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Mohamed E. I. Badawy
Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt

Nehad E. M. Taktak
Department of Tropical Health, High Institute of Public Health, Alexandria University, Alexandria, Egypt

Osama M. Awad
Department of Tropical Health, High Institute of Public Health, Alexandria University, Alexandria, Egypt

Souraya A. Elfiki
Department of Tropical Health, High Institute of Public Health, Alexandria University, Alexandria, Egypt

Nadia E. Abou El-Ela
Department of Tropical Health, High Institute of Public Health, Alexandria University, Alexandria, Egypt

Correspondence:
Mohamed E. I. Badawy
Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt

Larvicidal activity of temephos released from new chitosan/alginate/gelatin capsules against *Culex pipiens*

Mohamed E. I. Badawy, Nehad E. M. Taktak, Osama M. Awad, Souraya A. Elfiki, Nadia E. Abou El-Ela

Abstract
A new controlled release capsules based on chitosan, alginate, and gelatin crosslinked with glutaraldehyde and loaded with the insecticide temephos were prepared and assessed against *Culex pipiens* larvae. Capsules were characterized with respect to their size, shape, larvicide loading, swelling, and *in vitro* and *in vivo* release kinetics. Capsules were loaded with temephos 7.35% as determined by UV spectroscopy. The highest *in vitro* release amount of the insecticide was found at the first day. The *in vivo* release data showed that the capsules reached to a plateau at the first day with the highest weight of the capsules, resulting in a highest mortality (100%). These studies revealed that the release kinetic showed high efficacy against the larvae that showed 100% mortality up to 120 and 92 days with 0.05 g of capsules in the stagnant and running water experiments, respectively. It could be concluded that the new capsules based on the biodegradable polymers containing temephos could be effective as controlled release formulation against *C. pipiens* larvae for long time.

Keywords: Chitosan capsules, Temephos, Controlled release formulation, Swelling, Mosquitocidal activity, *Culex pipiens,* Biochemical study.

1. Introduction
Mosquitoes cause a serious public health importance as they can transmit more diseases than any other groups of arthropods and affect millions of people throughout the world [1, 2]. The females, as blood sucking insects, are vectors of a multitude disease of man and animals in different countries through transmission of pathogenic agents [3, 4]. Among these, *Culex pipiens* (Diptera: Culicidae) is the common and widely distributed mosquitoes in Egypt and has been incriminated as the main vector of bancroftian filariasis and the Rift Valley fever [5-8]. Bancroftian filariasis is considered the fastest spreading insect-borne disease of man in the tropics, affecting about 146 million people [9] and occurs where the environmental conditions favor insect vectors responsible for the transmission of diseases [10].
Efforts to control these diseases involve the use of various strategies directed against the primary vector and to the pathogen agent. Vector control has been the major method to control these diseases and the strategies including chemical, biological, and microbial have been used [11-14]. Chemical control is an effective strategy which is extensively used in daily life [1, 15]. There are many kinds of pesticides effectively against mosquitoes, including organochlorine, organophosphorus, carbamates, and pyrethroids [16-18]. Specific compounds for larval stages are one of the successful ways in reducing mosquito densities in their breeding places before they emerge into adults. Larviciding largely depends on the use of synthetic insecticides such as organophosphates for example, temephos, malathion and fenthion [2, 19]. The organophosphate temephos (Abate®), is a non-systemic larvicide, and one of the most used compounds in controlling of the mosquitoes globally [20, 21]. It exhibits very low mammalian toxicity, widely recommended and has been used for malaria control in India and Mauritius [22, 23]. Temephos has also been valuable in the Onechercerciasis control program in West Africa [24].
Because of the repetitive application with low rates of mosquitocides, controlled release technology has been developed in pesticides industry to combat the side effects caused by administering high doses of conventional pesticides [25, 26]. In addition, controlled release formulations have been used extensively to maintain an effective level of applied pesticides, extend pesticide residual activity, and reduce the application rates and cost, pesticide levels in the environment, and toxicity to mammals and nontarget organisms [22, 27].

Generally, it is considered that an efficient pesticide formulation should not only show an effective biological activity, it must also be safe to mammals and environmentally friendly [28]. Depending on the criterions, different pesticides delivery system has recently been developed [29, 30]. The pesticides are formulated in the form of wettable powders, emulsifiable concentrates, water solutions, powder or granules, aerosols or spray formulations. But such formulations have different degrees of health hazards ranging from respiratory exposure to penetration through the skin. Being larger in size, granules do not pose any of the upper mentioned health hazards but the formulation is not suitable enough to deliver in a convenient way for field application. Considering all these facts, encapsulation is the most efficient way of delivering and control release of pesticides, which reduce exposure to hazard [29, 31].

For environmentally friendly applications, fabrication of controlled release formulations depends on using of biodegradable materials instead of synthetic polymers such plastics. Biopolymers such as chitosan and alginate have been extensively investigated for encapsulation of biologically active compounds, as they are biodegradable, biocompatible, and low mammalian toxic [22, 32]. Therefore, the present study aims to prepare and develop a crosslinked interpenetrating polymeric network capsules based on chitosan, sodium alginate and gelatin for loading insecticide temephos as effective controlled release formulation against *C. pipiens* larvae. The capsules characterizations including scanning electron microscopy (SEM), FT-IR, physical properties, and swelling capacities were investigated in details. In addition, the *in vivo* effects of this insecticide on biochemical parameters such as acetyl cholinesterase (AChE), carboxyl esterase (CaE), and glutathione S-transferase (GST) was also examined.

