Original Article

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Development and characterization of local anti-inflammatory implantation for the controlled release of the hexane extract of the flower-heads of *Euryops pectinatus* L. (Cass.)

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ABSTRACT: A hexane extract of the flower-heads of *Euryops pectinatus* L. (Cass.) was formulated into local anti-inflammatory implantation patches with controlled release. Cross-linked sodium hyaluronate patches (F1-F3) and chitosan patches (F4-F6) were prepared by a casting/solvent evaporation technique. Morphological and mechanical characterizations including the components ratio, surfactant and the loaded amount of the hexane extract (50, 100, and 200 mg/kg b.wt.) were investigated. Release studies were performed during 24 h using a diffusion cell. Films with optimum *in vitro* release rate have been investigated for testing the anti-inflammatory activity and the sustaining effect of the formulations. The sustained anti-inflammatory effect of the hexane extract of *E. pectinatus* flower-heads from the selected films was studied by inducing paw edema in rats with 1% (w/v) carrageenan solution. The results indicated the compatibility of hexane extract with both sodium hyaluronate and chitosan patches forming yellowish transparent films. Based on variations in drug release profiles throughout the 24-h among the formulations (F1-F6) studies, F3 and F6 were selected for further investigation. When the films were applied 1 h before the subplantar injection of carrageenan in the hind paw of male Albino rats, formulation (F3) provided its maximum inhibition of paw edema in rats (91.3%) 4 h after edema induction whereas, formulation (F6) showed less inhibition after 4 h (70.6%). The previous two formulations (F3 and F6) produced potent results (95.3 and 89.5%, respectively) after 24 h when compared with a local market preparation containing 25% β-sitosterol used as positive control. Histophathological investigation was conducted for 1, 4, and 12 weeks to study the tissue response for the two formulations (F3 and F6) at the implantation site. Chemical investigation of the hexane extract was achieved for both unsaponifiable matter (USM) and fatty acid methyl esters (FAME) using gas liquid chromatography (GLC). The USM was dominated by *n*-pentacosane (14.40%), phytosterols (Cholesterol, Campesterol, Stigmasterol, β-sitosterol, α-amyrin) reached 33.44% and the FAME was dominated by Linoleinic (49.97%). Quality control of the local implantation was evaluated by GLC using cholesterol as an analytical marker and phytosterols as an active marker compared to the plain extract.

Keywords: *Euryops pectinatus* hexane extract, chemical investigation, local anti-inflammatory implantation, sodium hyaluronate, chitosan patches, *in vitro* release-*in vivo* study

1. Introduction

Medicinal plants constitute a source of raw materials used for treatment of a variety of illnesses. Although most of these plants in their natural state are not fit for administration, preparations suitable for administration are made according to modern pharmacopeia directions. *Euryops pectinatus* L. (Cass.), family Asteraceae, has been previously incorporated in the development of a novel leaf petroleum ether anti-inflammatory gel emulsion formulation and found to be safe and pleasant to use while capable of providing its activity. The selected formulation was polyethylene glycol 400, Tween 80, stearic acid, dimethicone, Carbopol 940 NF, and a water gel emulsion as a carrier in a topical drug delivery of 2% petroleum ether leaf extract (*1*).

In recent years, biodegradable polymeric systems have gained importance for design of drug delivery systems with different routes of administration (*2*), synthetic (polyesters, polyamides, and polyanhydrides) and natural (polyamino acids and polysaccharides) (*3*). Polysaccharide-based polymers represent a major

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class of biomaterials, which includes agarose, alginate, carrageenan, dextran, chitosan, and hyaluronic acid.

Hyaluronic acid is a naturally occurring macromolecular polysaccharide found in synovial fluid, extracellular matrices, connective tissues, and organs of all higher animals. More recently, hyaluronic acid has been investigated as a drug delivery agent for various routes of administration including ophthalmic, nasal, pulmonary, oral, parenteral, and topical (*4-6*). The physical properties of hyaluronic acid can be altered by controlled esterification with alcohols to produce a variety of dosage forms such as fibres, films, gels, sponges, gauzes, and pellets (*7,8*). For the purpose of preparing an extract-film, it would be more advantageous to use polysaccharides, biodegradable and environmentally benign materials as film-forming materials than synthetic polymers.

Chitosan, poly-β-(1,4)-2-amino-2-deoxy-D-glucose, is a hydrophilic biopolymer obtained industrially by hydrolyzing the aminoacetyl groups of chitin, which is the main component of shells, crabs, shrimp, and krill, by alkaline treatment. Chitosan has gained increasing importance in the pharmaceutical field owing to its good biocompatibility, non-toxicity and biodegradability (*9,10*). It is used in the food industry (*11*), in cosmetics and as a bioadhesive in numerous pharmaceutical applications in the form of beads, microspheres, and microcapsules, typically for the prolonged release of drugs (*12,13*). Chitosan films are usually prepared by chemical cross-linking with glutaraldehyde (*14*).

The film-forming property of chitosan has found many applications in tissue engineering and drug delivery by virtue of its mechanical strength and rather slow biodegradation (*10*). Some drug-loaded chitosan films are emerging as novel drug delivery systems, and films appear to have potential for local sustained delivery (*15,16*).

Poloxamers, a class of non-ionic surfactants, polyoxyethylene-polyoxypropylene block-type copolymers, exhibited mucoadhesive properties with many pharmaceutical applications. Poloxamer 407 (P407), which has a molecular weight of 12,000 daltons and a PEO/PPO ratio of 2:1 by weight, has been the most widely used of these copolymers (*17-19*).

Physicochemical properties such as moisture content, moisture uptake, and tensile strength were analyzed together with the thickness of the *E. pectinatus* flower-heads hexane extract-loaded films, and the extract release profiles. Furthermore, these patches have been examined for tissue response anti-inflammatory reactions by histological examination after implantation in rats.

The objective of this study is to prepare a novel local sustained anti-inflammatory implantation of sodium hyaluronate or chitosan together with a natural herbal extract of *E. pectinatus* flower-heads, for localized action based on implantable systems. The chemical composition of the hexane extract was investigated to suggest the compounds responsible for activity. Finally, marker compounds were chosen and their presence in the final formulation was monitored.

2. Materials and Methods

2.1. *Plant material*

Flower-heads of *Euryops pectinatus* L. (Cass.) were collected during the flowering stage (February-April 2008) from the Experimental Station of Medicinal Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Cairo, Egypt. The plant was authenticated by Terase Labib, Agriculture Engineer of Orman Garden, Giza, Egypt, and the identity was confirmed by the late Professor Dr. M. Abd-El Fattah Zaki, Professor of Plant Taxonomy, Faculty of Science, Cairo University. Voucher specimens were kept at the Herbarium Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

2.2. *Animals*

LD_{50} was determined using Albino mice weighing 20-25 g (*20*). For anti-inflammatory activity and histopathological studies, adult Albino rats of the Sprague Dawely and Wister strains weighing 100-120 g and 180-220 g respectively were used. Animals were obtained from the animal house colony, National Research Center (NODCAR Laboratory Animal Center), Cairo, Egypt, and kept on standard laboratory diet under hygienic conditions. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt, following the World Medical Association Declaration of Helsinki (WMA General Assembly, 1964).

