Research Paper

Characterization, Biocompatibility and Biological Properties of *Cissampelos pareira* Pectin-Based Hydrogel Patch Containing *Heliotropium indicum* Extract

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***Cissampelos pareira* and *Heliotropium indicum* have been used as traditional medicine for treating several disorders. Specifically, *Cissampelos pareira* simply forms a gel without additional compounds which result from mainly pectin sources. In this study, the pectin from *Cissampelos pareira* was used to develop a hydrogel patch by adding *Heliotropium indicum* extract. The characterization of pectin-based hydrogel patch and pectin-based hydrogel containing *Heliotropium indicum* patch was evaluated. The results revealed suitable characterization of pectin-based hydrogel patches in physical and mechanical properties. The pectin-based hydrogel patches also presented biological activities through antioxidant and anti-inflammatory activities and these effects were synergized in a pectin-based hydrogel containing *Heliotropium indicum* patches. Furthermore, the pectin-based hydrogel patches presented biocompatibility by non-cytotoxicity to fibroblast and macrophage cells. Fibroblasts can proliferate in the pectin-based hydrogel patches. According to the results, the pectin-based hydrogel patches are of great benefit in pharmaceutical and cosmeceutical applications.**

Key words: *Cissampelos pareira*, *Heliotropium indicum*, pectin, cytocompatibility, antioxidant, anti-inflammation

Cissampelos pareira (*C. pareira*) (family Menispermaceae) is a tropical climbing plant omnipresent in Asia, South America and East Africa. This plant is consumed as food and traditionally used to treat disorders of skin, inflammation and gastrointestinal diseases such as diarrhea, dyspepsia, and anorexia[1]. It has been reported the pharmacological properties of various parts of *C. pareira*. The root extract has been revealed antioxidant[2], anti-inflammatory[3], anti-nociceptive, and anti-arthritic[4] activities both of *in vitro* and *in vivo* studies. Furthermore, the whole plant extract of *C. pareira* has been reported analgesic and anti-inflammatory[5] as well as cytotoxic to the cancer cells[6]. Likewise, the leaf extract of *C. pareira* has also been possessed anti-arthritics activity[7]. The water extract of fresh leaves of *C. pareira* presents a specially property that can form a gel at room temperature in a short period time without the need for sucrose or calcium[8,9]. The main gelling composition of *C. pariera* leaves compose mainly of galacturonic acid and small amounts of neutral sugars which are defined as low methoxyl-pectin[10]. In addition, our previous study showed that the pectin from *C. pariera* leaves demonstrated the radical scavenging, and anti-inflammatory activities in Lipopolysaccharide (LPS) stimulated macrophage cells[11]. *Heliotropium indicum* (*H. indicum*) (family Boraginaceae) is distributed in the tropical and temperate parts of the world such as India, Africa, Bangladesh, Sri Lanka, Nepal, the Philippines, and Thailand. It has been commonly used in India in traditional medicine to treat fever, diarrhea, skin disorders, wound and ulcer[12]. In addition, various pharmacological properties of *H. indicum* Extracts (HIE) have been reported. Among these, many effects are valuable in wound

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healing promotion including antimicrobial and antioxidant activities[13,14]. In previous research, we have found that HIE given radical scavenging, anti-inflammatory activities in macrophages, and also growth factor promotion in fibroblasts[15]. The *in vitro* and cellular studies according to the previous studies which demonstrated that HIE promoted wound healing in animal models including excision (normal and infected), incision, dead space, and diabetic wound[16,17].

