Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

MODE OF ACTION OF 5-ACETYL-4-METHYLTHIAZOLE DERIVATIVES AS ANTIMICROBIAL AGENTS

(Mod Tindakan Sebatian Terbitan 5-Asetil-4-Metiltiazol Sebagai Agen Antimikrob)

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Received: 5 February 2021; Accepted: 29 March 2021; Published: xx April 2021

Abstract

Thiazole derivatives have been widely known to possess antimicrobial behaviour. The purpose of this study is to investigate the mode of antimicrobial actions of synthesised thiazole compounds on Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative bacteria (*Salmonella typhimurium*), and fungus (*Candida albicans*). Three synthesised thiazole compounds namely 5-acetyl-4-methyl-2-(3,4-dichloroaniline)-1,3-thiazole (**T1**), 5-acetyl-4-methyl-2-(4-aminophenol)-1,3-thiazole (**T2**), and 5-acetyl-4-methyl-2-(methyl-4-aminobenzoate)-1,3-thiazole (**T3**) were evaluated in the mode of action assays such as salt tolerance, time-killing, crystal violet and leakage 260/280 nm absorbing materials. The result showed that the **T3** compound exhibited the best performance for all tested assays at a concentration equal to 4 × MIC compared to **T1** and **T2**.

Keywords: thiazole, mode of action, antimicrobial

Abstrak

Terbitan tiazol telah dikenali umum sebagai sebatian yang mempunyai sifat antimikrob. Justeru, kajian ini dijalankan untuk mengkaji mod tindakan sebatian tiazol yang disintesis sebagai agen antimikrob terhadap bakteria Gram-positif (*Staphylococcus aureus*), Gram-negatif (*Salmonella typhimurium*) and fungus (*Candida albicans*). Tiga sebatian tiazol yang telah disintesis iaitu 5-asetil-4-metil-2-(3,4-dikloroanilina)-1,3-tiazol (**T1**), 5-asetil-4-metil-2-(4-aminofenol)-1,3-tiazol (**T2**) and 5-asetil-4-metil-2-(metil-4-aminobenzoat)-1,3-tiazol (**T3**) telah dikaji dan dinilai secara toleransi garam, masa pembunuhan, violet kristal dan kebocoran bahan serapan 260/280 nm. Keputusan kajian mendapati sebatian **T3** menunjukkan kebolehan sebagai agen antimikrob yang terbaik pada kepekatan 4 × MIC berbanding **T1** dan **T2**.

Kata kunci: tiazol, mod tindakan, antimikrob

Introduction

Serious diseases such as diarrhoea, infections to intravascular organ, nausea, inflammation, and abdominal pain caused by bacterial infection have led to the growing research trend in the development of treatment system [1, 2]. One of the main related research areas is antibiotic development [3]. The synthesised organic compound is known to be the most suitable candidate compared to inorganic and natural product extract due to its easy structural modification, high percentage yield, and prevention of natural resources depletion [4-8]. The main challenge for this option is to design an efficient compound that can solve the rising concern on antibiotic resistance. In recent years, thiazole has emerged as the subject of considerable interest in the field of pharmacology among researchers due to its excellent properties in biological fields since it can act as anti-inflammatory, antifungal, anticancer, antibacterial, anticonvulsant, and antiviral [9]. The manifestation of nitrogen and sulphur atoms in thiazole moiety has significantly increased its physiochemical properties to be biologically active in most biological areas [10]. A continuous effort from our previous work on synthesis, preliminary biological screening and molecular docking [11] has led to the evaluation of three thiazole derivatives. The mode of action assays such as salt tolerance, time-killing, crystal violet, and leakage 260/280 nm absorbing materials was performed on the derivatives.

Materials and Methods

Preparation of thiazole derivatives

Synthesis and characterisation of thiazole derivatives namely 5-acetyl-4-methyl-2-(3,4-dichloroaniline)-1,3-thiazole (**T1**), 5-acetyl-4-methyl-2-(4-aminophenol)-1,3-thiazole (**T2**), and 5-acetyl-4-methyl-2-(methyl-4-aminobenzoate)-1,3-thiazole (**T3**) were performed as previously described [11]. The chemical structure of T1, T2, and T3 is shown in Figure 1.

