

Study of Action of *Terfezia Boudieri* Chatin, a Wild Edible Desert Truffle from Egypt on Morphological Features and Ultra Structures of Certain Bacterial and Fungal Isolates



Biotechnology

KEYWORDS : Terfiziaboudieri; antimicrobial activity; bacterial fungal isolates; SEM; TEM.

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ABSTRACT

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to study the effect of aqueous, chloroform, acetone and methanol extracts of *Terfezia boudieri* on morphological features and ultra-structures of four isolates including Gram positive and Gram negative bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa* and filamentous *Aspergillus niger* and *Candida albicans*, where previously showed highly sensitive to these extracts, respectively. Great changes (aggregation, distortion, elongation, swollen) of morphological features and alteration of ultra- organelles measurements of tested fungal isolates were reported.

Introduction

From early stages of civilization, desert macro-fungi in forms of mushrooms and truffles have been used as food and medicine. Originally, these types of organisms were associated with Mediterranean region and were first recorded as poem in Egyptian temples as follows: "Without leaves, without buds, without flowers: yet they from fruit; as a food, as a tonic, as a medicine: the entire creation is precious. Thus, macro-fungi were considered as food and medicine for royalty, and that no normal citizens were allowed to consume this precious food. During Greek and Roman eras, they were imported from Libya and sold in southern part of the European continent (Honrubia *et al.*, 2007). In the southern part of African continent, the nomadic people of Kalahari Desert used truffles for millennia (Trappe, 1990).

Mushrooms are visible to the naked eye as they grow above the earth, whereas truffles grow underground in depth between 5 and 10cm. Truffles are usually collected by specialists who have special skills and experience to explore this type of flora. Sometimes, truffle collectors use some animals such as pigs and dogs to discover this type of geographical distribution. This is based on their high sensitivity to the characteristic truffle volatile compounds. Traditionally, mushrooms and truffles are taken as type of precious food and consumed either raw or cooked. In addition, they have been also applied as main component of folk medicine. This was based on the fact that they are rich source for proteins, amino acids, fatty acids, fiber, minerals, vitamins, terpenoids, sterols, flavor compounds, and carbohydrates as reported by many authors (Trappe, 1990 and Kala, 2009).

In general, not all types of macro-fungi are able to grow in the harsh environmental conditions of desert. The term "Desert Macro-fungi: Mushrooms and Truffles" is related to the nature and distribution of those species which can grow under arid and semiarid conditions. Thus, the geographical distribution of desert truffles in Africa and Middle East is related to countries around the Mediterranean such as Morocco, Algeria, Tunisia, Libya, Egypt, and Israel in addition to the countries of the Arabian Peninsula such as Jordan, Syria, Saudi Arabia, Iraq, Bahrain, and Kuwait. However, some types of desert truffles were also found in South Africa and Botswana. Generally, the growth of desert truffles requires an annual rainfall range between 50 and 380 mm. In North Africa, good yields of truffle are usually obtained if the rainfalls range between 70 to 120 mm. In addition, the time, quantity, and distribution of the rainfall play an important role in the quality of desert macrofungus growth. For example, to obtain good truffles in North African and Middle Eastern regions, it needs to get rainfall no later than the beginning of December whereas; in southern Europe it should not be later than the beginning of October (Lu *et al.*, 2004 and Morte *et al.*, 2009).

Nutritional value and chemical content (carbohydrates, organic fatty and phenolic acids compositions) as well as, antioxidant activity also the potency of *Terfezia boudieri* as antimicrobial activity against several pathogenic bacterial and fungal isolates and were previously investigated by Mekawey (2014). However, currently work aim to study the effect of , these extracts of *Terfezia boudier* ion

ultra - structures and morphological features of the most sensitive bacterial and fungal isolates using transmission and scanning electron microscope.

Materials and methods

Previously, fresh ascocarps of *T. boudieri* were purchased and identified in the agriculture research center - Cairo University in April 2014, Cairo Egypt. Two methods were carried out to extract the active compounds of *T. boudieri*.

