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Synthesis, antimicrobial and anticancer activities of a novel series of diphenyl 1-(pyridin-3-yl)ethylphosphonates

Mohamed F. Abdel-Megeed [a], Badr E. Badr [b], Mohamed M. Azaam [a], Gamal A. El-Hiti [a,c,*]

[a] Department of Chemistry, Faculty of Science, Tanta University, Tanta 31527, Egypt
[b] Department of Botany, Faculty of Science, Tanta University, Tanta 31527, Egypt
[c] School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, UK

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ABSTRACT

A novel series of diphenyl 1-(arylamino)-1-(pyridin-3-yl)ethylphosphonates **1–5** was obtained in high yields from reactions of 3-acetyl pyridine with aromatic amines and triphenylphosphite in the presence of lithium perchlorate as a catalyst. The structures of the synthesized compounds were confirmed by IR, ^{1}H NMR spectral data and microanalyses. Compounds **1–5** showed high antimicrobial 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, at low concentrations (10–100 μg/mL). Also, the synthesized compounds showed significant cytotoxicity anticancer activities against liver carcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF7). The lethal dose of the synthesized compounds was also determined and indicated that most compounds are safe to use.

1. Introduction

α-Aminophosphonic acids[1] have been found to exhibit a wide range of biological activities.[2,3] A large volume of research on their synthesis and biological activities has been reported during the last 20 years. A considerable number of α-aminophosphonate derivatives are known to be antiviral, antibacterial, antifungal, antimicrobial and anticancer.[4–15] Because of the increase of contamination and microbial infection by microorganisms and cancers diseases in the last few years there has been a great concern in various areas for producing new antimicrobial and anticancer safe agents. The microbial infection and cancer diseases are global problems because the cancer cells tend to resist anticancer treatments. This resistance comes from change in membrane permeability, lyses of used substances by lyses enzymes, neutralization of active groups and change in agent receptors. Antimicrobials agents attract both academics and industry due to their potential to provide a high quality, good yield and safety benefits against different diseases. Depending on the type of applications,[16] the safety of products is the major problem. In the early twentieth century, the revolutionary is a magic concept introduced by the pioneering work of Ehrlich highlighted the potential of targeted toxicity which would allow the lethal delivery of cytotoxic agents within malignant cells.[17] Since then, the design of drugs and prodrugs directed towards specific toxicity expanded by progressively understanding the unique features of cancer cells.[18] The target and safety of new products are the most common problems.

In continuation of our own interest in the synthesis of substituted aromatics[19–22] we have demonstrated efficient syntheses and/or ring system modification of various heterocycles including 3*H*-quinazolin-4-ones, quinoxalines, isoindolines, isoquinolines, isatins and acridinium ester.[23–35] It was of interest to see if α-aminophosphonates having a pyridine moiety could be produced efficiently. Therefore, we decided to attempt synthesis of various α-aminophosphonates in which different aromatic amines were used and investigate their antimicrobial and anticancer activities.

The present work was aimed to synthesize new α-aminophosphonates containing pyridine moiety with the hope that new anticancer agents could be developed. The general method for the synthesis of α-aminophosphonates involves a three components reaction of carbonyl compounds, amine and dialkyl phosphite in an organic solvent via one pot Mannich-type process.[36] We now report the successful synthesis of a range of diphenyl 1-(arylamino)-1-(pyridin-3-yl)ethylphosphonates and their antimicrobial activities against Gram-negative and Gram-positive bacteria and fungi and the in vitro anticancer activities against liver carcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF7).

* Corresponding author. Tel.: +44 2920870600.
E-mail addresses: el-hitiga@cardiff.ac.uk, gelhiti@yahoo.co.uk (G.A. El-Hiti).

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2. Chemistry

2.1. Synthesis of diphenyl 1-(pyridin-3-yl)ethylphosphonates 1–5

As a test case, the reaction of 3-acetylpyridine with aniline and triphenylphosphite in the presence of lithium perchlorate as a catalyst in dichloromethane (DCM) at room temperature was investigated (Scheme 1; Ar = Ph). When aniline (4 M equiv) was added a mixture of 3-acetylpyridine (2 M equiv) and lithium perchlorate (15 M equiv) followed by the addition of triphenylphosphite (3 M equiv) in DCM at room temperature for 24 h, the yield of **1** was 73% (Table 1) after crystallization from ethanol.