Hydrogels are insoluble hydrophilic materials which do not dissolve in water but swells considerably in an aqueous solution. They are popular groups of a wound dressing. The swelling property of hydrogels helps granulation tissues and epithelium in a moist environment. In general, hydrogels are transparent, maintain a moist wound environment, and are appropriate for wounds with low to moderate exudate[18]. Therefore, hydrogels have been widely used as wound dressing materials for dry chronic wounds, necrotic wounds, skin tears, surgical wounds, burn wounds, and pressure ulcers[19,20]. The forms of hydrogels are in the shapes of sheet hydrogel, amorphous gel, and impregnated gauze. Hydrogel sheets can be cut to fit around the wound due to their flexible nature without secondary dressing[21]. There are many hydrogels in the market such as sheet dressings, impregnated gauze, and water-based gels, Purilon™, Intrasite™, Nu-gel™ and Aquaform™ polymers. Due to their advantage in biological activities of *C. pareira* and *H. indicum* are promising natural alternatives that may extend to use in pharmaceutical and cosmeceutical applications. Therefore, this work aimed to evaluate the biological properties of *C. pareira* pectin-based hydrogel containing HIE through antioxidant and anti-inflammatory activity. Moreover, characterization and biocompatibility were also observed.

MATERIALS AND METHODS

Materials:

C. pareira leaves were collected from Thailand's Northeast province of Udonthani and *H. indicum* leaves were collected from Buri Rum province. Murine macrophage (RAW 264.7) and murine fibroblast (NIH 3T3) cell lines were procured from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, and trypsin-Ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco, USA. Trypan blue, resazurin, sodium nitroprusside, and DPPH were purchased from Sigma-Aldrich, USA. Griess reagent kit was purchased from Promega, USA.

Preparation of *C. pareira* pectin and HIE:

In this research, the method for *C. pareira* pectin extraction was modified from Singthong *et al.*[9], whereby the fresh leaves of *C. pareira* were first cleaned with water and dried at 45° for 24 h. The dried leaves were ground and kept in a desiccator at room temperature. The dry powder of *C. pareira* was mixed with distilled water (1:50 w/v) and adjusted to pH 3.8-4.0 followed by heating at 60° in a water bath for 60 min. The crude extract was filtrated and centrifuged at 10 000 rpm for 15 min. The supernatant was collected to concentrate to half of its volume in a rotary evaporator followed by precipitation in 95 % ethanol (w/v) for the final concentration of 70 %. The precipitate was freeze-dried and ground for powder of *C. pareira* pectin. The powder was kept in a desiccator for subsequent development the hydrogel patch.

For HIE, the leaves of the plant were cleaned with water and dried at 50° for 72 h. The dried leaves were ground and kept in a desiccator at room temperature. The dry powder was macerated in 70 % ethanol and shook at 250 rpm with a shaker for 24 h. The macerated mixture was filtrated before collect the supernatant to evaporate using a rotary evaporator. The crude extract of *H. indicum* leaves was stored at -20° until used.

Preparation of the pectin-based hydrogel patch:

Preparation of *C. pareira* pectin solution: *C. pareira* pectin powder was dissolved in distilled water at a concentration 5 mg/ml. The solution was centrifuged at 8000 rpm for 10 min. The supernatant was collected and kept at 4°.

Preparation of *H. indicum* solution: The *H. indicum* solution of 1 % w/w was prepared by weighing 1 g of *H. indicum* crude extract and then added 100 ml of propylene glycol. The mixture was mixed continuously for 30 min.

Preparation of Pectin-Based hydrogel (PB) patch and Pectin-based Hydrogel containing HIE (PH) patch: The patches were prepared by mixing 50 g of *C. pareira* pectin solution, 20 g of 10 %

polyvinyl alcohol, 10 g of 10 % gelatin, 5 g of 10 % carrageenan, and 15 g of propylene glycol (PB) or *H. indicum* solution (PH) using hot plate magnetic stirrers at 40° for 30 min. Two grams of the mixtures were poured into a petri dish (100×15 mm) and then dried in an oven at 60° for 12 h. After drying, PB and PH patches were stored away from moisture, heat and light.

Physical and mechanical characterization of the pectin-based hydrogel patch:

Color: The color of PB and PH patches was evaluated by organoleptic and spectrophotometer (ColorQuest XE, HunterLab, USA) based on CIEL*a*b* system. The color parameters were measured by the value of L* (black [0] to white [100]), a* (greenness [−] to redness [+]), and b* (blueness [−] to yellowness [+]).

Thickness: The thickness of PB and PH patches was measured using a digital vernier caliper at five locations (center, two points of edge, and two points within the patch) of three patches. The thickness of the PH was presented as mean±SD.