2.3. *Material for formulation*

Sodium hyaluronate from *Streptococcus equi* sp., chitosan, Poloxamer 407 were purchased from Sigma-Aldrich (St Louis, USA). Glacial acetic acid was obtained from LOBA Chemie (Mumbai, India). Glutaraldehyde was purchased from Merck (Darmstadt, Germany). Hematoxylin and eosin were from Fluka (Buchs, Switzerland) and BDH Chemicals (Poole, UK), respectively. Formalin was from Adwic (Cairo, Egypt). Vicryl® sutures 4-0 were from Ethicon (Livingston, UK). Spectra/Por® 3 dialysis membrane (cellophane membrane of MWCO: 3,500 daltons) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Betadine solution was from Nile Co. for Pharmaceuticals and Chemical Industries (El-Nile; Cairo, Egypt) under license from Mundipharma Laboratories (Basel, Switzerland). β-Sitosterol (25%; MEBO ointment) from Gulf Pharmaceutical Industries (Ras Al Khaimah, UAE) was used as standard material for

anti-inflammatory activity. Mebo cream was from Julphar, Gulf Pharmaceutical Industries. All other materials used were analytical grade.

2.4. *Preparation and analysis of hexane extract of E. pectinatus flower-heads*

Four hundred grams powdered flower-heads were exhaustively extracted by refluxing with hexane (4 L × 4). The hexane extract was filtered and evaporated to dryness under reduced temperature and pressure to afford the hexane extract. One gram hexane extract was saponified and the USM and FAME were prepared (*21*) and analyzed by GLC techniques. Qualitative GLC analysis of both USM and FAME were achieved by comparison of retention times of each peak from the sample with those of pure authentic materials. Quantitative GLC determination was carried out based on peak area measurements.

2.5. *Incorporation of the hexane extract into the different formulations*

Due to the hydrophobic character of the hexane extract of *E. pectinatus* flower-heads, one approach was used for its incorporation into sodium hyaluronate or chitosan films by using only Poloxamer 407 in the presence of the extract (Polox-Ext) at a ratio of 1:2 (w/w).

2.6. *Preparation of cross-linked sodium hyaluronate patches by solution casting (F1-F3)*

Initially, 1 g of sodium hyaluronate was hydrated in 40 mL of distilled water to obtain a 2.5% (w/v) polymer solution. The solution mixture was heated in a water bath at 37°C with stirring, and then 20 mL of 0.01 N HCl was added to the viscous solution. Glutaraldehyde cross-linked sodium hyaluronate patches were prepared by adding 20 mL of aqueous solution of 300 mM glutaraldehyde to the sodium hyaluronate solution. Finally, 20 mL of 80% (v/v) acetone was added and stirred without heating to avoid evaporation of acetone. After thorough mixing, 1 mL of an aqueous solution of Polox-Ext (equivalent to 50, 100, or 200 mg of *E. pectinatus* flower-heads) was added to the mixture and well stirred. The polymer casting solution was left to stand until all air bubbles had disappeared. The solution was cast into Petri dishes and allowed to air dry at room temperature for three days. The resulting film was peeled off, dried in vacuum (> 0.1 mmHg) for 8 h, cut into 2 × 2 cm^2 test sections and then stored in a desiccator. The prepared sodium hyaluronate formulations (F1-F3 and F7 as a plain sodium hyaluronate patch without extract) are listed in Table 1.

2.7. *Preparation of cross-linked chitosan patches (F4-F6)*

Chitosan films were produced using a casting solvent evaporation technique. Initially, chitosan (1.0 g) was dissolved in 40 mL of 1% (w/v) aqueous acetic acid solution. Glutaraldehyde cross-linked chitosan films were prepared by adding an aqueous solution of glutaraldehyde to this chitosan solution at a ratio of 6.9:1 (chitosan/glutaraldehyde, w/w) to initiate the reaction between the amino group of chitosan and the aldehyde group of glutaraldehyde (*22*). The mixture was mechanically stirred for 30 sec in order to mix and homogenize the solution and then the mixture was left standing at room temperature. The reaction appeared to be complete in less than 1 h. After sonication, 1 mL of aqueous solution of Polox-Ext (equivalent to 50, 100, or 200 mg of *E. pectinatus* flower-heads) was added to the mixture and well stirred. The mixture was left to stand until trapped air bubbles were removed, and poured on a glass plate. The films were dried for 48 h in an oven at 37°C, and further dried under vacuum at room temperature until constant weight was achieved. The dried films were cut into 2 × 2 cm^2 test sections. The chitosan patches formed were slightly yellow and turned bright yellow in color after storage at room temperature in a desiccator. All chitosan patches (F4-F6 and F8 as a plain chitosan patch without extract) are listed in Table 1.

2.8. *Evaluation of the physicochemical properties of the patches*

2.8.1. *Morphology examination*

The cross-sectional morphologies of the plain patches of sodium hyaluronate (F7) and chitosan (F8) as well as F3 and F6 patches containing 200 mg Polox-Ext were

Table 1. Formulations of hexane extract from *E. pectinatus* flower-heads

Formulations	Symbols	Contents
F1	SHA-a	Sodium hyaluronate patch containing 50 mg hexane extract
F2	SHA-b	Formulated 100 mg hexane extract in sodium hyaluronate patch
F3	SHA-c	Formulated 200 mg hexane extract in sodium hyaluronate patch
F4	CH-a	Formulated 50 mg hexane extract in chitosan patch
F5	CH-b	Formulated 100 mg hexane extract in chitosan patch
F6	CH-c	Formulated 200 mg hexane extract in chitosan patch
F7	SHA	Plain sodium hyaluronate patch without extract
F8	CH	Plain chitosan patch without extract

examined using an optic imaging system (Heidelberg Engineering, Heidelberg, Germany) consisting of a light microscope equipped with a color digital camera linked to a computer *via* an image-capturing board, where the Image-Pro Plus software package ver. 5.0.1 (Media Cybernetics, Bethesda, MD, USA) was installed.

2.8.2. *Moisture analysis*

Moisture determination is one of the most frequently performed analyses of the product. The water content directly influences the quality (*23-26*), processability, shelf life, and stability of a wide range of products (*27-30*). The patches were analyzed for their residual moisture content using a Karl Fischer titrator 787 KF Titrino (Metrohm USA Inc., Riverview, FL, USA). Each patch was inserted in the titration vessel containing dried methanol (HPLC grade) and titrated with the titrator after a stirring time of 2 min. Results are presented as means ± S.D. (n = 3).

