SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF NEW AMIDIC DERIVATIVES OF 5-NITROINDAZOL-1-YL ACETIC ACID ENCAPSULATED INTO ALGINATE/PECTIN PARTICLES

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New amidic compounds with biologic activity, derived from 5-nitroindazol-1-yl-acetic acid have been synthesised and their chemical structure was confirmed by elemental and spectral analysis (FT-IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry). The incorporation of some of the amides into sodium alginate and pectin based microcapsules, prepared by polymer ionotropic gelation in O/W emulsion determined the augmentation of their antibacterial potential against bacterial strains.

Keywords: 5-nitroindazol, amide, sodium alginate, pectin, microcapsules, antibacterial activity

INTRODUCTION

Sodium alginate and pectin are nontoxic, naturally occurring polysaccharides, which are used on a large scale in the pharmaceutical and biotechnology fields, the application in preparing controlled drug release systems being extensively exploited.[1] The cross-linking properties of sodium alginate and pectin with calcium ions have made possible their use as a matrix or membrane for the entrapment and/or delivery of a variety of drugs.[1-5]

It is thought that the chemical structure of pectin (the presence of hydroxyl, carboxyl and amide groups) is responsible for its mucoadhesivity towards gastrointestinal mucus,[6] depending on environmental pH. Resistant to the enzymes in the upper gastrointestinal tract, pectin can be digested in the colon.[7] In the case of orally administered drug delivery systems, the variation of environment pH all over the gastrointestinal tract (from acidic to alkaline) must be considered. Most research studies deal with designing drug vehicles responding to a rather large domain of pH.

Previous research reported the synthesis of 5-nitroindazole derivatives with various biological effects, such as antibacterial,[8] antituberculosis,[9] antipyretic[10] and even antitumoral.[11] Amidic derivatives are especially interesting, as amide pending groups are found in the chemical structure of various compounds effective in the antihelmitic,[12] antihistaminic,[13,14] antimicrobial,[15-18] antituberculosis,[19,20] antiinflamatory,[21,22] antiepileptic,[23] analgesic[24] and cytostatic[25-27] treatments. This paper is focused on the preparation of amide derivatives of 5-nitroindazol-yl-1-acetic acid and their encapsulation into pectin and alginate based microcapsules.

EXPERIMENTAL

Materials and methods

All reagents were used as purchased (Sigma-Aldrich, Fluka, Merck, S.C. Chemical Company S.A.). FT-IR and ^{1}H-NMR spectra were recorded using a FT-IR spectrophotometer (ATR) Brucker Tensor-27 and Brucker ARX 400 spectrometer (5 mm QNP probe; 1H/13C/31P/19F), respectively. Elemental analysis was performed on Exeter Analytical CE 440 elemental analyser. The melting points of the obtained compounds were determined using Mel-Temp melting point module, provided with digital thermometer. Particles size and morphology was evaluated using a Nitech Hitachi TM3000 scanning electron microscope and a Leica DM optical microscope. UV-vis analysis was performed using Nanodrop UV spectrophotometer.

Synthesis of amidic derivatives of 5-nitroindazol-1-yl acetic acid. General procedure and characterization

In a reaction flask provided with a refluxing cooler, equimolar (0.02 mol) quantities of ethyl ester of 5-nitroindazol-1-yl acetic acid and one of the following amines (monoethanol amine, diethanol amine, diethanol ethyl amine, isopropyl amine and alyl amine) were added in 100 mL anhydrous dioxane. The reaction mixture was maintained under reflux for 2-2.5 hours and then the excess of dioxane was removed by vacuum distillation. The amidic compounds were finally isolated upon cooling and purified by recrystallization from boiling ethanol.

Ethyl ester of 5-nitroindazol-1-yl acetic acid (I) was obtained from 5-nitroindazole and ethyl chloracetate in a solution of sodium ethoxyde.[9]

(β-hydroxyethyl)-amide of 5-nitroindazol-1-yl acetic acid (II)

