicrobial effects of metal nanoparticles are due to the damage of the cell wall of the microbial cells leading to leakage of the cell contents and finally cell death. Further studies are needed to investigate the efficacy of pharmaceutical preparation of ointments, skin lotions and synergistic therapy with other traditional antibiotics in the treatment of animal diseases.

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