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Original Article

Anti-Inflammatory Properties of Chitosan Hydrogels Containing *Satureja Khuzestanica Jamzad* Essential Oil in an Animal Model

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Abstract

Background and Aim: Nanoparticle shells protect bioactive compounds against the environmental conditions of light, heat, and oxygen, and are also used as carriers for targeted delivery. In the present study, the anti-inflammatory effects of a chitosan-based nanogel containing *Satureja Khuzestanica* essential oil (SKE:NP) were studied and compared with *Satureja Khuzestanica* essential oil (SKE).

Materials and Methods: Nanoparticles were prepared using the ionic gelation method. The anti-inflammatory effects of the chitosan-based hydrogel containing SKE, using the paw inflammatory model induced with 2.5% formalin at different times (0, 1, 2, 3, 4, and 24 h after subcutaneous injection), were evaluated.

Results: Nanoparticles with a particle size of 552.8 nm, a surface charge of 24.3 mV, and an encapsulation efficiency of 30.74% were synthesized. The results of this study showed that SKE:NP (50 mg/kg) could reduce inflammation at all times (0, 1, 2, 3, 4, and 24 h after subcutaneous injection). The hind paw was compared with diclofenac sodium at times (0, 1, 2, 3, 4 h after subcutaneous injection), and a significant decrease in paw inflammation was observed. The lowest amount was observed in the group receiving nanogel 50 mg (0.208 g/cm^3). In contrast, the amount of paw swelling in the group receiving SKE at a dose of 100 mg/kg two hours after the applied treatment was 0.441 g/cm^3 ($P<0.0001$).

Conclusion: A comparison of the results showed that SKE:NP had more anti-inflammatory effects than SKE. Explanation of the exact mechanism of this anti-inflammatory effect requires a separate investigation.

Keywords: Hydrogel, *Satureja Khuzestanica*, Carvacrol, Chitosan, Formalin test

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Introduction

Inflammation is an immunological defense response that protects an organism against injuries resulting from various internal and external stimuli such as microbial infections, allergies, and cancer (1). There is much evidence of the critical role of inflammation in numerous pathological states such as neurodegenerative and cardiovascular diseases, cancers, and diabetes mellitus (2). Clinical signs of

inflammation include fever, edema, redness, and pain. These symptoms are triggered by different inflammatory intermediaries produced by local or infiltrative cells, including interferons, prostaglandins (PGs), interleukins, nitric oxide, and histamine (3). Macrophages are strongly implicated in triggering and maintaining inflammation, and are stimulated by pro-inflammatory cytokines as well as PGE2 (4). The biosynthesis of PGs is mediated by the enzyme cyclooxygenase (COX), which is induced during inflammation and results in PGE2 overproduction (5, 6). The fact that the molecule is relevant to inflammation indicates that its inhibition provides a significant strategy for the development of anti-inflammatory and anti-nociceptive drugs. Nowadays, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to reduce inflammation by inhibiting the COX enzyme (7, 8). Despite their potent anti-inflammatory effects, NSAIDs exhibit a variety of adverse effects (9). Moreover, uncontrolled inflammation results in inflammatory disorders and organ dysfunction (1). Thus, it is critical to develop safe and effective anti-inflammatory agents. Naturally originating compounds have gained attention as promising targets in this regard. Over the years, polymeric nanoparticles (PNPs) have provided opportunities for biomedical delivery applications. PNPs exhibit a variety of advantages such as providing directed drug delivery, controlling drug release, protecting drugs against the environment, and promoting bioavailability and therapeutic efficiency (10). Various methodologies have been developed for synthesizing PNPs. Ionic gelation, freeze-drying, spray drying, and polyelectrolyte complexation are well-known methods for preparing PNPs (11). Chitosan, a linear polysaccharide polymer that comprises d-glucosamine and N-acetyl-d-glucosamine, is produced from chitin derived from crustaceans and insects (12). It is a non-toxic and biocompatible polymer with the ability to form films, membranes, gels, grains, fibers, and particles (13, 14). Chitosan exhibits antioxidant, anti-inflammatory, and antibacterial activities (15–17). Recently, chitosan nanoparticles have drawn scientist attention because they provide excellent physical and functional characteristics (18). Chitosan nanoparticles are generally developed by altering chitosan using different chemical procedures, including ionotropic gelation and spontaneous emulsification (14). *Satureja khuzestanica Jamzad* (SKJ) is an Iranian endemic plant of the Lamiaceae family which has traditionally been used as an anti-spasmodic and anti-nociceptive agent (19). The essential oil of SKJ is a colorless or pale yellow liquid containing phenolic compounds, including carvacrol and flavonoids (19, 20). Carvacrol, 2-methyl-5-[1-methylethyl] phenol, is the major active ingredient in the essential oil of SKJ (19, 21). Carvacrol has been reported to have antimicrobial, anti-inflammatory, and anti-thrombotic activities (22, 23). Its antimicrobial properties enable it to act as a food preservative of natural origin (19, 23). It is well known that chitosan is a suitable bioactive coating for naturally origin compounds that can improve their biological activities (24, 25). Considering the anti-inflammatory effects of chitosan and SKE, encapsulation of SKE essential oils (SKE:NP) in chitosan nanoparticles may allow the possibility of achieving a more effective and safer anti-inflammatory agent than NSAIDs. In this study, we examined the anti-inflammatory effects of SKE-loaded chitosan nanohydrogels compared with diclofenac in a formalin-induced paw swelling model.

