Full Paper

Synthesis and Antimicrobial Activities of a Novel Series of Heterocyclic α-Aminophosphonates

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Two series of novel α-aminophosphonates having heterocyclic moieties were synthesized in high yields. The structures of the newly synthesized compounds were confirmed by their elemental analyses, IR, ^{1}H NMR and MS spectral data. These compounds were screened for their antibacterial activities against *Escherichia coli* (NCIM2065) as a Gram-negative bacterium, *Bacillus subtilis* (PC1219) and *Staphylococcus aureus* (ATCC25292) as Gram-positive bacteria, and *Candida albicans* and *Saccharomyces cerevisiae* as fungi. The minimum inhibitory concentrations (MICs) of the synthesized compounds show high antibacterial and antifungal activities at low concentrations (10–1000 μg/mL). Furthermore, their lethal doses indicated that such compounds are safe for use as antimicrobial agents.

Keywords: α-Aminophosphonates / 2-Amino-4-chloro-6-methylpyrimidine / 3-Aminoquinazolin-4(3*H*)-one / Antimicrobial activities / Lethal dose

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Introduction

α-Aminophosphonates are among the most common and biologically active organophosphorus compounds [1–3]. α-Aminophosphonates and in particular the ones having heterocyclic moieties show very interesting biological activities and have been used as peptide mimics, inhibitors of serine hydrolase, enzyme inhibitors, antibiotics, antibacterial, antifungal, anticancer, anti-HIV and herbicidal agents [4–20].

Various synthetic processes have been developed for the production of α-aminophosphonates [21–27]. However, the most efficient method involves the one-pot Mannich-type [28] process of carbonyl compounds, amines and diphenyl phosphite in the presence of a Lewis acid catalyst. Such process is simple, general, high yielding and accommodates various substituents into aminophosphonates [29–32].

We have previously reported efficient syntheses of various biologically active heterocyclic compounds [33–40] as part of our continuing interest in organic synthesis. Recently, we have reported the successful synthesis, antimicrobial and anticancer activities of a novel series of α-aminophosphonates [41]. The present work was aimed to synthesize novel α-aminophosphonates containing quinazolin-4(3*H*)-one and pyrimidine moieties with the hope that new antimicrobial agents could be developed. We now report the successful synthesis of a range of α-aminophosphonates and their antimicrobial activities against Gram-negative and Gram-positive bacteria and fungi.

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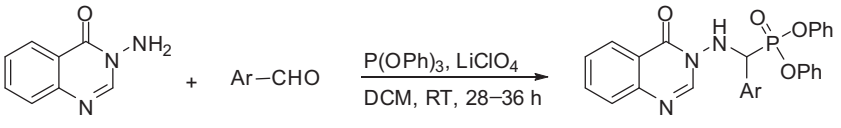
Results and discussion

Chemistry

Two series α-aminophosphonates were synthesized in which heterocyclic amines namely 3-aminoquinazolin-4(3*H*)-one (**1**) and 2-amino-4-chloro-6-methyl pyrimidine (**2**) were used. Reactions of 3-aminoquinazolin-4(3*H*)-one (**1**; 4 molar equivalents) with various aromatic aldehydes namely 2,4-dihydroxybenzaldehyde, 4-chlorobenzaldehyde and 4-nitrobenzaldehyde (2 molar equivalents) and triphenylphosphite (3 molar equivalents) in the presence of lithium perchlorate as a Lewis acid catalyst in dichloromethane (DCM) at room temperature for 28–36 h gave the corresponding diphenyl (aryl)(4-oxoquinazolin-3(4*H*)-ylamino)methylphosphonates **3**–**5** (Scheme 1) in 70–83% yields after crystallization from ethanol.



1 Ar = 2,4-di-HOC_6H_3, 4-ClC_6H_4, 3-$NO_2C_6H_4$ **3–5**

Scheme 1. Synthesis of diphenyl (aryl)(4-oxoquinazolin-3(4*H*)-ylamino)methylphosphonates **3–5**.

