ISSN No:-2456-2165

# *Centella asiatica* Extract Increased Expression of bFGF but not Sox-2 in Peptic Ulcer Model in Rats Induced by Indomethacin

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**Abstract:- Peptic ulcer is a disease that commonly occurs and spreads over the world and one of its causes is the use of long-term non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are also able to inhibit ulcer healing process. Madecassoside and asiaticoside are active ingredients in Centella asiatica extract which have wound healing effects by increasing angiogenesis and inhibiting the inflammatory process. The aim of this study was to determine the effect of Centella asiatica extract on the expression of bFGF and Sox-2 in the gastric Rattus novergicus model of peptic ulcer induced by indomethacin. The effect of Centella asiatica (CA) extract was seen in mice (Rattus novergicus) used as peptic ulcer models which were induced by indomethacin at a dose of 30 mg/kg Body Weight, then the extract was added by CA at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg for 7 days with a frequency of 1x/day. The experimental group was divided into 6 groups, namely negative control group, positive control group day 1st, positive control group day 8th, treatment group dose 100 mg/kg, 200 mg/kg and 400 mg/kg. The experimental animals were then sacrificed, then the expression of bFGF and Sox-2 in the gastric mucosal epithelium was assessed by doing immunohisto-chemistry. The results showed that CA was able to increase bFGF expression in rat gastric epithelial cells (p = 0.007) while Sox-2 expression showed no significant increase. These results indicated that Centella asiatica extract can help cure peptic ulcer by increasing bFGF expression in gastric epithelial cells which is important for angiogenesis, proliferation, and epithelial cell migration.**

***Keywords:-*** *bFGF, Extract of Centella asiatica, Indomethacin, Peptic Ulcer, Sox-2, Ulcer Healing.*

## I. INTRODUCTION

Peptic ulcer is a wound lesion that reaches the entire mucous and muscularis layers of the gastric and duodenal mucosa [1]. Several etiologies have been identified to be involved in the occurrence of peptic ulcer, one of the most common etiologies was the result of the use of long-term non-steroidal anti-inflammatory drug (NSAID) [1], [2]. This drug is not only capable of causing the effects of injury to the gastric mucosa, but also inhibiting the ulcer healing process [1].

When an ulcer occurs, the body has an ulcer healing mechanism which is a complex and ongoing process. This process is initiated by increasing growth factors, one of which is basic fibroblast growth factor (bFGF) which is important for the process of angiogenesis[3]. Furthermore, regeneration and repair of epithelium involving gastric stem cells with one of the markers is Sox2 (sex-determining region Y/SRY-related HMG box) [4].

Ulcer healing process is a process of tissue repair by itself after lesions are formed. The stages are sustainable and can be divided into several phases, namely hemostasis, inflammation, proliferation and remodeling [5]. Ulcer healing process is initiated by increasing the expression of growth factor one of which is bFGF which can stimulate epithelial cell proliferation and angiogenesis by increasing the re-inervation of newly formed micro blood vessels so that it is important for gastric ulcer healing [6]. bFGF has been detected in several gastrointestinal mucosal compartments, mainly in superficial epithelial cells along the gastrointestinal tract, in the basal lamina and extracellular matrix [6].

Stomach is also capable of carrying out cell renewal as a protective mechanism involving multipotent stem cells. This stomach stem cell can be found in the istmus

with one of the markers being Sox2 (sex-determining region Y/SRY-related HMG box) [4]. Sox2 expressed in the gastric gland epithelium has a function as an important regulator of stem cell maintenance, end-cell differentiation and control of homeostasis and tissue regeneration [7].

Centella asiatica is a short and creeping trunk plant that is known to have various physiological effects and can be used for wound healing[8]. Madecassoside and asiaticoside are active elements in Centella asiatica extract, which have wound healing activities by increasing collagen formation and angiogenesis and also able to inhibit the inflammatory process and improve capillary permeability [9], [10]. Because of its benefits to heal wound from extract, the researchers wanted to observe the ulcer healing effect of Centella asiatica (C. asiatica) extract on the gastric mucosa of wistar strain Rattus norvegicus rats induced by Indomethacin by looking at bFGF and Sox2 expression in rat gastric mucosa.

