Antimicrobial activities and mode of action of the selected novel thienopyrimidines derivatives 2-[2-(diphenylmethylene) hydrazino]-5-isopropyl--3-methylthieno [2, 3-d] pyrimidin-4-one

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Abstract

Background and purpose: *Although thienopyrimidines have an important antimicrobial efficacy, there is still a lack of understanding their antimicrobial mode of action. The current study aims to assess the antimicrobial activity of newly synthesized thienopyrimidines derivatives against some pathogens and to determine the mode of action of the most potent compound 2-[2-(diphenylmethylene) hydrazino]-5-isopropyl-3-methylthieno [2, 3-d] pyrimidin-4-one (compound no. 20).*

Materials and methods: *Newly synthesized thieno[2,3-d]pyrimidinediones compounds examined for antimicrobial activity against pathogenic organisms by cut plug and viable cell counting method. The minimum inhibitory concentration (MIC) of (compound no. 20), it's mode of action; cytotoxicity and LC_{50} were determined.*

Results: *One compound, compound no. 20 displayed potent activity. The growth inhibitory effect (M/C) of the compound no. 20 differed among the tested strains. The inhibition becomes stronger;* Candida albicans> Escherichia coli> Staphylococcus aureus. *The MIC for all the tested microorganisms was > 50 <100 µg/mL.* E. coli *and* C. albicans *recorded highest value of potassium flow. Compound no. 20 affects oxygen consumption and exhibited cytotoxicity against* E. coli. *LC50 value with brine shrimps was estimated at 48.736 µg/mL concentration.*

Conclusion: *It could be concluded that the mode of action of compound no. 20 may be due to disruption of the cytoplasmic membrane by action upon the membrane potentials, permeability or net surface charge of the microbial membranes by its physicochemical characteristic. This led to leakage of cellular components and eventually cell death. This will lead to improved antimicrobial formulations and to ensure the prevention of the emergence of microbial resistance.*

INTRODUCTION

Recently, the problems of environmental pollution and health attracted considerable attention. The increasing appearance of resistant strain of pathogenic bacteria to the classical antimicrobial agents is rapidly represents an alarming threat to public health. The most commonly encountered antibiotic-resistant bacteria, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and penicillin

and cephalosporin-resistant *Streptococci* has had a major impact on infections in both the hospital and community setting *(1)*. Thus, there is a pressing need for new efficacious antimicrobial agents that are capable of treating resistant bacterial strains.

Thienopyrimidines occupy a special position among fused pyrimidines compounds because they play an important role in diverse biological activities *(2)*. A number of derivatives of these compounds display therapeutic activity as antimicrobial *(3, 4)*, anti-inflammatory *(5)*, analgesic activities *(6)*, inhibition of cancer cell proliferation *(7)*, antidiabetic *(8)* and prevention of cartilage destruction in particular diseases *(6)*. Consequently, thienopyrimidines occupy a special position among these compounds. Thienopyrimidine derivatives are the one of outcome of the research in the field of therapeutic activity as antimicrobial *(9)*. They are structural analogs of biogenic purines and can be considered as potential nucleic acid antimetabolites *(10, 2)*.

In spite of the enormous antimicrobial activity of thienopyrimidines compounds and their derivatives that have been discovered, the mechanisms of action of these compounds are not yet reported *(1)*. According to the reports of antimicrobial activity for structurally unrelated thienopyrimidinones, the biocides and antibiotics have multiple target sites within the microbial cell and the overall damage to these target sites results in the bactericidal effect. Since the emergence of bacterial resistance to biocides or antibiotics and the suggestion that biocide and antibiotic resistance in bacteria might be linked, it became an important issue to understanding the mechanism(s) of action of a biocide *(11)*. Consequently we can consider the biocide and antibiotic mechanism(s) of action as a guide to understand the mechanism of action of thienopyrimidines compounds. Therefore, according to the bacterial structure; the overall mechanism(s) of action of any antimicrobial agents may be defined. Thus, there is three levels of interaction can be described: 1-interaction with outer cellular components; 2-interaction with the cytoplasmic membrane and 3-interactionwith cytoplasmic constituents. Despite this, a biocide acts at one or all three levels of interaction with the bacterial cells to produce its antimicrobial activity *(11)*.

The objective of this work was to study the antimicrobial activity of some newly synthesized thienopyrimidines derivatives and to investigate the mode of action of the most effective one, the compound no. 20 against Gram-positive, Gram-negative bacteria and pathogenic fungal species.