It was of interest to see if the reaction with other arylamines would be useful and general. Consequently, reactions of various arylamines, 3-acetylpyridine and triphenylphosphite in the presence of lithium perchlorate were carried out. Each reaction was conducted under identical conditions to that used for the production of **1**. The crude products were purified by crystallization from ethanol give α-aminophosphonates **1**–**5** (Scheme 1) in high yields (Table 1).

Table 1 indicated that the reaction represented in Scheme 1 is general, simple, high yielding, involves easy work-up and accommodates various substituents to produce various α-aminophosphonates.

The structures of α-aminophosphonates **1–5** were confirmed by IR, ^{1}H NMR spectroscopy and elemental analyses. The IR spectra of **1–5** are characterized by the presence of absorption bands within the 3387–3420 cm^{-1} region corresponding to the stretching vibrations of the NH groups. The bands within the 1591–1629 cm^{-1} region are due to the stretching vibration of the C=N groups of the pyridine moiety. While The absorption bands at the 1229–1287 cm^{-1} region are due to the symmetric stretching vibrations of the P=O groups and absorption bands within the 810–905 cm^{-1} region are attributed to the P–O–C groups.

The ^{1}H NMR spectra of α-aminophosphonates **1–5** showed a characteristic exchangeable singlet within the 9.52–6.94 ppm region due to NH proton. The CH_3 protons resonated as singlet within the 2.21–2.03 ppm region. The elemental analyses of **1–5** were consistent with the suggested structures. The structure of compound **5** was confirmed further by EI-mass spectrum and showed a molecular ion peak at m/z = 445.

3. Biology

3.1. 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.[37]

The synthesized α-aminophosphonates **1–5** were screened for their in vitro antibacterial and antifungal activities against

Table 1
Synthesis of diphenyl 1-(pyridin-3-yl)ethylphosphonates **1–5** according to Scheme 1

Product	Reaction time[a] (h)	Ar	Mp (°C)	Yield[b] (%)
1	24	C_6H_5	215–217	73
2	27	4-ClC_6H_4	120–122	81
3	22	4-MeC_6H_4	260–262	90
4	20	4-HOC_6H_4	180–182	85
5	30	C_6H_5NH	270–272	75

[a] Completion of the reaction was tested by the use of TLC.
[b] Yield of pure products as colorless crystals after crystallization from ethanol.

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 of antimicrobial testing are reported in Table 2.

The results recorded in Table 2 showed that compound **5** was the most active compound against all organisms. Such compound contains an extra NH group and produced from use of phenylhydrazine rather than aromatic amine. Compounds **2–4** showed high to moderate activities against bacteria and fungi. Compound **1** showed only moderate activities against the organisms tested. It is clear that substitution on the phenyl ring originated from arylamine tends to give better antibacterial and antifungal activities against the tested organisms (i.e., compounds **2–4**) than that obtained from aniline itself (i.e., compound **1**).

3.2. Minimum inhibitory concentrations (MICs)

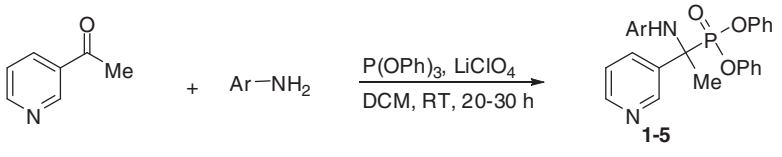
The minimum inhibitory concentrations (MICs) of the synthesized compounds **1–5** were determined for each antimicrobial agent by using agar diffusion method (Table 3). The inhibition zone was measured in triplicates in four different concentrations (10–1000 μg/mL) and the mean value ± standard deviation (SD) is recorded in Table 3.

Table 3 showed that all compounds showed high antimicrobial activities at low concentrations (10 μg/mL) for all microorganisms except for compounds **1** and **3** which showed activities at higher concentrations (100 and 50 μg/mL, respectively) in case of *E. coli* and *S. aureus*, respectively.

3.3. Anticancer activity and IC_{50} values

The cytotoxic activity of the tested aminophosphonates **1–5** was investigated using two types of cancer namely liver carcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF7) which are the most common ones in Egypt. The IC_{50} values were determined as the concentration of tested agents producing 50% decrease of cell survival. Figures 1 and 2 summarize the cytotoxicity data obtained by SRB assay.