## II. MATERIAL AND METHODS

### *A. Animals*

Male Wistar rats (150– 200 g), obtained from the Department of Pharmacology at the University of Brawijaya, were used in experiments. Rats were divided randomly into 6 groups of 3. The animals were maintained on standard pellet diet and tap water. Animal experiments were carried out following the guidelines of the Animal Ethics Committee of the institute.

### *B. Preparation of Ethanol Extracted from Centella asiatica*

Centella asiatica used is all parts of the plant that are cultivated above the ground. The material is then washed, cut into pieces, dried with a temperature of 40-60$^{o}$C to dry. The material is then crushed with a blender. The material was weighed 100 grams, soaked with a solvent of 900 ml to 1 liter. The mixture was shaken for 30 minutes, soaked overnight until it settled. The top layer of the mixture which is a mixture of solvents and active substances was then taken and put into the evaporation flask. The evaporation process was carried out at a temperature of 90$^{o}$C until the solvent flow stopped dripping on the holding pumpkin ($\pm$ 1.5 hours - 2 hours) and the extraction results were approximately 1/5 of the dry natural ingredients.

### *C. Indomethacin – induced Peptic Ulcer*

The experimental animals were fasted for 8 hours without being fed but allowed to drink, then positive control group day 1st, positive control group day 8th and treatment group was given indomethacin (Sigma) orally for induction of ulcers at a dose of 30 mg/kg. Experimental animals were left without food for 8 hours later [11]. Experimental models of peptic ulcer in the positive control group day 1st and negative control group were then tested by conducting cervical dislocation, followed by gastric organs' extraction. Positive control group rats on the 8th day were left alive and received ad libitum food and drinks. The treatment group 1, 2, and 3 received the first CA dose 8 hours after indomethacin administration with doses of 100 mg/kg body weight, 200 mg/kg body weight and 400 mg/kg body weight. Administration Was continued up to 7 times with a frequency of 1x/day. The positive control group rats on the 8th day and the treatment group were then sacrificed 24 hours after the last CA administration [10].

### *D. Histological preparation*

Specimen of the gastric from each rat was fixed in 10% buffered formalin and processed in a paraffin tissue processing machine. Section of stomach was made at a thickness of a 4 μm.

### *E. Immunohistochemistry bFGF and Sox-2*

Immunohistochemistry (Immunohistochemistry kit, ScyTek Laboratories Inc, USA) was used to measure bFGF and Sox-2 expression. Tissue slice samples with a thickness of 4 μm were warmed at a temperature of 60-80$^{o}$C for antigen retrieval and then deparaffinizated with xylol, then rehydrated by using decreased ethanol concentration. Samples were then incubated with Peroxidase Blocking for Image Analysis (ScyTek Laboratories Inc., ADA100) for 40 minutes at room temperature to block endogenous peroxidase activity. The slides were rinsed with phosphate buffer saline and again incubated with Super Block (ScyTek Laboratories Inc., AAA100) overnight at 4$^{o}$C. The slides were incubated with bFGF (Abcam, ab 16828) and Sox-2 (Santa Cruz Biotechnology, Inc, sc-365823) primary antibodies overnight for 4$^{o}$C then rinsed with phosphate buffer saline, followed by incubation with Anti-Polivalent HRP Polymer CRF (ScyTek Laboratories Inc., ABZ100) for 1 hour room temperature. The slides are then rinsed with PBS and rinsed again with distilled water until PBS was lost. Slides were incubated with DAB chromogen (ScyTek Laboratories Inc., ACB006) in the High Contrast substrate DAB (ScyTek Laboratories Inc., ACU100), observed expression on the target location was examined by microscopy until the response was marked with the formation of brown color on the target. Substrate reaction was stopped by inundating slides by using distilled water for 5 minutes, then rinsed them thoroughly. Slides were given Mayer's Hematoxylin counters (ScyTek Laboratories Inc., HMM125), observed with a microscope and rinsed with distilled water until they were clean.