MATERIALS AND METHODS

Synthesis of the thienopyrimidine derivatives compounds

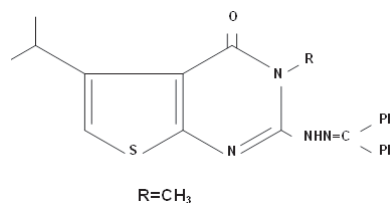The thienopyrimidine derivatives compounds were synthesized and kindly given by Prof. Hamed*et al. (12)*

Figure 1. *Structural formula of 2-[2-(diphenylmethylene) hydrazino]-5-isopropyl-3-methylthieno [2, 3-d] pyrimidin-4-one (compound no. 20).*

(Chemistry Department, Faculty of Science, Menoufiya University, Egypt). The thienopyrimidine molecular formula was $C_6H_4N_2S$. The procedures of the synthesis of these compounds were described in details by Hamed*et al. (12)*. The chemical structure of the most potent derivative compound no. 20 was 2-[2-(diphenylmethylene) hydrazino]-5-isopropyl-3-methylthieno [2, 3-d] pyrimidin-4-one. Its molecular formula was ($C_{23}H_{22}N_4OS$) as shown in Fig.1.

Antimicrobial screening

All the test organisms were obtained from the culture collection of the Microbiological Resource Center (MIRCEN), Faculty of Agriculture, Ain-Shams University, Cairo, Egypt.

Antibacterial activity

Two bacterial species represent both Gram-positive and Gram-negative strains were used to test the antibacterial activities of the target compounds: *S. aureus* as representative for the Gram-positive strains, while the Gram-negative strain was represented by *E. coli*. The bacteria were maintained on nutrient agar slopes.

Antifungal activity

Two pathogenic fungal species were used in the present study: *Fusarium oxysporum* and *C. albicans*. The fungi were obtained on czapek's Dox and sabouroud agar slopes, respectively.

The agar diffusion method

The antibacterial and antifungal activities of the synthesized compounds were screened against the following bacterial strains: *S. aureus* as representative for the Gram-positive strains, while the Gram-negative strain was represented by *E. coli* and the following fungi: *F. oxysporum* and *C. Albicans* using the agar diffusion method *(13)*. It was used to determine the antimicrobial activities of the prepared eleven thienopyrimidine compounds as described by El-Sayed *et al. (2)*. The assay medium flasks containing 50 mL of media for each test organism. They were allowed to reach 40 to 50 °C to be inoculated with 0.5 mL of the test organism cell suspension. The flasks were mixed well and poured each into a Petri dish (nine cm) and allowed to

solidify. After solidification, holes (0.5 cm in diameter) were made in the agar plate by a sterile cork poorer. The solutions of synthesized target compounds were prepared by dissolving 20 mg of each compound in two mL DMSO (Dimethyl sulfoxide). In these holes, 100 µL of each compound was placed using an automatic micropipette. The Petri dishes were left at 5 °C for one hour to allow diffusion of the samples through the agar medium and retard the growth of the test organism. Plates were incubated at 30 °C for 24 h for bacteria and 72 h of incubation at 28 °C for fungi. The antibiotic ampicillin trihydrate and the antifungal drug clotrimazole (Canasten) were used as positive controls, while DMSO was used as negative control. The diameters of inhibition zone were measured. The compounds which produced highest inhibition zone were further assayed at different concentrations in DMSO in order to quantify its inhibitory effect.

Minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is „the lowest concentration of an antimicrobial that prevents the visible growth of a microorganism after overnight incubation". The importance of the MIC test is that it contains a semi-quantitative process which gives an approximation to the least concentration of antimicrobial agent needed to prevent microbial growth. The compound no. 20 showing reasonable inhibition zones (≥ 20mm) were subjected to determine their minimal MIC value using the twofold serial dilution method recommended by the Clinical and Laboratory Standards Institute (14). The test organisms were grown in their suitable nutrient broth medium for bacteria and sabouraud's medium for fungi at 30 °C for 24 h for bacteria and 48 h at 28 °C for fungi. Two fold serial dilutions of the test compound no.20 were prepared using the proper nutrient broth to obtain concentrations 100, 50, 25 and 12.5 µg/mL. The broth tubes were seeded with the test bacteria (6 x 10^4) CFU/mL and (8 x 10^2) spores/mL for the test fungi. The incubation period was for 24 h at 30 °C for bacteria and 96 h at 28 °C for fungi.

