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BIOIMAGING ANALYSIS ON COLONIC TARGETING FORM OF XYLAN-MESALAMINE PRODRUG USING ADVANCED MICROSCOPIC TECHNIQUES

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ABSTRACT

Xylan could be applied as a drug targeting agent for colonic delivery due to the fact that the degradation of xylan occurs by the action of xylanases and xylosidases enzymes produced by bacteria in the colon. Xylan from pineapple stem waste was isolated and then conjugated with mesalamine (5-aminosalycylic acid/5-ASA), which is the golden standard drug for chronic colon inflammation [1], forming a prodrug as an attempt to lower the absorption as well as the release of the drug in the stomach and small intestine. Xylan-mesalamine prodrug was tested to treat rats that suffered colon inflammation caused by TNBS (2, 4, 6-Trinitrobenzenesulfonic acid) induction. The healing progress of the treated inflamed colon tissue after 15 days was evaluated by histopathological analysis using light microscope (LM) and scanning electron microscope (SEM). Particularly, the tissue colon analysis using SEM is able to give a clearer insight of colon morphology due to the fact that higher magnification, as compared to that of LM, can be applied in SEM. Therefore, the superior healing effect of xylan-mesalamine prodrug on the treated colon tissue could be elucidated more clearly using SEM, showing that xylan-mesalamine prodrug give a better healing outcome of chronic colon inflammation treatment as compared to free mesalamine.

Keywords: colon inflammation, mesalamine, prodrug, scanning electron microscope, xylan.

INTRODUCTION

Pineapple is naturally abundant especially in Subang, West Java, Indonesia; that is one of the largest pineapple source in Indonesia. During pineapple production, the crown and stem are trimmed off before peeling and then the fruit is removed for further processing. Pineapple wastes including peel, core, stem, crown and leaves; generally account for 50% (w/w) of total pineapple weight [2]. Waste disposal represents a growing problem since it is usually prone to microbial spoilage and it causes serious environmental problems. Further processing of pineapple wastes to be a useful product would be a powerful method to solve this massive agricultural waste problem. This approach would lead to a relevant increase in the sustainability of agriculture, not only in Indonesia but also all around the world.

Pineapple wastes are found to have potential to be converted into some value-added products. For example, the peel is a rich source of cellulose, hemicelluloses and other carbohydrates; it has been used to produce paper, banknotes, and clothes [2]. Aside from that, there is one promising substance can be obtained for pineapple stem waste named as xylan. Xylan has drawn considerable interest due to its potential for biodegradable packaging films and food coatings, as well as for its use on biomedical products [3]. Xylan is promising as a material for colon targeted drug delivery due to its good properties such as water-insoluble, acidic resistant, and degraded only by xylanase enzyme present in colon. The esterification of xylan via activation of the carboxylic acid with N, N"-carbonyldiimidazole has been carried out in order to produce prodrugs for ibuprofen release [4]. Xylan degradation occurs by the action of hydrolytic enzymes: xylanases and xylosidases. Those enzymes are produced by a number of organisms, such as bacteria, algae, fungi, protozoa, gastropods, and arthropods [5]. The degradation of xylan in ruminants has been well reported, while some human intestinal bacteria have been investigated for their ability to produce xylan-polymer degrading enzymes. Among those intestinal species that are able to degrade complex carbohydrates such as lactobacilli, bacteroides, and non-pathogenic clostridia have demonstrated the ability to produce xylan-polymer degrading enzymes [6]. The presence of those bacteria in the human colon is a promising situation for polymer-based colonic delivery.