### *F. Data collection*

Calculation of bFGF expression in epithelial cells was conducted by seeing the presence of brown color in the cytoplasm of epithelial cells. As for Sox-2 it appeared as brown in the cell nucleus. The results of each calculation were written on a worksheet and an average value per field of view was taken. Scavenging of Hematoxylene-Eosin was used as a structural comparison. In order to guarantee representation and reduce yield errors, observations of approximately 10 visual fields with 400x magnification were needed, each of which contained approximately 1500 cells [12], [13].

### *G. Statistical analysis*

The statistical significance of differences between groups was assessed by using Kruskal Wallis. A probability value of $p < 0.05$ was considered to be statistically significant.

ISSN No:-2456-2165

## III. RESULTS

*A. Ethanol extract of Centella asiatica increased expression of bFGF in epithelial mukosa gaster of peptic ulcer model rats*

This study showed that the ethanol extract of Centella asiatica was able to increase the expression of bFGF in the gastric mucosal epithelium of Rattus novergicus mice (p= 0,007)

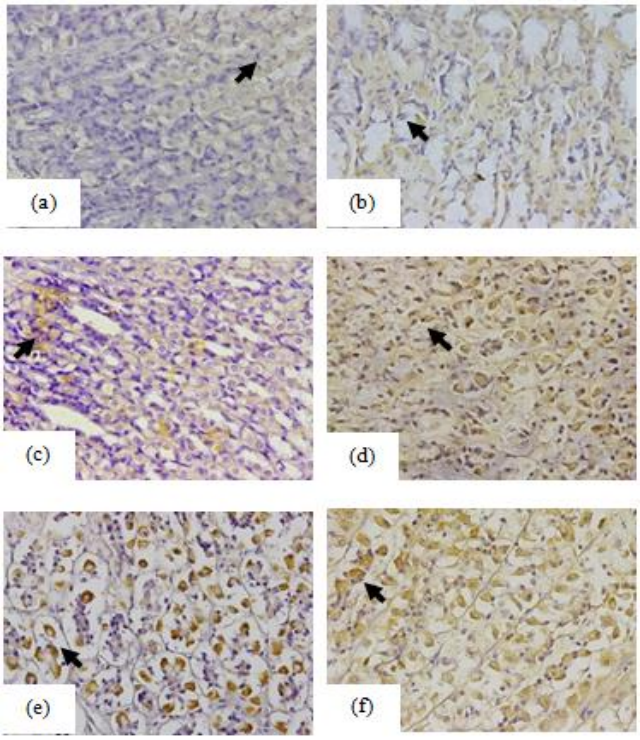


Fig 1:- Immunohistochemical staining of bFGF expression (400x magnification). (*Negative control group (a), positive control group day 1st (b), positive control group day 8th (c), treatment group dose 100 mg/kg (d), treatment group dose 200 mg/kg (e), treatment group dose of 400 mg/kg (f). The expression of bFGF was shown by an arrow indicating the cytoplasm of the mucous epithelial cells which were stained brown)*

Figure 1 shows that bFGF expression was seen in gastric mucosal epithelial cells. There was no difference in the expression of bFGF in the positive control group on the first day and the positive control group on the eighth day. In the positive control group on the first day there was a higher expression of bFGF compared with the negative control group, it appeared from the more number of brown colored cells in the positive control group the first day. The same results were also shown by the eighth positive control group compared to the negative control group. In the treatment group given Centella asiatica ethanol extract, both dosages of 100 mg/kgBB, 200 mg/kgBB and 400 mg/kgBB showed higher expression of bFGF compared to the positive control group on the first day and 8$^{th}$ day. This showed that administration Centella asiatica ethanol extract can improve the healing process of the ulcer by increasing the expression of bFGF.