Weight: The weight of the PB and PH patches was determined three of patches on an electronic balance and mean weight was calculated. The weight of the patches was presented as mean±SD.

pH measurement: The 2 g PB or PH patches were dissolved with 25 g deionized water and stirred until homogenous solution at 25°. The pH of solutions was determined using a pH meter (SP-2100, Suntex, Taiwan).

Swelling study: The PB and PH patches were cut in 2×2 cm size and dried at 60° for 48 h. The dried patches were kept in a beaker with 20 ml of pH 7.4 phosphate buffer at room temperature. The swollen patches were weighted in grams. The swelling ratio was calculated using the following formula:

Percentage swelling=$[(W_S-W_D)/W_D]\times100$

Where W_S=Weight of the swollen patches in gram and W_D=Weight of the dry patches in gram

Morphological study by scanning electron microscope: The morphology of the patches was examined by scanning electron microscopy (JEOL JSM-IT300, Oxford X-Max 20, USA). The PB and PH patches were cut into 10×10 mm pieces. Samples were gold coated and observed using an accelerating voltage of 2.0 kV.

Mechanical properties: The tensile strength and elongation of PB and PH patches were measured using a universal tester with sample size 1×5 cm, with a load cell of 50 newtons and 10 mm/min testing speed. The strength and elongation were presented as tensile strength and percentage elongation of mean±SD.

Heating-cooling cycle test: PB and PH patches were kept in the refrigerator at 4±2° for 24 h and in the hot air oven at 45±2° for 24 h per one cycle. The test was carried out for 7 cycles. Physical properties of PB and PH patches in terms of color and pH were evaluated at the end of the test.

Antioxidant activities of pectin-based hydrogel:

DPPH radical scavenging activity: The radical scavenging activity of PB and PH patches was modified from Norajit *et al.*[22]. The patches were cut into 1 g and dissolved in pH 7.4 phosphate buffer saline 5 ml. The dissolved patches were centrifuged, and the 75 µl of the supernatants were collected to mixed with 150 µl of 0.2 mmol/l 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (in methanol) and allowed to stand for 30 min without direct exposure to light. The absorbance was determined at 520 nm using a microplate reader. In addition, PBS and L-ascorbic acid were used as the negative and positive controls, respectively. The DPPH scavenging capacity of the experimental pectin is expressed as a percentage of DPPH radical inhibition as below, where OD is the optical density;

Percentage DPPH radical inhibition=[(OD without extract–ODwith extract)/ODwithout extract)×100

Nitric Oxide (NO) radical scavenging activity: The supernatants 1 ml were incubated with the NO donor (sodium nitroprusside) 10 mmol/l in a pH 7.4 phosphate buffer saline for 180 min. Approximately 100 µl of the resulting solution was withdrawn to react with a Griess Reagent kit (Promega, USA), whereby the solution was reacted with 20 µl sulfanilamide for 10 min and then 20 µl N-(1-napthyl)ethylenediamine dihydrochloride for another 10 min. The reaction mixture absorbance was measured at 560 nm and the NO concentrations were determined as the nitrite (NO_2^-) concentrations from the standard curve of a standard nitrite solution. PBS and L-ascorbic acid were used as the negative and positive controls, respectively. The NO scavenging capacity of the

experimental pectin was expressed as a percentage of nitrite production inhibition using the following formula;

Percentage NO_2^- production inhibition=[(NO_2^- without extract–NO_2^- with extract)/NO_2^- without extract]×100

Cell culture:

Murine macrophage (RAW264.7) and murine fibroblast (NIH3T3) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS and 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37° in a humidified atmosphere of 5 % CO_2. The macrophages were passaged twice a week using cell scraper. Fibroblasts were passaged twice a week using trypsinization with 0.25 % trypsin-EDTA. The cell viability was determined with 0.4 % trypan blue. Cells with more than 85 % viability were used in the experiments.

