# Antisalmonella Activity and Modes of Action of Extracts, Fractions and Cerevisterol from *Impatiens burtonii* against Multi-Drug Resistant *Salmonella*

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*This work was carried out in collaboration among all authors. Authors ADYP and FWTK carried out the experiments and contributed to the data analysis. Authors AGF, MN, SPCF and LAT contributed to the data analysis and helped in writing and editing the manuscript. Authors DG and RBT designed the study, supervised the work and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Typhoid fever is an endemic disease in developing countries, especially throughout Asia and Africa, which may lead to life-threatening conditions in the absence of adequate treatment.
**Objective:** This study was aimed at determining the *in vitro* activity of the crude extracts, fractions and compounds of *Impatiens burtonii* against drug sensitive and multidrug-resistant *Salmonella* strains.
**Methods:** Moreover, the modes of action of the most active compound, cerevisterol (**1**) were investigated on *Salmonella* TyphiATCC6539.The *in vitro* antisalmonella activity of the samples was evaluated by the broth microdilution method. Catalase Activity was done by measuring the high of the foam in the presence of hydrogen peroxide. Bacteriolysis, time kill-assay, nucleic acid and protein leakage potential of cerevisterol were carried out through spectrophotometric methods. Finally, time kill-assay and proton-ATPase pump activity were determined by bacterial count and pH measurement, respectively.
**Results:** The *In vitro* antisalmonella activity revealed that the hydroethanolic 75% crude extract of *Impatiens burtonii* showed the highest antisalmonella activity, with minimal inhibitory concentration (MIC) ranging from 32 to 512 µg/mL. Among the fractions of that extract, the dichloromethane (DM) fraction exhibited the highest activity, with MIC ranging from 16 to 32 µg/mL. Cerevisterol (**1**) isolated from the DM extract was the most active compound (8 ≤ MIC≤ 128 µg/mL). This study showed that cerevisterol is bacteriostatic against *Salmonella* Typhi. Moreover, cerevisterol induced the release of the nucleotides, inhibited H+-ATPase proton pumps, and reduced the catalase activity of *Salmonella* Typhi ATCC6539.
**Conclusion:** Finally, this study allowed to identify cerevisterol as a good candidate to tackle typhoid fever infections and mainly that caused by MDR (multidrug-resistant) strains.

## ABBREVIATIONS

CC      : *Column Chromatography*
TLC     : *Thin-Layer Chromatography*
UV      : *UltraViolet*
TMS    : *Tetra Methyl Silane*
ATCC   : *American Type Culture Collection*
MIC    : *Minimal Inhibitory Concentration*
MBC   : *Minimal Bactericidal Concentration*
INT     : *p-IodoNitrotetrazolium chloride*
SFR/CAM: *Society of Forest Reserve of Cameroon*
1H-NMR  : *Proton Nuclear Magnetic Resonance*
13C-NMR: *Carbon Nuclear Magnetic Resonance*
CHL    : *Chloramphenicol*
MHA   : *Mueller Hinton Agar*
MHB   : *Mueller Hinton Broth*

## 1. INTRODUCTION

"Typhoid fever is an endemic disease present in developing countries, especially throughout Asia and Africa"[1]. "It is caused by *Salmonella enterica* serovar Typhi (S. Typhi), an acute generalized pathogen of the mononuclear phagocyte system, intestinal lymphoid tissue, and gallbladder who is associated with poor sanitation and untreated water supply" [2]. Typhoid and paratyphoid fevers manifest one or two weeks following infection and the most clinical signs are fever and malaise, abdominal pain, headache, myalgias, nausea, anorexia, constipations diarrhea [3]. These manifestations can be life-threatening, for the patient in the absence of adequate treatment [4]. According to the World Health Organization, about 20 million cases of typhoid fever and more than 200.000 deaths occurred per year world wide [5]. In Cameroon, over the same period, 145.281 cases were recorded in 2015 compared to 173.603 cases in 2022 [6], reflecting a clear increase in the disease. Appropriate antibiotic therapy against salmonellosis can lower the risk of mortality to less than 1% [2]. Therefore, the misuse of these antibiotics currently yield to the selection of drug resistant Salmonella strains. Alarmingly, many reports have shown the increase of MDR strains mainly against fluoroquinolones, 3rd generation cephalosporins,

and ciprofloxacin [7]. Thus, it becomes essential to look for new sources of treatment to manage Salmonella infections, especially those caused by MDR strains.

Medicinal plants are currently used in various part of the world for the prevention, diagnosis, and treatment of various diseases [8-10]. "So, medicinal plants appear as a good sources for the search and development of novel antibacterial molecules from the plant kingdom effective against MDR bacterial infections are based on" [11-14].

*Impatiens burtonii* is a plant belonging to the family of Basalminaceae, which is used in traditional medicine to treat abdominal pains, constipation, diarrhea, indigestion, nausea" [15] and cough [16]. "Moreover, this plant is used in the treatment of female infertility and bacterial infections, hepatitis, feed for goats and pigs" [17]. "The rational of the present study comes to the fact that the leaves of *I. burtonii* are used to treat infections including bacterial infections. Recent chemical studies have elucidated phenolic acids, flavonoids, anthocyanins and alkaloid in *I. burtonii*" [15]. Previous biological studies have reported the antimicrobial [18], neuroprotective [16], antioxidant and antiepileptic [19] activities of *Impatiens burtonii*.

Herein, the antibacterial activity of extracts, fractions and compounds from the whole plant was determined on a panel of sensitive and MDR Salmonella strains. The modes of action of the most active secondary metabolite cerevisterol (**1**),were also evaluated.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals and natural products

Polymyxine (POLY), and ciprofloxacin (CIP), (Sigma-Aldrich, St. Quentin Fallavier, France) were used as reference antibiotics (RA). Dimethyl sulfoxide (Sigma-Aldrich) was used to dissolve tested samples. *P-Iodonitrotetrazolium* chloride (INT; Sigma-Aldrich) was used as microbial growth indicator. All solvents used for isolation and purification of bioactive components were of analytical grade.

#### 2.1.2 Plant materials

The whole plant of *Impatiens burtonii* was collected in October 2022 in Batcham, a village located in the West Region of Cameroon (7◦ 9′ 28.30″ N, 9◦ 27′ 2.28″ E). The plant was identified at the National Herbarium of Cameroon (HNC), Yaoundé, Cameroon, by Mr. Tchatchouang Ngansop Eric, a Botanist, where a voucher specimen was deposited under the number 16913/SRF/Cam.

#### 2.1.3 Microorganisms and culture media

Twelve microorganisms were used to determine the antibacterial activities of the extracts and fractions of *Impatiens burtonii.* Nine of them were multiresistant strains of *Salmonella* Typhi and Paratyphi collected in the stool of patients diagnosed typhoid fever positive in Bafoussam-Cameroon [21]. In addition, two sensitive clinical isolates including *Salmonella enterica* serovar Enteridis (SE) and *Salmonella enterica* serovar Typhimurium (STM) obtained from the "Centre Pasteur, Cameroun". Moreover, a sensitive strain of *Salmonella* Typhi ATCC6539 was obtained from American Type Culture Collection. The microorganisms were activated on Salmonella-Shigella Agar (SSA) (Accumix, Belgium) and stored at 4°C. Mueller Hinton broth (MHB) (Liofilchem, Italy) was used for the determination of Minimum Inhibitory and Bactericidal Concentrations (MIC).