The growth was measured with the spread-plate (viable cell counting) method. After 24 h of contact, one mL portions were removed and mixed with nine mL of the full-strength nutrient broth; then, decimal serial dilutions were prepared from these dilutions, and the surviving bacteria or fungi were counted by the spread-plate method. After inoculation, the plates were incubated at 30 °C and the number of colonies was counted after 24 h for bacteria and after 48 h for fungi at 28 °C. The ratio of the colony numbers for the media containing the compound no. 20 and those without this compound (M/C) was taken as the surviving cell number, and with this value, the antimicrobial activity was evaluated (15).

Leakage of potassium (K⁺)

Leakage of potassium was assayed as a mean to study membrane damage caused by the compound no. 20 as described by Strauch et al. (16). The two tested bacterial species grown in nutrient broth medium at 30 °C for 24 h. and the tested fungi grown in the broth sabouraud's medium at 28 °C for 96 h. The cells of the test organisms were collected during exponential phase of growth by centrifugation, washed three times with 0.9% (wt. /vol) NaCl and finally resuspended in 20 mL of the same solution. The compound no. 20 was added at sublethal dose concentration (50 µg/mL) to aliquots of the cultures and shake for 30 min. Samples (five mL) were taken and centrifuged immediately at 6000 g, and the supernatants were frozen. K⁺ concentration was determined using a flame photometer (clinical flame photometer 410). Negative control sample was not treated with the compound no.20.

Respiratory activity

The quantity of oxygen consumed by the living cells (respiratory activity) in the presence or absence of the sublethal dose of the thienopyrimidine compound was determined as described by Tapia et al. (17). The living cells of the tested bacterial species and fungi grown up to exponential phase were harvested by centrifugation (6000 g), washed with saline solution and resuspended to a concentration of one mg/mL cells (wet weight) in 50 mM phosphate buffer (pH 7.0).The cell suspension was added to a two mL chamber. The chamber then closed and incubated for five min. at room temperature with sublethal dose (50 µg/mL) of the compound no.20. Cell suspension without the sublethal dose of compound no.20 was used as control. The oxygen consumption was measured polarographically with oxygraph (Yellow Spring Instrument, Yellow Spring, OH, U.S.A.). The quantity of oxygen was determined as µmol of oxygen/mg wt. /h (18).

In Vitro Cytotoxicity of the compound no.20 against E. coli

The cytotoxicity of the compound no.20 was detected using the biochemical induction assay (BIA).The biochemical induction assay (BIA) is a bacterial test system specifically responsive to agents that directly or indirectly initiate DNA-damage. The BIA uses an E. coli strain, in which the lacZ gene encodes β-galactosidase (LacZ). When the culture is treated with a DNA-damaging agent, the enzyme β-galactosidase is detected by a suitable chromogenic substrate (19).

In this test a plate containing 10 mL of nutrient agar was seeded by E. coli BR 315 and incubated at 30 °C for two days. The plates were spotted with sublethal dose (50 µg/mL) of the compound no.20. After three hours of incubation the plate is overlaid with a chromogenic substrate (a layer of agar containing 6-bromo-2-naphtyl β-D-galactopyranoside). The production of red color around the test compound is used as an indication of DNA-damaging activity.

Effect of the compound no.20 on cell morphology of *E. coli*

The test organism *E. coli* was treated with the sub-lethal dose (50 µg/mL) of the compound no.20. The morphology of *E. coli* was examined microscopically after being incubated for 24 h. at 30 °C *(20)*.

LC$_{50}$ Determination of compound no.20

LC$_{50}$ is the lethal concentration required to kill 50% of the population. This bioassay involves subjecting three replicate groups of individuals to a range of concentrations (or doses) of a toxic compound and measuring the mortality after a defined time interval. The data are then plotted and the LC$_{50}$ is interpolated from the graph. Brine shrimps (*Artemia salina*) lethality bioassay was used to determine the LC$_{50}$ of compound no.20 according to the procedures described by *(21, 22)*. The advantages of this method are being rapid results, reliability, inexpensive and convenient assay *(21)*. Brine shrimps were hatched using brine shrimp eggs in a shallow rectangular dish filled with sea water(prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration. A plastic divider with several two mm holes was clamped in the middle of the dish to make two unequal compartments. The shrimp eggs were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated. After 48 h the phototropic hatching, active nauplii free from egg shells were collected from the illuminated compartment and used for the assay.

Stock solution of the compound no.20 were prepared by dissolving 20 mg of it in two mL of DMSO. Four different concentrations namely 10, 25, 50 and 100 µg/mL, were prepared in triplicate from the stock solution. 0.5 mL of each concentration was added to vials each containing 4.5 mL of brine solution. Thirty nauplii (larva) were transferred through a glass capillary and placed in each vial. A control was prepared for the compound being assayed by adding 4.5 mL of sea water and 0.5 mL of DMSO. The shrimps that survived were counted after 24 hours. The total death and percentage mortality (death) at each dose level and control were determined. The LC$_{50}$ value was calculated using the MS Excel 2007 to find regression equation (Y=mortality; X=concentrations). The LC$_{50}$ was derived from the best-fit line obtained *(23)*.