Test microorganisms

The cultured microorganisms that were used in this study include Gram-positive bacteria, Gram-negative bacteria, and fungus. The Gram-positive bacteria used was *Staphylococcus aureus* (ATCC25923) while *Salmonella typhimurium* (IMRS1406/08A) was selected as the Gram-negative bacteria. The fungus species used was *Candida albicans* (ATCC10231).

Preparation of Inoculum

Stock cultures of bacteria and fungus were grown in Nutrient Agar (NA) at 37 °C for 24 hours and Potato Dextrose Agar (PDA) at 27 °C for 48 hours, respectively. A sub-culturing process was carried out on both microbes to get single colonies at the stationary phase. After the formation of single colonies, the top of each colony was swabbed using a cotton swab and transferred into a Falcon tube containing 10 mL of Nutrient Broth (NB) and Potato Dextrose Broth (PDB) for bacterial strains and fungus, respectively. Incubation process was then performed for the bacterial strains at 37 °C for 24 hours and fungus at 27 °C for 48 hours before the measurement of optical density (OD) using a spectrophotometer.

Salt tolerances assay

The capability of cultured bacteria and fungus treated with **T1**, **T2,** and **T3** on agar appended with sodium chloride (NaCl) at 50g/L was studied by applying a previously published method [12] with some modification. The microbes suspension was treated with compounds and standard drugs (streptomycin and tetracycline for bacteria and clotrimazole for fungus) at the respective MIC and 2 × MIC values concentration. Meanwhile, untreated cultures were used as control (blank). Next, both treated and untreated cultures were incubated for 30 minutes at 37 ºC. The samples were then serially diluted and inoculated onto an agar plate of NA and PDA or NA-NaCl and PDA-NaCl. After the incubation process, the number of CFU of bacteria per millilitre (CFU/mL) on each NA/PDA-NaCl plate was counted and compared to the NA/PDA plate. The tests were done in triplicate and data were computed using Microsoft Excel 2013 and the results were expressed as mean ± standard deviation (S.D) with p <0.05 is considered as statistically significant.

(Note: NA and NA-NaCl for bacteria; PDA and PDA-NaCl for fungus)

Time-killing assay

Ammonium assay for the killing rate of bacteria and fungus by the compounds was carried out using a plating method [13] with Clinical and Laboratory Standards Institute (CLSI). The effect of the compounds on killing ability over the time intervals of 0, 30, 60, and 120 minutes of treatment was measured [14]. The test compound was added into 10 mL of inoculum suspension that is equal to 4 × MIC. As a control, suspension of microorganism without test compound was included in each trial. After each time interval, 100 μL of treated and untreated cultures were transferred onto an agar plate. The plate was then incubated at 37 °C for 24 hours (for bacteria) at room temperature for 48 hours (for fungus). After the incubation period, the growth of the colonies on the agar plate was counted at each time interval and compared with control. The experiments were performed in triplicates.

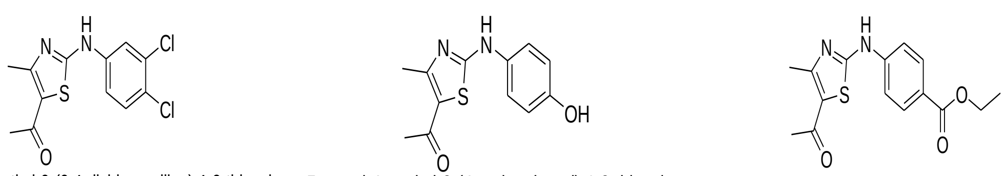
Crystal violet assay

The effect on membrane permeability of microorganism treated with **T1**, **T2,** and **T3** was assessed *via* crystal violet assay [15] using a staining method with some modification. Briefly, the cells were harvested upon exponential growth via centrifugation at 4500 × g for 5 minutes at 4 °C and washed twice prior to resuspension in phosphate buffer saline (pH 7.4) solution. Next, the compound was added into the cell suspension with different concentration of 2 × MIC and 4 × MIC values. The cell suspension without compound was marked as control whereas cells treated with 0.25M EDTA was used as the positive control. The antibiotics (Streptomycin: 0.62 μg/mL; Clotrimazole: 0.16 μg/mL) were also used in the treatment as standards. After incubated for 30 minutes at 37 ºC, the cells were harvested *via* centrifugation at 9300 × g for 5 minutes. Then, the cells were suspended in PBS containing 10 μg/mL of crystal violet and re-incubated for another 10 minutes. After that, the suspension was centrifuged at 13,400 × g for 15 minutes. Finally, the optical density (OD) value of the supernatant was measured using a UV-Vis spectrophotometer at 600 nm for bacteria and 494 nm for fungus [16]. The percentage of crystal violet uptake of all cells was calculated using the following formula in equation 1[17]:

$$\frac{\text{OD value of the sample}}{\text{OD value of crystal violet solution}} \text{ X } 100\% \qquad (1)$$

