***Original Research Article***

# Synthesis of Morphine Loaded Hydroxyapatite Nanoparticles (HAPs) and Determination of Genotoxic Effect for Using Pain Management


## Abstract

Morphine is used as a standard analgesic for intensive pain relief. It relieves acute and chronic pain by acting directly on the central nervous system and to treat myocardial infarction and shortness of breath. However, the use of morphine for the alleviation of chronic pain is controversial because of the its adverse side effects. The overall success of this medicine in chronic therapy is due to the long-term activity of the drug at a reasonable concentration. Nanoparticle-based carriers have emerged as a new class of drug delivery systems that can overcome traditional drug side-effect limitations by reducing toxicity to a minimum. In this study, a morphine-loaded HAPs drug delivery system was investigated. Fouirer Transform Infrared Spektrofotometre (FTIR) analysis was used to characterize typical functional groups found in the chemical composition of Hydroxyapatite Nanoparticles (HAPs) and morphine loaded HAPs (HAP+M). Scanning electron microscopy (SEM) and Transmission electron microscope (TEM) analyzes were performed to examine the size, morphology, and porosity of morphine loaded HAPs. The effects of pH on release of morphine-loaded HAPs was determined. In addition, it was investigated whether the morphine loaded HAP cell produced oxidative stress and genotoxic effect on DNA. Findings presented in this paper suggested that morphine-loaded HAPs have a promising future as a nanocarrier for pain treatment.


## ABBREVIATIONS

COMET **:** Single-cell Gel Electrophoresis
FTIR :Fouirer Transform Infrared Spektrofotometre
HA :Hydroxyapatite
HAP+M :Morphine loaded Hydroxyapatite Nanoparticles
HAPs :Hydroxyapatite Nanoparticles
HPLC :High performance liquid chromatography
IASP :International Association for the Study of Pain
SEM :Scanning electron microscopy
TEM :Transmission electron microscope
UISP :United States Pharmacopeia

WHO :World Health Organization
TAS :Total antioxidant status
TOS :Total oxidant status
OSI :The oxidative stress index

**Keywords:** Morphine, HAPs, delivery system, pain therapy.

## 1. INTRODUCTION

International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience linked to or associated with actual or potential tissue damage". Depending on the neuropsychological bases and duration of neuropathic, nociceptive, psychogenic, imaginary, acute and chronic, several different pain subtypes can be described [1]. Many people in the world suffer from chronic pain [2]. It is one of the most common reasons for individuals seeking medical treatment. If the chronic pain is not treated, either life quality may decrease or some dysfunctions such as physical and social may arise.

Accomplish pain management provides adequate analgesia without extreme side effects. The current pain management method of the World Health Organization (WHO) begins with non-opioid drugs, progresses with weak opioids and results in strong opioids [3]. The WHO also suggests adjuvant treatment with antidepressant drugs to help decrease anxiety associated with chronic pain. Pain management can be complicated by the dependence on the drugs used and drug side effects. Many factors can influence the drug trend, including genetic diversity, which can further complicate the management of these factors [4].Morphine is a powerful pain reliever and is used to treat severe pain such as surgery, serious injury, cancer-related pain, or heart attack. It is also used for other chronic pain types where weaker painkillers are no longer effective [4]. Morphine is marketed in the form of tablets, capsules, granules, injectable, and suppositories and can only be used by prescription. It is used as a standard analgesic for intensive pain treatment. It is used directly to relieve acute and chronic pain by acting on the central nervous system. It works by preventing the pain signals moving along the nerve from passing to the brain [5].

However, the use of morphine for the alleviation of chronic pain is controversial because of the adverse side effects such as addiction, respiratory depression, gastrointestinal effects, and urological effects. The most common side effects of morphine are constipation, feeling sick and insomnia. The use of morphine for the treatment of chronic pain can only be confirmed in patients who do not respond to other treatments. Because the long-term effects of overdose and continuous use of morphine can affect almost all organ systems of the body [5].

Over the past several decades, nanotechnology has emerged with momentum as a promising new solution to a range of previously unsolvable scientific and technological issues. Nanoparticles offer a massive range of properties and characteristics that can be finely tuned for many applications, from electronics to medicine. More recently, an exploration into their uses in the field of targeted drug delivery has gained popularity with many successes and advancements resulting [6,7,8]. Even with these successes, there has been a delay in the transfer of nanotechnology to the field of pain management. Nanoparticles are available in sizes that are well within the range of typical synaptic gaps through which neurons communicate, and their size also lends them towards possible passage through the blood-brain barrier, a system of tight gap junctions which prevent the passage of large, ionized molecules from entering the central nervous system [9,10]. These size advantages, coupled with the ease of surface modifications and highly tunable characteristics, suggest that the future of pain management lies within the field of nanotechnology.

Hydroxyapatite (HA) is a bioactive, osteoconductive chemical agent that is neither toxic nor immunogenic [11]. There are several applications of HA such as catalysis, fertilizers and pharmaceutical products, water treatment processes and bone and tooth repair. However, the applications areas are very restricted because of their fragility. Many studies have been carried out to modify HA with polymers since natural bone is a composite consisting essentially of nano-sized pinhole HA crystals (constituting about 65% of bones) [5] and collagen fibers [6]. Extremely thin HA powder has been used to increase the quality of HA [7]. In the literature, a number of methods such as Sol-gel [8,9], reverse microemulsion [10,11], hydrothermal [12], microwave hydrothermal [13], precipitation [14] and solid-state reaction [1] have been reported for HA synthesis. Nano-sized and weak agglomerated HA particles were produced by hydrothermal and microemulsion methods [7]. The most reported method for preparing HA particles is the precipitation method. This process is easy, economic

and appropriate for industrial production [7]. Ultrasonication is known to be particularly useful for disrupting aggregates and reducing size and polydispersity of nanoparticles [15].