Materials and Methods

Chitosan, tripolyphosphate, Tween 80, dichloromethane, sodium sulfate, acetic acid, and formalin were obtained from Merck Co.

Plant Preparation, Extraction and Identification

Satureja khuzestanica was obtained from Khorman Pharmaceutical Company. Extraction of essential oils in the laboratory was perfomed by hydrodistillation using a clevenger apparatus. The aerial parts, including the stems and leaves of the plant, were ground after they were completely dried at ambient temperature in the shade. Powdered *Satureja khuzestanica* (200 g) was extracted in a 3-liter volumetric flask containing 2 liters of water for 5h. It was then dehydrated using sodium sulfate and kept in a dark glass at 4 ℃. The essential oil compounds were identified using gas chromatography-mass spectrometry (GC-MS). SKE was analyzed on an Agilent 6890N coupled to an Agilent 5973 mass detector (USA) with an HP-5 Column, 30m × 0.25 mm (ID) × 0.25 µm and splitless injector temperature of 250°C. The temperature program was as follows: 60°C for 5 min, temperature increase at 10°C min^{-1} to 100°C

held for 5 min, and then temperature increase to 240℃. Helium (99.999%) was used as the carrier gas at a flow rate of 0.9 ml/min^{-1}.

Preparation of the Hydrogel

A chitosan solution (1%) was developed in 2% acetic acid for 2 h under stirring. Subsequently, 200 µl of Tween 80 was added to the chitosan in a paraffin bath at 45℃ and stirred for 2 h. Subsequently, 1ml essential oil of SKE diluted in methanol was added dropwise to the prepared sample in an ice bath and completely homogenized for 10 min using a homogenizer. One milliliter of 0.25% TPP solution, as a crosslinker, was added drop by drop to the chitosan solution and homogenized for 40 min using a homogenizer. The hydrogel was centrifuged at 4℃ and 9000 rpm for 15 min and then dried using a freeze dryer.

Encapsulation Efficiency

The dried nanogel was weighed (0.04 g), and 4 ml of 2 M HCl was added and placed in a bain-marie at 95 ℃ for 30 min. After cooling, 2 ml of ethanol was added to the nanogel and centrifuged (9000 rpm for 5 min). The supernatant was diluted with ethanol and its absorbance was read at 275 nm using a spectrophotometer. Encapsulation efficiency (EE) was calculated using Eq.

$$\left[\text{Encapsulation Efficiency}(\%) = 100 \times \left(\frac{\text{mass of loaded carvacrol}}{\text{mass of initial carvacrol}}\right)\right]$$

Release Test

A certain amount of SKE:NP was placed in a tube containing 5 ml of 40% ethanol and continuously stirred at room temperature. At certain time intervals, the samples were centrifuged at 9000 rpm and 25 ℃ for 5 min. Then, the supernatant (0.5 mL) was removed for measurement and immediately replaced with the solvent (0.5 mL). The absorbance of the samples was measured using a spectrophotometer at 275 nm. Finally, the cumulative percentage of the released amount was calculated using the following formula:

$$\text{Cumulative release}(\%) = \sum_{t=0}^{t} \frac{Mt}{M0} \times 100$$

The prepared nanoparticles were characterized by ultraviolet-visible spectrophotometry (UV-Vis), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and dynamic light scattering (DLS).