Leakage of 260 and 280nm-absorbing material assay

The assays were conducted to evaluate the leakage of materials into the microorganisms when exposed to thiazole compounds at a wavelength of 260 nm and 280 nm using a UV spectrophotometer [12] with some modification. The difference in absorption values used in this test was to estimate the release of metabolites of nucleic acid and protein [18]. Firstly, the cultured microorganism was centrifuged at 10,000 × g for 12 minutes at 4 °C prior to the twice washing step and resuspended with PBS (pH 7.4). Then, different concentrations of compounds at 2 × MIC and 4 × MIC values were added into 1 mL of cell suspension. Untreated cells were discernible as negative control and standard drugs were used as positive controls. All cells suspension was incubated for 60 minutes before centrifuged at 13,400 × g for 15 minutes. Finally, the optical density (OD) values of the supernatant measured at 260 and 280 nm were reflected as a percentage of cellular UV-absorbing materials released by the cells. The tests were performed in triplicates and the mean values were determined.



5-acetyl-4-methyl-2-(3,4-dichloroaniline)-1,3-thiazole **T1** | 5-acetyl-4-methyl-2-(4-aminophenol)-1,3-thiazole **T2** | 5-acetyl-4-methyl-2-(ethyl-4-aminobenzoate)-1,3-thiazole **T3**

Figure 1. The chemical structures of **T1**, **T2**, and **T3**

Results and Discussion

Salt tolerances assay

The effect of **T1**, **T2,** and **T3** compounds on the osmotic potential of bacteria and fungus cells was studied by investigating their ability to grow on agar plate supplemented with NaCl. In this assay, the antimicrobial effect of the compounds against the tested bacterial and fungal strains was investigated in treated and untreated agar with the salt solution. To conduct this assay, the concentration of MIC and 2 × MIC, which was able to inhibit the microbes were applied. The results of the salt tolerance assay of untreated and treated microbial are tabulated in Figure 2. The number of colonies was found to be reduced in all treated cells as the treatment concentration doubled. The sub-lethal damage of the cell membrane was seen by the reduction in the ability to form colonies on the agar supplemented with NaCl. Moreover, all treated cells showed a decreasing colony-forming ability as the treatment concentration increases. However, there was a slight difference in the number of survived colonies on NA/PDA and NA/PDA-NaCl agar. The range number of colonies survived on NA/PDA agar by seeing both concentrations applied for tested compounds towards all microbes were from 94×10^3 to 12×10^4 CFU/mL compared to untreated blank culture (control) at the range of 14×10^4 to 86×10^4 CFU/mL. In contrast, the range number of colonies that survived on NA/PDA-NaCl for both concentrations against microbial tested were 23×10^4 to 11×10^4 CFU/mL compared to control that was in the range of 78×10^4 to 13×10^4 CFU/mL.

From Table 1, it can be concluded that NaCl supplemented agar reduced the formation of the microbial colony. The addition of salt has affected the growth of microorganism by disturbing the metabolic process of microbial strains by changing the membrane arrangement and reducing their cell division [19]. Based on the assay, **T3** was found to be the most active compound towards all microbial tested on both media at MIC and 2 × MIC values. The number of colonies survived on NA/PDA-NaCl by *S. aureus*, *S. typhi*, and *C. albicans* treated with 2 × MIC value of **T3** were observed to be 54×10^3, 48×10^3 and 11×10^4 CFU/mL, respectively. In addition, **T1** displayed the weakest activity with a greater number of colonies survived equally to 18×10^4, 62×10^3, 13×10^4 CFU/mL, respectively.

