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ELABORATION OF BEESWAX MICROSPHERES LOADED BY PURE AND COMPLEXED NIFLUMIC ACID WITH β-CYCLODEXTRIN AS NOVEL LIPID-CONTROLLED DRUG RELEASE FORMULATIONS

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ABSTRACT. Controlled release systems based on natural lipid beeswax and loaded with Niflumic acid (NA) or inclusion complexes of NA with β-cyclodextrin are developed with the aim of simultaneously increasing the drug solubilisation, reducing the gastric side effects, and controlling the drug release. Simple and Green methods are used; firstly, the inclusion complexes of NA:β-CD (1:1) are prepared by physical grinding, co-evaporation, and co-precipitation. Secondly, the beeswax microspheres are elaborated by the hot-melt technique of microencapsulation using three emulsifying agents: PVA, Tween 20, and Tween 80. The formulations are analysed by FTIR, XRD, DSC, SEM, and optical microscopy. Depending on the complex type and surfactant agent, microencapsulation efficacy ranged from 50 to 96%. The *in-vitro* drug dissolution study in acidic fluid at pH = 1.2 and 37 °C showed different release profiles. So, the effect of surfactant is highlighted, and a remarkable efficacy of the combination of inclusion complex and microencapsulation using beeswax is perceived for simultaneously increasing the drug solubility and controlling the drug release.

KEY WORDS: Beeswax, Niflumic acid, β-Cyclodextrin inclusion complex, Microencapsulation, Drug delivery

INTRODUCTION

In drug delivery system development, lipid-based excipients include a large variety of products derived from vegetable oils, fatty acids, or waxes [1, 2]. The use of lipids as excipients has attracted long interest because of their various roles and benefits [3], such as their stability and efficacy [4], vectorization and targeting of the action's site [5], increasing drug solubilisation [6], prolonging and controlling the drug delivery [7], and particularly for their biocompatibility and biodegradability [8].

Beeswax is a natural lipid product that finds specific uses in pharmaceuticals, cosmetics, food, and other industries [9, 10]. Beeswax has a complex composition including various components classified in esters and di-esters of fatty acids (15-27%), free fatty acids (12-14%), fatty alcohols (1%), hydroxymonoesters (35-45%), hydrocarbons (12-16%) and others components such as exogenous molecules, linear wax monoesters, vitamins and minerals [10]. Many studies have demonstrated the beeswax benefits, such as its antimicrobial (antibacterial, antifungal) activity [11], anti-inflammatory activity [12-14], and anti-ulcer efficacy [15]. So, for these several beneficial properties of beeswax, our research is focused on its implication as biomaterial matrix for the preparation of microparticle systems loaded with niflumic acid.

Niflumic acid (NA) or 2-(ααα-trifluoro-m-toluidino) nicotinic acid is a poorly water soluble organic acid [16], it is known in the class of non-steroidal anti-inflammatory drugs (NSAIDs). In

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the pharmaceutical industry, the market is continuously growing because of its effective anti-inflammatory, analgesic, and antipyretic actions and the development of novel and innovative NA formulations [17].

NA is prescribed for rheumatic disorders, joint and muscle pain, post-surgery pain, and fever [16], and it has been shown to have an anti-cancer effect by combining it with ligands [18], an anti-fungal effect [19], and also its effectiveness compared to other NSAIDs has been affirmed [20].

Hence, by oral administration, NA causes side effects and gastrointestinal complications with severity ranging from simple dyspepsia, gastropathy, gastric bleeding leading to surgical intervention or death, depending on the presence of one or more risk factors such as age, history of peptic ulcer, and concomitant use of aspirin [21, 22].

To reduce these noticeable side effects and optimize the drug therapeutic action which ensure patient comfort, several strategies have been developed, including complexation with cyclodextrins [23-27] or with metals [28-31], crystallization by rapid microwave-assisted evaporation [32] or solvent diffusion/solvent evaporation [33], development of polymeric particles by microencapsulation process using cellulose derivative matrices [34] or by nozzle-free electrospinning technique using polyvinylpyrrolidone as excipient material [35].

Among these methods, the formulation by encapsulation is a promising strategy which is used in various domains to reduce or even eliminate adverse effects of active molecules given its ability to modify and control the release kinetics, to improve physicochemical properties and the targeting of the action's site, in addition to the control of problems and obstacles related to the drugs formulation (bitter taste, instability, etc.) [36-37].

By choosing the microencapsulation method and beeswax as a lipid coat material, the present research is intended to produce beeswax microspheres composed of pure NA or complexed NA to obtain controlled release formulations. The project consisted of improving the NA water solubility and consequently its bioavailability by producing β-cyclodextrin complexes. In addition, the effect of the combination of complexation and microencapsulation on the NA release profile is evaluated. So, complexes of NA and β-cyclodextrin are prepared according to three methods, i.e., physical grinding, co-evaporation, and co-precipitation, and the controlled release beeswax formulations are elaborated by the hot-melt technique of microencapsulation. As a result, the effect of the lipid matrix, surfactant agent, and the NA complexation on the drug release is highlighted in this paper.

EXPERIMENTAL

Chemicals

Niflumic Acid(NA) was purchased from Sigma-Aldrich, Tween 20 and polyvinyl alcohol (PVA), PVA-124 from BIOCHEM Chemopharma, and Tween 80 from Loba Chemie. Ethanol absolute (99.9% of purity) was provided by VWR BDH Chemicals. Beeswax originates from the west of Algeria and is purified by the fusion-solidification process.

Preparation of β-cyclodextrin:niflumic acid (NA:β-CD) complexes

Three complex formulations of NA with β-cyclodextrin at a 1:1 molar ratio of NA: β-CD are prepared by physical mixture (Cp1), co-precipitation (Cp2), and co-evaporation (Cp3).