2.8.3. *Moisture uptake*

A weighted film kept in desiccators at normal room temperature for 24 h was taken out and exposed to 84% relative humidity (saturated solution of potassium chloride) in a desiccator until a constant weight for the films was obtained. The percentage of moisture uptake was calculated as the difference between final and initial weight with respect to initial weight (*31*).

2.8.4. *Measurement of film thickness and mechanical properties*

Film thickness was measured using a micrometer (Mitutoyo, Kanagawa, Japan) with the smallest possible unit measurement count of 0.01 mm. Tensile strength of film was measured using tensile strength and compression tester (Tinius Olsen Model H1K-S tensile strength and compression tester; Tinius Olsen, Ltd., Surrey, UK). Film was secured with tensile grips, and a trigger force of 5 g was applied. The percent elongation % (ε) and tensile strength of the dried film were measured on an electronic tester machine.

2.9. *In-vitro release studies*

Cholesterol was chosen as an analytical marker. Determination of release rates of cholesterol in the Polox-Ext from different formulated sodium hyaluronate patches (F1-F3), as well as formulated chitosan patches (F4-F6) was carried out using a diffusion cell and cellophane dialysis membrane previously soaked for 24 h in the dissolution medium and stretched around one end of the tube. The whole tube was hanged into 100 mL glass beaker. The receptor is composed of 100 mL of ethanol/water (1:1, v/v) thermostatically adjusted to 37 ± 0.5°C and stirred at 50 rpm on a magnetic stirrer. Accurately weighed film of size 2 × 2 cm^2 of each tested formulation equivalent to 0.012 g hexane extract was introduced into the donor tube. At appropriate time intervals, aliquots of the solutions were withdrawn and the amount of cholesterol released from the Polox-Ext loaded patches were evaluated at 620 nm using a UV spectrophotometer (Shimadzu, Kyoto, Japan). Then, an equal volume of the same dissolution medium was added back to maintain a constant volume. Percent cholesterol released from different patch formulations was measured in duplicate and plotted against time.

2.10. *Kinetic analysis of the release data*

The data obtained from the release studies were kinetically analyzed to determine the mechanism and the order of drug release from different formulations (*32*). Linear regression analysis was done to test the goodness of fit of the data to the following models:

$$C_t = C_o - Kt \quad \text{(for zero-order kinetics)}$$

$$\text{Log } C_t = [-Kt/2.303] + \log C_o \quad \text{(for first-order kinetics)}$$

where C_t is the amount of drug remained to be released in time t. C_o is the initial amount of the drug. K is the first order rate constant.

$$Q = Kt^{1/2} \quad \text{(for Higuchi diffusion model) (33)}$$

where Q is the amount of the drug released in time t. K is the Higuchi dissolution constant.

2.11. *Anti-inflammatory activity*

The sustained anti-inflammatory activity was evaluated by the carrageenan-induced rat hind paw edema test (*34,35*). Male albino rats (n = 6) were topically treated with 200 mg/kg b.wt. hexane extract. One hour later, all animals had a subplanter injection of 0.1 mL of 1% carrageenan solution in saline in the right hind paw and 0.1 mL saline in the left hind paw. Four hours after topical administration, both hind paws were separately weighted to calculate the weight of edema. The percentage edema and inhibition were calculated according to the following equations respectively:

$$\% \text{ edema} = \frac{\text{wt. of right paw} - \text{wt. of left paw}}{\text{wt. of left paw}}$$

$$\% \text{ edema inhibiton} = (Mc - Mt) \times 100/Mc$$

where Mc is the mean edema in control rats and Mt is the mean edema in drug-treated animals. The positive control group was treated similarly with a commercial cream containing 25% β-sitosterol and the negative control was treated with saline.

For testing the different formulations, F3, F6, F7, and F8 were applied topically on the right hind paw and the animals were treated similarly to check for the sustained anti-inflammatory activity at intervals of 4, 12, and 24 h, respectively. The obtained results were statistically analyzed using the Student's *t*-test (*36*). Results with $p < 0.05$ were considered statistically significant.

2.12. *In vivo implantation studies of patches*

Biodegradation of patches was studied in healthy Wister rats (12 rats), weighting 180 to 220 g free of fungi and potentially pathogenic bacteria. The test periods were 7, 14, and 21 days. During the experiment, the rats had free access to food and water. The back of each rat was shaved with a hair clipper (Oster® animal electric hair clipper, model A5-000) and cleaned using betadine solution. Rats were anesthetized by inhalation of diethyl ether, and an incision was made in the back of the neck region with a scalpel. The implantation site was created by tunneling immediately beneath the skin, then films were inserted and the skin was sutured. Every animal received one patch whose sharp edges were rounded before implantation.

2.13. *Histopathological studies*

These studies were performed to examine the tissue response of the formulations F3 and F6 containing 200 mg extract at the implantation site. After 1, 4, and 12 weeks implantation period, 12 adult rats were divided into the following 3 groups (4 animals each): Group I, those who received F3; Group II, those who received F6; Group III, control sham surgery (without the implant). The same procedures were done as previously mentioned. The animals were humanely killed at time intervals of 1, 4 and 12 weeks by cervical dislocation and an incision was made in the implantation area. The tissue in which the patch was implanted was removed and stored in 50% formalin until processing. Subsequently, tissue processing involved dehydration through a graded series of alcohols (70, 80, 95, and 100%). To obtain thin sections (3-5 µm), tissues were embedded on the edge of paraffin blocks and were cut on a rotary microtome (Model RM2125; Leica Microsystems Wetzlar, Germany). The sections were deparafinized, rehydrated with graded alcohols (100, 95, 80, and 70%) and stained with hemotoxylin/eosin for microscopic examination (*37*). Sham surgery (without the implant) was used as a negative control group. Evaluation was done using light microscopy and photomicrographs of these sections were taken.

2.14. *Gas liquid chromatography (GLC) analysis*

Agilent Technologies 6890 N Network GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a dual flame ionization detector was used. The column used was HP-5 (5% phenyl methyl siloxane) coated capillary column, 30 cm × 320 µm, film thickness 0.25 µm.

For unsaponifiable matter (USM), the following conditions were adapted: the injector port temperature, 250°C; detector cell temperature, 300°C; carrier gas, nitrogen (30 mL/min); detector gas, hydrogen (30 mL/min) and air (300 mL/min). The column temperature was kept at 80°C and the injector temperature was kept at 80°C for 2 min, increased to 300°C at a rate of 10°C/min, then kept isothermally for 30 min.

For fatty acid methyl ester (FAME), the following conditions were adapted: the injector port temperature, 250°C; detector cell temperature, 280°C; carrier gas, nitrogen (30 mL/min) and air (300 mL/min). The column temperature was kept at 120°C for 2 min, increased to 250°C at a rate of 40°C/min, then kept isothermally for 230 min.

3. Results

3.1. *Morphological and physicochemical properties of formulations*

Morphological analysis of F3 and F6 patches by light microscope showed that the cross-section of both formulations was smooth and homogeneous without any micro-phase separation (Figure 1). This result indicates a good compatibility between the hexane extract and the matrix either sodium hyaluronate or chitosan.

