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Electron Microscopy Studies of the Effects of Garlic Extract Against *Trichophyton rubrum*

(Kajian Mikroskopi Elektron ke Atas Kesan Ekstrak Bawang Putih ke Atas *Trichophyton rubrum)*

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ABSTRACT

Trichophyton rubrum *is one of dermatophytes that penetrates keratinized tissues such as skin, hair and nail of human and animals. Recently, antifungal drugs such as imodazole and triazole was found to cause side effects, toxicity to patients and also not very efficient due to resistance to these drugs. As an alternative, some plants extract had been used to treat dermatophytes. This studies was done using Garlic extract (*Allium sativum*) to evaluate its effects on the growth of hypha of* Trichophyton *using Electron miscroscopy. Garlic had been known to posses antimicrobial, antiinflammatory, antithrombotic and antitumor activities. This studies found that garlic extract as low as 4 mg/mL inhibit the growth of hypha. Scanning electron microscopy studies revealed that hypha treated with garlic extract showed shrinkage, flat and cell wall demolition, similar to hypha treated with allicin (positive control) having rough surface, shrinkage and distortion. The tip of hypa became large after treatment with garlic extract. Transmission electron microscopy studies also found that hypha treated with allicin display cell wall thickening, local thickening, destruction of cytoplasmic content, mean while hypha treated with garlic extract exhibited cell wall thickening, disordered hyphal tip and desolution of cytoplasmic compartments and similar with hypha treated with allicin. These results showed that garlic extract and pure allicin could be use as an alternative to treat dermatophytes.*

*Keywords: Allicin; garlic extract (*Allium sativum*); hypha;* Trichophyton rubrum

ABSTRAK

Trichophyton rubrum *merupakan satu daripada dermatofit yang menyerang tisu berkeratin seperti kulit, rambut dan kuku manusia dan haiwan. Mutakhir kini, ubat-ubatan antikulat seperti imodazole dan triazole didapati menyebabkan kesan sampingan, beracun kepada pesakit dan penggunaan tidak berkesan disebabkan ketahanan kepada ubat-ubatan ini. Sebagai salah satu alternatif, beberapa ekstrak tumbuhan telah digunakan untuk merawat dermatofit. Kajian ini dilakukan dengan menggunakan ekstrak bawang putih (*Allium sativum*) untuk mengkaji kesannya ke atas pertumbuhan hifa* Trichophyton *menggunakan mikroskop elektron. Bawang putih telah dikenal pasti sebagai antimikrob, antiradang, antitrombotik dan antikanser. Hasil kajian mendapati ekstrak bawang putih dengan kepekatan paling rendah iaitu 4 mg/mL berupaya merencat pertumbuhan hifa. Kajian imbasan mikroskop elektron menunjukkan ekstrak bawang putih menyebabkan pengecutan dan pemipihan hifa, dinding selnya musnah dan hujung hifa menjadi besar. Hasil yang sama juga ditunjukkan oleh hifa yang dirawat dengan alisin (sebagai kawalan positif). Kajian pancaran mikroskop elektron pula mendapati hifa yang dirawat dengan alisin dan ekstrak bawang putih menunjukkan keputusan yang hampir sama dengan berlakunya penebalan dinding sel hifa, penebalan dalaman, penguraian kandungan sitoplasma dan pemusnahan kompartmen sitoplasma. Hasil penemuan kajian ini membuktikan ekstrak bawang putih dan alisin boleh diguna sebagai alternatif untuk merawat dermatofit.*

*Kata kunci: Alisin; ekstrak bawang putih (*Allium sativum*); hifa;* Trichophyton rubrum

INTRODUCTION

Dermatophytes are fungi that have the capacity to invade keratinized tissues such as skin, hair and nail of human and animals. One of the most frequently isolated dermatophytes is *T. rubrum* (Barros et al. 2007; Santos & Hamdan 2005). Antifungal drugs such as imodazole (ketoconazole) and triazole (fluconazole) groups has been used for the treatment of various fungal infection however, these drugs caused the side effects, toxicity to patients and resistance to drugs (Al-Mohsen & Hughes 1998; Odd et al. 2003; Pyun & Shin 2006).