The complex Cp1 is obtained by simple co-grinding of the NA with the β-CD manually in an agate mortar. Cp2 and Cp3 are prepared by the complexation in solution. So, two similar solutions are prepared with a 1:1 molar ratio of NA:-CD. Each one is obtained by adding drop wise NA solution composed of 0.8466 g of niflumic acid dissolved in 40 mL of absolute ethanol to a β-CD

solution previously prepared by dissolving 3.405 g of β-cyclodextrin in 100 mL of distilled water at 50°C under magnetic stirring (800 rpm) for 4 hours.

Cp2 is collected from a solution that is left in the refrigerator for 24 hours, then filtered and dried in desiccators, and Cp3 is obtained from the other solution, which is immediately evaporated using a BUCHI Rotavapor R-210 at 60°C.

Preparation of beeswax microspheres

Beeswax microspheres containing pure NA or NA: β-CD complexes are elaborated by the hot-melt technique of microencapsulation. 0.5 g of a pure NA or NA: β-CD complex is dispersed in 2 g of beeswax already melted using a water bath regulated at 90 °C. The obtained mixture is then emulsified in 120 mL of a hot aqueous solution (90 °C) previously prepared using 1% of surfactant (PVA or Tween 20 or Tween 80) under vigorous mechanical stirring at 800 rpm using a DLS stirrer. The emulsion is maintained for 20 min until the solution is cooled at room temperature; the solid microspheres are then recovered after filtration and washed several times with distilled water and left to dry at room temperature during 48 hours.

Determination of encapsulation efficiency and drug content

The drug content is determined by an extraction procedure in absolute ethanol; so, 5 mg of microspheres (or complex inclusion) are dispersed in 10 mL of absolute ethanol and placed in a hermetically sealed glass vial under magnetic stirring and heating at a temperature range of 40-50°C for 30 minutes, and then left under stirring for 24 hours at room temperature. The suspensions are filtered to remove the matrix, and the recovered solution is analysed by a UV-visible spectrophotometer UV-2401 PC SHIMADZU at λ_{max} = 341 nm (ε = 0.0157 mg^{-1} cm^{-1} L). The experiments are carried out in triplicate.

The drug content and encapsulation efficiency (EE) are calculated according to the following equations:

$$\text{Encapsulation efficiency (EE)} = \left(\frac{weight of extracted\ active\ substance}{initial\ weight\ of\ active\ substance\ used}\right) \text{X } 100 \quad (1)$$

$$\text{Drug content} = \left(\frac{weight\ of encapsulated\ (extracted) active\ substance}{weight\ of\ microspheres}\right) \text{X } 100 \quad (2)$$

The microencapsulation yield is the ratio between the weight of recovered microspheres or complexes after encapsulation or complexation and the weight of the initial materials.

Microparticle's size measurement

The size of microspheres is measured by optical examination of at least 450 microspheres of each formulation batch, using an optical microscope equipped with a camera and Optika Vision Lite 2.0 measuring software. The diameters of the microspheres are recorded in an Excel sheet and divided into classes with a center indicated by <di>. The statistical calculations of the frequencies, as well as the polydispersity of the formulations, are accomplished using the equations mentioned below:

A number mean diameter is $d_n = d_{10} = \frac{\Sigma nidi}{\Sigma ni}$ (3)

A volume mean diameter is $d_w = d_{43} = \frac{\Sigma nidi^4}{\Sigma nidi^3}$ (4)

Size distribution is $\delta = \frac{d_{43}}{d_{10}}$ (5)

Microspheres and complexes' characterization

Morphological aspects and the surface of microspheres and complexes are observed using the scanning electron microscope (Hitachi). The interaction between NA and β-cyclodextrin or beeswax is studied using IR-ATR (Alpha Bruker) in the range of wavenumber of 500-4000 cm^{-1}. For analysis, the spectra of pure NA, β-CD, beeswax, NA: β-CD complexes, and microspheres are recorded and discussed.

The crystallinity of the encapsulated active substance is illustrated by X-ray powder diffractometry (RIGAKU Ultima IV X-RAY DIFFRACTOMETER) in the diffraction angle range of 2θ between 0 and 50°. Also, by Differential Scanning Calorimeter, pure NA, β-CD, beeswax, NA-β-CD complexes, and microspheres are analysed using DSC-60 plus SHIMADZU, to detect stability and potential interactions between the active substance and the encapsulating materials, the spectra are recorded after samples heating in the temperature range from 25 °C to 250 °C.

In vitro drug release study

The dissolution tests are performed in triplicate for 6 hours. The *in vitro* release kinetics of NA is carried out by introducing 100 mg of microspheres or inclusion complex in an Erlenmeyer flask containing 900 mL of dissolution medium of pH = 1.2 ± 0.01 (obtained by adding 2 g of NaCl, 80 mL of 1N HCL in 1L of distilled water) under magnetic stirring (200rpm) and plunged in a bath at a constant temperature of 37°C.

Samples are withdrawn at different time intervals to monitor the kinetics; the taken volume is renewed by an equivalent volume of fresh dissolution medium. The absorbance is measured at a wavelength of 254 nm (Ɛ = 0.01041 $L.mg^{-1}.cm^{-1}$) using a UV 9200 SHIMADZU UV spectrophotometer.

Drug release kinetic modeling

In order to elucidate the drug release mechanism from all formulations, three models are explored for analysis [38]: The zero-order release kinetics related to a constant drug release independently of the concentration is expressed by:

$$Q_t = Q_0 + k_0 t \tag{5}$$

Where Q_t is the amount of drug released or dissolved at time t, Q_0 is the initial amount of drug in solution (it is usually zero), and K_0 is the zero-order release constant.
The simplified Higuchi Model illustrating the Fickian diffusion of a drug from an insoluble matrix is given by:

$$\frac{Q_t}{Q_0} = k_H\sqrt{t} \tag{6}$$

Q_t/Q_0: is the fractional drug released at time t, and k_H is the Higuchi dissolution constant.
Finally, the empirical equation of the Korsmeyer-Peppas Model is a mathematical tool for analyzing both Fickian and non-Fickian mechanisms of drug release from swelling as well as non-swelling polymeric delivery systems. The applied equation is:

$$\frac{\boldsymbol{Q_t}}{\boldsymbol{Q_0}} = k\ t^n \tag{7}$$

k is the release rate constant connected to structural and geometric characteristics of the dosage form, n is the release exponent, its value predicts of the transport mechanism of drug through the matrix [38, 39]; in fact, for spherical and non swellable system, n=0.43 indicates that the release

mechanism is governed by a Fickian diffusion, if $0.43 < n < 0.85$, the release mechanism is considered according to a non-Fickian diffusion or anomalous transport and both the diffusion and relaxation or erosion are involved. For n = 0.85 and above 0.85, the Case-II and super Case-II of transport are involved, respectively. Under the value of 0.5, the drug release mechanism is associated with the quasi-Fickian diffusion model.