Effect of the compound no.20 on the total protein of the tested organisms

The effect of the compound no.20 on total proteins of the tested organisms was determined using the method described by Lowery *et al*. *(24)*. The molecular weight of *C. albicans*, *E. coli* and *S. aureus* protein were determined using SDS-polyacrylamide gel electrophoresis according to Laemmli *(25)*.

Statistical analysis

The data were analyzed for statistical significance using analysis of variance (ANOVA) least significant difference (L.S.D) test at P value 0.05 *(26)*. All the experiments were carried out in triplicate otherwise it will be indicated

RESULTS

The antimicrobial activity of the tested thienopyrimidine compounds

The obtained results in Table 1 suggested that the antimicrobial activities of the eleven tested compounds showed normal and mild activity in comparison to the reference drugs ampicillin trihydrate and the antifungal drug clotrimazole (Canasten). From the eleven prepared thienopyrimidine compounds, the compound no.20 was the most effective one on Gram negative, Gram positive bacteria and *C. albicans*. The diameter of inhibition zones produced by it ranged from 20 to 25mm after 48h of incubation for bacteria and 96h for fungi. None of the eleven prepared thienopyrimidine compounds affect *F. oxysporum*.

Minimal inhibitory concentration (MIC)

The MIC of the compound no.20 result was illustrated in Fig.2. They showed that the inhibitory effect increased significantly by increasing in the concentration of
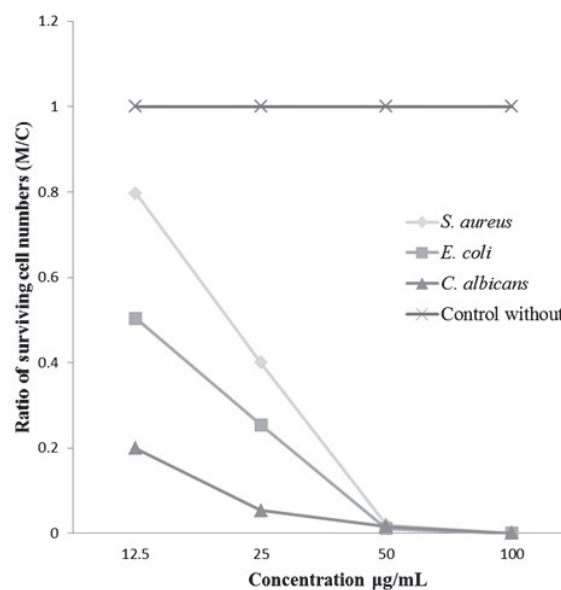
Figure 2. *The antimicrobial activity evaluated by surviving cell numbers at different concentration of the compound no.20*
*M: The colony numbers for the media containing the compound no.20, C: The colony numbers for the media without the compound no.20.* E. coli: Escherichia coli, S. aureus: Staphyloccous aureus, C. albicans: Candida albicans

Table 1. *The inhibitory effect of the tested thinopyrimidine compounds against different test bacteria on nutrient agar at 30 °C for 24 h and fungi on sabouroud agar at 28 °C for 96h by the cut plugs method.*

Thinopyrimidine compounds	Diameter of inhibition zones (mm)			
	Tested organisms			
	E. coli	*S. aureus*	*C. albicans*	*F. oxysporium*
3	nil	nil	nil	nil
4	12	12 ± 0.5	nil	nil
5	nil	nil	nil	nil
7a	6 ± 0.4	6 ±0.5	nil	nil
8	nil	nil	nil	nil
9	12 ± 0.2	12 ± 0.3	nil	nil
10a-h	nil	nil	nil	nil
11a-h	nil	nil	nil	nil
12a	nil	nil	nil	nil
14a	nil	nil	nil	nil
20	20± 0.3	25 ± 0.2	20 ± 0.2	nil
22a-d	nil	nil	nil	nil
Ampicillin trihydrate	20±0.3	22± 0.3	Not tested	Not tested
Clotrimazole	Not tested	Not tested	20 + 0.2	18
DMSO	nil	nil	nil	nil

E.coli: Escherichia coli, S. aureus: Staphyloccous aureus, C. albicans: Candida albicans, F. oxysporium: Fusarium oxysporium. Ampicillin trihydrate: antibacterial drug, Clotrimazole: antifungal drug. Nil: negative result (no inhibition). ±: standard deviation of the means. DMSO: Dimethyl sulfoxide.