### 2.2 Methods

#### 2.2.1 Preparation of crude extracts

The collected whole plant *of Impatiens burtonii* was cleaned, air-dried in the absence of UV radiation, and crushed using a blender (SINGSUNG, SINGAPOREE BL-530). One (1) kg of the total powder was removed and worm in water (2 L) which was remove from the heat source for 15 minutes for the infusion while for the decoction we mixed one kg of powder with 2L of water and allow both to boil for 15 minutes. Part (3,14 kg) of powder was also use to prepare hydroethanolic extracts (95%, 75%, 55% and 35%) according to the protocol described by Sokoudjou et al., [20]. After filtration, the obtained filtrates were evaporated under reduced pressure using a rotary evaporator to yield the extracts. The obtained extracts were stored at 4°C and were used for the evaluation of *in vitro* antisalmonella activity and for purification.

#### 2.2.2 Separation and bioactivity-guided fractionation

Part of the hydroethanolic extract 75% (158.6 g) was suspended in distilled water (300 mL) and successively extracted with equal volumes (500 mL) of dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc) and n-BuOH (saturated with water)

yielding after evaporation 99.6 g, 5.6 g and 9.4 g of fractions, respectively. Among these fractions, the $CH_2Cl_2$ fraction was the most active. An amount of 90.0 g of this fraction was then subjected to silica gel column chromatography using mixtures of *n*-hexane-EtOAc (1:0 to 0:1) and EtOAc-MeOH (1:0 to 1:1). Volumes of 400 mL were collected and combined based on their TLC profile to give six sub-fractions (A-F). These sub-fractions were further evaluated for their *in vitro* antisalmonella effects. The purification of 7.2 g of the most active sub-fraction (E) by repeated column chromatography using mixtures of *n*-hexane-EtOAc (1:1) as eluents, led to the isolation of compound **1** (100 mg). Sub-fraction F (7.1 g) was chromatographed over silica gel column eluted with EtOAc to yield compound **2** (80 mg). The antisalmonella properties these isolated compounds were also evaluated.

### 2.2.3 Determination of Minimal Inhibitory Concentration (MIC)

The MIC of extracts, fractions and compounds from *Impatiens burtonii* were determined in 96-wells microplates using rapid INT (iodo nitro tetra zolium chloride) colorimetric assay [20,22,23]. Briefly, each sample was dissolved in 5% Dimethyl-sulfoxide (DMSO)/MHB. The obtained solution was then added to 100 µL of MHB, and followed by two-fold serial dilution. Then 100 µL of inoculum ($1.5 \times 10^6$ CFU/mL) prepared in MHB were added to each well except the negative control wells. The plates were covered with a sterile plate sealer and incubated at 37°C for 18 h. The wells containing either MHB or MHB and 100 µL of inoculum served as control. After the incubation, 40 µL of INT (0.2 mg/mL) was added to each well and plates were re-incubated at 37°C for 30 min, and the MIC of each sample was recorded. MIC was defined as the lowest concentration of the sample that prevented change in color and exhibited complete inhibition of microbial growth.

### 2.2.4 Evaluation of the modes of action of cerevisterol

#### 2.2.4.1 Measurement of the bacteriolytic activity of cerevisterol

The lytic activity of cerevisterol on *S.* Typhi (ATCC6539) was evaluated according to the method described by Limsuwan et al., [24]. Briefly, 18 hours bacterial colonies were picked on agar and introduced into 0.9% NaCl solution. The resulting suspension was standardized to

have a turbidity corresponding to McFarland 0.5 standard ($1.5 \times 10^8$ CFU/mL). Then, 100 µL of cerevisterol was introduced into the wells of a microplate containing this suspension to have negative control, MIC, 2xMIC and 4xMIC as the final concentration in the wells. The resulting suspensions were shaken and the absorbances were immediately measured at 620 nm (T0). Then, the plates were incubated at 37°C with shaking and the absorbances were measured at 620 nm, respectively, every 2 hours intervals. Bacterial cell lysis was indicated by a decrease in OD 620 nm. A control with 1% DMSO was applied with the same conditions. All assays were carried out in triplicate.

#### 2.2.4.2 Time-kill assay

Time kill assay was performed using the method of Yadav et al., [25]. *S.* Typhi was grown on MHA media on Petri dish. Individual colony of bacterium was isolated from 24 h old culture and suspended in sterile normal saline. Density of bacterial suspension was adjusted to a 0.5 McFarland standard and the suspension was diluted 1:10 time in MHB. One milliliter (1 mL) of cerevisterol (1/2xMIC, MIC, 2 × MIC) and ciprofloxacin (MIC) was prepared in MHB followed by the addition of the above bacterial suspension. The resulting mixture was incubated at 28 ± 2°C for 24 h. A volume of 25 µl of sample was pipette out from each tube and spread over freshly prepared MHA Petri dishes at 0, 2, 4, 6, 8 ,10 and 12 h. Petri plates were incubated at 28 ± 2°C for 24 h in an incubator. Colonies on individual plates were counted and expressed as number of colony forming units/ml (CFU/mL). The killing rate was determined by plotting logarithm of the viable colony counts (CFU/mL) against time. The percentage reduction in total viable count of CFU was counted by using the formula:

$$Percentage\ reduction = \frac{Initial\ count - count\ at\ x\ interval}{initial\ count} \times 100$$

#### 2.2.4.3 Loss of 260 nm absorbing materials

The effect of cerevisterol on the nucleic acid leakage content was carried out as previously described [26] with slight modifications. The cultures of *S.* Typhi ATCC6539, in exponential growth phase in MHB were centrifuged at 3500 rpm for 30 min and the pallet was washed. The bacteria collected were resuspended in 10 mM PBS (pH 7.4) and incubated with cerevisterol at MIC for different times (0, 3, 6, 9, and 12 h). Bacterial strains incubated with PBS alone served as a control. The mixture was filtered

through a 0.2 *μ*m membrane to remove bacterial cells, and the absorbance of the filtrate was determined in a spectrophotometer (Biobase Bk-D590 Double Beam Scanning UV/Vis, China) at 260 nm. The test was carried out in triplicate.

### 2.2.4.4 Determination of protein content

The effect of cerevisterol on the protein content of *S.* Typhi(ATCC6539) was carried out as previously described [27]. Briefly, in 15 tubes each containing 9 ml of MHB, 0.5 ml of bacterial suspension (standardized to Mc Farland 0.5 scale) was added. Then, 0.5 mL of cerevisterol was added to each tube to obtain the following concentrations: 4xMIC, 2xMIC, MIC, and 1/2×MIC (each treatment was done in triplicate). The control tube was treated under the same conditions and received 0.5 mL of MHB instead of the compound. The tubes were incubated at 37°C with shaking. After 24 hours of incubation, centrifugation at 12,000 rpm for 3 minutes recovered the pellet that represented the bacterial cells. These cells were weighed and mixed with lysis buffer (10 mM Tris/HCl, pH 7.4; 100 mM EDTA, pH 7.4; 20 mM NaCl, 1% (w/v) SDS) at a rate of 40 mg bacteria per 500 μL buffer. Once the bacteria were lysed, the samples were centrifuged at 12,000 rpm for 3 minutes and the protein concentration was assessed in the supernatant by the Bradford method using a microplate reader (SoftMax Pro. USA). The blank was the lysis buffer. The protein content decreases compared to the control group not treated with the extract.