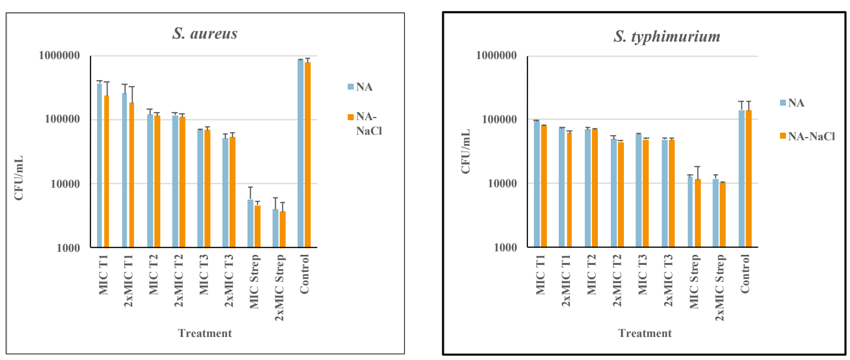
Time-killing assay

A time-killing assay was performed to determine the bactericidal and fungicidal effect of thiazole compounds as described in the previous literature [13]. This assay was widely applied for *in-vitro* analysis of new antimicrobial agents by delivering qualitative data on the pharmacodynamics relationships between bioactive compounds and their effects on microbes. The killing rate of the tested microbes by the compounds was determined by measuring the reduction in the number of colony-forming units (CFU/mL). A bactericidal effect can be defined as 99.9% killing of the final inoculums by observing the absence of colony growth. The results of the time-killing assay of the compounds were compared to the control towards microbial strains are shown in Figure 3 and Table 2. The results showed that all compounds exhibited similar patterns of killing kinetic profiles against all tested microbes. All growth reduction of treated cultured microbe significantly increased over time from 0 to 120 minutes as matched to the control. A treatment of microbial growth for compounds was observed to reach the endpoint at 60 minutes after incubation. The **T3** compound was found to be the most active compound with a significant killing ability towards all tested microbes by showing the lowest number of colonies survived after 30 minutes at a range of 23×10^2 to 60×10^2 CFU/mL. The highest number of colonies of 57×10^2 to 88×10^2 CFU/mL was recorded when treated with **T1,** which indicates a weak killing performance. In contrast, the control exhibited a different pattern by showing an increasing number of survived colonies ranging from 59×10^6 to 25×10^7 CFU/mL signifying a continuous growth of microbes after 30 minutes of treatment.

Crystal violet assay

The formation of a biofilm layer with a community of sessile bacterial cells and hydrated matrix provides a beneficial surface of planktonic bacteria by attracting nutrient and colonisation of macromolecules [20, 21]. Therefore, to study the ability of **T1**, **T2,** and **T3** to demolish the membrane of microbial strains or biofilms was performed using crystal violet assay [15] with some modification. In principle, the crystal violet solution has a weak ability in penetrating the outer membrane, but it can easily enter when the membrane is disrupted [22]. To conduct the experiment, all microbes were cultured before treated with the compounds and stained with crystal violet solution. The membrane permeability properties of the compounds were evaluated by measuring the percentage of crystal violet solution uptake after treatment. Figure 4 shows the percentage of CV uptake by microbial strains at a concentration of 2 × MIC and 4 × MIC. From the graphs, all compounds inhibited the formation of membrane for all tested microbes. It can be seen that the percentage of CV uptake by the microbial strains increased as the concentration of the compounds increased. In this assay, EDTA solution was used as a chelating agent that could modify the permeability of the outer membrane layer of a bacterial cell wall [22] and as external positive control. Streptomycin (for bacteria) and Clotrimazole (for fungus) were used as the internal positive control.

The results of the crystal violet assay were also matched with the untreated culture as a negative control. The results showed that the membrane layer of the microbial cell walls significantly increased in the crystal violet uptake after treated with the compounds compared to the negative control (untreated culture). Besides that, both external and internal positive controls also showed an increase uptake of crystal violet after treatment. **T3** showed the highest percentage of CV uptake towards all microbial strains tested such as *S. aureus*, *S. typhi*, and *C. albicans* at 4 × MIC with percentages of 49.4 %, 67.2 % and 67.9 %, respectively. Next, **T2** also exhibited good antibiofilm activity against microbes tested at similar concentration with percentage values of 47.1%, 65.0% and 65.7%, respectively. In contrast, **T1** showed the weakest CV uptake at a similar concentration of 4 × MIC against microbes with percentage values of 34.7 %, 51.4 % and 60.6 %, respectively. It can be concluded that the difference in the uptake of CV from the microbial cell walls is due to the destructive cell wall as their permeability changes [23].