In this report, xylan isolated from pineapple stem waste was conjugated to mesalamine (5-ASA) to form a prodrug for targeting the drug to the colon. Mesalamine is a golden standard drug for chronic colon inflammation or inflammatory bowel disease (IBD) that unfortunately lack of therapeutic outcome until now. Mesalamine is rapidly absorbed from the proximal part of gastrointestinal tract (GIT); therefore, orally administered mesalamine could leads to failure in IBD therapy. Various approaches have been investigated to reduce the release and then the absorption of the drug in the upper part of GIT and thereby facilitate quantitative drug delivery to the colon. These are including biodegradable polymers-based coating, pH-sensitive polymers-based coating, time-dependent formulations, forming biodegradable matrices, and

forming a prodrug [7, 8]. Among them, a prodrug, which is a pharmacologically inactive derivative of a parent drug molecule, is more promising. The reactivation of prodrug is unique and it is determined by the chemical form of the conjugation. The xylan-mesalamine prodrug is able to pass through the upper gastrointestinal tract in mostly intact, then cleaved by xylanases and xylosidases enzymes when reaching the colon to reactivate the drug.

A prodrug of mesalamine using xylan from pineapple stem waste was prepared and chemically characterized. The xylan-mesalamine prodrug tested in vivo both pharmacokinetic and bioactivity studies in animal models. Bioactivity study was performed on several groups of rats. The rats were induced with 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS) so that they suffered the IBD. The rats were orally given three different treatment for 15 days. One group of rats was treated with xylan only, another group was treated with mesalamine prodrug only, and the last group was treated with xylan-mesalamine prodrug. The colon sample was then obtained from the rats for histopathological analysis using light microscope (LM) and scanning electron microscope (SEM). These observations were performed to confirm a more successful colon targeting of xylan-mesalamine prodrug as compared to free mesalamine using similar dose and route of administration.

Imaging of biological materials is traditionally performed using LM up to at most around 1000 times magnification. SEM is an ideal technique to observe detailed morphological structure with higher magnification [9]. Careless biological sample treatment and preparation could potentially leads to an additional micro-structural damage and will result in wrong analysis and conclusion. All colon samples were carefully prepared by the same fixation and dehydration methods in this study in order to be able to have a good comparison of the structures of different samples obtained from SEM observation.

EXPERIMENTAL PROCEDURE

Materials

Pineapple stem waste from Dexa Laboratories of Biomolecular Sciences, Indonesia; NaOCl from Merck, Darmstadt, Germany; NaOH from Merck, Darmstadt, Germany; H2SO4 from Merck, Darmstadt, Germany; Mesalamine (5-ASA) from Sun Pharmaceutical Industries Ltd., Mumbai, India; formic acid from Merck, Darmstadt, Germany; dimethylformamide from Merck, Darmstadt, Germany; 1, 1-carbonyldiimidazole from Merck, Darmstadt, Germany; dimethyl sulfoxide from Merck, Darmstadt, Germany; triethylamine from Merck, Darmstadt, Germany; acetone from Avantor Performance Materials, LLC; HCl from Avantor Performance Materials, LLC; Xylene from Bratachem, Bandung, Indonesia; Ethanol from Merck, Darmstadt, Germany; and 2, 4, 6-trinitrobenzene sulfonic acid/TNBS from Sigma-Aldrich.

Animals

Pathogen free male Wistar rats 6-8 weeks old and weigh around 150-200g were provided by the School of Pharmacy, Bandung Institute of Technology, Indonesia. These rats were given sufficient daily food intake and unrestricted access to tap water.

METHODS

Extraction of Xylan and Xylan-Mesalamine Prodrug Formation

Xylan extraction was performed based on the procedure mentioned in a previous literature [10]. Pineapple stem powder was dispersed in water for 24 hours then mixed with NaOCl for 1 hour to remove the lignin, washed with distilled water, and dried. To extract the xylan, the dried mass was immersed in NaOH for 24 hours then dried. Xylan extract was mixed with H2SO4 until precipitated and then methanol was added. The precipitate was centrifuged and washed with methanol followed by distilled water, then the obtained xylan was dried.