the compound no.20. The test organisms was failed to grow completely with100 µg/mL concentration. Figure 2 proved that the MIC for all the tested microorganisms was >50<100 µg/mL. The growth inhibiting effect was quantitatively determined by ratio (M/C) of the surviving cell number, (M) in the medium containing the com-
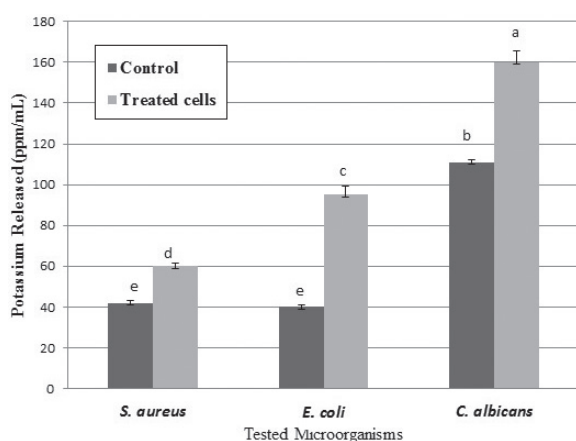
Figure 3. *The amount of potassium released under the effect of the compound no.20*
Control: Sample was not treated with the compound no.20, Treated cells: Cells treated with the compound no.20 at concentration 50 µg/mL (sublethal dose). PPm: Part per million. The results are the mean ± SD (n = 3). Means with the same letter are not significantly different.

pound no.20 to that without it (C). As shown in Fig.2, the growth inhibitory effect of the compound no.20 differed among the bacterial strains. The inhibition becomes stronger in (decreasing order) *C. albicans>E.coli>S. aureus.*

Leakage of potassium (K)

The effect of the compound no.20 upon the membranes of *S. aureus, E. coli* and *C. albicans* as indicated by potassium (K⁺) leakage is shown in Fig. 3. This figure indicated that compound no.20 has significant observable and marked leakage effects upon the tested organisms at 30 min. The highest value of potassium flow was recorded with *E. coli* and *C. albicans*, whereas the least flow was noticed with *S. aureus*. The results with the Gram- negative organism showed that the compound no.20 released higher amounts of potassium ion than in the Gram- positive organism.

Respiratory activity

Figure 4. showed that the respiratory activity of *E. coli, S. aureus* and *C. albicans* cells treated or untreated with the tested compound no.20 was evaluated. The compound no. 20 at the sublethal dose has almost no effect on *S. aureus* cells respiration where it gives values comparable to the control. However with *E. coli* and *C. albicans* the sublethal dose of compound no.20 increased significantly the respiratory activity to somehow as shown in Fig. 4 in comparable to the control.
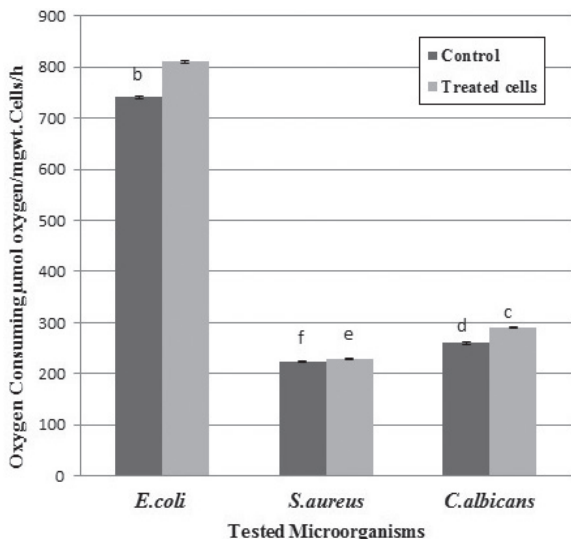
Figure 4. *The amount of oxygen consumed by the tested microorganisms under the effect of the compound no.20.*

Control: Sample was not treated with the compound no.20, Treated cells: cells treated with the compound no.20 at concentration 50 μg/mL (sublethal dose). The results are the mean ± SD (n = 3). Means with the same letter are not significantly different.