As an alternative, plant based or plant extract had been used to treat dermatophytes. One of the plant that has been used for many years is garlic. Garlic (*Allium sativum*) has been known as antimicrobial, antiinflammatory, antithrombotic and antitumor activities. *In-vitro* studies proved that garlic extract inhibited the growth of a large number of yeasts including *Candida* spp. and fungi such as *Coccidioides immitis* (Adetumbi et al. 1986; Appleton & Tansey 1975; Barone & Tansey 1977; Ghannoun 1988) and dermatophytic fungi *T. rubrum*, *T. mentagrophytes*, *T. verrucosum*, *Microsporum cani*s and *Epidermophyton flocossum* (Aala et al. 2010).

Di-allyl thiosulfinate (allicin), a sulphur-containing compound is the active component in garlic that can inhibit the growth of fungi and bacteria (Cavillito et al. 1994). Fresh aqueous garlic extract showed antifungal activity specifically against *A. fumigates, A. terreus, A. nidulans and A. niger* (Pai & Platt 1995). The inhibitory effects of fresh aqueous garlic extract against *Aspergillus* spp. was found at all concentration which is an increase in inhibition with an increase in fresh aqueous garlic extract. The activity of allicin against *Trichophyton* spp. is better than essential oils (biological compound) from *Allium* plants (Pyun & Shin 2006).

Many previous studies showed that plant extract inhibited fungal or bacteria's growth by calculating the MIC and FICI values and hyphal growth on the agar medium. However, there are very few studies regarding how these plant extracts react against fungal and bacteria. Thus, understanding the reaction of plant extract against fungi and bacteria at cellular level is crucial. To understanding this, electron microscopy studies is needed. Ghahfarokhi et al. (2004) who used SEM and TEM to study *T. rubrum* and *T. mentagrophytes*, reported that morphological abnormalities in hyphal compartments of these dermatophytes (which are caused by Allium extract) are associated with its fungistatic and fungicidal activities. Park et al. (2009) in studies of antifungal activities of four major constitutes of oil extracts (terpenes) against *T. mentagrophytes* used SEM and TEM result to explain the effects of the constituents on the gross morphology and ultrastructure of hyphae of *T. mentagrophytes*. The results underlined the antifungal activities of these four compounds against *T. mentagrophytes*. Thus, the aim of this study was to find out the ultrastructural characteristics of *T. rubrum* in response to aqueous garlic extract by SEM and TEM approach.

Materials and Methods

Preparation of antifungal agents and *T. rubrum* (ATCC- 10218)

Allicin which are used as positive control (Alexis-Biochemicals Co, San Diego, USA) was dissolved in 10 mg/mL in methanol/ water/formic acid (60: 40: 0.1) and stored at -20°C. Aqueous garlic extract (2 mg/mL and 4 mg/mL) was prepared from the fresh garlic bulbs with slight modification according to Ghahfarokhi et al. (2003). *T. rubrum* (ATCC- 10218) was used as a test fungus and cultured on sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, Michigan, USA) at 28°C for 14 days.

Culture conditions for microscopic observation

According to Park et al. (2009), minimal inhibitory concentration (MIC_{90}) used was as low concentration treatments for each allicin or garlic extract. Different concentrations of allicin: 6.25, 12.5 and 25.0 µg/mL and garlic extract: 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL were treated with *T. rubrum*. The mycelia samples were harvested for electron microscopic observation. To attain fungal mycelia from exposed to high concentration of allicin or garlic extract, sabouraud dextrose broth was used. Minimal fungicidal concentration (MFC) was used as high concentration treatments for each allicin or garlic extract. For treatment purposes, three plugs of *T. rubrum* which were inoculated in 100 mL liquid culture media (SDB) were pre-cultured in shaking incubator for 2 days. Then, allicin or garlic extract were added to the pre-culture medium and incubated for 3 more days. This study used 12.5 μg/ mL and 4 mg/mL for its selected concentration treatments of allicin and garlic extracts separately.