***In vitro* cytotoxicity test:** The cytotoxicity test of PB and PH patches was modified from Li *et al.*[23]. The patches were cut into 5×5 mm size and were UV irradiated in 12 well plates for 30 min. The irradiated patches were incubated with 1 ml of DMEM containing 10 % FBS and 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37° in a humidified atmosphere of 5 % CO_2 for 24 h. The conditioned medium was then removed for testing cytotoxicity in fibroblast cells. NIH3T3 fibroblast cells were plated in 96 well plate (2×10^5 cell/ml). The cells were treated with condition medium for 24, 48, and 72 h. The treated cells were determined cell viability using resazurin reduction assay, whereby the treated cells were incubated for 4 h at 37° in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 against 600 nm. The percentage of cell viability was calculated using the formula:

Percentage cell viability=[$(OD_{560}–OD_{600})_{with\ extract}$ $(OD_{560}–OD_{600})_{without\ extract}$]×100

***In vitro* cytocompatibility test:** To evaluate the cytocompatibility of PB and PH patches, the method was modified from Li *et al.*[23]. The PH patch was cut into 5×5 mm size and was UV irradiated in 12 well plates for 30 min. NIH3T3 fibroblast cells (4×10^5 cell/ml) were plated together with the patch for 24, 48, and 72 h. Finally, the morphology and proliferation of the cells were observed with an optical microscope.

Anti-inflammatory activity: Determination of anti-inflammatory activity of PB and PH patches was modified from Ninan *et al.*[24]. The RAW264.7 macrophage cells (4×10^5 cell/ml) were pretreated with the conditioned medium in 96 well plate and incubated at 37° for 24 h. The pre-treated cells were stimulated with 1 µg/ml of LPS and incubated for another 24 h. The NO concentrations were determined from NO_2^- in the stimulated-cell supernatant using a Griess reagent kit, whereby 100 µl of the supernatant was reacted with 20 µl sulfanilamide for 10 min and with 20 µl N-(1-napthyl)ethylenediamine dihydrochoride for another 10 min. The reaction mixture absorbance was measured at 560 nm and the NO concentrations were determined as the nitrite (NO_2^-) concentrations from the standard curve of a standard nitrite solution. DMEM and 100 µM of dexamethasone each with 1 µg/ml LPS were respectively used as the negative and positive controls.

Cell viability of the LPS-stimulated macrophage: The viability of the residual macrophage cells after the NO assay, given the conditioned medium was determined by resazurin reduction assay, whereby the residual cells were incubated for 2 h at 37° in 100 µl fresh DMEM containing 50 µg/ml resazurin. The reaction mixture absorbance was determined at 560 against 600 nm.

Statistical analysis:

Physical and mechanical characterization, antioxidant activities, and *in vitro* results were expressed as mean±Standard Error of the Mean (SEM) of triplicate experiments. Cell viability was analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey's (post hoc) using the SPSS 22.0 software and a value of $p<0.05$ was considered statistically significant. Descriptive statistics and mean±Standard Deviation (SD) were presented for physical and mechanical characterization.