As evident from the results represented in Figure 1 (A–E), the tested aminophosphonates **1–5** demonstrated cytotoxic effects against HepG2. Diphenyl 1-(pyridin-3-yl)-1-(*p*-tolylamino)ethylphosphonate **3** (Fig. 1C) was proved to be the most active cytotoxic



Ar = C_6H_5, 4-ClC_6H_4, 4-MeC_6H_4, 4-HOC_6H_4, NHC_6H_5

Scheme 1. Synthesis of diphenyl 1-(pyridin-3-yl)ethylphosphonates **1–5**.

Table 2
Antimicrobial activity (inhibition zones mm) for α-aminophosphonates **1–5**

Compound	Inhibition zone diameter[a] (mm)				
	Bacteria			Fungi	
	E. coli	*S. aureus*	*B. subtilis*	*C. albicans*	*S. cerevisiae*
1	13 ± 2	12 ± 2	15 ± 1	13 ± 1	14 ± 5
2	20 ± 2	21 ± 3	25 ± 3	23 ± 3	23 ± 3
3	22 ± 3	19 ± 3	15 ± 3	17 ± 2	16 ± 2
4	20 ± 1	21 ± 3	20 ± 3	15 ± 3	16 ± 2
5	32 ± 2	28 ± 4	26 ± 5	26 ± 4	27 ± 5

[a] DMSO was added to different organisms as control and showed no inhibition zone.

agent amongst the newly synthesized aminophosphonates, causing 50% inhibition of cell viability at low micromolar concentrations (13.3 μg/mL). Diphenyl 1-(2-phenylhydrazinyl)-1-(pyridin-3-yl)ethylphosphonate **5** was found to be the least active (Fig. 1E) and exhibited the highest IC_{50} (17.6 μg/mL). The compounds could be arranged according to their anticancer activities against HepG2 as follows: **3** > **2** > **1**, **4** and **5**.

The results represented in Figure 2 (A–E) confirmed that aminophosphonate **2** was the most active agent against MCF7 (Fig. 2B) and has the lowest IC_{50} value (17.6 μg/mL). While the least active aminophosphonate was compound **3** (Fig. 2C) and has the highest IC_{50} (20.2 μg/mL). The trend of anticancer activities against MCF7 was found to be as follows: **2**, **1** > **3**, **4** and **5**.

Generally, the liver cancer cell was found to be more sensitive to α-aminophosphonates **1–5** than the breast cancer cell in which the mean of IC_{50} was found to be 16.22 and 19 μg/mL, respectively. The IC_{50} of the standard compound (Doxorubicin) against HepG2 and MCF7 was found to be 3.73 and 2.97 μg/mL, respectively.

The mechanism of antimicrobial and anticancer effect of α-aminophosphonates **1–5** remains to be understood. The electrostatic attraction between the negatively charged bacterial, fungal, and cancer cells and positively charged substances may be the main role in binding and destruction of microbial and cancer cells. Also, α-aminophosphonates **1–5** have specific structures that associated with a tetrahedral structure which acting on cell metabolites by such a way which leading to cell death. Compounds **1–5** may interact with DNA or RNA or replicating enzyme that stop the cell proliferation. Several α-aminophosphonates are known as topoisomerase II inhibitors.[12] In similar manner compounds **1–5** may act as topoisomerase inhibitors.

3.4. The lethal dose

Cytotoxic anticancer substances have unique problems that come primarily from the lack of safety and side effects. Thus, the lethal dose of compounds **1–5** was examined using brine shrimp lethality bioassay. The cytotoxicity of synthetic compounds was determined by using Artemia which has been used for over 30 years in toxicological studies because they provide a quick, inexpensive and desirable alternative to testing on large animals. It is known that a positive correlation exists between Artemia lethality and human carcinoma cytotoxicty. In addition brine shrimp is used in many pre-screens for potential anticancer activity. Different concentrations of 10, 100 and 1000 μg/mL in vials containing 5 mL of water and twenty shrimp in each triplicate were used and survivors were counted after 72 h. The lethal dose of compounds **1–5** is shown in Figure 3 (A–E).

It is clear from Figure 3 that the number of living Artemia was decreased by increasing the concentration of the synthetic compounds. Compounds **1**, **4** and **5** have no effect on Artemia at low concentrations (10 and 100 μg/mL) and killed up to 60% of Artemia at a higher concentration (1000 μg/mL). Compound **2** reduced the living Artemia to 15% at low concentration (100 μg/mL) and able to kill 100% of Artemia at the higher concentration (1000 μg/mL). Compound **3** at low concentration (10 μg/mL) did not affect on Artemia, but at the higher concentration (1000 μg/mL) the number of living Artemia was reduced to 10%. In summary, compounds **1**, **4** and **5** are safe to be used because they exhibited high values of lethal dose. On the other hand, compounds **2** and **3** exhibited small values of lethal dose.