In vitro Cytotoxicity of the compound no.20 against *E. coli*

The compound no.20 showed some cytotoxicity against DNA genome of *E. coli,* where it gives a brown color (positive result) indicating the hydrolysis of 6-bromo2-naphthyl-β-galactopyranoside by β-galactosidase enzyme.
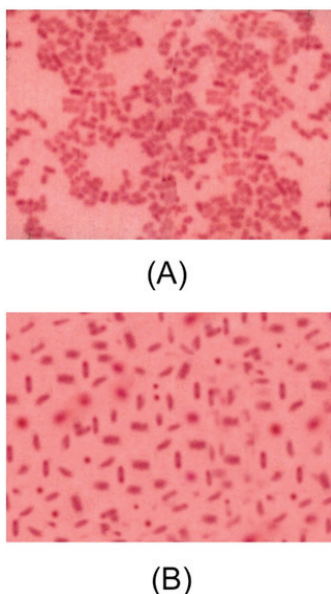

Figure 5. *Effect of the compound no.20 on cell morphology of E. coli cells stained with gram stain and grown in nutrient broth with sublethal dose (50 μg/mL) under Light microscopy (1000x). (A): Zero μg/mL, (B): 50 μg/mL (sublethal dose).*
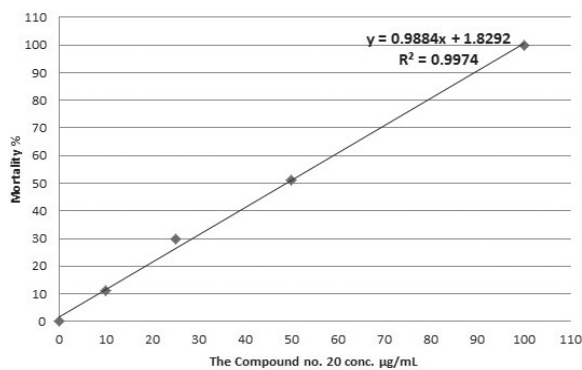

Figure 6. *Mortality percentage (%) curve of Brine shrimps* (Artemia salina) *nauplii exposed to different the compound no.20 concentrations for 24 h.*

Effect of the compound no.20 on cell morphology of *E. coli*

It was showed that the compound no.20 affect *E. coli* cell morphology, which was manifested as elongation and swollen of *E. coli* cells as shown in Fig.5.

LC$_{50}$ Determination of the compound no.20

The procedure determines LC$_{50}$ value in μg/ mL of the most effective compound no. 20 in the brine medium. The activity of the compound no.20 was manifested as toxic agent to shrimps. Figure 6 gave the cytotoxicity results for the compound no.20. In Fig.6 the result of regression analysis indicated that the mortality rate (Y) is
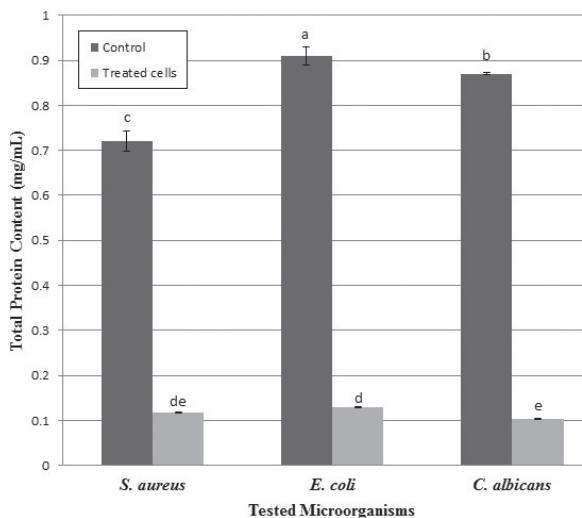

Figure 7. *Effect of the compound no.20 on the total protein content (mg/mL) of the tested organisms.*

Control: Sample was not treated with the compound no.20, Treated cells: cells treated with the compound no.20 at concentration 50 μg/mL (sublethal dose). The results are the mean ± SD (n = 3). Means with the same letter are not significantly different.
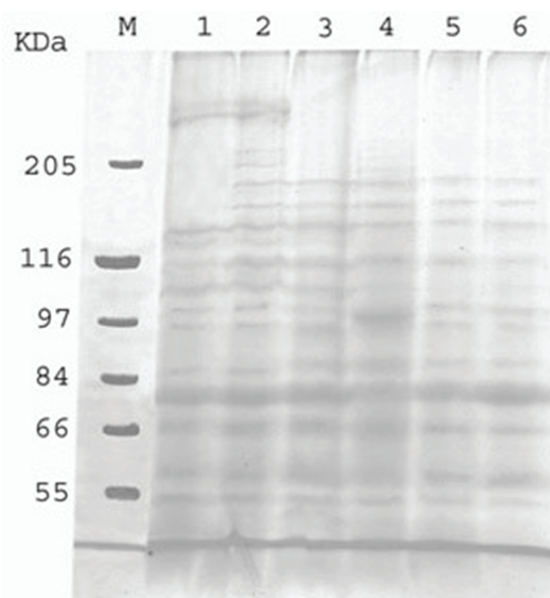
Figure 8. *Protein banding patterns of tested organisms under the effect of the compound no.20 at 50 μg/mL at 10% SDS- Polyacrylamide gel electrophoresis.*