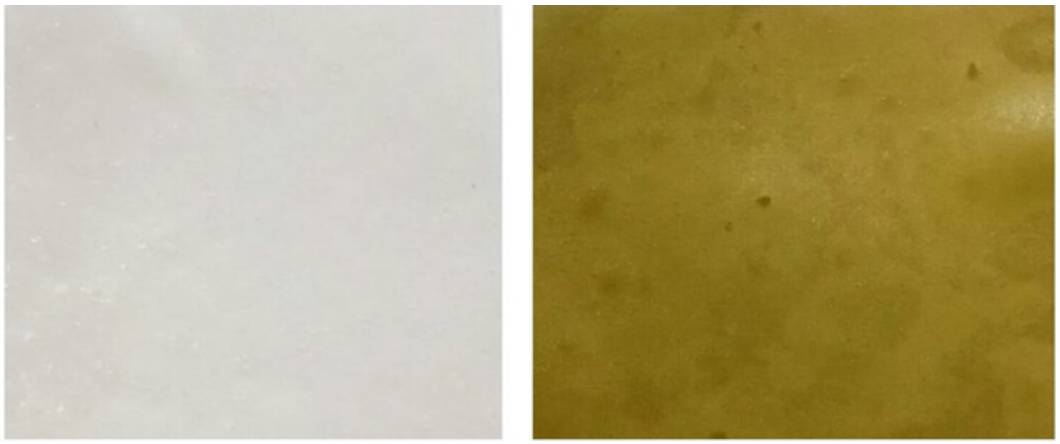
RESULTS AND DISCUSSION

The patches composed of low methoxyl pectin from *C. pareira*, gelatin, polyvinyl alcohol, gelatin, and carrageenan were formulated. Propylene glycol was used as a plasticizer and extracted solvent. A previous study suggested that pectin provided the same utilities of alginate which offers the possibility to control dissolution[25]. Moreover, pectin has been used as a texturizer or stabilizer in the food industry. Recently, pectin was interested in its use as an additive in the

drug delivery systems. Pectin-based formulations have developed in pharmaceutical applications for improving the properties for many reasons including higher drug loading efficiency, less pre-mature drug release, greater efficiency for the controlled release of peptide and protein drugs, increased biocompatibility and controllable swellability and degradability[26].

The characteristic of PB and PH patches is shown in fig. 1. For visual observation, the PB patch is translucent with white color while the PH patch is a dark-green color similar to HIE. The color of PB and PH patches were represented in L*, a*, and b* (Table 1). The color of the patch has a direct impact on the customer. PB patch showed a higher L* value than PH patch that represented lighter color. PH patch showed greenness with a higher a* value and quite a yellow color with higher b* value when compared with PB patch. The color is affected by the color of HIE. The thickness value affects the permeability and mechanical properties of the patch. PB and PH patches were not different in thickness and weight (Table 2). Thus, HIE was not affected by the thickness and weight of the patches. However, PH patches seem to be moist more than PB patches. That difference may be caused by HIE also. Both patches were smooth without pores and cracks on the surface. The pH of PB and PH patches are presented in Table 1. The pH of the PH patch was 6.13 which was more acidic than the PB patch significantly different due to the pH of HIE and propylene glycol, solvent for HIE. However, the pH value of both formulations was suitable and compatible for skin. Interestingly, the color and pH of the patches were not changed after stability testing. After stability testing, there was no visible change in the appearance of PB and PH patches. The color with L*, a*, and b* value and pH of the patches were not changed (Table 1).

The capacity of the dressing patch to hold exudate or donate fluid is one of the factors needed to be taken into consideration when selecting dressings[20]. However, hydrogels are suitable for dry wounds or those with minimal exudates[27]. In this study, the results show a high swelling ratio more than 90 % in PB and PH patches (Table 2).



A B

Fig. 1: Characteristics of (A): PB and (B): PH patches

TABLE 1: COLOR AND pH OF PB AND PH PATCHES BEFORE AND AFTER HEATING-COOLING CYCLE TEST

Patch	Before				After			
	L*	a*	b*	pH	L*	a*	b*	pH
PB	40.78±1.43	0.53±0.19	1.59±0.32	6.24±0.03	34.85±0.81	2.53±0.17	0.02±0.11	6.22±0.03
PH	31.56±0.66	1.24±0.04	7.31±0.55	6.13±0.01#	21.54±0.83	5.82±0.18	12.46±1.49	6.16±0.02#