The structures of α-aminophosphonates **3–5** were confirmed by various spectroscopic techniques including IR, ^{1}H NMR and mass spectral data and their purities were confirmed by elemental analyses. The IR spectra of **3–5** are characterized by the presence of absorption bands within the 3471–3399 cm^{-1} region corresponding to the stretching vibrations of the NH and/or OH groups. The absorption bands in the 1673–1659 cm^{-1} region are due to the symmetric stretching vibrations of the carbonyl groups, while the C=N groups absorbed within the 1598–1515 cm^{-1} region. The bands in the 1396–1363 cm^{-1} and 834–824 cm^{-1} regions are due to the stretching vibrations of the P=O and P–O–C groups, respectively. The ^{1}H NMR spectra showed a characteristic CH doublet signal (J = 16–18 Hz) within the δ = 7.60–5.59 ppm region. They also showed an exchangeable singlet signal that resonated in the δ = 10.56–9.45 ppm region due to the NH protons. The structures of **3–5** were confirmed further by mass spectral data. The electron impact and the electrospray mass spectra of **3–5** indicated the presence of molecular ion or pseudo molecular ion peaks ($[M-H]^+$). Moreover, the elemental analyses of **3–5** were consistent with the suggested structures (see Experimental Section for details).

Similarly, it was found that reactions of 2-amino-4-chloro-6-methyl pyrimidine (**2**) with aromatic aldehydes (2,4-dihydroxybenzaldehyde, 4-chlorobenzaldehyde and 4-nitrobenzaldehyde) and triphenylphosphite in the presence of lithium perchlorate in DCM under conditions similar to those used in Scheme 1 gave the corresponding diphenyl (aryl)(4-chloro-6-methylpyrimidin-2-ylamino)methylphosphonates **6–8** (Scheme 2) in 69–86% yield.

The structures of α-aminophosphonates **6–8** were confirmed by IR, ^{1}H NMR and mass spectral data. The purity of products was confirmed by their elemental analyses (see Experimental Section for details).

Antimicrobial activities

The antimicrobial agents available on the market have various drawbacks such as toxicity, narrow spectrum of activity and some also exhibit drug–drug interactions. In view of the high incidence of infections in immune compromised patients, demands for new antimicrobial agents with a broad spectrum of activity and good pharmacokinetic properties have increased [42].

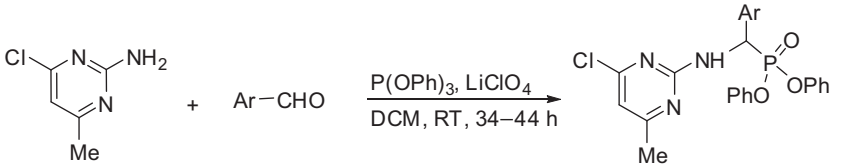
α-Aminophosphonates **3–8** along with their starting materials **1** and **2** were screened for their *in vitro* antibacterial and antifungal activities against *Escherichia coli* (NCIM2065) as a Gram-negative bacterium, *Bacillus subtilis* (PC1219) and *Staphylococcus aureus* (ATCC25292) as Gram-positive bacteria and *Candida albicans* and *Saccharomyces cerevisiae* as fungi. The inhibition zones were measured in triplicates and the results are reported in Table 1.

The results show that compounds **3–5** showed moderate antimicrobial activities except for *B. subtilis*. Compounds **6–8** showed high antimicrobial activities against all the tested organisms. Also, it is clear that α-aminophosphonates **3–8** are more active than the corresponding starting materials **1** and **2**.

Compounds **1–8** showed less antibacterial activities compared to the standard drug (ciprofloxacin; MIC = 5 μg/mL). On the other hand, compounds **3**, **6** and **8** showed high antifungal activities against *Candida albicans* compared to the standard drug (amphotericin B; MIC = 15 μg/mL). Also, compounds **4**, **6** and **8** showed high antifungal activities against *S. cerevisiae* compared to amphotericin B.

Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of compounds **1** and **2** and the newly synthesized α-aminophosphonates **3–8** were determined for each antimicrobial agent by using the agar diffusion method. The inhibition



2 Ar = 2,4-di-HOC_6H_3, 4-ClC_6H_4, 3-$NO_2C_6H_4$ **6–8**

Scheme 2. Synthesis of diphenyl (aryl)(4-chloro-6-methylpyrimidin-2-ylamino)methylphosphonates **6–8**.