HA acts as a prototype for bones and teeth and is also commonly used in medical implants [16]. HA is used in the bone as nano-sized needle-like crystals. HA can be used in a variety of forms including powder, granules, porous grains. It is necessary to characterize the HA powder depending on the desired application. Some parameters such as purity, crystallinity, and morphology can be controlled when the wet synthesis technique is used. Applications of morphologies can differ. For example, although spherical particles are used in thermal spray coating, needle-shaped or rod-shape are used in bone repair composite material [17]. Recently, nano-HA has attracted the attention of researchers thanks to the important role of HA in several biomedical applications. There are a few methods including chemical precipitation, spray drying, sol-gel to synthesize HA. The nanoparticle size of the HA crystal is an average length of 50 nm and is embedded in the collagen matrix in natural bone and teeth. Actually, collagen acts as a template in the collagen-controlled bio-mineralization process [18].

A long-acting product formulation from the morphine will have the potency of both patient rehabilitation and patient comfort. The overall success of this drug in chronic therapy is due to its long-term activity at a reasonable concentration around the action area. After entering the host, the nanoparticles, which have a reasonable density near the domain, function as a drug reservoir capable of releasing the drug for a long time in the bloodstream. This long-acting drug profile is used as a basis for long-term chronic drug action to the desired effect. For these reasons, there is a need to develop a morphine-loaded nanoparticle drug release system without any cytotoxicity threat. In addition, there is a need to be developed to release the morphine loaded drug slowly and in a controlled manner into the action zone.

In this study, the first time morphine loaded HAPs (HAP + M) were synthesized and partial characterization was performed. The effect of morphine release of pH was investigated and the genotoxicity of HAP + M was determined by comparing the comet assay and oxidative stress parameters.

## 2. MATERIALS AND METHODS

## 2.1. Materials

Poppy capsules were obtained from Opium Alkaloid Plant (Turkish Grain Board). The capsules were broken and their seeds are separated. Poppy capsules were dried at room temperature for 15 days in a dark room at room temperature. It was ground to the size of 80 mesh grain size before extraction. Morphine Reference Standard was obtained from the United States Pharmacopeia (USP). All chemicals used in all experiments were in analytical quality and in High performance liquid chromatography (HPLC) grades all solvents used for chromatographic purposes. All chemicals were purchased from Sigma Aldrich. 0.45 μm membranes (Millipore, Bedford) were used for the filtration of all solutions.

## 2.2. Extraction of morphine from poppy capsules.

Alkaloids are produced by using natural products or synthetically. Because of the low toxicity of natural products, they are preferred from the pharmaceutical industry. Therefore, the study is done by extracting morphine from poppy capsules. 100 g of dried and the ground sample was weighed into a 2 L of the beaker. 1000 ml of solvent (80 % Methanol + 20 % 0.1 M HCl) was added over the capsules. Morphine was extracted for one day by constant stirring. The mixture was filtered off 500 ml of solvent (80 % Methanol + 20 % 0.1 M HCl) for the second extraction was added on the poppy pulp. The mixture was filtered off. All extracts obtained from three extractions were combined for purification. The combined extract was evaporated under vacuum at 40 °C in a rotary evaporator to 200 ml of a total volume. 200 mL of concentrated aqueous extract was left. Concentrated extract was kept in the refrigerator at the temperature of (0-4 °C) for one day. The extracts were filtered and plant-derived wax, tannins, and oily substances were separated and thrown. A small amount of diatomaceous earth was added to remove the impurities in the concentrated extract solution to obtain a clearer extract, and the mixture was stirred at 50 °C for half an hour. The mixture was filtered through white band strainer paper. A more transparent extract was obtained.

The concentrated extract was extracted 3 times with petroleum ether to remove the vegetative oily substances in the concentrated extract. In each extraction, the aqueous concentrate extract and the petroleum ether were stirred for 15 minutes. It was left for 15 minutes and the phases were allowed to separate. The morphine-free petroleum ether phase was discarded. Active carbon is used to remove undesirable compounds that can cause color, quality, and property changes in liquids due to its enabling feature of final product in the

pharmaceutical industry to be uncolored and purified. For this, a small amount of activated carbon was added to the extract, stirred at 50 °C for half an hour and filtered. This process was repeated three times. Finally, the concentrated extract was evaporated to a saturation concentration in a rotary evaporator. The amount of morphine in the concentrated extract was determined by HPLC analysis [19].

### 2.3. Preparation of morphine loaded HAPs (HAPs) with precipitation method using ammonium phosphate and calcium nitrate solutions

HAPs were prepared using an aqueous precipitation technique. The molar concentration of calcium nitrate tetrahydrate and diammonium hydrogen orthophosphate was adjusted to have a theoretical value of the Ca/P ratio: 1.667. 0.156 M stock solution of diammonium hydrogen orthophosphate in demineralized water and 0.400 M stock solution of calcium nitrate tetrahydrate in absolute ethanol were used. These solutions were continuously mixed at a temperature of 70 °C for 4 h on the magnetic stirrer.100 mL of diammonium hydrogen orthophosphate solution was added to the beaker to synthesize morphine-loaded HAPs. The solution was heated to 70 °C. 50 mL of concentrated morphine solution was added over its. 100 mL of the calcium nitrate solution was added dropwise to this mixture for an hour. The mixture was stirred at 70 °C for 4 hours. The precipitated white colored nanoparticles were filtered using white band filter paper and washed three times with double distilled water and finally with ethanol. Nanoparticles powder were dried in an oven at 105 °C for 4 hours until it gets dry.

### 2.4. Characterization Analysis of HAPs

#### 2.4.1. Determination of Morphine Contents by HPLC Analysis

In our study, HPLC analyzes were used to determine that morphine content was loaded into HAP. Therefore, % purity control was not carried out in the extraction steps. Chromatographic analyzes were performed on an Agilent brand 1260 model HPLC instrument (Agilent, USA). The system includes a quaternary gradient pump, vacuum degasser, column thermostat, automatic sampler, and (UV/VIS) detector. The Chem Station software was used to collect and evaluate data. Chromatographic separation was performed with an ACE C18 column (5 µm, 150 mm * 4.6 mm I. D.). Mobile phase A was a solution of 5 % acetonitrile and mobile phase B was a mixture of acetonitrile: glacial acetic acid: trimethylamine in the ratio of (97.9: 2: 0.1, v / v). The flow rate of the mobile phase was 1 mL/min; Column thermostat temperature was maintained at 30 °C; Injection volume was 50

µl; Detection was carried out at 284 nm; Working time: 30 minutes. Elution was performed with the gradient: 0 min 10% solvent B; 0–5 min from 10 to 15% solvent B; 5–10 min from 15 to 20% solvent B; 10–20 min from 20 to 35% solvent B; 20–30 min from 35 to 10% solvent B [20].