Note: #Significantly difference at p<0.05 between sample

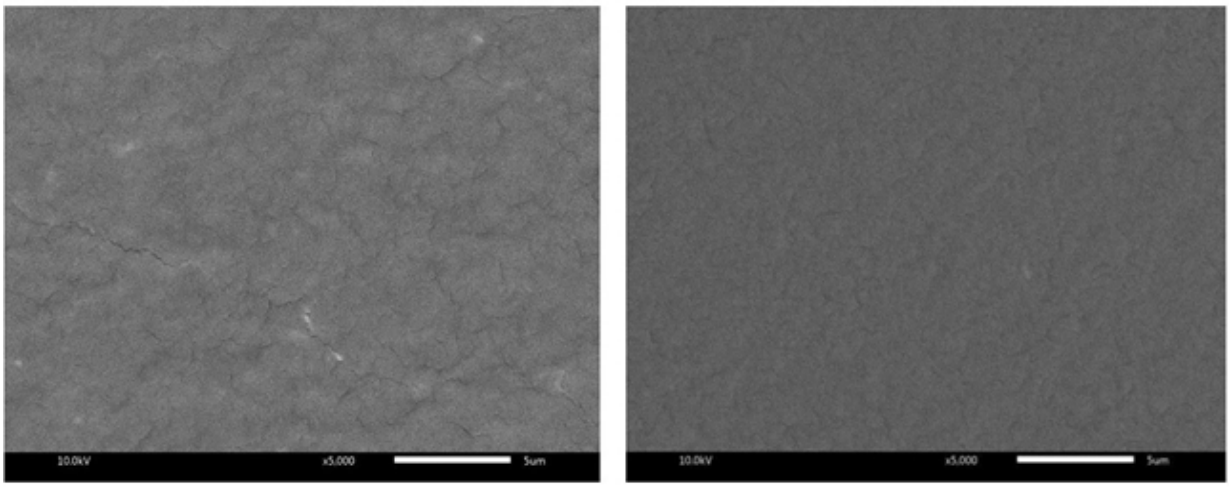
TABLE 2: THICKNESS, WEIGHT, AND SWELLING RATIO OF PECTIN-BASED HYDROGEL PATCHES

Patch	Thickness (mm)	Weight (mg)	Swelling ratio (%)
PB	0.68±0.02	4.61±0.01	106.82±26.54
PH	0.66±0.01	4.43±0.18	93.87±2.51

This result in close to the effectiveness of low methoxyl pectin/gelatin/carboxymethyl cellulose hydrogel films with a high fluid absorption capacity of approximately 90 %[28,29]. However, the PH patch revealed slightly lower fluid handling properties than the PB patch. HIE may be attributed to the reduction in the number of intermolecular cross-links between polymeric networks within films. Scanning electron microscopy examinations were carried out to get a better surface image of the patches. Fig. 2 showed the scanning electron microscopy of PB and PH patches. It was observed the surface of the homogeneous without bubbles of PB and PH patches. A patch containing HIE showed more structural continuity with chemical composition interaction. Therefore, the PH patch displayed a smooth surface more than the PB patch.

Mechanical properties of dressing or patch are important to be durable, stress-resistant, flexible, pliable, and comfortable to use in different parts of the body[21]. The tensile strength is the maximum tensile stress maintained by the patches during the tension test while the elongation at break is an indication of flexibility and extensibility of patches before breakage[30]. High elongation at break and tensile strength are suitable for patch application and handling purposes. From the results, PH and PB patches showed no significant difference in tensile strength (Table 3). However, incorporation of HIE into the patch significantly increased the percentage of elongation at break. Therefore, the PH patch is flexible more than the PB patch. Conversely, the PB patch presented withstand the stress than the PH patch. This is mainly caused by the interaction between polymers and polyphenolic compounds from HIE as well as green tea extract increased percentage elongation of chitosan films[30].

According to previous studies, *C. pareira* pectin and *H. indicum* ethanolic extract showed potent scavenging ability and anti-inflammatory property[11,15]. In this study, the antioxidant properties were evaluated through the scavenging performance of PB and PH patches. The results reveal that PB and PH patches significantly reduce DPPH and NO free radical, achieving the inhibition performance of 40.99±0.19 % and 13.24±0.30 %, and 61.57±3.05 % and 50.01±1.63 %, respectively (fig. 3). Interestingly, the addition of HIE significantly increased free radicals scavenging performance in pectin-based hydrogel patches. Likewise, *Citrus unshiu* (*C. unshiu*) peel pectin film alone showed lower antioxidant activity than *C. unshiu* peel pectin film containing *Salvia officinalis* leaf extract[31]. The antioxidant activities have also been found in alginate film containing ginseng extract[22] and gelatin-based containing *Caesalpinia decapetal*[32].