Residual moisture content from the different formulations are listed in Table 2. Plain sodium hyaluronate patch (F7) and plain CH patch (F8) contained the lowest water content equivalent to 4.93 ± 0.83%, 5.48 ± 0.84%, respectively, while F1 and F3 possessed the highest water content equivalent to 11.50 ± 2.03% and 11.37 ± 0.12%, respectively. Percentage of moisture uptake was calculated from the weight difference relative to the initial weight after exposing the prepared films to 84% relative humidity (saturated solution of potassium chloride). The results of the moisture uptake studies for different formulations are

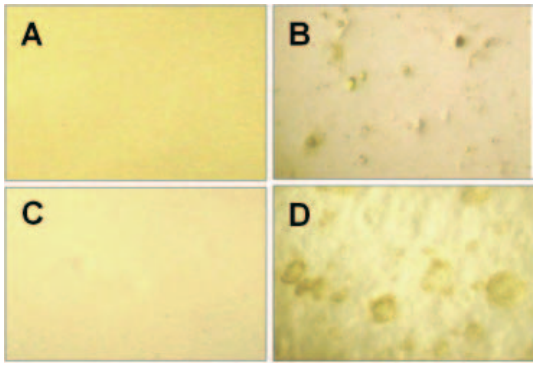

Figure 1. Typical microphotographs of F3 and F6 formulations. A, blank patches of sodium hyaluronate; **B**, sodium hyaluronate patch-F3 loaded hexane extract; **C**, blank patch of chitosan; **D**, chitosan patch-F6 loaded hexane extract.

Table 2. Physicochemical properties of the hexane extract of the flower-heads of *E. pectinatus* patches

Formulations	Water content[a] (%)	Moisture uptake (%)	Thickness[a] (mm)	Elongation[a] (%)	Tensile strength[a] (N)
F1	11.37 ± 0.12	41.9	0.224 ± 0.007	2.86 ± 0.09	11.95 ± 0.76
F2	10.45 ± 1.18	46.6	0.267 ± 0.004	2.28 ± 0.18	16.61 ± 0.21
F3	11.50 ± 1.03	51.3	0.286 ± 0.014	3.48 ± 0.22	18.61 ± 0.72
F4	7.00 ± 0.41	13.7	0.347 ± 0.020	0.71 ± 0.14	3.33 ± 0.28
F5	7.27 ± 0.41	21.4	0.290 ± 0.020	0.76 ± 0.01	5.60 ± 0.14
F6	7.60 ± 0.61	23.6	0.243 ± 0.021	2.18 ± 0.09	12.96 ± 0.25
F7	5.48 ± 0.83	34.2	0.247 ± 0.004	1.27 ± 0.42	4.10 ± 0.89
F8	4.92 ± 0.82	10.3	0.231 ± 0.020	0.90 ± 0.02	2.20 ± 0.05

[a] Data are presented as means ± S.D. (n = 3).

shown in Table 2. It was found to be in the range of 41.9 to 51.3% and the plain film gave 34.2% in sodium hyaluronate patches, while in case of chitosan patches it was found to be in the range of 13.7 to 23.6% and the plain film gave 10.3%.

Thickness of the films was measured by a Hans Schmidt micrometer. As shown in Table 2, the film thickness was between 0.22 ± 0.01 and 0.34 ± 0.02 mm. Mechanical strength of film is described in terms of tensile strength, and brittle films are characterized by a decrease in the percentage of elongation at break. Table 2 also shows the tensile strength and the elongation of extract-free and extract-loaded patches (F1-F8). Regarding the sodium hyaluronate patch, F3 showed a moderate tensile strength (18.61 ± 0.72 N) and high % elongation (3.48 ± 0.22%) in comparison with plain film sodium hyaluronate which gave a low tensile strength (4.10 ± 0.89 N) and low % elongation (0.9 ± 0.02%). On the other hand, regarding the chitosan patches, F6 gave less tensile strength (12.96 ± 0.25 N) and the % elongation was 2.18 ± 0.09% while those values in the plain film of chitosan were 2.20 ± 0.05 N and 0.9 ± 0.02%, respectively.

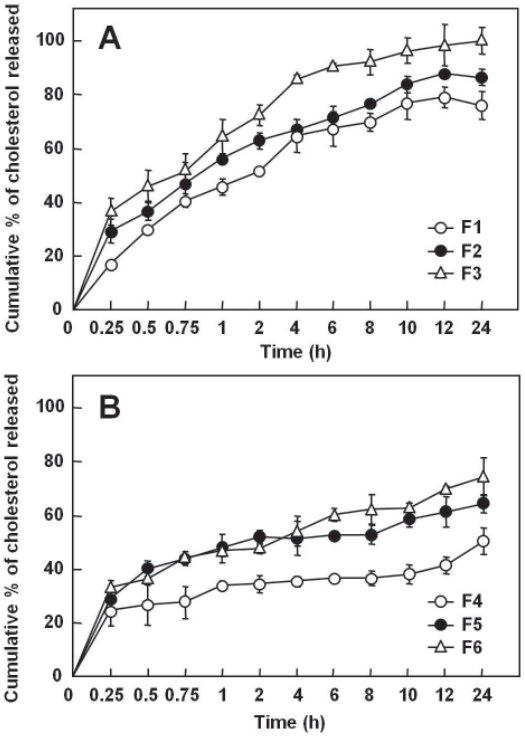

Figure 2. *In vitro* release of hexane extract of the flower-heads of *E. pectinatus* from various patches. (A) *In vitro* release from local sodium hyaluronate patches. Open circle, F1; closed circle, F2; open triangle, F3. **(B)** *In vitro* release from local chitosan patches. Open circle, F4; closed circle, F5; open triangle, F6.

3.2. *In vitro release studies*

Release studies from different patch formulations were performed using a diffusion cell. The calibration plot showed a linear relationship over the cholesterol concentration range of 5-40 µg/mL with a correlation coefficient of 0.999 (Y = 0.01026X + 0.00049). Preliminary tests of sodium hyaluronate patches (F1-F3) and the formulated extract of chitosan patches (F4-F6) together with different extract-loaded amounts (50, 100, and 200 mg, respectively) showed that when more extract was loaded, an increased cumulative release amount was observed (Figure 2). This suggests that more persistent release could be achieved by increasing the extract-loaded amount. Figure 2 also showed that the percent amount of cholesterol released to the receptor half-cell (Cr) by the rate of extract release decreased in the following order: F3 (100.0 ± 1.2% at 24 h) > F2 (88.0 ± 0.8% at 12 h) > F1 (79.1 ± 7.0% at 12 h) > F6 (73.8 ± 1.1% at 24 h) > F5 (63.8 ± 3.3% at 24 h) and F4 (50.0 ± 4.9% at 24 h). The kinetic analysis of the extract release data from different patch formulations was calculated by linear regression analysis according to zero and first order kinetics and a simplified Higuchi model. As shown in Table 3, the release follows the Higuchi kinetic model, suggesting that the diffusion dominated extract release had little or no drop in dissolution values throughout the test period.