Cream-white solid; yield: 87.5% (4.62 g); melting point: 192-193 °C. Anal. calcd. for $C_{11}H_{12}N_4O_4$: 50% C; 4.54% H; 21.21% N. Found: 50.49% C; 4.93% H; 21.58% N. FT-IR (ν cm^{-1}): 3125 (NH); 3300-3363 (OH); 1624 (C=O); 1334 (NO_2 symmetric); 1566 (NO_2 asymmetric); 1512 (C=N); 1078 (C-OH). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 3.12-3.16 (t, 2H, CH_2); 3.60-3.64 (m, 2H, CH_2); 5.12 (s, 1H, OH); 7.82-7.85 (d, 1H, Ar); 8.11-8.13 (d, 1H, Ar); 8.27 (s, 1H, Ar); 8.46 (s, 1H, Ar); 8.91 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 400 MHz), δ (ppm): 62.61 (CH_2); 65.27 (CH_2); 111.58; 119.81; 124.02 (Ar); 44.73; 135.15; 144.97; 147.52 (C-N); 169.87 (C=O). MS, m/z: 264 (M^+, 35%); 287 (M+Na, PB); 303 (M+K, 19%).

Di(β-hydroxyethyl)-amide of 5-nitroindazol-1-yl acetic acid (III)

White solid; yield: 84.74% (5.22 g); melting point: 123-125 °C. Anal. calcd. for $C_{13}H_{16}N_4O_5$: 50.65% C; 5.19% H; 18.18% N. Found: 51.02% C; 5.63% H; 18.47% N. FT-IR (ν cm^{-1}): 3414 (OH); 1622 (C=O); 1346 (NO_2 symmetric); 1517 (NO_2 asymmetric); 1484 (C=N); 1071 (C-OH). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 3.32-3.37 (t, 2H, CH_2); 3.48-3.49 (d, 2H, CH_2); 3.56-3.59 (t, 2H, CH_2); 3.66-3.67 (d, 2H, CH_2); 4.69 (s, 1H, OH); 5.13 (s, 1H, OH); 5.62 (s, 2H, CH_2); 7.75-7.77 (d, 1H, Ar); 8.27-8.29 (d, 1H, Ar); 8.38 (s, 1H, Ar); 8.92 (s, 1H, Ar). ^{13}C-NMR (DMSO-d_6, 400 MHz), δ (ppm): 54.57 (CH_2); 61.51(CH_2); 68.83 (CH_2); 111.14; 119.28; 121.09; 122.47 (Ar); 134.33; 141.83; 142.49 (C-N); 168.48 (C=O). MS, m/z: 309 (M+H, 55%); 331 (M+Na, 40%); 639 (2M+Na, PB).

β-Diethylaminoethyl-amide of 5-nitroindazol-1-yl acetic acid (IV)

White solid; yield: 78.84% (5.03 g); melting point: 119-121 °C. Anal. calcd. for $C_{15}H_{21}N_5O_3$: 56.42% C; 6.58% H; 21.94% N. Found: 56.75% C; 6.77% H; 22.25% N. FT-IR (ν cm^{-1}): 2970-3103 (NH); 1618 (C=O); 1339 (NO_2 symmetric); 1523 (NO_2 asymmetric); 1458 (C=N). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.90-0.94 (t, 6H, $2CH_3$); 2.42-2.47 (m, 6H, $3CH_2$); 3.12-3.17 (m, 2H, CH_2); 5.20 (s, 2H, CH_2); 7.80-7.83 (d, 1H, Ar); 8.08-8.11 (d, 1H, Ar); 8.26 (s, 1H, Ar); 8.40 (s, 1H, Ar); 8.92 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 400 MHz), δ (ppm): 15.75 (CH_3); 53.46 (CH_2); 63.17(CH_2); 111.38; 119.62; 123.77 (Ar); 134.33; 47.37; 56.45; 135.36; 145.08; 147.67 (C-N); 169.65 (C=O). MS, m/z: 320 (M+H, PB); 342 (M+Na, 12%); 358 (M+K, 26%).

Isopropyl-amide of 5-nitroindazol-1-yl acetic acid (V)

White solid; yield: 79.19% (4.15 g); melting point: 173-175 °C. Anal. calcd. for $C_{12}H_{14}N_4O_3$: 54.96% C; 5.34% H; 21.37% N. Found: 55.34% C; 5.71% H; 21.55% N. FT-IR (ν cm^{-1}): 2986 (NH); 1615 (C=O); 1336 (NO_2 symmetric); 1507 (NO_2 asymmetric); 1494 (C=N). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.18-1.22 (m, 6H, CH_3); 2.25-2.30 (m, 1H, CH); 5.51 (s, 2H, CH_2); 7.87-7.90 (d, 1H, Ar); 8.24-8.27 (d, 1H, Ar); 8.44 (s, 1H, Ar); 8.84 (s, 1H, Ar); 8.97 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 400 MHz), δ (ppm): 2.75 (CH_3); 66.47 (CH_2); 111.76; 119.44; 123.69 (Ar); 49.47; 134.97; 145.56; 147.61 (C-N); 169.22 (C=O). MS, m/z: 263 (M+H, 7%); 285 (M+Na, 31%); 547 (2M+Na, PB).