Table 1. Antimicrobial activities of compounds **1–8**.[a)]

Compound	Inhibition zone diameter (mm)[b)]					LD_{50} (μg/mL)
	Bacteria			Fungi		
	E. coli	*S. aureus*	*B. subtilis*	*C. albicans*	*S. cerevisiae*	
1	6 ± 1.1	10 ± 1.0	–	8 ± 0.8	10 ± 1.0	38 ± 3
2	8 ± 0.5	10 ± 1.0	8 ± 0.5	7 ± 1.0	10 ± 1.0	175 ± 12
3	20 ± 1.5	21 ± 3.0	–	14 ± 3.0	16 ± 0.2	2045 ± 205
4	10 ± 1.0	20 ± 1.9	–	12 ± 1.5	17 ± 1.0	175 ± 13
5	7 ± 1.0	18 ± 2.5	–	10 ± 1.1	14 ± 2.3	100 ± 8
6	20 ± 3.0	25 ± 4.0	10 ± 1.2	15 ± 2.0	22 ± 2.2	843 ± 76
7	10 ± 1.0	17 ± 2.0	9 ± 0.8	10 ± 0.9	14 ± 1.5	7841 ± 405
8	10 ± 1.2	18 ± 1.6	10 ± 1.7	17 ± 1.2	20 ± 1.9	4688 ± 229

[a)] The antimicrobial activities were measured at 10 μg/mL concentration.
[b)] DMSO was added to different organisms as control and showed no inhibition zone.

Table 2. Minimum inhibitory concentrations of compounds **1–8**.[a)]

Compd	Minimum inhibitory concentrations (MICs; μg/mL)					
	E. coli	*S. aureus*	*B. subtilis*	*C. albicans*	*S. cerevisiae*	Mean MICs (μg/mL)
1	1000 ± 98	1000 ± 98	1000 ± 98	1000 ± 100	1000 ± 100	1000 ± 98.8
2	1000 ± 101	100 ± 12	1000 ± 101	1000 ± 100	100 ± 14	640 ± 65.6
3	10 ± 1.2	10 ± 1.1	1000 ± 100	10 ± 1.0	50 ± 4.5	216 ± 21.5
4	100 ± 11	50 ± 5.0	1000 ± 100	100 ± 2.0	10 ± 1.0	252 ± 23.8
5	1000 ± 101	1000 ± 101	1000 ± 102	100 ± 1.0	1000 ± 98	820 ± 80.6
6	10 ± 1.0	10 ± 1.0	10 ± 1.0	10 ± 1.5	10 ± 2.0	10 ± 1.3
6	100 ± 10	50 ± 4.0	1000 ± 97	100 ± 9.0	50 ± 5.0	260 ± 25.0
8	100 ± 9.0	10 ± 0.7	100 ± 0.9	10 ± 1.2	10 ± 1.0	46 ± 2.6

[a)] The standard antibiotics were ciprofloxacin for bacteria (MIC = 5 μg/mL) and amphotericin B for fungi (MIC = 15 μg/mL).

zone was measured in triplicates in four different concentrations (10–1000 μg/mL) and the mean value ± standard deviation (SD) [43] is recorded in Table 2.

Table 2 shows that all compounds exhibited high antimicrobial activities at a concentration of 10–1000 μg/mL for all microorganisms. Compound **3** was found to be the most effective within the first series of α-aminophosphonates **3–5** in which the order of activity was as follows: **3** > **4** > **5**. For the second series (*i.e.*, compounds **6–8**) compound **6** was the most effective compound and the order of activity within this series was as follows: **6** > **8** > **7**.

The lethal dose

Cytotoxic anticancer substances have unique problems that come primarily from the lack of safety and side effects. Therefore, the cytotoxicity lethal doses (LD_{50}) of compounds **1** and **2** and the newly synthesized α-aminophosphonates **3–8** were determined on the larvae of *Artemia salina* using the brine shrimp lethality bioassay. The LD_{50} of compounds **1–8** are shown in Table 1.

It was found that the number of living *Artemia* was decreased by increasing the concentration of **1–8**. The lethal doses of the starting materials **1** and **2** were low (38 and 175 μg/mL, respectively).

Compound **3** has no effect on *Artemia* at low concentrations (10 and 100 μg/mL) and killed up to 85% of *Artemia* at a higher concentration (1000 μg/mL). Compounds **4** and **5** showed no effect on *Artemia* at lower concentration (10 μg/mL) and the number of living *Artemia* was reduced to 50–55% at a higher concentration (100 μg/mL). At an even higher concentration (1000 μg/mL) all *Artemia* were killed. Compound **3** was found to be the safest compound within the first series of α-aminophosphonates **3–5** in which the lethal dose was 2045 μg/mL. The lethal doses for compounds **4** and **5** were found to be 175 and 100 μg/mL, respectively.

Compounds **6**–**8** have no effect on *Artemia* at low concentrations (10 and 100 μg/mL). At a high concentration (1000 μg/mL), compound **6** reduced the living *Artemia* to 25%, while compounds **7** and **8** reduced the living *Artemia* to 35 and 15%, respectively. α-Aminophosphonates **6**–**8** were found to be very safe to be used since their lethal doses were high (843, 7841 and 4688 μg/mL, respectively).