3.3. *Anti-inflammatory activity in hexane extract of flower-heads of E. pectinatus L. (Cass.)*

LD_{50} of the hexane extract was 4.9 g/kg b.wt., which suggests the safety of the extract according to the Hodge and Sterner scale. Next, to examine anti-inflammatory activity, inflammation was induced by carrageenan, a sulphated polyanionic polysaccharide commonly used in experimental animals to screen for the effectiveness

Table 3. Analysis of release data of cholesterol in the hexane extract of the flower-heads of *E. pectinatus* from different patches (F1-F6) according to zero and first order kinetics and diffusion model

Formulations	Parameters	Zero order kinetics	First order kinetics	Diffusion model
F1	Slope	7.61	0.0780	23.9
	Intercept	29.0	1.45	14.1
	R^2	0.811	0.649	0.925
F2	Slope	6.42	0.0552	20.6
	Intercept	39.2	1.59	26.1
	R^2	0.745	0.662	0.889
F3	Slope	8.92	0.0608	27.6
	Intercept	44.7	1.65	27.9
	R^2	0.863	0.783	0.951
F4	Slope	1.87	0.0264	6.00
	Intercept	27.1	1.43	23.3
	R^2	0.686	0.661	0.821
F5	Slope	2.89	0.0297	9.69
	Intercept	38.7	1.58	32.3
	R^2	0.530	0.475	0.907
F6	Slope	4.16	0.0389	12.8
	Intercept	36.8	1.57	29.1
	R^2	0.892	0.829	0.973

Table 4. Anti-inflammatory activity of the plain and formulated patches of the hexane extract of the flower-heads of *E. pectinatus* L.

Formulations	4 h			12 h			24 h		
	Edema	% change	Potency[a]	Edema	% change	Potency	Edema	% change	Potency
Control (1 mL saline)	62.9 ± 2.4	–		63.1 ± 1.7	–		64.6 ± 1.8	–	
Hexane extract (200 mg)	31.3 ± 1.2[b]	50.2	78.9	–	–		–	–	
F3	26.3 ± 0.7[b]	58.2	91.3	26.9 ± 0.8[b]	57.4	88.3	23.9 ± 0.8[b]	63.0	95.3
F6	34.6 ± 1.1[b]	45.0	70.6	29.3 ± 0.7[b]	53.6	82.4	26.4 ± 07[b]	59.1	89.5
F7	33.7 ± 1.9[b]	46.4	72.8	31.5 ± 1.7[b]	50.1	77.1	30.7 ± 1.4[b]	52.5	79.4
F8	38.2 ± 0.9[b]	39.3	61.6	36.4 ± 1.8[b]	42.3	65.1	32.8 ± 1.4[b]	49.2	74.5
Commercial cream	22.8 ± 0.5[b]	63.8	100	22.1 ± 0.3[b]	65.0	100	21.9 ± 0.4[b]	66.1	100

[a] Potency denotes anti-inflammatory activity compared to commercial cream which contains 25% β-sitosterol.
[b] $p > 0.05$ compared to control saline ($n = 6$).

of anti-inflammatory drugs (*38*). Table 4 summarizes the anti-inflammatory activity of the hexane extract at a dose of 200 mg, as well as its incorporation in F3, F6 formulations and F7, F8 plain films for 4, 12, and 24 h. All formulations tested showed comparative anti-inflammatory activities in comparison to the commercial cream containing 25% β-sitosterol. The hexane extract revealed a potency of 78.9% after 4 h while the highest potencies were obtained from formulation F3 (91.3, 88.3 and 95.3%, respectively) followed by F6 (70.6, 82.4 and 89.5%, respectively) (Table 4). The F7 formulation (plain sodium hyaluronate patch without extract) showed 72.8, 77.1, and 79.4%, respectively, while the lowest potencies (61.6, 65.1, and 74.5%, respectively) were obtained from F8 (plain chitosan patch without extract) (Table 4).

3.4. *GLC analysis of USM and FAME in the hexane extract*

GLC analysis of USM in the hexane extract of flower-heads of *E. pectinatus* L. revealed the presence of a considerable amount of cholesterol (6.72%) and phytosterols such as campesterol (8.04%), stigmasterol, (9.91%), β-sitosterol (5.39%), and α-amyrin (3.38%) as shown in Table 5. Phytosterols are known to possess anti-inflammatory activity (*39-41*), thus contributing to the anti-inflammatory activity.

GLC analysis of FAME in the extract showed a high percentage of unsaturated fatty acids (85.89%) dominated by linoleinic acid (49.97%) and linoleic acid (33.54%) (Table 5). Linoleic acid is an n-3 polyunsaturated fatty acid (n-3 PUFAs) involved in many biological processes. Being an essential fatty acid, it is modified by the body to make lipoxins (anti-inflammatory mediators) and resolvins (reducing cellular inflammation) (*42*). Linoleic acid is the root of the ω-6 fatty acids family, suggesting that the anti-inflammatory activity of the hexane extract of *E. pectinatus* flower-heads may be ascribed to the presence of the high percentage of n-3 PUFAs (*43*) in addition to the presence of phytosterols.

Based on the aforementioned anti-inflammatory activity and release studies, local implantations (F3, F6) containing the 200 mg hexane extract were subjected to further experiments as described below.

Table 5. Major compounds identified using GLC analysis of USM and FAME in hexane extract of the flower heads of *E. pectinatus* L. (Cass.)

Major compounds identified	R_t (min)	Relative %
USM		
n-decane C_{10}	5.9	0.35
n-heneidecane C_{11}	7.9	0.45
n -tridecane C_{13}	10.7	0.47
n-pentadecane C_{15}	14.4	3.33
n-hexadecane C_{16}	15.5	4.33
n-heptadecane C_{17}	17.3	1.52
n-octadecane C_{18}	18.5	2.37
n- nonadecane C_{19}	19.7	1.03
n-eicosane C_{20}	20.5	3.23
n-heneicosane C_{21}	21.8	3.31
n-docosane C_{22}	23.6	6.87
n-tricosane C_{23}	24.2	9.33
n-tetracosane C_{24}	25.3	6.80
n-pentacosane C_{25}	27.3	14.40
n-hexacosane C_{26}	28.5	3.25
n-heptacosane C_{27}	29.4	5.50
Cholesterol	31.2	6.72
Campesterol	33.4	8.04
Stigmasterol	36.3	9.91
β-sitosterol	38.6	5.39
α-amyrin	39.5	3.38
Total identified compounds	99.98	
FAME		
Caprylic $C_{8:0}$	1.1	0.03
Capric $C_{10:0}$	1.2	0.42
Lauric $C_{12:0}$	1.6	0.21
Myristic $C_{14:0}$	1.9	4.39
Tetradecenoic $C_{14:1}$	2.9	0.96
Palmitic $C_{16:0}$	3.0	7.38
Margaric $C_{17:0}$	3.9	0.10
Stearic $C_{18:0}$	4.9	0.60
Oleic $C_{18:1}$	5.5	1.42
Linoleic $C_{18:2}$	6.7	33.54
Linoleinic $C_{18:3}$	8.9	49.97
Arachidic $C_{20:0}$	11.7	0.14
Behenic $C_{22:0}$	13.6	0.12
Total identified compounds	99.28	

GLC, gas liquid chromatography; USM, unsaponifiable matter; FAME, fatty acid methyl ester; Polox-Ext, Poloxamer 407 with hexane extract.