To analyze the amount of morphine in the morphine-loaded nanoparticle, 200 mg of nanoparticle was weighed into a 100 mL beaker. 20 ml of 0.1 molar HCl was added and dissolved in the ultrasonic bath. Transferred to a 50 ml balloon, the volume was completely deionized water. The amount of morphine in the resulting solution was analyzed by HPLC.

### 2.4.2. FTIR analysis

FTIR analysis was carried out to determine the various phosphate and carbonate functional groups in the synthesized HAPs. HAPs dried at 105 °C was analyzed for FTIR analysis. Spectrum Two model of Perkin Elmer brand FTIR Spectrometer was used in FTIR analysis.

### 2.4.3. Scanning electron microscopy (SEM) analysis

SEM analysis was performed to determine the morphological and grain sizes of the synthesized nanoparticles. Phenom brand ProX model SEM device was used in the analysis.

### 2.4.4. Transmission electron microscope (TEM) analysis

TEM analysis was performed to clarify the size, shape, morphology and internal structure of the nanoparticles. JEOL JEM-2100 transmission electron microscope (UHR) device was used in the analysis.

## 2.5. The effect of pH on morphine-loaded drug release

A NaCl / HCl solution with a pH of 1.2 (stomach pH) and 7.4 (intestinal pH) were prepared. The release tests were carried out at a temperature of 37 ± 0.5 °C (human body temperature) in a horizontal shaking kiln at 100 rpm. 200 mg powder of morphine loaded HAPs was weighed to a beaker of 100 mL and added 20 mL of the PBS solution. The morphine amount in the prepared medium is 14.2 ppm. Erlenmeyer was covered with aluminum foil and was placed in a shaker oven 100 rpm at 37 °C. 2 mL of samples was taken at every five hours. The samples were analyzed by HPLC after filtration through an injector filter [21]. Measurements were continued until the drug release was fixed.

### 2.6. Comet assay

The blood samples was collected from a healthy and non-smoking young donor at the age of 28. Leukocytes were isolated over Histopaque 1083 gradients by centrifugation at 2100 rpm for 20 min at 15 °C. The comet assay was performed under alkaline conditions according to Singh et al. (1988) with some modifications. Isolated human leukocytes (100 µL) were incubated with 100 µL different concentrations of HA and HA+M (5, 10 and 25 mg/mL) for 1 h at 37°C [22]. a Positive (30 mM $H_2O_2$) and a solvent control (1XPBS) were also included. Following the incubation, leucocytes were at 1600 rpm for 10 min at 25 °C. While supernatant was used for TAS and TOS determination, the pellet was used for Comet assay. The Comet assay protocol was done according to Avuloglu-Yilmaz et al. (2017) [23]. The analysis of comet scores was calculated as described by the Cigerci et al. (2015) [24].

### 2.7. Measurement of total oxidant status and oxidative stress index

TOS and TAS were determined spectrophotometrically using Rel Assay Diagnostic kit RL0024 and RL0017reading at 530 nm and 660 nm respectively by Elisa Thermo Scıentificıfic. (Chiaz marka ve ülke ismi) TAS and TOS value was calculated according to the following formula; TOS: (ΔAbsSample)/ (ΔbsStandard) X Conc. of standard TAS: ((ΔAbs H2O) - (ΔAbs Sample)) / ((ΔAbsH2O) - (ΔAbs Standart)). The oxidative stress index (OSI) of samples were determined with the ratio of TOS to TAS.

### 2.8. Statistical analysis

The scores were presented as means ± standard deviation. The levels of significance in different treatment groups were analyzed Duncan multiple range tests by using SPSS 23.0 version for Windows software. $P < 0.05$ was set as statistical significance.

## 3. RESULTS AND DISCUSSION

### 3.1 Morphine Contents of Morphine Loaded Nanoparticles by HPLC

As a result of HPLC analysis, the content of morphine in the poppy capsules was determined as 281,6 ppm and the content of morphine in the nanoparticle was determined as 14.2 ppm (Table 1). The HPLC chromatogram of HAPs was shown in Figure 1. Extra peaks except morphine which are seen in Fig. 1 is due to solvent. It was determined that morphine was loaded into HAP as pure.

**Table 1 The amount of morphine**

| Extraction stages | The amount of morphine (ppm) |
|---|---|
| First extraction | 281,6 |
| Second extraction | 490,5 |
| Third extraction | 2872,8 |
| Morphine in the nanoparticle | 14,2 |