Animals

We divided 35 male BALB/c mice weighing 25-30 g with 7 weeks of age into 7 groups as follows:

Group A: 10 µL of normal saline was injected into the hind paw of each animal.

Group B: 10 µL of 200mg/kg diclofenac diluted with normal saline was injected into the hind paw of each animal.

Group C: 10 µL of diluted 100mg/kg SKE was injected into the hind paw of each animal.

Group D: 10 µL of diluted 50mg/kg NPs was injected into the hind paw of each animal.

Group E: 10 µL of 25mg/kg SKE:NP was injected into the hind paw of each animal.

Group F: 10 µL of 50mg/kg SKE:NP was injected into the hind paw of each animal.

Group G: 10 µL of 100mg/kg SKE:NP was injected into the hind paw of each animal.

They were kept in light-dark cycles (12:12-h) at room temperature (22±2 ºC) with water and food access ad libitum. The mice were allowed to adapt to the laboratory situation 30 minutes before beginning the test. The protocol of the study was approved by the Ethics Committee of Lorestan University of Medical Sciences, Khorramabad, Iran (No. 92/17).

Formalin Test

This test was conducted using formalin. The tests were performed between 9 am and 5 pm at ambient temperature and in a stress-free environment. First, the volume of the animal paw was measured using a mercury plethysmograph. Then, 10 µL of 2.5% formalin was injected subcutaneously into the hind paw of each animal. The formalin injection happened 30 minutes after the injection of each drug in the hind paw considering its group. The percentage of inflammatory changes was investigated at distinct time points (0, 1, 2, 3, 4, and 24 h after subcutaneous injection). The volume of the hind paw was recorded and compared with the amount of edema.

Statistical Analysis

The data were analyzed using SPSS software (version 27), one-way analysis of variance (ANOVA), and paired sample tests. Statistical significance was set at $P<0.05$.

Results and Discussion

The chemical composition of SKE, as demonstrated by GC-MS analysis, has been shown in Table 1. According to our findings, 45 compounds were detected in SKE. Carvacrol was the main component of SKE, with an abundance of 60.61%.

Scanning Electron Microscopy (SEM) and the Particle Size

SEM images were obtained for the freeze-dried hydrogel samples. The images show that a completely compact and dense matrix was formed (Figure 1), which can be due to the creation of hydrogen or electrostatic bonds between the polyphosphate groups in tripolyphosphate (TPP) and the amino groups in chitosan. DLS analysis demonstrated that the particles synthesized using SKE showed an average size of 552.8 nm and a surface charge of 24.3 mV (Figure 2). The encapsulation efficiency of the SKE-loaded NPs was 30.74%.

Differential Scanning Calorimetry (DSC)

The DSC thermograms of SKE, nanoparticles (NPs), and SKE:NP have been shown in **Figure 3**, respectively. SKE exhibited an endothermic peak at temperatures below 124℃. The onset temperature for the thermogram of SKE was 46.13℃. The NPs did not show any peak. Decomposition of chitosan nanoparticles started after loading SKE at 120.87℃, and they showed an endothermic peak at 314℃. These results showed that encapsulation increases the thermal stability of the formulation.

Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

In the chitosan sample, the absorption area of 3500-3200 is related to O-H and N-H stretching, and the absorption area of 2924 can be related to C-H stretching

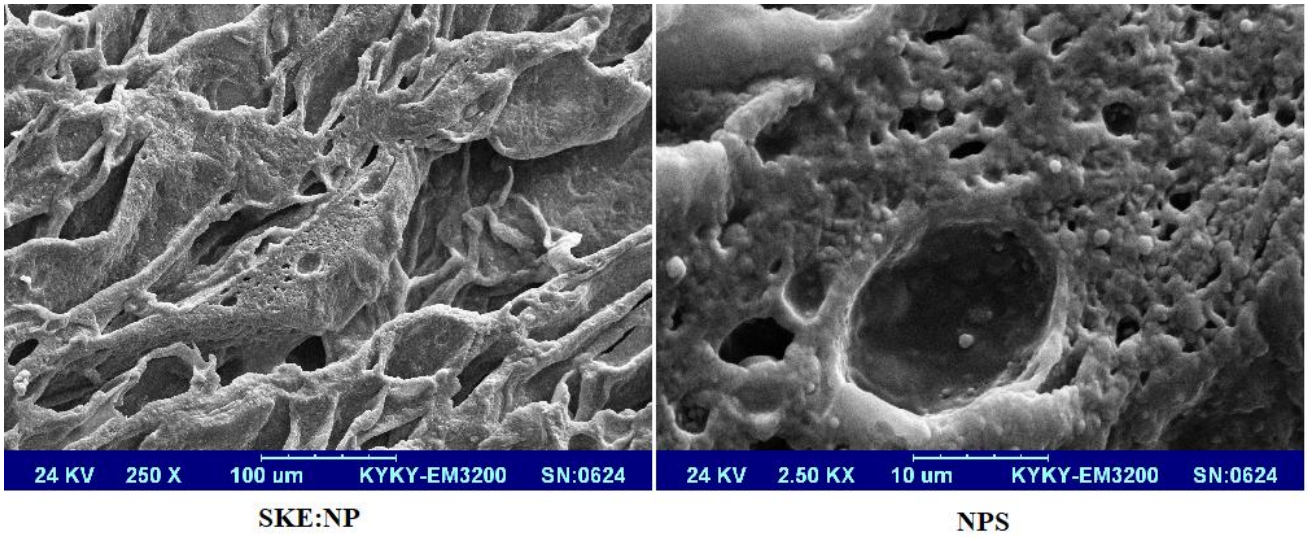

Figure 1. The scanning electron microscope (SEM) images of nanoparticles without SKE (NPs) and nanoparticles loaded with SKE (SKE:NP).

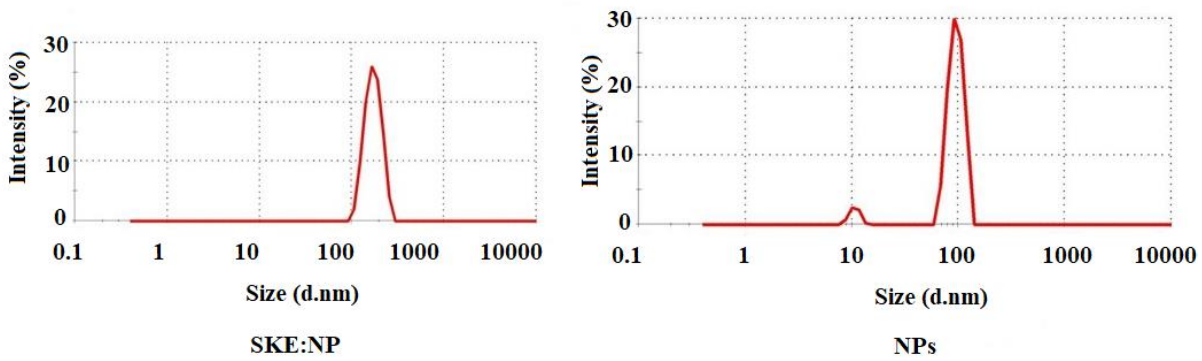

Figure 2. The size of nanoparticles without SKE (NPs) and nanoparticles loaded with SKE (SKE:NP) using DLS at 25 ℃.