The collective data of IC_{50} and LD_{50} for α-aminophosphonates **1–5** are recorded in Table 4. The results indicated that LD_{50} values were higher than IC_{50} values for all compounds against the two cell lines. The synthetic compounds can be arranged according to their IC_{50}/LD_{50} in the following order: **2** > **3** > **1**, **4** and **5** for both, HepG2 and MCF7 cell line. Clearly, compound **4** was found to be the safest compound to be used, while, compound **2** was the least safe one.

4. Conclusion

A convenient method for the synthesis of diphenyl 1-(arylamino)-1-(pyridin-3-yl)ethylphosphonates was developed. The newly synthesized compounds exhibit a remarkable inhibition of the growth of Gram-positive, Gram-negative bacteria and fungi at low concentrations. The cytotoxicities of the synthesized compounds showed significant cytotoxicity. In particular, the liver cancer cell was more sensitive than the breast cancer cell for all compounds. The lethal dose of the synthetic compounds indicated that most of the synthesized compounds are safe and are promising for their uses as in vivo antimicrobial and anticancer reagents.

5. Experimental

5.1. 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 KBr disk 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

Table 3
Minimum inhibitory concentrations (MICs) of α-aminophosphonates **1–5**[a]

Compound	Minimum inhibitory concentrations (MICs) (μg/mL)					
	E. coli	*S. aureus*	*B. subtilis*	*C. albicans*	*S. cerevisiae*	Mean MICs (μg/mL)
1	100 ± 12	10 ± 0.9	10 ± 1.0	10 ± 1.0	10 ± 1.0	28 ± 7.0
2	10 ± 0.9	10 ± 1.0	10 ± 2.0	10 ± 0.8	10 ± 0.8	10 ± 2.5
3	10 ± 1.3	50 ± 3.5	10 ± 1.3	10 ± 0.9	10 ± 0.6	18 ± 4.5
4	10 ± 1.2	10 ± 1.0	10 ± 2.0	10 ± 0.9	10 ± 0.5	10 ± 2.5
5	10 ± 1.0	10 ± 1.0	10 ± 1.0	10 ± 1.0	10 ± 1.0	10 ± 2.5