RESULTS AND DISCUSSION

Inclusion complexes and beeswax microspheres characterization

As described in the experimental part, NA:β-CD inclusion complexes are prepared according to three methods; the corresponding results are given in Table 1. Obviously, it is remarked that the drug inclusion in β-CD decreased for the co-evaporation method, and consequently, the stoichiometry or molar ratio of (guest: host) dropped to (1:1.41) for the complex formulation (Cp3). Regarding the co-precipitation inclusion method, the molar ratio of Cp2 is almost unchanged, but the yield mainly decreased.

Table 1. Results of (NA:β-CD) inclusion complexes characteristics (yield, drug content, encapsulation efficiency, and experimental molar ratio) prepared with an initial molar ratio of NA:β-CD (1:1).

Complex	Yield (%)	Drug content (%) ± ΔS (n=3)	EE (%) ± ΔS (n=3)	Experimental molar ratio of NA:β-CD
Cp1	100	20	100	1 :1
Cp2	54.1	19.20 ± 0.01	96.00 ± 0.04	1 :1.05
Cp3	92.4	15.03 ± 1.21	75.15 ± 6.35	1 :1.41

The entrapment of pure NA and also NA:β-CD inclusion complexes in Beeswax-based microspheres is conducted using three emulsifier agents of different hydrophily i.e PVA (HLB = 18), Tween20 (HLB = 16.7), and Tween80 (HLB = 15), since the surface agents can affect the surface tension and influence the drug formulation and solubility [40, 41]. The results of microencapsulation of pure NA and complexes in beeswax are gathered in Table 2.

By comparing pure NA beeswax microspheres (L1-L3), we noticed that using the two surfactants, Tween 20 and Tween 80, for L2 and L3 formulations, respectively, high drug contents and encapsulation efficiencies are obtained. So, the results confirm that these surfactant agents are good dispersants for beeswax material and prevent the drug transfer and migration to the external phase as reported in previous research [40, 41]. The low values of drug content and encapsulation efficiency observed using PVA (L1) can be explained by the high hydration of beeswax microspheres and the possible transfer of the drug to the external phase. The microspheres' mean size is very influenced by the emulsifier type, as shown in Table 2. By comparing L2 and L3 lots, Tween 80 led to larger microspheres. This can be explained by the high molecular weight of Tween 80, which can reduce the breakup phenomenon. However, L1 microspheres with a middle value of mean diameter (102µm) are obtained by using PVA.

Table 2. Beeswax microparticles characteristics (yield, drug content, encapsulation efficiency (EE), number mean diameter (d10), and size distribution) based on pure NA and NA:β-CD inclusion complexes and prepared with different surfactants.

Lot	Surfactant	Yield (%) ± ΔS (n=3)	Drug content (%) ± ΔS (n=3)	EE (%) ±ΔS (n=3)	d_{10} (µm)	δ
L1	PVA	72.6 ±4.0	10.18 ±0.37	50.90 ±1.83	102.3	1.43
L2	Tween 20	72.0 ±4.1	17.48 ±0.45	87.42 ±2.24	74.3	1.19

L3	Tween 80	74.5 ±1.6	18.15±0.32	90.76±1.61	121.1	1.41
L1 Cp1	PVA	72.2 ±4.4	4.41±0.37	76.61±7.31	105.5	1.34
L2 Cp1	Tween 20	73.6 ±3.0	4.50 ±0.30	89.98±5.98	88.1	1.37
L3 Cp1	Tween 80	74.8 ±1.8	4.05 ±0.44	81.01±8.78	90.4	1.31
L1 Cp2	PVA	71.2 ±4.1	4.62±0.11	96.33±2.17	105.3	1.35
L2 Cp2	Tween 20	73.5 ±2.6	4.03 ±0.26	84,00±5,37	72.8	1.36
L3 Cp2	Tween 80	76.5 ±6.8	3.83 ±0.26	79.71±5.42	96.7	1.34
L1 Cp3	PVA	73.3 ±3.3	2.55 ±0.36	67.80±9.49	116.1	1.25
L2 Cp3	Tween 20	69.0±2.9	2.07 ±0.11	55.14±2.95	82.3	1.25
L3 Cp3	Tween 80	70.4±4.4	2.05 ±0.13	54.73±3.55	97.5	1.29

Regarding NA:β-CD-based microspheres, the initial drug content didn't exceed 5%, and the experimental results varied from 2 to 4.5%, allowing for encapsulation efficiencies between 54 to 96%; the results are satisfactory. In this case, the type of complex affected the drug content significantly; encapsulation of Cp3 gave low drug contents, indicating the possible high solubility of the drug complex, which induced its transfer to the external phase during the microencapsulation process.

In addition, in contrast to pure NA microspheres, for the microspheres loaded by Cp2 or Cp3, the use of PVA as surfactant leads to the maximum of drug encapsulation compared with Tween 20 and Tween 80. The fact can be explained by the microparticles' size; it is demonstrated that the drug content increased when the droplet size increased [42].