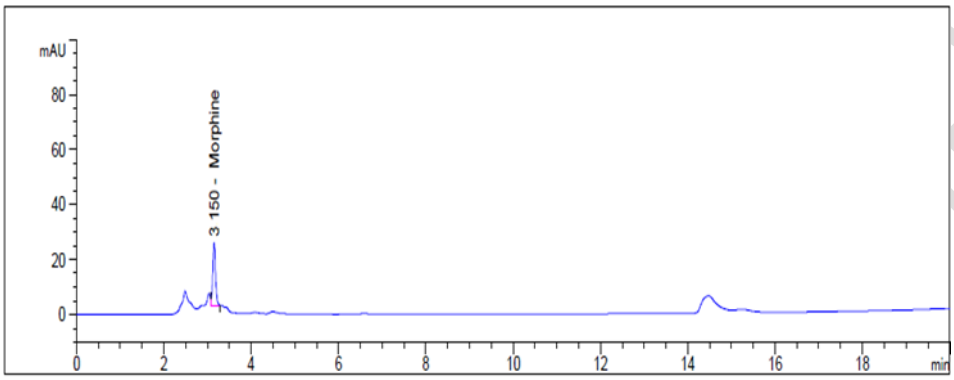



**Figure 1 HPLC chromatogram of morphine loaded HAPs.**

Functional groups associated with HA were identified by FTIR spectroscopy. The FTIR spectra of morphine loaded HAPs and HAPs were shown in Figure. According to Figure 2, the absorption bands at 1346 $cm^{-1}$ and 886 $cm^{-1}$ show the presence of $(CO_3)^{2-}$ in HA structure. The absorption bands at 1060 $cm^{-1}$ detected in the spectra are attributed $(PO_4)^{3-}$ groups. HA has been revealed by the absence of a large peak at 3550 $cm^{-1}$ attributed to the crystallization water and the water molecules trapped in the apatite unit cell. Although nonstoichiometric HA can contain some water molecules, stoichiometric HA cannot contain water molecules generally in its unit cell. Absorption bands at 3571 $cm^{-1}$ and 629 $cm^{-1}$ show the presence of hydroxyl ion in the apatite lattice. Absorption bands observed at 1124, 1060, 993, 886 and 562 $cm^{-1}$ show $(PO_4)^{3-}$ groups [25,26]. Misra et al (2011) determined morphine absorption bands in FTIR [27]. Similarly, in this study; FTIR spectrum of morphine-loaded HAPs was observed to contain different peaks (653.32, 789.75, 875.36, 1653.8, 3473, 3539 $cm^{-1}$) due to the morphine when compared to the spectrum of HAP (Fig 2).

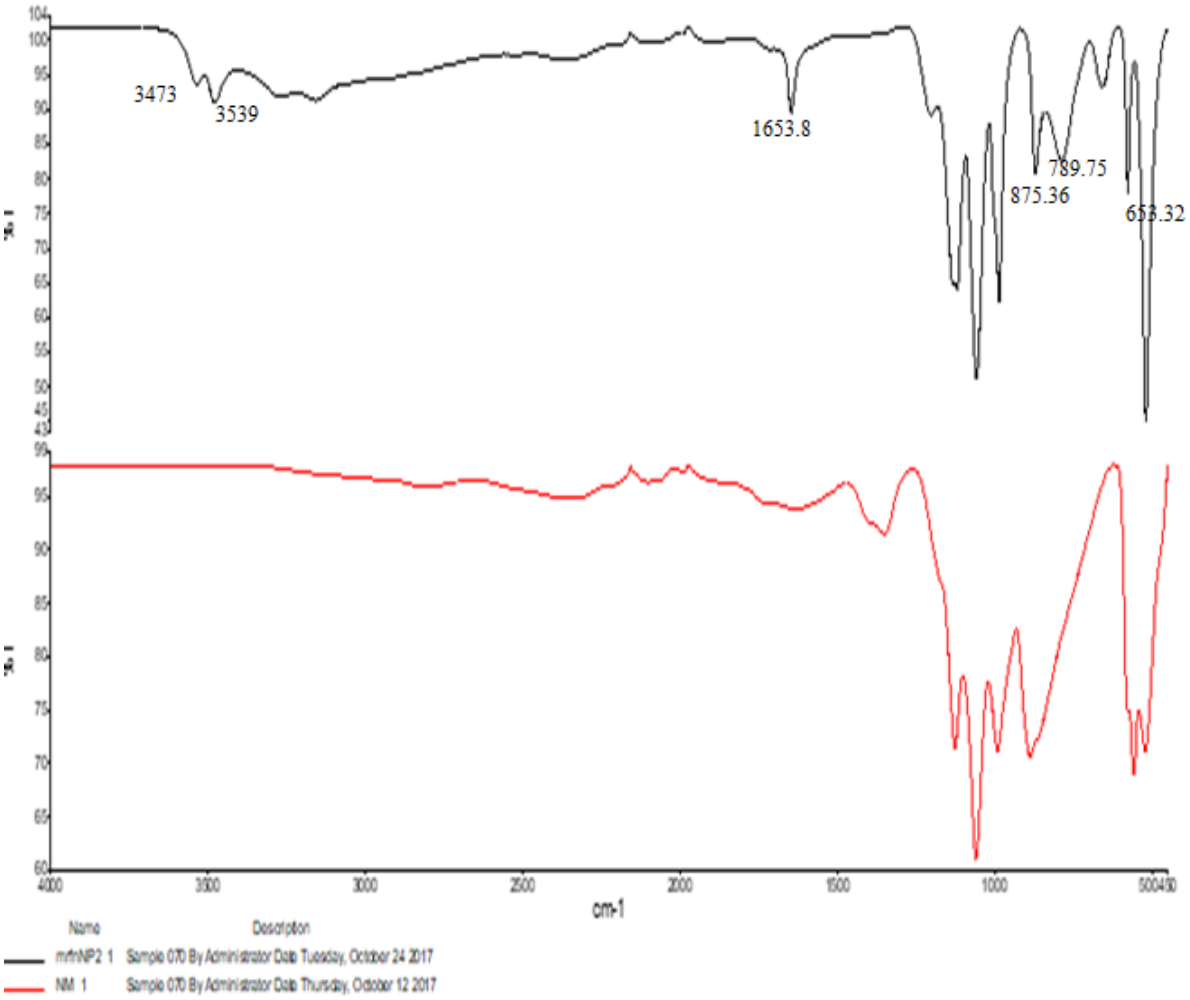



**Figure 2 FTIR spectrum of morphine loaded HA and unloaded HAPs, respectively.**

### 3.3. Scanning electron microscopy

Studies show that the morphology (Irregular, sphere, rod, needle, platelet, tube, fiber, filament, wire, whisker, strip, platelet, flower) and magnitude of HAP (3 nm sp 1000 μm) may vary depending on the synthesis method used [28,29,30].

The morphologies of the synthesized powders were observed by SEM and it was shown in Fig. 3. The samples are mostly composed of fine-grained and homogeneous particles. The produced spherical particles can be stacked at high levels, most of the particles are submicron and nano-sized, as shown in Fig. 3. Since HA provides a porous surface structure, the predominant size of the particles is in the range of 90-150 nm.