A-The collected *T. boudieri* fruits were cleaned and cut into small pieces and dried under shade at room temperature. The dried material was ground to fine powder using a mechanical blender and passed through 24 mesh sieves. *T. boudieri* powder (100 g) was extracted with 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The extract was filtered through cheese cloth to remove the major debris and the filtrate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was considered as crude aqueous extract of *T. boudieri* and stored at 4°C for experimental use (Aldebasi *et al.*, 2012).

B-Powdered fungus sample (30 g) was extracted with 250 mL of chloroform in a glass beaker for 8 h at 55°C on a hot plate. The resultant extract was concentrated using a rotary evaporator at 40°C at low pressure, and the desired phase was separated from the crude extract with chloroform. Later, the residue was extracted with acetone followed by methanol. After extractions were completed, all the semi-solid extracts were lyophilized and stored at 4°C (Doğan and Aydin, 2013).

Also, Previously antimicrobial activities of these extracts were examined their potency as antimicrobial activity against 10 pathogenic bacterial isolates; five Gram-negative bacteria and the others Gram positive bacteria. nine filamentous fungi and sex yeast isolates, the results were reported that all the tested bacterial and fungal isolates were affected by *T. boudieri* extracts. In addition, all Gram positive and unicellular fungi (yeasts) exhibited highest rate of sensitivity to *T. boudieri* aqueous extract while organic solvent extracts had great ability to inhibit the growth of Gram negative and filamentous fungi (Mekawey, 2014). However, aqueous extract had high effective on *Bacillus subtilis*, as well as *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* showed high sensitiv-

ity to chloroform acetone and methanol extracts, respectively so that all these organisms were recently studies their effectiveness to *T. boudieri* extracts under electron microscope.

Scanning Electron Microscopy (SEM):

Blocks of the investigated fungal isolate were prepared and examined for SEM at the National Research Centre, Dokki, Cairo, Egypt. Fixation and dehydration procedures were performed using the programmable LEICA EM TP tissue processor model (A-1170), where six to eight millimeter squares of agar with fungal growth were cut from the cultures. The squares were then fixed by immersion in 2% (w/v) aqueous osmium tetroxide (OsO₄) at 4°C for 12 h. Fixed material was allowed to attain at room temperature and then washed in distilled water (3 times, 10 min each) to remove excess of OsO₄ (Yamaguchi et al., 1992).

Fixed and washed materials were submerged and dehydrated through a graded, 10% steps, ethanol series from 10% to 90% and finally absolute ethanol. Dehydrated specimens were critical point-dried using pressure bomb. The critical point-dried specimens were then attached to 0.9 mm diameter copper stubs using a carbon adhesive. Specimens were gold-coated (nearly 50 nm thickness) using polar instruments Inc., Doylestown, PA with gold then examined using the high-vacuum mode of a JEOL JSM-35LV Scanning Electron Microscope (El-Meleigi, 1996).

Transmission Electron Microscopy (TEM):

Onem³ blocks of tested isolates as control and other treated with EHP compound were fixed with 3% glutaraldehyde – 1% paraformaldehyde at 4°C overnight and 1% osmium tetroxide at 4°C for 1hr., embedded in 2% agar, and dehydrated by a graded series of ethanol. For rapid freezing and freeze-substitution, living cells were sandwiched between two copper discs, rapidly frozen by plunging into propane slush in liquid nitrogen (Yamaguchi et al., 1992), and freeze – substituted in acetone containing 2% osmium tetroxide at -80°C for 2 days. For freeze substitution after glutaraldehyde fixation, cells were fixed in a mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30 – 60 min. or at 4 °C over night.

They are collected by centrifugation, rapidly frozen by propane slush, and freeze – substituted in acetone containing 2% osmium tetroxide at 80°C for 2 days. These differently fixed and dehydrated samples were them embedded in epoxy resin and polymerized at 60 °C for 24 hrs. Ultra-thin sections were cut to a thickness of 70 – 90 nm with a diamond knife on an ultra-microtome (Leica ultracut S) and mounted on copper grids. They are stained with uranyl acetate and lead citrate, coated with plasma – polymerized naphthalene support film and observed in JEM 12000EX TEM (Jeol, Tokyo) at 80kv at the National Research Centre, Dokki, Cairo, Egypt(Yamaguchiet al., 2005).. Measurements of microbial isolates morphological features and their ultra structures were performed by image analysis software (analysis 2.1) where the mean, minimum and maximum of 50 measurements for each case were taken.