Alyl-amide of 5-nitroindazol-1-yl acetic acid (VI)

Light yellow solid; yield: 71.15% (3.7 g); melting point: 168-169 °C. Anal. calcd. for $C_{12}H_{12}N_4O_3$: 55.38% C; 4.61% H; 21.53% N. Found: 55.73% C; 4.78% H; 21.81% N. FT-IR (ν cm^{-1}): 2947, 3095 (NH); 1622 (C=O); 1337 (NO_2 symmetric); 1533 (NO_2 asymmetric); 1490 (C=N). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.02-1.04 (d, 2H, CH_2); 1.18-1.22 (m, 1H, CH); 4.13-4.18 (m, 2H, CH_2); 5.51 (s, 2H, CH_2); 7.72-7.74 (d, 1H, Ar); 8.18-8.20 (d, 1H, Ar); 8.41 (s, 1H, Ar); 8.84 (s, 1H, Ar); 9.01-9.03 (d, 1H, NH). ^{13}C-NMR (DMSO-d_6, 400 MHz), δ (ppm): 47.34 (CH_2-N); 66.25 (CH_2); 111.63; 119.57; 120.68; 123.72 (Ar);

134.8 (CH=); 137.88; 145.71; 147.20 (C-N). MS, m/z: 260 (M^+, PB); 283 (M+Na, 12%); 299 (M+K, 11%).

Preparation of sodium alginate and pectin based microcapsules

Alginate/pectin particles were prepared using a polymer crosslinking O/W emulsion technique. O/W emulsion was obtained by adding dropwise 5 mL drug solution in benzyl alcohol (10 mg/mL) into 30 mL (1%; wt) polymer (pectin/alginate = 30/70; wt/wt) aqueous solution, in the presence of 2% (wt) Tween80, under vigorous stirring (UltraTurrax, 8000 rpm). After 20 minutes of emulsification, 30 mL $CaCl_2$ solution (0.1 M) were added dropwise for polymer crosslinking and the suspension was left under mild stirring (500 rpm) another 120 minute for polymer curing. Finally, the particles were recovered by decantation and then washed twice with distilled water and freeze dried overnight at 30 °C.

Particle swelling in aqueous environment

The swelling capacity of microcapsules at different pH was evaluated gravimetrically by incubating 15 µg blank particles into 1 mL aqueous solution at pH 4 and 6.8, at 37±0.5 °C for 24 hours. At predetermined time intervals, the suspension was centrifuged (10000 rpm; 8 minutes) and the swollen microcapsules accurately weighed. Then 1 mL swelling medium was added and the sample was vigorously agitated. The swelling ratio was calculated using Equation 1:

Swelling ratio=[(weight of swollen particles-weight of dry particles)/weight of dry particles] x 100

Determination of drug loading

Drug loading into alginate/pectin microcapsules was calculated using Equation 2:

Drug loading = Amount of incorporated active principle/initial amount of active principle.

The amount of drug incorporated into polymer particles was determined by UV-vis spectroscopy. Briefly, a known quantity of microcapsules was suspended in 1% (w/v) phosphate buffer solution (pH 7.4), under stirring, until complete disolvation of microparticles. Then, the drug was extracted into acetonitrile (30 minutes, under stirring). After centrifuging the mixture (10000 rpm; 15 minutes), the supernatant was analysed in triplicate by UV-vis spectroscopy, considering the drug standard curves in acetonitrile/phosphate buffer mixture.

***In vitro* drug release**

The release of incorporated drugs from alginate/pectin particles was studied in aqueous environment of different pH (4 and 6.8), under sink conditions. Approximately 50 µg of drug loaded microcapsules were introduced into cellulose dialysis bags and then suspended in 75 mL aqueous solutions under mild stirring (150 rpm) at 37 °C. At predetermined time intervals small aliquots of release medium were withdrawn and analysed by UV-vis. All determinations were made in duplicate.

Drug release efficiency was calculated, as follows:

Drug release (%)= Amount of released drug/amount of loaded drug .