In order to obtain xylan-mesalamine prodrug, 5-N-formyl-aminosalicylic acid synthesis (5-fASA) intermediate has to be synthesized first. Mesalamine (5-ASA) was dissolved in formic acid and then distilled water was added into the mixture. The resulting precipitate was collected by centrifugation, washed in distilled water and then dried in the oven to obtain 5-fASA. After, dimethylformamide and 1, 1-carbonyldiimidazole were slowly added to 5-fASA and the mixture was stirred for 1 hour. Xylan is mixed with dimethyl sulfoxide and triethylamine, then the xylan mixture were slowly added to the 5-fASA mixture after stirring. Further, acetone was also added to the mixture. The precipitate was collected by centrifugation then hydrolyzed with HCl for 10 minutes. Acetone was added to the final product, and then washed and dried in a vacuum oven at 60^{o}C until a constant weight was reached.

Activity Study of Xylan-Mesalamine Prodrug on TNBS-Induced Colitis Ulcerative

The activity study of xylan-mesalamine prodrug was done in colitis rat model induced by TNBS (80 mg/kg bodyweight) through rectal route. Five groups were applied in the activity study, which are (A) healthy colon or negative control, (B) inflamed colon or positive control, (C) xylan treated inflamed colon (192.96 mg/kg body weight/day), (D) mesalamine treated inflamed colon (180 mg/kg body weight/day), and (E) xylan-mesalamine prodrug inflamed colon (372.96 mg/kg body weight/day). All animals were induced for colitis with previously reported method [11]; except of those belonged to the negative control group. Animals were treated daily for 14 days, started at day 2 after TNBS induction to their respective groups. At day 15 following TNBS induction, all rats were sacrificed and the colon were washed with cold saline water. Washed colon groups was weighed, measured for length, and prepared for histopathological analysis using LM and SEM. Detailed study on the

performance of xylan-mesalamine prodrug for colonic targeting of mesalamine could be seen on the previously published report [12].

Histopathological Analysis Using Microscope

Colon groups samples were treated with following consecutive steps: formalin fixation, dehydration, and paraffin embedding. Subsequently, the paraffin embedded colon samples were sectioned using microtome with the final sample thickness of 3µm, then the sections are placed on top of cover glass slides. Microtome-sectioned paraffin embedded samples were then prepared for observation under LM and SEM. Further, microtome-sectioned paraffin embedded samples stained by hematoxylin and eosin for LM observation.

In order to perform SEM observation, microtome-sectioned paraffin embedded colon samples needs to be processed further. The first step is the deparaffinization of samples using xylene [13]. Deparaffinization procedure was performed by washing the samples with xylene for 4x30 minutes at 37^{o}C. Further, the samples were washed using 100% ethanol for 4x15 minutes at room temperature. The samples were then left to dry overnight at room temperature. Subsequently, the samples were gold coated using ion sputtering machine, with gold layer thickness of about 2.5nm, to increase sample conductivity. The samples observation was conducted with SEM (Hitachi SU3500) at operating voltage of 5kV. The advantage of this SEM sample preparation method is that the samples to be observed with LM and SEM could be prepared with similar procedure, so that the same section of sample could be observed with LM and SEM.

RESULTS AND DISCUSSIONS

Colon samples from each group were taken as shown in Figure-1. As shown, the inflamed colon (Figure-1b) has the most severe condition compared to other groups, either treated by free mesalamine (d) or the prodrug (e) which are healed better such that they have a better and more similar structure compared to that of the healthy colon (a). These results suggest that the xylan-mesalamine prodrug exhibited better effect due to higher accumulation of mesalamine on the colon compartment. Upon reaching colon, the prodrug is releasing a sufficient amount of free mesalamine to give a better therapeutic outcome [12]. To evaluate detailed microstructure of mucosal colon tissue and the influence of the therapies, LM as well as SEM were performed.