3.5. In vivo implantation and histopathological studies

To confirm *in-vivo* degradation of F3 and F6 patches, a time-dependent visual monitoring of film texture and integrity following implantation in Wister rats was performed. On days 7, 14 and 21, rats were humanely killed by cervical dislocation, and an incision was made in the site of implantation for examination of films. After 7 days of implantation, it was observed that the films were intact but turned white and opaque when compared with the corresponding control patches which were initially yellowish opaque. With an extension of implantation period beyond 14 days, the patch turned soft and delicate with loss of mechanical strength. On day 21, the patch commenced to get embedded into surrounding tissues completely. Although this experiment was qualitative in nature, the film's loss of integrity and identity over time confirmed that the implant was amenable to biodegradation.

Tissue response to implanted films was studied by the microscopic examination of these tissues in the implanted area after 1, 4, and 12 weeks. Inflammation was manifested in skeletal muscle sections through the appearance of multiple scattered dilated and congested blood vessels. The surrounding tissues showed mild to moderate inflammatory cellular infiltrate including blood monocytes, macrophages and a few mast cells (Figure 3). Lack of an increase in macrophages at the site of implantation suggests that inflammatory responses were either minimal or absent (*44*). Muscle tissues in Group I rats that were implanted with F3, showed normal muscle tissues with mild edema, mild dilated congested blood vessels, and mild cellular infiltration after one week of implantation (Figure 3A). The tissues in this group showed only mild edema and mild inflammation after 4 weeks (Figure 3B) and then showed neither inflammation nor edema after 12 weeks (Figure 3C). This suggests that the extract-sodium hyaluronate patch (F3) may reduce local inflammatory responses following implantation. Similarly, Muscle tissues in Group II rats that were implanted with F6, showed dilated congested blood vessels, inflammatory cells, and cellular infiltration after one week (Figure 3D). After 4 weeks, edema was still present in muscle tissues and blood vessels were congested (Figure 3E). After 12 weeks, the muscle tissues still showed congested blood vessels and mast cells (Figure 3F). In contrast, sections of control Group III rats that were treated with sham surgery showed normal tissue structure (Figure 3G). These results suggested that F3 exhibited a better treatment profile than F6 throughout the whole test period.

4. Discussion

Interest in phytopharmaceuticals has been augmented in recent years by the study of complementary and alternative medicine. Medicinal plants constitute a source of raw materials used for treatment of a variety of illness. Among these plants, *Euryops pectinatus* (L.) Cass. was previously investigated for the anti-inflammatory activity of its leaf petroleum ether extract (*1*). The leaf extract was incorporated in the development of novel leaf petroleum ether gel emulsion formulations and was found to be safe and applicable for use while capable of providing its activity (*1*). Similarly, the hexane extract of the flower-heads was screened for its anti-inflammatory activity. As a result, the hexane extract was found to be safe as it produced no adverse effect even in 10 times the therapeutic dose (LD_{50} = 4.9 g/kg b.wt.; effective dose 200 mg/kg b.wt.) (*45*). It showed a significant rapid anti-inflammatory effect (78.9%) after 4 h when compared to a commercial cream containing 25% β-sitosterol using the carrageenan-induced hind paw edema method. This stimulated the