2. Materials and methods

2.1. Chemicals and insecticide

Low molecular weight of acid-soluble chitosan (3.60×10^5 Da and 11% degree of acetylation), sodium alginate, gelatin (type A, from porcine skin, 300 bloom), glutaraldehyde (25%), bovine serum albumin (BSA), 4-nitrophenyl acetate (NPA), acetylthiocholine iodide (ATChI), 5,5′-dithiobis(2-nitrobenzoic acid (DTNB), L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), ethylenediaminetetracetic acid (EDTA), acetone, and acetonitrile were purchased from Sigma-Aldrich Co. (Spruce Street, St. Louis, MO, USA). Temephos 93%, *O*,*O*,*O*′,*O*′-Tetramethyl *O*,*O*′-sulfanediylbis(1,4-phenylene) diphosphorothioate was purchased from Kalyani Industries Pvt. Ltd. All other commercially available solvents and reagents were used without further purification.

2.2. Test insect and rearing

The *C. pipiens* third instar larvae were obtained from the susceptible reared strain of Research Institute of Medical Entomology, Ministry of Health, Dokki, Giza, Egypt and reared under laboratory conditions. About 400-600 larvae of *C. pipiens* were transferred to white enameled and shallow trays about 30 cm diameter containing 2-3 liters of de-chlorinated water. These trays were always covered with mesh screen to prevent oviposition by escaped adult mosquitoes and were maintained at 26±2°C and 70±5 %) RH with a 14:10 (L:D) photo-period. The Larvae were fed daily on biscuits and yeast power in the 3:1 ratio until pupation and water was replaced every two days. The pupae were transferred from the trays to plastic cups containing de-chlorinated water and were maintained in cages with netting cover wood frames (30×30×30 cm) until adults emerged. Adults were provided with 10% sucrose solution and females were fed on pigeon blood for four times a week [33]. The egg-rafts were placed in the white trays containing de-chlorinated water until hatching.

2.3. Dose-response larval bioassay for technical temephos

The acute toxicity of technical temephos (93%) was evaluated by mixing different concentrations, prepared in dimethyl sulphoxide (DMSO), with de-chlorinated tap water. The appropriate volume of dilution was added to 100 mL of de-chlorinated water in plastic cups to obtain the desired target concentrations (0, 2, 4, 6, 8, 10, 20, 30, and 40 ppb). Three replicates were tested for each concentration and controls were set up simultaneously with de-chlorinated water. The test cups were held at 25±2ºC and preferably a photoperiod of 12:12 (L:D). Larval mortality was recorded after 24 and 48 h of the exposure. Larvae that have pupated during the test period will negate from the test. The log dose-response curve was used for the determination of the LC_{50} and LC_{95} values according to the probit analysis [34]. If the mortality in the control was between 5 and 20%, the mortalities of the treated groups were corrected according to Abbott's formula [35].

2.4. Preparation of chitosan/alginate/gelatin capsules loaded with temephos

Chitosan/alginate/gelatin capsules were prepared in cross-linking with glutaraldehyde as follows. Chitosan was dissolved in 1% aqueous acetic acid solution until the solution was transparent to achieve a final chitosan concentration 10000 mg/L. Sodium alginate was dissolved in distilled water and stirred mechanically until dissolved completely. Glutaraldehyde solution was mixed with sodium alginate solution to obtain final concentrations 2% (w/v). Gelatin solution 2.5% (w/v) was prepared in distilled water. To form the capsules, 25 mL of 1% (w/v) sodium alginate solution in distilled water containing 2% (w/v) glutaraldehyde was placed in a beaker. 12.5 mL of the chitosan solution (1%, w/v) was mixed with 12.5 mL the gelatin solution (2.5%, w/v) and this mixture was then dropped by syringe (internal diameter = 0.5 mm) into the alginate / glutaraldehyde solution with gently stirring at 25°C. The product with spherical shape was formed immediately after the dropwise addition; after 1 h of reaction, the capsules formed were removed by sieving from the solution then washed with distilled water for several seconds, dried and kept in a desiccator until further use. Loaded capsules with insecticide temephos was prepared by the same method by mixing temephos (0.0625 g) dissolved in acetonitrile (3 mL) with chitosan solution.

2.5. Capsules characterizations

2.5.1. Capsule size measurement

Ten samples of the completely dried capsules from formulation were selected and their sizes were measured by using 6 inch LCD Digital Vernier Caliper/Micrometer Guage 150 mm (Shenzhen, China) with an accuracy of ± 0.02 mm/0.001 inch.

2.5.2. Scanning electron microscopy (SEM)

SEM was made with a JEOL JSM-5300 microscope (JEOL USA, Inc.) to observe the surface morphology of the capsules. The observations were performed at an accelerating voltage of

25 kV. The dry gel spheres were mounted on metal stubs with double-sided tape, sputtered with gold and viewed in the SEM.

2.5.3. FT-IR spectroscopy
The capsules were analyzed with FT-IR spectroscopy with KBr discs, in the range from 4000 to 400 cm^{-1}, with a resolution of 4.0 cm^{-1} on a Perkin Elmer FT-IR Spectrophotometer (Waltham, Massachusetts, USA).

2.5.4. Swelling properties
The swelling studies of loaded and unloaded capsules were carried out by the following method. The capsules water uptake was determined by immersing a definite weight (0.05 g) of capsules into distilled water and allowed to swell and the water gain being weighed at a different time intervals up to 10 days. Excess surface water was removed by being wiped with soft paper and the percentage of swelling was calculated by the following equation:

$$\text{Swelling (\%)} = \left[\frac{(W_t - W_o)}{W_o}\right] \times 100$$

Where W_t and W_o are the weights of the swollen sample at time t and that of the original sample, respectively. Experiments were performed in triplicate, and the data obtained were averaged.