Specimen preparation for SEM and TEM

The samples for SEM observation were prepared according to Park et al. (2009) and Iwasawa et al. (2009) methods with slight modification. The isolates were harvested after being treated with allicin or garlic extract. The specimen of each isolate was fixed with 4% (v/v) glutaraldehyde at 4°C overnight (12- 24 h). The fixed mycelia were washed three times (10 min each step) with 0.1 M sodium sacodylate buffer (pH7.4). The samples were post-fixed with 1% (v/v) osmium tetroxide at 4°C for 2 h. The postfixed mycelia were washed again three times (10 min each time) with 0.1 M sodium cacodylate buffer (pH7.4). Then, the post-fixed samples were dehydrated in a series of graded ethanol series (from 30 to 90%) each for 10 min and then dehydrated in absolute ethanol three times (15 min each). The dehydrated specimens were critical point dried (BAL-TEC, CPD 030, Germany) for 30 min. The dried sample was coated with gold (nm) using sputter coater (BAL- TEC, SCD 005, Germany). The specimens were then observed using electron microscope (Philips XL30- ESTM at 20 kV.

The samples for TEM observations were prepared according to Park et al. (2009) and Iwasawa et al. (2009) methods with slight modification. The isolates were selectively collected after being treated with allicin and garlic extract. A specimen for each isolate was fixed with 4% (v/v) glutaraldehyde at 4°C overnight. The fixed mycelia were washed three times (each for 10 min) with 0.1 M sodium cacodylate buffer (pH7.4). The samples were post-fixed with 1% (v/v) osmium tetroxide at 4°C for 2 h. The post- fixed mycelia were washed again three times (each time for 10 min) with 0.1 M sodium cacodylate buffer (pH7.4). Then, the post- fixed samples were dehydrated in an ascending acetone concentration from 35 to 95% (each for 10 min). The samples were then dehydrated in acetone 100% three times (each for 15 min). The dehydrated samples were infiltrated with acetone and resin combination twice for 1 and 2 h, respectively. Then, the samples were kept in 100% resin overnight. The samples embedded in Spurr's resin were then polymerized in oven (Memmert UIS, Western Germany) at 60°C for 24-48 h. The samples were cut into ultra-thin sections using ultramicrotome (Leica UCT, Austria). Sections were stained with uranyl acetate for 10 min followed by lead citrate for 10 min. The stained sections were observed using a

transmission electron microscope (Philips EM 400- HMG, Holland) at 80 kV. Untreated isolates were used as controls.

Results

Figure 1 shows that the radial growth of *T. rubrum* cultures on SDA medium treated with 6.25 µg/mL, 12.5 µg/mL of allicin and 2 mg/mL, 4 mg/mL of garlic extract decreased in comparison to the negative control. In addition, the inhibition of *T. rubrum* with allicin was more as compared to fresh aqueous garlic.

Figure 2 shows the comparison between normal and treated hypa with allicin and garlic extract. Observation using SEM of *T. rubrum* exhibit normal hypha shown as smooth, thick ring- shaped septum and stable surface decoration. Micrograph b shows hypha treated with allicin (12.5 µg/mL) was found to have rough surface, shrinkage and distortion. Micrograph c showed hypha treated with garlic extract (4 mg/mL) that shrink, a flat ribbon- shaped structure and with demolition of cell wall.

Figure 3 reveals normal hypha micrograph which are linear like and showed tapered apex. Micrograph b shows hypha treated with allicin (12.5 µg/mL) revealed an expanded hyphal tip whereas hypha treated with garlic extract (4 mg/mL) exhibits a large hyphal tip.

TEM studies of *T. rubrum* isolates treated with allicin and garlic extract are shown in Figure 4. Normal/ untreated hypha showed typical cell wall, cell membrane and organelles. In contrast, hypha treated with allicin (12.5 µg/mL) revealed cell wall thickening and local thickening. Meanwhile, hypha treated with garlic extract (4 mg/mL) exhibited wall thickening and disordered hyphal tip.