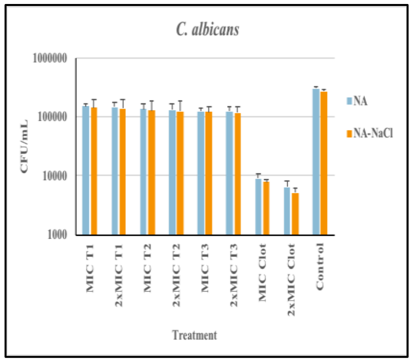


Figure 2. Salt tolerance by untreated (control) and untreated *S. aureus*, *S. typhi*, and *C. albicans* at a concentration equal to MIC and 2 × MIC of **T1**, **T2,** and **T3**

Table 1. Salt tolerance results of synthesised thiazole and antibiotics

Compound	**Agar Condition**	***S. aureus***	***S. typhi***	***C. albicans***
MIC **T1**	NA	37×10^4	94×10^3	15×10^4
	NA-NaCl	23×10^4	80×10^3	14×10^4
2 × MIC **T1**	NA	26×10^4	72×10^3	14×10^4
	NA-NaCl	18×10^4	62×10^3	13×10^4
MIC **T2**	NA	12×10^4	70×10^3	13×10^4
	NA-NaCl	11×10^4	69×10^3	12×10^4
2 × MIC **T2**	NA	11×10^4	50×10^3	13×10^4
	NA-NaCl	10×10^4	44×10^3	12×10^4
MIC **T3**	NA	70×10^3	59×10^3	12×10^4
	NA-NaCl	69×10^3	48×10^3	11×10^4
2 × MIC **T3**	NA	55×10^3	49×10^3	12×10^4
	NA-NaCl	54×10^3	48×10^3	11×10^4
MIC Strep	NA	56×10^2	12×10^3	-
	NA-NaCl	46×10^2	11×10^3	-
2 × MIC Strep	NA	40×10^2	11×10^3	-
	NA-NaCl	36×10^2	10×10^3	-
MIC Clot	PDA	-	-	90×10^2
	PDA-NaCl	-	-	78×10^2
2 × MIC Clot	PDA	-	-	65×10^2
	PDA-NaCl	-	-	52×10^2
Control	NA	86×10^4	14×10^4	-
	NA-NaCl	78×10^4	13×10^4	-
	PDA	-	-	30×10^4
	PDA-NaCl	-	-	27×10^4