2.6. Spectral analysis and estimation of temephos content in controlled release capsules
Absorption scans in the range of 190 to 340 nm were performed on 5 mg/L temephos in acetonitrile using U.V/Visible Spectrophotometer (Alpha- 1502. Laxco Inc, USA). The solvent only was used as blank. The maximum wave length (λ_{max}) appeared at 201 nm. Therefore, all absorbance measurements were performed at this wave length. For estimation the temephos content in capsules, a weight of 0.01 g of the capsules was immersed in acetonitrile (2.5 mL) and grinded to disintegrate the capsules. The vial was left at room temperature for 2 h and then filtered through Whatman filter paper No. 1. The filtrate of acetonitrile was used for UV analysis at 201 nm (λ_{max} of temephos). Blank capsules without temephos was extracted and used as a control.

2.7. The *in vitro* temephos release study
Four weights of capsules (0.005, 0.01, 0.02, and 0.05 g) were immersed in 100 mL of distilled water and stored at 25±2ºC. Two techniques were studied included the running and stagnant water daily. A sample of 0.5 mL was taken from each bottle at different time intervals and analyzed by UV spectroscopy at 201 nm until temephos non-detectable. The distilled water sample was used as a blank. The measured water samples were poured back into the dissolution cell to keep the volume constant.

2.8. The *in vivo* temephos release study
The *in vivo* temephos release against *C. pipiens* larvae was evaluated in two techniques. Firstly, bioassay in stagnant water was conducted as follows. Capsules (0.005, 0.01, 0.02, and 0.05 g) were placed in 100 mL of water containing 10 third instars of *C. pipiens* larvae, and their death was monitored as a function of time daily for a period of time without change of water but with fresh larvae [36, 37]. Experiments were run in three replicates, and a control sample was obtained by larvae being kept in water under the same conditions, but with unloaded beads. Secondly, the bioassay in running water which the solution and larvae were removed and discharged daily and the capsules were kept in a small portion of water. After that the water was replenished and fresh larvae were added, and a new death/survival count was carried out daily. The trial was repeated until a negligible mortality was obtained. The mortality percentages were recorded in two experiments.

2.9. Biochemical studies
Specific activities of AChE, CaE and GST were determined in surviving larvae after 24 h of exposing to LC_{50} of temephos. Larvae were homogenized using a glass/Teflon homogenizer in 10 mM NaCl (1%, w/v) Triton X-100, and 40 mM sodium phosphate buffer (pH 7.4) at 4°C. The homogenate was filtered through cheesecloth and centrifuged at 5000 rpm for 20 min at 4°C. The supernatant were used immediately for assaying AChE, CaE and GST or were stored at -20°C. All the experiments were performed in triplicate. Crude protein was determined according to Lowry *et al*. method [38] using BSA for standard curve. Activity of AChE was determined by the colorimetric method of Ellman and co-authors [39] using 0.075 M ATChI as a substrate. The assay medium (1.5mL total) consisted of 1,420 µL phosphate buffer (pH 8), 20 µL of the crude enzyme, 50 µL of 0.01 M DTNB, and 10 µL of 0.075 M ATChI. Over 10 min incubation at 37°C, the reaction was monitored using a Unico 1200 Spectrophotometer at 412 nm. The specific activity was expressed as ΔOD_{412} min^{-1}.mg $protein^{-1}$. CaE activity was assayed by spectrophotometer using the substrate NPA according to the method of Chanda and co-authors [40]. The assay medium (2 mL total) consisted of 1925 µL of 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 50µL of enzyme extract and 25 µL of 5 mM NPA. In this method, after 5 min of incubation at 25°C, the formation of yellow 4-nitrophenol was measured at 405 nm and plotted against a standard curve of 4-nitrophenol. The specific activity was expressed as ΔOD_{405} min^{-1}.mg $protein^{-1}$. GST activity was measured as described by Saint-Denis and co-authors [41] by mixing of 1650 uL of 100 mM phosphate buffer (pH 7.4) containing 1mM EDTA, 50uL of enzyme extract, 200 uL of 2.5 mM GSH, 100 uL of 1 mM of CDNB, as a substrate. The absorbance was measured at 340 nm. One unit of activity corresponded to the quantity of enzyme conjugating 1 mmol of GSH per min. The specific activity was expressed as ΔOD_{340}.min^{-1}.mg $protein^{-1}$. For all enzymes, blanks (reaction mixture free of crude enzyme sample) were periodically checked for non enzymatic activities; however, no significant activity was observed for all.

2.10. Statistical Analysis
Statistical analysis was performed using the IBM SPSS statistics version 21.0 software program (Statistical Package for Social Sciences, USA). Mortality percentages were calculated for each treatment bioassay methods and corrected using Abott's equation [35]. Means and standard error (SE) were determined from three independent replications performed for each treatment. The log dose-response curves were used in the determination of the LC_{50} value for the insect bioassay according to the probit analysis [34]. The 95% confidence limits were determined by the least-square regression analysis.

3. Results and discussion

3.1. Preparation of the capsules

In the present study, the reaction between chitosan (1%), sodium alginate (1%) crosslinked with glutaraldehyde (2%) and their combination with gelatin (2%) produced spherical capsules. The capsules were in yellowish-brown in color before drying and in brown in color after drying. The same method was also made in the presence of 5000 mg/L of the insecticide Temephos (technical grade 93%) to prepare the loaded capsules. The properties of the unloaded and loaded capsules are summarized in Table 1. It can be noted that the unloaded capsules are bigger in diameter (1004 µm) than that loaded with the temephos (954 µm) which gave 820 and 2650 capsule per gram, respectively. Capsules were loaded with 7.35% temephos, as determined by UV spectroscopy at 201 nm.