A B

Fig. 2: Characteristics of (A): PB and (B): PH patches

TABLE 3: MECHANICAL PROPERTIES OF PECTIN-BASED HYDROGEL PATCHES

Patch	Tensile strength (MP)	Elongation (%)
PB	74.27±11.28	34.90±3.08
PH	65.97±12.65	62.84±5.00*

Note: *Significantly difference at p<0.05 between sample

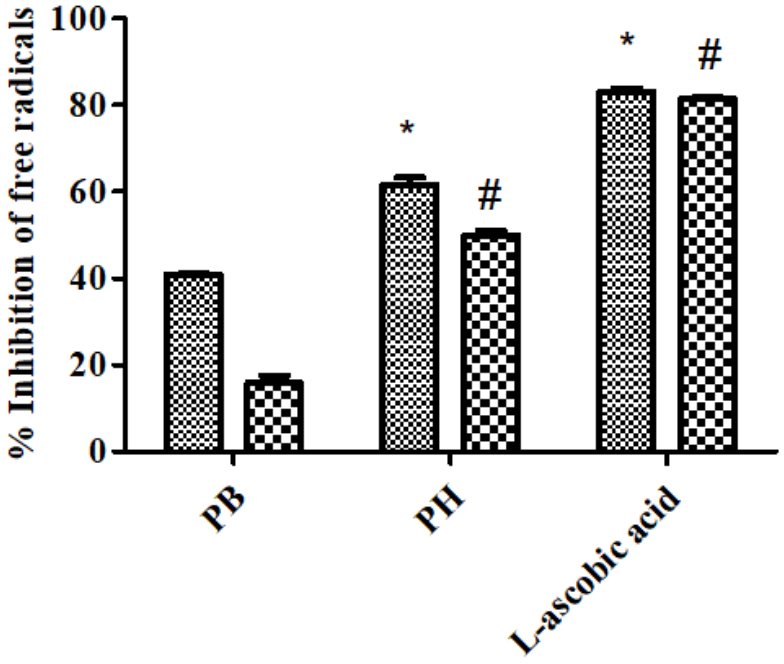


Fig. 3: DPPH and NO inhibition efficiency (%) of PB and PH patches; L-ascorbic acid is used as positive control. Values are means±SEM; *p<0.05, compared to PB DPPH and #p<0.05, compared to PB NO; ( ): DPPH; ( ): NO

Hydrogel dressings for wound treatment are nonreactive with biological tissue, permeable to metabolites, and are non-irritant[18]. The cytocompatibility of PB and PH patches was observed in fibroblast cells. The results of cytocompatibility revealed that PB and PH patches were non-toxic to the fibroblast cells. The cell viability of PB and PH patches treated fibroblast cells was close to untreated control at 24, 48, and 72 h (fig. 4). The cells surrounding the gels adhered to, spread, and grew on the cell culture plate after 24, 48, and 72 h of plating. Furthermore, results also showed that fibroblast cells can proliferation in PB and PH patches (fig. 5). These results indicated that the pectin-based hydrogel patch was non-cytotoxic to the cells. Then it benefits in product development.

Inflammation is the host defense mechanism against pathogens, tissue damage, or abnormal cells. In process of inflammation, NO plays an important role which acts as a signaling molecule. Macrophages are a major source of NO production during the inflammatory process[33]. This study focuses on the investigation of the anti-inflammatory properties of pectin-based hydrogel patch through NO inhibitory activity. The macrophage cells were stimulated with LPS to become activated macrophages. The activated macrophages can produce a high amount of NO. The inhibition of NO production refers to the anti-inflammatory activity of the patches. The result showed the decreasing of NO production in PB and PH patches under LPS-stimulated macrophages when compared with the positive control, dexamethasone (fig. 6). These results suggested that PB and PH patches demonstrated anti-inflammatory properties by inhibiting NO production in activated macrophages. NO inhibition was more pronounced in the PH treatment. Some previous studies reported anti-inflammatory activity in agarose hydrogel containing tannic acid and its benefit for wound healing[23]. Given the non-cytotoxicity of PB and PH patches, the pectin patch-treated cells could achieve high NO inhibition table 1e results indicated that the cell viability of macrophages was close to non-treated control cells.