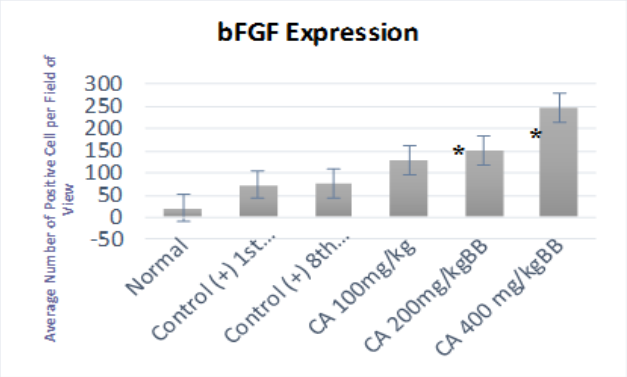


Fig 2:- Average bFGF expression (*Significance of p value if p < 0.05. The p value was significant compared to the group of positive control mice on the first day and eighth day (*).)*

Figure 2 shows an increase in the mean expression of bFGF in the gastric mucosal epithelial cells of the treatment group compared to the control group with a significant increase in the treatment group dose of 200 mg/kg body weight and 400 mg/kg body weight.

*B. Ethanol extract of Centella asiatica increased expression of Sox-2 in gastric mucosal epithelial cells of peptic ulcer model rats*

In this study there was no difference in the expression of Sox-2 in gastric mucosal epithelial cells in both control or treatment groups (Fig 3).

## IV. DISCUSSION

The administration of non-steroidal anti-inflammatory drugs (NSAIDs), in this study which was conducted by using indomethacin at a dose of 30 mg/kg, was able to cause peptic ulcers due to prostaglandin (PG) deficiency due to cyclooxygenase (COX) inhibition, both COX-1 and COX-2 [14]. Further prostaglandin deficiency caused gastric hypermotility [15], decreased blood flow in the gastric mucosa, decreased mucous layer formation, bicarbonate and phospholipid [16], increased mucosal permeability [15] and increased acid production [1]. This results in activation of inflammatory mediators, impaired repair and healing of the mucosa, forming peptic ulcers [1].

When peptic ulcer was formed, the body would make efforts to do healing. The process began with an increase in growth factors, one of which was bFGF / FGF-2. bFGF is expressed by normal gastric tissue in both mice and humans and it was reported that its expression increased when peptic ulcer occured [17], [18]. bFGF was bound by heparan sulphate proteoglycans (HSPG) which modulated bFGF activity on the cell surface, protected bFGF from inactivation by acids and proteolysis, and acted as a storage place to be released during injury[18]. In this study it was found that bFGF was also expressed by the negative control group but in low numbers and obtained an increase in expression compared to the positive control group on the first day (21.33 $\pm$ 14.61 vs. 73.83 $\pm$ 7.87, p = 0.069) and in the positive control group on the eighth day (21.33 $\pm$ 14.61 vs 77.17 $\pm$ 8.63, p = 0.055).