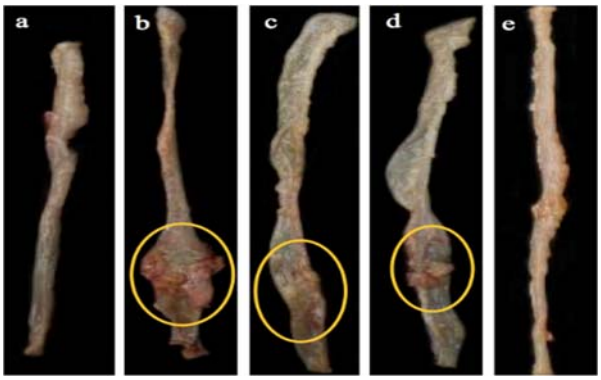

Figure-1. Visual observation of colon samples from each group: (a) Healthy colon, (b) Inflamed colon, (c) Xylan treated colon, (d) Mesalamine treated colon, (e) Xylan-mesalamine prodrug treated colon. Yellow circles indicate the swelling due to inflammation

Light Microscope Analysis of Hematoxylin/Eosin-Stained Colon

LM observation was performed to evaluate the overall morphology of each colon group. Figure-2 shows images of colon from each group at 40 times magnification. Figure-2(a) shows a healthy colon cross-section taken as a reference, where the successfully treated inflamed colon has reverted back to this healthy condition. As shown in Figure-2(b), the inflamed colon lost its crypts with major ulcer signs as indicated by the yellow box. Similar structure was also detected after IBD suffered rats were treated with xylan only (Figure-2c), more damaged area with deep mucosal erosion could be seen as indicated by yellow box and yellow arrow. Based on this observation, it is clearly seen that xylan is an inert material with no pharmacological activity. Damaged colon treated with free mesalamine (Figure-2d) demonstrated a good healing progress indicating by crypts disappearance, although minor shallow mucosal (ephitelium) erosion was still detected. In contrast, complete regeneration of colon morphology with very minor ephitelium erosion was demonstrated by xylan-mesalamine prodrug treatment (Figure-2e).

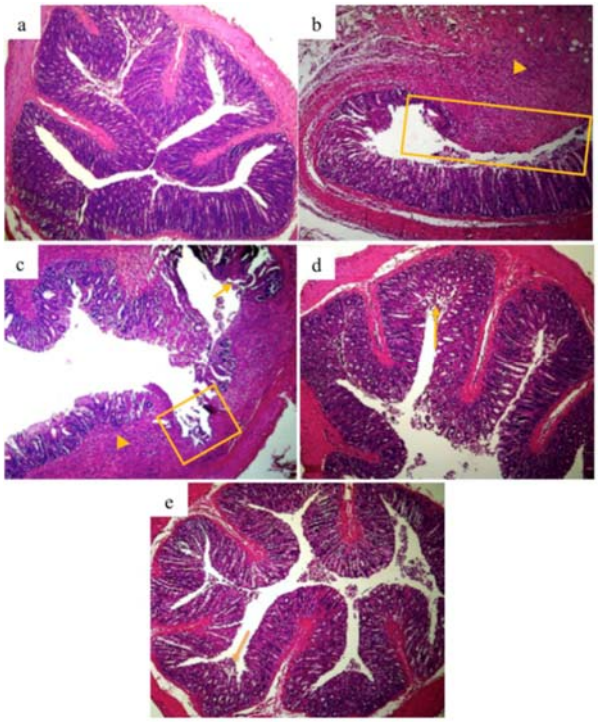

Figure-2. LM observation of colon samples from each group (a) Healthy colon, (b) Inflamed colon, (c) Xylan-treated colon, (d) Mesalamine-treated colon, (e) Xylan-mesalamine prodrug-treated colon. Yellow box in (b) shows ulceration. Yellow box in (c) shows large mucosal erosion. Arrows indicate mucosal erosion. Magnification of 40x.

Observation of colon morphology using LM with higher magnification of 400 times as shown by Figure-3 was performed to evaluate the detailed both mucosal erosion and colon damage after disease induction as well as the effect of treatments. As depicted in the figure, both inflamed and xylan treated colon clearly indicated tissue rupture (yellow arrows, Figure-3b and 3c), respectively. The treatments either using free mesalamine (Figure-3d) or xylan-mesalamine prodrug healed the tissue damage significantly with superior healing was presented by the prodrug. However, due to limitation of LM, more detailed information regarding the successful treatment is missing. Therefore, SEM was applied to obtain clear difference of healing progress between free mesalamine and xylan-mesalamine prodrug treatments.