Results

Study the effect of *T. boudieri* extracts on morphological features and ultra-structures of many bacterial and fungal isolates are investigated using scanning and transmission electron microscopes. *Bacillus subtilis* (Gram positive

bacteria), *Pseudomonas aeruginosa* (Gram negative bacteria), *Aspergillus niger* (filamentous fungi), and *Candida*

albicans (unicellular fungi), showed high sensitivity to aqueous,

chloroform, acetone and methanol extracts; respectively

so that all these organisms are subject to SEM & TEM to study the effect of a *T. boudieri* extracts on their morphological and

their structures. Scanning electron micrographs reveal the high sensitive of bacterial isolates used to *T. boudieri* extracts. Many changes are detect in cells of *Bacillus subtilis* and *Pseudomonas aeruginosa* when treated by aqueous and chloroform extracts, respectively. Both bacteria isolates are unable to keep their form, many distortion in shape and their sizes dimension are reduced, whereas the length and width of normal cells of *Bacillus subtilis* measure 1.2X0.6µm and became 0.56X0.29µm after stress effect (Photos 1 “A,B,C,D”). On contrast, sizes dimension of *Pseudomonas aeruginosa* are increased from 0.85X0.43 µm to 1.44X 0.64 µm after stress condition (photos 2 “A,B,C,D”). Also very enlarged and tall cells chains are produced in both bacterial isolates after treatment with *T. boudieri* extracts (arrows in photo 1&2 “C,D”).

Transmission electron micrographs revealed many changes in ultra-structures of *Bacillus subtilis* and *Pseudomonas aeruginosa* under the same stress such as most of cells became empty; cell wall became thinner (normal *Bacillus subtilis* and *Pseudomonas aeruginosa* cells wall measures 110.5 and 162.9 µm in diameter, respectively and became 67.4 and 76.1 µm in diameter, respectively after treatment (photos 1 & 2 “E, F, G, H”). On the other hand, separation and shrinking in cell membrane is clearly observe, and many pigment vacuoles are formed (arrows in photo 1&2 “G,H”).

Similar, scanning electron micrographs reveal the high sensitive of *A. niger* against acetone extract of *T. boudieri* (photo 3 “A, B, C, D”). The fungus was able to keep its aspergillate form but with alteration of all morphological features. However, conidia increased in diameters from 2.74 to 3.67 µm, vesicles became distorted and smaller by 11.3 µm. Sterigmata became shorter in length and swollen whereas different distorted sterigmata shapes were observed; enlarged, slender as well as diminished. As well as, several changes on the morphological structure of *Candida albicans* by methanol extract of *T. boudieri* can be summarized as, aggregation of *C. albicans* cells was observed while many distortion was obtained in many *C. albicans* cells like unregularly shape, swollen beside their number were decreased and pseudo-mycelium was disappeared. Also, SEM results show the remarkable changes on the morphological structure of conidia whereas most of yeast cells were greatly increase in sizes from 3.5X2.75 to 10.27X8.48 (photo 4 “A, B, C, D”).

In the present study, action of *T. boudieri* extract on the sub-cellular organelles of *A. niger* and *C. albicans* was investigated using TEM microscopy. The present study declared that, several changeable and distortions of sub-organelles of *A. niger* were obtained under acetone and methanol extracts of *T. boudieri* stress. On general, cell wall and cell membrane were slightly decreased in thickness, on contrast, great reductions in mitochondria and nucleus diameter was observed. Photos 3 & 4 (“E, F, G, H”) showed that all fungus and yeast cells parts appeared inside semi-empty and all sub-organs were distorted in their shape, separation and shrinking of cell membrane away from cell wall was observed. Oil drops was appeared and pigment was precipitated around cell membrane. Number of vacuoles was decreased while very large vacuole was showed. Finally, thick sheath was appeared around the conidia.