### 2.2.4.5 Evaluation ofH⁺-ATPases mediated proton pumps activity

The effect of cerevisterol on the proton pump activity of *S.* Typhi (ATCC6539) has been monitored by acidification of the external environment of bacteria [28]. "Briefly, 500 μL of a bacterial suspension obtained from an 18 h bacterial culture was introduced into MHB to obtain a volume of 50 mL of bacterial culture and then incubated at 37°Cfor 18 h. The resulting culture was centrifuged 3500*g* for 10min. The resulting pellet was washed with distilled water, then with 50 mM KCl and resuspended in 50 mL of 50 mM KCl. The suspension was then stored at 4°Cfor 18 h for glucose deprivation and the pH was adjusted to 6.4 by adding HCl and/or NaOH. In 4 mL of this solution, 0.5 mL of cerevisterol solution dissolved in 3% DMSO and distilled water was added to obtain the concentration of cerevisterol equal to the MIC. After 10 min of pre-incubation at 37°C, the acidification of the

medium was initiated by adding 0.5 mL of 20% of glucose solution, the rapid catabolism of which will be accompanied by the release of protons in the medium. Then, the pH of the medium was measured every 10 min for 1 h (60 min). For this test, ciprofloxacin was used as the positive control. The noted pH values made it possible to draw curves of variation of the pH as a function of time. Any inhibition of the acidification of the medium in the presence of cerevisterol has been attributed to an inhibitory effect on the functioning of H+-ATPases pumps by the compound" [28].

### 2.2.4.6 Evaluation of catalase activity in S. Typhi (ATCC6539)

"Fresh colonies of *S.* Typhi (ATCC6539) were inoculated in 5 mL sterile tryptone soy broth (TSB) and followed by incubation at 37◦C in a shaker overnight to set up untreated culture controls. Similarly, fresh colonies of *S.* Typhi (ATCC6539) were grown in 1 mL of cerevisterol (MIC) and 4 mL of tryptone soy broth overnight. Then, 100 μL of the respective test samples corresponding to O.D. of 0.1 was transferred into a test tube. Subsequently, 100 μL of 1% Triton X-100 (InvitrogenTM, Thermo Fisher Scientific, Loughborough, UK) and 100 μL of 30% (*v/v*) $H_2O_2$ were added to the tube. After mixing thoroughly, the tubes were incubated at room temperature for 5 min"[29]. "After the reaction stopped, the height of the foam remained constant was measured using a ruler" [30]. Result was expressed as percentage change using the following formula.

$$\% \text{ change} = \left[\frac{\{\text{ height of foam (exposed cells)} - \text{ height of foam (unexposed control)}\}}{\text{height of foam (unexposed control)}}\right] * 100$$

### 2.2.5 Statistical analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA) and presented as mean±standard deviation (SD) of three replications. The levels of significance, considered at $p < 0.05$, were determined by Waller–Duncan test using the Statistical Package for the Social Sciences (SPSS) software version 22.0.

## 3. RESULTS

### 3.1 Structure Elucidation

The structures of the isolated compounds (Fig. 1) were elucidated on the basis of spectroscopic data (¹H-NMR and ¹³C-NMR, HSQC, and HMBC; Supplementary materials Fig. S1-S5). By

comparison of their data with those reported in the literature, they were identified as cerevisterol (**1**) [31] and *β*-sitosterol -3-*O*-*β*-D-glucopyranoside (**2**) [32].

Cerevisterol (1): 1H-NMR (600 MHz, DMSO-d6): δ 5.33 (d, J = 5.4 Hz, H-7), 5.20 (dd, J = 7.2, 15 Hz, H-23), 5.16 (dd, J = 7.2, 15 Hz, H-22), 4.06 (m, H-3), 3.60 (m, H-6), 1.07 (s, H-19), 1.00 (d, J = 6.6 Hz, H-21), 0.90 (d, J = 7.2 Hz, H-28), 0.82 (d, J = 6.6 Hz, H-27), 0.80 (d, J = 6.6 Hz, H-26), 0.58 (s, H-18); 13C-NMR (150 MHz, DMSO-d6): δ 144.0 (C-8), 135.4 (C-22), 132.2 (C-23), 117.5 (C-7), 76.0 (C-5), 73.7 (C-6), 67.7 (C-3), 56.0 (C-17), 54.8 (C-14), 43.8 (C-13), 43.5 (C-9), 42.8 (C-24), 40.4 (C-28), 39.2 (C-4), 39.2 (C-12), 37.1 (C-10), 33.1 (C-25), 33.0 (C-1), 30.9 (C-2), 27.9 (C-16), 22.9 (C-15), 22.1 (C-11), 21.1 (C-27), 19.9 (C-26), 19.6 (C-21), 18.8 (C-19), 17.6 (C-28), 12.33 (C-18).

β-sitosterol 3-O-β-D-glucopyranoside (2): White amorphous powder, mp = 289-291°C (Lit: 290-292°C[33].

### 3.2 MICs of Extracts, Fractions, and Compounds from *Impatiens burtonii*

The antibacterial activity of crude extract, fractions, and compounds from the whole plant of *Impatiens burtonii* was evaluated through the determination of their MICs against twelve bacteria strains and isolates of *Salmonella*. The results obtained were depicted in Tables 1–2. The crude 75% hydroethanolic extract had the highest activity, with MICs ranging from ranging from 32 to 512 μg/mL. Among the obtained fractions, dichloromethane and ethyl acetate fractions showed the best anti-salmonella activity. Therefore, the dichloromethane fraction was further subjected to silica gel column chromatography to yield six sub-fractions. Among them, sub-fractions E and F showed the highest inhibitory activity, with a MIC of 16 μg/mL and 32 μg/mL against *Salmonella* Typhi, Typhimurium and *Salmonella* Enteritidis (Table 2).

The isolated compounds showed the highest activity on the salmonella tested (8 ≤ MIC ≤128 μg/mL). The MICs of the reference antibiotic, ciprofloxacin were ranged from 0.5 to 512 μg/mL. It appears that the activity of isolated compounds is greater than those of the crude extracts and fractions.

### 3.3 Bacteriolysis

Fig. 2 showed the bacteriolytic effect of compound **1** (cerevisterol) at different concentrations (MIC, 2×MIC and 4×MIC) on *Salmonella* Typhi(ATCC6539) as a function of time. Analysis of this figure revealed a decrease in absorbance in bacterial cells treated with different concentrations of cerevisterol compared to untreated cells. The treatment of the bacteria with cerevisterol at MIC, 2xMIC and 4xMIC caused cell lysis after 4 h. The bacteriolytic effect of compound **1** was significant at 4xMIC during the whole experiment.
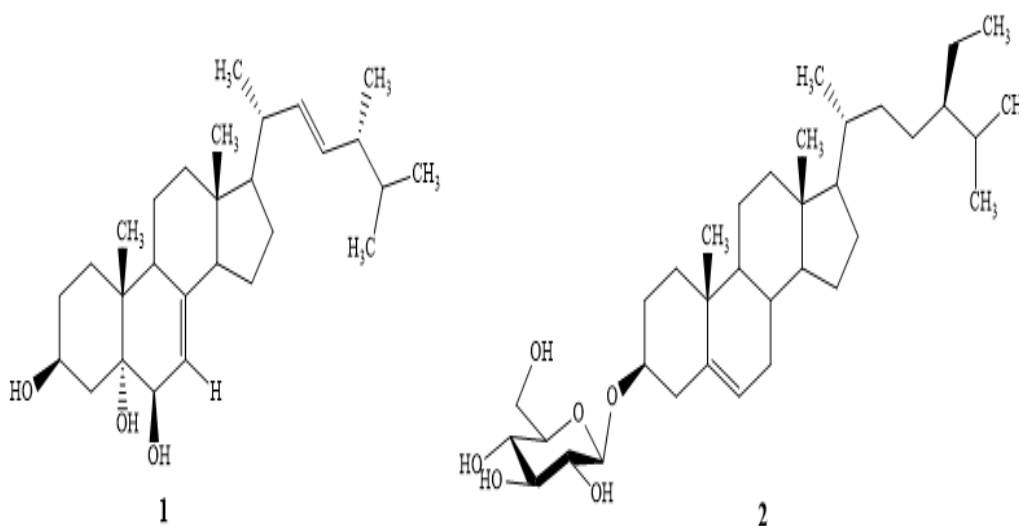