Lanes: M: high molecular weight mass protein standard, 1: Protein pattern of untreated E. coli (control), 2: Protein pattern of E. coli treated with the compound no.20, 3: Protein pattern of untreated S. aureus (control), 4: Protein pattern of S. aureus treated with the compound no.20, 5: Protein pattern of untreated C. albicans (control) and 6: Protein pattern of C. ablicans treated with the compound no.20.

positively correlated the concentration (X) having a regression coefficient (R) close to one. The number of dead shrimps increased in response to increase in the compound no.20 concentrations. An LC_{50} value of the compound no. 20 for brine shrimps was estimated at 48.736 μg/mL concentration from the regression equation.

Effect of the compound no.20 on the total protein of the tested organisms

The amount of the total protein of *E. coli*, *S. aureus* and *C. albicans* cells treated with the compound no. 20 were decreased as compared with control as shown in Fig.7. Gel electrophoresis of the total protein indicated that there is a difference in protein banding pattern at 205-116 KDa in *E. coli* and also at 97 KDa in *S. aureus* as compared with untreated cells. However, there is no difference in protein banding pattern in *C.albicans* treated with the compound no. 20 compared with untreated cells as shown in Fig.8.

DISCUSSION
The antimicrobial activity of the tested thienopyrimidine compounds

Although, thieno[2,3-d]pyrimidines had been extensively explored for their varied biological activity, the com-

pounds reported here had not yet been prepared in previous literature. In this paper, the compound no.20 possess abroad spectrum of antimicrobial activities. These results agreed with that obtained by many researchers (27,3,28). The results indicated that thienopyrimidines are interesting heterocyclic compounds and a number of derivatives of these compounds display therapeutic activity as antibacterial and antifungal agents (5,1,29,9).

Minimal inhibitory concentration (MIC)

The MIC of the compound no.20 for all the tested microorganisms was >50 <100 μg/mL. Other researchers found that cyclopenteno and cyclohexeno [b]thieno[2,3-d]-3,4-dihydropyrimidine-4-one displayed reasonable activity (MIC values 6.25-25 mg/L) against both Gram-positive and Gram-negative bacteria (30,1). Chambhare *et al.* (3), proved that furanyl-thieno[2,3-d]pyrimidin-4-ones displayed MIC values in the range of 4-100 mg/L against a collection of Gram-positive and Gram-negative microbe. Some novel thienopyrimidine and triazolo-thienopyrimidines showed MIC values in the range of 11- 512 μg/mL (6). As well as, they also displayed antimicrobial activates towards the test organisms (*Pseudomonas aeruginosa*, *S. aureus*, *Bacillus subtilis*, *E. coli*, *C. albicans* and *C. parapsilosis*).It is obvious that the MIC depends mainly on the tested compound as well as used organisms. The growth inhibiting effect (M/C) obtained by Kenawy *et al.* (31) coincide with that of the compound no.20 in this study. They found that the growth inhibitory effect M/C of polymer IX became stronger in the order of *B. subtilis*<*E. coli* <*P. aeruginosa*< a fungus (*Trichophyton rubrum*). Hence, the novel compound no.20 is considered as potent antibacterial agent (2, 32, 33).

Leakage of potassium (K)

The treatment of Gram- negative and positive bacteria with the compound no.20 at sublethal dose lead to release of higher amounts of potassium ion with the Gram- negative than the Gram- positive organism. Marked leakage of cytoplasmic material is considered indicative of gross and irreversible damaged to the cytoplasmic membrane (34).

Respiratory activity

The effect of the compound no.20 on bacterial respiration was similar to that recorded with nisin on *E. coli* and *S. aureus* (35). Poly (chlorethyvinylether-co-vinylbenzoyl-chloride) increased respiration for *C. albicans* and *C. tropicials (20)* as that observed with the effect of the compound no.20 on *C. albicans* respiration. This effect may be indicates that many chemical compounds interfering directly with electron transfer or uncoupled oxidative phosphorylation from electron transport. So, the aerobic synthesis of ATP is inhibited and the oxygen uptake is not inhibited (17). Also, it may be indicates a slight effect on

the plasma membrane and increasing cells respiration for exhausting the energy compound present inside the cell before its death *(20)*.