For these formulations, the effect of the surfactant agent on the mean diameter (d_{10}) is also notable. In fact, Tween 20 leads to the smaller microspheres, but PVA, in this case, allowed for a larger size of droplets. So, the effect of surfactant on the microspheres' size is not similar for pure NA (hydrophobic) and NA complexes (hydrophilic) based microspheres.

For all formulations, the yield didn't exceed 76 %; the losses can be related to the adhesion of the beeswax organic phase to the vessel when it is poured into an aqueous solution.

The SEM images of pure NA and NA:β-CD complexes, which are given in Figure 1, showed the particles' aspect and morphology. NA particles are rod-shaped, CP1 showed an amorphous structure with bulk agglomerates; however, the morphology of CP2 and CP3 particles appeared more regular with a cylindrical form.

The SEM micrographs of microspheres given in Figure 2 showed spherical microparticles that are gathered particularly in NA:-CD-based microspheres; the surface is rough and wrinkled for all beeswax microspheres.

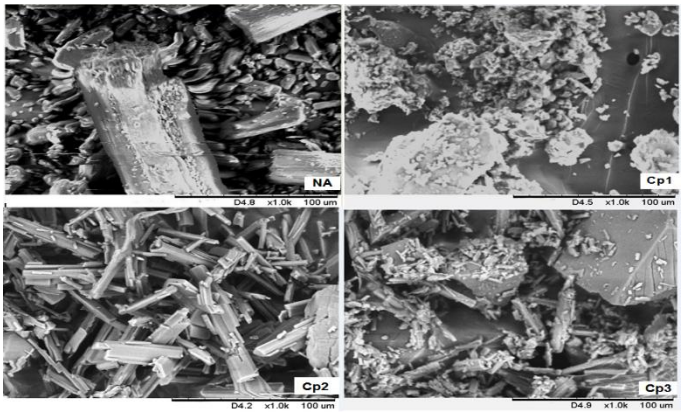

Figure 1. SEM photographs of pure NA and NA:β-CD complexes: physical mixture (Cp1), co-precipitate (Cp2) and co-evaporate (Cp3).

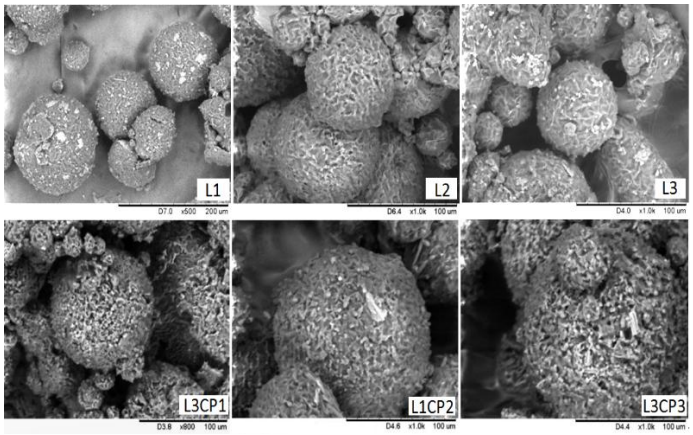

Figure 2. SEM photographs of Pure NA-beeswax microspheres batches obtained using PVA (L1), Tween 20 (L2), Tween 80 (L3), and some NA:-CD beeswax microspheres: Physical mixture complex and Tween 80 (L3Cp1), co-precipitate complex and PVA (L1Cp2), and co-evaporate complex and Tween 80 (L3Cp3).

The investigation of FTIR results needed the interpretation and comparison of the FTIR spectra of pure NA, inclusion complexes, beeswax, and microspheres (Figure 3).

From Figure 3, the major characteristic bands in the FTIR spectrum of pure NA are the C=O of the carboxylic acid band at 1659 cm^{-1}, in the wave number range of 2500-3520 cm^{-1}, a broad band including the O-H acid, N-H amine band, and aromatic C-H functions is observed. The aliphatic and aromatic C-N bands are found at wave numbers of 1282 and 1444 $cm^{-1,}$ and C-H aromatic bending at 768 cm^{-1}.

According to the IR investigation of Niflumic acid molecule [25, 34, 43], the principal characteristic IR-bands appeared in complexes and beeswax microspheres. In fact, the absorption peaks of the bending aromatic =C-H in the range of 765-776 cm^{-1} of pure NA are found in all the spectra of complexes and microspheres. The NA spectrum revealed C-F bands at 694 cm^{-1} and 1173 $cm^{-1,}$ which appeared clearly in complexes and beeswax microspheres (Figure 3).

In the FTIR spectrum of β-CD (Figure 3), some prominent absorption bands are identified, such as stretching vibration of O-H at 3273cm^{-1}, of CH and CH_2 groups at 2800-3000cm^{-1}, of C-OH and C-O-C groups at 1075 and 1152 cm^{-1} [44].

The molecular interactions between the host (CD) and guest molecules (drug) can be revealed by an FTIR study. In general, the IR absorption or intensities of some bands can change after the complexation and inclusion of the drug molecule into the CD cavity [25].

The inclusion complexes' IR spectra showed a notable decrease in the NA peaks' intensities, in all complexes, band shifts are observed as mentioned in Figure 3, which indicates host–guest interactions occurring and confirming the NA-based inclusion complex formation