**Fig. 1. Chemical structures of compounds 1 and 2**
*(1): cerevisterol; (2): β-sitosterol -3-O-β-D-glucopyranoside*

**Table 1. MIC of the crude extract and partition fractions from *Impatiens burtonii* whole plant against different test microorganisms**

| Tested samples | *Salmonella* strains/isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ST1r | ST2r | ST3r | SPA1r | SPA2r | SPB1r | SPB2r | SPB3r | SPCr | STSs | STMs | SEs |
| EtOH 95% | 512 | 512 | 1024 | 1024 | 1024 | 1024 | 1024 | 1024 | 512 | 256 | 256 | 256 |
| EtOH 75% | 128 | 256 | 512 | 256 | 128 | 256 | 128 | 256 | 256 | 64 | 64 | 32 |
| EtOH 55% | 512 | 1024 | 1024 | 512 | 512 | 256 | 512 | 1024 | 256 | 128 | 256 | 256 |
| EtOH 35% | 1024 | 1024 | 512 | 1024 | 512 | 512 | 1024 | 1024 | 512 | 512 | 256 | 256 |
| Aqueous macerated | 1024 | 1024 | 512 | 1024 | 1024 | 512 | 1024 | 512 | 1024 | 512 | 512 | 256 |
| Aqueous decoction | 1024 | 1024 | 512 | 1024 | 1024 | 1024 | 512 | 1024 | 512 | 128 | 256 | 256 |
| Aqueous infused | 1024 | 512 | 1024 | 512 | 1024 | 1024 | 1024 | 512 | 1024 | 256 | 256 | 256 |
| $CH_2Cl_2$ | 128 | 256 | 64 | 128 | 64 | 128 | 128 | 64 | 64 | 32 | 16 | 16 |
| EtOAc | 256 | 256 | 512 | 256 | 128 | 128 | 256 | 128 | 512 | 64 | 32 | 64 |
| n-BuOH | 1024 | 512 | 1024 | 1024 | 512 | 1024 | 1024 | 1024 | 512 | 128 | 128 | 128 |
| Cip | 128 | 256 | 512 | 128 | 256 | 256 | 128 | 512 | 128 | 0,5 | 0,5 | 0,5 |

*EtOH: Hydroethanolic Extract, MIC: Minimal Inhibitory Concentration; $CH_2Cl_2$: Dichloromethane; EtOAc: Ethyl acetate; n-BuOH: n-Butanol; Cip: Ciprofloxacin; ST1r, ST2r, ST3r: Multi-Resistant isolate of Salmonella Typhi; SPA1r, SPA2r, SPB1r, SPB2r, SPB3r, SPC: Multi-Resistant isolate of Salmonella Paratyphi A, B and C; STS: Sensible strain of Salmonella. TyphiATCC6539; STM: Sensible Strain of Salmonella enterica Typhimirium SE: Salmonella Enterica Serovar Enteridis*

**Table 2. MIC of the sub-partition fractions and isolated compounds from *Impatiens burtonii***

| Tested samples | *Salmonella* strains/isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ST1r | ST2r | ST3r | SPA1r | SPA2r | SPB1r | SPB2 r | SPB3r | SPCr | STSs | STMs | SEs |
| A | 512 | 512 | 256 | 1024 | 256 | 1024 | 1024 | 512 | 512 | 128 | 128 | 64 |
| B | 512 | 1024 | 512 | 1024 | 512 | 512 | 1024 | 1024 | 512 | 64 | 128 | 128 |
| C | 256 | 128 | 256 | 128 | 256 | 128 | 128 | 128 | 256 | 32 | 64 | 128 |
| D | 512 | 1024 | 256 | 64 | 256 | 256 | 512 | 128 | 256 | 128 | 64 | 64 |
| E | 128 | 64 | 256 | 256 | 64 | 128 | 128 | 64 | 128 | 16 | 32 | 32 |
| F | 64 | 128 | 128 | 64 | 64 | 32 | 128 | 64 | 64 | 16 | 16 | 16 |
| 1 | 32 | 32 | 128 | 64 | 64 | 32 | 64 | 32 | 32 | 8 | 16 | 8 |
| 2 | 1024 | 512 | 1024 | 256 | 1024 | 512 | 1024 | 512 | 1024 | 128 | 256 | 256 |
| Cip | 128 | 256 | 512 | 128 | 256 | 256 | 128 | 512 | 128 | 0,5 | 0,5 | 0,.5 |

*MIC: Minimal Inhibitory Concentration; Cip: Ciprofloxacin; ST1r, ST2r, ST3r: Multi-Resistant isolate of Salmonella Typhi; SPA1r, SPA2r, SPB1r, SPB2r, SPB3r, SPC: Multi-Resistant isolate of Salmonella Paratyphi A, B and C; STS: Sensible strain of Salmonella. Typhi ATCC6539; STM: Sensible Strain of Salmonella enterica Typhimirium SE: Salmonella Enterica serovar Enteridis Sub-dichloromethane fraction (A); Sub-dichloromethane fraction (B); Sub-dichloromethane fraction (C); Sub-dichloromethane fraction (D); Sub-dichloromethane fraction (E); Sub-dichloromethane fraction (F). Cerevisterol (1); (MIC) β-sitosterol -3-O-β-D-glucopyranoside (2)*
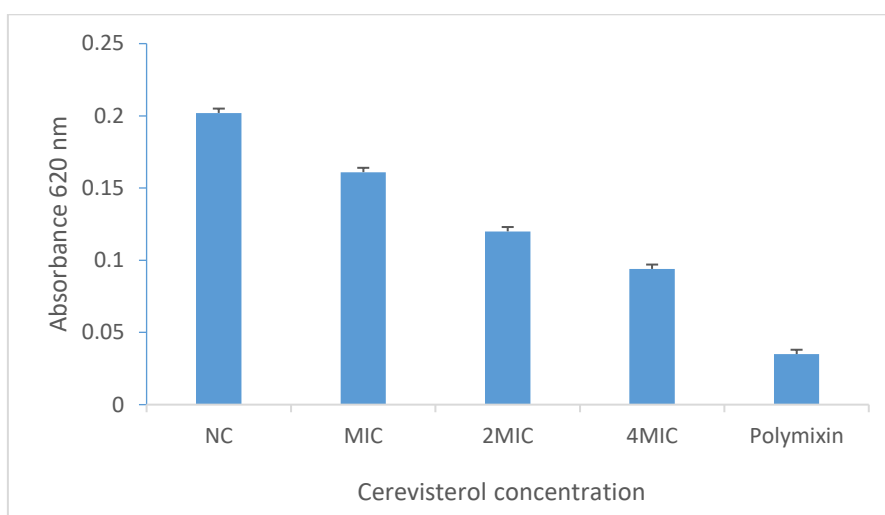
**Fig. 2. Effect of cerevisterol at different concentrations and the lytic activity of *Salmonella* Typhi(ATCC6539) as a function of time**