In Vitro Cytotoxicity of the compound no.20 against *E. coli*

The cytotoxic effect of the compound no.20 against *E. coli* coincide with that obtained by *(11)*. They observed that the cytoplasmic membrane disruption is often exemplified by the leakage of intracellular components, potassium (K+) followed by inorganic phosphates, pool of amino acids and materials absorbing at 260 nm, nucleic acids and proteins. Moreover, the compound no.20 changed cell morphology in *E. coli* cells. Saloman and Farias *(36)* found that microcin 25; a peptide antibiotic caused *E. coli* cell filamentation. Moreover, poly (methyl methacrylate co-vinylbenzoylchloride) altered cell morphology which was manifested as aggregated and swollen yeast cells (*C. albicans* ATTCC 2091) *(20)*. Simõesc *et al.* *(18)* found that interference on bacterial cell cycle occurs at high ortho-phtalaldehyde concentrations and cell division is compromised as the cellular septation seems not to occur. Therefore, a cellular elongation takes place in *P. fluorescens* cells. Consequently, the formation of long forms of bacteria following antimicrobial treatment may be an indication of cell wall damage *(11)*.

LC$_{50}$ Determination of the compound no.20

Compound no.20 is manifested as toxic agent to brine shrimps. The result of LC$_{50}$ value of the compound no.20 for brine shrimps coincides with many other studies. The LD$_{50}$ values of the active compound of a series of 2-substituted amino-3-aminocyclopenteno or cyclohexeno[b] thieno[2,3-d]-3,4-dihydropyrimidin-4-ones ranged from 25-250 µg/mL *(30)*. The cytotoxicity activity of the polar fractions of *Cochlospermum tinctorium* extracts using the brine shrimp lethality bioassay method were determined by linear regression analysis method and it was 240µg/ mL for 80 % acetone extract and 437µg/mL for n-butanol extract *(37)*. The compound no.20 showed potent biocide activity against brine shrimp due to their lower cytotoxicity that agreement with preliminary anti-microbial screening and the minimum inhibitory concentration (MIC). This finding is in agreement with that of Fayed and Ghanem *(29)*.

Effect of the compound no.20 on the total protein of the tested organisms

The result obtained was in agreement with that obtained by Ali *et al.* *(38)*. They found that treatments of MCF-7 cells with 1/10 of LC$_{50}$ of three thienopyrimidine derivatives decline the total protein, DNA and RNA content compared with MCF-7 cells harvested from control.

The protein banding pattern of *E. coli* and *S. aureus* treated cells with the compound no.20, give results agreed

with many researchers *(39, 40)*. They found that thienopyrimidine derivatives inhibits dihydrofolate reductase (DHFR) which plays an essential role in carbon metabolism, which is critical to the biosynthesis of DNA, RNA, and the essential amino acid methionine. However, the effect of the compound no.20 on protein banding pattern in *C. albicans* coincide with that obtained with Mahmoud and Aly *(20)*. They found that the effect of synthesized polymers modified poly (methylmethacrylate-co-vinylbenzoylchloride) and a modified linear poly (chloroethylvinylether-co-vinylbenzoylchloride) on *C. albicans* indicated no clear effects in the phosphopeptidomannan molecular weight with main bands at 45, 25 and 15 kDa. The only differences were in the protein concentrations.

Conclusion: it could be concluded that the compound no.20 has antimicrobial activity and cytotoxicity against the tested organisms. Moreover, the present results lead us to speculate that the compound no.20 antimicrobial mode of action may be due to disruption of the cytoplasmic membrane which is often exemplified by the leakage of intracellular components, potassium (K+) followed by pool of amino acids and materials absorbing at 260 nm, nucleic acids and proteins. It is generally accepted that the bacterial outer envelope is responsible for the different microbial responses to biocidal challenges *(11)*. Active agent can induce damage to the cytoplasmic membrane by action upon the membrane potentials, bound enzymes or permeability and this lead to leakage potassium ion, and other cellular components and eventually cell death *(41, 42, 43)*. Furthermore, anther speculation of mode of action is that, the physicochemical characteristic of the compound no.20 may be match with lipid composition and net surface charge of the bacterial membranes. Therefore, this cause its penetration and membrane leakage subsequently, this process can change the morphology of the bacterial cell *(34)*. Thus, the ultimate goal of study the mechanisms of action of antimicrobial compounds will help to improve the bactericidal activity of a product, to confirm that biocides are employed in the most advantageous manner and to ensure the prevention of the emergence of microbial resistance. Furthermore, understanding the target sites of the antimicrobial compounds will lead to improved antimicrobial formulations *(11, 1)*.

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