To futher provide evidence of the effect of garlic and allicin, Figures 5(b) and 5(c) were included. Figure 5 shows a regular untreated hypha showing normal cell

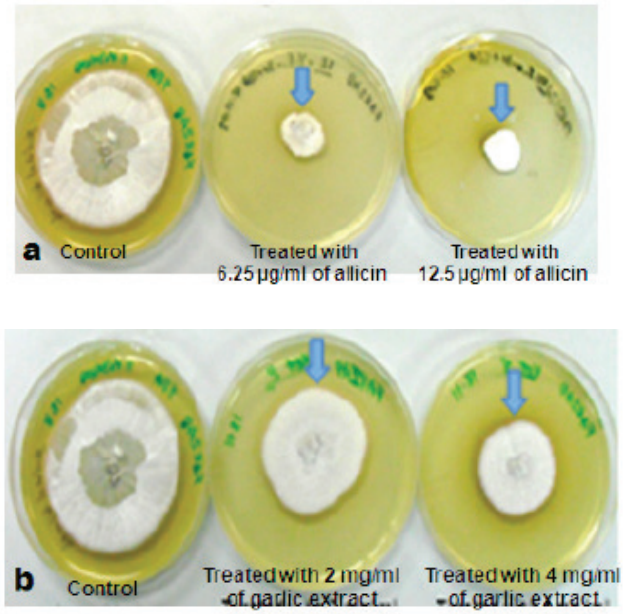

FIGURE 1. The effects of allicin (a) and fresh garlic extract (b) on the cultures of *T. rubrum*. The radial growth of *T. rubrum* colonies was reduced compared with the control

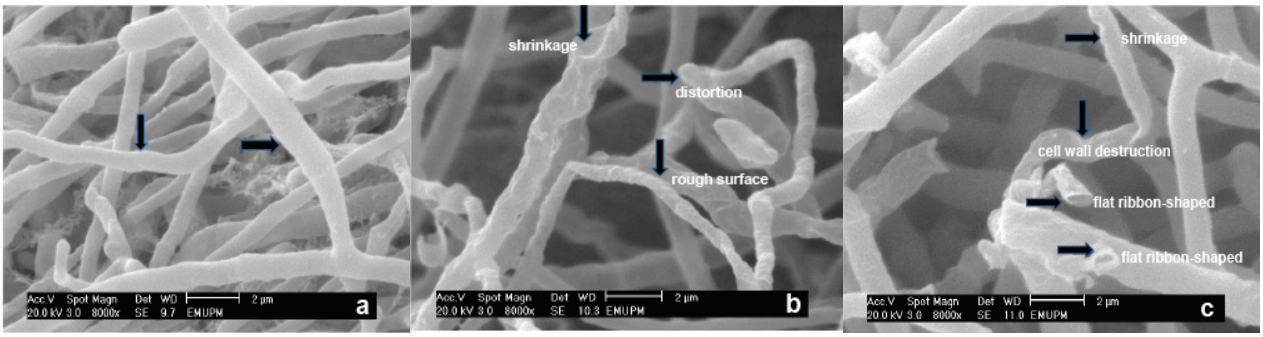

FIGURE 2. (a) Scanning electron micrographs of *T. rubrum*. A normal hypha was observed with smooth, thick ring-shaped septum and a stable surface decoration, (b) scanning electron micrographs of *T. rubrum* treated with allicin (12.5 µg/mL) showing hypha with rough surface, shrinkage and distortion and (c) scanning electron micrographs of *T. rubrum* treated with garlic extract (4 mg/mL) showing hypha with shrinkage, flat ribbon-shaped and cell wall destruction