*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum inhibitory concentration. The MICs of the extract and cerevisterolagainst S. Typhi ATCC6539 were 64 µg/mL and 8 µg/mL, respectively*
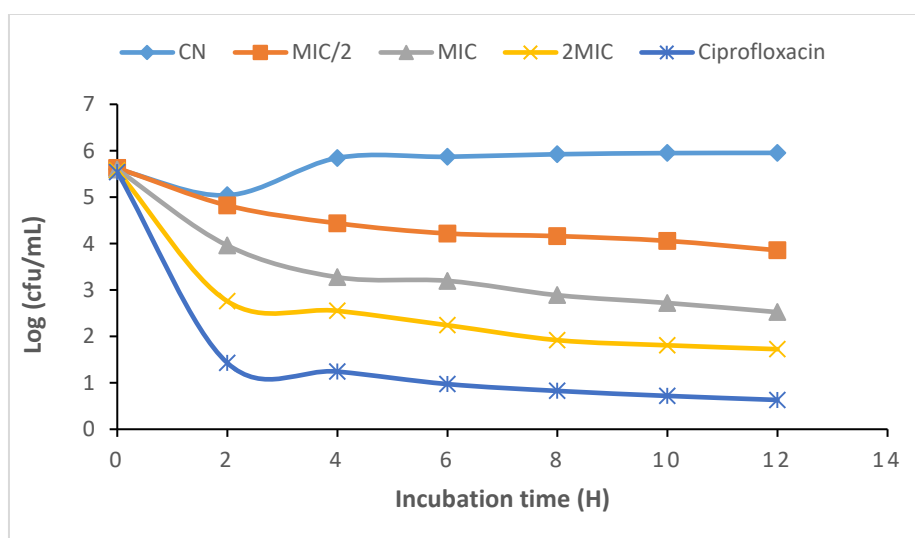


**Fig. 3. Time-kill assay curves of *Salmonella*Typhi(ATCC6539) after treatment with cerevisterol**
*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum inhibitory concentration. The MIC of cerevisterolagainst S. Typhi ATCC6539 was 8 µg/mL*

## 3.4 Time-Kill Assay

Time kill assay of cerevisterol *againstSalmonella* Typhi(ATCC6539) is demonstrated in Fig. 3. Complete killing of *Salmonella* Typhi (ATCC6539) cells treated with 2×MIC, MIC, and MIC/2 of the cerevisterol occurred within 4, 8, and 12 h after treatment with cerevisterol, respectively. The growth rate of 1/2 MIC treated culture was lower than the control until 12 h and subsequently reached the same level as the control.

## 3.5 Effect of Cerevisterol on the 260 Nm-Absorbing Materials

Cerevisterol caused significant increases in the OD 260 nm in all bacterial suspensions. This was observed from the third hour of incubation with a maximum effect at 12 hours when compared to the respective negative control culture (Fig. 4). The effects of cerevisterol were lower than the one of polymyxin used as standard controls.
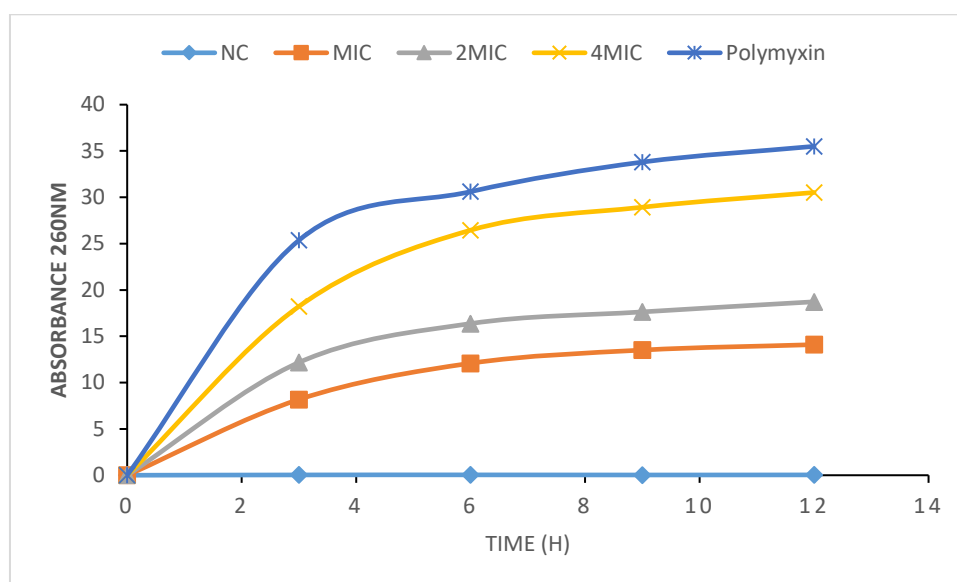
**Fig. 4. Nucleotide leakage from bacterial suspensions treated with MIC of cerevisterol**
*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum Inhibitory Concentration. The MIC of cerevisterolagainst S. Typhi ATCC6539 was 8 µg/mL*
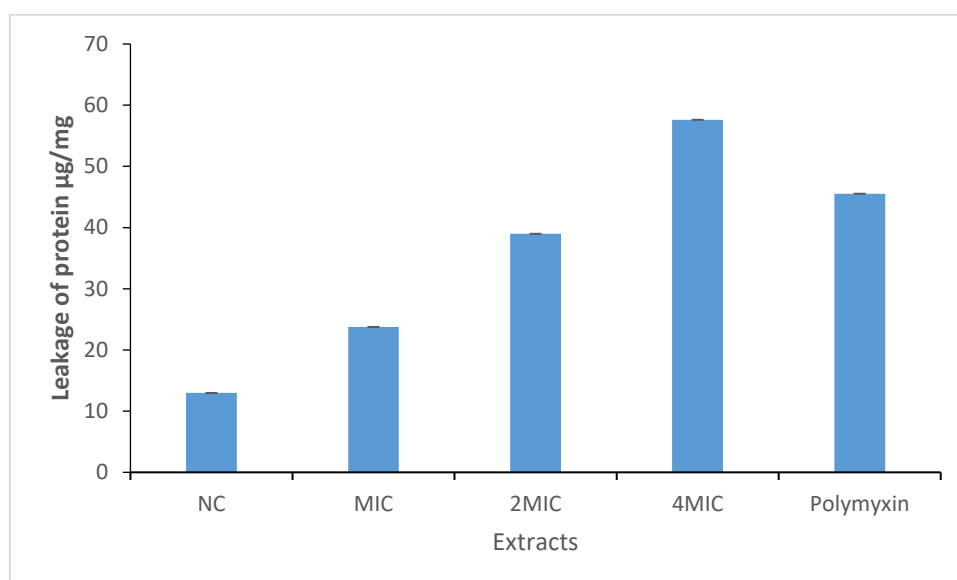


**Fig. 5. Leakage of proteins from bacterial suspension treated with cerevisterol**
*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum Inhibitory Concentration. The MIC of cerevisterolagainst S. Typhi ATCC6539 was 8 µg/mL*

## 3.6 Effect of Cerevisterol on the Protein Leakages

The leakages of proteins were significantly ($p < 0.05$) highest in the bacterial suspensions treated with cerevisterol when compared to the untreated cells (Fig. 4). This result suggested that cerevisterol accelerates the escape of proteins from the bacterial cytoplasm. Furthermore, cerevisterolinduced the highest leakages of proteins at 4 MIC (Fig. 5) in *Salmonella* Typhi (ATCC6539) suspension following.