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

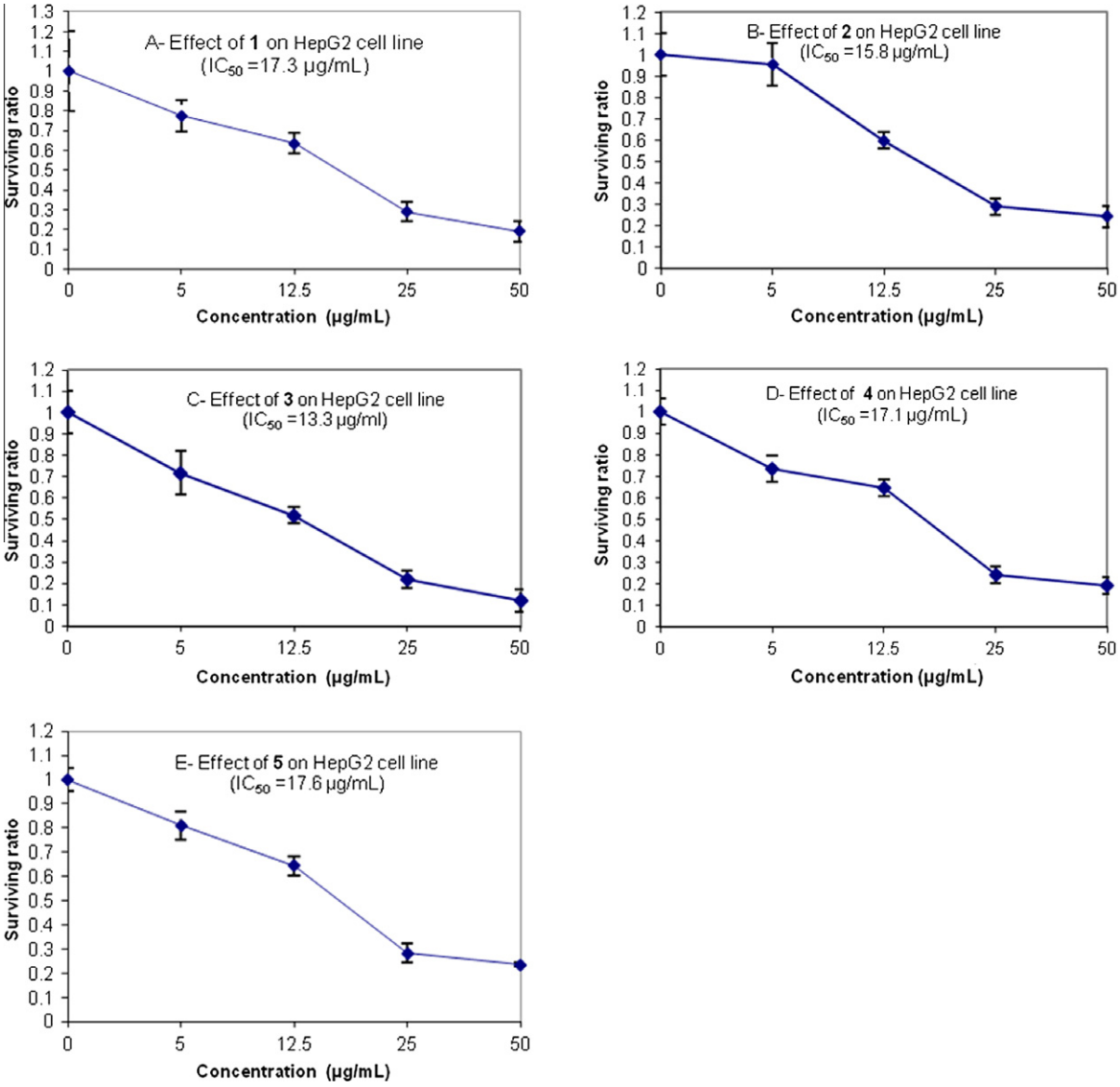


Figure 1. The effect of various concentrations (0–50 µg/mL) of α-aminophosphonates **1**–**5** on liver cancer cell line (HepG2) in vitro.

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 spectrum for compound **5** was recorded at energy 70 eV with a 7070 EQ mass spectrometer. 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} sheet (0.2 mm) with chloroform/acetone (5:2 by volume) or petroleum ether (40–60 °C)/acetone (5:2 by volume) as developing eluents. The spots were detected with UV Lamp Model UV GL-58. Reagents and solvents were used from commercial sources without purification.

5.2. Synthesis of diphenyl 1-(arylamino)-1-(pyridin-3-yl)ethylphosphonates 1–5

Arylamine (4.0 mmol) was added to a stirred mixture of 3-acetylpyridine (0.242 g, 2.0 mmol) and a solution of $LiClO_4$ in DCM (3.0 mL, 15.0 mmol; 5 M). The mixture was stirred at room temperature for 10 min before triphenylphosphite (0.93 g, 3.0 mmol) was added. The mixture was stirred at room temperature for 22–30 h before H_2O (10 mL) and DCM (10 mL) were added. The organic phase was separated and the aqueous layer was extracted with DCM (2×10 mL). The combined organics were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to give the crude product which was recrystallized from ethanol to give pure product.

5.2.1. Diphenyl 1-(phenylamino)-1-(pyridin-3-yl)ethylphosphonate 1

IR (KBr): 3411, 1628, 1287, 812 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.58 (br s, exch., 1H, NH), 7.52–6.90 (m, 19H, Ar-H), 2.09 (s, 3H, CH_3); Anal. Calcd for $C_{25}H_{23}N_2O_3P$: C, 69.76; H, 5.39; N, 6.51; P, 7.20. Found: C, 69.86; H, 5.25; N, 6.80; P, 7.18.

5.2.2. Diphenyl 1-(4-chlorophenylamino)-1-(pyridin-3-yl)-ethylphosphonate 2

IR (KBr): 3421, 1622, 1271, 817 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.11 (br s, exch., 1H, NH), 8.80–6.51 (m, 18H, Ar-H), 2.03 (s, 3H,