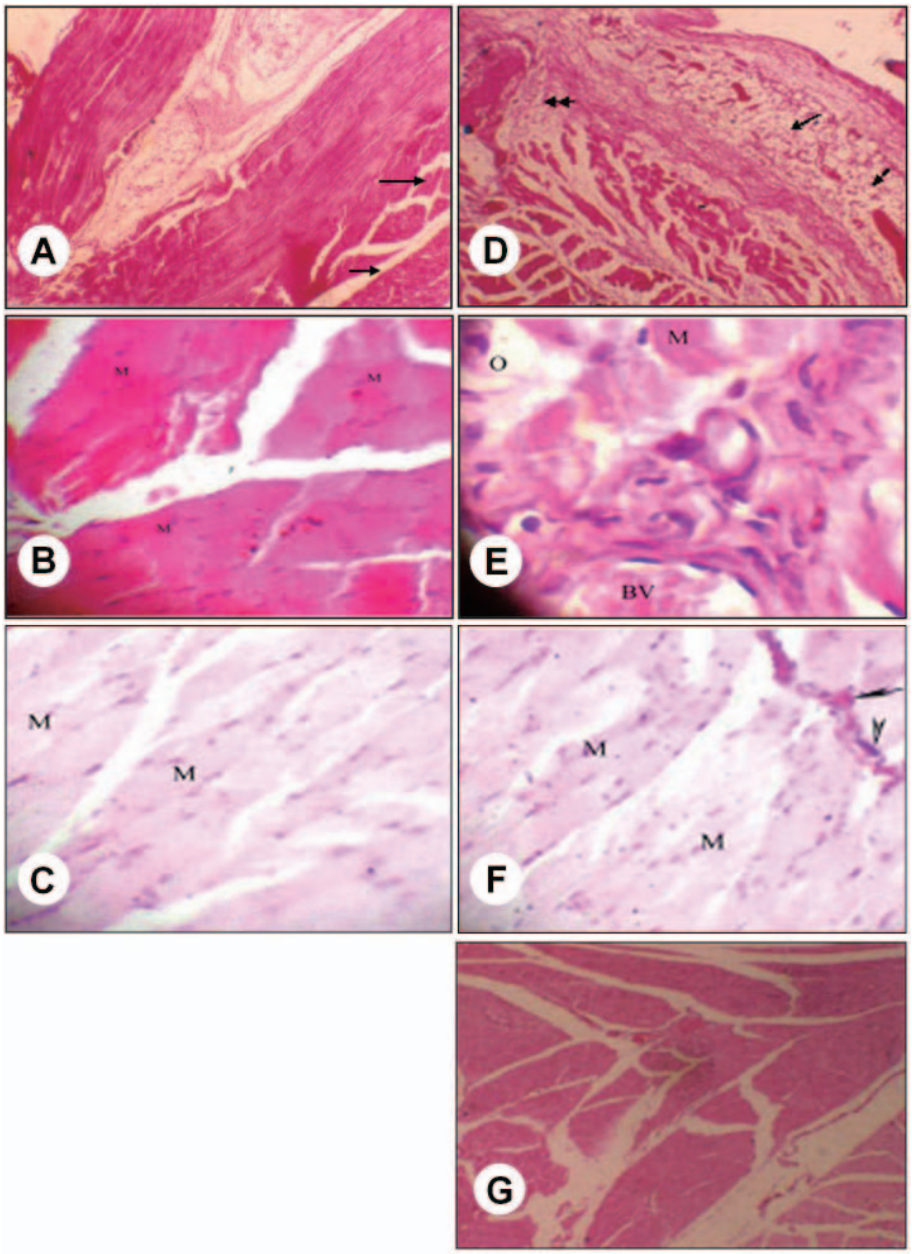

Figure 3. Typical photographs of rat muscle tissues after F3 or F6 implantation. (A) At one week after F3 implantation, mild edema was observed (arrow). **(B)** At 4 weeks after F3 implantation, mild edema and mild inflammation were observed. **(C)** At 12 weeks after F3 implantation, normal muscle tissue appeared. **(D)** At one week after F6 implantation, dilated congested blood vessel, inflammatory cells (arrow), and cellular infiltration (double arrow) were observed. **(E)** At 4 weeks after F6 implantation, edema (M) in muscle tissue (M) and congested blood vessel (BV) were observed. **(F)** At 12 weeks after F6 implantation, congested blood vessel (arrow) and mast cells (arrow head) were observed. **(G)** A typical photograph of muscle tissue of Sham-operated rat as a reference (group III), showing normal muscle structure. Original magnifications: **A**, **B**, **C**, and **E**, ×200; **D**, **F**, and **G**, ×100.

authors to formulate the hexane extract into local anti-inflammatory implantations with controlled release. Cross-linked sodium hyaluronate patches (F1-F3) and chitosan patches (F4-F6) were prepared by a casting/solvent evaporation technique.

Concerning the film's structure and morphological characterization, our study showed a good mechanical properties and good compatibility between the matrix film and the hexane extract (Table 2). The kinetic analysis of the extract from different formulations calculated by linear regression followed the Higuchi kinetic model (Table 3).

Based on optimum release and highest potencies, formulations F3 and F6 were chosen for further histopathological investigation to examine tissue response to the extract incorporated within different excipient bases. Formula F3 showed neither inflammation nor edema after 12 weeks (Table 4), suggesting that the extract-sodium hyaluronate patch may reduce local inflammatory responses following implantation. These patches could lead to a successful application for localized drug delivery in an implantation site.

Chemical investigation of the hexane extract by GLC prompted us to discuss the probable anti-inflammatory

mechanism of action. There are two phases of carrageenan-induced inflammatory reaction: an early phase starting from 2-5 h after carrageenan injection which results from serotonin, brady-kinin and histamine liberation while the late phase is associated with the release of prostaglandins (*46*). As shown in Table 4, the formulated hexane extracts, especially F3, significantly inhibited each phase of edema in rats, suggesting that the extract had a non-selective effect on the release of these mediators.

Quality control of herbal extracts, either plain or formulated, still remains a challenge because of the high variability of chemical compounds in the extracts. The therapeutic effects of herbal medicines are based on the complex interaction of numerous ingredients in combination which are totally different from those of chemical drugs. In a preliminary study, the authors have chosen cholesterol as an analytical marker that serves solely for analytical purposes based on an easy method of determination (*47*). The relative percentage of cholesterol in the plain hexane extract and F3 calculated by GLC was found to be 6.72% and 6.58%, respectively, with a 97.9% recovery. By choosing another active marker phytosterol, which contributes to the therapeutic activity, it was found to be 33.44% in the plain extract and 32.57% in F3 with a 97.4% recovery.

In conclusion, the formulation F3 (200 mg hexane extract in a sodium hyaluronate patch) may offer a suitable anti-inflammatory implantation with controlled release which can be used for the benefit of phytopharmaceutical treatment.

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References

1. El-Alfy TS, Michel CG, Nesseem DI, Sleem AA. A study of the composition, bioactivity and formulation of the petroleum ether extract of the leaves of *Euryops pectinatus* (L.) Cass. cultivated in Egypt. Bull Fac Pharm Cairo Univ. 2005; 43:85-95.
2. Angelova N, Hunkeler D. Rationalizing the design of polymeric biomaterials. Trends Biotechno. 1999; 17:409-421.
3. Pillai O, Panchagnula R. Polymers in drug delivery. Curr Opin Chem Biol. 2001; 5:447-451.
4. Kafedjiiski K, Jetti RK, Föger F, Hoyer H, Werle M, Hoffer M, Bernkop-Schnürch A. Synthesis and *in vitro* evaluation of thiolated hyaluronic acid for mucoadhesive drug delivery. Int J Pharm. 2007; 343:48-58.
5. Kim A, Checkla DM, Dehazya P, Chen W. Characterization of DNA-hyaluronan matrix for sustained gene transfer. J Control Release. 2003; 90:81-95.
6. Fraser JR, Laurent TC, Laurent UB. Hyaluronan: Its nature, distribution, functions and turnover. J Intern Med. 1997; 242:27-33.
7. Rastrelli A, Beccaro M, Biviano F, Calderini G, Pastorello A. Hyaluronic acid esters, a new class of semisynthetic biopolymers: Chemical and physico-chemical properties. In: Clinical Implant Materials (Heimke G, Soltesz U, Lee AJC, eds). Elsevier, Amsterdam, Netherland, 1990; pp. 199-200.
8. Benedetti LM. New biomaterials from hyaluronic acid. Med Device Technol. 1994; 5:32-37.
9. Lim ST, Martin GP, Berry DJ, Brown MB. Preparation and evaluation of the *in vitro* drug release properties and mucoadhesion of novel microspheres of hyaluronic acid and chitosan. J Control Release. 2000; 66:281-292.
10. Ma J, Wang H, He B, Chen J. A preliminary *in vitro* study on the fabrication and tissue engineering applications of a novel chitosan bilayer material as a scaffold of human neofetal dermal fibroblasts. Biomaterials. 2001; 22:331-336.
11. Tokuyasu K. Utilisation of chitin and chitosan in food industry. J Jpn Soc Food Sci Technol (Nippon Shokuhin Kagaku Kogaku Kaishi). 1999; 46:356-360.
12. Sezer AD, Akbuga J. Release characteristics of chitosan treated alginate beads: I. Sustained release of a macro-molecular drug from chitosan treated alginate beads. J Microencapsul. 1999; 16:195-203.
13. He P, Davis SS, Illum L. Sustained release chitosan microspheres prepared by novel spray drying methods. J Microencapsul. 1999; 16:343-355.
14. Illum L. Chitosan and its use as a pharmaceutical excipient. Pharm Res. 1998; 15:1326-1331.
15. Miyazaki S, Yamaguchi H, Takada M. Pharmaceutical application of biomedical polymers. XXIX. Preliminary study on film dosage form prepared from chitosan for oral drug delivery. Acta Pharm Nord. 1990; 2:401-406.
16. Chandy T, Sharma CP. Biodegradable chitosan matrix for the controlled release of steroids. Biomater Artif Cells Immobilization Biotechnol. 1991; 19:745-760.
17. Brüsewitz C, Schendler A, Funke A, Wagner T, Lipp R. Novel poloxamerbased nanoemulsions to enhance the intestinal absorption of active compounds. Int J Pharm. 2007; 329:173-181.
18. Lin H, Gebhardt M, Bian S, Kwon KA, Shim CK, Chung SJ, Kim DD. Enhancing effect of surfactants on fexofenadine. HCl transport across the human nasal epithelial cell monolayer. Int J Pharm. 2007; 330:23-31.
19. Bromberg L, Alakhov V. Effects of polyether-modified poly (acrylic acid) microgels on doxorubicin transport in human intestinal epithelial Caco-2 cell layers. J Control Release. 2003; 88:11-22.
20. Buck WB, Osweiter GD, van Gelder AG. Clinical and diagnostic veterinary toxicology. 2nd Edition, Kendall/ Hund Publishing Company, Dubuque, IA, USA, 1976.
21. Johnson AR, Davenpart JB. Biochemistry and Methodology of Lipids. John Wiley and Sons, New York, NY, USA, 1971; p. 35.
22. de Fávere VT, Hinze WL. Evaluation of the potential of chitosan hydrogels to extract polar organic species from nonpolar organic solvents: Application to the extraction of aminopyridines from hexane. J Colloid Interface Sci. 2009; 330:38-44.
23. Serrano C, Monedero E, La Puerta M, Portero H. Effect of moisture content, particle size and pine addition on quality parameters of barley straw pellets. Fuel Processing Technology. 2011; 92:699-706.