## 3.7 Effect of Cerevisterolon Proton-ATPase Pumps

"Plasma membrane H+-ATPases (H+-pumps) are the primary active transporters that

translocate protons to the outside of each cell, providing the electrical and chemical energy that drives solute transport" [34]. "Thus, any inhibition of these pumps equally inhibits the growth of the bacteria" [35]. "In, this study, the acidification was slightly modified by the tested samples (mainly cerevisterol at 2 × MIC) compared to the control, which showed a significantly increased acidification of the medium with time" [35] (Fig. 6). This suggests that inhibition of the plasma membrane proton pump may be one of the modes of action of cerevisterol and its active ingredient compound against *S.* TyphiATCC6539.
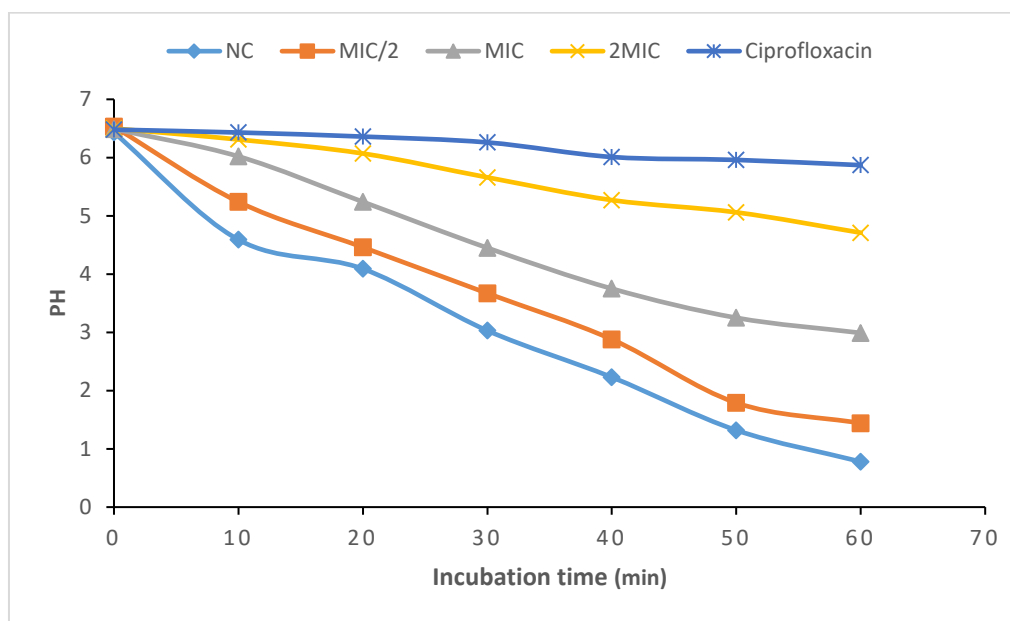


**Fig. 6. Effects of cerevisterolon proton-ATPasepumps**
*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum Inhibitory Concentration. The MIC of cerevisterolagainst S. Typhi ATCC6539 was 8 µg/mL*
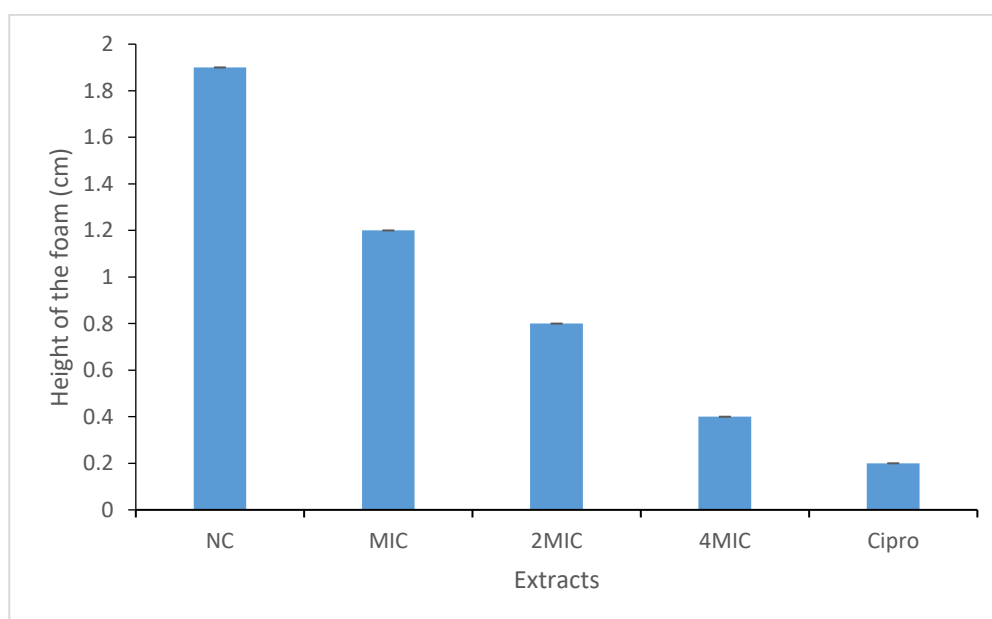


**Fig. 7. Effect of cerevisterol on catalase activity in *Salmonella* Typhi ATCC6539 treated cells**
*Each point represents the mean ± SD; n = 3 (number of repetitions). MIC: Minimum Inhibitory Concentration. The MIC of cerevisterolagainst S. Typhi ATCC6539 was 8 µg/mL*

## 3.8 Effect of Cerevisterolon Catalase Activity

The catalase activity of *S.* Typhi *(ATCC6539)* was visually determined using the hydrogen peroxide method. The height of foam (cm) was measured. Fig. 7 displays the effect cerevisterol on the catalase activity in *S.* TyphiATCC6539. Cerevisterol treated cells revealed a decrease in catalase activity as the height of foam reduced considerably, from 1.9 cm in the control (untreated cells) to 0, 4 cm in cerevisterol cells. Ciprofloxacin used as a reference drug showed a reduction in catalase activity with 0.2 cm of foam height (Fig 7).

## 4. DISCUSSION

The secondary metabolites secondary metabolites like polyphenols, flavonoids, and tannins from plants have been proven to possess antimicrobial activity amongst which the antibacterial activity [17, 36]. "Herein. We have evaluated the *in vitro* activity of the crude extracts, fractions and compounds of *Impatiens burtonii* against drug sensitive and multidrug-resistant *Salmonella* strains. The antibacterial activity of plant material and derived compounds have been established based on the Cut-off values of MICs" [11]. Based on these values, the crude extract of *Impatiens burtonii* 75% showed strong antibacterial activity (MIC <100 μg/mL) among all the crude extracts tested. Additionally, cerevisterol isolated from the most active fraction (CH$_2$Cl$_2$ fraction) showed significant (MIC <10 μg/mL) to moderate (moderate if 10 <MIC ≤100 μg/mL) antibacterial activity. However, we should be considered element like the activity spectrum and level of resistance of the studied strains. As this plant is commonly used in traditional medicine to treat bacterial infections [17], the obtained results are supplementary data indicating the possible used of 75% hydroethanolic extract of *I. burtonii* whole plant or its active ingredient cerevisterol as potential agents against *Salmonella* Typhi ATCC6539 infections including typhoid fevers.