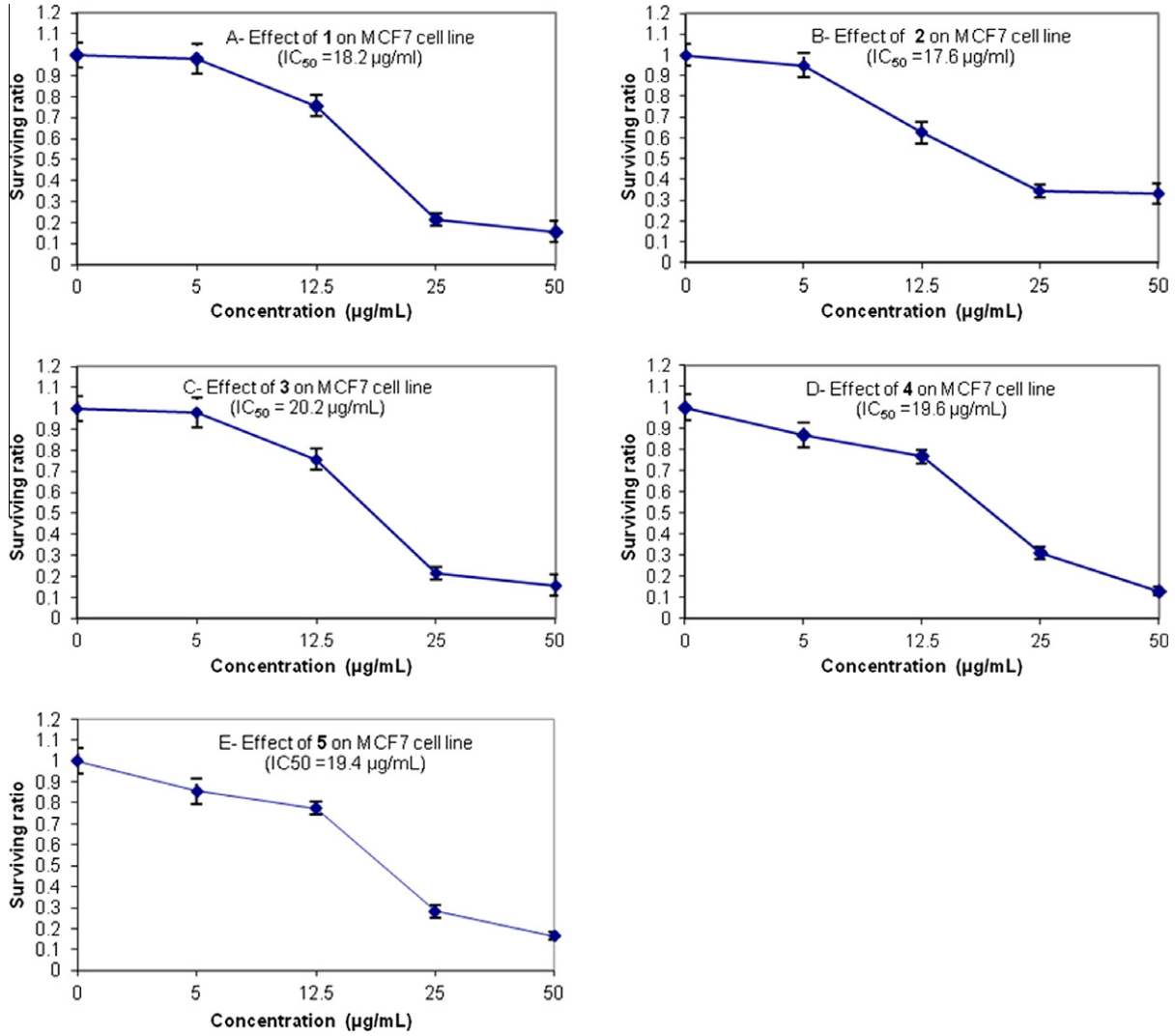


Figure 2. The effect of various concentrations (0–50 µg/mL) of α-aminophosphonates **1**–**5** on breast cancer cell line (MCF7) in vitro.

CH_3); Anal. Calcd for $C_{25}H_{22}N_2O_3PCl$: C: 64.59, H, 4.77; N, 6.03; P, 6.66. Found: C, 64.70; H, 4.22; N, 6.45; P, 6.55.

5.2.3. Diphenyl 1-(pyridin-3-yl)-1-(*p*-tolylamino)ethylphosphonate 3

IR (KBr): 3415, 1629, 1279, 810 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.12 (br s, exch., 1H, NH), 8.82–5.80 (m, 18H, Ar-H), 2.34 (s, 3H, CH_3), 2.11 (s, 3H, CH_3); Anal. Calcd for $C_{26}H_{25}N_2O_3P$: C, 70.26; H, 5.67; N, 6.30; P, 6.97. Found: C, 69.98; H, 5.62; N, 6.37; P, 6.95.