Toxicity evaluation

The toxicity of the synthesized amide derivatives and drug loaded polymer particles was evaluated following their intraperitoneal administration as suspensions in Tween80 to groups of 14 mice (20±5 g), according to Karber method.[28] Mice were monitored and their mortality after 7 days was noted; thus, calculating LD_{50}.

The evaluation of antimicrobial activity was performed on standard microbial strains, such as *Staphylococcus aureus* ATCC-25923, *Bacillus subtilis* ATCC-6638, *Bacillus cereus* ATCC-10876, *Escherichia coli* ATCC-25922 and *Salmonella Entiritidis* P1131, from the Bacterial Library of the Institute of Public Health of Iasi (Microbiology Laboratory), according to NCCLS international standards.[29]

The strains were grown on 1% glucose containing gelose media, thermostated at 37 ºC. Bacterial suspensions (1/100) in sterile physiological saline serum were inoculated into previously sterilized growth media. Then, different drug amounts (at concentrations of 250, 500 and 1000 µg/mL growth medium) were dissolved in dimethylformamide (solvent which has no influence on the microbial growth) and the formed solutions (100 mL) were mixed with the bacterial growth media and sterilized for 20 minutes at 120 ºC. Two control batches were used: growth medium with dimethylformamide and growth medium with no drug or dimethylformamide. Microbial growth was evaluated 24 and 48 hours after inoculation.

RESULTS AND DISCUSSION

Synthesis of active principles

Amidic derivatives were synthesised using the ethylic ester of 5-nitroindazol-1-yl-acetic acid (I) as intermediate,[9] which was condensed with various amines: monoethanol amine, diethanol amine, diethanol ethyl amine, isopropyl amine and alyl amine (Figure 1). The reaction was performed by refluxing the components mixture for 2-2.5 hours, in anhydrous dioxane.

The structure proposed for the synthesised compounds (II-VI), based on chemical analysis, was confirmed by spectral data (FT-IR, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy). For amides II and IV-VI, FT-IR spectra show at 2947-3363 cm^{-1} absorption bands characteristic of –NH-; intense

bands specific to carbonyl groups appear at 1615-1624 cm^{-1} in all spectra.

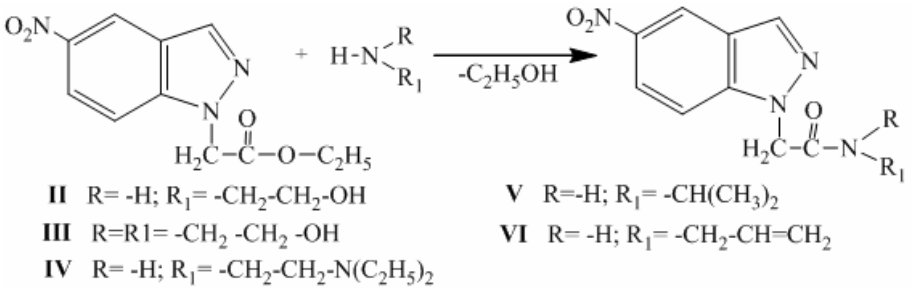

Figure 1: Synthesis of amides of 5-nitroindazol-1-yl-acetic acid

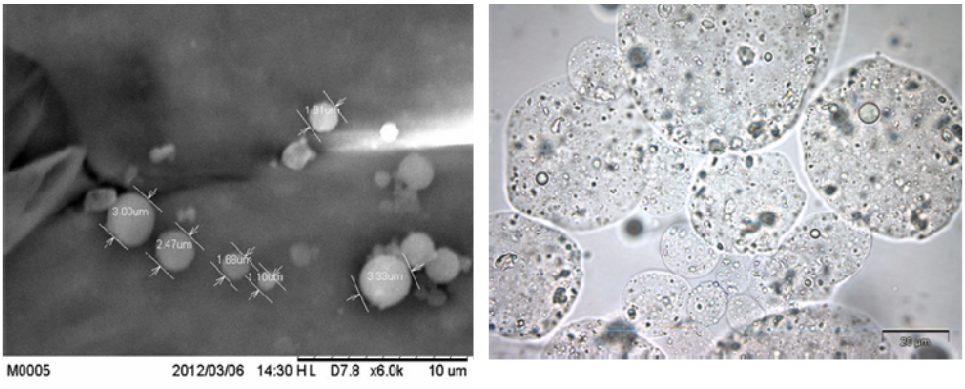

Figure 2: SEM image (left) and optical microscopy image (right) of alginate/pectin microcapsules in dry state and dispersed in distilled water, respectively