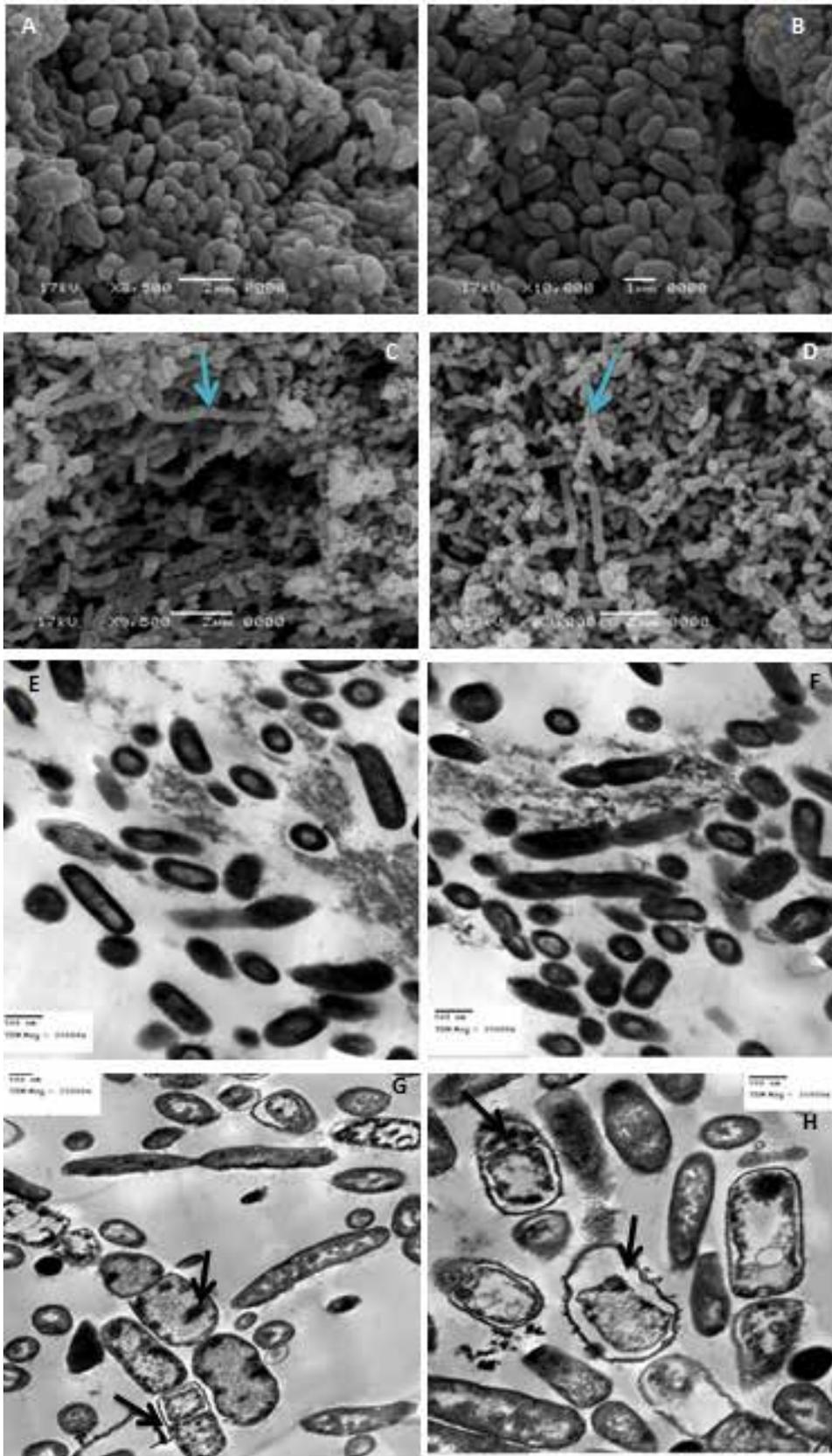


Photo 1: Electro-micrographs of *Bacillus subtilis*

A,B: SEM of normal cell (control); C, D: SEM of treated cells with aqueous extract of *Terfiziaboudier*
E,F: TEM of normal cell (control); G, H: TEM of treated cells with aqueous extract of *Terfiziaboudieri*