Table 1: Properties of chitosan/sodium alginate/gelatine capsules loaded and unloaded with the mosquitocide temephos

Parameters		Unloaded capsules	Capsules loaded with temephos
Components ratio, % (Ch:Alg:Gel:Glut: temephos)		1:1:10:2:0	1:1:10:2:7.35
Capsule color		Yellow	Brown
Weight before drying (g)		17.68	10.33
Weight after drying (g)		1.53	0.840
Capsule no./g		820	2650
Diameter (µm)		1004±32	954±29
Flexibility	Before drying	Flexible	Flexible
	After drying	Hard	Hard

Ch: Chitosan, Alg: Alginate, Gel: Gelatin and Glut: Glutaraldehyde.

In this work, it was necessary to modify chitosan structure with alginate and crosslinking with glutaraldehyde as it has been reported that the mechanical strength of chitosan capsules is low, thus limits its use as a controlled release formulation [32, 42]. Chitosan reacts with alginate via ionic interaction between the carboxyl residues of alginate and the amino terminals of chitosan [43]. The interaction could also be via intermolecular hydrogen bonding as described by Ngah and co-authors [44] who studied the reaction mechanism between starch and alginate in crosslinking. This complex reduces the porosity of the alginate capsules and decreases the leakage of the encapsulated substances. Bhan and co-authors [28] encapsulated temephos at ratios of 1 to 16% and imidacloprid at 1 to 8% within biodegradable and biocompatible polyethylene glycol in different ratios. Nnamonu and co-authors [45] prepared and characterized slow release formulations of imazaquin (a herbicide) in starch and chitosan beads reinforced with alginate. They obtained highly porous spherical beads with diameter of 2310-2530 µm. A chitosan and cashew gum beads containing insecticide dichlorvos [2,2-dichlorovinyldimethyl phosphate (DDVP)] prepared by Paula and co-authors [37] resulted loading ratio of DDVP from 5.15 to 71.4%. The chitosan and chitosan/ cashew gum beads had average masses of 2.56 and 2.47 mg and average diameters of 1570 and 1760 µm, respectively.

3.2. Scanning electron microscope (SEM) analysis

SEM photographies of loaded and unloaded capsules are shown in Figure 1. The capsules were initially studied under a light stereo microscope before and after drying (Figure 1A and 1B, respectively) to distinguish their characteristics. They appeared spherical in shape and yellow gold in color before drying and turned to brown after drying. Under SEM, the unloaded capsules were characterized irregular in shape with longitudinal cavity (Figure 1C) and their surfaces morphology were examined under power of magnification 35000x and appeared a lot of pores and wrinkles (Figure 1D). After loading with the insecticide temephos, the capsules appeared under SEM as regular oval in shape with cavity (Figure 1E) and its surface morphology under power of magnification 35000x seemed more rough with a lot of scars (Figure 1F). This confirmed that the loading of the temephos on the capsules fill all pores. It was found that the capsules were characterized elliptical and uniform in shape, which indicates a good compatibility between chitosan, alginate and gelatin biopolymers. The capsules diameter was ranged from 950 to 1000 µm in size.

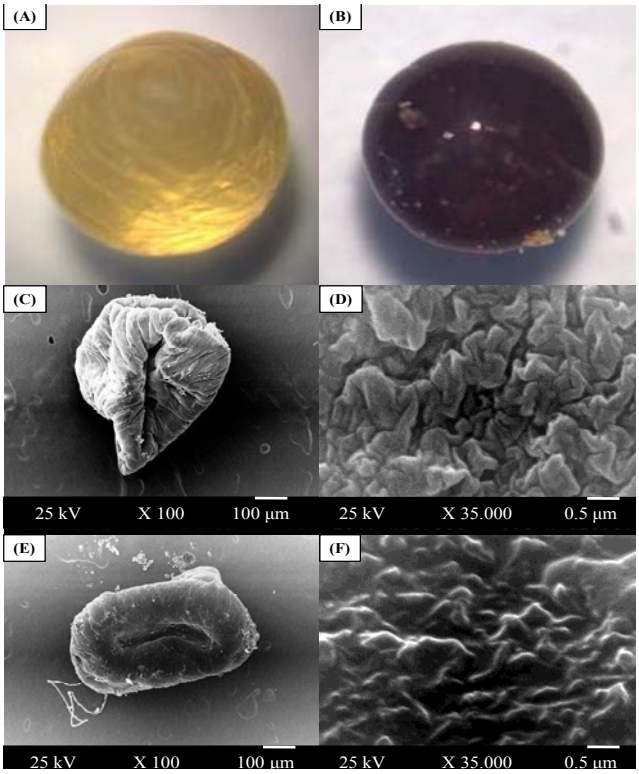

Fig 1: Stereo optical microscope morphology of whole shape (magnification, X10) of the wet capsules prepared from chitosan (1%), alginate (1%), glutaraldehyde (2%) and gelatine of 2.5%, before (A) and after (B) drying. SEM photograph of unloaded capsules (C) and its surface morphology (D); and SEM photograph of loaded capsules with temephos (E) and its surface morphology (F). Scale bar 100 µm and magnification x100 for whole capsules and scale bar 0.5 µm and magnification x35000 for their surface ……. morphologies.

3.3. FT-IR spectroscopy

Figure 2 shows the FT-IR spectra of the unloaded (A) and loaded capsules (B). In both spectra, the absorption band at 3405 and 3391 cm^{-1}, respectively were recorded in this work due to the hydroxyl group O-H stretching vibrations which is characteristic of natural polysaccharides. The weak absorption band at 2930 (Figure 2A) and 2949 cm^{-1} (Figure 2B) represent the -OH stretching vibration. The strong asymmetric stretching absorption band at 1649 cm^{-1} in both spectra is due to the presence of carboxylate anions in sodium alginate and chitosan. In the spectrum of the loaded capsules, it can be

noted that new strong absorption bands at 1486, 1212, 1037, 935 and 839 cm^{-1}. The strong stretching absorption band at 1037 cm^{-1} appeared only in the loaded capsules spectrum due to P=S of temephos. The absorption band at 839 cm^{-1} is assigned to P-O-C and the absorption band at 1486 cm^{-1} is assigned to CH_3 asymmetric stretching in the temephos structure. Absorption band at *935* cm^{-1} is assigned to P-O-R and the band at 1212 cm^{-1} is assigned to C-O.