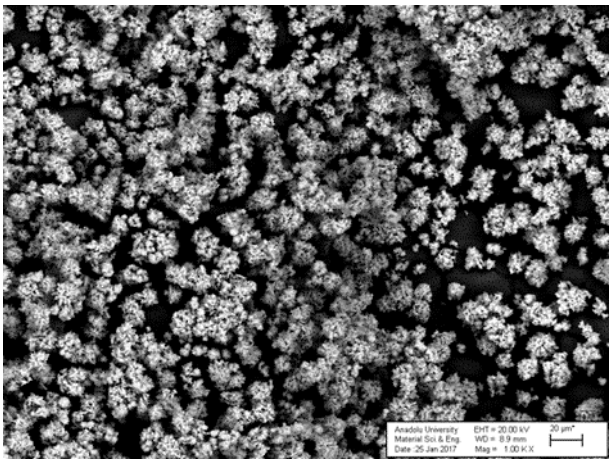
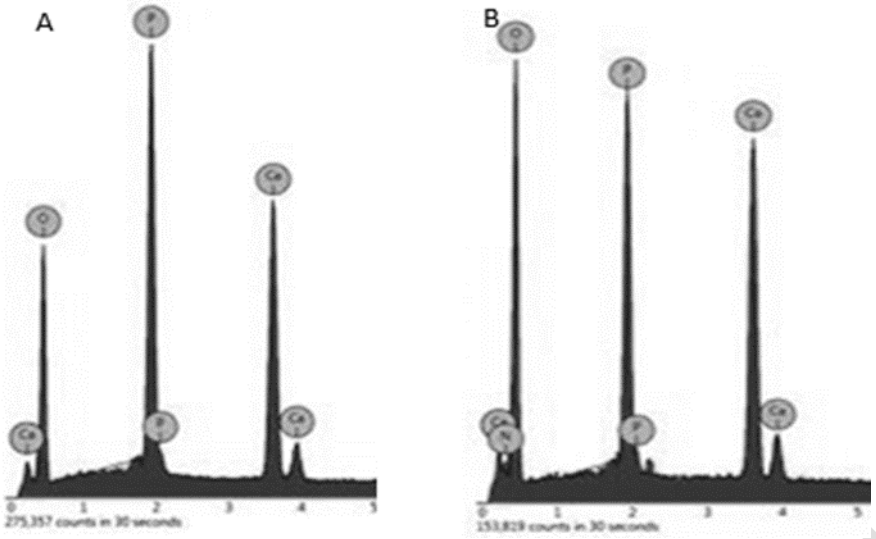


**Figure 3 SEM image of morphine loaded HAPs**

The crystal structures of apatite have been studied in details [31]. The HAP lattice consists of $Ca^{2+}$, $PO_4^{3-}$ and $OH^-$ ions distributed over two mirror symmetric halves of the unit cell [32]. As a result of point analysis, Ca and P ions were detected in empty HAP (Fig 4). These ions in the structure of hydroxyapatite [$Ca_5 (PO_4)_3 (OH)$] are proof that the desired HAP is formed as shown Fig 5.. The structure of morphine ($C_{17}H_{19}NO_3$) contains nitrogen ions (fig 5). As a result of the SEM point analysis, the determination of the nitrogen with charged particles has shown that morphine is loaded with morphine into HAP (fig 4). In this study, SEM point analysis shows that both the empty HAP and the morphine loaded HAP are obtained.

There are few articles about the mechanisms of involvement of biomolecules on the surface of HAP particles. Therapeutic agents can interact with the surface of the nanoparticles in two different ways. One is through detachable covalent connections and the other through physical interactions. The amino or hydroxyl groups on the surface of the nanoparticles are effective in covalent binding. Physical interactions such as electrostatic, hydrophobic/hydrophilic and affinity ones can lead to coupling of drug molecules with the surfaces of nanoparticles [33].

In a single cell unit of HAP, there are 10 $PO_4^{3-}$ groups of a unit cell, two remain inside and eight at the periphery. The positively charged morphine molecules we have obtained are most likely bound by weak electrostatic bonds within the hexagonal structure of the HAP nanoparticles. Binding occurred between the negative charges of the polarized morphine ends and the positively charged phosphate ions in the HAP.




A; HAPs B; HAP+M

**Figure 4 Point analysis of empty (A) and morphine loaded (B) nanoparticle**

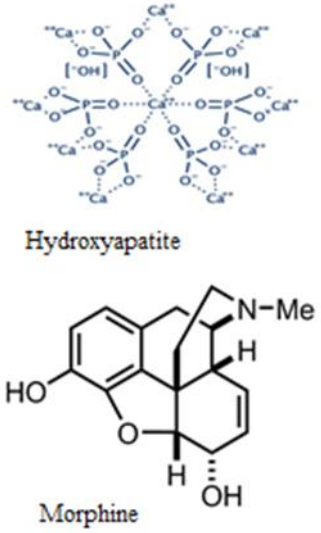



**Figure 5 Chemical structuree of HA and morphine**

### 3.4. Transmission electron microscopy

Figure 6 shows TEM images of morphine loaded HA nanoparticle samples. The results demonstrated that the shape of morphine loaded HA nanoparticle samples is a spherical shape. Nanoparticle sizes were observed to be average 100 nm.

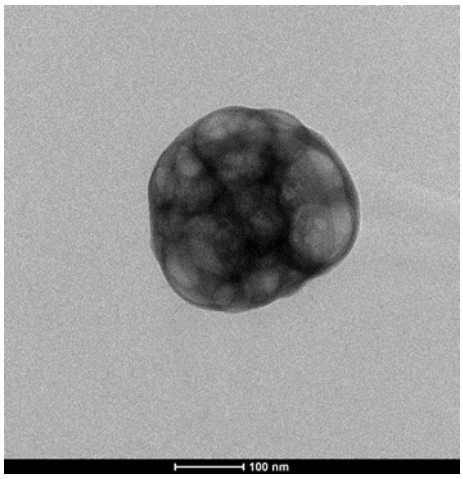



**Figure 6 TEM images of morphine loaded HA nanoparticle samples.**

### 3.5. Comet Assay

Since genotoxicity tests of many commonly used drugs are found to be positive, it has become mandatory to screen for mutagenic and carcinogenic potentials of new drugs. It has been reported that morphine causes a significant increase in micronucleus count and DNA damage depending on the dose [34]. Morphinehas a genotoxic effect through reactive oxygen species (ROS) causing oxidative stress like other opioids [35,36,37]. In addition, Morfin causes DNA damage by inhibiting oxidative stress enzymes such as Glutathione peroxidase, Glutathione, Superoxide dismutase [38].