Also, symmetric and asymmetric vibrations of NO_2 are shown at 1334-1346 cm^{-1} and 1507-1566 cm^{-1}, respectively. The presence of OH group in the hydroxyethyl-amides II and III is shown in FT-IR spectra by large absorption bands at 3300-3414 cm^{-1}. ^{1}H-NMR spectra prove the presence of various structural groups. In the aliphatic area, CH_2 and CH_3 groups can be identified. The signal specific to NH proton appears at 8.91-9.03 ppm, to aromatic protons at 7.72-8.84 ppm and to OH at 4.69-5.13 ppm. ^{13}C-NMR spectra show signals at 111.14-123.77 ppm attributed to heterocyclic carbons and at 53.45-68.83 ppm attributed to aliphatic carbons. C-N group and C=O are proved by signals at 44.73-147.67 and at 168.48-169.87 ppm, respectively. Mass spectroscopy also confirmed the proposed chemical structures for II-VI. All spectra show signals after additions of sodium (M+Na) at m/z values of 287, 639, 342, 547 and 283, of which some are base peaks. The peaks specific to protonated molecules are also shown in the spectra.

Preparation of polymer microcapsules

Polymer microcapsules have been prepared by ionotropic gelation of sodium alginate and pectin with calcium chloride. Benzyl alcohol has been used as an organic solvent for the synthesised active principles; this polar solvent presents low toxicity and bacteriostatic and antipruritic properties. Spherical shaped particles with sizes of about 1-3 µm were obtained. Optical images (Figure 2) show the polymer particles in swollen state as aqueous suspensions.

Being polyelectrolyte, alginate and pectin are susceptible to swelling properties, sensitive to pH variations. The ability of the particles to swell in acid media was evaluated by the gravimetric method during 24 hours, by suspending them in aqueous solutions of pH 4 and 6.8, simulating, thus, the conditions of the gastrointestinal tract. Higher swelling degrees were noticed while increasing the pH of the aqueous medium. After 24 hours, the alginate/pectin particles swelled ~350 times their weight in simulated intestinal

conditions (pH 6.8), favouring drug release by diffusion and/or particle disintegration.

(β-hydroxyethyl)-amide of 5-nitroindazol-1-yl acetic acid (II) and di(β-hydroxyethyl)-amide of 5-nitroindazol-1-yl acetic acid (III) were loaded into alginate/pectin particles in relatively high ratios, compared to the initial amount of active principle. Amide II and amide III were found at 61.88% and 84.73%, respectively, in the polymer matrix. Drug loss could be caused by repeated particle washings.

Table 1
Toxicity evaluation of active principles and drug loaded particles

Compound	LD_{50} (mg/kg body)	Administration
II	8450	Intraperitoneal on mice
III	8870	
IV	7740	
V	7380	
VI	7250	
Microparticles loaded with II	8640	
Microparticles loaded with III	9114	

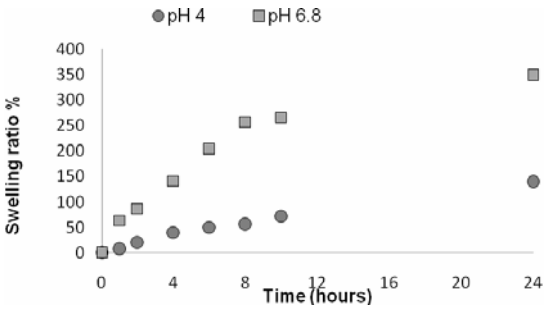

Figure 3: Swelling ratio of alginate/pectin microcapsules in aqueous environment of different pH

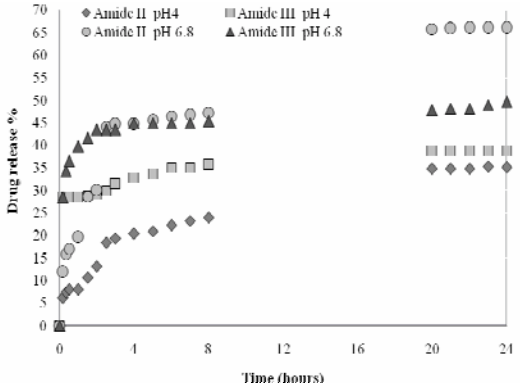

Figure 4: Drug release profiles from polymer particles

The addition of pectin to the alginate matrix to the formation of particles was done in order to delay or sustain drug release. It has been reported that the addition of pectin to alginate enhances the rate of drug release in acidic environment, also influencing the cross-linking degree.[30]

Drug release and particle swelling studies were performed in acidic media, simulating gastrointestinal conditions. A more efficient drug release has been noticed at pH 6.8, than at pH 4; more than 66% of the included active principle (amide II) was released after 24 hours. In all cases, a significant drug release was noticed in the first hour, followed by a slower sustained release. This could be explained by the initial rapid release of the drug found on the external side of polymer particles, followed by drug diffusion from the core of the polymer particles. Moreover, drug releasing profiles seem to follow particle swelling tendency.