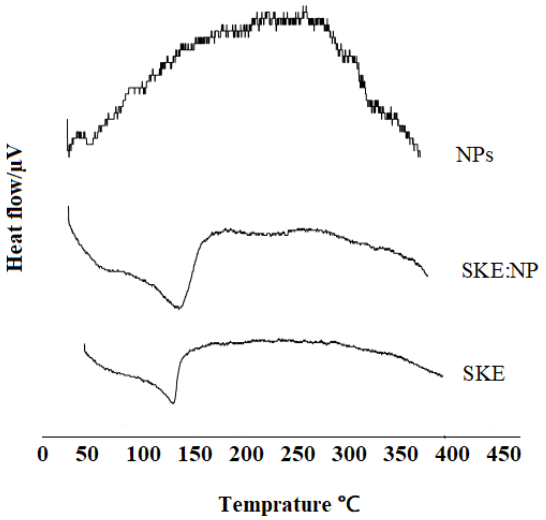

Figure 3. Differential scanning calorimetry (DSC) Thermograms of *Satureja Khozestanica* essential oil (SKE), nanoparticles (NPs) and nanoparticles loaded with *Satureja Khozestanica* essential oil (SKE:NP) under nitrogen degradation.

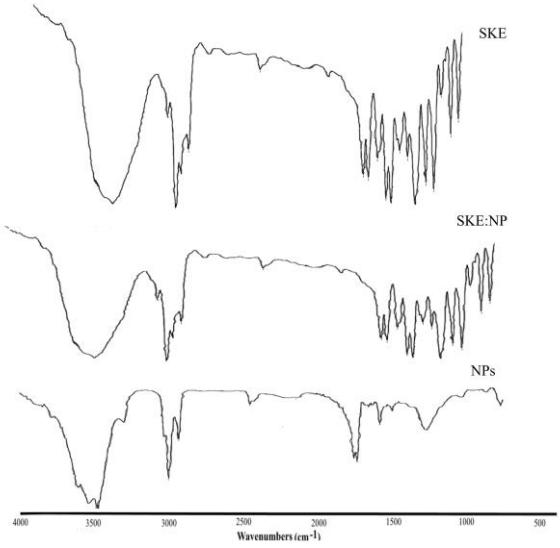

Figure 4. Analysis of the samples by Fourier transform infrared spectroscopy: *Satureja Khozestanica* essential oil. SKE, nanoparticles without SKE (NPs) and nanoparticles containing SKE (SKE:NP). The numbers indicate the wavenumbers of the main groups in the sample components.

(Figure 4). Moreover, the stretching vibrations in the 1638 region contributed to Amid I (C=O stretching). Bending absorptions of Amid II (N-H bonding) groups in the chitosan sample were also observed at approximately 1617 cm^{-1}. The strong absorption of the P-O stretching group, which can be seen in the range of 1214, happened at a lower frequency than the absorption of this group in the combination of chitosan, which could be a reason for the development of new hydrogen bonds between the OH groups and the compounds in the sample resulting from the combination. In the IR spectrum of SKE, there is a strong absorption in the 3415 and 2962 regions, which is associated with the stretching vibrations of the O-H bond and stretching of the C-H of the aromatic rings. Despite the fact that the FTIR spectra of the products obtained by adding SKE were similar to those of the chitosan particles, an increased was observed in the intensity of the CH stretching peak as the initial SKE content increased. This implies the augmentation of the incorporated SKE content as a function of its primary content. Thus, the CH stretching peak was utilized as a probe band for indirect determination of the loaded SKE content.

Release Kinetics

After sampling from the microtubes containing the nanogel, the amount of carvacrol released was