5.2.4. Diphenyl 1-(4-hydroxyphenylamino)-1-(pyridin-3-yl)-ethylphosphonate 4

IR (KBr): 3387, 1593, 1229, 833 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.00–6.94 (m, 19H, Ar-H and NH), 6.74 (s, exch., 1H, OH), 2.20 (s, 3H, CH_3); Anal. Calcd for $C_{25}H_{23}N_2O_4P$: C, 67.26; H, 5.19; N, 6.27; P, 6.94. Found: C, 67.79; H, 5.22; N, 6.30; P, 6.92.

5.2.5. Diphenyl 1-(2-phenylhydrazinyl)-1-(pyridin-3-yl)ethylphosphonate 5

IR (KBr): 3420, 1591, 1247, 905 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.52 (br s, exch., 1H, NH), 9.02–6.71 (m, 20H, Ar-H and NH), 2.21 (s, 3H, CH_3); EI-MS: *m/z* (%) 445 (M^+, 25), 402 (6), 325 (14), 215 (10), 141 (11), 94 (46); Anal. Calcd for $C_{19}H_{19}N_4O_5P$: C, 67.41; H, 5.43; N, 9.43; P, 6.95. Found: C, 67.38; H, 5.93; N, 8.96; P, 6.93.

5.3. Antimicrobial activities

5.3.1. Test microorganisms

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

5.3.1.2. 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* is mostly involved in Urinary infection, wound, ulceration and septicemia. *S. aureus* is the mild stone of Gram-positive bacteria and it is a causative agent of pneumonia, meningitis and food poisoning.

5.3.1.3. Fungi. Pathogenic fungi spatially yeasts are responsible for a number of diseases in human, 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.