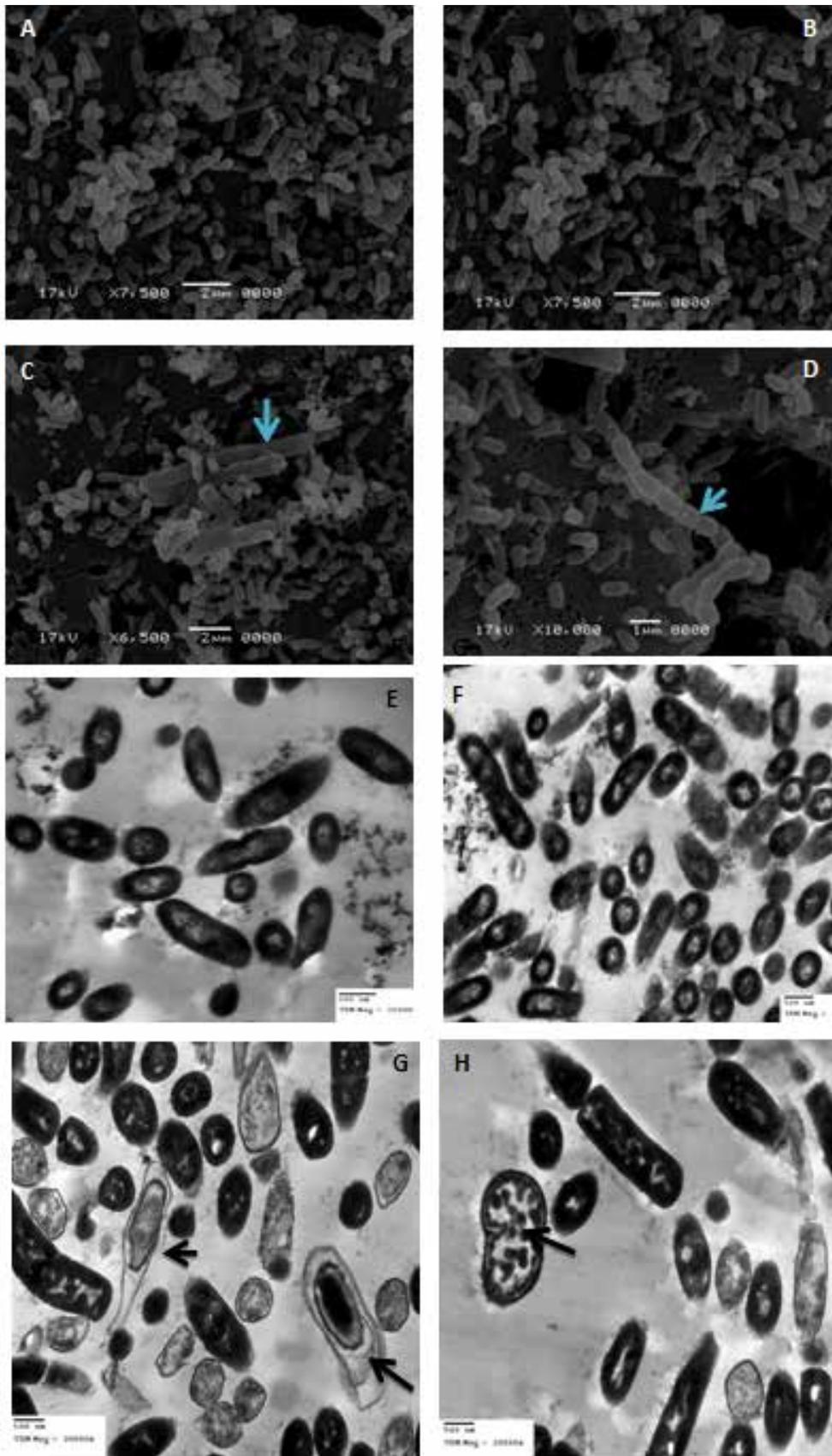


Photo 2: Electro-micrographs of *Pseudomonas aeruginosa*

A,B: SEM of normal cell (control); C, D: SEM of treated cells with chloroform extract of *Terfziaboudier*
 E,F: TEM of normal cell (control); G, H: TEM of treated cells with chloroform extract of *Terfziaboudieri*