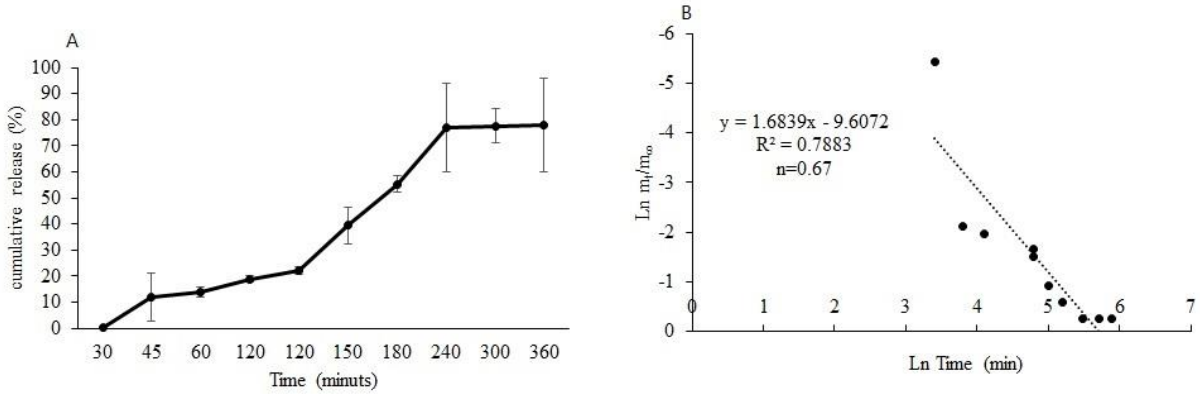

Figure 5. Release kinetics of nanoparticles containing SKE (SKE:NP) at 25°C: (A) cumulative carvacrol release profile; (B) fitting of the data using the Korsmeyer-Peppas mathematical model. Values represent the mean of the three experiments.

Table 1: The chemical components of *Satureja khozestanica* essential oil (SKE).

No.	RT	KI	Area (%)	Compound Name
1	7.33	841.6	0.17	2-methyl ethyl ester butanoic acid
2	7.37	843.7	0.17	Ethyl isovalorate
3	9.2	928.7	0.3	α-thujene
4	9.44	938.2	1.91	α-pinene
5	9.85	954.6	0.23	Camphene
6	10.1	964.5	0.05	2-methylbuthyl propionate
7	10.56	982.9	0.25	β-pinene
8	10.71	988.8	2.38	β-Myrcene
9	10.83	993.6	0.14	dehydro-1,8-cineole
10	11.21	1008	0.72	α-phellandrene
11	11.4	1015	0.22	(MS)
12	11.54	1020	2.34	α-Terpinene
13	11.77	1028	9.66	P-cymene
14	11.89	1032	0.95	limonene
15	11.94	1034	0.68	β-phellandrene
16	11.98	1036	0.53	1,8-cineole
17	12.25	1045	0.05	E-β-Ocimene
18	12.68	1061	4.04	γ-Terpinene
19	13.06	1075	0.05	trans-linalool oxide
20	13.52	1091	1.44	p-cymenene
21	13.72	1099	1.77	linalool
22	15.25	1152	0.09	camphor

23	16.14	1184	1.79	4-terpineol
24	16.66	1202	0.62	α-terpineol
25	17.51	1233	0.55	(MS)
26	17.83	1244	0.51	Carvacrol methyl ether
27	18.26	1260	0.05	d-carvone
28	19.12	1291	0.74	thymol
29	19.67	1311	**60.61**	**carvacrol**
30	20.24	1332	0.09	(MS)
31	21.03	1362	0.13	Eugenol
32	21.33	1373	0.08	carvacrol acetate
33	21.99	1398	0.04	n-tetradecane
34	22.99	1437	0.56	trans caryophyllene
35	23.18	1445	0.14	trans-α-Bergamotene
36	24.52	1498	0.06	n-pentadcane
37	24.96	1516	2.44	β-Bisabolene
38	25.74	1548	0.2	trans-α-bisabolene
39	26.94	1598	0.06	n-hexadecane
40	27.08	1604	0.22	caryophyllene oxide
41	29.22	1698	0.04	heptadecane
42	34.78	1959	0.57	palmtic acid
43	41.93	2300	0.59	(MS)
44	42.35	2315	0.92	(MS)
45	43.78	2382	0.83	(MS)
		99.98 %		Total

calculated at 30, 45, 60, 120, 150, 180, 240, 300, and 360 min using UV-Vis spectroscopy at 275 nm (Figure