There is clear evidence that drugs can lead to oxidative stress. Oxidation on DNA results in various lesions such as abasic zones and single or double helix fractures.

There is clear evidence to implicate drug-induced oxidative stress as a mechanism of toxicity. Oxidation of DNA leads to the formation of lesions including oxidized bases, abasic sites, and DNA single- and/or double-strand breaks. One of the reliable techniques for determining oxidative DNA damage is single-cell gel electrophoresis (comet) assay [39]. COMET test is preferred by a number of researchers in toxicity studies due to its precision, speed and economy. In particular, showing DNA damage is a very useful and successful technique. Shafer et al. (1994) observed dose- dependent, significant increases in the frequency of comet tails of fragmented DNA when cells were treated with morphine ($5 \times 10^{-9}$ /$10^{-7}$M) [40]. According to Even though indirect measurements of oxidative stress level indicates the generation of ROS by HAP, no significant effects associated with ROS mediated cellular

damage was evident suggesting the levels of ROS generated is not crossing the threshold level which the system could manage [41].

In this study, the genotoxic effect of morphine-loaded HAP (HAP+M) was evaluated by measuring the values of both comet and oxidative stress parameters (TAS, TOS and OSI). The effect of HAP and HAP+M on DNA damage is given to Table 2. All tested concentrations increased DNA damage in a dose-dependent manner for HA (r=-0.891p=0.01) and for HAP+M (r=-0.905 p=0.01). The significant DNA damage was induced after HA except for 5 mg/mL and after HAP+M at 25 mg/mL. While the highest DNA damage was observed the positive control (271.67±4.37), the lowest one observed in the control group (0.33±0.33). 25 mg/L of HAP+M significantly reduced DNA damage compared to HAP.

**Table 2 Protective effect of HO leaf extract against to $H_2O_2$**

| **Treatment** | **DNA Damage (Arbitrary Unit ±SD)*** |
|---|---|
| Control | $0.33\pm0.33^{a}$ |
| 30 µM $H_2O_2$ | $271.67\pm4.37^{b}$ |
| 25 mg/L HAP | $110.67\pm2.33^{c}$ |
| 10 mg/L HAP | $8.33\pm0.67^{d}$ |
| 5 mg/L HAP | $1.67\pm0.33^{ae}$ |
| 25 mg/L HAP+M | $6\pm0.67^{de}$ |
| 10 mg/L HAP+M | $1\pm0.58^{ae}$ |
| 5 mg/L HAP+M | - |

* Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation

HAP; HA nanoparticle, HAP+M; morphine loaded HA nanoparticle

No significant difference was observed between the 5 mg / mL HAP + M and control group. When the concentration increased, total oxidant capacity increased and total antioxidant capacity decreased. 10 and 25 mg / mL of HAP and 25 mg / mL of HAP + M were found to be statistically significant compared to the control group (Table 3).

**Table 3. Total oxidant and antioxidant capacity**

| | TAS (mmol Trolox Equiv./ L.) | TOS (mM $H_2O_2$ Equiv. / L.) | OSI |
|---|---|---|---|
| Control | 19.14±3.15[a] | 5.54±0.17[a] | 3.54±0.17[a] |
| 30 µM $H_2O_2$ | 5,21±0,25[b] | 19±4,25[b] | 10.54±0.15[b] |
| 25 mg/ mL HAP | 8,12±0,21[e] | 14±1,23[e] | 8.21±0.49[e] |
| 10 mg/ mL HAP | 12±1,71[d] | 10±1,51[d] | 5.95±0.18d |
| 5 mg/ mL HAP | 16,57±0,41[a,c] | 7,01±1,11[a,c] | 4.52±0.6[a,c] |
| 25 mg/mL HAP+M | 15,90±1,11[c,d] | 8,10±1,11[c,d] | 6.04±0.78[d] |
| 10 mg/mL HAP+M | 18,11±1,47[a,c] | 5,11±0,47[a,c] | 5.4±2.11[c] |
| 5 mg/mL HAP+M | 20,13±1,05[a] | 4,15±0,15[a] | 3.65±0.73[a] |

### 3.7. The effect of pH on morphine-loaded HAP release

The graph showing drug release in pH:1.2 as a function of time was given in Figure 7. Morphine-loaded HAPs were found to release a maximum of 69.3 % at the end of 30 hours in pH:1.2.

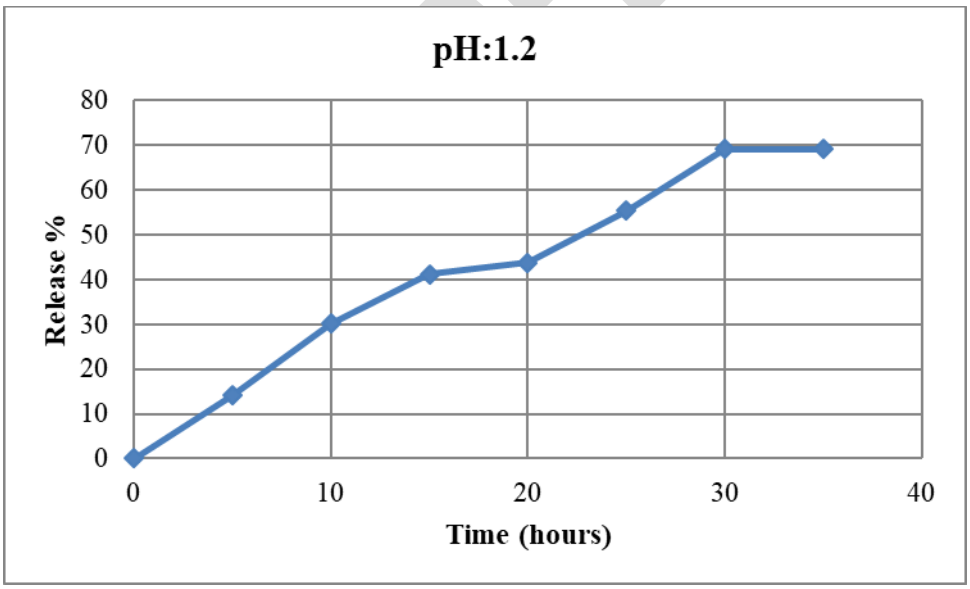



**Figure 7 The graph showing drug release in pH:1.2.**

The graph showing drug release in pH:7.4 as a function of time was given in Figure 8. Morphine-loaded HAPs were found to release a maximum of 7.0 % at the end of 95 hours in the intestinal environment.