Note. Strep: Streptomycin; Clot: Clotrimazole

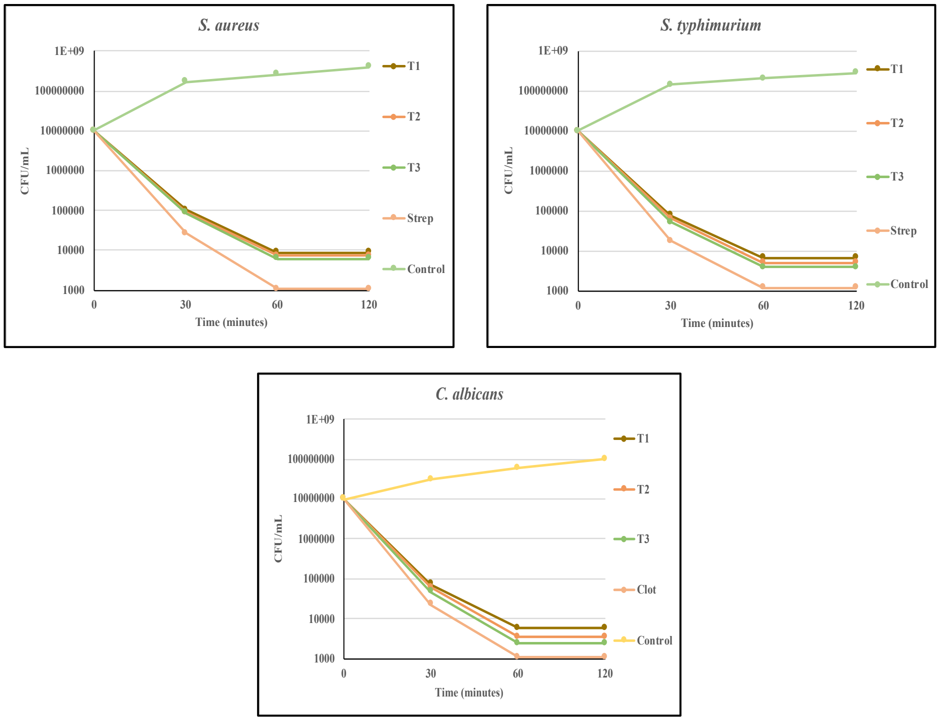


Figure 3. Time-killing curves of untreated (control) and treated *S. aureus*, *S. typhi*, and *C. albicans* at a concentration equal to 4 × MIC of thiazole compounds

Table 2. Salt tolerance results of synthesised thiazole and antibiotics

Compounds	Time (Minutes)	*S. aureus*	*S. typhi*	*C. albicans*
T1	30	10×10^4	79×10^3	73×10^3
	60	88×10^2	68×10^2	57×10^2
	120	88×10^2	68×10^2	57×10^2
T2	30	89×10^3	65×10^3	59×10^3
	60	73×10^2	51×10^2	34×10^2
	120	73×10^2	51×10^2	34×10^2
T3	30	85×10^3	53×10^3	46×10^3
	60	60×10^2	39×10^2	23×10^2
	120	60×10^2	39×10^2	23×10^2
Streptomycin	30	27×10^3	17×10^3	-
	60	11×10^2	12×10^2	-
	120	11×10^2	12×10^2	-
Clotrimazole	30	-	-	22×10^3
	60	-	-	10×10^2
	120	-	-	10×10^2
Control	30	17×10^7	14×10^7	30×10^6
	60	25×10^7	20×10^7	59×10^6
	120	39×10^7	28×10^7	99×10^6

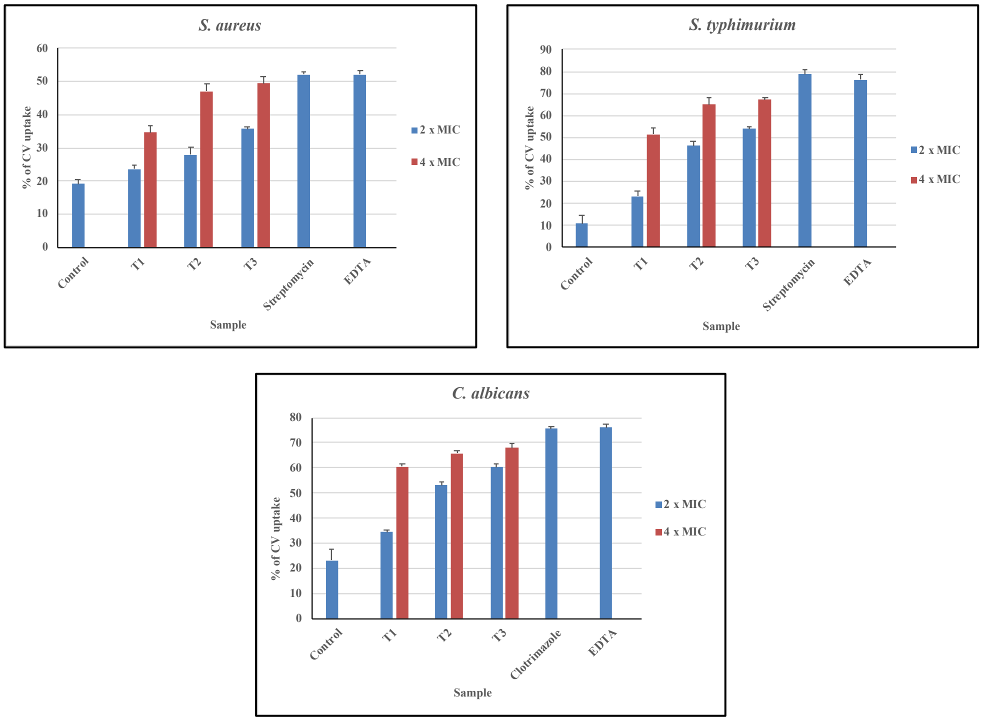


Figure 4. Percent of crystal violet uptake by untreated (control) and treated microbes at a concentration equal to 2 × MIC and 4 × MIC of **T1**, **T2** and **T3**, 0.25 M EDTA, and standard drugs

Leakage of 260 and 280nm-absorbing material assay

The assay was carried out to investigate material loss from the microbial strains when treated with the compounds. The leakage of material cells of nucleic acid and protein were analysed using a UV spectrophotometer at a wavelength of 260 and 280 nm, respectively. The results showed that higher absorption values indicate more loss of material cells from the microbial strains tested. Figure 5 shows the absorbance values of material cells (nucleic acids) of all microbes at 260 nm.