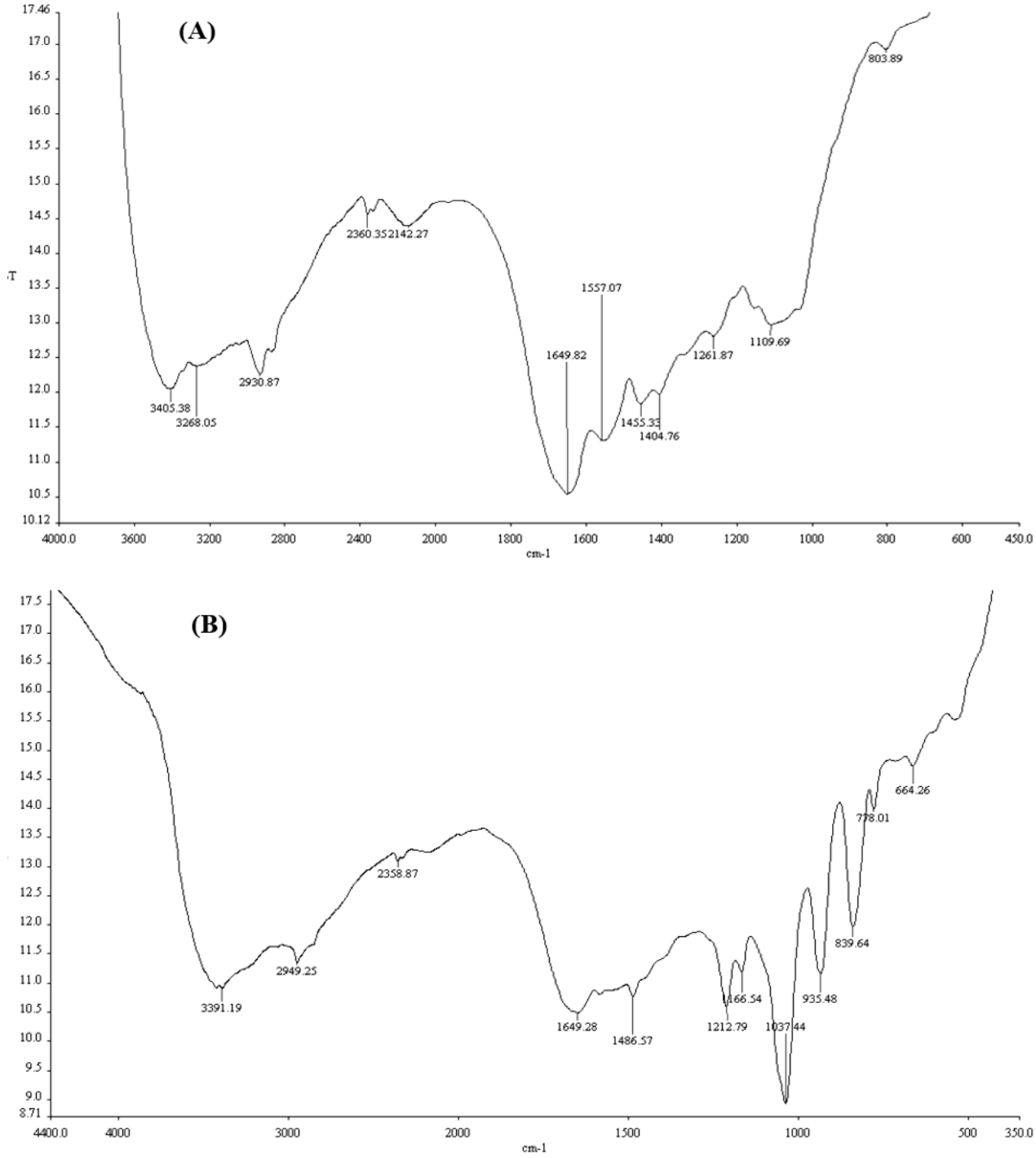

Fig 2: FT-IR spectra of unloaded **(A)** and loaded **(B)** capsules with temephos.

3.4. Swelling study

The swelling capacity of loaded and unloaded capsules is shown in Figure 3 during ten days of the experiment. The unloaded capsules gradually increased in their weight and exhibited a higher water absorption rate than the loaded capsules through the first six days. The capsules rapidly swelled within the first 24 h. The highest swelling degree was obtained at the third day (170%) however it was decreased to 80% on seventh day then remained constant up to tenth day. However, it can be noted that the swelling of the loaded capsules tend to be more stable along the experiment (80 to 100%).

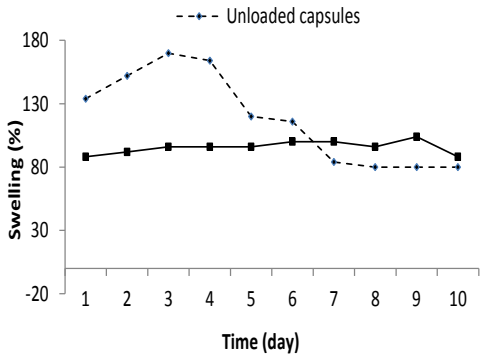

Fig 3: Swelling kinetics of loaded and unloaded capsules with temephos during ten days.