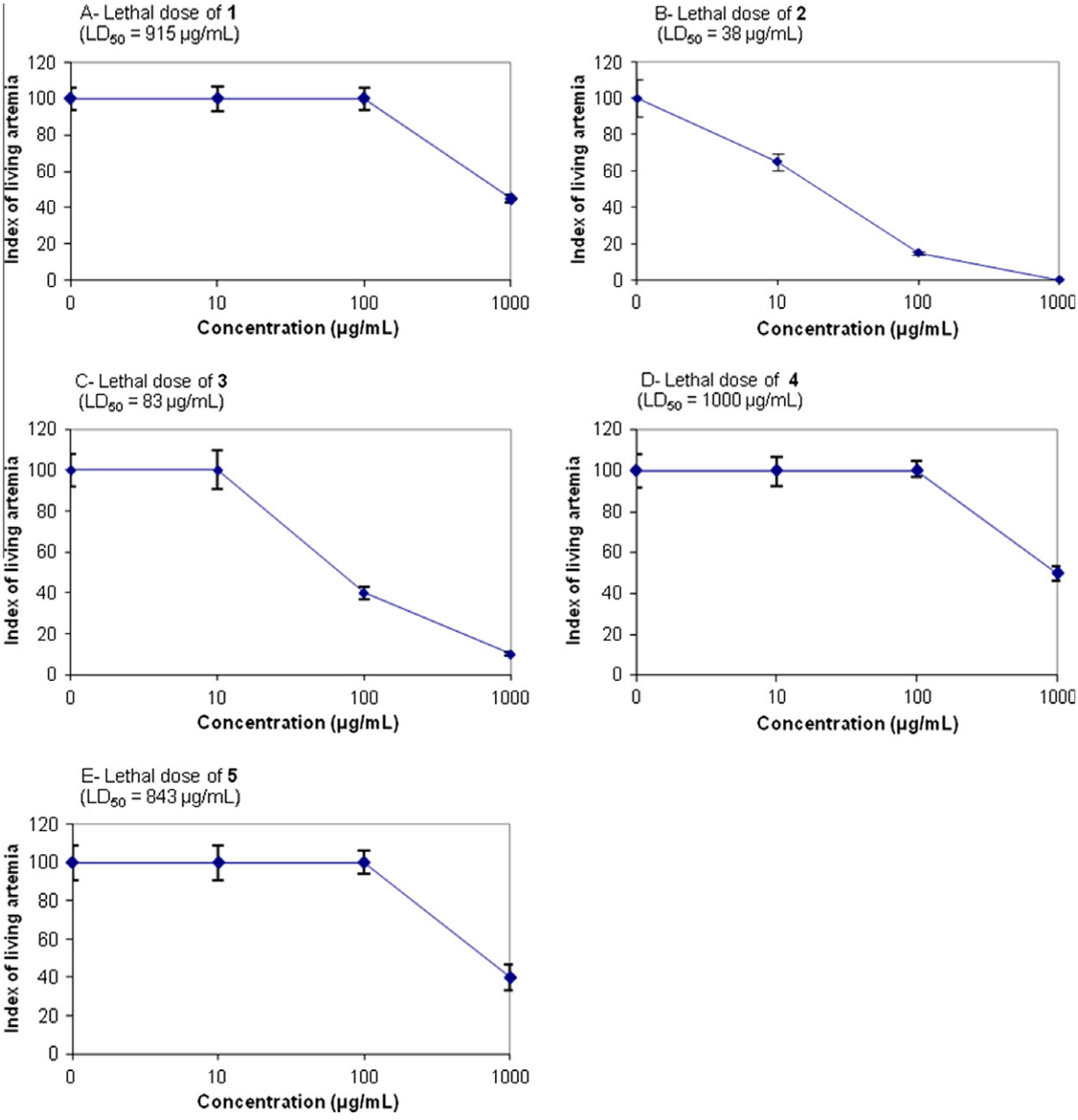


Figure 3. The lethal dose of various concentrations (0–1000 µg/mL) of α-aminophosphonates **1**–**5** with Artemia.

Table 4
The IC_{50}/LD_{50} values of α-aminophosphonates **1**–**5** against HepG2 and MCF7 cell lines

Compd	IC_{50} (µg/mL)		IC_{50}/LD_{50}		LD_{50} (µg/mL)
	HepG2	MCF7	HepG2	MCF7	
1	17.3 ± 1.8	18.2 ± 1.7	0.019	0.020	915 ± 67
2	15.8 ± 1.6	17.6 ± 1.8	0.415	0.463	38 ± 2.6
3	13.3 ± 1.2	20.2 ± 1.8	0.160	0.243	83 ± 6.6
4	17.1 ± 1.7	19.6 ± 1.7	0.017	0.020	1000 ± 87
5	17.6 ± 1.6	19.4 ± 1.9	0.020	0.023	843 ± 66

5.3.2. 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 synthetic 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.[38]

5.4. Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentration (MIC) was determined by agar diffusion assay using filter paper disc method. The MICs

were determined for synthetic 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, 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 antimicrobial activities of the tested samples were determined by measuring the diameter of zone of inhibition expressed in millimeter. The inhibition zones were measured in triplicates and expressed as mean ± SD.[39]

5.5. Anticancer activities

In vitro potential cytotoxicities of the synthesized compounds were tested using the colorimetric method. Cytotoxic activity of α-aminophosphonates **1–5** were tested against cancer cells (5 mg/mL in DMSO), obtaining medium inhibitory concentration (IC_{50}) values in μg/mL, using a dilution method in a 96-well plate. After 72 h incubation, absorbance was measured at 546 nm. DMSO was used as negative control and Doxorubicin (DOX) as positive control, both at the same concentration as the synthesized compounds. Compounds **1–5** were dissolved in DMSO and subjected to cytotoxic evaluation against two cancer cell lines namely liver carcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF7). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was dissolved in saline to make a concentration of 5 mg/mL as a stock solution. Cancer cells (5×10^5 cells) suspended in 200 μL of MEM medium containing 10% fetal calf serum were seeded into a 96 well culture plate. After 24 h of preincubation at 37 °C in a humidified atmosphere of 5% CO_2 to allow cell attachment, various concentrations of tested solution (0, 12.5, 25, and 50 μg/mL) were added and then incubated for 72 h under the above conditions. At the end of the incubation, 50 μL of tetrazolium reagent was added to each well and then incubated at 37 °C for 4 h. The supernatant was decanted and DMSO (100 μL) was added to allow formosan solubilization. The optical density (OD) of each well was detected by ELIZA microplate reader (Meter tech. Σ 960, USA) at 564 nm. Each determination represents the mean of four replicates. The 50% inhibition concentration (IC_{50}) was determined by curve fitting.[40]

5.6. The lethal dose

Brine shrimps lethality bioassay is very simple bench-top assay used to measure cytotoxicity of plant extracts as well as synthetic compounds. Brine shrimp eggs, available commercially and being used as fish food. Evaluation of natural products and synthetic compounds by using brine shrimp describes not only cytotoxicity but also anticancer, antiviral, insecticidal and pesticidal potential. The cytotoxicity lethal dose (LD_{50}) of the synthesized compounds was determined to the larvae of *Artemia salina* using brine shrimp lethality bioassay. 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.[41]

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