Medicinal plant extracts as well as the isolated compounds are known to possess different mechanisms of action against pathogenic bacteria [10, 37]. In this study, the mechanisms of action of cerevisterol where studied on *Salmonella* Typhi ATCC6539 lysis. The treatment of *Salmonella* Typhi(ATCC6539) with cerevisterol resulted in cell lysis after 4 hours of treatment. This suggest that it mechanism of action may be associated with cell wall and membrane damage. In fact, many isolated compounds such carvacrol and 1,8-Cineole were reported to damage the bacteria cell membrane [26].

To determine how quickly cerevisterol acts on *Salmonella* Typhi(ATCC6539) time-kill study was performed. Time-kill study is defined as the rate of killing by a fixed concentration of an antimicrobial agent and is one of the methods for determining tolerance. Cerevisterol at concentrations of 1/2xMIC, MIC, and 2xMIC(MBC) demonstrated a ≥ 4 log10-cfu killing (93.9 %) at 4, 8, and 12 h, respectively. This indicates that cerevisterol was bacteriostatic. Previously, Nirwaan and co-authors [38] also reported the bacteriostatic effect of compounds isolated from the leaves of *Melastoma malabathricum* on S. Typhi*,S. aureus* and *E. coli*. It is known that it is advantageous to have drugs with bacteriostatic effect rather than the bactericidal action depending on the disease status [39]. Thus, cerevisterol with bacteriostatic effect can have a clinical value.

"The intracellular compartment is made up of nucleic acids and derivatives, but also proteins, which are the main cellular components. An increased absorption at 260 nm in the extracellular medium indicates the presence of nucleic acids or derivatives, and consequently, reflects a loss in membrane integrity" [39, 40]. Our results showed a concentration-dependent increase of the absorbance at 260 nm in cerevisterol treated bacteria compared to control, suggesting the release of nucleic acids which is intracellular materials out of the bacteria. Moreover, this observation further suggests the contribution of cerevisterol to the alteration of the *Salmonella* Typhi ATCC6539 membrane leading toits death [41]. "The ability of cerevisterolto alter the bacterial cell membrane through the leakage of cytoplasmic materials such as sugar, proteins, and nucleic acids was further demonstrated"[42].

"Plasma membrane H+-ATPases (H+-pumps) are the primary active transporters that translocate protons to the outside of each cell, providing the electrical and chemical energy that drives solute transport" [33]. "Thus, any inhibition of these pumps equally inhibits the growth of the bacteria [34]. In, this study, cerevisterol at 2×MIC showed a high activity. This suggests that inhibition of the plasma membrane proton pump may be one of the modes of action of cerevisterol against *Salmonella Typhi* ATCC6539" [43].

"Many pathogens are known to produce catalase enzymes to protect themselves from hydrogen peroxide, a defense mechanism commonly used by the host's immunity" [29]. "Previous studies have reported that catalase-deficient mutant pathogens are more sensitive to oxidative stress and attack by the host immune system" [44,45,46]. "Our investigation on the effect of cerevisterol on catalase activity in *Salmonella* Typhi (ATCC6539)has provided a deeper insight into the inherent mechanisms of bacterial pathogenicity and resistance to oxidative stress. It was observed that in *Salmonella* Typhi(ATCC6539), cerevisterol-treated samples displayed a 60% decrease in catalase activity was observed. It could be anticipated that the decrease in enzymatic activity may be due to the presence of phytochemicals such as thymol, carvacrol, and other phenolic acids which are known to possess antibacterial properties and to inactivate bacterial enzyme systems as one of their potential modes of action" [47,48].

## 5. CONCLUSIONS

In this work, the hydroethanolic crude extract of *Impatiens burtonii* revealed significant antibacterial activity against MDR of *Salmonella*. Cerevisterol isolated from the main active fraction showed impressive activity against the tested bacteria. The action of cerevisterol may be associated to cell wall and membrane damage. Overall, cerevisterol may have potential to be used as therapeutic agent against *Salmonella* Typhi ATCC6539 infections including typhoid fevers.

## DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding authors upon reasonable request.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

## ACKNOWLEDGEMENTS

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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## SUPPLEMENTARY

**Fig S1-S5**: Spectroscopic data ($^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$H COSY, HSQC, and HMBC) for cerevisterol (**1**) (**S**upplementary file).

**Spectroscopic data ($^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$H COSY, HSQC, and HMBC) for cerevisterol Cerevisterol (1)**: 1H-NMR (600 MHz, DMSO-d6): δ 5.33 (d, J = 5.4 Hz, H-7), 5.20 (dd, J = 7.2, 15 Hz, H-23), 5.16 (dd, J = 7.2, 15 Hz, H-22), 4.06 (m, H-3), 3.60 (m, H-6), 1.07 (s, H-19), 1.00 (d, J = 6.6 Hz, H-21), 0.90 (d, J = 7.2 Hz, H-28), 0.82 (d, J = 6.6 Hz, H-27), 0.80 (d, J = 6.6 Hz, H-26), 0.58 (s, H-18); 13C-NMR (150 MHz, DMSO-d6): δ 144.0 (C-8), 135.4 (C-22), 132.2 (C-23), 117.5 (C-7), 76.0 (C-5), 73.7 (C-6), 67.7 (C-3), 56.0 (C-17), 54.8 (C-14), 43.8 (C-13), 43.5 (C-9), 42.8 (C-24), 40.4 (C-28), 39.2 (C-4), 39.2 (C-12), 37.1 (C-10), 33.1 (C-25), 33.0 (C-1), 30.9 (C-2), 27.9 (C-16), 22.9 (C-15), 22.1 (C-11), 21.1 (C-27), 19.9 (C-26), 19.6 (C-21), 18.8 (C-19), 17.6 (C-28), 12.33 (C-18).