According to the results of this study, pectin-based hydrogel patches revealed suitable physical and mechanical characterization. The pectin-based hydrogel containing HIE patches increases in antioxidant and anti-inflammatory properties. Furthermore, the pectin-based hydrogel patches also presented cytocompatibility and non-cytotoxicity to fibroblast and macrophage cells. These results suggested the benefit of pectin-based hydrogel patches in pharmaceutical and cosmeceutical applications. However, *in vivo* toxicity and efficacy are further study.

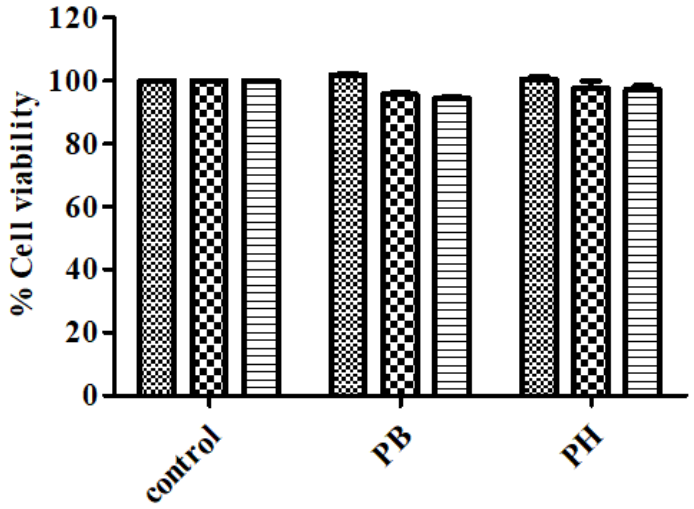


Fig. 4: Cell viability of PB and PH patches treated cell for 24, 48 and 72 h; ( ): 24 h; ( ): 48 h; ( ): 72 h

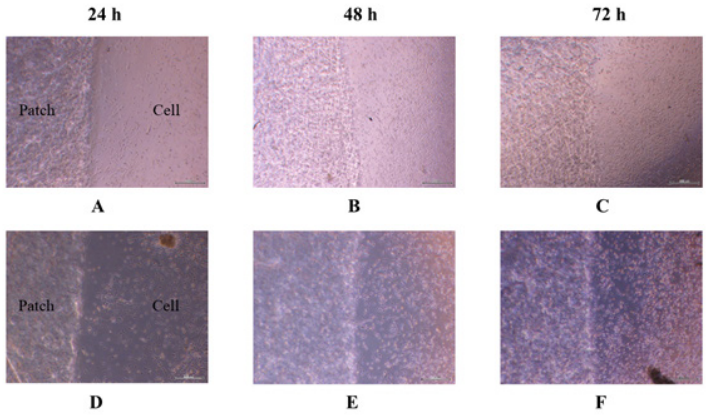


Fig. 5: The morphology of fibroblasts cells contacted with PB (A, B, C) and PH (D, E, F) patches at 24, 48 and 72 h

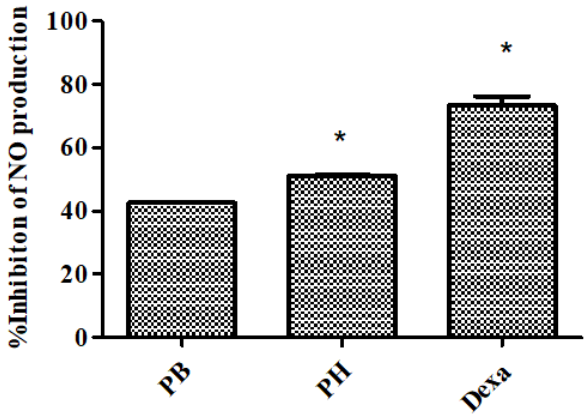


Fig. 6: Effects of pectin-based hydrogel patches on production of NO in LPS-stimulated macrophages. Values are mean±SEM; *denote p<0.05, compared to PB patch; Dexamethasone is used as positive control

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Conflicts of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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