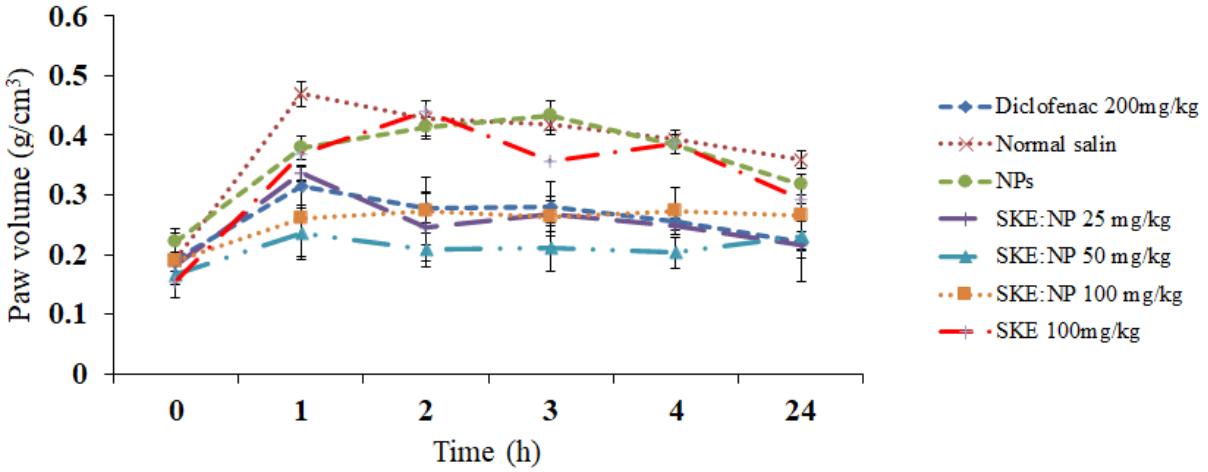

Figure 6. A comparison of the decrease in paw volume after the induction of inflammation in different groups, including Diclofenac (200 mg/kg), normal saline, NPs, SKE:NP 25 mg/kg, SKE:NP 50 mg/kg, SKE:NP 100 mg/kg, and SKE 100 mg/kg.

5). It was shown that 77.84% of the initial carvacrol was released after 360 min. The Korsmeyer-Peppas mathematical model was used to calculate (n), in which a value of 0.67 was finally obtained. This value indicates a non-Fickian release, which can be a combination of two processes, i.e. diffusion and relaxation of the hydrogel.

Formalin Test

The results of the variance analysis of the data (Table 2) showed that the comparison of the averages in different nanogel formulations with and without SKE, the comparison of the averages at different times, and the interaction effects of different nanogel formulations at different times after the induction of inflammation on paw swelling were statistically significant at a probability level of less than 0.0001%. The results indicated that the highest and lowest amounts of paw swelling were related to the group receiving normal saline (0.377 g/cm^3) and nanogel (0.208 g/cm^3), respectively (Figure 6).

This study is the first investigation of the effects of SKJEO-loaded chitosan nanohydrogels on paw edema in mice following formalin administration. The results of this study indicated that the encapsulation of SKE in chitosan nanoparticles considerably increased its anti-inflammatory effects on paw edema, comparable to that of diclofenac. The physical and functional characteristics of chitosan make it a suitable carrier for biomedical delivery (18, 26).

Several studies have shown that the encapsulation of biological compounds in chitosan nanoparticles increases their biological activities (19, 27). In this regard, we speculated that encapsulation of SKE in chitosan nanohydrogels would result in a more intensive anti-inflammatory activity in mice with paw edema. The physicochemical characteristics of the prepared nanoparticles showed successful synthesis of the SKE-loaded chitosan nanohydrogel and uniform distribution of nanoparticles in the nanoformulation. Overall, smaller nanoparticles allow for better cellular uptake, distribution, surface/volume ratio, and colloidal stability (28, 29). Intriguingly, a small size of 552.8 nm was observed for the prepared SKE-loaded chitosan nanohydrogels. Furthermore, a release test showed that 77.84% of the initial carvacrol was released after 360 min. The Korsmeyer–Peppas mathematical model determined the pattern of carvacrol release from the chitosan nanohydrogel, indicating a release pattern including diffusion and relaxation of the nanohydrogel. It has been indicated that the release pattern of chitosan nanoparticles is influenced by different factors such as the nanoparticle shape and size, polymer molecular weight, and interaction of the nanoparticles with the used polymer (27, 30). Formalin-induced inflammatory paw model is a well-known method for investigating the anti-inflammatory effects of drugs or other compounds (31, 32). Formalin injection into the animal paw results in paw edema in a dose-dependent manner, reaching its highest level approximately 3 h after formalin administration (31). In a previous study, Amanlou *et al.* (33) found that the anti-inflammatory activity of the

Table 2: Paw volume after the induction of inflammation in different groups receiving normal saline, diclofenac, NPs, and SKE:NP.