Conclusion

We developed a convenient process for the synthesis of various α-aminophosphonates having quinazolin-4(3*H*)-one and pyrimidine moieties in high yields. The antimicrobial activities of the newly synthesized compounds show high activities against Gram-positive, Gram-negative bacteria and fungi at low concentrations. Their lethal doses indicated that the synthesized compounds are safe and are promising for their use as *in vivo* antimicrobial agents.

Experimental

General experimental

Melting point determinations were performed by the open capillary method using an Electrothermal MEL-TEMP II apparatus and are reported uncorrected. IR spectra were recorded on a Perkin-Elmer 1430 spectrophotometer using the KBr disc technique. ^{1}H NMR spectra were recorded on a Bruker AC400 spectrometer operating at 400 MHz. The spectra were recorded in DMSO-d_6. Chemical shifts δ are reported in parts per million (ppm) relative to TMS. Assignments of signals are based on integration values and expected chemical shift values and have not been rigorously confirmed. EI mass spectra were recorded at energy 70 eV with a 7070 EQ mass spectrometer. Electrospray (ES) analyses were performed on a ZQ4000 spectrometer in both positive and negative ionization modes. Microanalysis was performed by analytical service at both the Universities of Tanta and Cairo, Egypt. Analytical thin layer chromatography (TLC) was performed on EM silica gel F_{254} sheets (0.2 mm) with petroleum ether (40–60°C)/acetone (5:2 by volume) as a developing eluent. The spots were detected with a UV lamp model UV GL-58. Reagents and solvents were obtained from commercial sources and used without purification.

3-Aminoquinazoline-4(3*H*)-one (**1**) was prepared according to the literature procedure, mp 208–210°C (lit. 209–210°C) [44]. 2-Amino-4-chloro-6-methylpyrimidine (**2**) was purchased from Aldrich Chemical Company; mp of 184–186°C.

Chemistry

*General procedure for the synthesis of α-aminophosphonates (**3**–**8**)*

A mixture of **1** or **2** (4.0 mmol) and a DCM solution of $LiClO_4$ (3 mL, 5.0 M; 15.0 mmol) in DCM (10 mL) was stirred for 2 min and the aromatic aldehyde (2.0 mmol) was then added. After 10 min, triphenylphosphite (0.93 g, 3.0 mmol) was added and the mixture was stirred at room temperature for 28–44 h. Water (10 mL) was added and the organic phase was separated and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to give the crude product which was recrystallized from ethanol to give the pure products **3**–**8** in high yields as white solids.

*Diphenyl (2,4-dihydroxyphenyl)(4-oxoquinazolin-3(4H)-ylamino)methylphosphonate (**3**)*

Reaction time: 28 h, yield: 83%; mp: 240–242°C; IR (KBr): 3471 (NH/OH), 1659 (C=O), 1515 (C=N), 1363 (P=O), 834 (P–O–C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 10.56 (br s, exch., 1H, NH), 9.14 (s, exch., 1H, OH), 9.01 (s, exch., 1H, OH), 8.54–7.60 (m, 18 H, Ar–H), 6.41 (d, J = 17 Hz, 1H, CH); EI-MS: m/z (%) 514 ([M−H]$^+$, 25), 430 (19), 146 (59), 76 (100); Anal. Calcd. for $C_{27}H_{22}N_3O_6P$ (515.45): C, 62.91; H, 4.30; N, 8.15; P, 6.01. Found: C, 62.92; H, 4.32; N, 8.19; P, 6.05.

*Diphenyl (4-chlorophenyl)(4-oxoquinazolin-3(4H)-ylamino)methylphosphonate (**4**)*

Reaction time: 30 h, yield: 75%; mp: 170–172°C; IR (KBr): 3424 (NH), 1672 (C=O), 1595 (C=N), 1396 (P=O), 825 (P–O–C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.45 (br s, exch., 1H, NH), 8.65–7.74 (m, 19H, Ar–H), 7.60 (d, J = 16 Hz, 1H, CH); ES$^+$–MS: m/z (%) 519 ([M^{37}Cl]$^+$, 2), 517 ([M^{35}Cl]$^+$, 6), 376 (100), 339 (16), 249 (7), 237 (26), 232 (13), 229 (11); Anal. Calcd. for $C_{27}H_{21}ClN_3O_4P$ (517.90): C, 62.62; H, 5.09; N, 8.11; P, 5.98. Found: C, 62.72; H, 4.87; N, 8.15; P, 5.95.