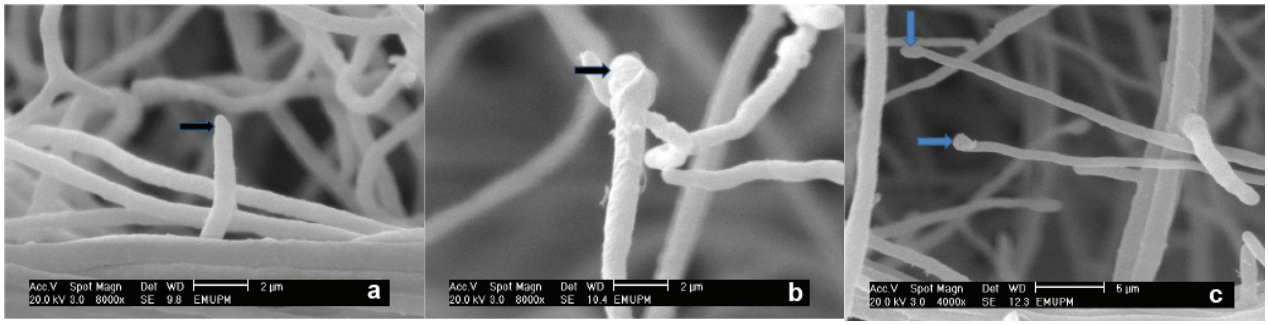

FIGURE 3. (a) Scanning electron micrographs of *T. rubrum*. Control hypha showed linearlike and tapering apex, (b) scanning electron micrographs of *T. rubrum* treated with allicin (12.5 µg/mL) showing hypha with expanded hyphal tip and (c) scanning electron micrographs of *T. rubrum* treated with garlic extract (4 mg/mL) showing hypha with large hyphal tip

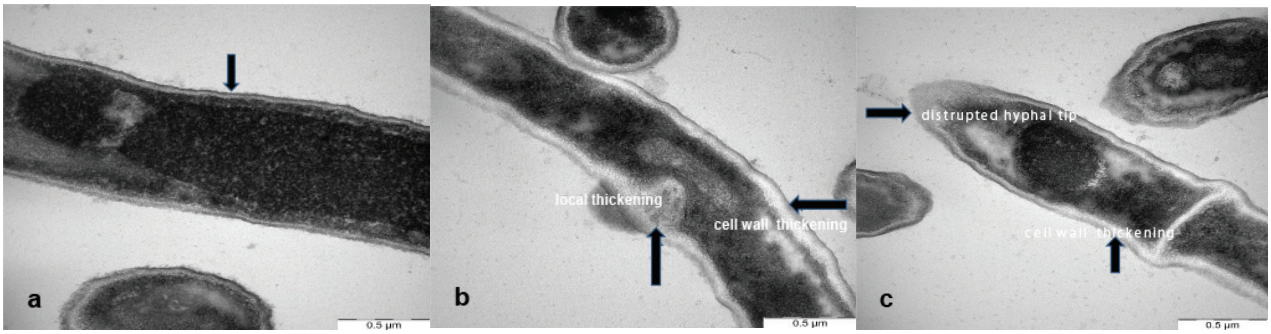

FIGURE 4. TEM studies of a longitudinal section of *T. rubrum*. (a) normal untreated hypha was described by typical cell wall, cell membrane and organelles, (b) hypa treated with Allicin (12.5 µg/mL) which display cell wall thickening and local thickening and (c) hypa treated with garlic extract (4 mg/mL) shows wall thickening and disrupted hyphal tip. Scale bar=0.5 µm

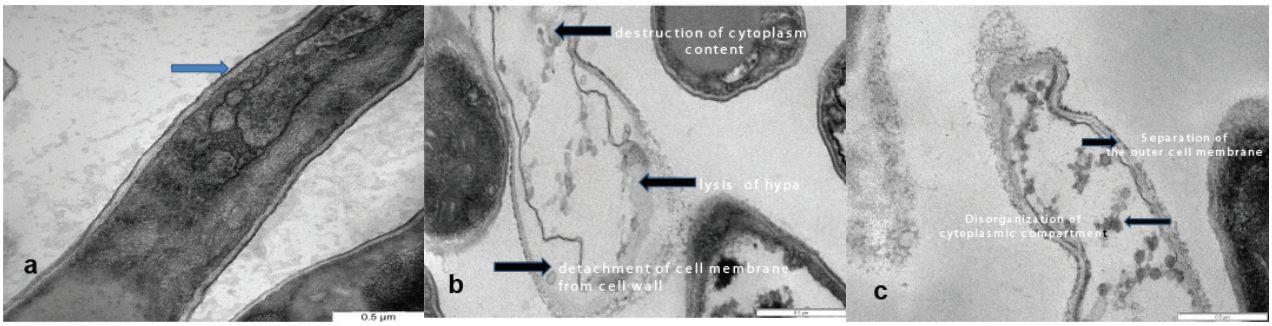

FIGURE 5. TEM of a longitudinal section of *T. rubrum*. (a) A typical untreated hypha was described by normal cell wall, cell membrane and organelles, (b) hypha treated with allicin (12.5 µg/mL) shows total detachment of the cell membrane from the cell wall, destruction of cytoplasm content, and hypha which seemed to undergo lysis and (c) hypha treated with garlic extract (4 mg/mL) shows separation of the outer cell membrane and its disconnection from the cell wall, disorganization and desolation of cytoplasmic compartments. Scale bar=0.5 µm