ISSN No:-2456-2165

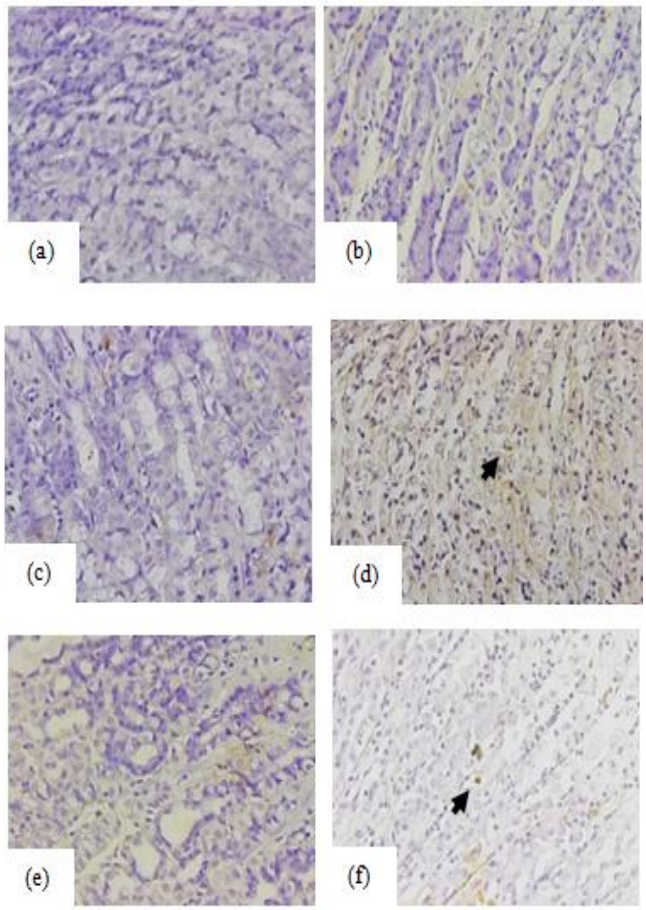


Fig 3:- Immunohistochemical staining of Sox-2 expression (400x magnification). (*Negative control group (a), positive control group first day (b), positive control group 8th day (c), treatment group dose 100 mg/kg (d), treatment group dose 200 mg/kg (e), treatment group dose of 400 mg/kg (f). The expression of Sox-2 is shown by an arrow indicating the nucleus of the mucous epithelial cells which are stained brown)*

bFGF is a cytosolic protein expressed by rat and human gastric and duodenal mucosa [17], [18]. Expression of bFGF is seen in mucosal epithelial cells, goblet cells, mucous neck cells, parietal cells, chief cells and basal gland cells [17]. In this study it was also obtained that the expression of bFGF appeared in form of brown stains in the cytoplasm of gastric mucous cells, spreading from mucosal epithelial cells on the surface to basal glandular cells of the gastric mucosa (Figure 1).

Centella asiatica ethanol extract with the active ingredient madecasoside and asiaticoside has wound healing activities both in vivo and in vitro through angiogenesis [19]. In addition this substance is also able to inhibit the inflammatory process which is important in the early pathogenesis of the formation of peptic ulcer [9]. Research by Cheng et al showed that Centella asiatica extract was able to stimulate blood vessel formation and regeneration of mucous cells during the stage of gastric ulcer healing [10]. It is known that angiogenesis is an important part of the wound healing process. Angiogenesis helps heal wounds by improving the stylisation in the wound area, to deliver oxygen and nutrients during the healing process including for reepithelialization [20]. Centella asiatica extract also plays a role in the proliferation phase in the process of wound healing. In this phase the process is dominated by the formation of granulation tissue and epithelialization, closely related to growth factors, one of which is bFGF[21]. The effect of Centella asiatica extract in aiding peptic ulcer healing can be caused by the induction of bFGF expression because of the important role of bFGF in the healing process of peptic ulcer [10].

bFGF can affect the same cells to proliferate and migrate, one of which is when regeneration occurs to close the wound. Through the same FGF receptor, bFGF is able to play a role in mitogenesis and cell mobility [20], [22], [23]. bFGF affects cell proliferation by providing a mitogenic effect through the Ras-MAP kinase pathway which includes ERK 1/2 [24], [25]. The effect of migration by bFGF is due to activation of the Src pathway and the MAP kinase pathway through p38 [24]. The ability of bFGF in the wound healing process is proven by a study by Ernst et al which explains that the use of exogenous bFGF can accelerate the process of tissue repair in healing gastric ulcer through complex interactions of cell matrices, cell proliferation, cell migration and angiogenesis [6].