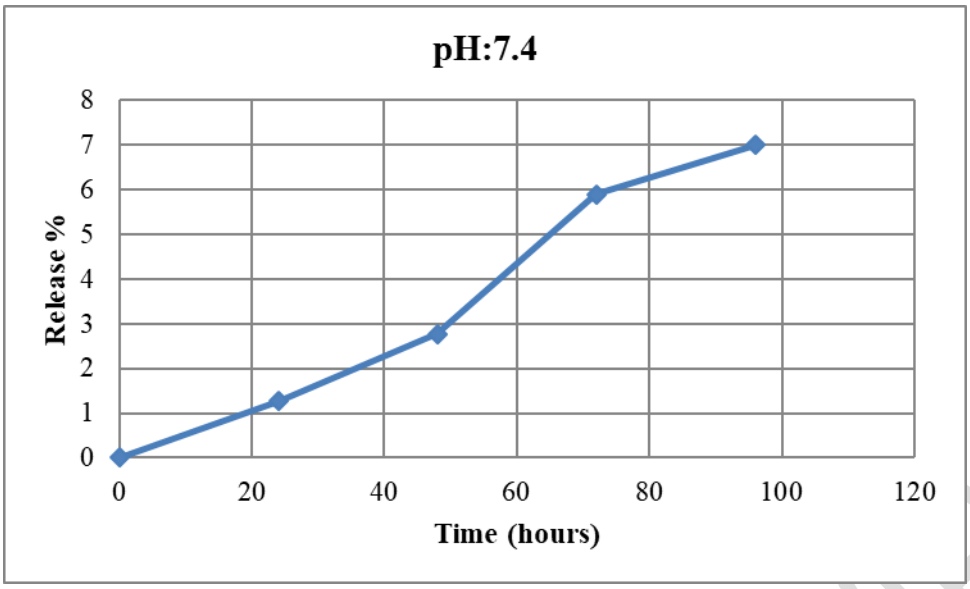



**Figure 8 The graph showing drug release in the intestinal environment.**

Matsumoto et al (2004) reported that release of protein in Ph 4 was higher than Ph 7 .0 at protein loaded HAP. This is because solubility of HA is greatly affected by the pH. In general, a more acidic environment causes HA to become more soluble, while a less acidic environment makes HA less soluble.

## 4. Conclusion

The development of morphine-based controlled release formulations for chronic pain management is an extremely important issue. Other options should be used for drug delivery aiming at obtaining effective, safe and innovative products. The literature has shown that binding of morphine to particulate systems not only provides sustained and controlled release of the drug but also provides a superior or equivalent analgesic profile and reduced side effect formation from free radicals. HA appears to be an interesting alternative to future studies, considering the wide range of advantages of nanoparticles and the lack of study of morphine. Current studies are still not enough to revive the production of new products containing morphine-loaded nanoparticles in the pharmaceutical industry.

In this study, it was determined that HAP can be used as a nongenotoxic morphine transport system. However the drug load of the carrier can be increased and controlled release is

achieved with modifications to the HAP molecule. Subsequent studies should be based on release modeling of the morphine charged HAP nanoparticle at in vivo and invitro media.

## REFERENCES


1. Bonica JJ.The need of a taxonomy. Pain. 1979; 6:247-248.
2. Furlan AD, Reardon R, Weppler C. Opioids for chronic noncancer pain: a new Canadian practice guideline. Can Med Assoc J. 2010; 182:923–30
3. Hardt J, Jacobsen C, Goldberg J, Nickel R, Buchwald D. Prevalence of chronic pain in a representative sample in the united states. Pain Med. 2008; 9:803-812
4. Kroenke K, Krebs E,Wu J, Bair MJ, Damush T, Chumbler N, et al. Stepped care to optimize pain care effectiveness (scope) trial study design and sample characteristics. Contemp Clin Trials. 2014; 34: 270-281
5. Martin C, De Baerdemaeker A, Poelaert J, Madder A, Hoogenboom R, Ballet S. Controlled-release of opioids for improved pain management. Materials Today. 2016; 19(9): 491–502
6. Woodle MC. Sterically stabilized liposome therapeutics.Adv. Drug Deliv. Rev. 1995; 16(2–3): 249–265
7. Anselmo AC, Mitragotri S. (2014). Cell-mediated delivery of nanoparticles: Taking advantage of circulatory cells to target nanoparticles. J. Control. Release. 2014; 190: 531–541.
8. Rossi F, Ferrari R, Papa S, Moscatelli D, Casalini T, Forloni G, Perale G, and Veglianese P. Tunable hydrogel-Nanoparticles release system for sustained combination therapies in the spinal cord. Colloids Surfaces B Biointerfaces. 2013; 108:169–177
9. Choi YS, Lee MY, David AE, Park YS. Nanoparticles for gene delivery: therapeutic and toxic effects. 2014; Mol. Cell. Toxicol.10 (1): 1–8
10. Bao G, Mitragotri S and Tong, S. Multifunctional nanoparticles for drug delivery and molecular imaging. Annu. Rev. Biomed. Eng. 2013; 15:253–82
11. Khaled RM, Hanan HB, Zenab M El-Rashidy. In vitro study of nano-HA/chitosan–gelatin composites for bio-applications. Journal of Advanced Research . 2014; 5: 201–208