**Fig. S1. $^1$H NMR spectrum of compound 1 (DMSO-*d6*, 600 MHz)**

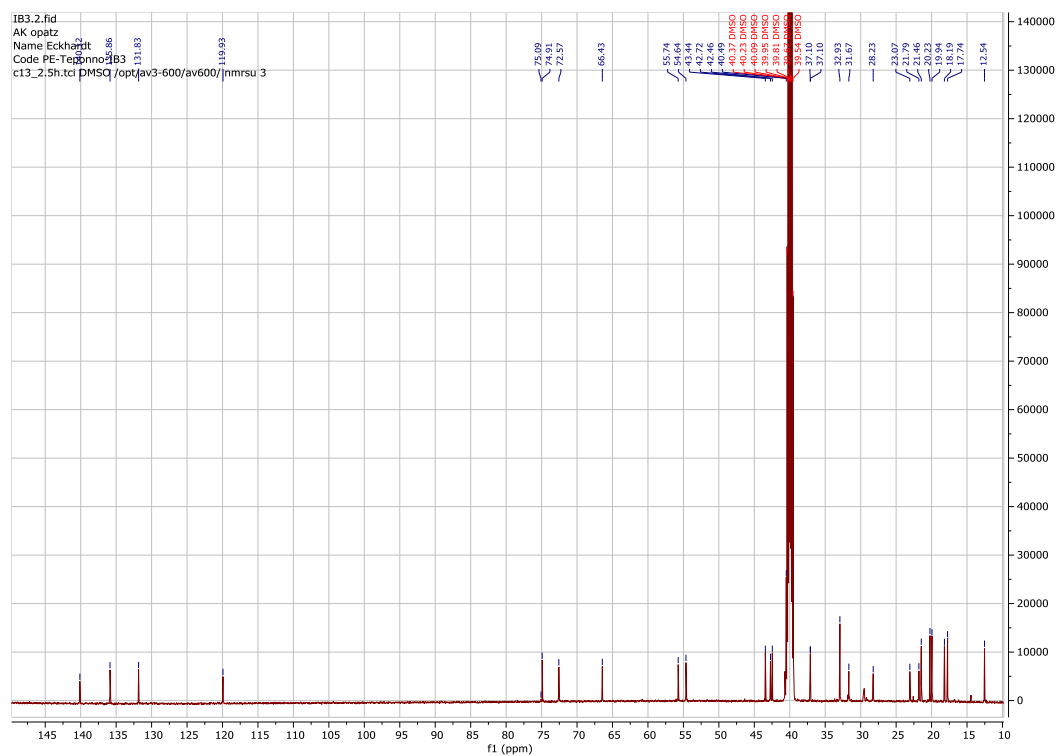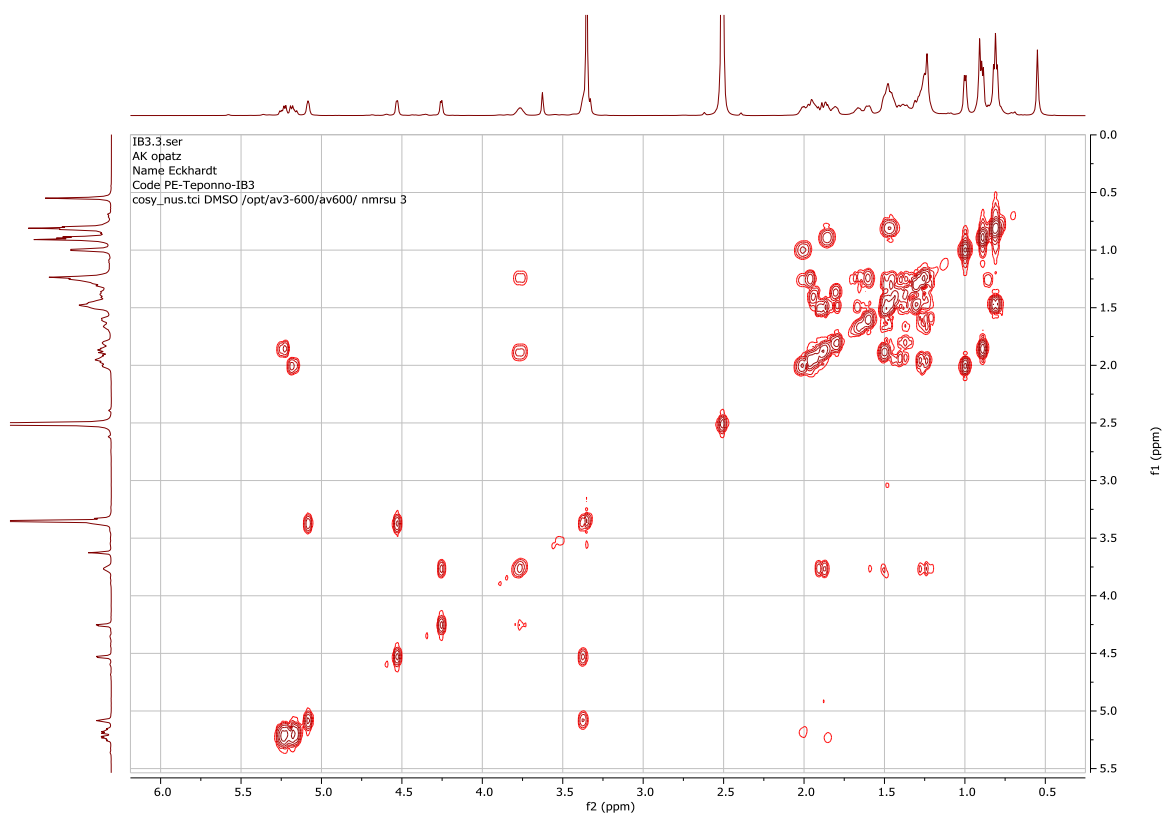**Fig. S2. ¹³C NMR spectrum of compound 1 (DMSO-*d6*, 150 MHz)**



**Fig. S3. ¹H-¹H COSY spectrum of compound 1 (DMSO-*d6*)**
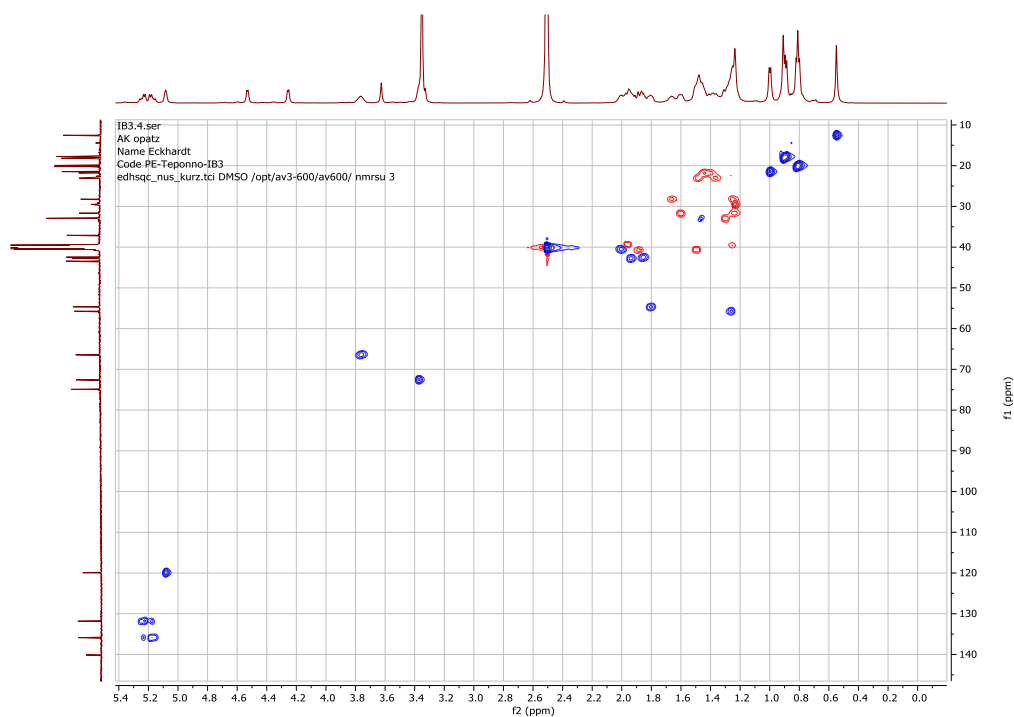
**Fig. S4. HSQC spectrum of compound 1 (DMSO-*d6*)**



**Fig. S5. HMBC spectrum of compound 1 (DMSO-*d6*)**