From Figure 5, doubled the concentration of the compounds, a greater leakage was observed due to the loss of absorbing material of nucleic acid. Among all compounds, the highest absorbance values of OD260 were recorded at 0.77, 0.65 and 0.74 of *S. aureus*, *S. typhi* and *C. albicans*, respectively when exposed to **T3** at a concentration of 4 × MIC. Whilst **T3** compound also showed the highest absorbance values at a concentration of 2 × MIC compared to **T1** and **T2** towards microbial strains tested, which were 0.62, 0.57 and 0.67, respectively. Moreover, the second highest absorbance values were recorded for **T2** against similar microbial strains of *S. aureus*, *S. typhi*, and *C. albicans* were 0.62, 0.57 and 0.67, respectively but at 4 × MIC concentration. In contrast, when compared with **T1** at the concentration of 4 × MIC, the lowest absorbance values were analysed at 0.66 (*S. aureus*), 0.57 (*S. typhi*) and 0.58 (*C. albicans*), respectively. It can be assumed that the highest values of OD_{260} were recorded with **T3** whereas the lowest OD_{260} values were seen for **T1**, indicating that **T3** treatment released the highest amounts of nucleic acids.

Next is the results of the leakage of proteins materials at 280 nm-absorbing materials of microbial strains. The findings of the assay are shown in Figure 6. A similar trend to 260 nm-absorbing material results was observed when the concentrations of the compounds doubled. The higher absorbance values were detected due to the greater leakage of proteins. From the graphs, **T3** showed the greatest OD_{280} absorbance values, which were 0.79, 0.78 and 0.83 towards *S. aureus*, *S. typhi* and *C. albicans*, respectively at a concentration of 4 × MIC. **T1** also showed the weakest absorbance values of 0.65, 0.68 and 0.74, respectively at a similar concentration.

It can be concluded that microbial suspension treated with **T1**, **T2,** and **T3** lost their absorbing materials significantly at 260 and 280 nm through a destroyed cytoplasmic membrane that released nucleic acids and proteins. This could be explained by the chemical structure of compounds that comprise of two parts. The first group is heterocyclic thiazole containing nitrogen and sulphur atoms that are attached to the amine at the 2-position to another group linked to an aryl group that plays a role in the mode of action of the assay. The heterocyclic thiazole and aryl groups possess hydrophilic and hydrophobic properties, respectively. Therefore, the compounds can be considered as amphiphilic molecules with two parts of hydrophilic (polar group) and hydrophobic (non-polar group) possibly to interrupt the microbes cell membrane resulting in cell lysis.