***In vivo* biologic activity**

The evaluation of the biologic activity for the synthesised amide derivatives (II-VI) and polymer particles loaded with compounds II and III consisted in determining the toxicity and antibacterial effect of the new amides of 5-nitroindazol-1-yl acetic acid (II-VI) and alginate/pectin particles loaded with compounds II or III (Table 1).

Toxicology data show that all synthesised amide derivatives are practically nontoxic, even higher LD_{50} values being noted for alginate/pectin based particles loaded with compounds II and III, due to the drug protection by biocompatible and nontoxic polymer matrix.

Connections between the chemical structure of the newly synthesised amide derivatives and their potential biologic activity were established by evaluating their antimicrobial activity (Table 2). Under experimental conditions, the amide derivatives (II-VI) and the polymer particles loaded with II and III are biologically active at small concentrations (250 μg/mL) against *B. subtilis* and *B. aureus*. No antimicrobial activity was registered in the case of *S. aureus* and *E. coli*.

Table 2
Antibacterial spectrum of amide derivatives (II-VI) of 5-nitroindazol-1-yl acetic acid and polymer particles loaded with compounds (II) and (III)

Compound	Time (hours)	*S. aureus*			*B. subtilis*			*B. cereus* (μg/ml)			*E. coli*			*S. entiritidis*		
		1000	500	250	1000	500	250	1000	500	250	1000	500	250	1000	500	250
II	24	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+
	48	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+
III	24	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+
	48	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+
IV	24	+	+	+	-	-	-	-	-	-	+	+	+	-	-+	-+
	48	+	+	+	-	-	-	-	-	-	+	+	+	-+	-+	-+
V	24	+	+	+	-	-	-	-	-	-	+	+	+	-	-+	-+
	48	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
VI	24	+	+	+	-	-	-	-	-	-	+	+	+	-+	+	+
	48	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
II loaded particles	24	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-
	48	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-
III loaded particles	24	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-
	48	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-
Control batch	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

- No bacterial growth; -+ Moderate bacterial growth; + Normal bacterial growth

The development of *S. entiritidis* was found to be inhibited by (β-hydroxyethyl)-amide of 5-nitroindazol-1-yl acetic acid (II) and di(β-hydroxyethyl)-amide of 5-nitroinzalol-1-yl acetic acid (III) 24 and 48 hours after inoculation at a drug concentration of 1000 μg/mL. alginate/pectin based particles loaded with compounds II and III presented biologic activity against *S. entiritidis* at much lower concentrations (250 μg/mL) over 48 hours, than the respective amides in free form. Thus, drug encapsulation into polymer particles determined the enhancement of antibacterial activity, in comparison with the free from of the compounds. β-diethylaminoethyl)-amide of 5-nitroinzalol-1-yl acetic acid (IV) and isopropyl-amide of 5-nitroindazol-1-yl acetic acid (V) determined only moderate inhibition of bacterial growth over a period of 48 hours at high concentrations.

CONCLUSION

New amidic compounds with antibacterial activity have been synthesised starting from ethyl ester of 5-nitroindazol-1-yl acetic acid. Their chemical structure was confirmed by elemental and spectral analysis (FT-IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry). All active principles proved to be nontoxic and to present antimicrobial activity against *B. subtilis* and *B. cereus* at small concentrations and against *S. entiritidis* at higher concentrations.

There have been prepared sodium alginate and pectin based microparticles of around 1-3 μm by polymer ionotropic gelation with calcium chloride in O/W emulsion. The incorporation of two amide derivatives into the polymeric particles proved an increase of their antibacterial potential against *S. enteritidis* at lower concentrations, compared to their form. *In vitro* drug release profiles seem similar to particle tendency to swell in aqueous media, an initial rapid drug release being noticed, followed by a slower, sustained release.

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