*Diphenyl (3-nitrophenyl)(4-oxoquinazolin-3(4H)-ylamino)methylphosphonate (**5**)*

Reaction time: 36 h, yield: 70%; mp: 190–192°C; IR (KBr): 3399 (NH), 1673 (C=O), 1526 (C=N), 1393 (P=O), 824 (P–O–C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.61 (br s, exch., 1H, NH), 8.65–7.53 (m, 19H, Ar–H), 5.92 (d, J = 16 Hz, 1H, CH); EI–MS: m/z (%) 528 (M$^+$, 2), 493 (11), 419 (23), 345 (72), 270 (88), 197 (100) and 150 (92); ES$^+$–MS: m/z (%) 528 (M$^+$, 52), 474 (12), 415 (16), 403 (13), 295 (9), 243 (15), 202 (100), 188 (17), 147 (16); Anal. Calcd. for $C_{27}H_{21}N_4O_6P$ (528.45): C, 61.37; H, 4.01; N, 10.60; P, 5.86. Found: C, 61.35; H, 4.03; N, 10.63; P, 5.85.

*Diphenyl (4-chloro-6-methylpyrimidin-2-ylamino)-(2,4-dihydroxyphenyl)methylphosphonate (**6**)*

Reaction time: 34 h, yield: 86%; mp: 280–282°C; IR (KBr): 3369 (NH/OH), 1596 (C=N), 1397 (P=O), 761 (P–O–C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 9.92 (s, exch., 1H, OH), 9.59 (s, exch., 1H, OH), 7.46–6.13 (m, 14H, Ar–H), 5.91 (br s, 1H, NH), 5.59 (d, J = 18 Hz, 1H, CH), 2.23 (s, 3H, CH_3); EI–MS: m/z (%) 498 ([M^{37}Cl−H]$^+$, 2), 496 ([M^{35}Cl−H]$^+$, 6), 247 (6), 211 (46), 119 (31), 94 (43), 76 (100); Anal. Calcd. for $C_{24}H_{21}ClN_3O_5P$ (497.87): C, 57.90; H, 4.25; N, 8.44; P, 6.22. Found: C, 57.92; H, 4.22; N, 8.45; P, 6.24.

*Diphenyl (4-chloro-6-methylpyrimidin-2-ylamino)-(4-chlorophenyl)methylphosphonate (**7**)*

Reaction time: 38 h, yield: 78%; mp: 155–157°C; IR (KBr): 3369 (NH), 1596 (C=N), 1397 (P=O), 827 (P–O–C) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.82–7.03 (m, 15H, Ar–H), 6.84 (br s, exch., 1H, NH), 6.24 (d, J = 16 Hz, 1H, CH), 2.32 (s, 3H, CH_3); ES$^+$–MS: m/z (%) 567 ([M^{37}Cl$_2$+MeCNNa]$^+$, 23), 565 ([M^{37}Cl^{35}Cl+MeCNNa]$^+$, 70), 563 ([M^{35}Cl$_2$+MeCNNa]$^+$, 100), 526 ([M^{37}Cl$_2$+Na]$^+$, 18), 524 ([M^{37}Cl^{35}Cl+Na]$^+$, 35), 522 ([M^{35}Cl$_2$+Na]$^+$, 20), 504 ([M^{37}Cl$_2$]$^+$, 5), 502 ([M^{37}Cl^{35}Cl]$^+$, 12), 500 ([M^{35}Cl$_2$]$^+$, 15), 160 (35), 145 (37), 112 (7); Anal. Calcd. for $C_{24}H_{20}Cl_2N_3O_3P$ (500.31): C, 57.62; H, 4.03; N, 8.40; P, 6.19. Found: C, 57.63; H, 4.06; N, 8.42; P, 6.22.