24. Chen FL, Wei YM, Zhang B. Chemical cross-linking and molecular aggregation of soybean protein during extraction cooking at low and high moisture content. LWT-Food Sci Technol. 2011; 44:957-962.
25. Angellier-Coussy H, Gastaldi E, Gontard N, Guillard V. Influence of processing temperature on the water vapour transport properties of wheat gluten based agromaterials. Ind Crops Prod. 2011; 33:457-461.
26. Ashour T, Georg H, Wu W. An experimental investigation on equilibrium moisture content of earth plaster with natural reinforcement fibres for straw bale buildings. Applied Thermal Engineering. 2011; 31:293-303.
27. Skendi A, Papageorgiou M, Costas GB. Influence of water and barley β-glucan addition on wheat dough viscoelasticity. Food Res Int. 2010; 43:57-65.
28. Stefanelli D, Goodwin I, Jones R. Minimal nitrogen and water use in horticulture: Effects on quality and content of selected nutrients. Food Res Int. 2010; 43:1833-1843.
29. Weinberg ZG, Yan Y, Chen Y, Finkelman S, Ashbell G, Navarro S. The effect of moisture level on high-moisture maize (Zea mays L.) under hermetic storage conditions – *in vitro* studies. J Stored Prod Res. 2008; 44:136-144.
30. van der Oever MJA, Beck B, Müssig J. Agrofibre reinforced poly(lactic acid) composites: Effect of moisture on degradation and mechanical properties. Compos Part A Appl Sci Manuf. 2010; 41:1628-1635.
31. Amnuaikit C, Ikeuchi I, Ogawara K, Higaki K, Kimura T. Skin permeation of propranolol from polymeric film containing terpene enhancers for transdermal use. Int J Pharm. 2005; 289:167-178.
32. Basak SC, Rahman J, Ramalingam M. Design and *in vitro* testing of a floatable gastroretentive tablet of metformin hydrochloride. Pharmazie. 2007; 62:145-148.
33. Higuch WI. Analysis of data on the medicament release from ointment. J Pharm Sci. 1962; 51:802-804.
34. Winter CA, Risley EA, Nuss GW. Carrageenan-induced edema in hind paw of the rat as an assay for inflammatory drugs. Proc Soc Exp Biol Med. 1962; 111:544-547.
35. Vogel HG, Voge WH. Drug Discovery and Evaluation. Springer-Verlag, Berlin, Heidelberg, Germany, 1997.
36. Snedecor WG, Coshran GW. Statistical Methods. Iowa State University Press, Ames, IA, USA, 1971.
37. Horisawa E, Hirota T, Kawazoe S, Yamada J, Yamamoto H, Takeuchi H, Kawashima Y. Prolonged anti-inflammatory action of DL-lactide/glycolide copolymer nanospheres containing betamethasone sodium phosphate for intra-articular delivery system in antigen-induced arthritic rabbit. Pharm Res. 2002; 19:403-410.
38. Brestel EP, McClain EJ, Castranova V. Carrageenan stimulates reduction of nitroblue tetrazolium by human neutrophils without membrane depolarization, myeloperoxidase secretion, or increased oxygen consumption. Inflammation. 1986; 10:425-434.
39. Gupta MB, Nath R, Srivastava N, Shanker K, Kishor K, Bhargava KP. Anti-inflammatory and antipyretic activities of β-sitosterol. Planta Medica. 1980; 39:157.
40. García MD, Sáenz MT, Gómez MA, Fernández MA. Topical antiinflammatory activity of phytosterols isolated from *Erynguim foetidum* on chronic and acute inflammation models. Phytother Res. 1999; 13:78-80.
41. Mehtiev AR, Misharin Alu. Biological activity of phytosterols and their derivatives. Biomed Khim. 2007; 53:497-521. (in Russian)
42. Reddy AC, Lokesh BR. Studies on anti-inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on carrageenan-induced inflammation in rats. Ann Nutr Metab. 1994; 38:349-358.
43. Massimo C, Francesco E, Salvatore G, Alessandra M, Gianmario A, Aurelia T, Luigi M. Fatty acids profile and anti-inflammatory activity of *Nonea setosa* R. et S. Phytother Res. 2006; 20:422-423.
44. Mathiowitz E. Characterization of delivery systems, surface analysis and controlled release systems. In: Encyclopedia of Controlled Drug Delivery. John Wiley and Sons, New York, NY, USA, 1999; pp. 261-269.
45. Rowan AN. Of mice, models, and men: A critical evaluation of animal research. State University Albany Press, New York, NY, USA, 1984.
46. Zhu ZZ, Ma KJ, Ran X, Zhang H, Zheng CJ, Han T, Zhang QY, Qin LP. Analgesic, anti-inflammatory and antipyretic activities of the petroleum ether fraction from the ethanol extract of *Desmodium podocarpum*. J Ethnopharmacol. 2011; 133:1126-1131.
47. Francis EL, Arthur T Jr, Scanlan JT. Spectrophotometric determination of cholesterol and triterpene alcohols in wool wax. Anal Chem. 1953; 25:1497-1499

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