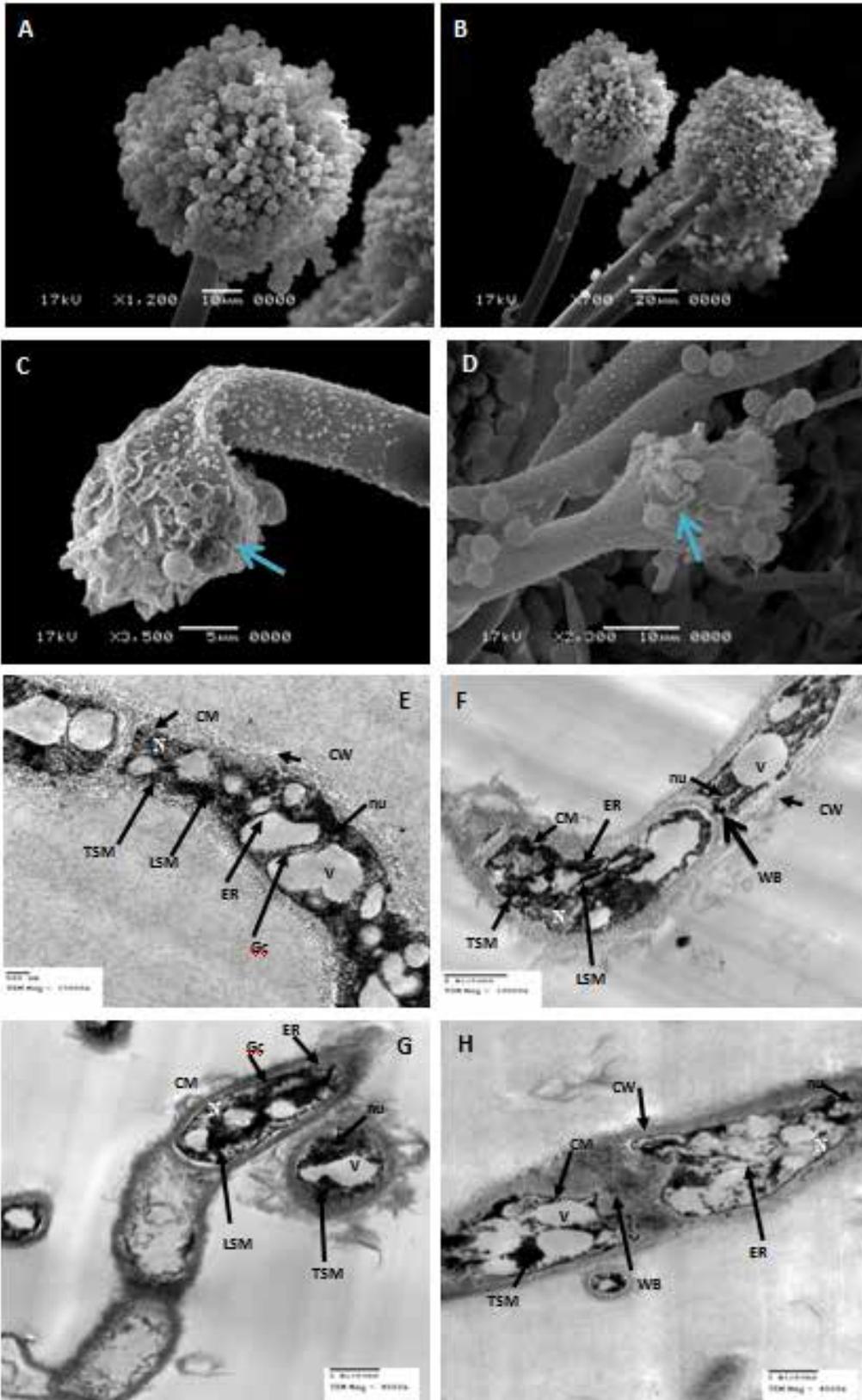


Photo 3: Electro-micrographs of *Aspergillusniger*

A,B: SEM of normal cell (control); C, D: SEM of treated cells with acetone extract of *Terfizia boudieri*