In this study the results were also found to be appropriate, namely an increase in bFGF expression in indomethacin-induced peptic ulcer groups given Centella asiatica ethanol extract at a dose of 100 mg / kgBB, 200 mg / kgBB and 400 mg / kgBB, with the highest increase in expression in the group a dose of 400 mg / kgBB. The positive control group in this study was allowed to stay alive until the eighth day to compare the ulcer healing process by their own abilities compared to mice that had been given Centella asiatica extract. The results showed that there was a significant increase in bFGF expression in the treatment group given Centella asiatica extract 200 mg / kgBB ($p = 0.048$) and a dose of 400 mg / kgBB ($p = 0.002$) when compared to the positive control group on the eighth day.

When peptic ulcer occurs which damages the barrier of the gastric mucosal epithelium, the body attempts to regenerate through the proliferation and differentiation of stem cells and progenitors for renewal of mucosal epithelial cells within days to months [26]. Several stem cell markers in the stomach have been known, one of which is Sox-2, a transcription factor that is important for homeostasis and regeneration [27]. Research has so far discussed much about the role of Sox-2 in early embryonic cells and their regulation in the fetus [7]. Arnold et al. found that Sox-2 is also a marker of stem cells in several adult epithelial tissues including the stomach both in the forestomach area, corpus and pylorus [7].

Sox-2 through immunohistochemistry appears to be expressed in the nucleus cell and generally its expression is increased in cancer cases, for example in gastric cancer [27]. The study by Aihara et al showed that Sox-2 was

expressed in the cytoplasmic and nucleus compartments in the area of rat gastric isthmus, and was sporadically expressed as well as in other studies [28], [29]. Sox-2 expression in the normal stomach is found to be very low, only one to two cells per gland, different from other stem cell markers in the stomach [7]. In this study, Sox-2 appeared to be expressed in nucleus cell and cytoplasm of epithelial gastric mucosa isthmus cells with very low expression, which was only found in the number of one or two cells both in the control group and the treatment group.

Centella asiatica extract has been known to have a role in the proliferation phase in the process of wound healing. But in this study there was no increase in Sox-2 expression in the treatment group. The study by Aihara et al. who looked at the healing of gastric ulcers from the 8th day to 8 months after the ulcer, found an increase in Sox-2 expression in the gastric mucosa on day 30 [29]. In this study, peptic ulcer induction was performed and Centella asiatica extract was given for 7 consecutive days and rats were sacrificed on the eighth day.

Cell proliferation in the process of ulcer healing is an important aspect. This process can occur by involving epithelial cells at the edge of the ulcer or at a later stage followed by the involvement of stem cells to contribute to proliferation and differentiation [26]. In this study, Sox-2 as one of the gastric epithelial stem cell markers did not appear to be involved in the ulcer healing process. This may indicate that the ulcer healing process is sufficient only with the proliferation and migration of epithelial cells around the ulcer and does not require the involvement of Sox-2. Activated pathways can involve Smad 2/3 which can lead to proliferation without involving Sox-2 in the process [30].

This study also showed the presence of Sox-2 expression in the cytoplasm. Sox-2 is expressed in the cytoplasm as a result of nucleated nuclear export as a negative regulation of this protein followed by Sox-2 ubiquitination. The existence of the Sox2 protein does not always indicate that this protein has worked at a certain time until this protein trans-locates to the nucleus to activate the target gene [28]. To work to affect cell proliferation, Sox-2 needs to translate to the nucleus by phosphorylation and undergo nuclear import, this process is closely related to AKT kinase [31], [32].

## V. CONCLUSION

Based on the results of the study, it can be concluded that Centella asiatica extract is able to help cure peptic ulcer in Rattus novergicus rats by increasing bFGF expression in gastric mucosal epithelial cells which causes proliferation and migration of mucous epithelial cells. In this study it did not show an increase in Sox-2 expression which could be due to the duration of the study that was too short or indeed the role of stem cells involved in the ulcer healing process because it had been sufficient by the proliferation mechanism of surrounding epithelial cells.

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