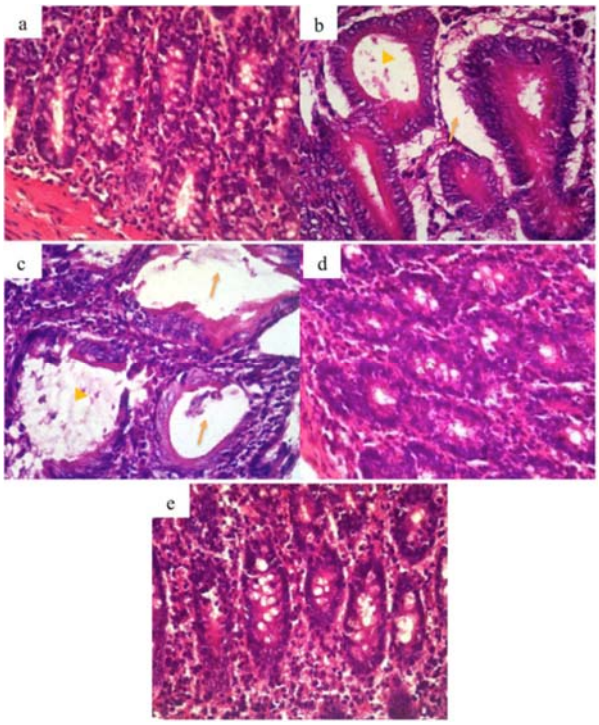

Figure-3. LM observation of colon samples from each group (a) Healthy colon, (b) Inflamed colon, (c) Xylan-treated colon, (d) Mesalamine-treated colon, (e) Xylan-mesalamine prodrug-treated colon. Yellow box in the image shows ulceration. Arrows show mucosal erosion. Magnification of 400x.

SEM Analysis to Evaluate the Influence of the Treatments to the Surface Morphology

In order to observe more detailed structural morphology of these colon samples, especially to find out the clear effect of xylan-mesalamine prodrug treatment, observation using SEM was conducted. Figure-4 shows the SEM pictures of mucosal colon tissue from each group with 4000 times magnification. As depicted in Figure-4(a), a healthy colon shows a densely packed tissue with smooth surface. While, a very severe damage on the tissue and a large part of spherical inflammatory cells are clearly exposed on the untreated inflamed group (Figure-4b). In xylan treated group (Figure-4c), several fractures pointed by yellow arrows on the image and also some spherical cells are exposed as indicated by the red box. These small fractures still appears in mesalamine-treated group shown on the areas pointed by yellow arrows (Figure-4d); however, these fractures are minor compared to that in the untreated or xylan treated groups. In addition, the spherical tissues were not exposed as compared to xylan treated group, indicating denser matrix i.e. healing process was detected. Major improvement of healing process was observed in group treated with xylan-mesalamine prodrug (Figure-4e), indicated by very minor fractures and a compact matrix.