Treatments	Paw volume (g/cm^3)						P value
Groups	T=0h	T=1h	T=2h	T=3h	T=4h	T=24h	Level of significant
Normal saline	0.335	0.470	0.419	0.395	0.360	0.193	<.0001
Diclofenac (200mg/kg)	0.191	0.314[a]	0.279 [a]	0.280 [a]	0.255 [a]	0.220 [a]	<.0001
SKE (100mg/kg)	0.155	0.370 [a]	0.441 [b]	0.356 [a,b]	0.386	0.292	<.0001
NPs	0.220	0.379 [a]	0.412 [b]	0.433 [b]	0.384 [a,b]	0.317 [b]	<.0001
SKE:NP 25 mg/kg	0.181[a,]	0.336 [a]	0.245 [a,c]	0.269 [a,c]	0.248 [a,c]	0.216 [a,c]	<.0001
SKE:NP 50 mg/kg	0.166	0.235 [a,b,c]	0.208 [a,c]	0.212 [a,b,c]	0.203 [a,b,c]	0.231 [a]	<.0001
SKE:NP 100 mg/kg	0.190	0.261 [a,c]	0.273 [a,c]	0.263 [a,c]	0.272 [a,b,c]	0.264 [a]	<.0001

[a] Significant difference compared with normal saline
[b] Significant difference compared with diclofenac
[c] Significant difference compared with the NPs

hydroalcoholic extract of *Satureja khuzestanica* was greater than that of indomethacin. In another study, *Satureja hortensis* essential oils were reported to produce anti-inflammatory effects during the paw-edema test (34). These studies strongly confirm the anti-inflammatory effects of Satureja species. Interestingly, our results indicated that SKE showed noticeable anti-inflammatory effects in mice with paw swelling, and encapsulation of SKE in chitosan nanohydrogel increased its anti-inflammatory effects. These results are in accordance with those of other studies that have indicated the anti-inflammatory effects of Satureja species essential oils (33, 34). Moreover, we found that SKE was rich in carvacrol, flavonoids, and terpenoids. These compounds may be responsible for the anti-inflammatory activities of the SKE-loaded chitosan nanohydrogel in our rodent model. Furthermore, the anti-inflammatory properties of chitosan should also be considered (17).

Our findings indicated that SKE-loaded chitosan nanoparticles at different doses (25, 50, and 100 mg/kg) considerably reduced paw volume after formalin injection. A dose of 50 mg/kg exhibited the greatest anti-inflammatory effect on paw swelling. Formalin-induced inflammatory paw swelling has been shown to upregulate COX2 and increase inflammatory mediators (35, 36). Several studies have highlighted the anti-inflammatory effects of carvacrol as the main component of SKE, which reduces inflammatory mediators through suppressing COX2 (37, 38). Moreover, Jalalvand *et al*. reported that *Satureja khuzestanica* essential oils and carvacrol attenuated LPS-induced inflammatory response by inhibiting COX2 (39). Interestingly, Chou *et al*. delineated that chitosan remarkably inhibited the COX2 pathway (40). Taken everything into consideration, the anti-inflammatory activities of the SKJEO-loaded chitosan nanohydrogel might be related to its inhibitory effects on the COX2 pathway. The possible inhibitory effects of the SKJEO-loaded chitosan nanohydrogel on the COX pathway require further investigation in our rodent model.

Conclusion

In summary, our findings indicated that SKE-loaded chitosan nanohydrogels could exert noticeable anti-inflammatory effects in mice with formalin-induced paw swelling. Furthermore, the encapsulation of SKE in chitosan nanohydrogels could enhance its anti-inflammatory activities. Interestingly, the SKE:NP chitosan nanohydrogel was more effective than diclofenac in reducing paw swelling following formalin administration. Additionally, SKE-loaded chitosan nanoparticles at a dose of 50 mg/kg could exhibit the greatest anti-inflammatory effects on paw edema compared with other doses (25 and 100 mg/kg).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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