*Diphenyl (4-chloro-6-methylpyrimidin-2-ylamino)-(3-nitrophenyl)methylphosphonate (**8**)*

Reaction time: 44 h, yield: 69%; mp: 121–123°C; IR (KBr): 3271 (NH), 1568 (C=N), 1352 (P=O) and 825 (P–O–C) cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.75–6.98 (m, 15H, Ar–H), 6.74 (br s, exch., 1H, NH), 6.04 (d, $J = 15$ Hz, 1H, CH), 2.49 (s, 3H, CH_3); ES^-–MS: *m*/*z* (%) 549 ($[M^{37}Cl+^{37}Cl]^-$, 11), 547 ($[M^{37}Cl+^{35}Cl$ or $[M^{35}Cl+^{37}Cl]^-$, 52), 545 ($[M^{35}Cl+^{35}Cl]^-$, 75), 511 ($[M^{37}Cl-H]^-$, 5), 509 ($[M^{35}Cl-H]^-$, 18), 491 (21), 433 (37), 397 (53), 339 (19), 257 (100), 233 (40); Anal. Calcd. for $C_{24}H_{20}ClN_4O_5P$ (510.87): C, 56.43; H, 3.95; N, 10.97; P, 6.06. Found: C, 55.91; H, 3.67; N, 10.67; P, 6.05.

Antimicrobial activities

Tested microorganisms

Gram-negative bacteria

After the Gram-staining procedure, Gram-negative cells appear pink. The Gram-negative bacterium used in this study was *E. coli* which is known as the backbone example for Gram-negative bacteria and to cause urinary infection, wound infection and gastroenteritis.

Gram-positive bacteria

The thick cell wall of a Gram-positive organism retains the crystal violet dye used in the Gram-staining procedure, so the stained cells appear purple under magnification. Gram-positive bacteria used in this study were *B. subtilis* and *S. aureus*. *B. subtilis* are mostly involved in urinary infection, wound, ulceration and septicemia. *S. aureus* is the milestone of Gram-positive bacteria and it is a causative agent of pneumonia, meningitis and food poisoning.

Fungi

Pathogenic fungi especially yeasts are responsible for a number of diseases in human and animals. A number of pathogenic strains of fungi are represented in *C. albicans* and *S. cerevisiae*. The tested organisms were obtained from the culture collection of Bacteriology Unit, Department of Botany, Faculty of Science, Tanta University, Egypt.

Media used and antimicrobial assay

Nutrient and Sabouraud's broths, nutrient and Sabouraud's agar were used for growing and maintaining the tested bacteria and yeast, respectively. The antimicrobial spectrum of the synthesized compounds was determined as powdered samples by the cut-plug method on plates seeded with the tested bacteria (*E. coli*, *B. subtilis* and *S. aureus*) on nutrient agar, which contained per liter: peptone (3 g), beef extract (5 g), NaCl (5 g) and agar (20 g) at pH 7. The test was also performed on plates seeded with *C. albicans* and *S. cerevisiae* on Sabaraud's agar that contained per liter: glucose (40 g), peptone (10 g) and agar (20 g) at pH 6.0. After solidification, the wells were made and each was filled with powdery compounds (10 mg). The plates were then incubated at 30°C for 24–48 h, after which the diameters of the inhibition zones were measured. Compounds which produced the highest inhibition zones were selected and assayed further at different concentrations in suspensions to quantify their inhibitory effects. Nutrient and Sabouraud's broths were used in activation of organisms [45].

Determination of minimum inhibitory concentrations (MICs)

The MIC was determined by agar diffusion assay using the filter paper disc method. The MICs were determined for the synthesized compounds against *E. coli*, *S. aureus* and *B. subtilis* as bacteria and *C. albicans* and *S. cerevisiae* as yeasts. It was carried out by impregnation of different concentrations of synthesized compounds (0, 10, 50, 100 and 1000 μg/mL) in DMSO as a solvent and then placed on filter paper discs of the same diameter (5 mm). The agar plate dilution method was used to inoculate the bacteria and yeasts used in the plate. Nutrient agar medium was seeded with 100 μL of inoculum size (5×10^5 for bacteria and 4×10^4 for yeasts). The impregnated discs containing the tested samples of different concentrations were placed on the agar medium seeded with tested microorganisms. Standard antibiotic discs (ciproflaxcicin, 5 μg/mL for bacteria and 15 μg/mL amphotericin B for yeasts) and blank discs (impregnated with DMSO) were used as positive and negative control. The plates were then incubated at 37°C for 24–48 h to allow maximum growth of the microorganisms.

The lethal dose

Brine shrimps lethality bioassay is a very simple bench-top assay used to measure the cytotoxicity of plant extracts as well as of synthesized compounds. Brine shrimp eggs are available commercially and are used as fish food. Different concentrations of each compound (10, 100 and 1000 μg/mL) were suspended in 5 mL vials containing saline solution and 20 shrimps. Three replicates were used for each concentration and living larvae were counted after 72 h. All data were expressed as mean ± SD [46].

The authors have declared no conflict of interest.

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