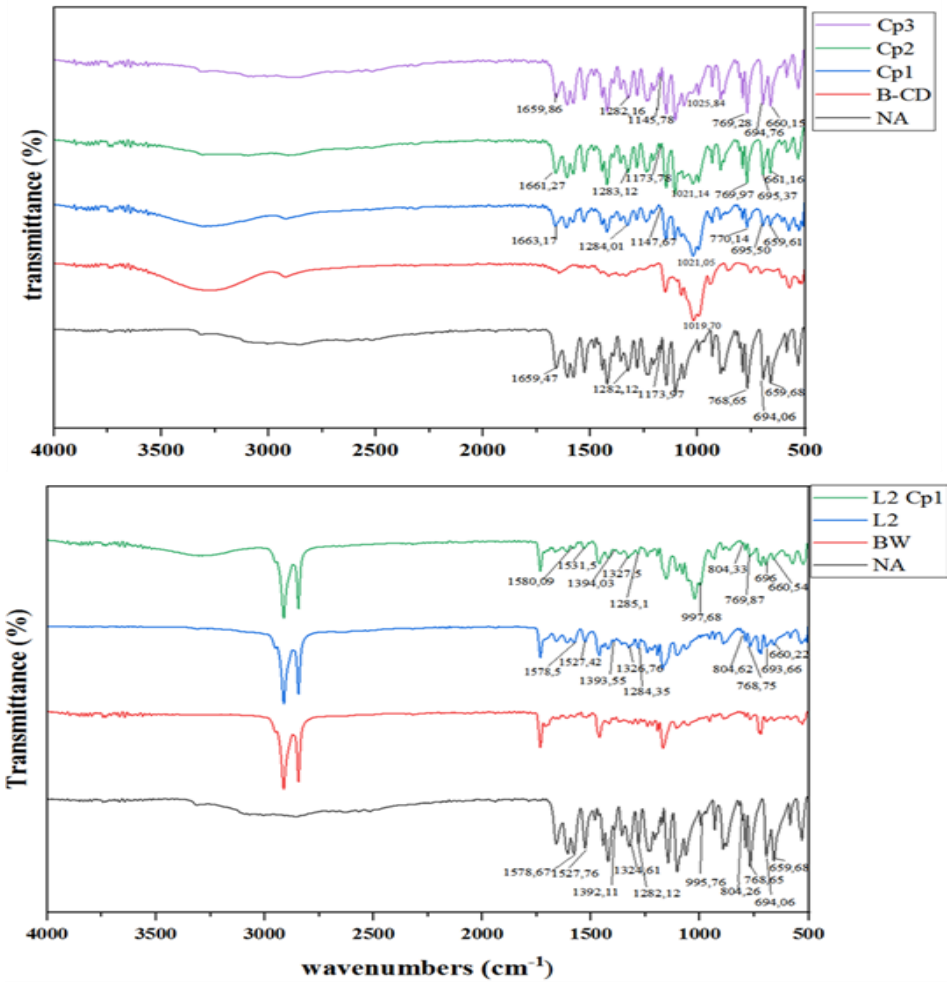

Figure 3. Infrared spectra of pure NA, β-CD, NA:β-CD complexes obtained by physical mixture (Cp1), co-precipitation (Cp2), and co-evaporation (Cp3), and pure NA-beeswax microspheres prepared with Tween 20 (L2) and NA:β-CD beeswax microspheres composed of physical mixture complex and prepared with Tween 20 (L2Cp1).

The FTIR beeswax spectrum (Figure 3) showed major bands of νC–H alkanes at 2954 and 2848 cm^{-1}, δC–H at 1472 cm^{-1}, and γCH_2 in long-chain alkanes at 719 cm^{-1}. Also, at 1733 cm^{-1}, a corresponding band of νC=O of ester linkages between the fatty acids and glycerol backbone in beeswax appeared [41].

The given example of the FTIR spectrum of beeswax microspheres of L2 batch in Figure 3 showed that some of the NA bands are hidden by the beeswax material bands and appear with very low intensities. In the L2CP1 spectrum, the C-OH and C-O-C bands at 1075 and 1152 cm^{-1} of the β-CD band are clearly visible. It is also noted that no new adsorption band is readable, so no chemical interaction between Niflumic acid and beeswax is detected; the same conclusion was reported by other studies [40, 41].

The X-ray diffraction pattern of pure NA (Figure 4) showed crystalline character of the drug molecule; it displayed characteristic peaks at 8.7°, 12°, 13.5°, 16.9°, 19.3°, 21.9°, 23.8°, 25.6°, 26.4°, 29.8°, and 37.3°; these values are close to those reported in other research [25, 34]. Moreover, complex formation and interaction between host and guest are proved by the appearance of new peaks in CP1, CP2, and CP3 patterns as shown in Figure 4, and also the difference between them indicates a difference in the interaction behavior.

The DRX pattern of L1 and L1CP1 microspheres (Figure 4) displayed intense peaks at 2θ 22.0 and 24.4° and weak peaks at 19.9 and 36.6° corresponding to beeswax. [41]. Some of the NA peaks appeared, such as 8.7°, 13.4°, 19.5°, 23.8°, 25.6°, 26.4°, 29.7°, but with very low intensity in the L1 pattern, indicating the reduction of the NA crystalline zone. However, in L1CP1, only the beeswax peaks appeared, indicating the total loss of the NA crystalline form or resulting from the low drug content.