wall, cell membrane and organelles. Exhibits a hypha allicin (12.5 µg/mL) treatment produced total detachment of the cell membrane from the cell wall together with the demolition of cytoplasm content and hypha undergoning lysis. Hypha treated with garlic extract (4 mg/mL) exhibited disconnection of the exterior cell membrane and separation from the cell wall in addition to disorganization and desolation of cytoplasmic compartments (Figure 5(c)).

DISCUSSION

This study found that both aqueous garlic extract and allicin inhibited the growth of hyphae. However, Allicin was found to be more effective in inhibiting the growth of hyphae in low concentration (12.5 µg/ml) as compared with garlic extract at a much higher concentration i.e 4 mg/mL. In general, SEM studies of hypha in response to Allicin and aqueous garlic extract only showed slight different as shown in Figures 2 and 3. This could be due

to the purity of the active compound, allicin in Allicin is higher (99%) than aqueous garlic extract. It can be seen that aqueous garlic extract contains other active compounds such as ajoene and several other organosulfideds which are produced when fresh garlic is crushed (Block et al. 1984). Studies by Jakobsen et al. (2012) had identified the sulfur-containing compound ajoene present in fresh garlic that was extracted by bio-assay-guided fractionation. Previous study by Yoshida et al. (1987) found that ajoene showed antifungal activity like allicin. Their studies found hypha treated with ajoene (20 μg/mL) exhibited flat ribbon like structure or surface demolition. However, the mechanism of ajoene was not clear. It is predicted that ajoene may damage the cell wall of fungi.

The TEM studies proved the ability of Allicin and aqueous garlic extract to reacts on the cell wall and ultrastructural of hyphae as shown in Figure 5. Generally both Allicin and aqueous garlic extract had the ability to damage, disorganized, desolution of cytoplasm content and also detachment of cell membrane from the cell wall. Thus, these findings led to understanding the mechanism of active compound on hyphae. Allicin and its derivatives contain sulfide molecules that are able to interact with portions of proteins that also contain sulfur. As a result, allicin and its related chemicals can disrupt the function of certain proteins that are essential for fungi to grow. According to Willis (1956), allicin even though in very low concentration (5×10^{-4} to 5×10^{-5} M) is able to inhibit many important metabolic enzymes, especially those having reactive –SH groups. As a result, the growth of hyphae is retarded. Barone and Tansey (1977) proposed the mode action of allicin as anticandidal. They proposed that allicin disrupts cell metabolism primarily by inactivating protein by oxidation of essential thiols to the disulfide, inhibiting the activity of sulfhydryl compounds such as cycteine or glutathione by combining with them, resulting in statis or cell death and noncompetitively inhibiting enzyme function by oxidation of the binding to –SH groups at allosteric sites.

Study by Adetumbi et al. (1986) found that aqueous garlic extract inhibit the growth of yeast, *Candida albicans*. They proposed that fresh garlic extract block lipid synthesis and inhibit both protein and nucleic acid synthesis, thus leading to static growth of yeast. However, the mechanism how fresh garlic extract block lipid, protein and nucleic acid synthesis were not discussed. Garlic extract also contain allylamines, a class of antifungal compounds containing a chemical group naturally found in garlic. This compound causes accumulation of squalene monooxygenase, an important enzyme used in the formation of the fungal cell wall. This lead to blockage of the formation of ergosterol which is essential for synthesis of the fungal cell wall (Gupta & Porter 2001).

CONCLUSION

Garlic extract and Allicin possess antifungal activity which inhibits the hyphal growth of *T. rubrum*. The morphological and cellular modifications of *T. rubrum* treated by both extracts observed using SEM and TEM that demonstrated the antifungal activity of these two extracts. Garlic extract and Allicin could be used for proposed for antifungal treatment.

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