12. Wang F, Li M, Lu Y. A simple sol-gel technique for preparing HA nanopowders. Mater. Lett. 2005; 59:916-919
13. Kim I, Kumta PN. Sol-gel synthesis and characterization of nanostructured HA powder. Mater. Sci. Eng. B.2004; 111: 232-236
14. Sun Y, Guo G, Wang Z, Guo H. Synthesis of single-crystal HAP nanorods. Ceram. Int. 2006; 32: 951-954
15. Koumoulidis GC, Katsoulidis AP, Ladavos AK, Pomonis PJ, Trapalis CC, Sdoukos AT, Vaimakis TC. Preparation of HA via microemulsion route. J. Colloid Interface Sci. 2003; 259: 254-260
16. Yin G, Liu Z, Zhan J, Ding F. and Yuan N. Impacts of the surface charge property on protein adsorption on HA. Chemical Engineering Journal. 2002; 87(2): 181– 186.
17. Kramer E, Podurgiel J and Wei M. Control of HA nanoparticle morphology using wet synthesis techniques: Reactant addition rate effects. Materials Letters.2014; 131: 145 – 147.
18. Mostafa AA, Oudadesse H, Mohamed MB, Foad ES, Le Gal Y and Cathelineau G. The convenient approach of nanoHA polymeric matrix composites. Chemical Engineering Journal. 2009; 153(1): 187 – 192.
19. Taş CA, Korkusuz F, Timuçin M, Akkaş N. An investigation of the chemical synthesis and high-temperature sintering behaviour of calcium HA and tricalcium phosphate (TCP) bioceramics. J Mater Sci Mater Med.1997; 8:91-6.
20. Endlová L, Laryšová A, Vrbovský V, Navrátilová Z. Analysis of Alkaloids in Poppy Straw by High-Performance Liquid Chromatography, IOSR Journal of Engineering. 2015; 05:1-7.
21. Gün M. Aljinat-Kitosan Nanopartiküllerin Kolşisin Salımında Kullanılmasının Araştırılması, Yüksek Lisans, Kimya Anabilim Dalı Programı Adnan Menderes Üniversitesi Fen Bilimleri Enstitüsü.2013
22. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988; 175:184– 191.
23. Avuloğlu Y, Yüzbaşıoğlu D. Evaluation of genotoxic effects of 3-methyl-5-(4-carboxycyclohexylmethyl)-tetrahydro-2H-1,3,5-thiadiazine-2-thione on human peripheral lymphocytes. Pharmaceutıcal bıology,.2017; 55(1): 1228–1233
24. Ciğerci IH, Liman R, Özgül E, Konuk M. Genotoxicity of indiumtin oxide by Allium and Comet tests. Cytotechnology. 2015; 67:157–163

25. Feng W, Li MS, Lu YP. and Qi YX, Liu YX. Synthesis and microstucture of HA nanofibers synthesized at 37oC, Materials Chemistry and Physics.2006; 95: 145-149
26. Cengiz B. Hidroksiapatit Nanoparçacıklarının Sentezi, Yüksek Lisans, Kimya Mühendisliği Anabilim Dalı, Fen Bilimleri Enstitüsü. 2007
27. Misra N, Dwivedi , Pandey AK and Trived S. Vibrational analysis of Two Narcotic Compounds- Codeine and Morphine - A comparative DFT study. Der Pharma. Chemica.2011; 3(3):427-448
28. Padmanabhan SK, Balakrishnan A, Chu MC, Lee YJ, Kim TN, Cho SJ. Sol–gel synthesis and characterization of HA nanorods. Particuology. 2009;7:466–470.
29. Shojai MS, Khorasani MT, Khoshdargi ED, Jamshidi A. Synthesis methods for nanosized HA with diverse structures. Acta Biomater. 2013; 9:7591–7621
30. Mobasherpour I, Heshajin MS, Kazemzadeh A, Zakeri M. Synthesis of nanocrystalline HA by using precipitation method. J Alloys Compd. 2007; 430:330–333.
31. Naray SS. The structure of apatite $(CaF)Ca_4(PO_4)_3$. Z Kristallogr. 1930; 75:387–398.
32. Mondal S, Dorozhkin S, Pal U. Recent progress on fabrication and drug delivery applications of nanostructured HA .Wiley Interdiscip Rev Nanomed Nanobiotechnol.2018;10(4): doi: 10.1002/wnan.1504.
33. Kong L, Mu Z, Yu Y, Zhang L, Hu J. Polyethyleneimine- stabilized HAPs modified with hyaluronic acid for targeted drug delivery. RSC Adv 2016, 6:101790–101799.
34. Li Jih-Heng and Lin Lih-Fang .Genetic toxicology of abused drugs: a brief review. Mutagenesb vol.13 no.6 pp.557-565, 1998
35. Zhang YT, Zheng QS, Pan J, Zheng RL. Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. Basic Clin Pharmacol Toxicol. 2004; 95:53-8.
36. Sharp BM, Keane WF, Suh HJ, Gekker G, Tsukayama D, Peterson PK. Opioid peptides rapidly stimulate superoxide production by human polymorphonuclear leukocytes and macrophages, Endocrinology. 1985; 117:793-5.
37. Slupphaug G, Kavli B, Krokan HE. The interacting pathways for prevention and repair of oxidative DNA damage, Mutat Res. 2003; 531:231-51.
38. Skoulis NP, James RC, Harbison RD, Roberts SM. Depression of hepatic glutathione by opioid analgesic drugs in mice. Toxicol Appl Pharmacol. 1989; 99:139-47.

39. Damian GD, Elizabeth AM, Judith MH, and Ruth R. Drug-Induced Oxidative Stress and Toxicity. J Toxicol. 2012, Article ID 645460, 13 pages. doi: 10.1155/2012/645460

40. Shafer DA , Xie Y. Detection of opiate- enhanced increases in DNA damage, HPRT mutants, and the mutation frequency in human HUT- 78 cells. Environmental and Molecular Mutagenesis. 1994; 23:37-44.

41. Remya NS, Syama S, Sabareeswaran A, Mohanan PV. Investigation of chronic toxicity of hydroxyapatite nanoparticles administered orally for one year in wistar rats. Materials Science and Engineering: C.2017; 76: 518-527