The swelling ability of the capsules is an important factor to evaluate its property, used as scaffold materials for controlled temephos release in water to play an active role as a larvicide for a long period. Swelling property refers to the hydrophilic groups and more positively charged amino groups along of the biopolymer chains lead to much expanding polymer network [46, 47]. In addition, the swelling behavior of the capsules mainly results from the electrostatic repulsion between the negatively charged carboxyl groups on alginate and gelatin. Furthermore, it is known that only the primary amine groups of lysine and arginine residues on the gelatin may reacting with glutaraldehyde, while each glucosamine unit of chitosan can reacting with glutaraldehyde and lysine and arginine residues in gelatin are much less. On the other hand, the chitosan has more average number of primary amine groups than of the gelatin in the reaction with the glutaraldehyde [48, 49]. Paula and his research group [37] prepared chitosan/cashew gum beads loaded with insecticide dichlorvos. They confirmed that the unloaded and loaded beads did not differ significantly in their swelling characteristics. However, in the current study we found that the unloaded capsules showed high swelling degree than the loaded. This result may be referring to the complex formation between the polymers that reduces the porosity of the capsules and decreases the sorption of the water into capsules.

3.5. Temephos *in vitro* and *in vivo* release kinetics

Chitosan capsules were loaded with 3.4% temephos as determined by UV spectrophotometer at λ_{max} 201 nm. A calibration curve given by the following equation: Absorbance = +0.129 C (mg/L), with 0.992 correlation coefficient. This equation was used for temephos determination. Figure 4 shows the *in vitro* release of temephos (mg /100 mL water) from prepared capsules in the running **(A)** and stagnant water **(B)** daily during a month and 40 days, respectively. On the first day, the highest quantity of temephos (0.74 mg) released in 100 mL water from the highest weight of capsules (0.05 g) in the running water followed by 0.59, 0.40, and 0.17 mg/100 mL water for 0.02, 0.01, and 0.005 g capsules, respectively (Figure 4A). After that, the quantities of the temephos released from all samples (0.005, 0.01, 0.02, and 0.05 g) significantly decreased with change of water daily. The temephos released reached to none detected by the UV spectrophotometric method on tenth day and at day 12 for 0.01 g of the capsules, while with 0.02 and 0.05 g, temephos continue to be detected up to twenty-one day.

In stagnant water experiment, the release kinetics of temephos from the capsules is shown in Figure 4B. The quantities of temephos released were increased rapidly from the four weights of the capsules (0.005, 0.01, 0.02, and 0.05 g) on the first day (0.20, 0.41, 0.61, and 0.74 mg/100mL, respectively). Moreover, the release was increased to the maximum values at seventh day (0.28, 0.47, 0.69, and 1.0 mg/100 mL for 0.005, 0.01, 0.02, and 0.05 g, respectively). After that it was decreased gradually to none detected at day 25, 26, 27, and 40 for 0.005, 0.01, 0.02, and 0.05 g of the capsules, respectively. It can be noted that, the quantities of temephos released were highly accumulated in stagnant water than running water experiment.

The *in vivo* release behaviour of temephos according to the larval mortality percentages for different weights of capsules (0.005, 0.01, 0.02, and 0.05 g) is shown in Figure 5 in running water (A) and stagnant water (B) experiments. In the two types of experiments, mortality percentage recorded 100% from the first day for all weights of capsules. The highest weight of capsules (0.05 g) exhibited 100% larval mortality up to the day 92 in the running water, but in stagnant water still achieved 100% mortality up to the day 120. This amount showed long effect in larval mortality (30% on 134 day) in the running water however, showed high activity up to 211 day (60%) in the stagnant water experiment. The capsule weights of 0.005 and 0.01 g showed gradually decreasing in the mortality up to zero at 36 day and 92 day in running and stagnant experiments, respectively. At the weight of 0.02 g, the larval mortality gradually decreased from 100% to zero on 43 day in the running water experiment. However, in stagnant water, the mortality still 100% throughout 43 day then decreased to 40% on 190 day and still effective (20% mortality) up to 211 day.

This result is in agreement with the results obtained previously by Paula and co-authors [37] who reported that dichlorvos [2,2-dichlorovinyldimethyl phosphate (DDVP)] loaded in chitosan and cashew gum beads resulted the highest larval mortality of *Aedes sp* with high masses of beads used. Mulla and co-authors [50] studied the *in vivo* release of Bti tablets and a zeolite granules formulation of temephos (1%) during 6 months against *A. aegypti*. They reported that 0.37 g of Bti/50 L of water provided control for about 90-112 days, whereas the temephos formulations at 5 g/50 L of water (100,000 ppm) yielded almost 100% control for more than 6 months. On the contrary, Toma and co-authors [51] prepared a Bti tablet formulation containing (34000 ppm) was reported to induce 100% larval mortality of *A. albopictus* after 24 h; however, the larvicidal activity lasted only about 48 h. Our study was also in agreement with Bhan and co-authors [28], who studied the encapsulation of temephos and imidacloprid on biodegradable and biocompatible polyethylene glycol in different ratios. They studied this formulation against *C. quinquefasciatus* and found that the encapsulated temephos was more toxic than the encapsulated imidacloprid with LC_{50} values of 0.013, 0.010 and 0.003 mg/L after 24, 48 and 72 h, respectively.