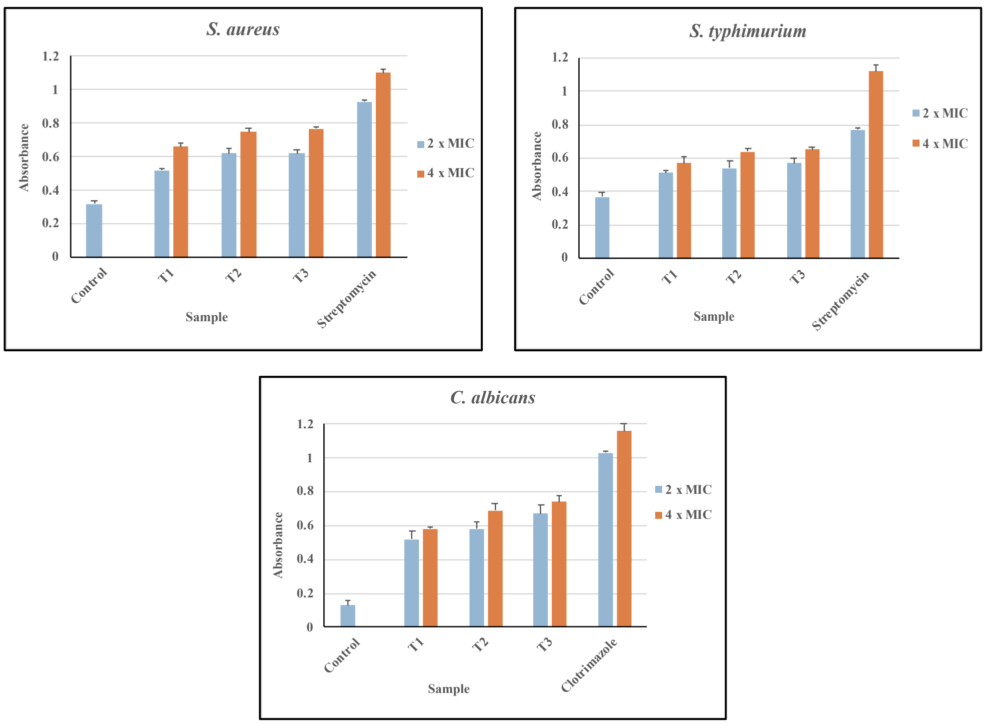


Figure 5. Leakage of extracellular 260 nm-absorbing material from untreated and treated microbial strains with compounds and standard drugs at a concentration equal to 2 × MIC and 4 × MIC

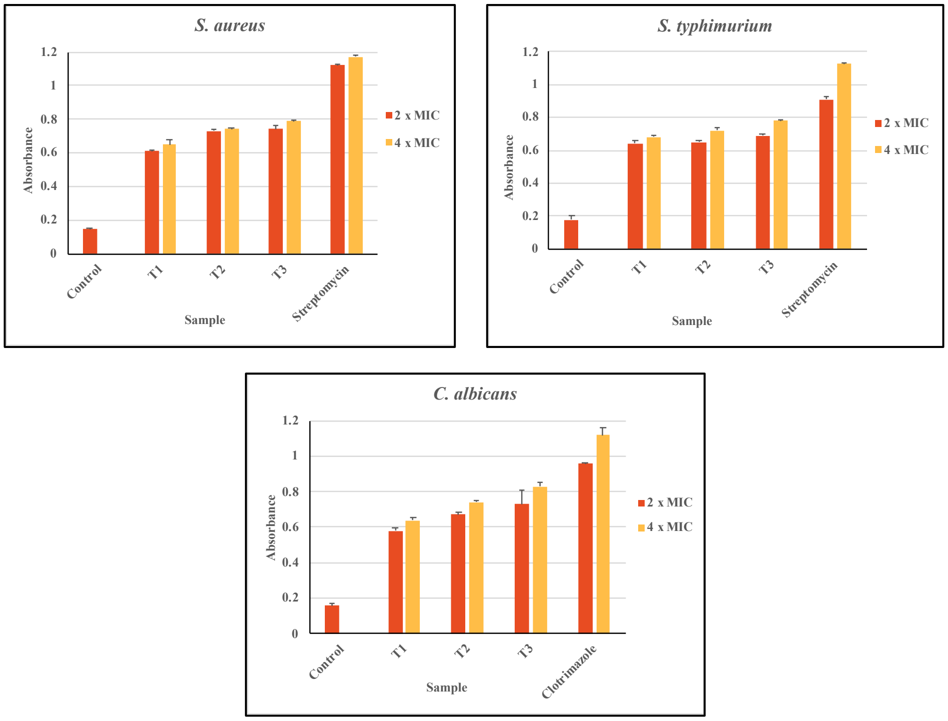


Figure 6. Leakage of 280 nm-absorbing materials from untreated and treated microbial strains with compound and standard drugs at a concentration equal to 2 × MIC and 4 × MIC

Conclusion

In conclusion, this work has demonstrated that 5-acetyl-4-methylthiazole derivatives (**T1**-**T3**) were successfully evaluated for their mode of action assays. From the experimental data, **T3** exhibited the best performance against tested bacterial and fungal strains compared to **T1** and **T2** in all tested assays.

Acknowledgement

We gratefully acknowledge grants from the Ministry of Education Malaysia FRGS14-15-0392, and Department of Chemistry and Biotechnology, Kulliyyah of Science, International Islamic University Malaysia (IIUM) for providing the essential laboratory facilities.

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