E,F: TEM of normal cell (control); G, H: TEM of treated cells with acetone extract of *Terfizia boudieri*

*** Cell Wall (CW); Cell Membrane (CM); Transverse Section of Mitochondria (TSM); Longitudinal Section of Mitochondria (LSM); Nucleus (N); nucleolus (nu); Vacuole (V); Multivesicular Bodies (MvB); Golgi cisternae (Gc); Budding scare (BS); Endoplasmic Reticulum (ER)**

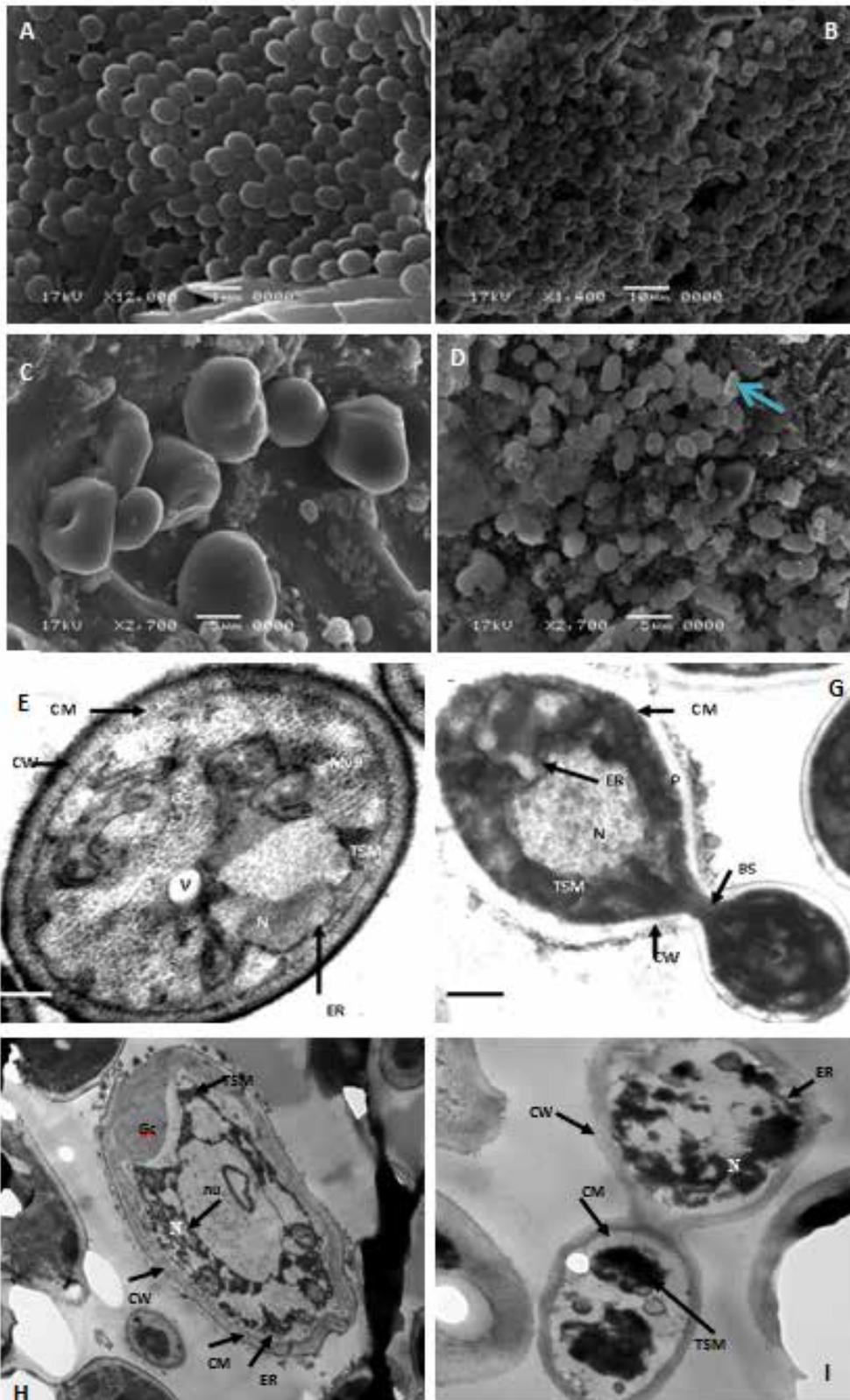


Photo 4: Electro-micrographs of *Candida albicans*

A,B: SEM of normal cell (control); C, D: SEM of treated cells with methanol extract of *Terfizia boudieri*