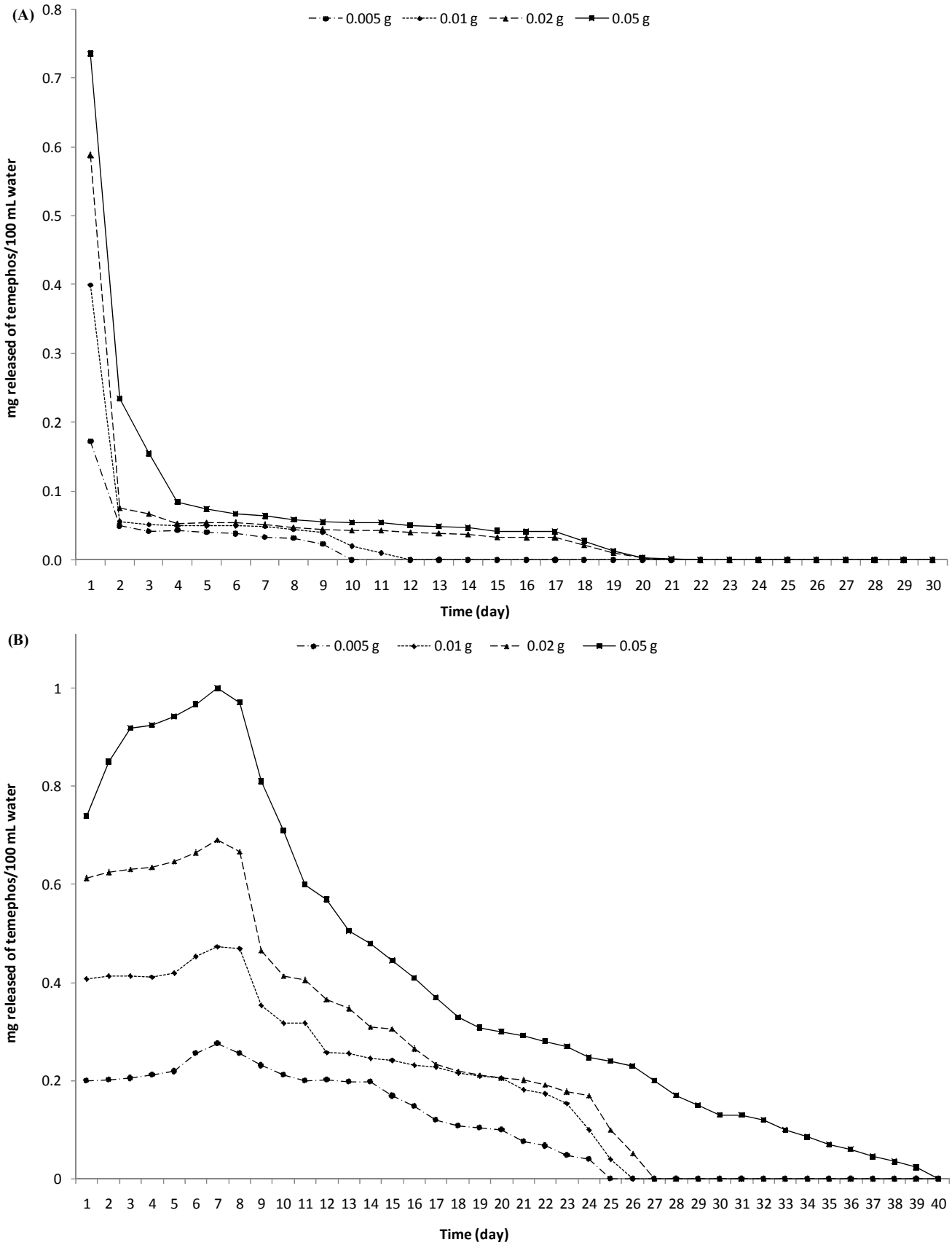

Fig 4: *In vitro* release kinetics of the larvicide temephos by UV spectrophotometric assay in running (A) and in stagnant water (B).
The *in vivo* release behavior of temephos according to the larval mortality percentages for different weights of capsules