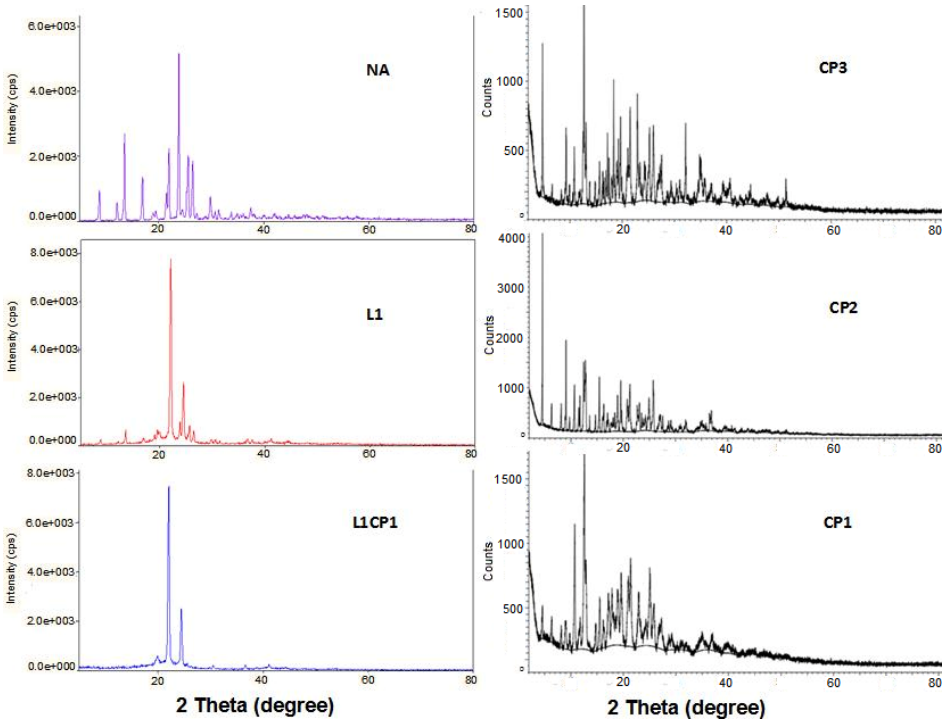

Figure 4. X-ray diffraction patterns of pure NA, NA: β-CD complexes obtained by physical mixture (Cp1), co-precipitation (Cp2), and co-evaporation (Cp3), and pure NA-beeswax microspheres batch prepared using PVA (L1) and NA:β-CD beeswax microspheres composed of physical mixture complex and prepared with PVA (L1Cp1).

The interaction between the drug and the encapsulation material can also result in a change in thermal behaviour. DSC analysis is shown in Figure 5. The DSC thermogram of pure NA clearly illustrated a sharp and intense melting profile at 207.47°, close to that reported in the literature [25, 34, 45]. This peak appeared in the three complexes with a low shift. In addition, the DSC thermograms of the complexes showed a larger, more pronounced shift of the melting peak of pure -CD, which appeared at 130°. So, the heating profiles also confirmed the complex formation and interaction between NA and β-CD.

Also, Figure 5 showed a heating profile pointed at 69 °C related to the melting peak of beeswax, and the melting peak of NA is clearly distinguished in the DSC thermograms of L1; however, it is hidden in the L1CP2 thermogram, probably due to the low concentration of NA in these microparticles.

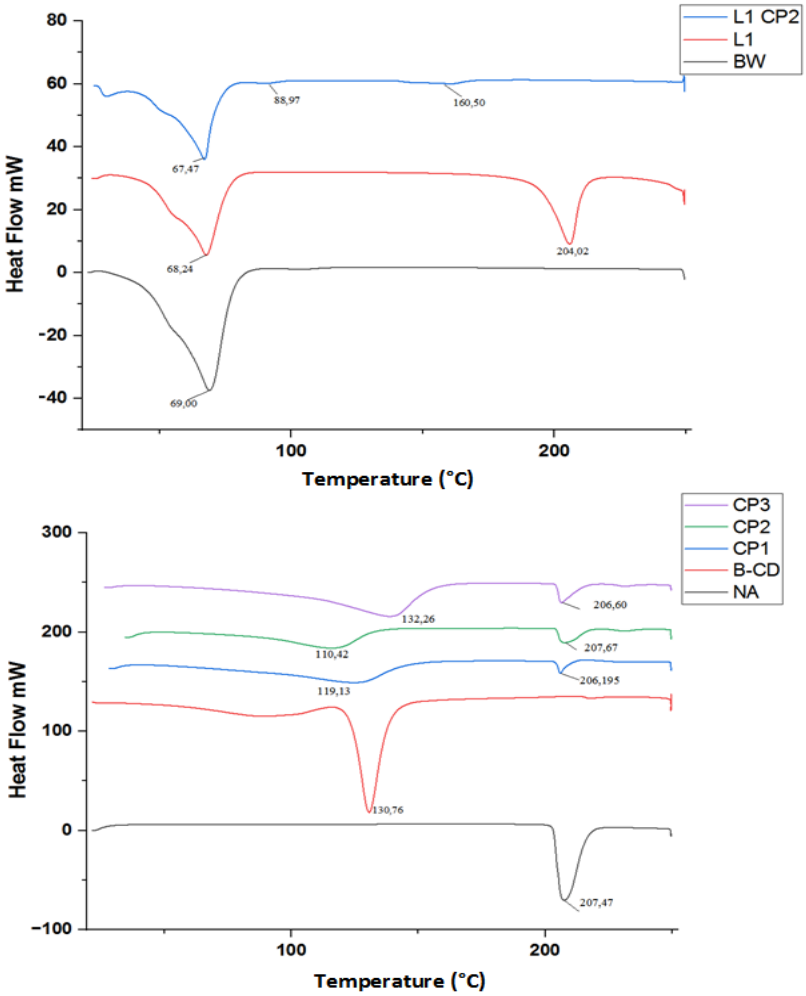

Figure 5. Heating profiles by DSC analysis of pure NA, NA: β-CD complexes obtained by physical mixture (Cp1), co-precipitation (Cp2), and co-evaporation (Cp3), and Pure NA-beeswax microspheres batch using PVA (L1) and NA:β-CD beeswax microspheres composed of co-precipitate complex and prepared with PVA (L1Cp2).

In-vitro drug dissolution investigation

Firstly, pure NA and its complexes are assessed by the study of dissolution profile in simulated gastric fluid at pH = 1.2 in order to evaluate the influence of complexation on NA solubility. From Figure 6, it is clearly distinguished that NA dissolution from NA:β-CD complexes is higher than that of pure NA; the results proved that the inclusion of the NA guest molecule in the β-CD host

molecule improved the drug dissolution significantly. In fact, various studies confirmed that the inclusion of poorly water-soluble drug molecules in cyclodextrins' cavity enhances the drug solubility and bioavailability [25-26, 44, 46]. By comparing the three complexes CP1, CP2, and CP3 (Figure 6), it is noticed that the complexes resulting from co-evaporation and precipitation (CP2 and CP3) exhibited a higher drug dissolution than CP1, which is obtained by a physical mixture.