E,F: TEM of normal cell (control); G, H: TEM of treated cells with methanol extract of *Terfizia boudieri** Cell Wall (CW); Cell Membrane (CM); Transverse Section of Mitochondria (TSM); Longitudinal Section of Mitochondria (LSM); Nucleus (N); nucleolus (nu); Vacuole (V); Multivesicular Bodies (MvB); Golgi cisternae (Gc); Budding scare (BS); Endoplasmic Reticulum (ER)

Discussion

The most interesting finding is the detection of no literature about studying on the action of *T. boudieri* extracts on the morphological features and ultra-structures of bacteria and/or fungi were found to support the present results, thus we can compare the action of present natural products with action of previous plants and/or fungi, so that we recommended further investigation occur to prove mentioned results.

Our results agree with those of Younis et al (2009) reported that three edible mushroom strains belonging to family Pleurotaceae (*Pleurotus ostreatus*, *Pleurotus sajorcaju* and *Pleurotus eryngii*) were assayed *in vitro* for their antimicrobial activities using three solvents for extraction including ethanol, methanol and water from the fruiting bodies of the three *Pleurotus speciosa* against twenty fungal species including yeast and filamentous fungi as well as ten bacterial species including gram positive and negative bacteria. High affective microorganisms were subject to SEM and TEM assay and shrinkage of cell membrane and all distortion of all sub organs were observed.

Also, plant consider nature source can effect against fungal and bacterial isolates such as, Kurita *et al.* (1981) who suggested that, plant components have fungicidal action on *Candida albicans*. Plant components, however induced damages causing the death of a great number of yeasts. *Plantago major* would seem, therefore, to be the major agent in yeast degradation. The action of plant extract on membranous structure, described by Moulinet *al.* (1986) who reported that, plant extract components might be degraded the components of cell wall, and that explained the reduction of cell wall of the cells of *C. albicans*. These results in accordance with Shraideh *et al.* (1998) who declared that, ultrastructural modifications were essentially reflected in the decreased in the number of mitochondria and most of cytoplasmic membrane distorted in its shape.

Neil and Geoffrey (1995) who reported that pure antifungal compound acts by binding to a specific sterol in cytoplasmic membrane of sensitive fungi, induce excessive permeability of the plasma membrane allowing leakage of essential molecules. Antifungal agent able to react with the bounds in organs and suppressed their functions so that fungal growth was stopped. On the otherhand, granules formed between cell wall and plasma membrane as action of components of *Hammada elegans* these granules may be lomasomes *organs.lomasomes* have been recognized in many species of fungi (Kucers *et al.*, 1997).

Conclusions and Future Prospects

Africa and Middle East are very rich regions of unique types of macro-fungi. Both truffles and mushrooms of this area of the world produce wide variety of interesting bio-active compounds of high medical value and were used for millennium in the treatment of different diseases. The main drawbacks for their application in modern medicine and for production in industrial scales are based on four main facts. First, most of these types of organisms are not cultivable in green house and thus their availability is seasonal and highly affected by climate change. The second fact is the wide variability of the bioactive ingredient contents which are highly dependent on collection time, procedure, season, and environment. Third, based on the chemical composition of both of mushrooms and truffles, they have high capacity to accumulate high concentration of heavy metals and radio active isotopes. Thus, special consideration should be taken into account when collected from polluted areas. The fourth fact is the lack of standard testing protocols to guarantee the quality and the efficacy of the fungal product. Thus, more research is required to solve the above mentioned problem to increase the use of wild macro-fungus in medical applications. This will change in part the current medical practice using chemically synthesized compounds of many side effects.

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