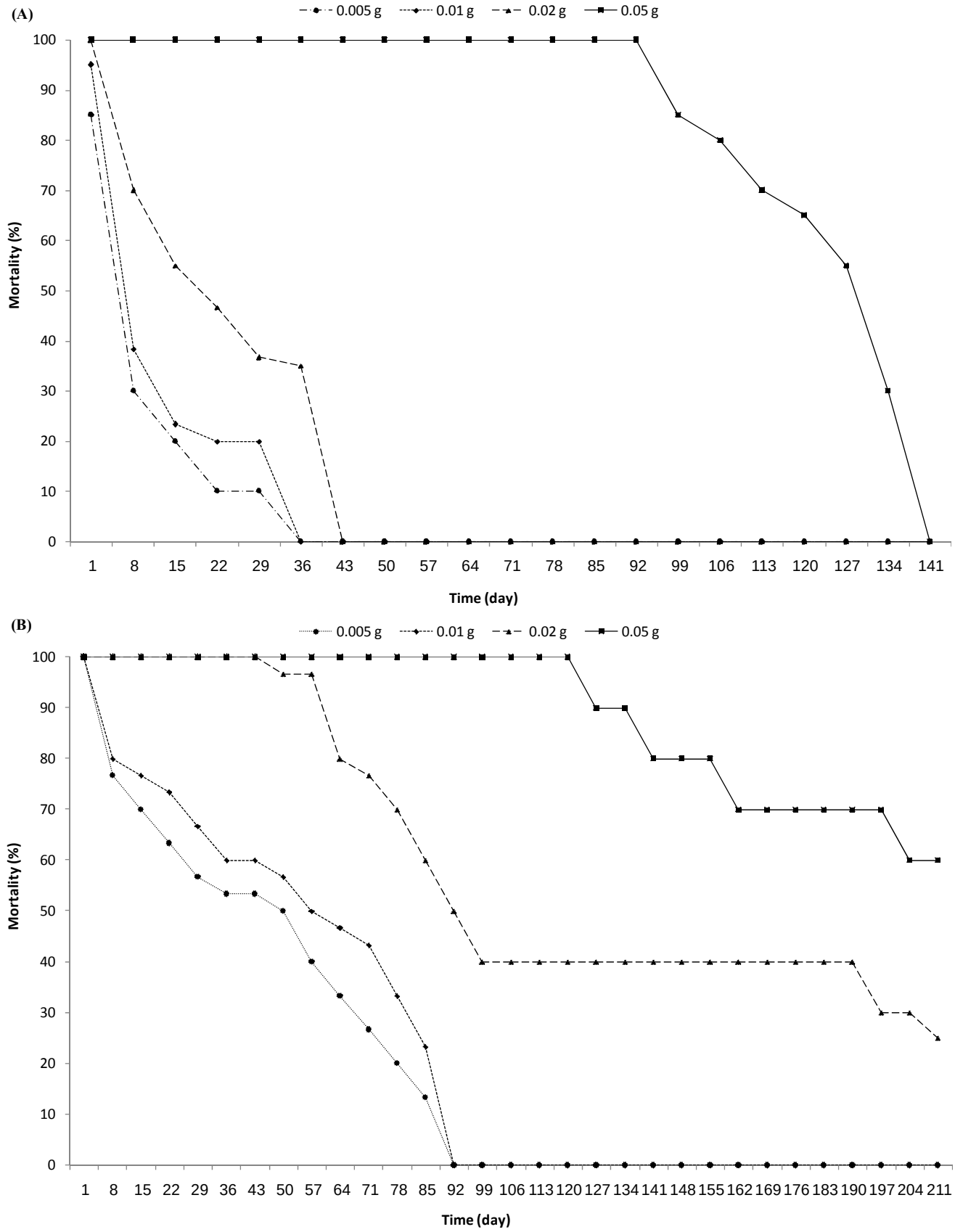

Fig 5: *In vivo* release kinetics according to mortality percentage for different weights of capsules (0.005, 0.01, 0.02, and 0.05 g) loaded with temephos (7.35%) calculated by U.V spectroscopy for a period of time. **(A):** In running water (exchange of larvae and water together) and **(B)**: in stagnant water (exchange of larvae only).

3.6. Biochemical studies

The dose response test of the technical insecticide temephos against *C. pipiens* showed LC_{50} value at 0.01 mg/L. This result confirmed the results obtained by Al-Sarar and co-authors [52], who reported that this value was 0.0059 mg/L against *Culex spp*. and Rey and co-authors [53] found it was 0.03, 0.15, and 0.07 mg/L for *A. Albopictus, A. Stephensi*, and *C. Pipiens*, respectively.

To explore some biochemical actions, the effect of the LC_{50} value on AChE, CaE and GST isolated from treated larvae of *C. pipiens* for 24 h was studied. The data are shown in Table 2 as specific activity (ΔOD. min^{-1}. mg $protein^{-1}$) and as inhibition percentages for each enzyme. The untreated larvae of *C. pipiens* have specific activity 5.49, 2.98, and 1.23 for AChE, CaE and GST, respectively. However, temephos reduced the specific activities to 0.88, 0.59, and 0.57, respectively for the same enzymes, respectively. It can be noted that the capsules loaded with temephos caused the highest inhibition percentage in the case of AChE (83.91%) than CaE (80.31%) and GST (53.23%). This result proved that the temephos as an organophosphorus insecticide is specific inhibitor of AChE. Multiple forms of AChE, which confer varying degrees of resistance, have been found in a variety of arthropods [54-56]. Our study is in agreement with the results obtained by Chen and co-authors [57] who studied non-specific esterases, mixed function oxidases, glutathione-S-transferases, and AChE and they found that the specific activities of AChE in *Ae. aegypti* larvae ranged from 0.08 to 0.13, from 0.19 to 0.2 for CaE, and 0.07 to from 0.15 foe GST at 10 mg/L formulated temephos. Vaughan and co-authors [58] found that paraoxon (an organophosphorus insecticide) gave 90% inhibition of AChE in *Ae. Aegypti*. Fourcy and co-authors [59] used AChE and CaE as biomarkers to assess the effects of Abate1500e (a.i. temephos) and Vectobac 112 AS (a.i. endotoxins of *Bacillus thuringiensis* var. israelensis, Bti) in Nereis (Hediste) diversicolor. Esterase inhibition revealed a marked impact of temephos and found that exposure to temephos resulted in decreased esterase (both AChE and CaE) activities in the worms collected either 24 or 72 h after treatment. AChE was less sensitive than CaE to pesticide exposure. This may be due to either a higher sensitivity of CaE to temephos, otherwise in our study CaE was less sensitive than AChE.

Table 2: Biochemical effects of temephos at LC_{50} value (0.01 mg/L) on the AChE, CaE, and GST in *C. pipiens* larvae after 24 h of the treatment

Treatment	Specific activity (ΔOD. min^{-1}. mg $protein^{-1}$) ± SE			Inhibition (%) ± SE		
	AChE	CaE	GST	AChE	CaE	GST
Control	5.49±0.2	2.98±0.08	1.23±0.09	0.00	0.00	0.00
LC_{50} (0.01 mg/L)	0.88±0.04	0.59±0.04	0.57±0.05	83.91±0.69	80.31±1.26	53.23±3.8

AChE: Acetyl coline esterase, **CaE:** Carboxyl esterase, **GST:** Glutathione S- transferase.

4. Conclusions

The biopolymer capsules based on a combination of chitosan, alginate, and gelatin were successfully prepared to envelop the most potent insecticide temephos for sustaining its release against *C. pipiens* larvae. Temephos release kinetics was evaluated by *in vitro* and *in vivo* assays. Both trials were achieved in running and stagnant water daily during a long period. The results proved that the prepared capsules exhibited high residual activities against the larvae for a long time to exceed six months. These results, together with a simple preparation technique, indicate the applicability of these biodegradable capsules as environmentally friendly materials for temephos carrier and controlling its release.

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