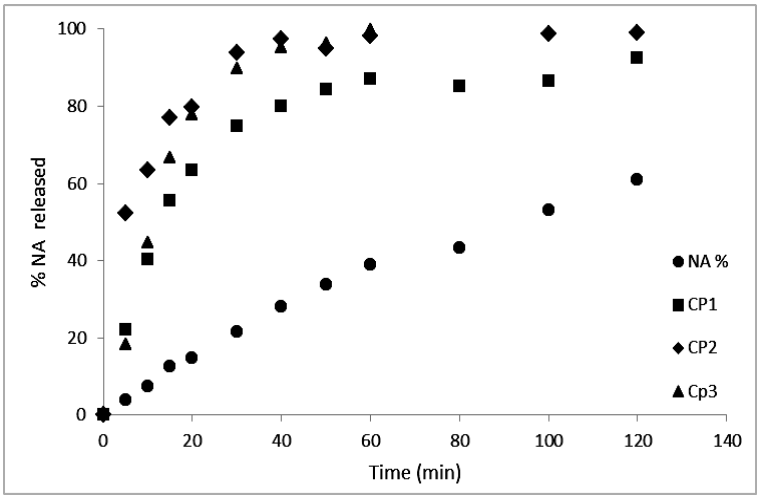

Figure 6. Dissolution profiles of Pure NA and NA: β-CD inclusion complexes obtained by physical mixture (Cp1), co-precipitation (Cp2), and co-evaporation (Cp3) in simulated gastric fluid at pH = 1.2.

Secondly, to obtain variable sustained release profiles, the pure NA and NA:β-CD complexes are incorporated in beeswax as a lipid matrix. The drug dissolution from the obtained microparticles is performed in a simulated gastric liquid, and the NA release profiles are given in Figure 7. The drug release was substantially slow from the formulations composed of pure NA. Regarding these formulations L1, L2, and L3 (Figure 7-A), it is noticeable that the drug is faster released from L1 than L2 or L3; the results can be explained by the drug content and concentration. In fact, as given in Table 3, the drug content in L1 is lower than in L2 and L3, so for L1, the drug concentration in solution is lower, inducing its fast dissolution and transfer to the aqueous phase. In addition, by comparing L2 and L3 formulations, the drug contents are almost similar, but the drug release difference is notable. In this case, the results can be related to the microparticles' size and, evidently, the emulsifier effect. In fact, L2 microparticles are smaller than L3 microparticles (Table 2) and exhibited a higher drug release; this is common because for the smaller microspheres, the surface area in contact with aqueous solution is higher, which induced a rapid drug dissolution and transfer [47]. In summary, the surfactant agent influences the beeswax microspheres' characteristics, especially the size and the drug content, and consequently it highly affects the drug release.

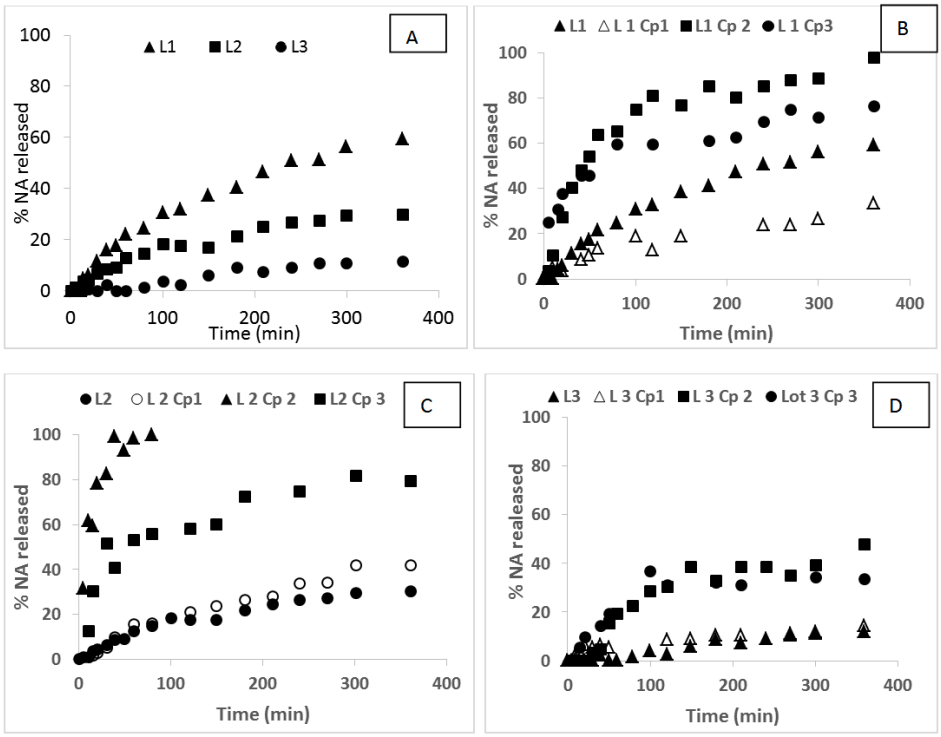

Figure 7. NA release profiles of all beeswax formulations in simulated gastric fluid at pH=1.2: A: Pure NA-beeswax microspheres obtained using PVA (L1), Tween 20 (L2), and Tween 80 (L3), B: beeswax microspheres prepared using PVA and composed of pure NA (L1) and NA:β-CD complexes: physical mixture (L1Cp1), co-precipitate (L1Cp2) and co-evaporate L1Cp3, C: beeswax microspheres prepared using Tween 20 and composed of pure NA (L2) and NA: β-CD complexes: physical mixture (L2Cp1), co-precipitate (L2Cp2) and co-evaporate (L2Cp3), D: beeswax microspheres prepared using Tween 80 and composed of pure NA (L3) and NA:β-CD complexes: physical mixture (L3Cp1), co-precipitate (L3Cp2) and co-evaporate (L3Cp3).

The microencapsulation of NA:β-CD inclusion complexes in beeswax leads to microspheres with various release profiles. Also, the drug dissolution from these nine batches of microspheres is studied and plotted as shown in Figure 7-B, C, and D. The results showed that the Cp1-based microspheres (L1Cp1, L2Cp1, and L3Cp1) gave lower release rates; the results are supported by the values of the NA fractional release after 2 hours given in Table 3 where the %NA released didn't exceed 21%.