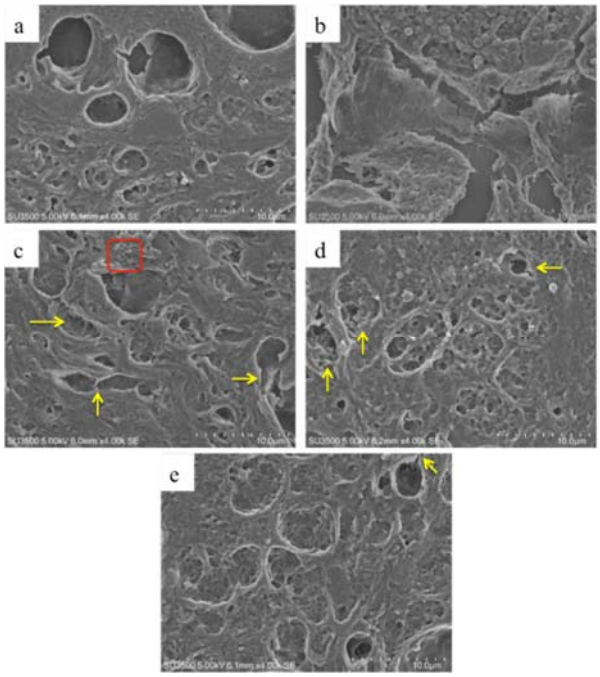

Figure-4. SEM images of colon samples from each group: (a) Healthy colon, (b) Inflamed colon, (c) Xylan-treated colon, (d) Mesalamine-treated colon, (e) Xylan-mesalamine prodrug-treated colon. Yellow arrows show some fractures on the tissue. Red box shows the area with exposed spherical inflammatory cells. Magnification of 4000x.

SEM observation at a higher magnification of 8000 times was performed to confirm the superior effect of the xylan-mesalamine prodrug on the inflamed colon. The detailed SEM analysis of colon tissues is presented in Figure-5. As seen, major tissue damages and serious fractures are clearly detected in the untreated inflamed and xylan treated animal, as shown in Figure-5b and 5c respectively. These pathological signs were reduced significantly when the animals treated with the drug (Figure-5d and 5e). In particular, the xylan-mesalamine prodrug treatment removed the fracture completely (Figure-5e). Although the colon density did not completely recover as shown in healthy animal, this data postulates the potential use of the pineapple stem waste xylan to direct the mesalamine to the colon for more effective therapy.

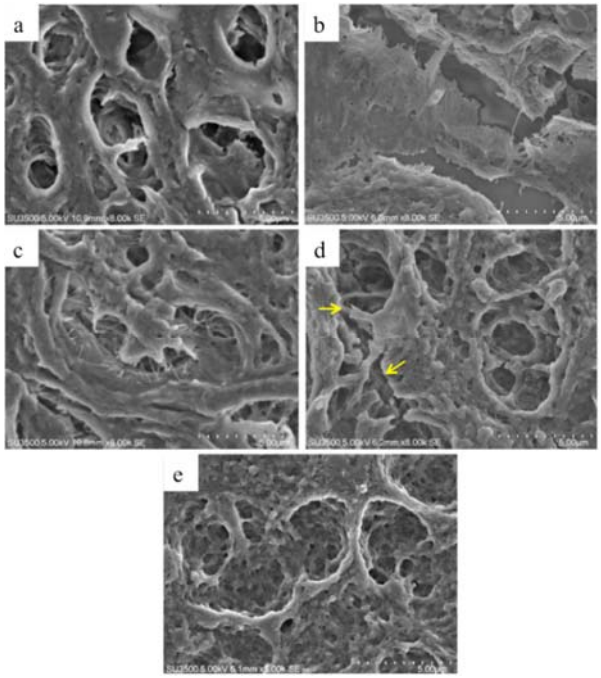

Figure-5. SEM images of colon samples from each group: (a) Healthy colon, (b) Inflamed colon, (c) Xylan treated colon, (d) Mesalamine treated colon, (e) Xylan-mesalamine prodrug treated colon. Yellow arrows show some small fractures on the tissue. Magnification of 8000x.

CONCLUSIONS

Pineapple stem waste xylan demontrates a potential benefit for medical application to form xylan-mesalamine prodrug as a colon targeting agent. Xylan-mesalamine prodrug give a better outcome of chronic colon inflammation treatment as compared to free mesalamine. This is due to the xylan could prevent the release and the absorption of mesalamine at the upper part of gastrointestinal tract and only release the mesalamine once it has already reached the colon. The improvement healing progress of inflamed colon tissue after xylan-mesalamine prodrug treatment is proven by LM and confirmed by SEM observation. Better mucosal colon tissue morphology in animals treated with xylan-mesalamine prodrug is clearly exhibited under SEM observation at 8000 times magnification. Bioimaging by SEM is therefore a powerful tool to expose more detailed histological analysis to give an elucidation of the colon mucosal tissue condition.

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