Concerning the beeswax microspheres composed of complexes Cp2 and Cp3, the results plotted in Figures 7-C and 7-D displayed a very fast release of NA from the corresponding formulations. In fact, microspheres based on Cp2 gave the highest drug release, especially for L2Cp2, where the drug release reached 100% before 100 minutes of contact time. However, the microspheres based on CP3 exhibited intermediate release profiles. In conclusion, the microencapsulation of the physical mixture of β-cyclodextrin and NA in beeswax didn't change notably the drug release profile suggesting that the complex is probably destroyed during the microencapsulation process. Though the effect of inclusion complexes obtained by co-evaporation and co-precipitation on the drug dissolution and release is very significant. As

reported in Table 3, for these formulations, the fractional release of NA after 2 hours reached 60% for Cp3-based microspheres and achieved a total release for Cp2 microspheres. So, the NA dissolution is actually improved using these complexes Cp2 and Cp3 obtained respectively by co-precipitation and co-evaporation techniques.

To identify and discuss the drug release mechanism through microspheres, the release data are plotted according to the zero-order, Higuchi, and Korsmeyer–Peppas models. The results are reported in Table 3, and the adequate release model is selected from among these mathematical models, giving higher correlation coefficients (R^2). By comparing the zero and Higuchi models, the highest correlation coefficient corresponded to the Higuchi model. Then, the NA release is mostly governed by the diffusion mechanism. The "n" value of the Korsmeyer-Peppas model is $0.43 > n > 0.85$, in major cases, indicating that the mechanism of transport is considered as non-Fickian diffusion. For some beeswax formulations, the case-II of transport is more suitable since $n > 0.85$.

The available experimental data indicate that beeswax can significantly influence the NA release behavior, particularly by reducing dissolution rate and producing a release profile consistent with diffusion-controlled kinetics. While the gastro-protective activity of beeswax components has been proven [14, 15], nevertheless, conclusions regarding the gastro-protective efficacy of these formulations cannot be considered definitive without preclinical evidence.

The *in vitro* release data suggest that beeswax-based formulations may produce a more gradual input of drug into the systemic circulation, which could theoretically reduce Cmax. Also, the *in vitro* dissolution rate profile can be used to develop a one-point sampling strategy for predicting bioavailability, but cannot be directly equated with *in vivo* pharmacokinetics, since gastric emptying, food effects, dissolution conditions, and intestinal absorption may substantially modify the final exposure profile [48-49]. Therefore, these findings should be interpreted as preliminary, and further in vivo, pharmacokinetic, and safety studies are required.

Table 3. Results of NA% released after 2 hours from all formulations in gastric simulated medium pH 1, 2, and the data kinetic analysis and modeling according to zero-order, Higuchi, and Korsmeyer-Peppas models.

Lot	Zero-order		Higuchi		Korsmeyer-Peppas		NA% ± SD (n=3)
	K_0 min^{-1}	R^2	K_H $min^{-1/2}$	R^2	n	R^2	After 2 hours
L1	0.0162	0.946	0.0371	0.994	0.77	0.964	31.8±3.8
L2	0.0149	0.919	0.0187	0.984	0.82	0.938	17.7±1.5
L3	0.0064	0.910	0.0083	0.910	1.05	0.925	02.6±0.7
L1Cp1	0.0033	0.922	0.0167	0.945	0.58	0.929	12.7±2.9
L1Cp2	0.0099	0.705	0.0991	0.978	1.18	0.979	81.3±0.9
L1Cp3	0.0032	0.847	0.0435	0.980	0.28	0.975	59.3±5.1
L2Cp1	0.0053	0.956	0.0259	0.986	0.92	0.950	21.1±5.7
L2Cp2	0.0338	0.676	0.0994	0.873	0.61	0.895	total
L2Cp3	0.0033	0.799	0.0461	0.846	0.49	0.836	58.0±0.4
L3Cp1	0.0011	0.891	0.0059	0.920	0.39	0.856	08.5±0.1
L3Cp2	0.0043	0.779	0.0291	0.870	0.89	0.773	30.5±3.4
L3Cp3	0.0017	0.851	0.0200	0.938	0.53	0.928	30.8±13.8

CONCLUSION

Beeswax has shown beneficial and therapeutic activities for humans, and it is selected in the present study as a biomaterial matrix to produce controlled drug delivery systems loaded with NA, a poorly water-soluble active molecule. Firstly, pure NA is incorporated in beeswax using the hot-melt technique of microencapsulation without using any organic solvent; the effect of the

emulsifier agent on the microsphere's properties and consequently the drug release is studied. For these formulations, the drug content varied from 10 to 18%, and the microparticle size ranged from 74 to 121µm. The results showed that Tween 80 gave the highest encapsulation efficiency, but the corresponding formulation discharged the active agent slowly, in contrast to PVA. Secondly, three NA:β-CD inclusion complexes are produced in order to improve the drug solubility and then dispersed in beeswax microspheres by a microencapsulation process. These formulations exhibited variable release profiles depending on the inclusion complex type. In fact, the microencapsulation of complex NA:β-CD resulting from physical mixture did not induce a change in the drug release, especially for microspheres prepared using Tween 20 and Tween 80. However, the microencapsulation of inclusion complexes produced by co-evaporation and co-precipitation leads to microspheres with variable drug release rates; rapid and total in some cases. According to the Higuchi model, the drug release is governed by diffusion in major cases, and the release constant kH varied from 0.0059-0.0994 min-1/2, suggesting variable release rates. In conclusion, the beeswax formulations based either on NA or NA:-CD inclusion complexes are obtained using an environmentally friendly process and are promising systems for controlling the drug release and reducing its gastric side effects. In addition, the beeswax microspheres based on β-CD complex can be recommended for NA gel development.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding this article.

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